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Multigenerational Epigenetic Adaptation of the Hepatic Wound-Healing Response

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Abstract

We asked if ancestral liver damage leads to heritable reprogramming of hepatic wound-healing. We discovered that male rats with a history of liver damage transmit epigenetic suppressive adaptation of the fibrogenic component of wound-healing through male F_1 and F_2 generations. Underlying this adaptation was reduced generation of liver myofibroblasts, increased hepatic expression of antifibrogenic PPAR-γ and decreased expression of profibrogenic TGF-β1. Remodelling of DNA methylation and histone acetylation underpinned these alterations in gene expression. Sperm from rats with liver fibrosis were enriched for H2A.Z and H3K27me3 at PPAR-γ chromatin. These sperm chromatin modifications were transmittable by adaptive serum transfer from fibrotic rats and were induced in stem cells exposed to myofibroblast-conditioned media. A myofibroblast secreted soluble factor therefore stimulates heritable epigenetic signatures to sperm so as to adapt fibrogenesis in offspring. Humans with mild liver fibrosis display *PPAR-γ* promoter hypomethylation compared with severe fibrotics, thus lending support for epigenetic regulation of fibrosis.

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Wound-healing is a complex multi-step and multi-cellular process occurring in all metazoans that operates to restore tissue architecture and function following trauma or damage caused by environmental insults. During embryogenesis mammals repair wounds with remarkable efficiency and without scar (fibrosis) formation[1, 2]. By contrast, the ageing mammal is susceptible to either impaired repair in the form of chronic wounds, or to uncontrolled wound-healing characterised by the progressive deposition of fibrotic tissue. In humans such deviations from optimal tissue repair underlie the development of life-threatening and age-associated pathologies including cancers and tissue fibrosis[3–5]. It is assumed that a combination of genetic and environmental factors dictate observed differences in the susceptibility of individuals to either impaired or uncontrolled wound-healing and their associated pathological consequences. However, the nature of the genetic traits and epigenetic mechanisms that impact on the efficiency of wound-healing and its outcomes remain poorly defined.

Liver cirrhosis is an example of a complex pathology that is highly variable within the patient population[6–8]. The majority of patients with chronic liver disease (CLD) have pathologies with the potential to progress to cirrhosis, but only a minority (10–20%) of patients will ever go onto develop this life threatening end-stage disease. Mouse models suggest that genetic influences on disease progression in CLD are complex and likely to involve interactions of multiple low-impact variants. An alternative hypothesis is that exposure to environmental factors (including microbes and xenobiotics) may trigger adaptive epigenetic mechanisms that impact on the regulation of hepatic wound-healing. Adaptive epigenetic mechanisms may include alterations in DNA methylation, post-translational modifications of histones and control of gene expression by non-coding RNAs[9–13]. As these epigenetic signatures may become stable there is potential for non-genetic transmission of adaptive traits by both mitotic and meiotic routes, with the latter enabling intergenerational inheritance of epigenetically programmed traits. In this study we tested the hypothesis that population variability in hepatic wound-healing may be influenced by environmentally-induced adaptive traits transmitted between generations via heritable epigenetic signatures.

RESULTS

Ancestral liver damage promotes adaptation of hepatic wound-healing

To test our hypothesis we developed a model for investigating multigenerational influences on liver fibrosis using outbred adult male rats (Figure 1a). By selecting outbred animals we mitigate against genetic traits impacting on wound-healing. Our rationale for studying transmission through the male line was to avoid influences from maternal factors either within somatic components of the oocyte or arising from the *in utero* environment, the latter being known to have major epigenetic influences on offspring[14]. The model involved repeated injury of male rats by the hepatotoxin carbon tetrachloride (CCl_4) to induce a state of chronic wound-healing resulting in fibrosis[15, 16]. Cessation of injury allowed spontaneous resolution of fibrosis before the animals were paired with uninjured females for breeding (Supplementary Figure 1a and 1b). By repeating this process in the F_1 offspring and with the inclusion of control sham (olive oil) injured animals we generated four groups of F_2 males

with distinct ancestral histories of liver disease (Figure 1a). Group A had no history of injury in the F_1 or F_0 paternal lines. Group B was derived from injured F_1 fathers and uninjured F_0 grandfathers. Group C had a history of injury at F_0 but not in the F_1 generation. Group D were derived from male lines with injuries at both F_1 and F_0. All four adult F_2 groups were then repeatedly injured with CCl_4 and culled 24hrs after the final CCl_4 administration. Elevated serum liver enzymes (ALT and AST) were observed for all four groups with no significant differences between groups indicating similar degrees of hepatocyte damage (Supplementary Figure 1c). From H&E stained sections (Figure 1b) and pathology grading there were no obvious differences in necrosis or inflammation between groups (Supplementary Figure 1d). Cyp2E1, the major cytochrome p450 protein involved in metabolism of CCl_4 to its reactive trichloromethyl metabolite[17], was expressed at similar levels between groups A and D as was Cyp3A4 which is also implicated in CCl_4 metabolism (Supplementary Figure 1e). Therefore, ancestral liver injury and fibrosis has no heritable influence on CCl_4 metabolism or its induction of hepatocellular damage.

To assess wound-healing, liver sections were stained with Sirius Red (SR) which selectively reacts with fibrotic (type I and III) collagens. Representative SR stained sections (Figure 1b) illustrate for group A the expected network of bridging fibrotic septae. Morphometric analysis of SR stained sections revealed reduced levels of fibrotic matrix in groups B, C and D relative to A (Figure 1c). For groups C and D, where fibrosis had been established at F_0 generation, bridging fibrosis was rarely observed. Relative to group A, a modest reduction in hepatic pro-collagen I transcript expression was detected for group B, with 50% and 90% reduced expression observed for Groups C and D respectively, the latter reaching statistical significance (Figure 1d). We conclude that ancestral liver damage can trigger heritable adaptive mechanisms for suppression of the hepatic wound-healing response. Moreover, since for group C the disease trigger for this adaptation was in the F_0 and not F_1 it appears that this adaptive mechanism can be transmitted in a transgenerational as well as intergenerational manner. Since the only biological material passing between each successive generation is that contained within sperm it is likely that epigenetic modifications either in the mature sperm or germ cells are responsible for intergenerational transmission of adaptation to liver fibrosis. Since in our model animals were mated 14 days after cessation of injury we do not know if an extended delay to enable a new spermatogenic wave to occur would result in loss of the adaptive trait. If this was the case, then adaptation would be transient and mechanistically restricted to events in the mature sperm.

Adaptive suppression of hepatic myofibroblast activation

Next we determined the cellular basis for the influence of ancestral liver damage on wound-healing. The major cellular drivers of liver fibrosis are smooth muscle α-actin (αSMA) positive myofibroblasts. Myofibroblasts are rare in the uninjured liver but accumulate in diseased liver mainly via transdifferentiation (activation) of hepatic stellate cells, which adopt a highly proliferative and profibrogenic phenotype[18, 19]. αSMA positive myofibroblasts were observed within tracts of bridging fibrotic septae and in the proximity of necrotic, ballooning hepatocytes (dark patches in the stained tissue)(Figure 2a). Manual counting of αSMA positive cells revealed reduced numbers of myofibroblasts in groups B, C and D relative to group A (Figure 2b). Livers from animals in groups C and D had

respectively 50% and 30% the number of myofibroblasts in group A livers, which was reflected by reduced abundance of hepatic αSMA mRNA (Figure 2c). Possible explanations for lower numbers of myofibroblasts in groups B–D included a developmental reduction in numbers of hepatic stellate cells prior to injury or defects in the inflammatory response required for activation of hepatic stellate cells[19]. Desmin is a marker for both quiescent and activated hepatic stellate cells suitable for quantification of total numbers of these cells in normal and diseased liver[20]. Sham injured group A and D livers contained similar numbers of desmin positive cells, i.e. approximately 8 cells/field (Figure 2d and f). CCl_4 injury increased these numbers to 26 and 13 desmin positive cells/field in group A and D respectively (Figure 2e and f). These data argue against a developmental influence on numbers of hepatic stellate cells and instead suggest modulation of their activation is influenced by ancestral liver damage. As hepatic TNFα was expressed at similar levels in groups A to D it seems unlikely that there are gross differences in the hepatic inflammatory response (Figure 2g). Moreover, manual counts of macrophages (CD68), T lymphocytes (CD3) and neutrophils did not indicate differences in inflammatory cell recruitment between all four groups of rats (Supplementary Figure 2a). While these data do not completely exclude modulation of inflammation they are more supportive of an intrinsic reprogramming of the hepatic stellate cell phenotype that suppresses transdifferentiation.

To begin to interrogate the molecular basis for modulation of hepatic stellate cell transdifferentiation we selected candidate genes based on their role in wound-healing. Quiescent hepatic stellate cells have an adipogenic-like phenotype controlled in part by nuclear receptors PPAR-γ and PPAR-α which are repressed during transdifferentiation[21]. PPAR-γ expression is repressed by DNA-methylation and MeCP2-dependent chromatin remodelling that is critical for generation of the myofibroblast phenotype[22]. Hepatic expression of PPAR-γ was elevated in groups B, C and D compared with A (Figure 3a), and immunoblotting confirmed raised levels of PPAR-γ protein in group D vs A livers (Figure 3b). This differential expression of PPAR-γ is relevant since ectopic re-expression in myofibroblasts can reverse their phenotype into a more quiescent state[21, 23]. Additionally, we observed increased expression of PPAR-α in group C and D (Figure 3a). In contrast hepatic TGF-β1, a highly profibrogenic factor induced during hepatic stellate cell activation[24, 25], was reduced 2-fold in group D compared to A and trends towards reduced TGF-β1 expression also noted in groups B and C (Figure 3a). By contrast, there were no differences in expression of the TGF-β pseudoreceptor Bambi or the TGF-β1 signalling molecules SMADs 1–6, whereas inhibitory SMAD7 was elevated 5-fold in group D compared with A (Figure 3c). cDNA array analysis identified additional genes displaying differential expression between groups A and D (Supplementary Figure 2b). Of those genes that were either significantly over- or under-expressed in group D several have been implicated as regulators of fibrogenesis and/or hepatic stellate cell activation including Matrix Metalloproteinase 3 (MMP3 = +3.3 fold), IGF Binding Protein 1 (IGFBP1 = +2.8 fold), Secreted Phosphoprotein 1 (SPP1 = +2.5 fold), One Cut homeobox 1/HNF6 (ONECUT1 = −4.8 fold) and Suppressor of Cytokine Signaling 2 (SOCS2 = −2.4 fold)[26, 27]. We conclude that liver damage has an intergenerational influence on the transcriptional control of multiple genes in hepatic stellate cells.

Renal fibrosis is not influenced by ancestral liver damage

A question arising from our observations was whether fibrosis in the liver has a global impact on wound-healing in future generations. To address this we generated F_1 adult male rats that either had history of CCl_4-induced parental liver fibrosis (PLF) or control parental sham injury (PSI) (Figure 4a). Renal injury was then induced in PLF and PSI groups using the unilateral ureteral obstruction (UUO) model. Histological SR (Figure 4b and 4d) and αSMA (Figure 4c and 4e) stains revealed no differences in collagen deposition or myofibroblast accumulation respectively between the two groups of animals. Since the PLF group historically resemble group B from the multigenerational model in that these animals will have inherited a suppressed hepatic wound-healing response associated with reduced expression of collagen I (Figure 1c and 1d) and αSMA (Figure 2b and c).

From this experiment we conclude that ancestral liver injury does not have a global impact on fibrogenesis. However, more extensive investigations employing a variety of tissue injury models in multiple organs, and preferably with F_2 generations, will be required to establish if the adaptive response is tissue restricted. A further caveat is that because the nature of the tissue injury and repair mechanism differ profoundly between the CCl_4 and UUO models, it will be important in future studies to determine if distinct injury, inflammatory and wound-healing events influence the adaptive process.

Epigenetic modifications regulate hepatic myofibroblast activation

Despite not yet having definitive evidence for the molecular basis of the adaptive response, the observation that hepatic expression of PPAR-γ and TGF-β1 were modulated in injured offspring is important since both genes are important regulators of the hepatic stellate cell phenotype. We therefore reasoned that examining the epigenetic profile of these two genes should provide further mechanistic insights. Preliminary experiments using DNA methylation-sensitive restriction enzyme mapping indicated hypomethylation of PPAR-γ and hypermethylation of TGF-β1 genes in group D compared with group A (Supplementary Figure 3a and b). To refine the methylation profiling and identify differences in methylation at specific CpG sites we developed quantitative sequence-specific CpG methylation (pyrosequencing) assays for the rat PPAR-γ, PPAR-α and TGF-β1 genes (Supplementary Figures 4a–c). Pyrosequencing across the PPAR-γ promoter identified altered methylation at five consecutive CpG sites within a 20bp sequence (Figure 5a). At the three central sites in this sequence (CpG2, CpG3 and CpG4) reduced methylation was found in groups B, C and D. In each case group D was associated with the lowest level of methylation at these sequences, in the case of the most central CpG sequence (CpG3) we detected 7% methylation in group A compared with 2% in group D. Similar analysis of the PPAR-α promoter identified a single CpG site which was hypomethylated in groups B, C and D (Figure 5b). Pyrosequencing of the TGF-β1 gene identified four CpG sites displaying modest increases in methylation which for two adjacent CpG sites in the intron 1 region reached statistical significance (Figure 5c). Since DNA methylation is in part regulated by DNA methyltransferases we measured their hepatic expression of Dnmt1, Dnmt3a or Dnmt3b but observed no differences (Supplementary Figure 5a). We also failed to detect differences for CpG methylation at the PPAR-γ promoter in splenic tissue (Supplementary Figure 5b). This finding suggests that remodelling of DNA methylation is to a degree tissue-

specific, although without a wider examination of other organs and tissues we cannot yet state whether this is a liver-specific process.

One way in which DNA methylation may regulate gene transcription is by modelling basal states of histone acetylation which impact on the accessibility of chromatin for transcription factors[28]. Quantitative chromatin immunoprecipitation (qChIP) assays revealed higher enrichment of AcetH3 at the PPAR-γ promoter in group D compared with group A, whereas AcetH3 at the TGF-β1 promoter was reduced in group D relative to A (Figure 5d). Hence the observed adaptive changes in expression of fibrogenic regulators are underpinned by remodelling of DNA methylation and associated histone acetylation such that antifibrogenic PPAR-γ is expected to become more transcriptionally permissive whereas profibrogenic TGF-β1 would be predictive to be less permissive for transcription.

Sperm chromatin modifications correlate with wound-healing adaptation

We next investigated potential mechanisms by which the damaged liver may exert an intergenerational influence on hepatic stellate cell gene expression and wound-healing. Since sperm represent the only biological material that transfers from injured animals to offspring in our model, a critical question to address is how liver fibrosis communicates epigenetic adaptation to sperm. Pilot studies failed to detect DNA methylation remodelling events in the testes of F_1 fathers similar to those we described at the PPAR-γ and TGF-β1 genes in F_2 livers. While this preliminary observation does require further investigation, we reasoned that because DNA methylation is globally erased once during spermatogenesis and then again during embryonic development[29, 30], chromatin modifications in sperm may be implicated. We were particularly interested to focus on chromatin signatures that have an influence on annotation of DNA methylation. In this regard, the presence of the histone variant H2A.Z is reported to be mutually exclusive with DNA methylation and its incorporation into nucleosomes may suppress CpG methylation during embryo development[29, 30]. Genome-wide studies in embryonic stem cells reveal that H2A.Z is enriched at the promoters of genes functioning in cell differentiation and development[31]. Interestingly the majority of genes occupied by H2A.Z are targets for the transcriptional repressor Polycomb proteins including the PRC2 proteins that regulate H3K27 trimethylation (H3K27me3)[31]. Since we have reported previously that PPAR-γ is a Polycomb regulated gene[22] we were intrigued to determine whether H2A.Z is incorporated into chromatin at the PPAR-γ promoter in sperm in response to liver fibrosis. Of note, although the majority of histones are replaced with protamines during development of sperm, a significant proportion of the genome of mature sperm remains associated with histones including variant H2A.Z[32, 33]. Sperm were isolated from male rats that had been injured with CCl_4 or vehicle control for 4 weeks and then allowed to recover for 2 weeks, during which time wound-healing fully resolves. Crosslinked qChIP was then used to quantify incorporation of H2A.Z and H3K27me3 into chromatin at the PPAR-γ promoter (Figure 6a). Both histone signatures were associated with the PPAR-γ promoter and were found at increased levels from sperm of rats recovering from fibrosis relative to controls. H3K27me3 was only modestly enriched, whereas H2A.Z was found at 6-fold higher levels suggesting that the variant histone is incorporated into PPAR-γ chromatin as a consequence of liver damage. To confirm this observation we carried out an identical analysis in

chromatin prepared from the sperm of rats injured by bile duct ligation (BDL), a surgical liver injury model which causes severe tissue damage and fibrosis but by injury mechanisms which are distinct from the CCl_4 model[34]. We again observed a modest enrichment of H3K27me3 and a substantive 4-fold increased incorporation of H2A.Z (Figure 6b). From these data we suspected that liver damage results in the accumulation of a soluble factor in serum that can modify chromatin structure either in germ stem cells or/and mature sperm. To test this idea we repeated the qChIP analysis of histone signatures in sperm from uninjured rats that had undergone repeated serum transfers from rats that had been injured with CCl_4 for 4 weeks prior to a 48hrs recovery to ensure clearance of the chemical and its active metabolites (Figure 6c). Remarkably, serum transfer induced a modest increase in PPAR-γ-associated H3K27me3 and a 15-fold enrichment of H2A.Z. ChIP assays at the TGF-β1 gene did not demonstrate alterations in chromatin associated H2A.Z but detected reduced H3K27me3 both in sperm from CCl_4 injured rats and sperm from animals in the serum transfer experiment (Supplementary figure 6).

We next asked if hepatic stellate cell-derived myofibroblasts may be a source of the soluble mediator of PPAR-γ chromatin remodelling. Isolated primary hepatic stellate cells will undergo myofibroblast transdifferention when cultured on plastic and in full media[18, 35]. We therefore collected media conditioned by cultured, activated rat hepatic stellate cells (HSCs) and added this to cultures of rat bone marrow-derived mesenchymal stem cells (rMSCs). ChIP analysis showed a 3-fold enrichment of H3K27me3 and a 4-fold increase in recruitment of H2A.Z to the PPAR-γ promoter (Figure 6d). To determine the relevance of these data for human liver disease we next collected conditioned media from cultures of quiescent or activated primary human hepatic stellate cells and exposed three independently prepared and phenotyped human MSCs (hMSCs) to the media (Figure 6e and supplementary figure 7a–c). We again observed increased association of H3K27me3 and H2A.Z with the PPAR-γ chromatin in hMSC exposed to conditioned media from activated human HSCs compared with quiescent HSC cultures, suggesting that the factor/s responsible for observed histone modifications are released by activated HSCs. Ideally these studies would have been carried out with mature sperm (or germ cells), but these data are indicative of secretion of a soluble factor from activated hepatic stellate cells that has the ability to modify chromatin in cell types that are not of hepatic origin.

Finally, we were eager to determine if there is any clinical relevance of epigenetic remodelling with liver fibrosis. Specifically we asked if differences in PPAR-γ promoter methylation can be observed between human livers with mild (Kleiner score 0–2) or severe (Kleiner score 3–4) fibrosis. DNA was prepared in a blinded manner from archived liver biopsy samples from a well characterised cohort of males between the ages of 46 to 65 with biopsy proven non-alcoholic fatty liver disease (NAFLD) (for details see supplementary figure 7d). Pyrosequencing targeting two me-CpG sites in the human PPAR-γ promoter demonstrated hypermethylation (approx 10% higher at both dinucleotides) associated with severe versus mild fibrosis (Figure 6f). These latter data lend support to the idea that wound-healing in the human liver may be influenced by epigenetic signatures.

DISCUSSION

The concept that epigenetic signatures can be inherited in an intergenerational fashion is supported by recent experimental studies. Alterations in germ cell DNA methylation caused by *in utero* exposure to vinclozolin mediate transgenerational transmission of adult-onset pathologies in multiple organs[36]. Ng *et al* recently reported that feeding adult male rats a high fat diet led to insulin-resistance in female offspring associated with alterations in the expression of 642 β-cell genes[37]. Feeding a low protein diet to male mice altered global CpG methylation patterns in offspring associated with adaptation of hepatic lipid and cholesterol metabolism[38]. Here we report that hepatic wound-healing may also be subject to intergenerational adaptation, such that an ancestral history of liver fibrosis appears to suppress fibrogenesis in future generations. The adaptive effect on fibrogenesis was apparent within a single generation but was more pronounced where liver fibrosis had been present in successive F_0 and F_1 animals suggestive of a cumulative process. Intriguingly we were unable to demonstrate adaptation of wound-healing outside the liver since renal injury triggered similar fibrogenic responses in the offspring of paternal parents with, or without, history of liver fibrosis. While it is tempting to speculate that this apparent tissue-specific adaptation may be important for ensuring appropriate control of matrix remodelling events during embryogenesis and for maintaining optimal tissue repair responses in adult organs; it is important to note that our studies were limited to a single injury model in the kidney of F_1 generation males and so it is premature to unequivocally rule out an adaptive impact on extra-hepatic tissues.

At the cellular level, a reduction in the number of hepatic stellate cells that underwent myofibroblast transdifferentiation was the most obvious difference we observed in the liver of adapted offspring. This effect was associated with changes in the expression of fibrogenic proteins and of a number of genes known to influence the hepatic stellate cell phenotype. By contrast we did not observe any underlying modification of the injury or inflammatory response, although it remains possible that subtle changes in the function or distribution of immune cell populations may have influences not examined here. Furthermore, we have yet to identify the changes in gene expression which are responsible for suppression of hepatic stellate cell transdifferention in adapted animals. Answering each of these remaining questions will require detailed *ex-vivo* phenotypic characterisation of isolated and purified hepatic non-parenchymal cells from F_1 and F_2 generations. Despite these mechanistic limitations of our study, we demonstrated that the injured livers of F_2 generation adapted males displayed a relative over-expression of the master transcriptional repressor of hepatic stellate cell transdifferentiation PPAR-γ, combined with relative under-expression of the major autocrine/paracrine fibrogenic growth factor TGF-β1. These expression changes were associated with remodelling of DNA methylation at specific CpG dinucleotide sequences in the promoter regions of both genes, with hypomethylation versus hypermethylation for PPAR-γ and TGF-β1 respectively. Hypomethylation in the anti-fibrogenic PPAR-α gene promoter supported a role for DNA methylation in the adaptive process and indicated the need in future studies to adopt genome-wide approaches to map the influences of ancestral fibrosis on remodeling of the hepatic stellate cell methylome. A further limitation of our DNA methylation analysis was the use of genomic DNA from whole liver tissue. It is well

established that DNA methylation can be annotated in a cell-specific manner[39–42]. Hence the relatively modest methylation changes we detected at the PPAR-γ and TGF-β1 loci may reflect a restricted mechanism of methylome remodelling targeted to hepatic stellate cells which are only a minor cellular component of the liver, even in the diseased state. Genome-wide DNA methylation analysis of purified populations of individual types of liver cells will be important to determine if methylome remodelling is indeed cell-specific and would also facilitate a more mechanistic evaluation of the molecular basis for the adaptive response to liver fibrosis.

Among the major unanswered questions arising from this work are i) the identity of the factor (or factors) present in the serum of fibrotic male rats that communicates adaptive epigenetic modifications in sperm, and ii) the mechanism(s) that transmit these modifications in sperm to liver cells in the adult offspring. Despite the genome-wide erasure of DNA methylation marks during sperm development[43, 44], it remains possible that remodelling of DNA methylation in germ cells (or sperm) may occur and that fibrosis-induced modifications are retained into the adult in the manner described for imprinted loci[45, 46]. While not ruling out this latter possibility we have offered an alternative, or possibly contributory, mechanism involving modulation of chromatin remodelling in sperm or germ cells. Spermatogenesis in rodents is accompanied by extensive chromatin modifications involving histone acetylation, methylation, phosphorylation and ubiquitination, and the recruitment of histone variants which occurs prior to the removal and replacement of the majority of histone proteins with protamines to enable nuclear condensation in the head of late spermatids[47]. Despite near to complete re-packaging of the sperm genome with protamines, there is increasing evidence that regions of the rodent and human sperm genome remain associated with histones bearing epigenetic modifications resembling those found at the same genome sites in somatic cells[48]. This retention of histone signatures may provide an epigenetic history of spermatogenesis. Here we have shown that liver fibrosis, or exposure of uninjured animals to serum from rats with liver fibrosis, induces chromatin changes at the PPAR-γ gene in sperm involving increased association with the repressive polycomb mark H3K27me3 and the variant histone H2A.Z. The mechanistic association of these chromatin modifications with the adaptive phenotype in offspring remains to be determined, but it is noteworthy that H2A.Z has been implicated in so-called “epigenetic memory” and is antagonistic for DNA methylation[29]. In future work it will be important to establish how H2A.Z is recruited to the PPAR-γ gene in response to liver fibrosis, which other genes in the sperm genome it becomes associated with, and the functional relationship of H2A.Z in sperm with the adaptive modifications in DNA methylation and histone signatures found in the liver of adult offspring. An interesting discovery in the present study was that media conditioned by cultured myofibroblastic rat and human hepatic stellate cells can stimulate H2A.Z recruitment to the PPAR-γ gene in stem cells. This suggests that hepatic stellate cells may provide a source of the putative serum factor(s) that communicates epigenetic modifications to maturing sperm and that the cell culture model provides a useful experimental system to identify the soluble factor(s) and its mechanism of action.

The intergenerational adaptation of hepatic wound-healing which we have described may have wider biological implications and potential clinical relevance. With respect to clinical

relevance, we have provided preliminary evidence for an association of PPAR-γ promoter DNA hypomethylation with milder fibrotic lesions in humans with NAFLD. However, as yet we have no evidence that this epigenetic state was influenced by ancestral events in parents/grandparents or that it is functionally associated with disease progression. More extensive epigenetic profiling in larger populations will be required to determine if PPAR-γ methylation status is associated with fibrosis progression. Gathering evidence for transgenerational influences on liver fibrosis will be very challenging requiring prospective epigenetic studies in families of liver disease patients. Whether the adaptive mechanisms we have described are directed via transient epigenetic events that are remodelled with each new wave of spermatogenesis or are stabilised within germ cells remains to be established. This latter question will be important to resolve prior to any clinical investigations aimed at investigating mechanisms of transgenerational inheritance in humans. Speculating on the biological implications, the liver has a remarkable intrinsic capacity to rapidly, and repeatedly, regenerate lost or damaged parenchymal tissue to maintain its function[49]. As such it can be argued that deposition of fibrotic tissue is detrimental to liver repair while the organ retains its regenerative ability, which in the case of rodents and humans is maintained during the reproductive years. Intergenerational down-regulation of liver fibrogenesis may therefore have the biological advantage of ensuring greater fitness of future generations exposed to the environmental pressures of potent liver toxins.

Online methods

Animal use- Authors hold appropriate licences for animal experiments which were issued/ approved by local ethical committee and UK Home Office.

CCl_4 injury model and breeding protocol- We carried out chronic CCl_4 injury for 4 weeks as previously described[50]. Adult male rats were either sacrificed 1 day after the last CCl_4 treatment, or allowed to recover for 2 weeks after which time they were bred with females. Male offspring was mixed so that father from each treatment group had a male offspring in each of the relevant group in the next generation thus avoiding the possibility of genetic influence over phenotype- five fathers from olive oil treatment group in F0 had a representative male offspring each in the olive oil and CCl_4 treatment groups in F1 and so on.

Immunohistochemistry- We cut and stained liver sections from all animals with Sirius Red, hematoxylin and eosin or FITC conjugated antibody targeting αSMA as previously described[51]. Desmin, macrophage, lymphocyte and neutrophil staining were carried out using anti-desmin antibody D33 (Abcam- ab8470), mouse anti rat CD68 (Serotec- MCA341R), rat anti human CD3 (Serotec- MCA1477) and Naphthol AS-D Chloroacetate (Specific Esterase) Kit (Sigma-Aldrich). Percent area collagen I was determined using Leica QWin V3 imaging software. Necrosis and inflammation were scored using a scale as outlined in Supplementary Figure 1 legend.

Quantitative RT-PCR and western blotting- We carried out qPCR as published previously[22] with the primers listed in Supplementary Table 1. Western blot was done as previously

described[22] using 1 μg/ml anti PPAR-γ antibody (Abcam, ab19481), anti-cytochrome p450 2E1 antibody- (Abcam, ab28146)and anti-cytochrome p450 3A4 antibody (Abcam, ab3572).

Bile duct ligation (BDL)- We performed bile duct ligation/sham as previously described[48]. Briefly, adult male rats were individually anaesthetised by inhalation using 2–3% isofluorane mixed in oxygen. We removed fur from the whole of the abdominal area which was then cleaned and central midline laparotomy incision made from the base of the sternum towards the abdomen (~1cm). We separated bile duct from portal vein and hepatic artery then passed 3 pieces of 5.0 mersilk (Ethicon) under the bile duct and tied each piece of mersilk into a double knot, locating two knots above and one below the place where the bile duct is to be severed. We cut the bile duct and closed the abdomen using an inverted interrupted suture.

Sperm isolation and chromatin preparation- Epididymis and connected vas deferens were detached from testis and seminal vesicle on both sides. Mature sperm was isolated by gently shaking isolated epididymis which resulted in sperm being ejaculated out of the end of attached vas deferens into a cell culture dish containing warm PBS. Purity of sperm was confirmed by microscopy. Chromatin was made from fresh sperm using standard crosslinking method (3 minutes crosslinking in 1% final concentration of fresh formaldehyde at room temperature) and sonication in Diagenode water bath sonicator (10 cycles of 30s on, 20s off at high power). Antibodies used in ChIP were raised against H3K27me3 (Diagenode, pAb-069-050), H2A.Z (Abcam, ab4174) or ChIP control antibody (Abcam). We carried out sperm ChIP as follows- 10μgs of sperm sonicated chromatin was diluted 1 in 10 with ChIP dilution buffer (1.1% Triton X- 100, 1.2mM EDTA, 16.7mM Tris-HCl, pH 8.1, 167mM NaCl) and precleared by incubation with blocked Staph A membranes. Precleared chromatin was incubated with anti H2A.Z, anti H3K27me3 or isotype matched control antibody overnight at 4 degrees C. We added one hundred microliters of blocked Staph A membranes to ChIP reactions for two hours at 4 degrees C, then samples washed, eluted and genomic DNA purified as previously outlined[22]. Quantitative PCR was carried out as previously described[22]. Primer sequences are available on request. Liver tissue ChIP was carried out as in[52].

Promoter region and CpG island estimation- The promoter regions of the genes were estimated using Genomatix Gene2 promoter (Genomatix Software Gmbh, Ann Arbor, MI, USA). Cpg islands were determined by EMBOSS CpG Plot tool (http://www.ebi.ac.uk/Tools/emboss/cpgplot/).

Induction of Unilateral Ureteric Obstruction (UUO)- We induced experimental UUO in adult male rats aged 10 weeks. Rats were individually anaesthetised by inhalation using 2–3% isofluorane mixed in oxygen. A ventral laparotomy incision was made along the linea alba, extending from just above the symphysis pubis to the xyphi sternum. The left ureter was located and ligated three times with 6/0 Mersilk (Ethicon). A double ligature was placed at the superior end of the ureter and one at the inferior end to prevent backflow of urine. A cut was made between the two inferior ligatures. We isolated obstructed and control kidneys from animals 10 days following the operation.

Isolation of genomic DNA- We extracted genomic DNA from rat livers using phenol-chloroform extraction method. Briefly, we added 250 microliters of lysis buffer (125mM NaCl, 12.5mM EDTA, 25mM Tris-HCL pH 7.5, 0.5% SDS) to material that genomic DNA was isolated from, then we added 0.5% [v/v] β-Mercaptoethanol and 200 micrograms proteinase K. We then incubated samples overnight at 55 degrees C, centrifuged at top speed for 5mins and transfer supernatant to a new DNAse free tube, added two volumes of phenol-chloroform, vortexed and centrifuged at top speed for 5mins, transfered aqueous layer to a new tube and add two volumes of 100% ethanol and 2μl of 3M sodium acetate. We incubated samples at −80 for 1 hour, centrifuged the tubes for 15mins at top speed, removed ethanol, air dried pellet on bench and re-suspended DNA in 40μl of Tris-EDTA buffer.

Human male subjects' liver biopsies, genomic DNA isolation, bisulfite treatment and pyrosequencing- Retrospective non-alcoholic fatty liver disease (NAFLD) adult male patient samples were obtained from archives of local Health Trust under a current ethical approval (approval number 06/Q0905/150). Paraffin embedded subject liver biopsy tissue was dewaxed and genomic DNA obtained as described for rat tissues. 2μg of genomic DNA were bisulfite treated by EZ DNA methylation kit (Zymo Research, Irvine CA). Bisulfite modified DNA was amplified by primers either described in Supplementary Table 2 or by Rn-Ppparg-03 and Rn-Ppara-02 PM PyroMark CpG assay (PM00549535, PM00581903 Qiagen). 10 μl of PCR product was used in pyrosequencing reaction. The percentage methylation at each CpG sites was measured by Pyromark Q96 MD (Qiagen) and analysed by PyroQ-CpGTM 1.0.6 software (Biotage, Uppsala, Sweden)[42, 53].

Isolation of rat and human primary hepatic and mesenchymal stem cells- Primary human and rat hepatic stellate cells (HSC) were generated as before[50, 51]. Rat mesenchymal stem cells (MSC) were obtained by culturing isolated bone marrow from adult male rat femurs in MesenCult medium (StemCell technologies) for 2 weeks when they were cultured for 72hrs with conditioned media collected from 48hrs culture of activated rat HSCs. Human bone marrow stem cells were isolated from human bone marrow mononuclear cells (Lonza Biosciences)[54]. Cells were grown and expanded in monolayer culture in Mesenchymal Stem Cell Growth Medium Bullet Kit (Lonza) supplemented with 5ng/ml fibroblast growth factor-2 (R&D Systems Europe Ltd). The phenotype of all donors of MSC was tested by flow cytometry on a FACSCanto II system (Becton Dickinson) using a human MSC Phenotyping Kit (Miltenyi Biotec), with positive staining for CD73, CD90, CD105 and negative staining for CD14, CD20, CD34, and CD45 (Supplementary Figure 7a–c). Experiments were performed using cells at passage 5–7, and all experiments were repeated with cells from 3 donors (21–24 years of age). Donor MSCs were cultured for 72hrs with conditioned media collected from 24 and 48hrs culture of quiescent and activated human HSCs respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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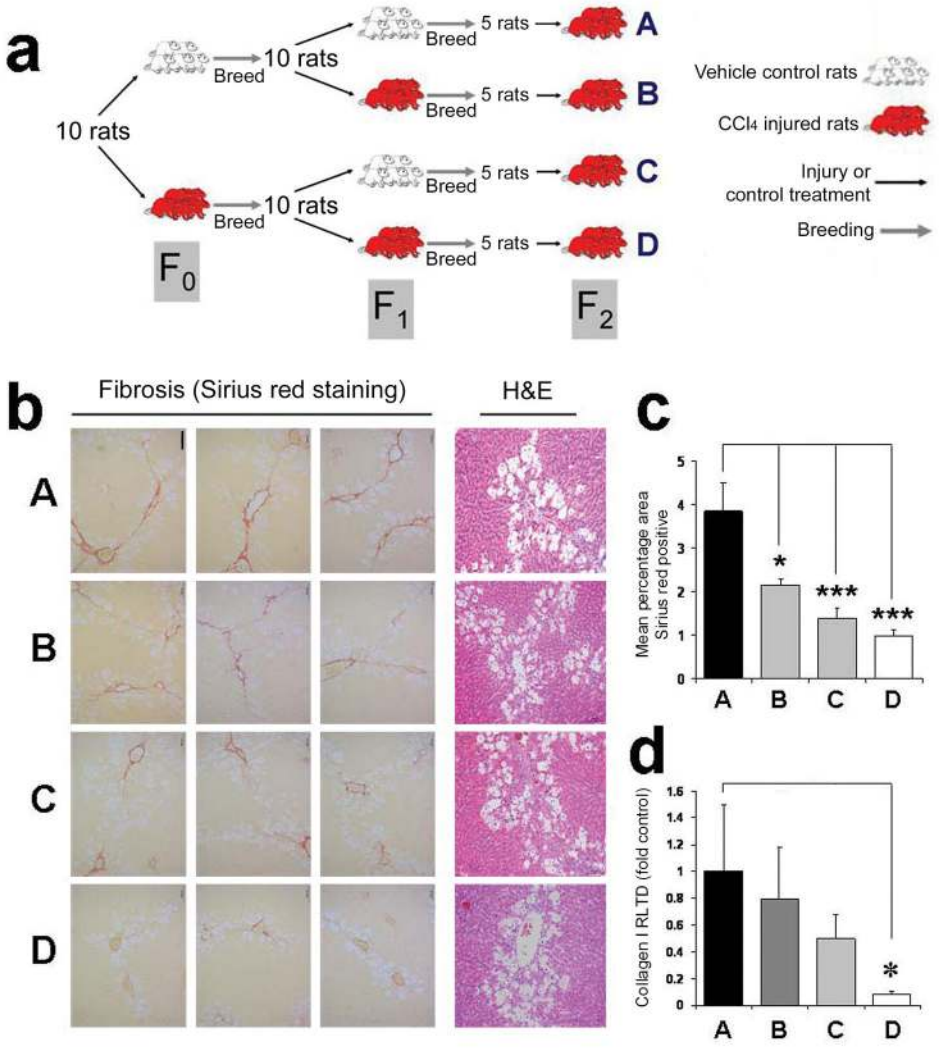

Figure 1. Presence of liver injury in male ancestors reduces liver fibrogenesis in F_2 male offspring

a) Adult outbred male rats were divided into vehicle control or a chronically injured liver group. Five rats from each group were harvested at peak fibrosis while another 5 from each group were bred following complete recovery from liver injury. This procedure was repeated with F_1 generated males to produce F_2 males which were then divided in groups A (no injury in F_0 or F_1), B (Injured F_1, uninjured F_0), C (injured F_0, uninjured F_1) and D (injury in both F_0 and F_1). Groups A-D were chronically injured and tissues collected at peak fibrosis (control uninjured group also included). b) Representative images of Sirius Red (collagen deposition) stained peak fibrosis livers from groups A to D. Panel in each row shows three separate animals. Scale bar, 100μm. Right hand side panel- representative H&E stained livers showing comparable level of liver injury in all groups. c) Percentage area collagen I and d) mRNA level of collagen I expressed as relative level of transcription difference (RLTD) in peak fibrosis livers of groups A to D rats. Error bars are means ± s.e.m (n=5). Statistical analysis; one way parametric analysis of variance (ANOVA), Tukey-Kramer post test. (*) $p<0.05$, (***) $p<0.001$.

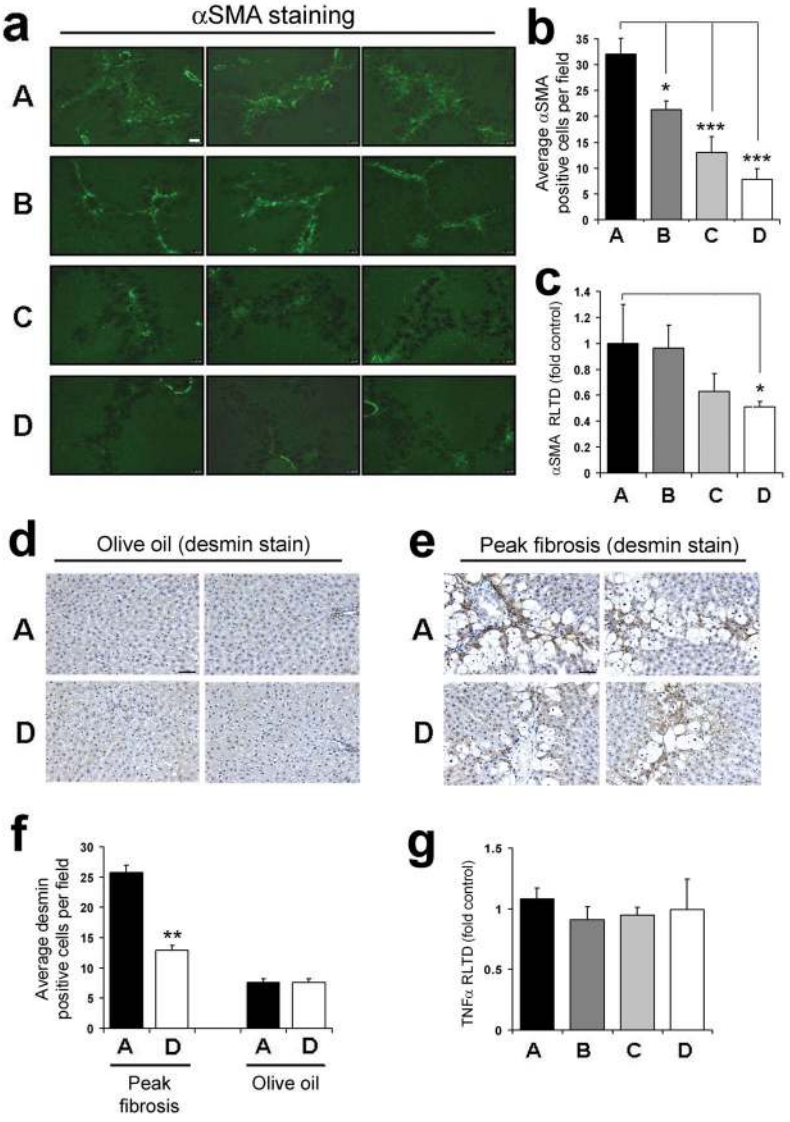

Figure 2. Paternally transmitted adaptation from fibrogenic response in chronic injury is mediated via a decrease in αSMA positive myofibroblasts in the liver

a) Representative images αSMA stained peak fibrosis livers from groups A to D. Panel in each row shows three separate animals Scale bar, 100μm. b) Average number of αSMA positive cells and c) mRNA level of αSMA expressed as relative level of transcription difference (RLTD) in peak fibrosis livers of groups A to D rats. Error bars are means ± s.e.m (n=5). Statistical analysis; one way parametric analysis of variance (ANOVA), Tukey-Kramer post test. (*) $p<0.05$, (**) $p<0.01$ and (***) $p<0.001$. d–e) Representative images of desmin stained olive oil control (d) and peak fibrosis (e) livers from groups A to D. Panel in each row shows two separate animals Scale bar, 50μm. f) Average number of desmin positive cells in control or injured groups A and D. Statistical analysis; two-tailed Student's t-test, (**) $p<0.01$. g) mRNA level of TNFα expressed as relative level of transcription difference (RLTD) in peak fibrosis livers of groups A to D rats. Error bars are means ± s.e.m (n=5).

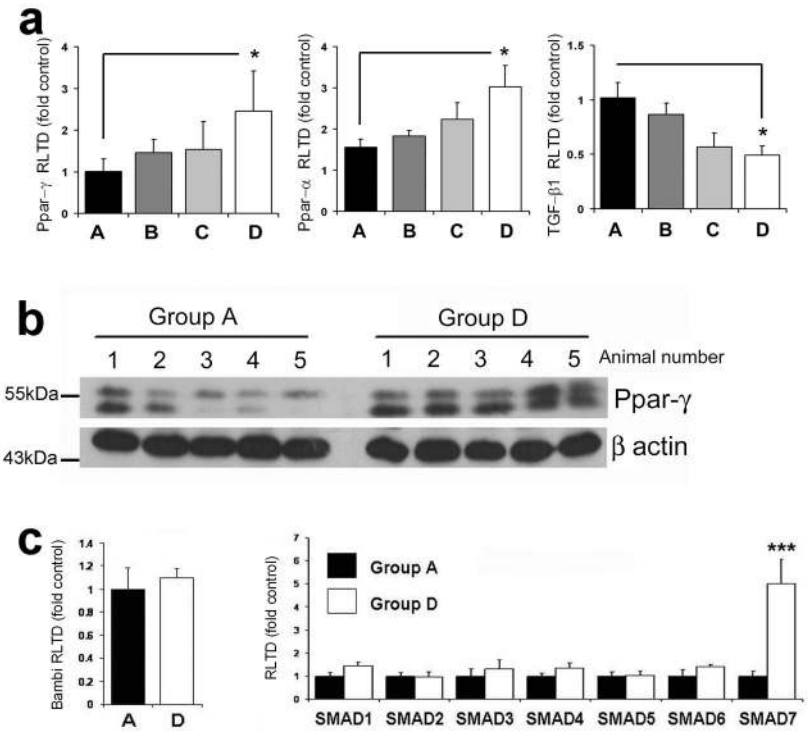

Figure 3. Protected animals have altered expression of profibrogenic and antifibrogenic genes in their livers

a) mRNA level of PPAR-γ, PPAR-α and TGF-β1 expressed as relative level of transcription difference (RLTD) in peak fibrosis livers of groups A to D rats. Error bars are means ± s.e.m (n=5). Statistical analysis- one way parametric ANOVA, Tukey-Kramer post test for A to D groups where (*) indicates $p<0.05$. b) Western blot detection of PPAR-γ and β actin in group A and D peak fibrosis livers c) mRNA level of Bambi and SMADs 1 to 7 expressed as relative level of transcription difference (RLTD) in peak fibrosis livers of groups A to D rats. Error bars are means ± s.e.m (n=5). Statistical analysis; Student's t-test, (***) indicates $p<0.001$.

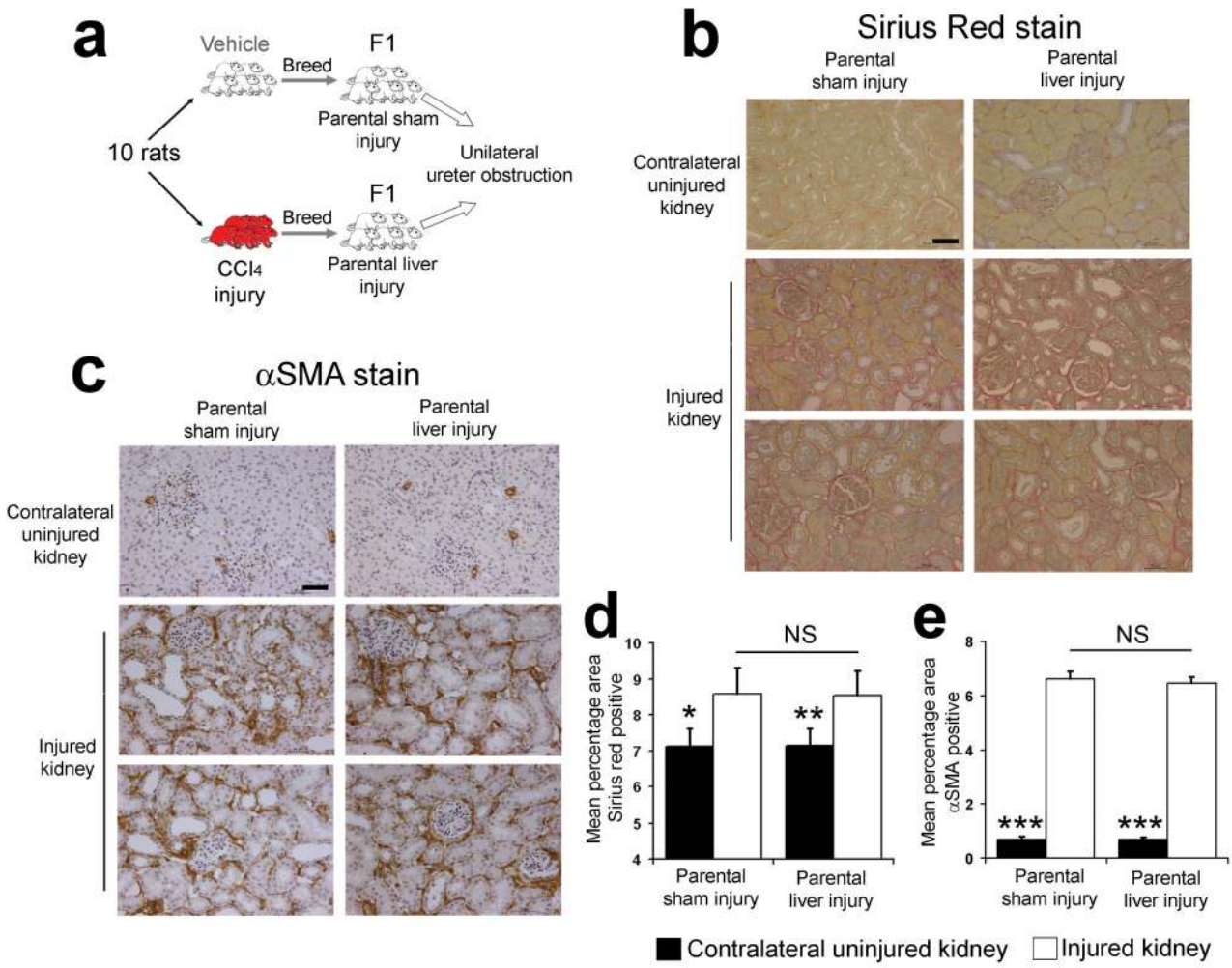

Figure 4. Ancestral injury influenced fibrogenic response is limited to liver

a) Adult outbred male rats were divided into vehicle control or a chronically injured liver group. Five rats from each group were bred following complete recovery from liver injury. Males produced from the breeding cycle (F1) were subjected to unilateral ureter obstruction (UUO) and fibrotic and control kidneys harvested 7 days after the surgery (n=10). b) and c) Sirius red (b) and αSMA (c) staining in contralateral uninjured or obstructed kidneys in control and ancestral liver injury groups of rats. d) and e) Percentage area Sirius Red (d) and αSMA (e) positive in contralateral uninjured or obstructed kidneys in control and ancestral liver injury groups. Error bars are means ± s.e.m (n=7). Statistical analysis; Student's t-test, where (*) in d) indicates p=0.041, (**) p=0.028 and in e) (***) indicates p<0.001.

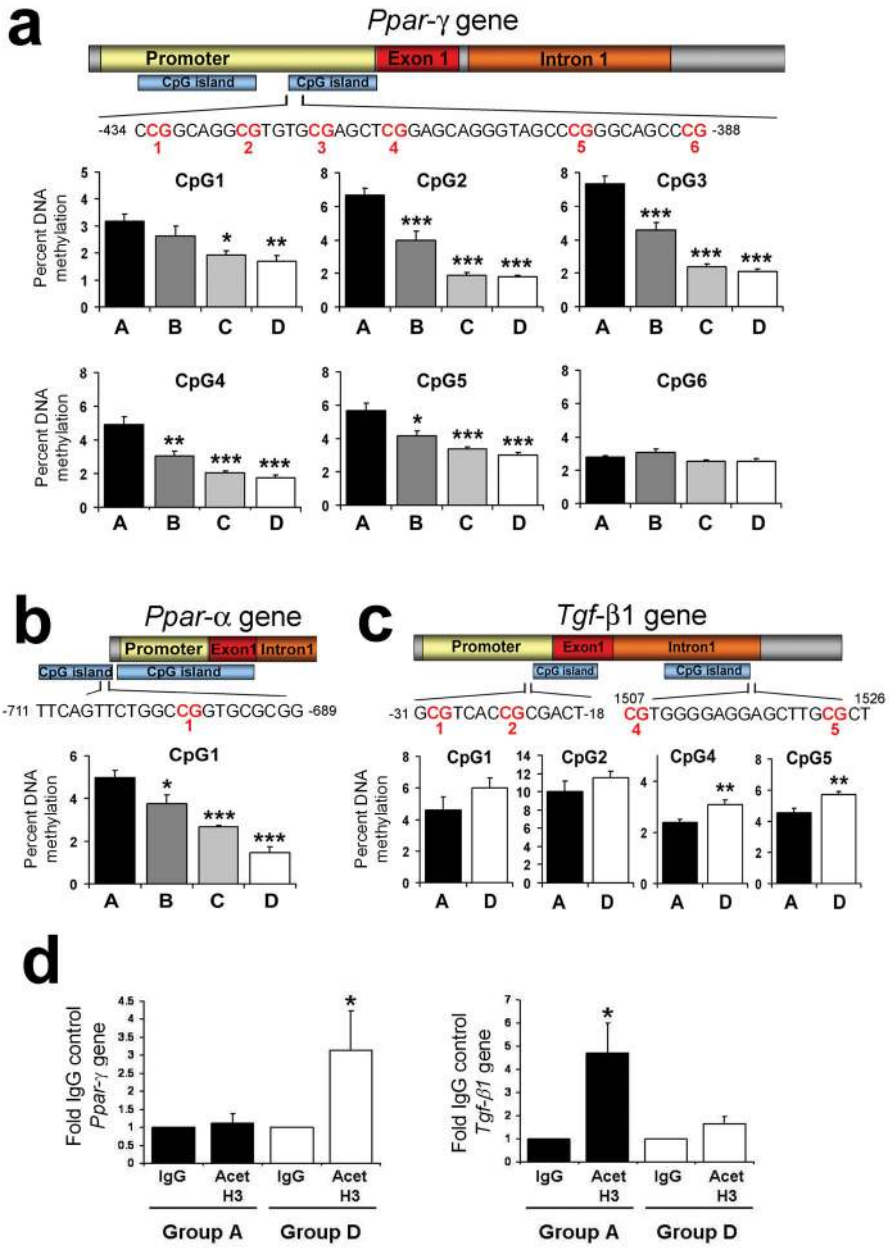

Figure 5. Altered expression of profibrogenic and antifibrogenic genes in the livers of protected animals is underpinned by differences in DNA methylation

a) DNA methylation at particular CG dinucleotides within PPAR-γ promoter in peak fibrosis livers from A to D groups of rats determined by pyrosequencing. Position of the differentially methylated CGs is shown in the schematic drawing above the graphs. Differences are expressed as percentage DNA methylation (n=5). Statistical analysis; one way parametric ANOVA, Tukey-Kramer post test. (*) $p<0.05$, (**) $p<0.01$ and (***) $p<0.001$. b) and c) Pyrosequencing as in a) determined DNA methyation in PPAR-α promoter (b) TGF-β1 promoter and intron 1 (c). Position of the differentially methylated CGs is shown in the schematic drawing above the graphs. Differences are expressed as percentage DNA methylation (n=5). Statistical analysis; one way parametric ANOVA,

Tukey-Kramer post test. (*) $p<0.05$ and (***) $p<0.001$ for PPAR-α and two tailed Student's t-test, (**) indicates $p<0.01$, for TGF-β1. d) ChIP analysis of acetylated H3 enrichment at the PPAR-γ gene promoter (left panel) and TGF-β1 promoter (right panel) in peak fibrosis livers of groups A to D rats. Results were expressed as fold control IgG (n=5). Statistical analysis; Student's t-test, $p=0.0159$ (*) and $p=0.0278$ (*) for PPAR-γ and TGF-β1 respectively.

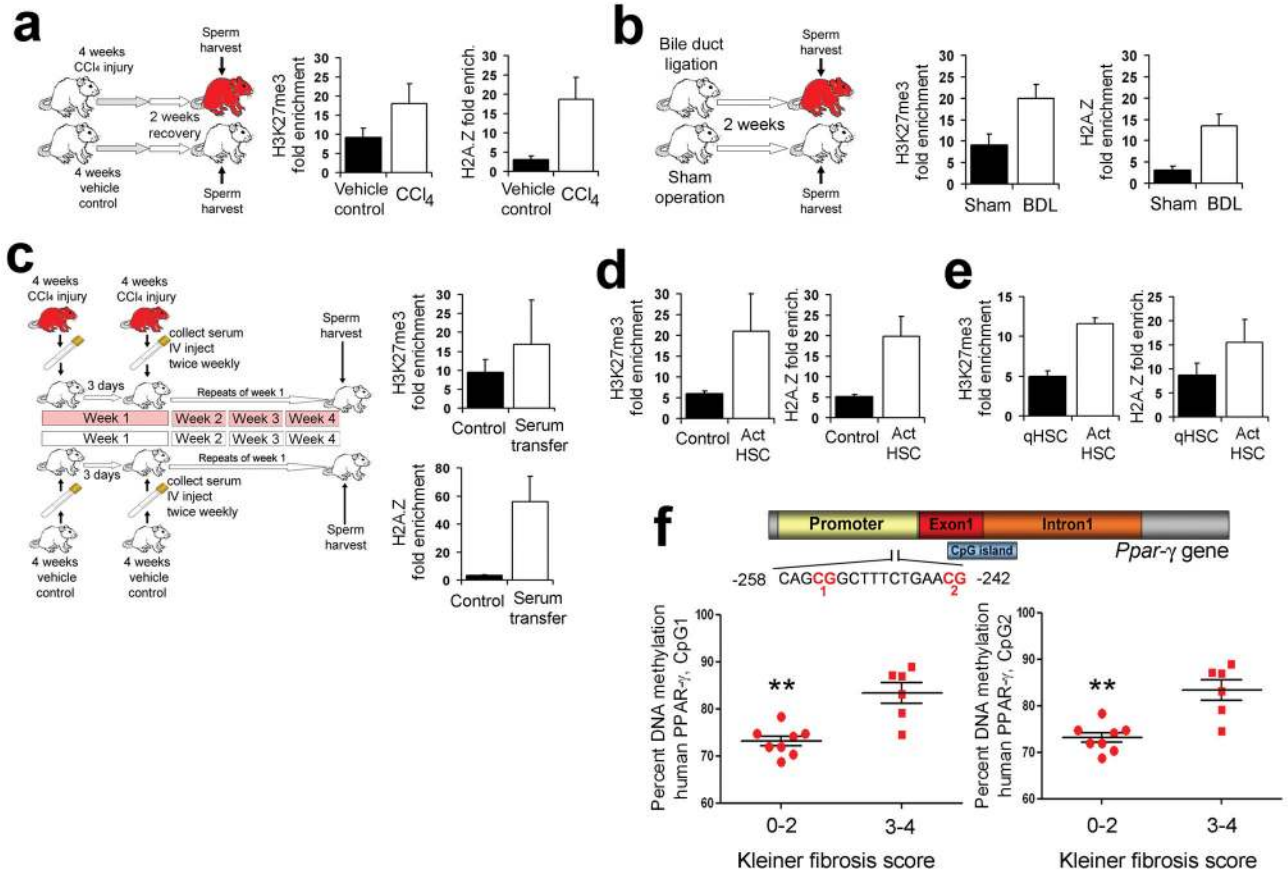

Figure 6. Extrahepatic transmission of epigenetic modifications and evidence for modifications in DNA methylation at fibrogenic regulator gene associated with liver disease progression in humans

a–b) ChIP analysis of trimethylated H3 lysine 27 (H3K27me3) and histone variant H2A.Z enrichment at the rat PPAR-γ gene promoter in mature sperm collected from male adult rats that were given chronic CCl_4 or olive oil (control) for 4 weeks, then recovered for 2 weeks (n=5). All ChIP results in a) to e) are expressed as fold control isotype matched antibody. b) ChIP analysis as in a) was carried out on mature sperm isolated from male adult rats that underwent bile duct ligation (BDL) or sham operation (control) 15 days previously. c) ChIP analysis as in a) was carried out on mature sperm isolated from rats that received twice weekly intravenous serum transfers (for four weeks total) from control or rats that were given CCl_4 for 4 weeks and serum collected 48hrs following last injection (n=6) d) ChIP analysis as in a) was carried out on primary rat mesenchymal stem cells which were treated with control or 48hrs conditioned activated HSC media for 72hrs (n=3). e) ChIP analysis as in a) was carried out on human PPAR-γ gene promoter in primary human mesenchymal stem cells which were treated with quiescent (day 1) or activated HSC (day 15) conditioned media for 72hrs (n=3). f) DNA methylation at particular CG dinucleotides within human PPAR-γ promoter in NAFLD patients liver biopsy tissues was determined by pyrosequencing. Position of the differentially methylated CGs is shown in the schematic

drawing above the graphs. Differences are expressed as percentage DNA methylation Statistical analysis; Mann Whitney test, where p=0.0013 for CpG1 and p=0.0047 for CpG2.