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Titel der Masterarbeit

Mouse Models of Human Diseases:

„New Tools for Mouse Genome Engineering“

and

„Evaluation of the Role of MKK7 in LPS/GaIN Induced Hepatitis“

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Abstract

Breast cancer is the most common cancer among women worldwide. Nevertheless only few good breast cancer mouse models exist so far. In order to generate a novel *in vivo* breast cancer model, two new tools for mouse genetics were developed. First, female stem cells were characterized and chimeras from those female stem cells carrying the Polyoma Middle T (PyMT) oncogene were derived successfully. In the future, this cell line will be used for laser assisted injection into 8-cell stage embryos to rapidly produce F0 mice that are fully derived from the embryonic stem cells (ESCs) and therefore can be used directly for phenotyping without further breeding. Second, to facilitate the generation of mice targeted at the ROSA26 locus, an exchange vector system was developed, that drastically reduces time and effort needed to target transgenes into ESCs. Using this system mouse lines can be generated, that express different shRNAs from the ROSA26 locus, in order to knock down candidate breast cancer genes on a sensitized background.

A second project aimed to examine the role of MKK7 in the LPS/GalN induced hepatitis model. The MKK7-JNK-MAPK pathway is known to play an important role in TNF α -induced liver inflammation. Hepatocyte specific deletion of MKK7 did not affect the extent of liver damage following LPS/GalN treatment. Likewise, survival as well as serum transaminase levels (ALT and AST) were found to be similar between control mice and mice lacking MKK7 in the liver and the hematopoietic system, indicating a similar hepatic damage. Furthermore, loss of MKK7 in the liver and the hematopoietic system was associated with a reduced basal expression of *c-Jun* mRNA. However, upon LPS/GalN treatment normal induction of *c-Jun* mRNA was observed. Interestingly, no induction of c-Jun protein was observed in knockout mice, indicating that the loss of MKK7 rather affects either mRNA stability, translation, protein stability or posttranslational modifications. Taken together, loss of MKK7 in the liver and the hematopoietic system in the context of LPS/GalN induced hepatitis was shown to affect the expression of certain genes (*c-Jun*, *Tnfa*) and proteins (c-Jun) but not the overall outcome after induction of hepatitis.

Kurzfassung

Brustkrebs ist die häufigste Krebserkrankung bei Frauen weltweit. Trotzdem stehen bis heute nur wenige gute Mausmodelle für Brustkrebs zur Verfügung. Zwei innovative Entwicklungen sollen helfen, einen *in vivo* Brustkrebs-Screen zu ermöglichen. Die Stammzellqualitäten einer weiblichen embryonalen Stammzelllinie wurden nachgewiesen und chimäre Mäuse, welche das Polyoma Middle T (PyMT) Onkogen exprimieren, wurden erfolgreich erzeugt. In der Zukunft sollen diese embryonalen Stammzellen (ESZ) in Mausembryos im 8-Zellstadium injiziert werden. Das ermöglicht die Herstellung von F0 Mäusen, die vollständig von den ESZs abstammen und somit eine Analyse des Phänotyps in der ersten Generation erlauben. Um das Generieren von ESZs mit Manipulationen im ROSA26 Locus zu erleichtern, wurde ein Exchange Vector-System entwickelt, um den Zeit- und Kostenaufwand zur Herstellung ROSA26-mutanter Mäuse drastisch zu reduzieren. Mit Hilfe dieses Systems sollen mehrere Mauslinien erzeugt werden, die verschiedene shRNAs in ihrem ROSA26 Locus tragen. Diese shRNAs werden folgendermaßen gewählt, dass sie verschiedene Moleküle der PyMT-Signalkaskade ausschalten und dadurch möglicherweise den Verlust des onkogenen Potenzials von PyMT verursachen. Bei der eigentlichen Anwendung im *in vivo* Brustkrebs-Screen werden die shRNAs in die PyMT-exprimierenden ESZs eingebracht, um die Funktionsweise von PyMT im Zusammenhang mit der malignen Transformation in der F0-Generation zu erforschen.

Ein zweites Projekt hatte zum Ziel, die Rolle der Stresskinase MKK7 im LPS/GalN induzierten Maushepatitismodell zu analysieren. Es ist bekannt, dass der MKK7-JNK-MAPK-Signalweg von großer Bedeutung für die Entstehung von TNF α -stimulierter Hepatitis ist. Hepatozytenspezifische Deletion von MKK7 hatte keinen erkennbaren Einfluss auf das Ausmaß des Leberschadens nach Behandlung mit LPS/GalN. Auch bei Mäusen mit einer Deletion von MKK7 in der Leber als auch im hämatopoietischen System waren die Serumtransaminaselevel (ALT und AST) unverändert gegenüber der Kontrollgruppe. Des Weiteren konnte eine reduzierte Basalexpression von *c-Jun* mRNA bei diesen Knockoutmäusen festgestellt werden, wohingegen die Transkription von *c-Jun* mRNA in Folge der Hepatitisinduktion unverändert gegenüber den Kontrolltieren war. Interessanterweise wurde ein vollständiger Verlust der Induktion von c-Jun-Protein in Knockoutmäusen erfasst. Diese Beobachtungen deuten auf einen Einfluss von MKK7 auf die mRNA-Stabilität, Translation, Proteinstabilität oder posttranslationale Modifikationen hin. Zusammenfassend konnte gezeigt werden, dass Verlust von MKK7 in der Leber und dem hämatopoietischen System im Kontext von LPS/GalN-induzierter Hepatitis einen Einfluss auf die Expression bestimmter Gene (*c-Jun*, *Tnfa*) und Proteine (c-Jun) hat.

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1 PART 1: New Tools for Mouse Genome Engineering

1.1 Introduction

1.1.1 Mouse genome engineering

Over the last decades the mouse (*Mus musculus*) has become the premier genetic vertebrate model (Nguyen & Xu 2008; Glaser et al. 2005). Humans and mice display a high overall similarity in terms of physiology, anatomy, metabolism as well as tissue structure and organization and mice can develop diseases, that closely resemble the pathologies seen in human patients (Raymond & Soriano 2006). With the completion of the mouse and human genome sequences, the importance and power of the mouse as a genetic model for human biology became even more evident. Both genomes are highly similar and for almost every human gene a murine counterpart can be found (Gunter & Dhand 2002). Moreover, 96 % of these genes are located within syntenic regions of mouse and human chromosomes, indicating that the arrangement is evolutionary conserved. The median amino acid sequence identity of the encoded proteins of all analyzed orthologous gene pairs is 78.5 % (Boguski 2002). Thus, the mouse is a powerful tool to define gene function that underlies human physiology and pathology with respect to comparative genomics.

Historically, research was contingent on mice with naturally occurring spontaneous mutations. These mutations were identified by an altered phenotype and then the genotype was analyzed using primarily forward genetic approaches (Nguyen & Xu 2008). In contrast, reverse genetic technologies alter specific genes of the genome in order to determine their function by examining the resulting phenotypes (Figure 1). During the last years many powerful tools to genetically modify mice have been developed, some of them will be discussed in the next sections.

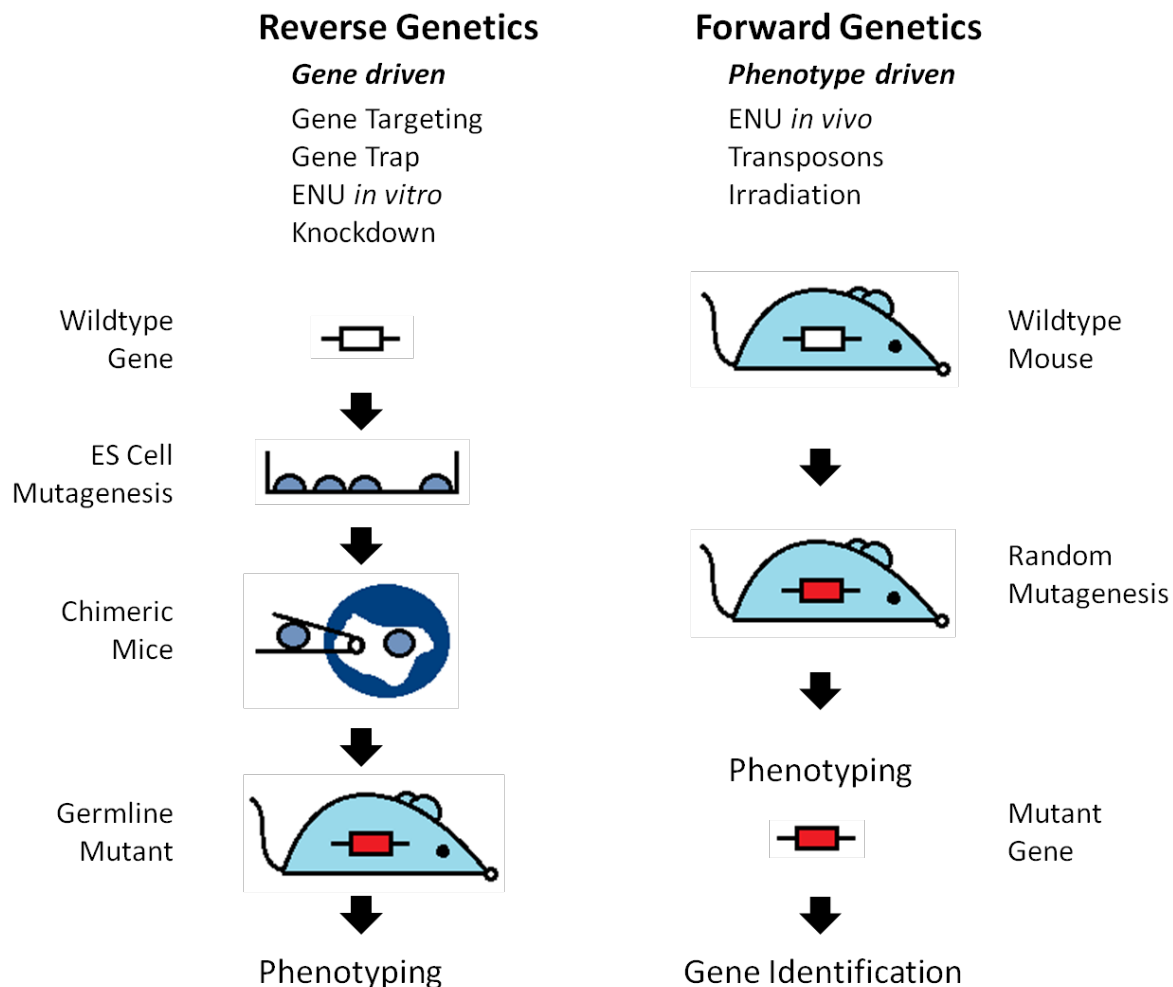


Figure 1: Comparison of reverse and forward mutagenesis strategies. For the reverse genetics approach a preselected gene is manipulated *in vitro* in embryonic stem cells (ESCs) that are used to generate a mutant mouse strain. The phenotypic characterization of the mutant should reveal the essential, nonredundant function of the targeted gene. By contrast, forward genetics randomly mutagenizes a large number of genes *in vivo*. Upon breeding of the founder generation relevant mutant offspring are identified by phenotypic screening. The altered gene must be identified by genetic mapping and sequence analysis. Figure and Figure legend adapted from (Kühn & Wurst 2009).

1.1.1.1 Tools for mouse mutagenesis

Reverse genetic methods include amongst others gene targeting, gene trapping, *in vitro* chemical mutagenesis and RNAi mediated gene knockdown (Kühn & Wurst 2009). Gene trapping vectors inactivate and report the expression of the trapped gene and provide a molecular tag for its identification (De-Zolt et al. 2009). Both, gene trapping and chemical mutagenesis can be used to hit a large number of genes *in vitro* in order to establish libraries of mutant embryonic stem cells (ESCs). These libraries can then be screened by qPCR or sequencing to identify clones that harbor mutations in a specific gene of interest (Kühn & Wurst 2009).

With gene targeting it is possible to introduce defined modifications as deletions, insertions or point mutations at specific locations in the mouse genome (Tessarollo et al. 2009). This is achieved by homologous recombination (HR) between a targeting vector and the endogenous locus. As HR of the vector occurs infrequently, there need to be ways to discriminate between HR and random integration. The most commonly used positive selection marker is a neomycin (neo) resistance gene cassette, which confers resistance to geneticin (G418). Furthermore, screening methods such as Southern blotting and long-range PCR are needed to prove the correct HR at a defined locus (Simon et al. 2009). As the construction of a gene-targeting vector by PCR and standard cloning techniques is time consuming, methods have been developed that allow a much faster assembly of targeting vectors (Kühn & Wurst 2009). One of these methods is recombineering in *Escherichia coli* (*E. coli*). This method utilizes functions encoded either by λ phage or cryptic phage Φ 12 to induce rapid and highly efficient HR (Lee et al. 2009).

There are two considerable disadvantages concerning conventional gene targeting approaches. First, a gene deletion may result in embryonic lethality. Second, by exerting its effect in multiple cell and tissue types, a gene ablation could create a complex phenotype in which it is difficult to discriminate between the direct function in a particular tissue and secondary effects resulting from altered gene function in other tissues. To avoid these problems, strategies for conditional gene deletion have been developed. These strategies make use of site-specific recombinases (SSRs), which are derived from recombination systems of yeast or bacteriophages (Wang 2009; Simon et al. 2009). SSRs bind and cleave specific short DNA sequences, exchange two involved DNA strands and rejoin the DNA strands, thereby rearranging DNA segments between two strands. Thus the SSR systems can be seen as “switches”, allowing activation or silencing of the desired gene (Birling et al. 2009). The expression of the recombinase can be achieved using viruses or by crossing the mice to a recombinase expressing transgenic mouse line. The most widely used system in mice is the Cre/loxP system (Gu et al. 1993). Another very similar system is the Flp/frt system (Dymecki 1996). Although the Flp/frt system could be used instead of the Cre/loxP system to make a conditional deletion, this is done quite rarely because the number and diversity of available Cre transgenic lines is much higher than that of Flp transgenic lines. The Flp/frt system is therefore still mainly used to remove the selection markers (Simon et al. 2009). The recently discovered Dre/rox system acts in a similar way as the aforementioned systems (Anastassiadis et al. 2009). The Φ C31 integrase mediates recombination between attP and attB sites to produce attL and attR sites, which are inert to further Φ C31-mediated recombination events (Birling et al. 2009). Thus, the Φ C31 integrase is the ideal tool for transgene insertion or cassette exchange.

Recombination can be controlled spatially and temporally by expressing the recombinase under the control of a tissue or cell type specific promoter or fused with the ligand-binding domain of either the estrogen, glucocorticoid or progesterone receptor (Wang 2009; Feil et al. 1996; Kellendonk et al. 1996). In the latter situation, the fusion protein is sequestered in the cytoplasm in absence of the adequate steroid. Upon ligand binding, it translocates to the nucleus, free to mediate recombination. In order to prevent binding and interference of endogenous steroids, mutations have been introduced in the ligand binding domains, allowing induction only by special synthetic steroids, such as tamoxifen (Birling et al. 2009). Another way to control transgene expression is the tetracycline (Tet)-inducible expression system, which exists in two basic variants, the tTA (Tet-off) system and the rtTA (Tet-on) system (Sun et al. 2007).

RNA interference (RNAi) can serve as an alternative to classical gene targeting by HR. It can be used for rapid functional genomics and also facilitates large-scale screens (Glaser et al. 2005). RNAi is a sequence-specific gene silencing process at the mRNA level. In mammalian cells, long double-stranded RNAs (dsRNA; >30 bp) lead to an interferon response that causes a global inhibition of protein synthesis and non-specific mRNA decay. Small interfering RNAs (siRNAs; <30 bp) on the other hand are able to direct the knockdown of specific mRNAs without interferon activation (Hitz et al. 2009). This mechanism can be used as an efficient method to silence gene expression in mammalian cells either transiently by transfection of siRNAs or stably by transcription of short hairpin RNAs (shRNAs) from expression vectors or retroviruses (Rubinson et al. 2003). Retroviral delivery or pronuclear DNA injection can lead to a random integration of the expression vector, which makes it impossible to control the number of integration events. However, single-copy RNAi constructs can be placed into the ROSA26 locus of ESCs (Hitz et al. 2009). By targeting of genes to the ROSA26 locus, ubiquitous expression of the transgene during development and in the adult is facilitated (Soriano 1999). The amount of shRNA transcribed from one single vector sequence integrated into the ROSA26 locus is sufficient for efficient gene knockdown (Hitz et al. 2007). The shRNA transgenic progeny can be either directly phenotyped or, in case of conditional shRNA vectors, crossed to a Cre transgenic strain (Hitz et al. 2009).

1.1.1.2 Pluripotent stem cells for mouse engineering

The basis of generating mice specifically altered in a certain locus are pluripotent stem cells. There is no uniformly accepted definition for stem cells (Potten & Loeffler 1990). One possible definition says that a cell has to comply with three requirements to be considered as a “stem cell”. First, it is not specialized for any particular function. Second, under proper conditions the cell is able to differentiate

into a more specialized cell type. And third, it can also reproduce itself indefinitely (Hemmat et al. 2010). The process of generating different daughter cells - one cell that is committed to lineage-specific differentiation and one stem cell - is called asymmetric cell division, which is considered as a hallmark of all stem cells (Knoblich 2008). Totipotent and pluripotent cells are equal in their ability to form all cell types of the embryo and adult, including germ cells, but only totipotent cells are additionally capable to give rise to extra-embryonic structures (Mummery et al. 2011b). Totipotent cells can be derived while the fertilized egg is still a zygote and during the first four blastomere cleavage phases (Hemmat et al. 2010). Pluripotent stem cells can be derived from various sources (Yu & Thomson 2008).

In the 1950s scientists began the study of teratocarcinomas, which are malignant germ cell tumors that comprise undifferentiated as well as a differentiated components including all three germ layers (Yu & Thomson 2008). As these tumors develop very rarely, real progress in this field could only be made when in 1954 Stevens and Little found a high incidence of teratocarcinomas in male 129 mice. In 1970 Kahan and Ephrussi managed to isolate stem cells from teratocarcinomas that could be stably propagated *in vitro* and during the early 1970s the first mouse embryonal carcinoma (EC) cell lines could be established and used to study development (Yu & Thomson 2008; Stevens & Little 1954; Kahan & Ephrussi 1970). These EC cells resemble undifferentiated ESCs of the mouse blastocyst stage embryo in many respects (Mummery et al. 2011a). Some EC cell lines are even capable of contributing to various somatic cell types upon injection into mouse blastocysts, but most EC cell lines have limited developmental potential and contribute only poorly to chimeric mice, which probably is due to the accumulation of genetic changes during malignant transformation (Yu & Thomson 2008).

Mouse ESCs are the most widely used source of pluripotent stem cells. *In vivo* pluripotent cells are present only transiently in the early embryo, as they quickly differentiate into various somatic cells. In 1981 scientists first managed to derive ESC lines from the inner cell mass (ICM) of mouse blastocysts (Evans & Kaufman 1981; Martin 1981). As other pluripotent cell lines, ESCs can be cultured indefinitely, differentiate into a wide variety of cell types *in vitro* and form teratocarcinomas when injected into mice. Their tremendous advantage is that they are karyotypically normal (euploid) and contribute at high frequency to all kind of tissues in chimeras, including germ cells. These characteristics make them the ideal tool for introducing modifications into the mouse germline (Yu & Thomson 2008; Mummery et al. 2011a; Evans & Kaufman 1981; Martin 1981). However, these cells have an innate tendency to differentiate into specialized cell types that compose the embryo. For the culture and experimental use of ESCs it is therefore essential to inhibit this differentiation process. This is achieved by the set-up of sophisticated culture conditions. The two most important features of the ESC culture settings are the co-culture with “feeder” cells, which are mitotically inactivated mouse

embryonic fibroblasts (MEFs) and the addition of leukemia inhibitory factor (LIF) to the media (Mummery et al. 2011a).

Another source of pluripotent stem cells are primordial germ cells (PGCs) (Lensch et al. 2006). These cells are precursors of the later germ cells and are formed even before the gonads are present. During ontogeny the PGCs migrate through the developing embryo towards the newly formed gonads. During this migration they can be isolated and cultured *in vitro* (Mummery et al. 2011a). These PGC derived cells are called embryonic germ cells (EGCs). EGCs can proliferate indefinitely and give rise to cells of all germ layers plus the germline, however there are some differences between ESCs and EGCs such as cytokine requirements (Lensch et al. 2006) and their epigenetic state (Yu & Thomson 2008). EGCs but not ESCs retain certain epigenetic features of the PGCs, such as genome-wide demethylation and erasure of genomic imprints (Labosky et al. 1994; Tada et al. 1997). There is also the possibility to isolate pluripotent stem cells from the neonatal or adult testis, so called spermatogonial stem cells (Lensch et al. 2006).

In 2006 Kazutoshi Takahashi and Shinya Yamanaka presented the possibility of generating induced pluripotent stem cells (iPSCs) from mouse embryonic and adult fibroblast cultures (Takahashi & Yamanaka 2006). Because iPSCs from adult tissues circumvent the ethical and legal issues of human embryonic stem cells, they are considered as a big hope for the development of stem cell based therapies (Kelly et al. 2011). A lot of progress has been achieved during the last years in terms of ways of reprogramming as well as characterization and potential use of iPSCs (Okita & Yamanaka 2010; Yamanaka & Blau 2010). Nevertheless I will not discuss this topic in more detail due to space limitations.

1.1.1.3 Generation of mutant mice

With today's knowledge and techniques we are able to manipulate the genome of ESCs and transfer the cells into an early embryo where the ESCs contribute to the inner cell mass and thereby give rise to a genetically modified mouse (Nguyen & Xu 2008). The most common method to produce genetically manipulated mice is the generation of chimeras by injection of the ESCs into host blastocysts or morula stage embryos (Plück & Klasen 2009a). In addition there is the possibility to produce chimeras by the generation of aggregation embryos (Plück & Klasen 2009b). Chimeras are by definition composed of host and donor cells. All methods start with the isolation of embryos from superovulated donor mice and end with the transfer of the manipulated embryos to pseudo-pregnant recipients (Wells 2002). Although chimera production in general is relatively easy and efficient,

obtaining germline transmission can still be a difficult and tedious task, where the quality of the used ESCs is of high importance (Wells 2002).

A great majority of all established mouse ESC lines is of male origin, thus these cells have 38 autosomes plus an X and a Y chromosome. In XX ESCs both X chromosomes are active, which appears to be disadvantageous for ESC propagation (Smith 2001). During the establishment of an XX ESC line one of the two X chromosomes is lost very frequently (Zvetkova et al. 2005). If male ESCs are injected into a female blastocyst they may drive the embryo to develop into a male. This process is called sex conversion and leads to a distortion of the gender distribution by producing a high proportion of male offspring (Auerbach 2009).

Using classical gene-targeting protocols, it takes about 1 year to establish a new mutant mouse line. By using the tetraploid complementation method it is possible to generate fertile adult mice, entirely derived from the donor ESCs (Wang et al. 1997). This is achieved by aggregating of ESCs with tetraploid four-cell stage embryos. While the ESCs are not or poorly able to form the extra-embryonic tissues, only the tetraploid cells are excluded from differentiating into embryonic tissues because of their aneuploidy. Thus, the embryo will be exclusively derived from the donor ESCs and the extra-embryonic tissues will develop from the tetraploid cells (Wang et al. 1997). However, as this method is very inefficient, it is still not at the stage of providing an alternative in generating genetically modified mice compared to the chimeric route (Wells 2002). The “VelociMouse” method offers also a fast and efficient way to generate mutant mice (Poueymirou et al. 2007). To generate Veloci mice, laser-assisted injection of ESCs into eight-cell stage embryos was found to efficiently yield F0 generation mice that are fully derived from the injected ESCs. This can be done with male or female, inbred or hybrid ESC lines. The generated F0 mice are healthy, exhibit highly efficient germline transmission and, most importantly, can be used directly for phenotypic analyses (Poueymirou et al. 2007).

1.1.2 Aims of the project

Breast cancer is the most common female cancer in the world, affecting about 10 % of women in industrialized countries (Szpirer & Szpirer 2007). Therefore we wanted to develop an *in vivo* breast cancer model by combining two innovative tools in order to rapidly assess the function of candidate cancer genes. Firstly, female F0 mice developing breast cancer will be generated by using female ESCs carrying an oncogene and laser-assisted 8-cell stage microinjection. In a second step, a targeting event will be performed in the ROSA26 locus of the oncogenic female ESCs to generate a switchable expression cassette, fused either to a Cre-controlled stop cassette or to the Tet-on/off system flanking shRNA sequences. Thus, different shRNAs can be inserted to knock down various candidate genes by

taking advantage of the endogenous RNAi machinery. Using these approaches, the effect of the inserted gene on breast cancer can be rapidly assayed *in vivo* (Figure 2).

To accomplish these aims, a female ESC line was established, characterized for stemness qualities and the oncogene Polyoma Middle T (under control of the MMTV promoter) was targeted to these cells. Two independent clones were injected into blastocysts in order to prove the stem cell function. In addition, to facilitate ROSA26 targeting, an exchange vector system was developed and its functionality demonstrated. The construct should allow the production of transgenic mice in an efficient, rapid and cost-effective manner. The advances achieved will significantly reduce time and costs associated with functional gene analysis in the mouse.

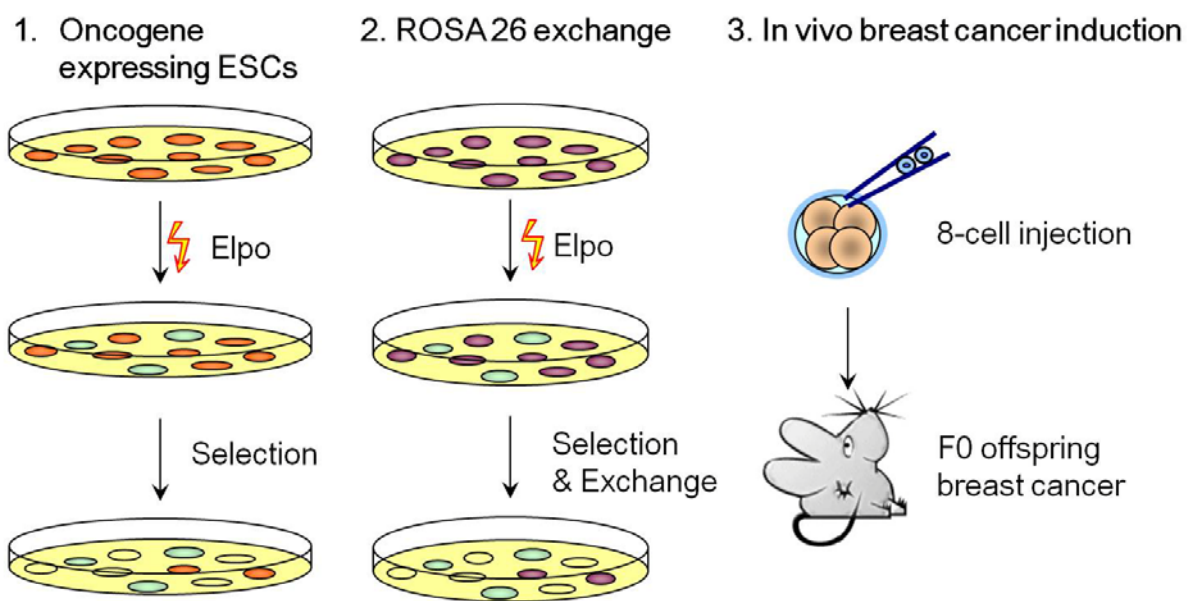


Figure 2: Development of an *in vivo* breast cancer screening system using female ESC lines. [A] Double targeted ESCs carrying an oncogene and a shRNA construct are used for 8-cell injection, which allows analysis of the F0 offspring.

1.2 Results

1.2.1 Generation of a novel ROSA26 inducible exchange vector system

The exchange vector system was designed as a tool for rapid and efficient generation of inducible transgenic mouse models by targeting the *ROSA26* locus. A detailed cloning procedure is summarized in the Materials and Methods section. The docking vector (DV; Figure 3A) contains 5' and 3' *ROSA26* homologous arms that flank a combined CMV (cytomegalovirus) and chicken β -actin promoter (CAG), a single *loxP* site, a promoterless hygromycin (*hygro*) resistance gene flanked by two *attP* sites and a reporter gene (IRES-EGFP-RpA). The exchange vector (EV) contains a promoterless β geo cassette (*lacZ* combined with a *neo* resistance gene) followed by a stop signal, a *loxP* site and the gene of interest (GOI). This cassette is flanked by two *attB* sites (Figure 3B).

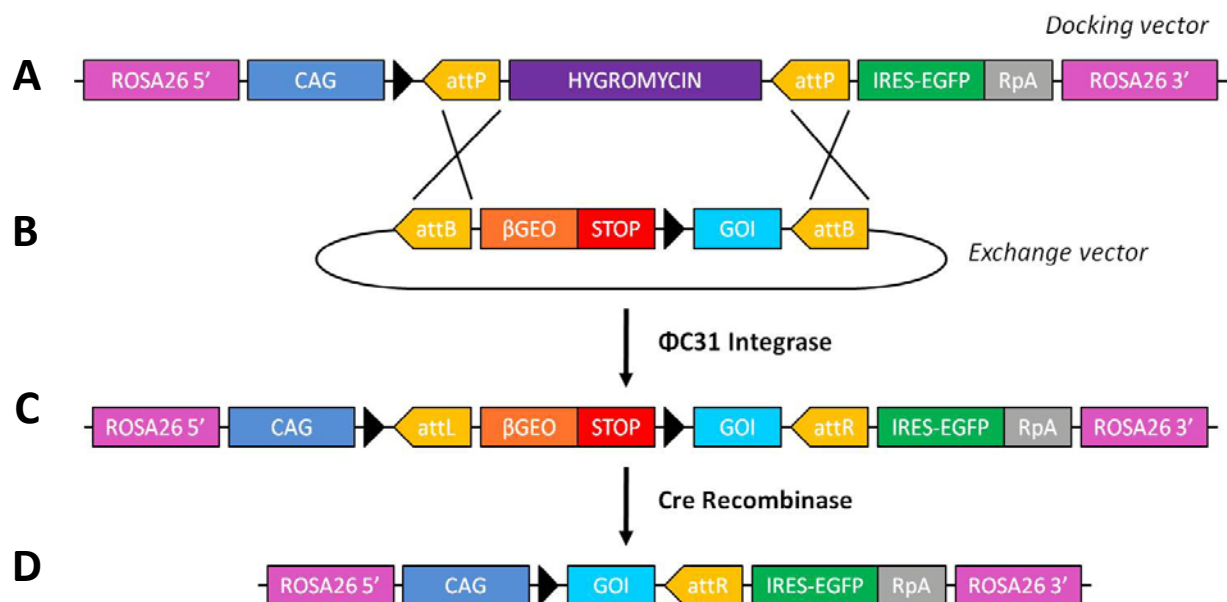


Figure 3: Schematic drawing of the docking vector (DV) **[A]** the exchange vector (EV) **[B]** as well as the targeted locus upon Φ C31 integrase mediated exchange **[C]** and Cre mediated recombination **[D]**. ROSA26 5' and ROSA26 3' are the homologous parts of the genomic *ROSA26* locus. CAG, coding sequence for the cytomegalovirus promoter fused with the chicken β -actin promoter; IRES-EGFP, internal ribosome entry site followed by the coding sequence for enhanced green fluorescent protein; β GEO-STOP, combined *lacZ* and *neo* resistance genes followed by a 3xpolyA-STOP cassette; GOI, gene of interest; RpA, rabbit polyA; black triangles, *loxP* sites; *attB* and *attP* sites, direct the recombination mediated by Φ C31 integrase; *attL* and *attR* sites remain after recombination, but are not further recognizable by Φ C31 integrase.

To introduce the DV construct into stem cells, male ESCs (clone A9) were electroporated and 33 *hygro* resistant ESC clones were picked and expanded for analysis. These clones were then screened for homologous recombination by Southern blot analysis and six correctly targeted clones (termed ROSA26-DV hereafter; 18 % efficiency) were identified (Figure 4A). One of the six ROSA26-DV cell lines was chosen and co-electroporated with the EV plasmid and the Φ C31 integrase. Upon co-transfection, an exchange between the *attB/attP* sites occurs, that could be detected via the conversion of *hygro* to *neo* resistance (Figure 4B, C). Only two *neo* resistant clones survived (ROSA26-DV-EV1 and ROSA26-DV-EV2), which could be explained by an inappropriate ratio of the EV and the Φ C31 integrase. PCR analyses of clone ROSA26-DV-EV2 confirmed the presence of the *neo* gene and the loss of the *hygro* gene (Figure 4B). By contrast, PCR analysis of ROSA26-DV-EV1 resulted in detection of both resistance genes. The reason for that could be explained by random integration of the EV or a mixed population of cells. To further determine the antibiotic resistances, both cell lines were cultured in media supplied with either G418 (300 μ g/ml) or hygromycin (150 μ g/ml), respectively and cells were counted over a period of 3 days. The results of the growth curve are shown in Figure 4C. As expected, ROSA26-DV-EV2 cells died in hygromycin containing media and survived in G418 supplemented media. Interestingly, ROSA26-DV-EV1 cells showed the same survival characteristics. Therefore one could assume that clone ROSA26-DV-EV1 also underwent correct cassette exchange. Next, ROSA26-DV-EV1 and ROSA26-DV-EV2 clones were analyzed for lacZ staining. As expected, all colonies of ROSA26-DV-EV1 as well as ROSA26-DV-EV2 turned blue upon substrate addition (Figure 4D). The final step of the validation of the exchange vector system was to test the functionality of the *loxP* sites. Upon adenoviral delivery of Cre, the *loxP* flanked stop cassette is deleted and expression of the gene of interest (GOI) and EGFP is induced. EGFP expressing cells were indeed readily detected in Adeno-Cre transfected cells (Figure 4E). Taken together, we have successfully developed a ROSA26 locus based exchange vector system that can be used to efficiently and rapidly target a gene of interest into a defined endogenous locus.

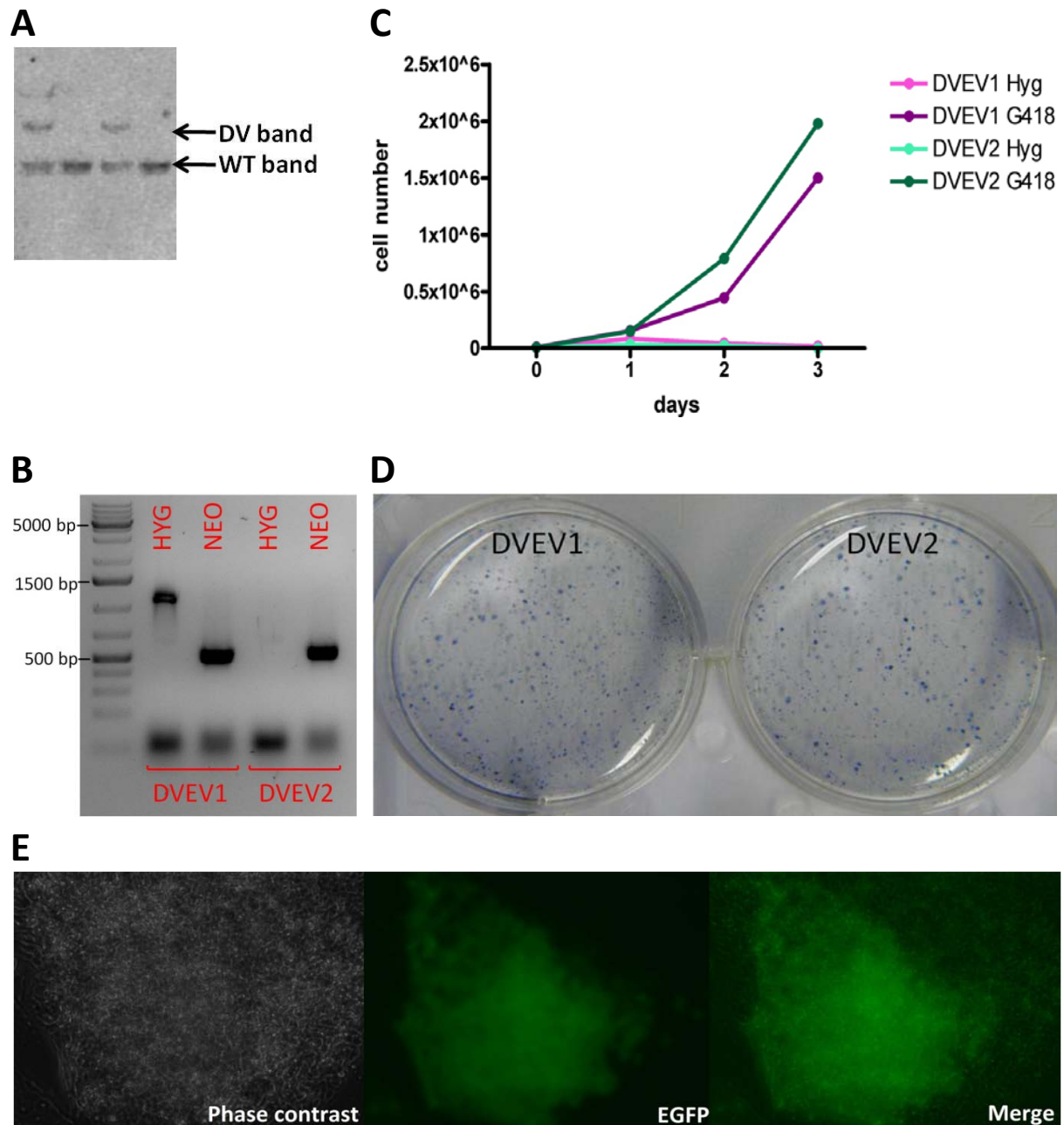


Figure 4: Generation and analysis of ROSA26-targeted ESCs. **[A]** Southern blot analysis of ROSA-DV targeted ESC clones to confirm integration of the DV construct via homologous recombination. Six clones were found to be targeted correctly (18 %). Southern blots of 2 targeted and 2 non-targeted ESC clones are shown exemplarily. **[B]** The recombination between the DV and the EV could be verified by PCR for the neomycin (*neo*) and hygromycin (*hygro*) resistance cassettes, respectively. 1kb Plus DNA Ladder (Fermentas) is shown for size comparison. *Hygro* PCR product = 1.2kb; *Neo* PCR product = 500bp. **[C]** Test of the antibiotic resistances by supplying the cells with media containing G418 or hygromycin, respectively. **[D]** Blueo-Gal staining of cultured ROSA-DV-EV cells indicates effective Φ C31 integrase mediated exchange. **[E]** Deletion of the stop cassette upon Cre-mediated recombination in ROSA-DV-EV cells. Fluorescence micrograph of EGFP-expressing cells is shown.

1.2.2 Characterization of the female A2 embryonic stem cells

The female ESC line A2 (F1 129 x BL6) has been generated by Erwin Wagner (CNIO, Spain) and Anton Wutz (Wellcome Trust Centre for Stem Cell Research, Cambridge, UK). Eleven ESC lines were generated in total, five male (A1, A7, A8, A9 and A11) and six female (A2, A3, A4, A5, A6 and A10). The male or female karyotype of the ESC lines was proven via Southern blot analysis. As shown in Figure 5A, ESC clone A1 shows the male reference bands, whereas in clones A2 and A3 the Y chromosome was absent.

Next, female XX A2 ESCs were further characterized for stem cell characteristics and pluripotency. One of the derived male ESC lines, A9, served as a reference for the stemness characterization. Macroscopically, female A2 cells form distinct colonies with well-defined borders similar to A9 cells (Figure 6A). Alkaline phosphatase (AP) staining, which is a classical stem cell marker (Wobus et al. 1984), showed blue colored colonies on a layer of unstained fibroblasts without any signs of differentiation (Figure 6B). Many genes are known to be primarily expressed in undifferentiated stem cells. Among the most important stem cell markers are *Oct3/4*, *Nanog* and *Sox2* (Imamura et al. 2006). Gene expression analysis of known stemness markers further revealed similar expression patterns between A2 and A9 ESC lines (Figure 6C).

An assay for pluripotency is the generation of embryoid bodies (EBs). Pluripotent stem cells aggregate when cultured in suspension, resembling to some extent the earliest events in embryogenesis. The inner cells of the EBs start to differentiate and are supposed to be able to form all three germ layers (Keller 1995). Importantly, A2 cells indeed formed EB aggregates in suspension culture (Figure 7A). qPCR analyses further confirmed that marker genes specific for the endodermal (*Brachyury*, *Afp2*, *Hnf*, *Foxa2*), ectodermal (*Sox2*) and mesodermal (*Sox2*, *Vimentin*, *Cdx2*) lineage are expressed in EBs generated from the female ESCs (Figure 7B). The differences in expression levels between female A2 and control male A9 ESCs are due to the fact that differentiation in EBs occurs stochastically towards one of the three germ layer lineages. These data show that the female ESC line A2 displays stem cell characteristics, comparable to the established control ESC line A9.

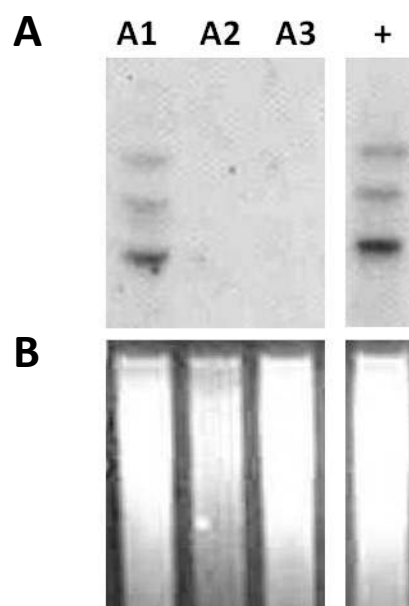


Figure 5: Sex determination of newly derived ESC lines. [A] Southern blot detection of the Y chromosome in DNA samples of different ESC clones. A Y chromosome was detected in clone A1, whereas it could not be detected in clones A2 and A3. [B] The gel picture of the same experiment is shown to prove the successful loading and digesting of genomic DNA. Genomic DNA of the tail of a male mouse was used as a positive control (+). Both figures by courtesy of Uta Moehle-Steinlein.

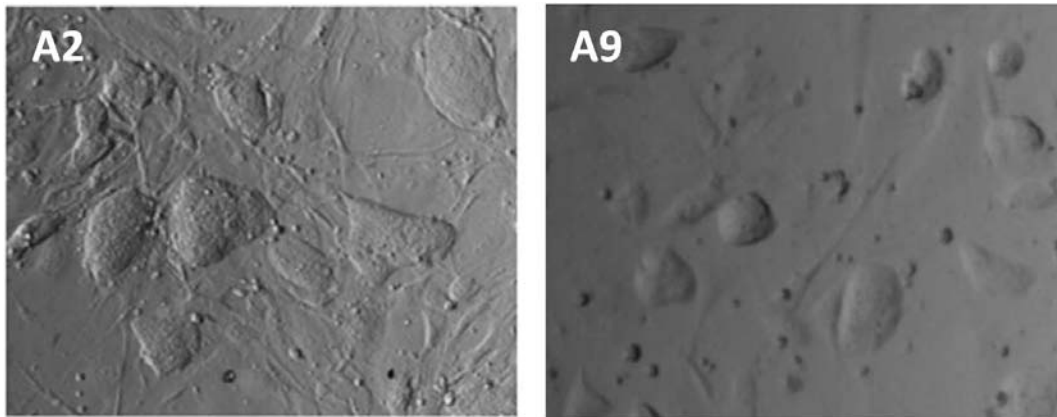
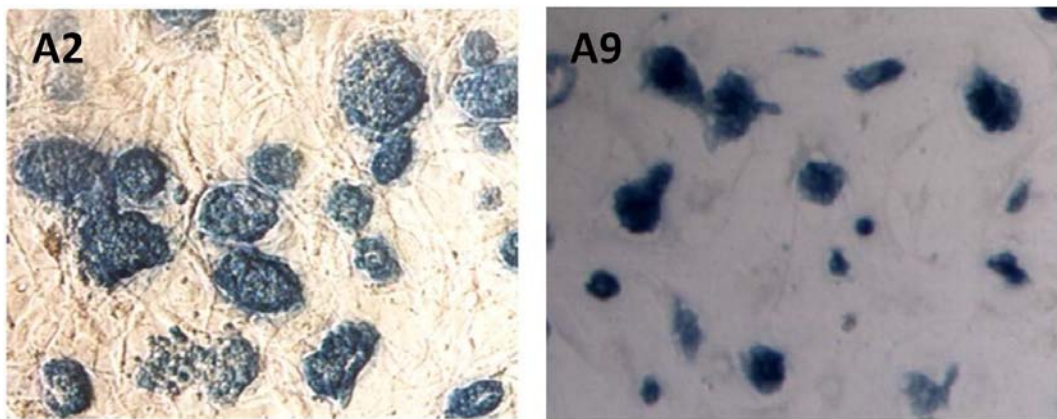
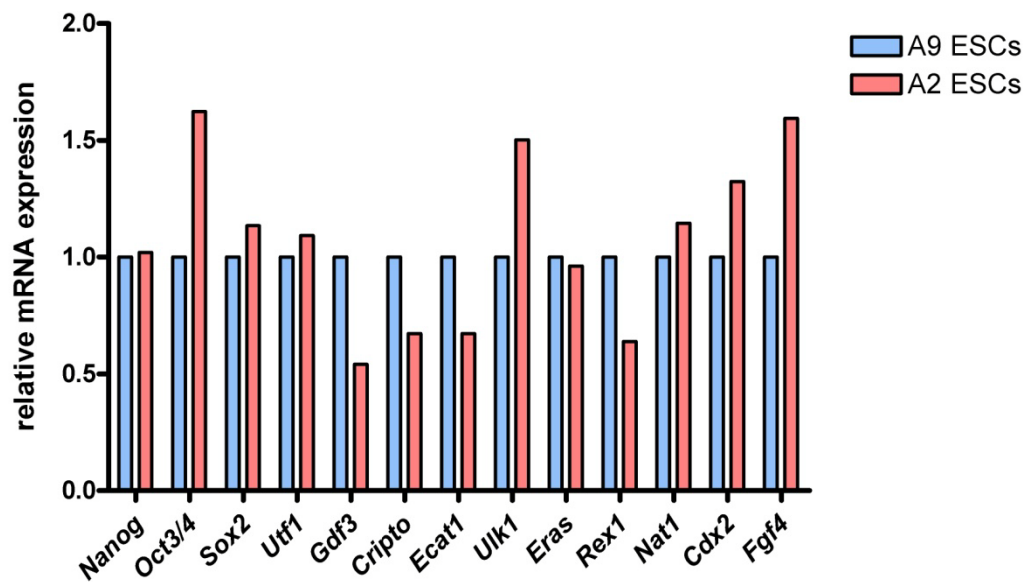
A**B****C**

Figure 6: Characterization of the female ESC line A2. [A] Phase contrast micrograph of A2 and A9 cells. A2 ESCs show a morphology typical for stem cells. [B] Alkaline phosphatase staining of A2 and A9 cells indicates the undifferentiated state of both cell lines. [C] Similar levels of expression of stem cell markers in A2 and A9 cells. Expression of the β -actin gene was used to normalize the amount of the indicated transcripts. PCR reactions were done in duplicates. Data are shown as mean values.

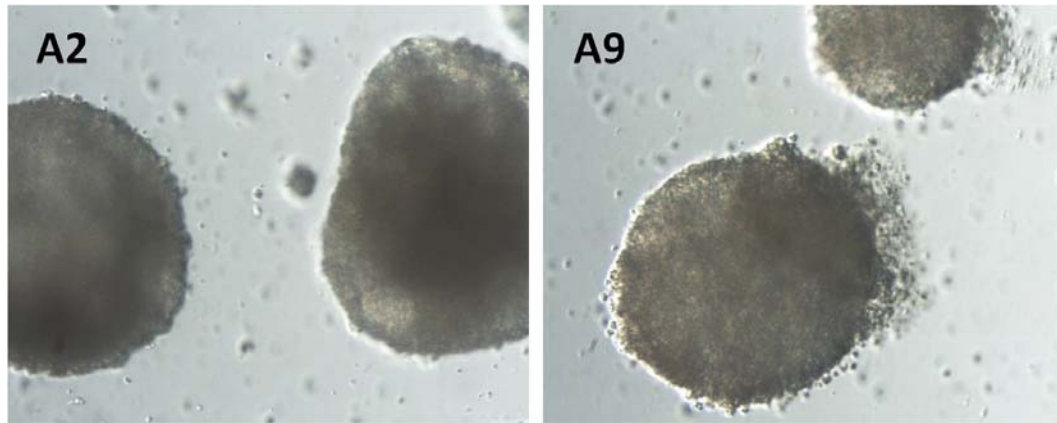
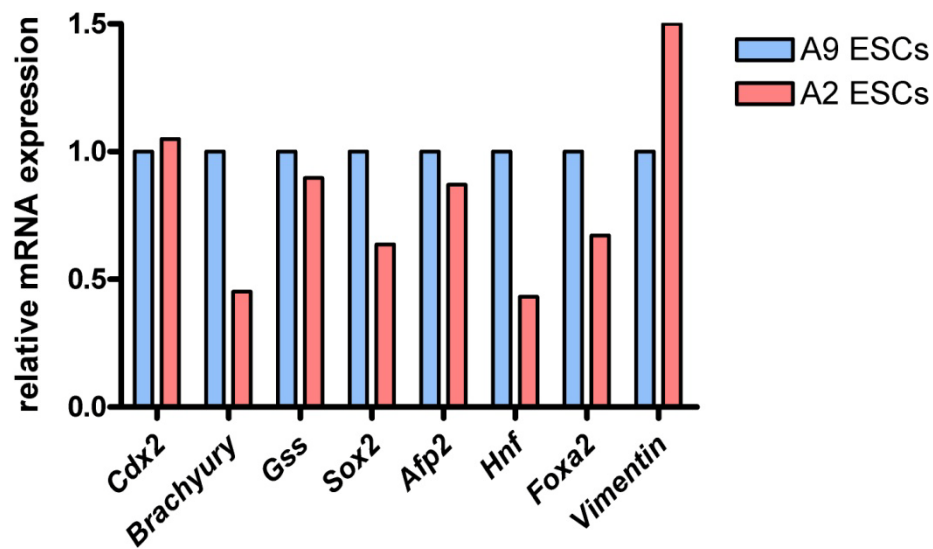
A**B**

Figure 7: Analysis of embryoid bodies (EBs) generated from female A2 ESCs. [A] Female A2 and male A9 cells aggregated to EBs when grown in suspension culture. **[B]** Gene expression analysis of EBs using qPCRs to detect genes specific for each of the three germ layers: endodermal (*Brachyury*, *Afp2*, *Hnf*, *Foxa2*), ectodermal (*Sox2*) and mesodermal (*Sox2*, *Vimentin*, *Cdx2*) lineage. Expression of the β -actin gene was used to normalize the amount of the transcripts. PCR reactions were done in duplicates. Data are shown as mean values.

1.2.3 Generation and characterization of MMTV-PyMT-IRES-CRE-puro mice using female embryonic stem cells

The most important proof of pluripotency of a cell line is to test its ability to generate chimeras that contribute to the germline. Additionally it was important to show, that A2 ESCs can be efficiently targeted. Therefore a MMTV-Polyoma Middle T (PyMT) oncogenic construct (Figure 8A) was delivered to A2 ESCs. MMTV-PyMT is known to be a potent oncogene inducing mammary tumors with a short latency and in a pregnancy-independent fashion in mice (Guy et al. 1992; Lin et al. 2003). Of note, the original construct was modified to a bicistronic construct where the oncogene PyMT and the IRES-CRE are under the control of the MMTV promoter (donation by William Muller). In addition, a PGK (phosphoglycerate kinase) promoter driven puromycin resistance selection cassette that can be excised by the Fip recombinase was added to generate the final vector MMTV-PyMT-IRES-CRE-puro (Figure 8A).

MMTV-PyMT-IRES-CRE-puro was delivered to A2 ESCs by electroporation and ESCs were selected for puromycin resistance. Transgenic clones were identified by PCR and two positive low-copy number clones (A2/22 and A2/23) were selected and injected into C57BL/6 blastocysts to generate chimeras. Chimerism was assessed by coat color (Figure 8B). No live-born mice were derived from clone A2/23, whereas clone A2/22 yielded high percentages of chimeric mice (Figure 8C). The contribution of transgenic ESCs to the chimeras ranged from 10 to 100 %. High-percentage female chimeras were backcrossed to C57BL/6 males for germline transmission. The F1 generation was assayed by PCR for the presence of the transgenes *PyMT* and *Cre* (Figure 8D). The PCR results showed that germline transmission of the MMTV-PyMT-IRES-CRE-puro was successful. Moreover, the transgene was inherited in a Mendelian fashion.

Although PyMT is known to be a potent oncogene (Lin et al. 2003) no tumor development could be detected in viable female F1 transgenic mice up to the age of 9 months. Additionally, RNA of various tissues from 9-months old MMTV-PyMT-IRES-CRE-puro transgenic female mice was prepared and PCR for *PyMT* expression was performed (Figure 9). Variable expression within the transgenic lines was observed ranging from strong expression in all tissues examined (mouse 1) to no detectable expression (mouse 3). In summary, we could demonstrate that the female ESC line A2 can be genetically manipulated and that it has the capability to contribute to the germline of chimeric mice. This opens new possibilities for usage of female ESCs for further genomic engineering approaches.

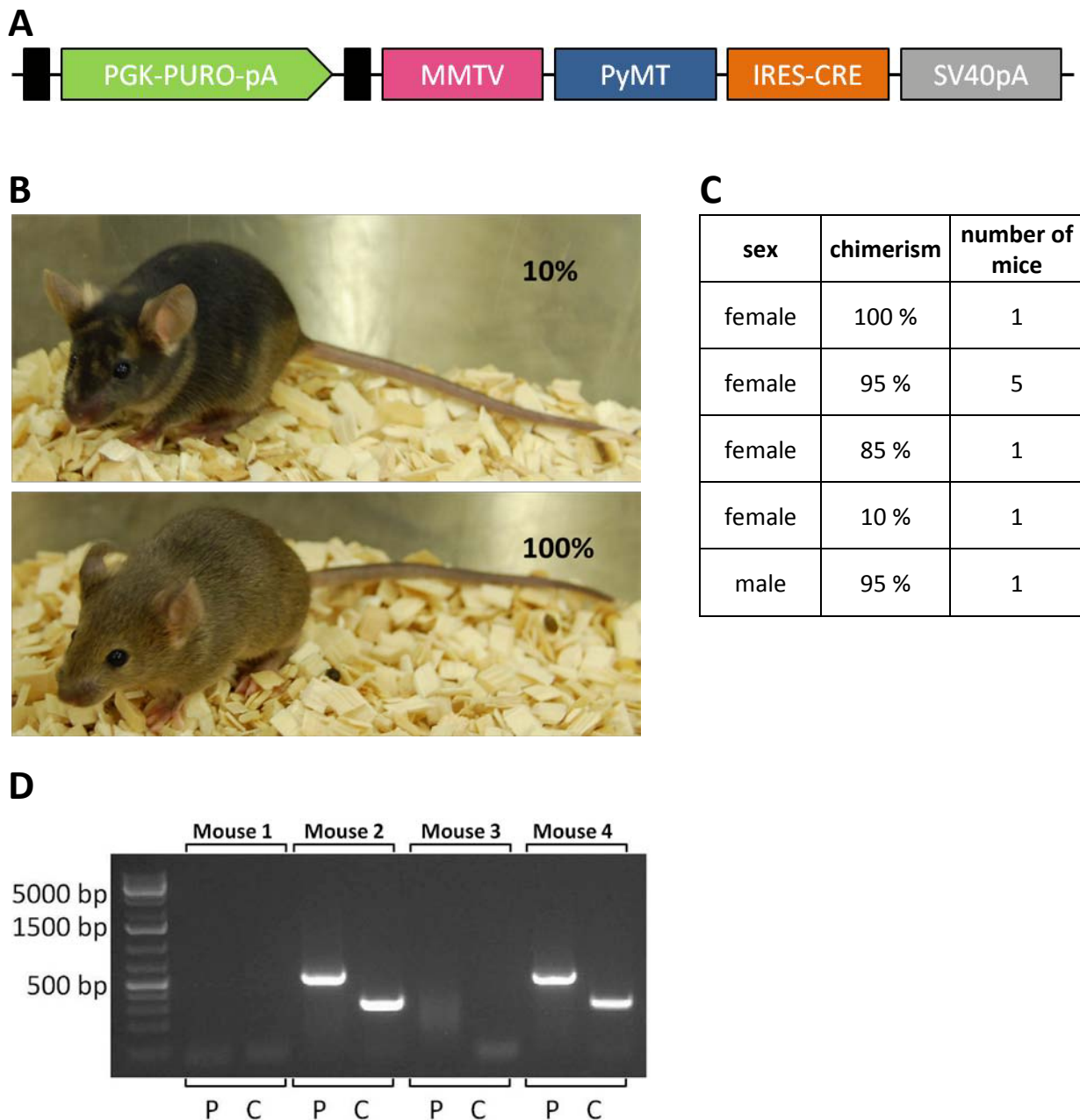


Figure 8: Generation of MMTV-PyMT transgenic mice from female A2 ESCs. **[A]** Schematic drawing of the PyMT transgene construct. PGK-PURO-pA encodes a phosphoglycerate kinase promoter (PGK), a puromycin resistance gene (PURO) and a terminal polyadenylation signal (pA). This cassette is flanked by FRT sites, which are recognition sites for the Flp recombinase. The remainder of the polycistronic plasmid codes for the mouse mammary tumor virus promoter (MMTV), the Polyoma Middle T oncogene (PyMT), the Cre recombinase with an internal ribosome entry site (IRES) and a SV40-virus polyadenylation signal. Black boxes, Frt sites. **[B]** Successful generation of chimeras as indicated by coat color. The contribution of transgenic ESCs to the mouse in the upper panel is estimated to be 10 %, whereas the mouse shown in the lower panel exhibits nearly 100 % chimerism. **[C]** A2 ESCs contributed to chimerism to different extents. **[D]** Verification of the presence of the transgene in the F1 generation by PCR detection of *PyMT* (P) and *Cre* (C) genes. 1kb Plus DNA Ladder (Fermentas). PyMT PCR product = 550 bp; Cre PCR product = 300 bp;

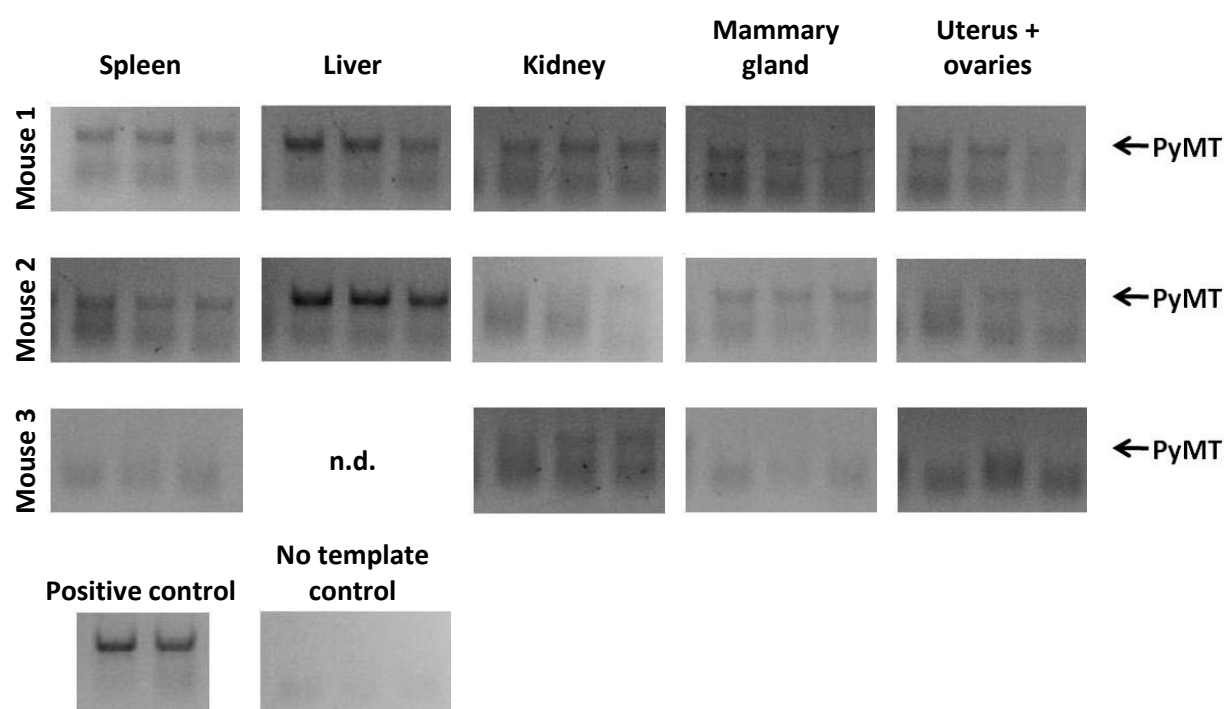


Figure 9: PCR analysis of MMTV-PyMT-IRES-cre-puro transgenic female mice. *PyMT* expression was detected in different tissues. PCR was performed on whole-organ lysates. A mosaic expression of the transgene was observed. PCR reactions were done in triplicates. n.d. = not determined. Plasmid DNA containing the MMTV-PyMT-IRES-cre-puro construct was used as a positive control. *PyMT* PCR product = 550 bp;

1.3 Discussion

The aim of this part of the thesis was to develop a novel *in vivo* breast cancer model using female ESC lines. A so called exchange vector system for rapid and efficient exchange of transgenes or shRNAs in mouse ESCs was generated. This system should allow for rapid generation of mice bearing a transgene in their ROSA26 locus. Upon generation of a cell line carrying a docking vector (DV) in the ROSA26 locus, such cells can be further targeted with different exchange vectors (EV), delivering the actual gene of interest. Ideally, the gene of interest can be inserted into the EV plasmid by one single cloning step. Upon electroporation of the ROSA26-DV cell line with an EV construct and the Φ C31 integrase, the gene of interest is inserted into the ROSA26 locus. Furthermore, the functionality of all parts of the exchange vector system could be proven: the EV was integrated by Φ C31 integrase mediated cassette exchange, which altered the antibiotic resistance of the cell line as expected; the activity of the β -galactosidase was demonstrated and the stop cassette was shown not to be leaky, as the reporter gene located downstream was not expressed. Moreover, the β GEO-stop cassette was removed by an adenoviral approach which could be demonstrated by the expression of the reporter gene EGFP. To generate mice with a whole-body knock-in of the transgene, the stop cassette can be removed by *in vitro* Cre recombination. In addition, the cells can be used directly for generation of tissue specific or inducible knock-in of the gene of interest, followed by crossing the generated mice to Cre-transgenic lines. In terms of *in vitro* functionality, the exchange vector system is ready to be used for generating mice. The ability to generate germline competent chimeras has yet to be tested in order to exclude detrimental effects of the multiple manipulation steps.

The stem cell characteristics of the female ESC line A2 were determined by different approaches such as alkaline phosphatase staining, gene expression analysis and EB formation. However, the most important prove of the stemness was the successful generation of chimeric mice. A2 cells were randomly targeted with an expression vector delivering the oncogenic construct MMTV-PyMT-IRES-CRE-puro and female chimeric mice were successfully derived. The chimerism ranged between 10 and 95 %. Moreover, the transgene could be passed on the germline of the offspring. Although PyMT is known to act as a potent oncogene leading to early onset mammary hyperplasia and malignant progression associated with pulmonary metastases (Lin et al. 2003), no aberrant growth of mammary gland tissue could be detected in our mice so far. The site of integration as well as the gene dosage could account for this missing oncogenic potential. We detected expression of PyMT in various MMTV driven tissues, however, the gene dosage might be too low to lead to oncogenic transformation. The transgenic ESC clone used for blastocyst injection was chosen because of its low copy number,

whereas in the original publication the transgene was delivered to the embryo by pronuclear microinjection, thereby potentially integrating multiple copies into the genome (Guy et al. 1992). Indeed, in the publication by Guy et al. low amounts of middle T antigen RNA were mentioned not to be associated with apparent growth disturbance. Moreover, the transgene was expressed in a mosaic pattern. Mosaicism for MMTV-controlled transgenes has been reported previously (Wagner et al. 2001). In the future, different transgenic founder lines have to be analyzed in more detail to identify mouse strains that can be used as breast cancer models. It might be also necessary to generate new chimeras by using an ESC clone with a higher copy number of the transgene to increase the gene dosage of PyMT. Nevertheless, the stem cell characteristics of the female ESC line A2 could be successfully proven and this cell line can now be used for further experiments. In the future, these ESCs will be delivered to 8-cell stage mouse embryos by laser-assisted injection (Poueymirou et al. 2007) as this method yields F0 mice that are fully derived from the ESCs and can therefore be used directly for phenotyping, thereby greatly accelerating research.

The ultimate ambition of these projects is to establish a novel tool for breast cancer research in mice. In contrast to some cancers, e.g. colon cancer, breast cancer comprises a spectrum of diseases that are genetically poorly defined. Various mouse models that harbor mutations in various signaling molecules and pathways that are implicated in different stages of tumor development have been generated to gain knowledge about the signaling events responsible for this disease (Watson 2002). Most mouse models of breast cancer take advantage of the MMTV promoter, yet other promoters are used as well (Taneja et al. 2009). Mouse strains used for breast cancer research are mostly designed to exhibit loss or gain of function mutations or amplifications of genes that have been found to be important in human breast cancers, as for example members of the EGFR family, cyclin D1 or Ras (Taneja et al. 2009). However, to further define the complex interactions of multiple genes in the process of oncogenesis, transgenic mice which harbor different genetic alterations have to be crossed with each other (Green et al. 2002). Our exchange vector system should facilitate this task. Double transgenic mice that express the randomly integrated PyMT construct as well as different shRNAs from the ROSA26 locus will be generated. The shRNAs will be designed to interfere with downstream targets of PyMT as well as with other molecules that possibly interact with PyMT driven malignant transformation. Comparison of the PyMT-only mice with PyMT-shRNA mice will help to understand the relevant signaling pathways. Furthermore, other oncogenes can be studied by this method as well. Taken together, two valuable tools for genetic research could be established. The combination of the exchange vector system with transgenic mice will open up many possibilities to greatly expand our understanding of the processes underlying the initiation, promotion and progression of breast cancer.

2 PART 2: Evaluation of the Role of MKK7 in Hepatitis

2.1 Introduction

The liver is the largest metabolic organ of the human body and performs crucial functions within the carbohydrate, protein and lipid metabolism (Faller & Schünke 2008). Moreover, it is important for detoxification of endogenous and exogenous substances (Faller & Schünke 2008). The 4 most common types of liver disease are cirrhosis, fatty liver, hepatitis and liver cancer (Sun & Karin 2008). Hepatitis and liver cancer are among the most serious public health problems worldwide. Hepatitis stands for “inflammation of the liver”, which can be caused by viral infections, toxins, alcohol and autoimmune syndromes (Sun & Karin 2008). In order to develop and improve therapies of these diseases, it is of great importance to understand the underlying pathogenic mechanisms at a molecular level.

2.1.1 Mouse models of acute liver injury

Over the last 40 years a lot of knowledge has been gained concerning the understanding and treatment of acute hepatic failure (AHF). This progress is largely due to the use of different animal models that reflect the clinical, biochemical and histological pattern of the syndrome seen in man (Rahman & Hodgson 2000). The most common species used for hepatitis research are pig, dog, rabbit, rat and mouse (Tuñón et al. 2009), but each model often only reflects a particular aspect of AHF (Rahman & Hodgson 2000). Small animals as mouse and rat offer ways to study the molecular events underlying AHF but for the development of artificial liver support systems larger animals as pig and dog are being used (Tuñón et al. 2009).

In 1991 Terblanche and Hickman suggested 6 main criteria for a suitable AHF animal model (Terblanche & Hickman 1991; Tuñón et al. 2009; Rahman & Hodgson 2000):

- Reversibility – suitable treatment may reverse and improve survival
- Reproducibility – reproducible end-points are required to standardize the model
- Death from liver failure – should reflect the human clinical pattern and death should be a direct result of the insult to the liver
- Therapeutic window – time for treatment should be available between insult and death to be assessed for its effect
- Adequate animal size – size large enough to allow blood and tissue analysis to take place serially
- Minimum hazard to personnel – minimum risk for operators and associated staff

2.1.1.1 A surgical model – Partial hepatectomy

The liver has the unique ability to repair itself after suffering loss of tissue mass from surgical resection or pathogenic and toxic factors (Fausto 2000). This regeneration is achieved by the re-entry of quiescent hepatocytes into the cell cycle (Mitchell et al. 2005). In response to liver injury, hepatocytes begin to grow to compensate for the lost cells and stop growing when the liver mass reaches within 10 % of the original liver mass (H. Cao et al. 2009). Partial hepatectomy (PH) provides a unique tool to study the regulation of proliferation and organ regeneration *in vivo* (Mitchell & Willenbring 2008). Two-thirds partial hepatectomy (70%) is the most studied model of liver regeneration in mice (Cao et al. 2009). Mice are able to regenerate their original liver mass within 2 weeks following 65-70 % PH (Cao et al. 2009). 90 % PH is considered to be the upper limit for a liver regeneration model in mice, as larger resections produce lethal hepatic failure (Tuñón et al. 2009). In humans liver regeneration can be also observed in split-liver transplantation, where the size of the donor's liver doubles within 7 days, and the recipient's between 7-14 days after surgery (Cao et al. 2009). The main disadvantages of this surgical method are the poor reproducibility and the reliance on surgical expertise. Another drawback is the inability of the model to recreate the inflammatory milieu that exists in acute liver failure (Newsome et al. 2000).

2.1.1.2 Chemical models

Galactosamine

D-galactosamine (GalN) is a selective hepatotoxin which, when metabolized via the galactose pathway in the liver, causes serious metabolic alterations and hepatic necrosis (Tuñón et al. 2009; Newsome et al. 2000). GalN blocks the transcriptional activity of nuclear factor- κ B (NF- κ B) (Ni et al. 2008). NF- κ B is activated by tumor necrosis factor α (TNF α) and acts as a suppressor of the apoptotic effects of TNF α (Kucharczak et al. 2003). GalN is commonly used in murine models of acute liver damage in combination with Concanavalin A or lipopolysaccharide (LPS) (Ni et al. 2008).

Concanavalin A

An initial event in the pathophysiology of a variety of liver diseases is the activation of T lymphocytes, which in turn interact with macrophages, leading to a release of proinflammatory cytokines such as TNF α and interferon gamma (IFN γ) (Künstle et al. 1999). Upon treatment with the T-cell mitogen Concanavalin A (ConA), TNF α and other hepatotoxic cytokines are secreted by T lymphocytes. ConA alone causes a mild liver injury that is followed by regeneration (Ni et al. 2008). Hepatocyte death is evident with the increased liver enzymes in the blood (Ni et al. 2008). The combination of ConA with

GalN leads to a more severe and irreversible liver damage with high mortality. In this model, a potent activation of apoptosis with significantly increased caspase activities is observed (Ni et al. 2008).

Lipopolysaccharide (LPS)

Another way of inducing TNF α is by treatment with LPS which stimulates resident macrophages in the liver, the Kupffer's cells (Chensue et al. 1991). Administration of LPS to rodents leads to endotoxic shock (Morrison & Ryan 1987). To provoke LPS-induced liver damage without affecting other organs, LPS is injected at subtoxic amounts into GalN-sensitized animals (Mizuhara et al. 1994). Combined treatment with LPS and GalN leads to significant apoptosis of hepatocytes and irreversible liver damage with a high mortality (Ni et al. 2008).

Acetaminophen

The commonly used drug acetaminophen (paracetamol) is known to be able to produce hepatic damage. Acetaminophen overdoses cause a large proportion of cases of AHF in Western countries, whereas the main causes of AHF in developing countries are viral illnesses, particularly infections with hepatitis B and E viruses (Lee 2008). The toxic effects of acetaminophen are dose-dependent (Newsome et al. 2000) and can be increased by fasting, cytochrome P-450 inducing drugs and alcohol consumption (Whitcomb & Block 1994). The hepatic detoxifying metabolism of acetaminophen is related to species and age, therefore different studies on this drug produce greatly heterogeneous results (Gregus et al. 1988). The major disadvantages of acetaminophen models are poor reproducibility and the varying time-lag between damage induction and death of the research animals (Newsome et al. 2000).

Carbon tetrachloride

Carbon tetrachloride (CCl₄) is a hepatotoxin that causes acute liver injury (Tuñón et al. 2009). It has also been widely used to induce chronic liver damage by repeated administration of low doses. Injection of CCl₄ can serve as a model for hepatic cirrhosis, liver fibrosis and cancer (Tuñón et al. 2009; Weiler-Normann et al. 2007). In the liver, CCl₃ radicals are formed by cytochrome P450 isozymes (Wong et al. 1998) which then induce hepatocyte damage and activation of hepatic stellate cells, leading to the production of extracellular matrix (Weiler-Normann et al. 2007).

2.1.1.3 Viral models

Although viral hepatitis is one of the main causes of AHF (Bernuau et al. 1986), the use of infective agents to develop animal models of AHF has been rather unsuccessful until now. There have been

studies with transgenic mice overexpressing virus B hepatitis proteins and with mice infected with murine hepatitis virus strain 3 (MHV-3), which shed some light on virus induced AHF mechanisms (Tuñón et al. 2009). As human HBV and HCV only infect humans and chimpanzees (Chayama et al. 2011), the analysis of both viruses has long been hampered by the absence of a small animal model. Transgenic mice that express HBV-DNA and export virus particles into the serum have been used to study antiviral agents. But as the viral life cycle is not established in this model, it is inappropriate to investigate the whole spectrum of HBV infections and the related hepatitis (Chayama et al. 2011). In recent years progress has been made by the development of human hepatocyte chimeric mice that are susceptible to HBV and HCV infections (Tsuge et al. 2005; Hiraga et al. 2007).

2.1.2 TNF-induced signaling in hepatocytes

The main mediators of liver inflammation are cytokines such as TNF α , interleukin-1 β (IL-1 β) or IFN γ , as well as oxidative stress radicals and bile acids (Jaeschke et al. 2002). Exposure to these agents will eventually cause hepatocyte cell death, which can contribute to acute liver injuries. On the other hand insufficient apoptosis of hepatocytes and unrestrained proliferation within the context of chronic inflammation can promote the development of liver cancer (Pikarsky et al. 2004). One of the earliest events in hepatic inflammation is the induction of TNF α (Mizuhara et al. 1994), which then triggers a cascade of other cytokines that cooperate in recruiting inflammatory cells (Ramadori et al. 2008), killing hepatocytes and initiating a wound-healing response (Bradley 2008; Wullaert et al. 2007). Although TNF α is involved in the pathology of various inflammatory liver diseases, it was also shown that TNF is necessary for liver regeneration, for instance following partial hepatectomy (Akerman et al. 1992). Thus, the proinflammatory cytokine TNF α acts as a key regulator of the crucial balance between hepatocyte proliferation and hepatocyte cell death (Wullaert et al. 2007).

TNF α can be bound by two plasma membrane receptors, TNF-receptor 1 (TNF-R1) and TNF-R2, where TNF-R1 is the main receptor for soluble TNF and TNF-R2 generally mediates the effects of the membrane-bound TNF precursor (Maeda et al. 2003). The majority of biological effects in the liver is mediated by TNF-R1 but it was also shown, that TNF-R2 can play a crucial role in certain conditions (Küsters et al. 1997). Pathways that can be induced by TNF-R1 include the activation of the transcription factor nuclear factor- κ B (NF- κ B) (Cao et al. 1999), initiation of MAPK cascades as well as cell death (Micheau & Tschopp 2003). This broad range of different signaling pathways initiated by TNF-R1 is accomplished by the recruitment of various adapter proteins to its cytoplasmic part (Liu et al. 1996; Wullaert et al. 2007). TNF α signaling starts with the rapid formation of complex I which consists of TNF-R-associated death domain (TRADD), TNF-R-associated factor 2 (TRAF2) and receptor

interacting protein 1 (RIP1) (Chang et al. 2006). This complex then further recruits TGF β -activated kinase 1 (TAK1) which then activates NF- κ B signaling. NF- κ B target genes are often associated with cell survival and proliferation (Hayden et al. 2006). As TAK1 is a member of the MAP3K family it also activates p38 and JNK cascades (Inokuchi et al. 2010). Subsequently by dissociation of complex I from the receptor and recruitment of Fas-associated death domain (FADD) and pro-caspase-8, signaling complex II is formed (Micheau & Tschopp 2003). The clustering of pro-caspase-8 allows its autoactivation and the thereby activated caspase-8 is then able to proteolytically activate several effector caspases (Juo et al. 1998; Yang et al. 1998). However, if NF- κ B has been activated during complex I formation, it prevents caspase-8 activation by complex II through induction of c-FLIP. c-FLIP is an adapter protein with a pseudo-caspase domain, which specifically inhibits caspase-8 (Chang et al. 2006). In cells that lack substantial NF- κ B activity, formation of complex II results in caspase-8 activation and ultimately leads to apoptosis (Chang et al. 2006). In the case of hepatocytes, however, a second pathway is required for TNF-induced apoptosis, which involves an abrupt increase of the permeability of the inner mitochondrial membrane and release of cytochrome c (Bradham et al. 1998).

2.1.2.1 MAP kinase signaling cascades

Cells have to be able to respond to extracellular stimuli and environmental changes in order to survive and perform their functions. This response is often mediated by the activation of transcription factors, which then induce the appropriate cellular process (Keshet & Seger 2010). As most extracellular agents cannot cross the plasma membrane, intracellular signaling pathways transmit the signals to various cytoplasmic and nuclear targets. Such pathways often act by sequential phosphorylation events called protein kinase cascades. This type of signaling mechanism is used by the mitogen-activated protein kinase (MAPK) signaling cascades, which will be described in the following sections (Keshet & Seger 2010).

MAPK signal transduction pathways are highly conserved from yeast to humans (Avruch 2007) and are among the most widely used activation pathways of eukaryotic cell regulation (Kyriakis & Avruch 2001). Different MAPK pathways are utilized by all eukaryotic cells to respond coordinately to multiple and divergent inputs. In mammals MAPKs can be activated by a broad spectrum of different stimuli, which include hormones, growth factors, inflammatory cytokines and environmental stresses such as radiation, osmotic shock or ischemic injury (Krishna & Narang 2008). Cells detect those signals through different receptor families that are coupled to MAPK pathways, such as receptor tyrosine kinases (RTKs) or G protein-coupled receptors (GPCRs) (Goldsmith & Dhanasekaran 2007; McKay & Morrison 2007). Many cellular activities can be regulated by those pathways, for example gene expression, cell

cycle progression and cell growth, cellular metabolism, motility or apoptosis (Johnson & Lapadat 2002). Today about 70 genes are known that encode for almost 200 distinct components that compose the MAPK system. This multiplicity is needed to facilitate the specificity and tight regulation, which are hallmarks of these cascades (Keshet & Seger 2010).

The most typical feature of the architecture of all MAPK pathways is the central three-tiered kinase cascade. This cascade consists of three sequentially acting kinases: a MAPK, a MAPK kinase (MAPKK, MAP2K, MKK) and a MAPK kinase kinase (MAPKKK, MAP3K, MEKK) (Ferrell 1996). MAP3Ks are Ser/Thr kinases which are activated by upstream Ras and Rho family GTPases in response to an extracellular stimulus (Kyriakis & Avruch 2001). MAP3Ks in turn phosphorylate and thereby activate the downstream MAP2Ks. These kinases are dual specificity kinases, which means that they can phosphorylate MAPKs on threonine and tyrosine on a conserved Thr-X-Tyr motif in order to activate them (Dhanasekaran & Premkumar 1998). Activated MAPKs are then able to phosphorylate their target substrates on serine or threonine residues only if those are followed by proline. Their substrates can be transcription factors, other kinases (MAPK activated kinases, MKs) or proteins like cytoskeletal proteins (Krishna & Narang 2008). Important mammalian MAPK cascades are extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase 1–3 (JNK1–3), p38MAPK α , β , γ , δ (p38 α – δ) and ERK5 (Qi & Elion 2005). Each of these cascades can regulate distinct but sometimes overlapping cellular processes. Misregulation of this highly sophisticated network often leads to diseases such as cancer, diabetes or inflammation (Keshet & Seger 2010).

2.1.2.2 MKK7

MKK4 and MKK7 are MAPK kinases that are crucial for the transduction of signals to the downstream stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) as they are the only known molecules that directly activate JNK (Tournier et al. 1997; Nishina et al. 1997). Both, MKK4 and MKK7 are activated in response to a variety of cellular stresses, including UV and γ -irradiation, hyperosmolarity, T cell receptor stimulation or inflammatory cytokines (Yao et al. 1997). The activity of MKK4 and MKK7 is increased following phosphorylation at serine and threonine residues within a Ser-X-Ala-Lys-Thr motif in their activation loops by multiple MAPK kinase kinases, including MEKK1–4, members of the mixed lineage kinase (MLK) family, apoptosis signal-regulated kinase 1 (ASK1) and TGF β -activated kinase 1 (TAK1) (Wang et al. 2007). Activated MKK4 and MKK7 in turn activate JNK by dual phosphorylation of its Thr-Pro-Tyr motif. Although both MAPK kinases are dual specificity kinases, MKK4 preferentially phosphorylates the tyrosine residue, whereas MKK7 phosphorylates the threonine residue within the Thr-Pro-Tyr motif (Fleming et al. 2000). *In vitro* and *in vivo* studies support the model of a synergistic activation of JNK by both MKK4 and MKK7 (Asaoka & Nishina 2010).

The murine gene encoding MKK7 encodes 14 exons that can be alternatively spliced to generate different isoforms (Tournier et al. 1999). There are three possible N-termini (α , β and γ isoforms) as well as two different C-termini (1 and 2 isoforms). The physiological relevance of different MKK7 isoforms remains to be proven and elucidated (Asaoka & Nishina 2010). In the adult mouse MKK4 and MKK7 are ubiquitously expressed in all organs, however there are notable differences in the pattern of expression during embryogenesis between MKK4 and MKK7, indicating their distinct functions in development (Wang et al. 2007). *Mkk7*^{+/-} mice appear normal and fertile without any obvious defects. *Mkk7*^{-/-} embryos die between E11.5 and E13.5 of severe anemia associated with impaired hepatogenesis (Wada et al. 2004). The same is seen for *mkk4*^{-/-} fetuses which additionally show severe hemorrhage in the region of the liver at E12.5 associated with increased apoptosis (Nishina et al. 1999; Wang et al. 2007). Mouse embryonic fibroblasts (MEFs) derived from *mkk7*^{-/-} embryos show decreased proliferation from passage 4-5 onwards plus premature senescence. JNK activation is impaired at the basal level as well as upon stimulation and overexpression of the second JNK activating kinase MKK4 cannot substitute for the loss of MKK7 (Wada et al. 2004). Moreover, the activation of JNK in response to proinflammatory stimuli as TNF α and IL-1 is almost completely lost in *mkk7*^{-/-} cells but only reduced by about 50 % in *mkk4*^{-/-} cells (Tournier et al. 2001). These data indicate that while MKK4 is required for optimal JNK activation, MKK7 is essential for the stimulation of JNK activity by proinflammatory cytokines (Tournier et al. 2001).

2.1.2.3 JNK

Two major MAPK pathways are the c-Jun NH₂-terminal kinase (JNK) and the p38 MAPK pathways, which are both termed stress-activated protein kinase (SAPK) pathways as they are activated by various genotoxic and environmental stresses (Hibi et al. 1993; Han et al. 1994; Wagner & Nebreda 2009). These pathways have important functions in inflammation and tissue homeostasis by controlling cell proliferation, differentiation and survival of specific cell types (Kyriakis & Avruch 2001). Three genes are encoding JNK proteins, *mapk8* encodes JNK1, *mapk9* encodes JNK2 and *mapk10* encodes JNK3. All genes are alternatively spliced giving rise to at least ten isoforms (Gupta et al. 1996). JNK signaling is regulated by recognition of conserved motifs between MKKKs and MKKs as well as between MKKs and MAPKs and by scaffold proteins that assemble the MKKK-MKK-MAPK module in larger protein complexes (Mooney & Whitmarsh 2004). At least 20 MAPK kinase kinases are known of which at least 14 activate the MKK4/MKK7-JNK axis, demonstrating the importance of JNK pathways in the cellular response to external stimuli (Johnson & Nakamura 2007).

Depending on stimulus and cell type, JNKs can phosphorylate a number of activator protein-1 (AP-1) components, including c-Jun, JunB, JunD and activating transcription factor 2 (ATF2) (Bode & Dong 2007), thereby regulating various target genes that contain AP-1 binding sites, including genes that control cell cycle, survival, apoptosis, metalloproteinases and nuclear hormone receptors (Wagner & Nebreda 2009). In addition to transcription factors JNKs can also phosphorylate non-transcription factors such as Bcl-2, Bcl-xL, paxillin and MAP2 (Nishina et al. 2004). Apart from their kinase activity JNK proteins also function to target some of their own substrates for degradation. For instance, c-Jun is marked for ubiquitinylation and subsequent degradation when associated with inactive JNK (Bode & Dong 2007). Depending on stimulus and the strength and duration of JNK activation, the cellular response has diverse outcomes, which range from induction of apoptosis to increased survival (Wagner & Nebreda 2009). For instance, whereas the early transient phase of JNK activation (<1 h) corresponds to cell survival, the later and more sustained phase of JNK activation (1-6 h) mediates TNF α -dependent apoptosis (Ventura et al. 2006).

2.1.2.4 AP-1 transcriptional complexes

The AP-1 transcription factors are dimeric complexes that are composed of members of the Jun, Fos, ATF and MAF (musculoaponeurotic fibrosarcoma) protein families (Curran & Franza 1988; Kataoka et al. 1994). AP-1 complexes can be formed by many different combinations of hetero- and homodimers, and these combinations determine the transcriptional targets that are regulated (Hai & Curran 1991). c-Jun appears to be especially important for hepatocytes, although it is expressed in a variety of different tissues (Eferl et al. 2003). For instance, it has been shown that in chimeric mice generated from c-Jun deficient ESCs, ESC derivatives can be found in all organs but were absent among hepatocytes (Eferl et al. 2003). The function of c-Jun in hepatocytes seems to depend on their differentiation stage, as fetal hepatocytes require c-Jun for cell survival, whereas differentiated hepatocytes rather require it for cell cycle progression (Eferl et al. 2003). The importance of c-Jun in the liver is also demonstrated by the fact that it is strongly expressed in human livers with severe acute hepatitis whereas livers without signs of inflammation express low levels of c-Jun (Hasselblatt et al. 2007). c-Jun expression in inflamed livers is predominately localized to hepatocytes rather than inflammatory cells. Furthermore, it was shown that liver-specific *c-Jun* deletion leads to impaired liver regeneration following PH (Behrens et al. 2002). Thus, c-Jun is a key regulator of hepatocyte proliferation (Stepniak et al. 2006).

2.1.3 Aims of the project

Over the last years and decades, multiple studies have addressed the role of TNF-MKK7-JNK pathway in liver inflammation (Figure 10). In 2003 Maeda et al. stated that ConA-induced liver damage is considerably reduced in *Jnk1*^{-/-} as well as in *Jnk2*^{-/-} single knockout mice (Maeda et al. 2003). However in a publication from 2009 by the group of Roger J. Davis this could not be confirmed. They detected similar hepatic damage in wild-type as in *Jnk1* or *Jnk2* single knockout mice. Moreover, they showed that Mx 1-Cre⁺ *Jnk1*^{fl/fl} *Jnk2*^{-/-} mice exhibit a strong protection against ConA induced hepatitis. In addition they could find the same protection using the LPS/GalN hepatitis model. This protection against hepatitis was found to be associated with reduced mortality, reduced serum transaminase activity as well as decreased expression of AP1-related genes and *Tnfα* mRNA in the liver and decreased amounts of serum TNFα (Das et al. 2009). The key conclusion of this publication was that JNK is not required for TNFα-mediated death of hepatocytes but JNK is essential for the expression of TNFα by hematopoietic cells that promote hepatitis (Das et al. 2009). In 2007 Hasselblatt et al. published that mice lacking c-Jun in hepatocytes display increased liver cell death and mortality upon ConA injection. This phenotype was caused by the impaired expression of nitric oxide synthase 2 (Nos2) and a thereby reduced production of nitric oxide (NO), a hepatoprotective agent (Hasselblatt et al. 2007). The aim of my project was to analyze the potential function of MKK7 in immune cells or in liver cells using the LPS/GalN acute hepatitis model (Figure 10).

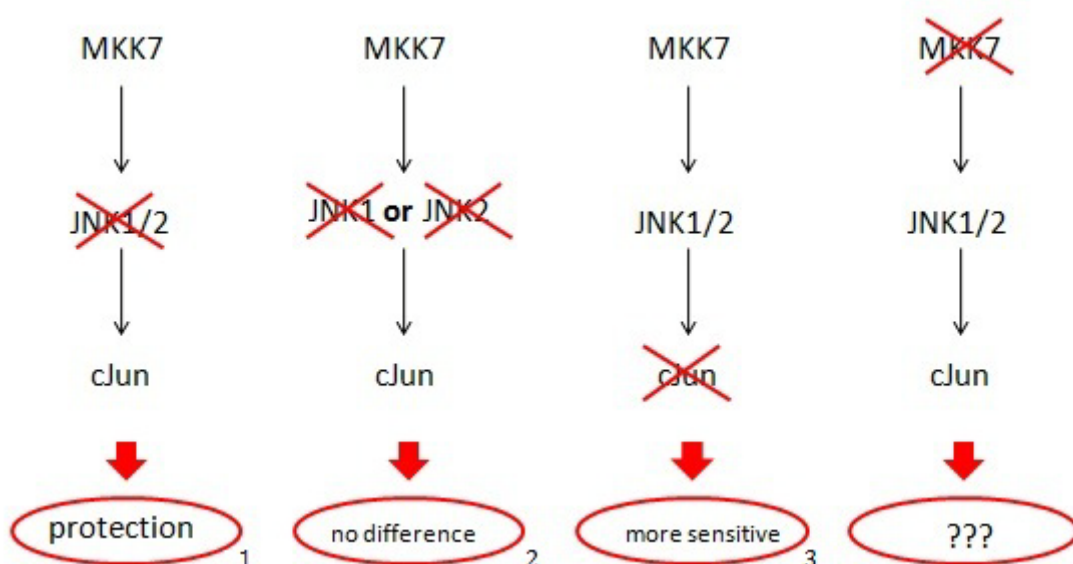


Figure 10: Different components of the MKK7-JNK pathway have been examined in the context of liver injury. The Figure gives an overview of the different outcomes in liver injury models when one or another player of the same signaling cascade is genetically inactivated. 1,2 (Das et al. 2009); 3 (Hasselblatt et al. 2007)

2.2 Results

2.2.1 Liver and hematopoietic specific deletion of MKK7

To test the role of MKK7 in the LPS/GalN liver injury model, we crossed MKK7 fl/fl mice to either Alfp-Cre or Mx1-Cre transgenic mice. To achieve a functional knockout of MKK7, exons 3 to 11 are deleted upon Cre recombination (Schramek et al. 2011). In the Alfp-Cre construct the Cre recombinase is under the control of both the mouse albumin regulatory elements (promoter and enhancer) and the α -fetoprotein (AFP) enhancers, thus mimicking the genomic organization of the mouse albumin gene (Kellendonk et al. 2000). In mice and other mammals the albumin gene is located upstream of the AFP gene and its expression is thought to be influenced by the AFP enhancers located between both genes (Kellendonk et al. 2000). In mice the expression of the albumin gene starts during embryogenesis shortly after the appearance of the liver bud (E9.5) (Kellendonk et al. 2000).

Loss of MKK7 protein was determined by Western blot analysis in liver lysates of Alfp-Cre MKK7 Δ/Δ mice (termed *MKK7 Δ^{Alfp}* hereafter), Alfp-Cre MKK7 $\Delta/+$ mice (termed *MKK7 $\Delta/+^{Alfp}$* hereafter) and control MKK7fl/fl mice (termed *MKK7^{Ctrl}* hereafter) (Figure 11). Of note, MKK7 protein was still detectable at low levels in *MKK7 Δ^{Alfp}* livers. This is licit as the Alfp-Cre transgene is expressed only in hepatocytes and biliary duct cells but not in nonepithelial cells of the liver, such as endothelial and Kupffer cells (Kellendonk et al. 2000).

In Mx1-Cre mice the expression of the Cre recombinase transgene is controlled by the promoter of the mouse Mx1 gene (Kühn et al. 1995). Mx1 is part of the defense to viral infections and remains silent in healthy mice. Upon application of interferon α (IFN- α) or IFN- β or of

synthetic double-stranded RNA (polyinosinic-polycytidylic acid – poly(I:C)) the promoter can be transiently activated to high amounts of transcription in various tissues (Kühn et al. 1995). Mx1-Cre deletes, amongst others in the liver and the hematopoietic system (Kühn et al. 1995). Western blot confirmed no detectable MKK7 expression in Mx1-Cre MKK7 Δ/Δ (termed *MKK7 Δ^{Mx1}* hereafter) liver samples following poly(I:C) injections (3 injections, 13 mg/kg) (Figure 11).

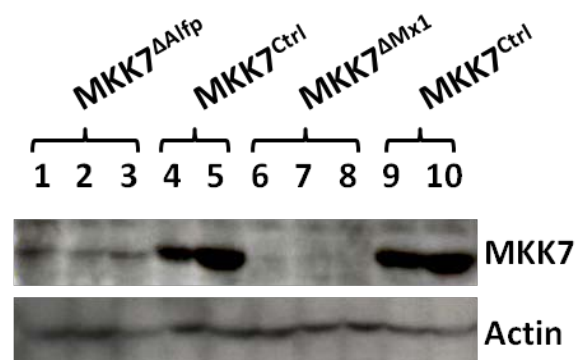


Figure 11: Deletion of MKK7 in the liver. Deletion efficiency in *MKK7 Δ^{Alfp}* and *MKK7 Δ^{Mx1}* mice was detected by Western blot analysis of whole liver lysates. *MKK7 Δ^{Alfp}* mice (lanes 1-3), *MKK7 Δ^{Mx1}* mice (lanes 6-8) and *MKK7^{Ctrl}* mice (lanes 4, 5, 9, 10). MKK7 protein is strongly reduced in *MKK7 Δ^{Alfp}* livers and not detectable in *MKK7 Δ^{Mx1}* livers.

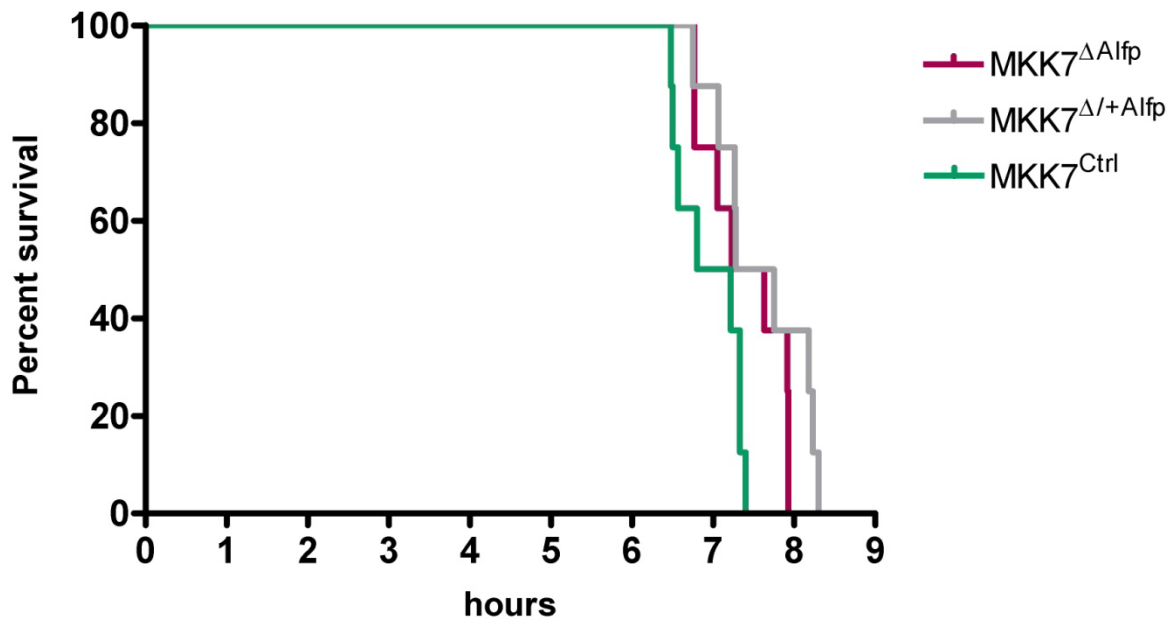
2.2.2 Hepatocyte-specific deletion of MKK7 does not protect against LPS/GalN induced hepatitis

Injection of LPS/GalN induces severe apoptosis of hepatocytes and irreversible liver damage with high mortality (Ni et al. 2008). At a lethal dose of LPS/GalN (10 µg/kg LPS, 1 g/kg GalN) mice die within 6 to 8 hours after injection. In Figure 12A the survival curves of LPS/GalN treated mice of different genotypes are shown. We classified end points according to the following symptoms: ruffled fur, partly-closed eyes, no spontaneous movements, no erecting after being put on the side. When these symptoms were observed, mice were euthanized for ethical reasons. Overall, the survival curves (Figure 12A) indicate that all genotypes are equally susceptible to LPS/GalN induced liver injury.

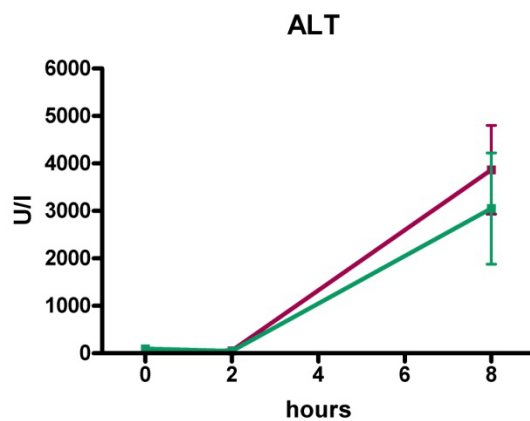
Liver injury can be detected by measurement of serum ALT (alanine aminotransferase) activity levels. A similar, but less specific marker is AST (aspartate aminotransferase) in the serum (Ozer et al. 2008). Both serum transaminases play important roles in amino acid metabolism and gluconeogenesis (Ozer et al. 2008). ALT is primarily expressed in the liver and to a lower extent in skeletal muscle and heart, whereas AST is expressed in heart, brain, skeletal muscle and liver. Damaged cells release their contents into the extracellular space where they ultimately enter blood circulation (Ozer et al. 2008).

No increase in ALT or AST levels was detectable two hours, whereas a strong induction of ALT and AST occurred 8 hours after LPS/GalN injection (Figures 12B and 12C). The induction of AST levels was found to be similar in *MKK7^{Ctrl}* and *MKK7^{ΔAlfp}* animals. Taken together, the levels of ALT and AST in the serum upon LPS/GalN treatment were found to be equal in *MKK7^{ΔAlfp}* and *MKK7^{Ctrl}* mice, indicating a similar liver injury.

A



B



— MKK7^{Ctrl}
— MKK7 Δ Alfp

C

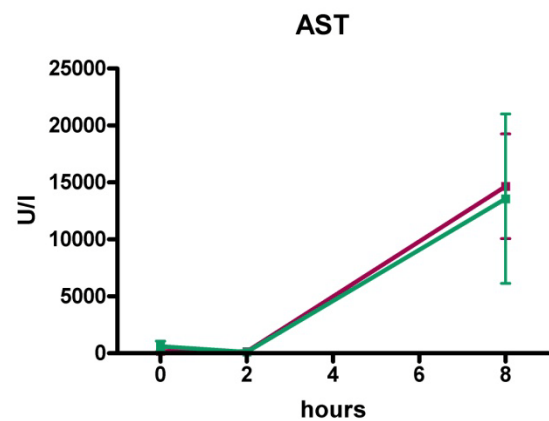


Figure 12: Survival curves and ALT/AST levels upon LPS/GalN treatment in *MKK7 Δ Alfp* mice. [A] Survival curves of Alfp-Cre *MKK7 Δ/Δ* mice (*MKK7 Δ Alfp*), Alfp-Cre *MKK7 $\Delta/+$* mice (*MKK7 $\Delta/+$ Alfp*) and control *MKK7^{fl/fl}* mice (*MKK7^{Ctrl}*) upon treatment with LPS/GalN (10 μ g/kg; 1g/kg). *n* = 8. To detect liver injury, serum ALT [B] and serum AST [C] activities were determined. Mice were sacrificed after 2 and 8 hours after LPS/GalN challenge. The baseline (time 0) was set by analysis of PBS-treated mice. PBS *MKK7^{Ctrl}* *n* = 4; PBS *MKK7 Δ Alfp* *n* = 4; LPS 2 hours *MKK7^{Ctrl}* *n* = 7; LPS 2 hours *MKK7 Δ Alfp* *n* = 6; LPS 8 hours *MKK7^{Ctrl}* *n* = 8; LPS 8 hours *MKK7 Δ Alfp* *n* = 8; Data are shown as mean values $\pm$ SEM; T-test: not significant. U/l = units per liter. SEM = standard error of the mean.

We next monitored liver damage of LPS/GalN treated mice histologically. PBS-treated mice showed normal, healthy liver architecture (Figure 13, upper panels), whereas in LPS/GalN treated animals massive bleeding into the parenchyma was observed throughout the whole organ (Figure 13, lower panels). The degree of tissue damage was found to be similar between $MKK7^{Ctrl}$ and $MKK7^{\Delta Alfp}$ mice. To specifically determine the apoptosis rate, TUNEL stainings of liver sections were performed (Figure 14A). For the quantification of apoptotic cells a computed algorithm was established with support of the BioOptics department at IMBA/IMP. In brief, this program is able to count and distinguish between blue dots (DAPI-stained nuclei; one dot is equal to one cell) and green dots (one dot is equal to one apoptotic cell stained by the TUNEL reaction). The quotient of apoptotic cells to all cells indicates the percentage of apoptosis. As shown in Figure 14B apoptosis upon LPS/GalN treatment was similar in the livers of $MKK7^{\Delta/+Alfp}$ and $MKK7^{\Delta Alfp}$ mice. Thus, treatment with LPS/GalN showed similar hepatic damage, as measured by survival, serum transaminase levels, histological analyses and apoptotic rates, suggesting that hepatocyte specific MKK7 deficiency does not confer protection in this liver injury model.

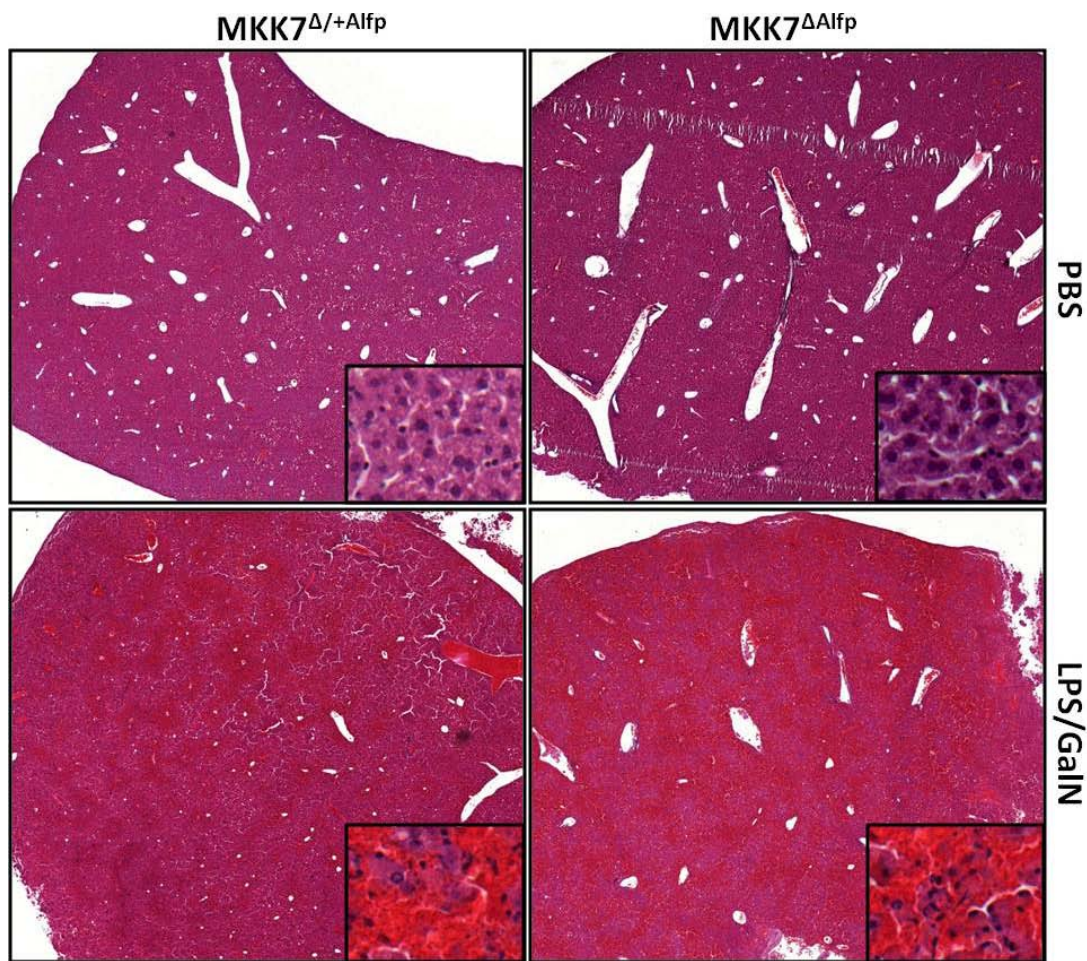


Figure 13: Histological analysis of livers upon LPS/GalN treatment. Representative H&E stainings of liver sections of PBS or LPS/GalN (10μg/kg; 1g/kg) treated $MKK7^{\Delta/+Alfp}$ and $MKK7^{\Delta Alfp}$ mice are shown. Massive bleeding was seen in LPS/GalN treated livers of $MKK7^{\Delta/+Alfp}$ and $MKK7^{\Delta Alfp}$ mice (see inserts). Samples were taken 8 hours after injections.

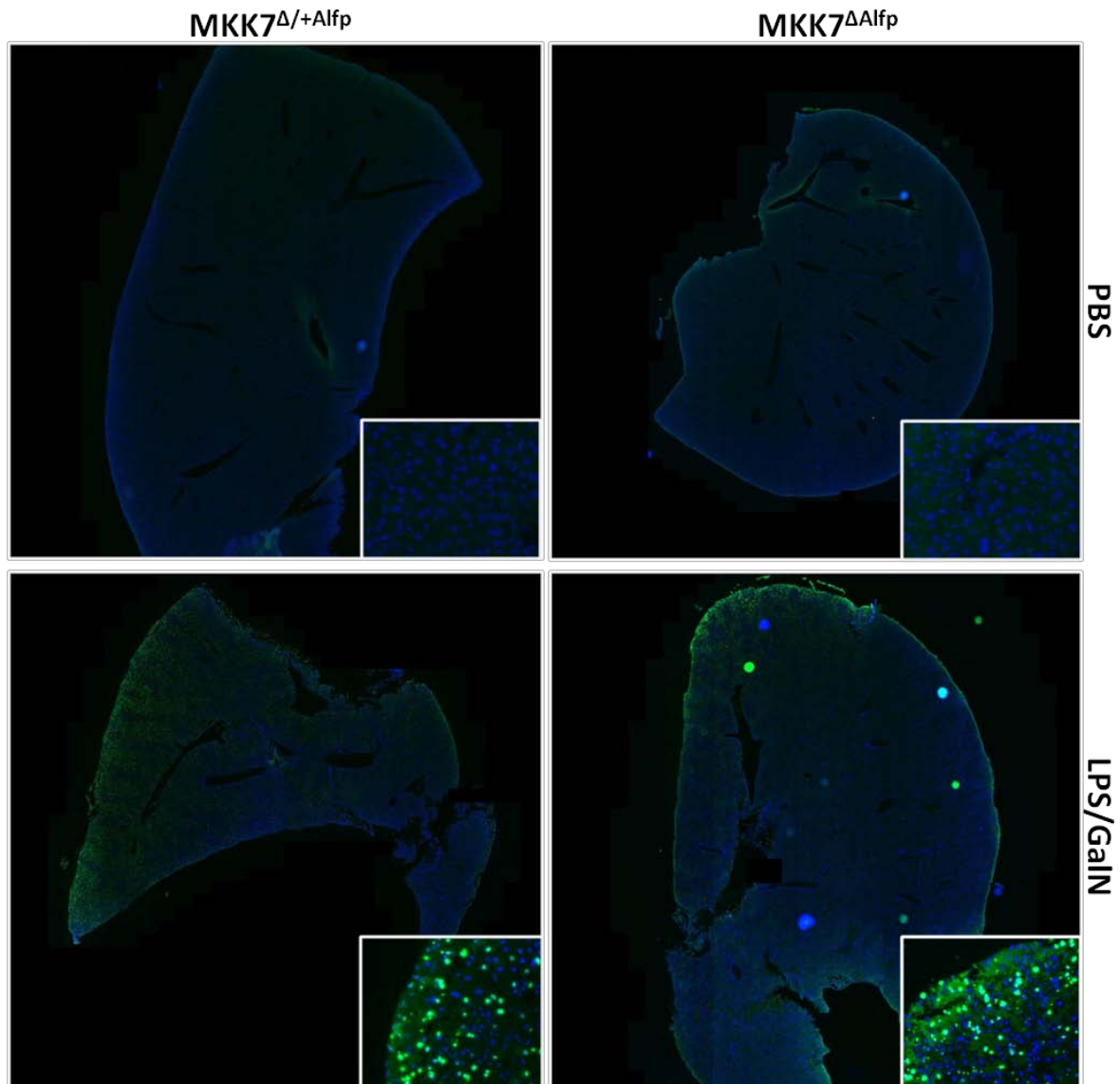
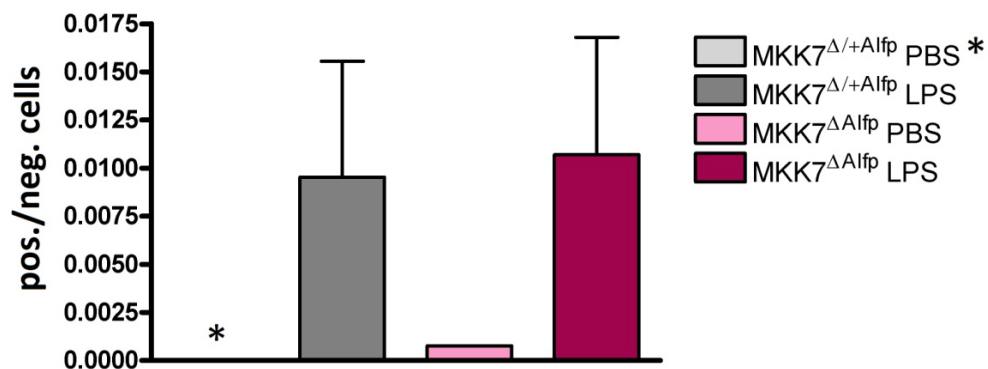
A**B**

Figure 14: Evaluation of apoptosis by TUNEL staining. [A] TUNEL stainings of liver sections to determine the extent of apoptosis in mice that received PBS and mice challenged with LPS/GalN. Apoptotic cells are stained green, all nuclei are stained blue (DAPI). **[B]** Quantification of apoptotic cells: Average proportions of apoptotic cells in 3 liver sections of 4 *MKK7 $\Delta/+Alfp$* livers and 4 *MKK7 $\Delta Alfp$* livers were calculated. Total cell numbers were determined by DAPI staining. Data are shown as mean ratios $\pm$ SEM of apoptotic (green) cells divided by non-apoptotic (blue) cells. PBS n = 1; LPS n = 4. Samples were taken 8 hours after PBS respectively LPS/GalN injections (10 μ g/kg; 1g/kg). SEM = standard error of the mean.

2.2.3 Gene expression and cytokine analysis in *MKK7^{ΔAlfp}* livers

To elucidate expression of signaling molecules in the liver upon LPS/GalN challenge, qPCR analyses were performed 2 and 8 hours after LPS/GalN injection. In *MKK7^{Ctrl}* mice expression of *Mkk7* was down-regulated 2 hours after the injection and was slightly increased at 8 hours (Figure 15). By contrast, *Gadd45β*, a specific inhibitor of MKK7 (Papa et al. 2004), showed the opposite expression pattern, as *Gadd45β* mRNA was strongly induced after 2 hours and down-regulated to basal levels after 8 hours. Importantly, there was no difference in *Gadd45β* induction and expression between *MKK7^{Ctrl}* and *MKK7^{ΔAlfp}* liver samples.

The MKK7-JNK stress pathway regulates gene transcription through phosphorylation of multiple transcription factors, including c-Jun (Davis 2000). The basal expression of *c-Jun* and *JunB*, but not *JunD* mRNA was increased in *MKK7^{Ctrl}* livers compared to *MKK7^{ΔAlfp}* livers, albeit these differences were statistically not significant. All three AP-1 members were down-regulated 2 hours upon LPS/GalN treatment in the livers of *MKK7^{Ctrl}* mice, whereas mRNA levels remained unchanged within the first two hours in *MKK7^{ΔAlfp}* livers. Two hours after LPS/GalN challenge the level of *JunD* mRNA was significantly higher in *MKK7^{ΔAlfp}* samples than in control samples (p-value 0.036). Between 2 and 8 hours *c-Jun* and *JunB* mRNA levels increased in livers of both genotypes. The expression level of *JunD* mRNA after 8 hours was elevated compared to the 2-hours time point only in *MKK7^{Ctrl}* samples. The amount of *JunD* mRNA in *MKK7^{ΔAlfp}* livers remained unchanged throughout all examined time points.

As *Tnfa* expression is critical for the development of liver injury in the LPS/GalN model (Wullaert et al. 2007) and is furthermore induced, amongst other activation pathways, by the MKK7-MAPK cascade (Tournier et al. 2001), *Tnfa* expression levels were determined. *Tnfa* mRNA was found to be induced in the liver 2 hours after treatment. *Tnfa* expression levels seemed to be higher in *MKK7^{ΔAlfp}* mice, however this difference was not significant. In *MKK7^{Ctrl}* as well as in *MKK7^{ΔAlfp}* mice the expression of *Tnfa* was reduced below basal levels 8 hours after treatment (Figure 15). By contrast, on the protein level a mild induction of TNFα was seen only in *MKK7^{Ctrl}* mice 2 hours following LPS/GalN treatment (p = 0.057). 8 hours after LPS/GalN challenge TNFα protein levels were similar between *MKK7^{Ctrl}* and *MKK7^{ΔAlfp}* mice (Figure 16A). The cytokine interleukin-6 (IL-6) also plays an important role in the regulation of the acute-phase response to injury and infection by activating, amongst others, MAPK cascades (Heinrich et al. 2003). IL-6 protein levels were increased 2 hours after injections and decreased 8 hours after LPS/GalN challenge in *MKK7^{Ctrl}* and in *MKK7^{ΔAlfp}* mice (Figure 16A). Finally, the level of phosphorylated JNK2 (p-JNK2) in liver homogenates was determined 2 hours after induction of hepatic damage (Figure 16B). p-JNK2 levels were found to be equal between *MKK7^{Ctrl}* and *MKK7^{ΔAlfp}* mice and no induction could be detected at this time point. Taken together, the expression and induction of *Gadd45β* and the acute phase cytokines TNFα and IL-6 was found to be similar between

MKK7^{Ctrl} and *MKK7^{ΔAlfp}* mice. Expression of *Mkk7*, *c-Jun*, *JunB* and *JunD* mRNA was downregulated 2 hours upon LPS/GalN treatment in *MKK7^{Ctrl}* animals, whereas LPS/GalN had no apparent effect on the expression of these genes in *MKK7^{ΔAlfp}* liver tissues.

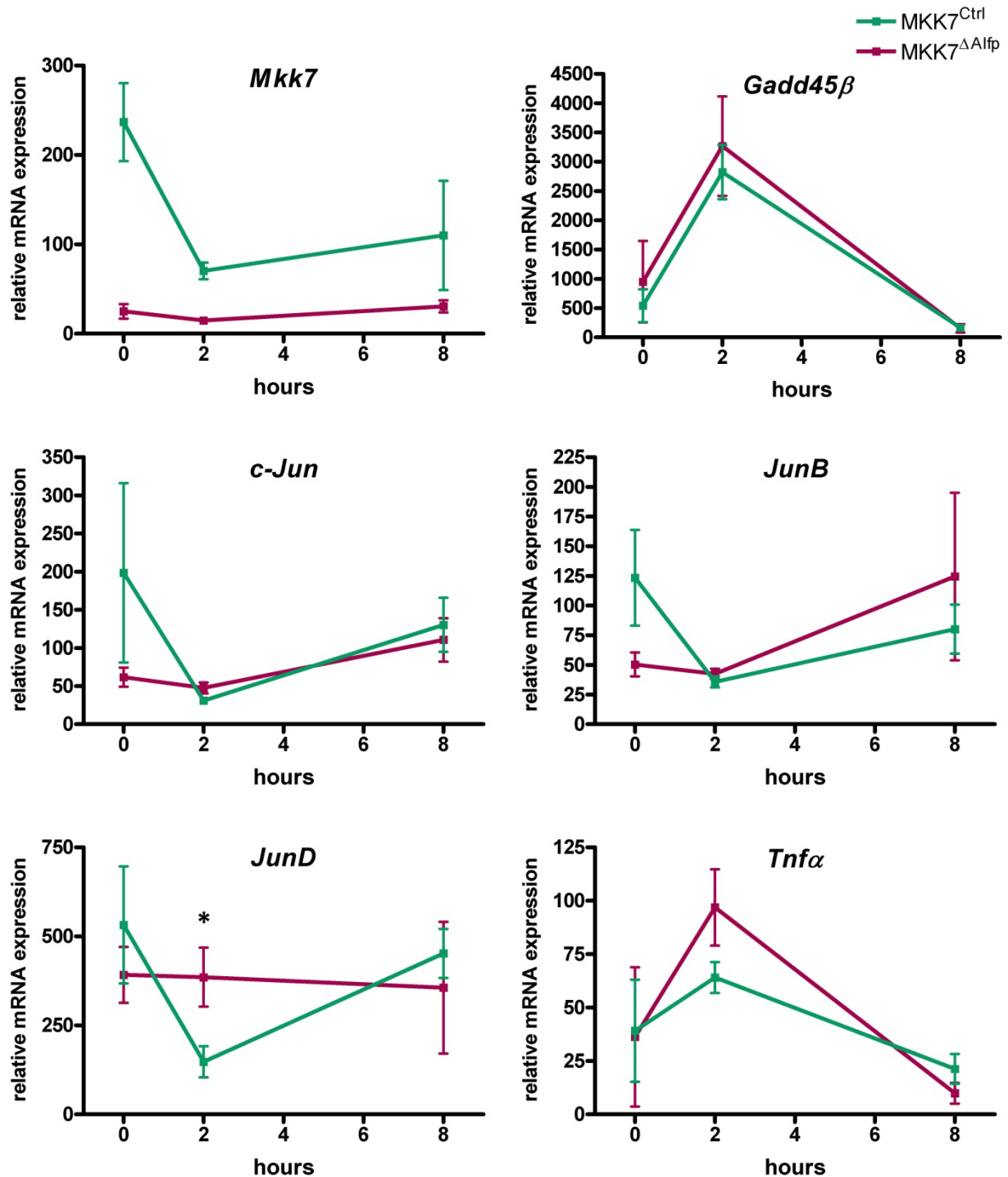


Figure 15: Gene expression analysis of whole livers of LPS/GalN treated and untreated *MKK7^{Ctrl}* and *MKK7^{ΔAlfp}* mice. Gene expression levels upon LPS/GalN (10μg/kg; 1g/kg) treatments were determined using qPCR. Mice were sacrificed after 2 and 8 hours. The baseline (time 0) was set by analysis of PBS-treated mice. PCR reactions were done in triplicates. Expression of the β -actin gene was used to normalize the amount of the indicated transcripts. PBS *MKK7^{Ctrl}* n = 4; PBS *MKK7^{ΔAlfp}* n = 4; LPS 2 hours *MKK7^{Ctrl}* n = 6; LPS 2 hours *MKK7^{ΔAlfp}* n = 5; LPS 8 hours *MKK7^{Ctrl}* n = 3; LPS 8 hours *MKK7^{ΔAlfp}* n = 3; Data are shown as mean values $\pm$ SEM. T-test: significant differences (p < 0.05) are indicated by asterisks. SEM = standard error of the mean.

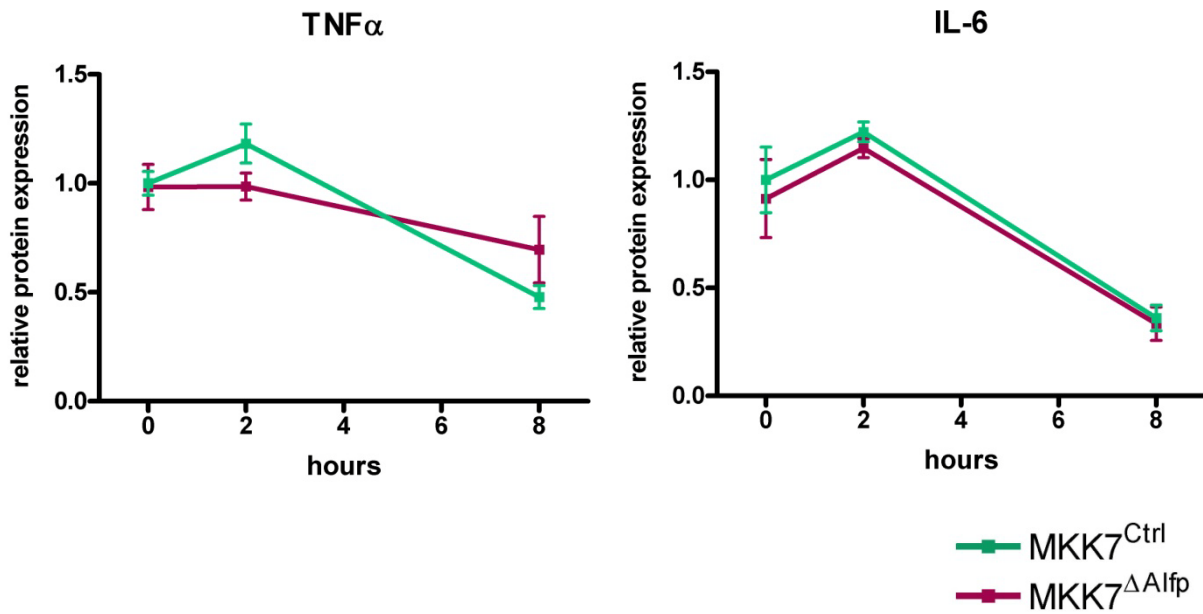
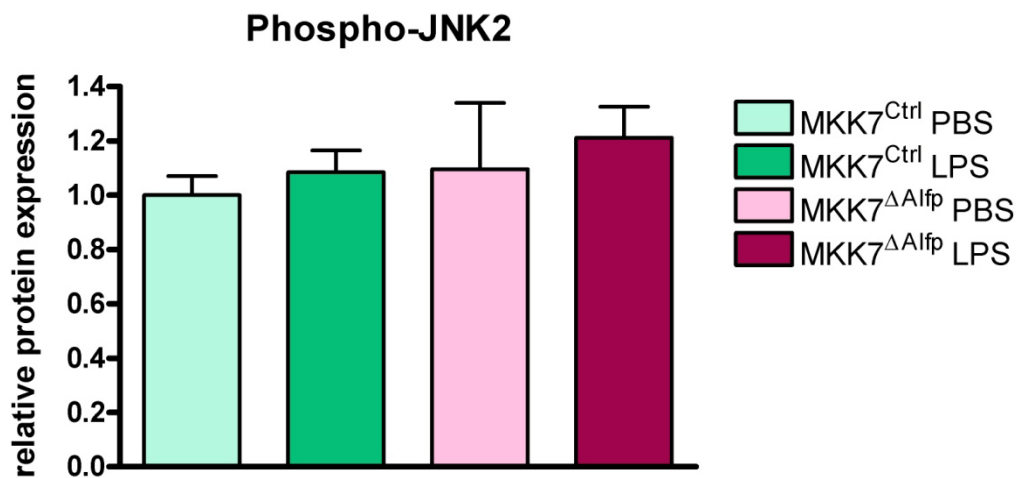
A**B**

Figure 16: Determination of $\text{TNF}\alpha$, IL-6 and p-JNK2 protein levels in liver homogenates. [A] $\text{TNF}\alpha$ and IL-6 protein levels in livers of $\text{MKK7}^{\text{Ctrl}}$ and $\text{MKK7}^{\Delta\text{Alfp}}$ mice was measured by ELISA at the indicated time points after LPS/GalN challenge. The baseline (time 0) was set by analysis of PBS-treated mice. Reactions were done in triplicates. PBS $\text{MKK7}^{\text{Ctrl}}$ n = 4; PBS $\text{MKK7}^{\Delta\text{Alfp}}$ n = 4; LPS 2 hours $\text{MKK7}^{\text{Ctrl}}$ n = 7; LPS 2 hours $\text{MKK7}^{\Delta\text{Alfp}}$ n = 6; LPS 8 hours $\text{MKK7}^{\text{Ctrl}}$ n = 6; LPS 8 hours $\text{MKK7}^{\Delta\text{Alfp}}$ n = 3; Data are shown as mean values $\pm$ SEM. T-test: not significant [B] Levels of phosphorylated JNK2 in liver lysates 2 hours after LPS/GalN (10μg/kg; 1g/kg) and PBS treatment. Reactions were done in triplicates. PBS $\text{MKK7}^{\text{Ctrl}}$ n = 2; PBS $\text{MKK7}^{\Delta\text{Alfp}}$ n = 2; LPS 2 hours $\text{MKK7}^{\text{Ctrl}}$ n = 7; LPS 2 hours $\text{MKK7}^{\Delta\text{Alfp}}$ n = 6; LPS 8 hours $\text{MKK7}^{\text{Ctrl}}$ n = 6; LPS 8 hours $\text{MKK7}^{\Delta\text{Alfp}}$ n = 3; Data are shown as mean values $\pm$ SEM. T-test: not significant. SEM = standard error of the mean.

2.2.4 Deletion of MKK7 in the hematopoietic system and the liver

Next, the role of hematopoietic cells in the LPS/GalN liver injury model was examined by using Mx1-Cre MKK7 Δ/Δ animals (termed *MKK7 Δ^{Mx1}* hereafter). The same dose as for the Alfp-Cre mice was used (10 μ g/kg LPS, 1 g/kg GalN). The survival curves of *MKK7 Δ^{Mx1}* and *MKK7^{Ctrl}* mice upon LPS/GalN injection are shown in Figure 17A. The observed tendency for a protective function of MKK7 deficiency against LPS/GalN induced liver damage is not yet significant ($p = 0.06$). Furthermore, serum transaminase activity (ALT and AST) was not found to be different between *MKK7^{Ctrl}* and *MKK7 Δ^{Mx1}* mice when samples were analyzed 8 hours after LPS/GalN challenge (Figure 17B).

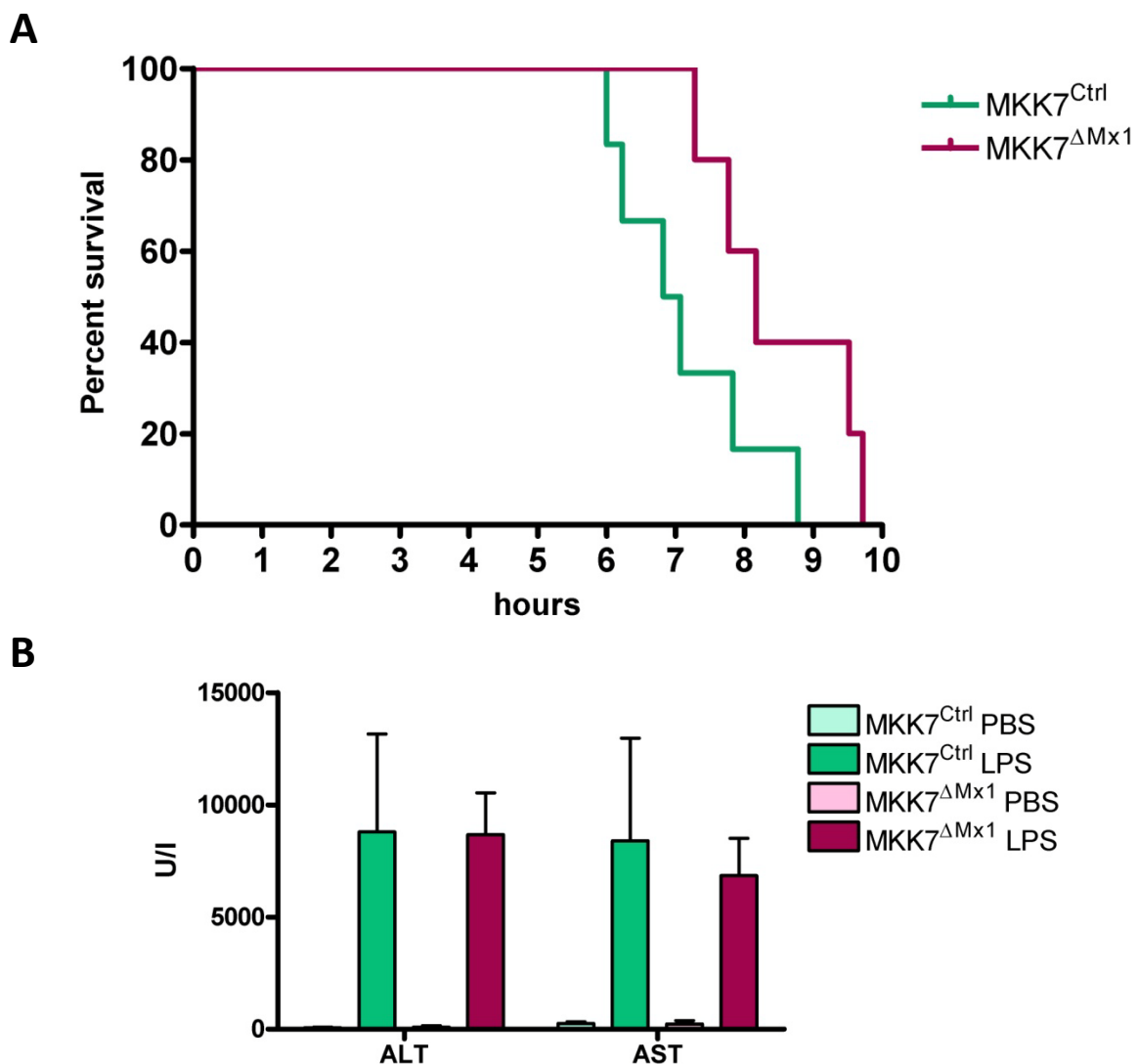


Figure 17: Survival curves and ALT/AST levels upon LPS/GalN treatment in *MKK7 Δ^{Mx1}* mice. [A] Survival curves of *MKK7 Δ^{Mx1}* and *MKK7^{Ctrl}* mice upon LPS/GalN (10 μ g/kg; 1g/kg) injection. *MKK7^{Ctrl}* $n = 6$; *MKK7 Δ^{Mx1}* $n = 5$. [B] ALT and AST levels of PBS treated *MKK7 Δ^{Mx1}* and *MKK7^{Ctrl}* mice compared to LPS/GalN treated *MKK7 Δ^{Mx1}* and *MKK7^{Ctrl}* mice. PBS *MKK7^{Ctrl}* $n = 3$; PBS *MKK7 Δ^{Mx1}* $n = 2$; LPS *MKK7^{Ctrl}* $n = 5$; LPS *MKK7 Δ^{Mx1}* $n = 4$; Data are shown as mean values $\pm$ SEM. T-test: not significant. Samples were taken 8 hours after injections. U/l = units per liter. SEM = standard error of the mean.

Moreover, no obvious difference in the extent of injury was seen in H&E stained liver sections of LPS/GaIN challenged control and *MKK7^{ΔMx1}* mice (Figure 18). Due to strong background fluorescence of red blood cells, apoptosis could not be assayed by TUNEL staining. Hence, an ELISA, detecting cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) which are a hallmark of apoptotic cell death (Bortner et al. 1995), was performed (Figure 19). No significant difference in the extent of apoptosis between *MKK7^{Ctrl}* and *MKK7^{ΔMx1}* livers could be determined. Taken together, no significant differences between *MKK7^{Ctrl}* and *MKK7^{ΔMx1}* mice were observed when analyzing survival times, serum transaminase levels and liver histology.

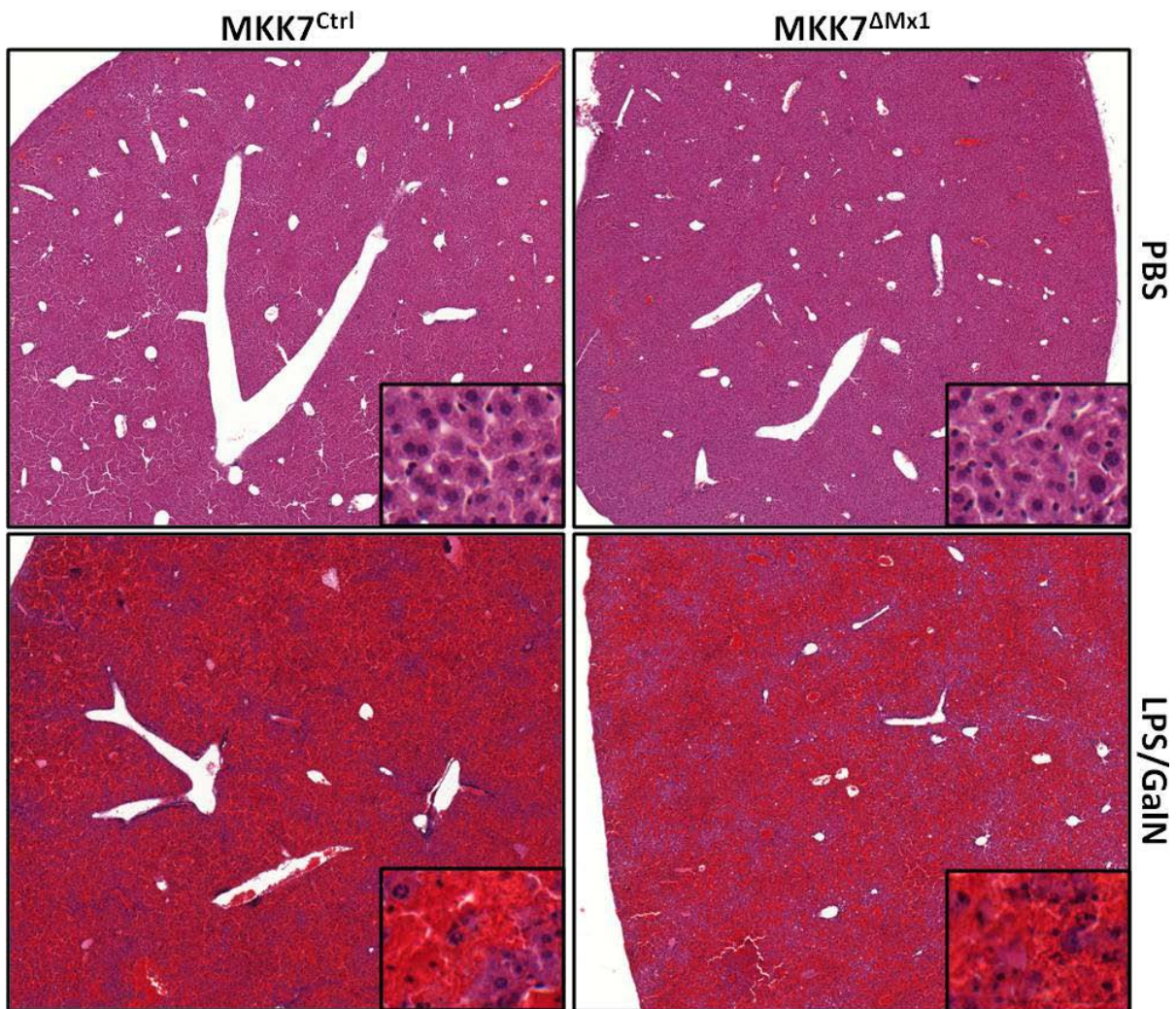


Figure 18: H&E stained liver sections. Liver sections of *MKK7^{Ctrl}* and *MKK7^{ΔMx1}* mice, treated either with PBS or LPS/GaIN (10μg/kg; 1g/kg) were prepared and stained with H&E. Massively injured livers were seen for both genotypes, without any obvious disparities (see inserts). Samples were taken 8 hours after injections.

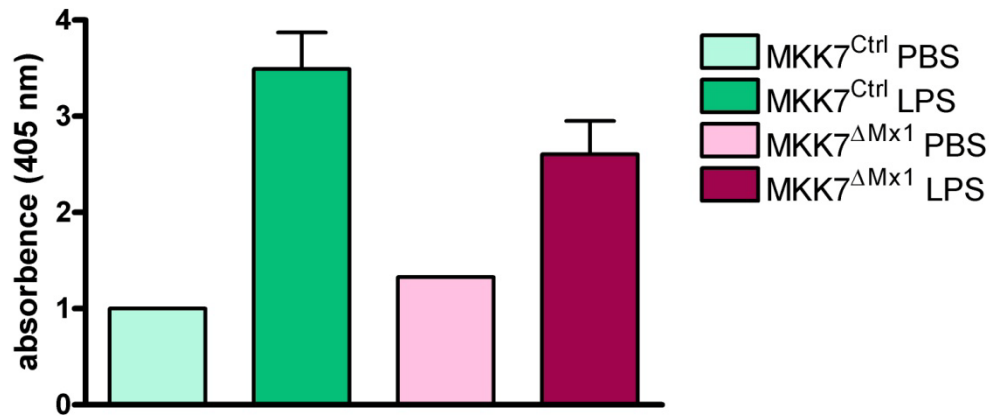


Figure 19: Determination of apoptotic liver damage: Mono- and oligonucleosomes, which are a typical sign for apoptosis, were detected in lysates of *MKK7^{Ctrl}* and *MKK7^{ΔMx1}* livers. Reactions were done in triplicates. PBS n = 1; LPS n = 5. Data are shown as mean values ± SEM. All samples were taken 8 hours after PBS respectively LPS/GaIN (10μg/kg; 1g/kg) injections. SEM = standard error of the mean.

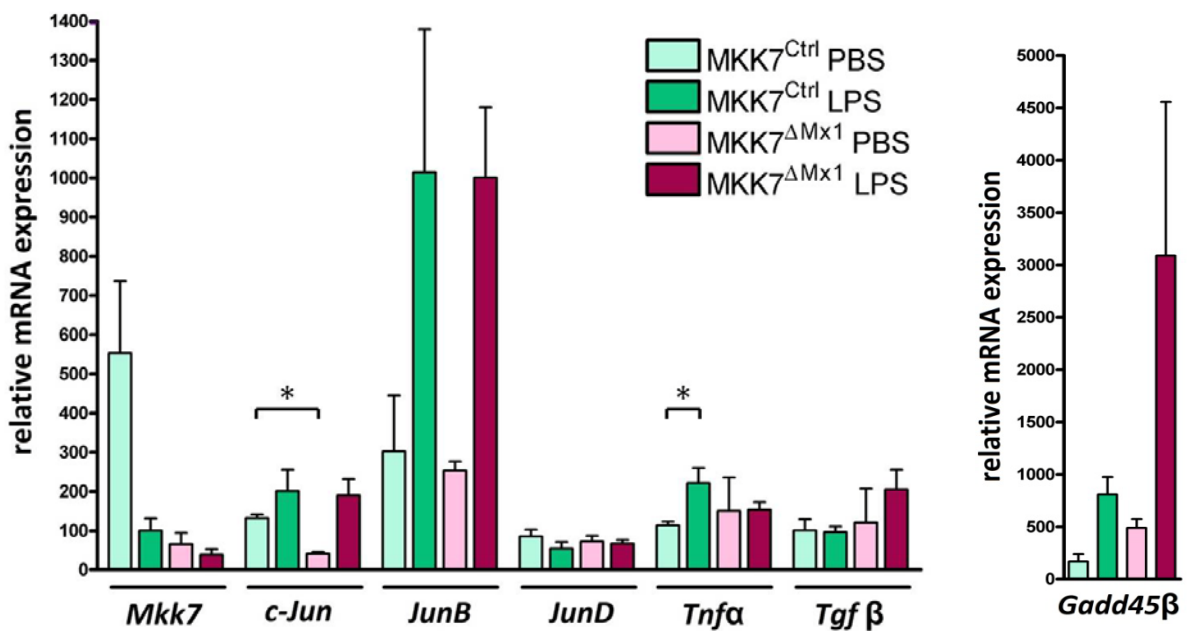


Figure 20: Gene expression analysis of livers of *MKK7^{Ctrl}* and *MKK7^{ΔMx1}* animals. Gene expression in livers of PBS treated and LPS/GaIN (10μg/kg; 1g/kg) treated *MKK7^{Ctrl}* and *MKK7^{ΔMx1}* mice was examined by qPCR. PCR reactions were done in triplicates. Expression of the β-actin gene was used to normalize the amount of the indicated transcripts. PBS *MKK7^{Ctrl}* n = 4; PBS *MKK7^{ΔMx1}* n = 2; LPS *MKK7^{Ctrl}* n = 6; LPS *MKK7^{ΔMx1}* n = 5; Data are shown as mean values ± SEM. T-test: significant differences (p < 0.05) between the genotypes are indicated by asterisks. Samples were taken 8 hours after injections. SEM = standard error of the mean.

2.2.5 Loss of MKK7 in *MKK7^{ΔMx1}* livers affects c-Jun protein expression

Next, gene expression analysis of the liver samples was performed 8 hours after LPS/GalN treatment (Figure 20). In *MKK7^{Ctrl}* mice *Mkk7* was down-regulated and *Gadd45β* was induced following hepatic injury. The residual *Mkk7* expression in *MKK7^{ΔMx1}* samples is very likely due to a contamination with genomic DNA. As well as in control mice, *Gadd45β* was strongly induced in *MKK7^{ΔMx1}*. The basal level of *c-Jun* mRNA was reduced in *MKK7^{ΔMx1}* liver tissues compared to *MKK7^{Ctrl}* mice ($p = 0.0009$), whereas the induction seemed to be unaffected. *JunB* induction was also found to be similar between *MKK7^{Ctrl}* and *MKK7^{ΔMx1}* mice whereas *JunD* mRNA expression levels remained constant upon LPS/GalN treatment for both genotypes. Next, c-Jun levels were analyzed on the protein level (Figure 21A). In liver homogenates of PBS treated mice a single band of about 43 kDa was detected. In *MKK7^{Ctrl}* mice that developed liver inflammation a second, elevated band appeared, which indicates an altered phosphorylation state (Wang et al. 2010). In LPS/GalN treated *MKK7^{ΔMx1}* mice this second band was absent, indicating that c-Jun was not phosphorylated (Figure 21A).

Interestingly, no *Tnfα* mRNA induction could be detected in *MKK7^{ΔMx1}* tissues at the examined time point, whereas the amount of *Tnfα* transcript was significantly increased in inflamed *MKK7^{Ctrl}* livers ($p = 0.039$). The expression of *Tgfbβ*, which is an important fibrosis marker (Schuppan et al. 1999), was unaffected upon LPS/GalN treatment (Figure 20). Next, TNFα, IL-6 and phospho-JNK2 protein levels were examined (Figure 21B and 21C). For TNFα as well as for IL-6 a reduction was detected 8 hours after LPS/GalN treatment as expected. The level of phospho-JNK2 was reduced in LPS/GalN treated *MKK7^{ΔMx1}* animals compared to *MKK7^{Ctrl}* animals. Thus, loss of MKK7 has no apparent effect on late transcriptional induction of the *c-Jun* gene but the loss of MKK7 affects either *c-Jun* mRNA stability, translation, c-Jun protein stability or posttranslational modifications of c-Jun protein.

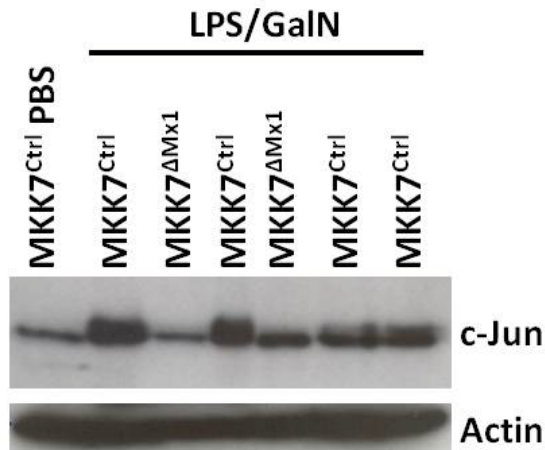
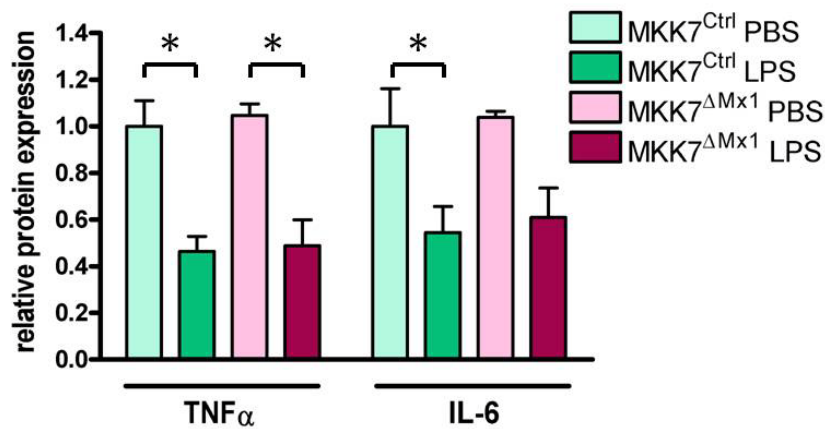
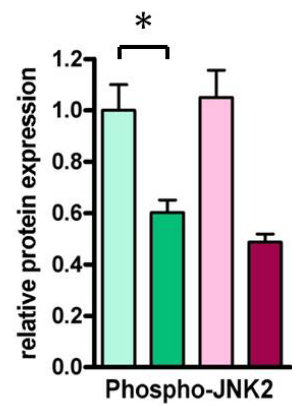
A**B****C**

Figure 21: Determination of protein levels in liver homogenates. [A] Western blotting of total c-Jun was performed on liver lysates of *MKK7^{Ctrl}* and *MKK7^{ΔMx1}* mice that received PBS injections or were challenged with LPS/GalN 8 hours earlier. [B] Homogenates of livers were analyzed for their relative levels of TNF α and IL-6 by ELISA. [C] Phospho-JNK2 protein in liver lysates. Reactions were done in triplicates. PBS *MKK7^{Ctrl}* n = 4; PBS *MKK7^{ΔMx1}* n = 2; LPS *MKK7^{Ctrl}* n = 6; LPS *MKK7^{ΔMx1}* n = 5; Data are shown as mean values $\pm$ SEM. T-test: significant differences (p < 0.05) are indicated by asterisks. Samples were taken 8 hours after PBS respectively LPS/GalN (10 μ g/kg; 1g/kg) injections. SEM = standard error of the mean.

2.3 Discussion

Over the last years and decades, multiple studies on the role of the TNF α -MKK7-JNK pathway in various hepatitis models have been performed. TNF α is a pleiotropic cytokine as it can affect cell proliferation as well as programmed cell death (Bradham et al. 1998). The signaling cascades around JNK and NF- κ B were of special interest as both are induced by TNF α (Song et al. 1997). TNF α induced TNFR triggering leads to the activation of NF- κ B transcription factors, ultimately promoting cell survival (Papa et al. 2004). The outcome of JNK activation depends on the magnitude and duration of JNK activity. Whereas transient activation of the JNK cascade signals cell growth and differentiation, a sustained activation triggers cell death (Ventura et al. 2006). The pro-survival function of NF- κ B involves a suppression of the sustained JNK activation (Tang et al. 2001). Thus, the biological outcome of TNF α stimulation is dictated, in part, by the balance between the opposing activities of NF- κ B and JNK (Papa et al. 2008; Wullaert et al. 2007).

Contradictory results were published from single *Jnk1* or *Jnk2* knockout mice. Whereas Maeda et al. reported a considerably reduced ConA-induced liver damage in *Jnk1*^{-/-} as well as in *Jnk2*^{-/-} single knockout mice (Maeda et al. 2003), Ni et al. observed a protective effect only for liver injury caused by ConA/GalN but not by ConA alone (Ni et al. 2008). In either *Jnk1* or *Jnk2* deficient mice, cytochrome c release as well as caspase activation were reduced upon ConA/GalN treatment compared to wild type mice, leading to less severe hepatic damage and reduced mortality (Ni et al. 2008). In 2009 Das et al. published that no difference was observed in whole-body single knockouts of either *Jnk1* or *Jnk2* in the ConA model, whereas a partial protection was found to be associated with the *Jnk1* or *Jnk2* deficiency in the LPS/GalN model, suggesting that JNK1 and JNK2 play redundant roles during the pathogenesis of hepatitis. Mx1-Cre *Jnk1*^{fl/fl} *Jnk2*^{-/-} mice exhibited a strong protection against ConA induced hepatitis (Das et al. 2009). The same effect was seen in the LPS/GalN model. This protection against hepatitis was found to be associated with reduced mortality, reduced serum transaminase activity as well as decreased expression of AP-1 related genes and *Tnf α* mRNA in the liver and decreased amounts of serum TNF α . The key conclusions were that JNK is not required for TNF α mediated death of hepatocytes but JNK is essential for the expression of TNF α by hematopoietic cells that promote hepatitis (Das et al. 2009). The differences between the various hepatitis models might explain these discrepancies. For example, the key regulator TNF α is produced by different cell populations in the LPS and ConA model and ConA induces only necrosis whereas LPS/GalN leads to necrosis and apoptosis (Ni et al. 2008). Furthermore the knockout strategy has to be considered, as there were whole-body knockouts as well as tissue and cell type specific knockouts. Thus, variations in

experimental setups may contribute to the inconsistent results concerning the JNK pathway in the context of acute hepatitis.

To shed more light on this pathway, the relevance of the upstream activator of JNK, MKK7, in acute hepatitis was examined. Previously it has been shown that disruption of MKK7 is sufficient to prevent JNK activation caused by proinflammatory cytokines such as TNF α (Tournier et al. 2001). Therefore *Mkk7^{fl/fl}* mice were crossed to either Alfp-Cre transgenic mice or Mx1-Cre transgenic mice, respectively. Previously it has been shown in our lab, that ConA challenge of Mx1-Cre *Mkk7^{fl/fl}* mice resulted in increased serum transaminase levels, whereas no change was observed with Alfp-Cre *Mkk7^{fl/fl}* mice. However, the overall survival and hepatic damage in Mx1-Cre *Mkk7^{fl/fl}* mice was found to be similar to wildtype mice, indicating that MKK7 does not protect against ConA induced hepatitis (unpublished data).

In this study, Alfp-Cre *Mkk7^{fl/fl}* and Mx1-Cre *Mkk7^{fl/fl}* mice were subjected to LPS/GalN treatments. On the basis of the publication by Das et al. no effect was expected to be seen with the Alfp-Cre *Mkk7^{fl/fl}* (*MKK7 ^{Δ Alfp}*) mice, whereas a protective effect of Mx1-Cre mediated deletion of the floxed allele (*MKK7 ^{Δ Mx1}*) was anticipated. Indeed, no prolonged survival or altered serum transaminase levels could be detected in *MKK7 ^{Δ Alfp}* mice as compared to *MKK7^{Ctrl}* animals upon LPS/GalN injections. Furthermore, quantification of TUNEL stainings revealed an equal extent of apoptotic liver damage in knockout and control animals. Thus, hepatocyte specific deletion of MKK7 does not confer protection from LPS/GalN induced hepatitis.

LPS/GalN challenge of *MKK7 ^{Δ Mx1}* mice revealed a mean survival time of 8.48 ± 1.28 hours, whereas the mean survival time for *MKK7^{Ctrl}* animals was 7.12 ± 1.28 hours. However, this result is not statistically significant as the p-value is 0.06. Interestingly no difference in serum transaminase levels could be detected and histological analyses indicated a similar liver damage in control and mutant mice. Although additional experiments need to be performed, the survival curves suggest a trend for a protective effect of hematopoietic deletion of MKK7.

Gene expression analyses revealed that the basal expression of *c-Jun* was reduced in *MKK7 ^{Δ Alfp}* and *MKK7 ^{Δ Mx1}* liver tissues, whereas upon treatment with LPS/GalN normal *c-Jun* induction occurred. Interestingly, no induction of c-Jun protein or c-Jun phosphorylation could be detected by Western blot analyses in *MKK7 ^{Δ Mx1}* livers. The other examined AP-1 factors, *JunB* and *JunD*, displayed the same mRNA expression patterns in *MKK7^{Ctrl}* and *MKK7 ^{Δ Mx1}* mice. Two hours after LPS/GalN challenge a downregulation of *JunD* mRNA was observed in *MKK7^{Ctrl}* livers, while the expression was constant in *MKK7 ^{Δ Alfp}* livers. TNF α and IL-6 protein levels in the liver were reduced 8 hours upon LPS/GalN injections and no differences were observed between knockout and control tissues. These low protein levels are consistent with the previously reported kinetics of TNF α and IL-6 expression following LPS

challenge. TNF α expression peaks 1 hour after LPS challenge, IL-6 expression after 3 hours and both proteins disappear rapidly after reaching maximum expression (Chensue et al. 1991). Therefore TNF α as well as IL-6 protein levels have to be assayed at earlier time points. The same reduction after 8 hours was seen for phospho-JNK2.

Other studies focused on the JNK pathway in the context of liver regeneration. To understand the crosstalk between NF- κ B and JNK, the authors examined a putative mediator of this crosstalk, Gadd45 β , in the context of partial hepatectomy (PH) (Papa et al. 2008). In contrast to ConA or LPS challenge, PH normally induces a transient, but not prolonged, JNK activation via TNFR signaling (Kamata et al. 2005), which is required for hepatocyte proliferation (Schwabe et al. 2003). Gadd45 β is known to be rapidly induced by PH (Su et al. 2002) and, moreover, is known to directly interact with the upstream activator of JNK, MKK7, thereby blocking its catalytic activity (Papa et al. 2004). Gadd45 $\beta^{-/-}$ mice were found to exhibit impaired hepatocyte proliferation and increased cell death following PH. Interestingly, Gadd45 β ablation did not affect hepatotoxic JNK signaling after ConA or LPS challenge, although both treatments are known to induce Gadd45 β expression (Papa et al. 2008). Consistent with this study, *Gadd45 β* upregulation upon LPS/GalN challenge was detected in our experiments. No significant difference in the expression level of *Gadd45 β* was detected between control and mutant mice. This specific role of Gadd45 β mediated JNK suppression during liver regeneration may be explained by the differences between transient (PH) and sustained (ConA, LPS) JNK activation. One difference is the involvement of reactive oxygen species (ROS). Whereas ROS are elevated following prolonged JNK activation, this elevation is not involved in the transient induction of JNK elicited by PH (Kamata et al. 2005; Papa et al. 2008).

Furthermore, TNF-R1 and c-Jun deficiency were shown to be associated with defective liver regeneration following PH (Yamada et al. 1997; Behrens et al. 2002) and *Mkk7* deficient mouse embryonic hepatoblasts were found to display impaired proliferation (Wada et al. 2008). These findings suggest that MKK7 might be involved in liver regeneration. To further examine the functions of MKK7 in liver regeneration, PH experiments using MKK7 Δ ^{Alfp} as well *MKK7 Δ ^{Mx1}* mice should be performed in the future.

Taken together, no definite role for MKK7 in hepatitis was found. Nevertheless, our results partly reflect the data from *Jnk1/Jnk2* double mutants, however, the observed phenotype is very mild and more experiments are needed to further explore this pathway in hepatitis.

3 Materials and Methods

3.1 Project „New Tools for Mouse Genome Engineering“

3.1.1 ES cell culture

Mouse embryonic stem cells (ESCs) were cultured on feeder cells on gelatinized cell culture dishes at standard conditions (37°C, 5 % CO₂). As feeder cells, mouse embryonic fibroblasts that have been arrested mitotically by treatment with mitomycin C were used. All culture media were filter sterilized (0.45 µm) before usage. Culture medium for ESCs was exchanged every day. When the desired density was reached, cells were washed with PBS twice and then incubated with trypsin (0.05 %) for 5 minutes at 37°C to detach from the surface of the cell culture dish. The enzymatic activity of trypsin was stopped by resuspending the cells in culture medium. Cells were pelleted at 1150 rpm for 5 minutes, resuspended in culture medium and then plated at the desired density. Alternatively cells were resuspended in 1 ml of freezing medium and frozen at -80°C or in liquid nitrogen.

ESC culture medium

DMEM
10 % ESC tested FCS
1x Penicillin/Streptomycin
2 mM L-Glutamin
1 mM Sodium pyruvate
1x Non-essential amino acids
50 nM beta-Mercaptoethanol
103 U/ml LIF

Fibroblast culture medium

DMEM
10 % FCS
1x Penicillin/Streptomycin
2 mM L-Glutamin

Freezing medium

90 % FCS
10 % DMSO

Gelatine

0.1 % Gelatine
PBS

3.1.2 Characterization of stem cells

3.1.2.1 Alkaline phosphatase staining

After washing cells 2 times with PBS, AP substrate solution (Vector Shields) was prepared according to the protocol and cells were incubated with for 20-30 minutes at 37°C in the dark. The staining solution was then removed, cells were washed with PBS and analyzed using a brightfield microscope.

3.1.2.2 DNA preparation

To derive DNA from cultured cells, plates were washed with PBS twice, covered with ESC lysis buffer and incubated at 37°C overnight. The next day the contents of the wells were transferred to 2 ml microcentrifuge tubes and filled to 500 µl with ESC lysis buffer. Upon adding 200 µl NaCl (5 M) tubes were shaken vigorously and centrifuged at 13.000 rpm for 10 minutes. Supernatants were transferred to fresh tubes, 500 µl isopropanol was added and tubes were inverted several times to precipitate the DNA. The samples were centrifuged at 13.000 rpm for 5 minutes, supernatants discarded and the DNA pellets washed with 70 % ethanol. After another centrifugation and removal of the ethanol, pellets were air dried and dissolved at 65°C for 30 minutes in 50 to 100 µl TE buffer.

ESC lysis buffer

1 mM Tris/HCl pH 8.0
5 mM EDTA
100 mM NaCl
1 % SDS
0.5 mg/ml Proteinase K

TE buffer

10 mM Tris/HCl pH 8.0
1 mM EDTA

3.1.2.3 RNA preparation

To reduce the number of feeder cells in the sample, cells were trypsinized and transferred to a new cell culture dish. After about half an hour, most feeder cells have attached to the surface, whereas most of the stem cells are still floating. The cell suspension was then centrifuged, the supernatant removed and the pellet either processed immediately or frozen at -20°C. For RNA extraction, the RNeasy Mini Kit (Qiagen) was used. RNA concentrations were measured by use of the NanoDrop 1000 Spectrophotometer (Peglab).

3.1.2.4 cDNA synthesis

For synthesis of first-strand cDNA templates from the purified RNA, Ready-To-Go™ You-Prime First-Strand Beads (GE Healthcare) were used. 2 µg total RNA were transcribed using Oligo(dT)18 primer (Fermentas) at the recommended concentration.

3.1.2.5 Quantitative real-time PCR (qPCR) of stem cell specific genes

qPCRs were performed on MyiQ and iQ5 Real Time PCR Detection Systems (Bio-Rad) using the iQ™SYBR® Green Supermix (Bio-Rad). Reaction set up, used primers and program are indicated below. Analysis was done in Microsoft Excel® by the delta-CT method. Results were normalized to β -actin expression. cDNA transcribed from RNA, which was extracted from feeder cells, was used as a negative control for stemness markers.

qPCR mix

12.5 μ l iQ mix
2 μ l primer mix (forward + reverse, 10 μ M each)
0.5 μ l nuclease-free water
10 μ l cDNA (1:40 dilution)

Program:

50°C	10 min		
95°C	5 min		
95°C	10 sec		
58°C	30 sec	real time detection	45 cycles
65°C	30 sec		
95°C	1 min		
65°C	10 sec	melting curve: 61 cycles; + 0.5°C per cycle	
15°C	∞		

Name	Sequence
Nanog_fwd	AGGGTCTGCTACTGAGATGCTCTG
Nanog_rev	CAACCACTGGTTTTCTGCCACCG
Oct3/4_fwd	TCTTTCCACCAGGCCCCCGGCTC
Oct3/4_rev	TGCGGGCGGACATGGGGAGATCC
Sox2_fwd	TAGAGCTAGACTCCGGGCGATGA
Sox2_rev	TTGCCTTAAACAAGACCACGAAA
Utf1_fwd	GGATGTCCCGGTGACTACGTCTG
Utf1_rev	GGCGGATCTGGTTATCGAAGGGT
Gdf3_fwd	GTTCCAACCTGTGCCTCGCGTCTT
Gdf3_rev	AGCGAGGCATGGAGAGAGCGGAGCAG
Cripto_fwd	ATGGACGCAACTGTGAACATGATGTTTCGCA
Cripto_rev	CTTTGAGGTCCTGGTCCATCACGTGACCAT
Ecat1_fwd	TGTGGGGGCCCTGAAAGGCGAGCTGAGAT
Ecat1_rev	ATGGGCCGCCATACGACGACGCTCAACT
Ulk1_fwd	GGAGACCTGGCTGACTACCTGCA
Ulk1_rev	CCTGACAGTGTCTTCACTCAGTGT
Eras_fwd	ACTGCCCCCATCAGACTGCTACT
Eras_rev	CACTGCCTTGTA CTGGGTAGCTG
Rex1_fwd	ACGAGTGGCAGTTTCTTCTTGGGA
Rex1_rev	TATGACTCACTTCCAGGGGGCACT

Nat1_fwd	ATTCTTCGTTGTCAAGCCGCCAAAGTGGAG
Nat1_rev	AGTTGTTTGCTGCGGAGTTGTCATCTCGTC
Cdx2_fwd	GGCGAAACCTGTGCGAGTGGATGCGGAA
Cdx2_rev	GATTGCTGTGCCGCCGCCGCTTCAGACC
Fgf4_fwd	CGTGGTGAGCATCTTCGGAGTGG
Fgf4_rev	CCTTCTTGGTCCGCCCGTTCTTA

3.1.2.6 Generation and analysis of embryoid bodies

For generation of embryoid bodies (EBs), ESCs were trypsinized for 5 minutes and 50 to 200 μ l of the cell suspension were transferred onto bacterial grade culture dishes with ESC medium without LIF. Medium was exchanged every day. After 7 days in culture, the EBs were spun down, RNA was prepared and cDNA was synthesized as described in sections 3.1.2.3 and 3.1.2.4. qPCRs using the primers listed below were performed in order to confirm the presence of cells of all 3 germ layers within the EBs. For reaction set up and temperature protocol see section 3.1.2.5.

Name	Sequence
Cdx2_fwd	GGCGAAACCTGTGCGAGTGGATGCGGAA
Cdx2_rev	GATTGCTGTGCCGCCGCCGCTTCAGACC
Brachyury_fwd	CATGTA CTCTTTCTTGCTGG
Brachyury_rev	GGTCTCGGGAAAGCAGTGGC
Gss_fwd	GCGAGCAGCTGGCCAGGAAGGTGCAC
Gss_rev	CAGCTAGCTCCTCGTTGCTTTCTC
Sox2_fwd	GAAGGGGAGAGATTTTCAAAGAGATACAAG
Sox2_rev	CCAGATCTATACATGGTCCGATTCCC
Afp2_fwd	CCTATGCCCCCTCCCCATTC
Afp2_rev	CTCACACCAAAGCGTCAACACATT
Hnf_fwd	TGCCCTCTCACCTCAGCAATG
Hnf_rev	CCCCTCAGCACACGGTTTTG
Foxa2_fwd	GTATGCTGGGAGCCGTGAAG
Foxa2_rev	CAGCGCCACATAGGATGAC
Vimentin_fwd	GCACTAACGAGTCCCTGGA
Vimentin_rev	GTGGGTGTCAACCAGAGGA

3.1.3 Analysis of transgenic mice

Genomic DNA for genotyping was obtained by incubating tail or ear biopsies overnight at 55°C with 16 μ l of tail buffer plus 4 μ l proteinase K. To inactivate the enzymatic activity of proteinase K, the lysate was heated at 99°C for 10 minutes the next day. After diluting the DNA with 180 μ l water the sample was used directly for PCR reactions without further purification. The presence of the transgene was

proven by PCR detecting PyMT and Cre sequences. All buffers used, primer pairs and programs are listed below.

Tail buffer

100 mM Tris/HCl pH 8.5
5 mM EDTA
200 mM NaCl
0.1 % SDS

10X PCR buffer

200 mM Tris pH 8.9
300 mM KCl
100 mM (NH₄)₂SO₄
15 mM MgCl₂

PCR mix

36 µl H₂O
5 µl 10X PCR buffer
4 µl dNTPs (2.5 mM)
1 µl primer 1 (10 µM)
1 µl primer 2 (10 µM)
1 µl Taq polymerase
2 µl template DNA

Programs:

PyMT

95°C	1.5 min	40 cycles
95°C	30 sec	
58°C	30 sec	
72°C	1 min	
72°C	5 min	
4°C	∞	

Cre

95°C	1.5 min	35 cycles
95°C	10 sec	
55°C	10 sec	
72°C	25 sec	
4°C	∞	

Name

PymTsense
PymTantisense
Cre1
Cre2

Sequence

GGAAGCAAGTACTTCACAAGG
GGAAAGTCACTAGGAGCAGGG
CTGCCACGACCAAGTGACAGCAATG
GCCTTCTCTACACCTGCGGTGCTAA

In order to detect PyMT mRNA expression, female mice were sacrificed and dissected. Organs were harvested and snapfrozen on dry ice. RNA was prepared and transcribed into cDNA as described in sections 3.1.2.3 and 3.1.2.4. PyMT PCR was performed as described above using the generated cDNA as templates.

3.1.4 The exchange vector system

3.1.4.1 Molecular cloning

Generation of the docking vector (DV): A modified hygromycin resistance gene was cut out of the plasmid pBA14 by the enzymes BglII and NheI and inserted into the corresponding sites of plasmid pCcall21E. The newly derived construct was ligated via Ascl and AsiSI and cloned into the vector pROSA-modified to obtain the docking vector plasmid.

Generation of the exchange vector (EV): A β GEO-STOP-loxP cassette was amplified from the plasmid pCcall-modified via PCR by use of the primers “NheI Δ 5' β geo_FWD” and “ Δ 5' β geo_REV”, thereby adding BglIII and NheI overhangs. The PCR reaction was run on a 1 % agarose gel and the PCR product was purified by use of the QIAquick Gel Extraction Kit (Qiagen). The purified DNA was then cloned into a commercial (TOPO® TA Cloning® Kit (with pCR®2.1-TOPO®) with One Shot TOP10 Chemically Competent E. coli, Invitrogen) vector. The plasmid TOPO- β GEO was verified by DNA sequence analysis. A small linker oligonucleotide (called TV1) was inserted into the KpnI and BglIII sites of a modified version of pBlue. pBlue-TV1 and TOPO- β GEO were both cut with BglIII and NheI and then ligated. After digesting the resulting plasmid with BglIII and SacII, a second linker oligonucleotide (TV2) was incorporated. The cloning steps are outlined in Figure 22.

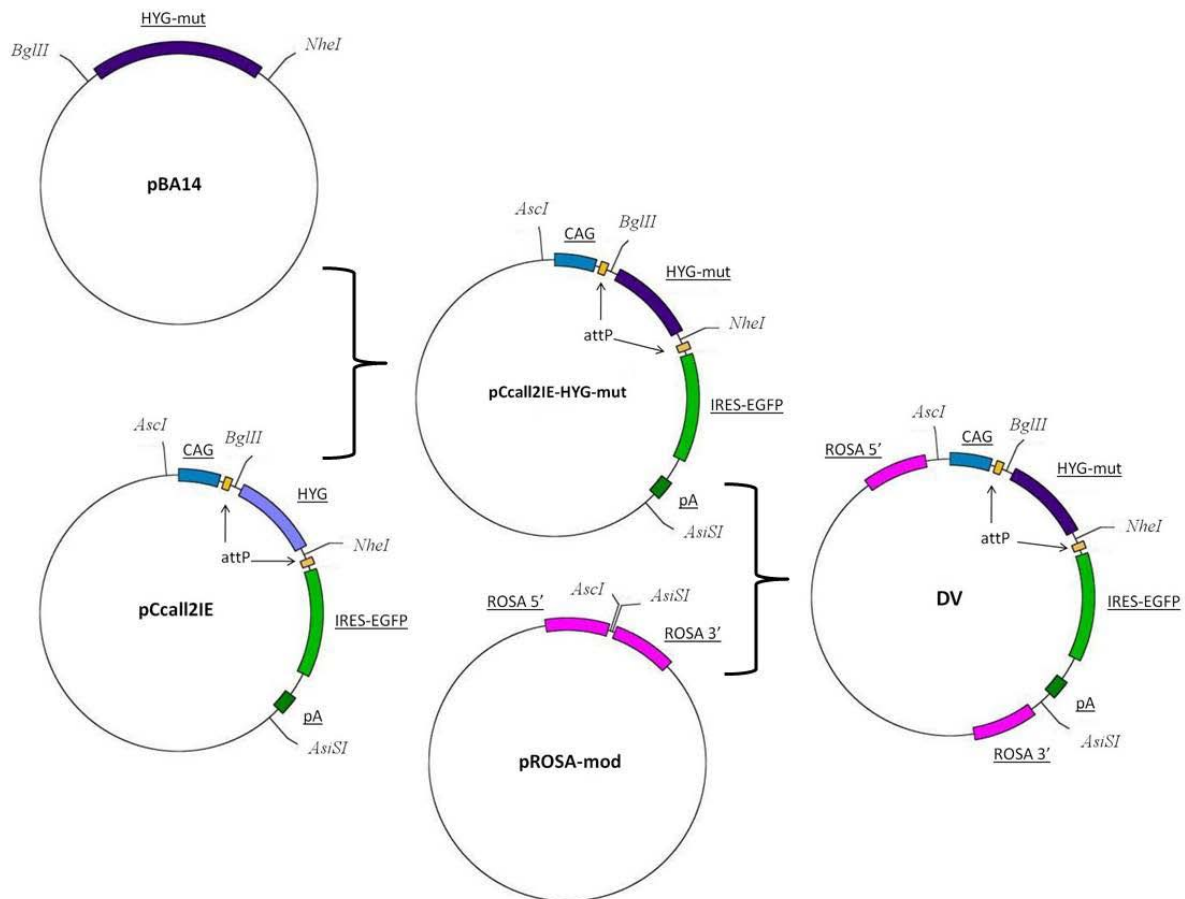


Figure 22: Generation of the docking vector. Schematic drawing of the plasmids used to generate the docking vector construct. HYG, hygromycin resistance gene; HYG-mut, mutated hygromycin resistance gene; CAG coding sequence for the cytomegalovirus promoter fused with the chicken β -actin promoter; IRES-EGFP, internal ribosome entry site followed by the coding sequence for enhanced green fluorescent protein; pA, polyadenylation signal; attP sites, direct the recombination mediated by Φ C31 integrase; AscI, AsiSI, BglIII, NheI, restrictions enzymes;

Bacterial culture

DNA fragments were ligated with a T4 DNA ligase (Roche) overnight at 16°C. Reactions were set up in a total volume of 10 to 30 µl. The amounts of vector and insert were chosen by setting them in a molar ratio of 1:3, 1:5 or 1:7. The next day the ligated plasmid was transformed into chemically competent E.coli (Turbo). In brief, competent bacteria were thawed on ice, 2 µl of ligation reaction were added and contents were mixed gently by flicking the tube. After incubating on ice for up to 30 minutes, a heatshock was done (45 seconds, 42°C). Bacteria were chilled on ice, 250 µl of LB medium was added and bacteria were incubated at 37°C for 1 hour on a shaker. Then the bacterial suspension was plated on LB agar plates with ampicillin and incubated overnight at 37°C. Next, single colonies were picked and inoculated in 2 ml LB medium containing 50 µg/ml ampicillin. Following another overnight incubation (37°C, under agitation) plasmid DNA was extracted by use of the QIAprep Spin Miniprep Kit (Qiagen). The derived samples were analyzed by PCR or restriction digests to proof the successful ligation. 50 to 500 ml of LB medium were inoculated with positive cultures, incubated overnight and plasmid DNA was prepared by use of QIAGEN Plasmid Midi or Maxi Kits.

Linker Hybridisation

To insert a small DNA fragment into a plasmid, the sense and the antisense oligonucleotide were mixed 1:1 and then diluted with water 1:200. The reaction was heated to 95°C for 5 minutes on a heating block. The block was then switched off and the tube was kept on the block until it reached room temperature. Following that, the linker was put on ice until further use. The linker and the plasmid were then ligated as described above.

Name	Sequence
NheIΔ5'βgeo_fwd	TTGCTAGCGCGGATCCCGTCGTTTTACA
Δ5'βgeo_rev	TTCTCGAGCGGGTACCCAAGATCTATAACTT
Linker	
TV1 sense	CCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTAGCTAGCA
Linker	
TV1 antisense	GATCTGCTAGCTACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGGGTAC
Linker	GATCTTCTAGACTCGAGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTAC
TV2 sense	CGC
Linker	
TV2 antisense	GGTACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGCTCGAGTCTAGAA

3.1.4.2 Preparation of plasmids for electroporation

100 µg docking vector DNA were digested with 10 µl PaeI restriction enzyme (New England Biolabs) overnight at 37°C. Next, the plasmid DNA was purified by phenol-chloroform (P/C) precipitation. 1

volume of P/C was added to the DNA and the tube shaken vigorously. To separate the aqueous phase from the organic phase the mixture was centrifuged at 13.000 rpm for 5 minutes. The upper phase was transferred to a fresh microcentrifuge tube and 1/10 volume of sodium acetate (3M, pH 5.2) and 2 volumes of ethanol (100 %) were added, followed by another centrifugation. The supernatant was removed and the DNA pellet washed with ethanol (70 %) two times. After about 20 minutes air drying 50 µl of PBS were added and the pellet was dissolved at 37°C for 1 hour.

3.1.4.3 Electroporation of A9 ESCs with docking vector

For targeting A9 cells with the docking vector (DV) construct, 1×10^7 cells were resuspended in 1 ml medium and transferred to an electroporation cuvette (Gene Pulser® Cuvette, 0.4 cm electrode gap; Bio-Rad) together with 30 µg of purified DV DNA. The cuvette was inserted into the GenePulser XCell (Bio-Rad) and electroporation was performed by one single pulse (260 V, 500 µF). Cells were then kept on ice for 10 minutes to recover and plated on 9 cell culture plates (Ø 10 cm) with hygromycin-resistant feeder cells and ESC medium. As a negative control about 1×10^6 cells in 1 ml medium without vector DNA were treated in the same way and seeded on 1 plate. Selection for hygromycin resistant clones was started the next day by supplying the cells with medium containing 100 µg/ml hygromycin. Selection medium was changed every day. After 2 to 3 days cell death could be observed. Selection was continued until no living cells were left on the negative control plate and colonies of resistant cells with round shape and distinct borders had formed on the other plates. Nine days after electroporation resistant clones were picked with a 20 µl pipette under a stereomicroscope. Picked clones were first kept in PBS in 96-well plates with round bottoms (U96 MicroWell™ Plates, Nunc), then treated with trypsin and brought to single-cell suspension by pipetting. Upon stopping the trypsin reaction by adding ESC medium, cells were transferred to 96-well cell culture dishes with feeder cells under continuous hygromycin treatment. When the desired density was reached, cells were split to 2 new 96-well plates with feeders and 2 96-well plates with gelatin only. As soon as the cells grown on feeders were dense enough, they were washed, trypsinized and 120 µl of 96-well freezing medium were added to each well followed by vigorous pipetting. Plates were wrapped with Parafilm, paper towels and aluminum foil, and frozen at -80°C. Cells grown on gelatin only were expanded to 24-wells for DNA preparation for Southern blot analysis. Here cells were cultured until confluent, lysed and DNA was extracted as described in section 3.1.2.2.

96-well freezing medium (1 well)

15 µl DMSO

25 µl ESC medium without antibiotics

80 µl FCS

3.1.4.4 Southern blot analysis

30 µl of each prepared DNA sample was digested with 5 µl BamHI (Roche) overnight at 37°C. The next day samples were run on a 0.7 % agarose gel with TAE buffer. Following electrophoresis the gel was first shaken gently in denaturation buffer for half an hour at room temperature and then in neutralization buffer for 20 minutes. The membrane (Porablot Membrane NY Plus, Macherey-Nagel) was soaked in water before assembly. Then the blotting stack was built as shown in Figure 23. The next day the stack was disassembled and the membrane was shaken in 4x SSC buffer for 20 minutes before it was air dried and UV cross-linked. The membrane was prehybridized with hybridization buffer (PerfectHyb Plus Hybridization Buffer, Sigma) for 1 hour at 65°C. Meanwhile the probe, complementary to the 5' arm of the ROSA26 locus (donation of C. Hartmann), was prepared. 20 to 50 ng of the probe were brought to a volume of 50 µl with TE buffer and denatured at 99°C for 5 minutes. Upon chilling on ice the probe was labeled with 5 µl dCTP-³²P using Ready-to-Go DNA Labeling Beads (GE Healthcare) according to the protocol. The probe was then purified (illustra ProbeQuant™ G-50 Micro Columns, GE Healthcare), boiled at 99°C for 3 minutes and added to the membrane. Hybridization was done overnight at 65°C. The next day the membrane was washed with wash buffer two times for half an hour and the radioactive signal was detected using a phosphorimager screen.

TAE buffer

40 mM Tris acetate

5 mM Sodium acetate

1 mM EDTA

Denaturing buffer

0.5 M NaOH

1.5 M NaCl

Neutralisation buffer

0.05 M NaPi pH 7.2

SSC buffer

150 mM NaCl

15 mM Sodium citrate

Washing buffer

0.02 M NaPi pH 7.2

1 % SDS

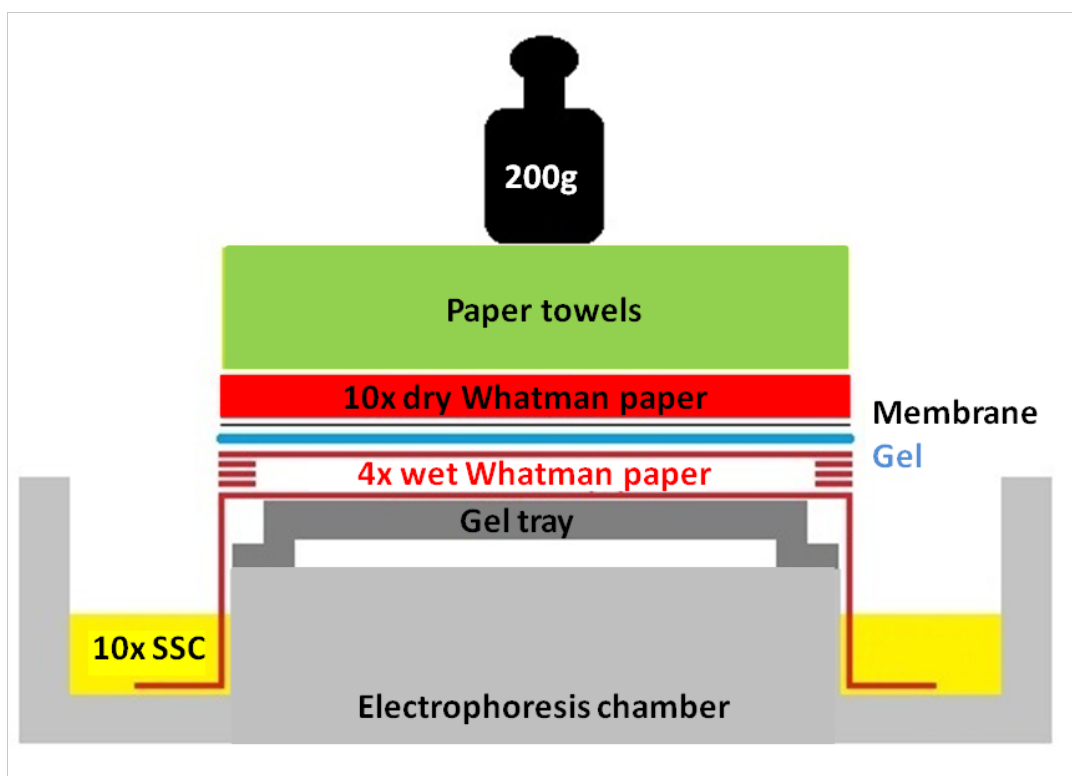


Figure 23: Southern Blotting stack. Schematic drawing of the construction of the blotting stack.

3.1.4.5 Electroporation of targeted A9 cells with exchange vector

Frozen plates were thawed quickly at 37°C and the respective clones were expanded. The clones were expanded to a cell number of 1×10^7 and the exchange vector together with a plasmid coding for the Φ C31 integrase (EV : integrase = 1:3) was delivered to the cells by electroporation. Cells were then plated on G418-resistant feeder cells and selection with G418 (300 μ g/ml) was started the next day. Neo-resistant clones were expanded and frozen.

3.1.4.6 Proof of successful exchange by PCR

Clones were tested for the presence of the hygromycin or neomycin resistance genes in their genomes by performing PCR. The PCR reaction mastermix was described in section 3.1.3, primer sequences and the temperature protocols are indicated below.

Programs:

Neo			Hygro		
95°C	1.5 min	35 cycles	95°C	1.5 min	35 cycles
95°C	30 sec		95°C	30 sec	
52°C	30 sec		58°C	30 sec	
72°C	1 min		72°C	1 min	
72°C	5 min		72°C	5 min	
4°C	∞		4°C	∞	

Name	Sequence
Neo_fwd	GTGGAGAGGCTATTCGGCTATGACT
Neo_rev	CTTGAGCCTGGCGAACAGTTCGGCT
Hyg_fwd	GGGCGAGATCTGCGGATCTCAAAAAGC
Hyg_rev	TGGGGCGCTAGCCTATTCCTTTGCCCTCG

3.1.4.7 *LacZ staining*

To show the activity of the enzyme β -galactosidase, lacZ staining was performed. Cells that express β -galactosidase will turn blue when the substrate “Bluo-Gal” is added. Medium was removed and plates were washed with PBS twice. Cells were fixed with 4 % PFA for 10 minutes at RT and then washed again with PBS. Cells were then covered with staining solution, and plates were wrapped with Parafilm and incubated at 37°C without CO₂ overnight. The next day, the staining solution was removed, plates were washed and analyzed.

500 mM K3 Stock

3.28 g K₃ (Fe(III)(CN)₆)
20 ml H₂O

500 mM K4 Stock

4.24 g K₄ (Fe(II)(CN)₆)
20 ml H₂O

40 mg/ml Bluo-Gal Stock

100 mg Bluo-Gal
2.5 ml Dimethylformamide

LacZ Staining Solution

10 ml PBS
2 ml K3 stock
2 ml K4 stock
0.5 ml Sodium deoxycholate stock solution (1 %)
0.1 ml MgCl₂ (1 M)
10 μ l NP-40
1.25 ml Bluo-Gal stock
fill with PBS to 50 ml

3.1.4.8 *Infection of DV-EV cells with AdenoCre*

In order to remove the cassette between the loxP sites in the double-targeted cells (DV-EV), cells were infected with an adenovirus carrying a Cre transgene (Ad5CMVCre-eGFP, University of Iowa Gene Transfer Vector Core, 3 x 10¹⁰ PFU/ml). Different amounts (1 μ l, 0.5 μ l, 0.25 μ l, 0.1 μ l) of virus were

added to 4×10^4 cells in 100 μ l ESC medium in a microcentrifuge tube followed by incubation at 37°C and 5 % CO₂ for six hours. Infected cells were washed twice with PBS and plated on 2 6-wells with feeder cells. The following days plates were analyzed for EGFP expression under a fluorescence microscope (488 nm).

3.2 Project “Evaluation of the Role of MKK7 in Hepatitis”

3.2.1 Mice

Alfp-Cre mice (Kellendonk et al. 2000), Mx1-Cre mice (R Kühn et al. 1995) as well as MKK7 fl/fl mice (Schramek et al. 2011) have been published previously. All mice were bred and maintained in the animal facility of IMBA according to an ethical animal license protocol complying with the current Austrian law.

3.2.2 Genotyping

Preparation of genomic DNA from tail or ear biopsies and the PCR mastermix are described in section 3.1.3. Primers used and protocols are indicated below. By use of the primers MKK7_wt/fl_fwd and MKK7_wt/fl_rev the presence of the wild type or the floxed allele could be detected. This reaction could either give a 234 bp band, representing the wild type allele, a 304 bp band, representing the floxed allele or, in a heterozygous case, both bands. In Mx-1 Cre mice with at least one floxed allele, a delta allele is generated upon induction of the Cre transgene. This was verified by use of the primers MKK7-/-_fwd and MKK7-/-_rev, which amplify a product of 300 bp length. The same primer pair (Cre1 and Cre2) was used to detect the Mx-1 Cre as well as the Alfp-Cre transgene. All used buffers, primer pairs and programs are listed below.

Name	Sequence
MKK7_wt/fl_fwd	CTGCCTGTAGCATGCCCGAGCTGTC
MKK7_wt/fl_rev	AGCTGTCTCATCTGTGCACCTCCCAGC
MKK7-/-_fwd	ATGCAGGCCATTGGGAAGTACCAAG
MKK7-/-_rev	AGAAAAATGAAGCCCGACTGTGCCT
Cre1	CTGCCACGACCAAGTGACAGCAATG
Cre2	GCCTTCTCTACACCTGCGGTGCTAA

Programs:

MKK7 wt/fl	MKK7 -/-	Cre
94°C 1.5 min	94°C 1 min	95°C 1.5 min
94°C 20 sec	94°C 45 sec	95°C 10 sec
68°C 30 sec	63°C 45 sec	55°C 10 sec
72°C 5 min	72°C 45 sec	72°C 25 sec
4°C ∞	72°C 5 min	4°C ∞
	4°C ∞	

3.2.3 Poly(I:C) injections of Mx-1 Cre mice

To induce the expression of the Cre transgene controlled by the Mx-1 Cre promoter, 6 to 12-week-old mice were injected intraperitoneally (i.p.) with poly(I:C) (Invivogen, 13 mg/kg) three times (day 1 – day 3 – day 5). Further experiments were started 2 weeks later.

3.2.4 LPS/GalN injections

In order to induce hepatic damage, mice received i.p. injections of LPS/GalN (LPS: 10 µg/kg, GalN: 1 g/kg). In control-treated animals only the carrier solution (PBS) was administered. Depending on the specifications of the experiment, mice were either observed until they died because of the liver injury or were sacrificed at defined time points.

3.2.5 Collection of blood and measurement of ALT/AST levels

Wherever possible blood was collected from the portal vein, transferred to EDTA-coated tubes and kept on ice until further processing. After centrifugation at 3000 rpm for 15 minutes, serum was transferred into fresh tubes and stored at 4°C (short term) or -20°C (long term). 50 µl of serum were diluted with 150 µl water and ALT and AST levels were determined by use of the Cobas c 111 analyzer (Roche). When necessary, higher dilutions were prepared.

3.2.6 Collection of tissues

Livers were removed and a part of each organ was snap-frozen on dry ice and stored at -80°C for later assays. One lobe of the liver of each mouse were put into a fixative (Roti®-Histofix 4 %) and kept at 4°C overnight.

3.2.7 Preparation of protein extracts from liver and spleen

Protein extracts were produced using the homogenizer Precellys 24 (PqLab) together with the recommended tubes and ceramic beads. Depending on the further use, tissues were either lysed in ProteoJET™ Mammalian Cell Lysis Reagent from Fermentas (ELISA) or in RIPA buffer (Western blot analysis) after adding Halt Protease and Phosphatase Inhibitor Cocktail (Pierce). After homogenization,

lysates were centrifuged (13000 rpm, 15 minutes, 4°C) and supernatants transferred to fresh 1.5 ml tubes. Protein extracts were stored at -80°C.

RIPA buffer

150 mM NaCl
50 mM Tris/HCl pH 8.0
1 % Triton X-100
0.5 % Sodium deoxycholate
0.1 % SDS

3.2.8 Measurement of protein concentrations

Protein concentrations were determined by use of the Bradford method. 900 µl of water were combined with 200 µl Bradford reagent (Bio-Rad) and 1 ml of that was transferred to a disposable cuvette. 2 µl of protein lysate were added and mixed well with the diluted Bradford reagent by repeatedly inverting the cuvette. The optical density (OD) at a wave length of 595 nm was measured by an Eppendorf BioPhotometer.

3.2.9 Western blot analysis

3.2.9.1 SDS-PAGE

Proteins contained in the extracts were separated according to their size on a denaturing polyacrylamide gel (SDS-PAGE). The components of the separating gel were added to water, mixed, filled into disposable cassettes (Invitrogen) and topped with isopropanol. Following polymerization, isopropanol was removed, the stacking gel was cast on top of the separating gel and combs with the desired pocket size and number were inserted.

Seperating gel – 12 % (1 minigel)

1.55 ml H₂O
2.875 ml Tris/HCl 1.0 M pH 8.8
3.0 ml AA
37.5 µl SDS 20 %
37.5 µl APS 10 %
5.0 µl TEMED

Stacking gel – 4 % (1 minigel)

1.5375 ml H₂O
0.625 ml Tris/HCl 0.5 M pH 6.8
0.335 ml AA
12.5 µl SDS 20 %
12.5 µl APS 10 %
2.5 µl TEMED

20 to 100 µg of protein was used per sample. Protein samples were prepared by diluting the protein lysates with water to the desired volume (20 µl for 12-well combs, 50 µl for 10-well combs) and adding 5X Laemmli buffer containing 3 % β-mercaptoethanol. Prior to use the samples were boiled at 99°C for

5 minutes. Gels were placed into the electrophoresis equipment (Invitrogen XCell SureLock™ Mini-Cell) and the chamber was filled with 1X SDS PAGE Running buffer. Samples were loaded carefully and gels were run at 80 volts until the dye front entered the separating gel and then at 120 V until the dye front reached the bottom of the gel. PageRuler™ Prestained Protein Ladder (Fermentas) was used as a size reference.

5X Laemmli Buffer

0.15 M Tris/HCl pH 6.8
5 % SDS
25 % Glycerol
0.01 % Bromphenol Blue

1X SDS PAGE running buffer

25 mM Tris base
190 mM Glycine
0.1 % SDS

3.2.9.2 Western blotting

After electrophoresis cassettes were opened and gels placed in transfer buffer. Transfer membranes (Millipore Immobilon Transfer Membrane) were cut to the same size as the gel, activated in methanol for 5 minutes and put into transfer buffer. 4 pieces of Whatman® paper were prepared and soaked in transfer buffer just prior to use. The blotting sandwich (2x paper – membrane – gel – 2x paper) was assembled and put into the blotting device (Bio-Rad Trans-Blot® Semi-Dry) according to the instructions. Proteins were transferred to the membrane by applying 22 V for 30 minutes. After transfer the membrane was rinsed with wash buffer PBS-T and blocked with 5 % BSA in PBS-T for 1 hour at 4°C under agitation. After incubation, the membrane was rinsed shortly with wash buffer and the primary antibody was added (anti-MKK7 1:1500, anti-β-actin 1:8000; both in 5 % BSA in PBS-T). The membrane was kept at 4°C on a shaker over night. The following day the membrane was washed extensively before the secondary antibody was added (anti rabbit-HRP to detect MKK7; anti mouse-HRP to detect β-actin; both 1:10.000 in 5 % dry milk in PBS-T). Incubation was done at room temperature with agitation for 1 hour. The membrane was then washed extensively and protein expression detected using the Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare) according to the instructions. The resulting chemoluminescent signal was visualized on autoradiography films (Amersham Hyperfilm ECL , GE Healthcare). Detection of c-Jun protein was done in the same way with some minor modifications. Blocking was performed in 5 % milk in PBS-T for 1 hour at room temperature under agitation. The c-Jun antibody (Cell Signaling) was diluted 1:500 in 5 % BSA in PBS-T and incubated over night at 4°C. To detect the primary antibody, HRP-labeled anti-rabbit antibody was used at a dilution of 1:5000 in 5 % milk in PBS-T for 1 hour at room temperature.

Transfer buffer

25 mM Tris Base
190 mM Glycine
20 % Methanol

Wash buffer PBS-T

PBS
0.1 % Tween20

3.2.10 ELISA

All ELISAs were performed using kits from R&D Systems according to the manufacturer's instructions. For determination of TNF- α or IL-6 levels liver lysates were diluted 1:30 with Reagent Diluent whereas spleen lysates were diluted 1:15. Sample preparation for detection of phospho-JNK was done as described in the manual. Measurement of the ODs at 450 and 560 nm wave length were performed on a microplate reader (Tecan). Cell death ELISAs (Cell Death Detection ELISA PLUS, Roche) were performed according to the instructions.

3.2.11 RNA isolation

Purification of total RNA from liver and spleen tissue was performed using the RNeasy Mini Kit (Qiagen) according to the protocol. Tissues were homogenized together with 350 μ l Buffer RLT supplied with 3.5 μ l β -mercaptoethanol using the Peqlab Precellys 24. After disruption of the tissue the manufacturer's instructions were followed. RNA was eluted in 40 μ l RNase-free water and concentrations were determined using the NanoDrop 1000 Spectrophotometer (Peqlab).

3.2.12 cDNA synthesis and qPCR

cDNA synthesis and qPCR were performed as described in section 3.1.2.4 and 3.1.2.5, respectively. A list of the used primers is shown below.

Name	Sequence
MKK7-RTPCR_fwd	TCCAGATCCCACCAAGCCTGACTATG
MKK7-RTPCR_rev	AATGACTGGAAGTCCCCTGAGAAGCC
cJun-RTPCR_fwd	GGGACACAGCTTTCACCCTA
cJun-RTPCR_rev	GAAAAGTAGCCCCAACCTC
JunB-RTPCR_fwd	AGACACAGGCGCATCTCTGA
JunB-RTPCR_rev	CTGCTGAGGTTGGTGTAGAC
JunD-RTPCR_fwd	TTGAGGGTCTTGACTTCTCCTCCA
JunD-RTPCR_rev	TTTCGCGGGTGCGCCGGGCCCCCTGG
iNOS-RTPCR_fwd	GGCAGCCTGTGAGACCTTTG
iNOS-RTPCR_rev	CATTGGAAGTGAAGCGTTTCG
Gadd45b-RTPCR_fwd	CCAGTCGTTCTGCTGCGACAATGACAT

Gadd45b-RTPCR_rev	CCTCCACCAAGCCTTGGCTTTTCCAGGAA
TNFa-RTPCR_fwd	AACTAGTGGTGCCAGCCGAT
TNFa-RTPCR_rev	CTTCACAGAGCAATGACTCC
TGFb-RTPCR_fwd	CACCGGAGAGCCCTGGATA
TGFb-RTPCR_rev	TGTACAGCTGCCGCACACA
b-actin-RTPCR_fwd	CCTGAACCCTAAGGCCAACCG
b-actin-RTPCR_rev	GCTCATAGCTCTTCTCCAGGG

3.2.13 Embedding of fixed tissues

After fixation overnight (Roti®-Histofix 4 %), tissues were first transferred to 70 % ethanol, then placed into tissue embedding cassettes and subjected to a series of different alcohol concentrations finally being infiltrated with paraffin. This process was done automatically by the Tissue-Tek® VIP® 5 Vacuum Infiltration Processor (Sakura). Tissues were then embedded into paraffin blocks and cooled for sectioning.

3.2.14 H&E staining

5 µm thick sections were layered on glass slides and dried overnight at 53°C. Sections were then dewaxed and stained (hematoxylin and eosin) automatically, mounted and sealed with a coverslip.

3.2.15 TUNEL staining

Paraffin sections were prepared as described above. After dewaxing they were stained with the In Situ Cell Death Detection Kit with Fluorescein (Roche) according to the protocol. For membrane permeabilisation a proteinase K solution (20 µg/ml in Tris-HCl, pH 7.5) was used. Sections were mounted in VECTASHIELD Mounting Medium with DAPI or VECTASHIELD HardSet Mounting Medium with DAPI (both Vector Laboratories) and protected with coverslips. Slides were scanned with the Mirax Scanner (Zeiss) and apoptotic areas were quantified by a computed algorithm developed by the BioOptics Facility of IMP-IMBA.

4 List of Abbreviations

AA	Acrylamide
AB	Antibody
AFP	α -fetoprotein
AHF	acute hepatic failure
ALT	alanine aminotransferase
AP	alkaline phosphatase
AP-1	activator protein 1
APS	ammonium persulfate
ASK1	apoptosis signal-regulated kinase 1
AST	aspartate aminotransferase
ATF2	activating transcription factor 2
CCl ₄	carbon tetrachloride
CMV	cytomegalovirus
ConA	Concanavalin A
DAPI	4',6 -diamidin-2-phenylindol
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DV	docking vector
EB	embryoid body
EC	embryonal carcinoma
EDTA	ethylenediaminetetraacetic acid
EGC	embryonic germ cell
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ERK1/2	extracellular signal-regulated kinase 1/2
ESC	embryonic stem cell
EV	exchange vector
FADD	Fas-associated death domain
FCS	fetal calf serum
GalN	D-galactosamine
GFP	green fluorescent protein
GOI	gene of interest
GPCR	G protein-coupled receptor
GTP	guanosine-5'-triphosphate
HBV	hepatitis B virus
HCV	hepatitis C virus
HE	hematoxylin and eosin
HR	homologous recombination
ICM	inner cell mass
IL-1 β	interleukin-1 β
IL-6	interleukin-6
INF γ	interferon gamma

iPSC	induced pluripotent stem cell
IRES	internal ribosome entry site
JNK	c-Jun NH2-terminal kinase
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
MAF	musculoaponeurotic fibrosarcoma
MAP2K	mitogen-activated protein kinase kinase (= MAPKK, MKK)
MAP3K	mitogen-activated protein kinase kinase kinase (= MAPKKK, MEKK)
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
MHV-3	murine hepatitis virus strain 3
MKK4	mitogen-activated protein kinase kinase 4
MKK7	mitogen-activated protein kinase kinase 7
MLK	mixed lineage kinase
MMTV	mouse mammary tumor virus
NF- κ B	nuclear factor- κ B
NO	nitric oxide
nos2	nitric oxide synthase 2
OD	optical density
PBS	Phosphate buffered saline
PGC	primordial germ cell
PGK	phosphoglycerate kinase
PH	partial hepatectomy
poly(I:C)	polyinosinic-polycytidylic acid
PyMT	Polyoma Middle T
RIP1	receptor interacting protein 1
RNAi	RNA interference
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
siRNA	small interfering RNA
SSR	site-specific recombinase
TAK1	TGF β -activated kinase 1
TEMED	Tetramethylethylenediamine
TGF β	transforming growth factor β
TNF-R1	tumor necrosis factor receptor 1
TNF-R2	tumor necrosis factor receptor 2
TNF α	tumor necrosis factor α
TRADD	tumor necrosis factor receptor-associated death domain
TRAF2	tumor necrosis factor receptor-associated factor 2
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling assay

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6 Curriculum Vitae

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Work Experience

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