**Transcriptome-wide analysis suggests that temporal changes in the relative contributions of hyperplasia, hypertrophy and apoptosis underlie liver growth in pregnant mice**

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**Running title:** Cellular mechanisms in gestational liver growth

**Summary sentence:** Gestational liver growth involves hyperplasia, hypertrophy and apoptosis which differ in contribution with progressing pregnancy.

**Key words:** Microarray, DNA methylation, hydroxyl DNA methylation, bisulphite sequencing

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**Abstract**

Maternal liver undergoes structural and metabolic changes during pregnancy to meet the

demands of the developing fetus. In rodents, this involves increased liver weight, but the

mechanism remains unclear. To address this, we analysed the histology, gene expression and

DNA methylation of livers of non-pregnant and pregnant C57/BL6 mice. Gestational liver

growth in pregnant mice was accompanied by increased hepatocyte area and lower cell density (days 14,18). Expression of cell proliferation markers was increased on days 14 and 18. 115 genes were differentially expressed on day 14 and 123 genes on day 18 (79 on both days). Pathway analysis indicated that pregnancy involves progressive increase in cell proliferation and decreased apoptosis. This was confirmed using archived data from the FVB wild-type mouse liver transcriptome. Four differentially DNA methylated and two differentially DNA hydroxymethylated regions identified on days 14 and 18 by methylome-wide analysis, but were not associated with altered gene expression. Long interspersed nuclear element-1 hypomethylation on days 14 and 18 was accompanied by increased ten-eleven translocase-2 and decreased DNA methyltransferase 3a and 3b expression. These findings suggest that gestational liver growth involves increased mitosis and hypertrophy, and decreased apoptosis contingent on pregnancy stage. Such changes may involve repetitive sequence, but not gene specific, DNA methylation.

**Introduction**

Pregnancy involves adaptions to maternal liver metabolism to meet the nutritional and metabolic demands of the mother and the developing fetus [1-3]. In rodents and other small mammals, such metabolic adaptions [4-6] are accompanied by a marked increase in liver size to increse the functional capacity of the organ [7-9]. Gestational changes in liver weight are related to the demands of the conceptus such that in rodents litter size explains over 50% of the variation in the liver weight of the dam [10]. Thus characterisation of the mechanisms that underlie gestational liver growth is important for understanding the reproductive physiology of rodents and other species that exhibit increased liver weight during pregnancy. Furthermore, although the liver of pregnant women does not increase in size during pregnancy [11], rodents are often used as models of human pregnancy. Understanding the regulation of liver growth in rodents may provide important insights into the findings of experiments using such models.

The mechanisms responsible for pregnancy-induced liver growth are poorly understood, but may include changes in hepatocyte size (hypertrophy), proliferation (hyperplasia) or apoptosis, or a combination of these processes. There is conflicting evidence about whether hepatocyte hypertrophy or hyperplasia, or both, are involved. This may reflect, at least in part, differences in experimental design. Hypertrophy without hyperplasia has been reported in some studies [12, 13] and pregnancy has been shown to increase the regenerative capacity of the liver, primarily as the result of hypertrophy rather than hyperplasia [7, 14]. Moreover, hypertrophic liver cells can undergo increased proliferation during the *post-partum* period, which suggests that mitosis may be suppressed during pregnancy [14]. These findings are consistent with increased expression of the cell cycle activation marker Ki67 in pregnant liver without an increase in mitotic figures [10]. In contrast, others have shown that liver DNA content at term is almost twice that of non-pregnant rats and correlated positively with liver weight [15]. This suggests that pregnancy-induced cell cycle activation can progress through mitosis. Furthermore, comparison of the rat liver transcriptome at term pregnancy with non-pregnant animals showed changes in several genes that were consistent with active mitosis [15]. However, neither the overall effect on the transcriptome nor the pathways affected were reported. Furthermore, limiting analysis to term pregnant and non-pregnant liver prevented characterisation of gene regulation with increasing gestational age.

Epigenetic processes confer regulation of transcription across different timescales [16, 17]. Importantly, such processes exhibit plasticity and can be modified by endocrine factors [18] although the underlying mechanisms are not understood. DNA methylation has been suggested to represent the equilibrium of methylation reactions [19] catalysed by DNA methyltransferases (Dnmts) [17] and demethylation catalysed by Ten Eleven Translocases (Tets) [20]. Altered epigenetic regulation of specific genes represents one possible mechanism by which the maternal liver adapts to pregnancy. However, the effect of pregnancy on epigenetic processes in the liver has not been described.

The present study tested hypothesis that liver growth in pregnancy in mice involves changes in the expression of the transcriptome in pathways associated with hypertrophy, cell proliferation and apoptosis. We measured hepatocyte size and the expression of the liver transcriptome at different gestational ages in mice. Furthermore, in order to investigate the mechanisms underlying any pregnancy-associated changes in the gene expression, we measured the DNA methylation and hydroxymethylation status of the maternal liver genome.

**Materials and Methods**

**Ethics**

The study was carried out in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act (1986) under Home Office Licence number 30/2884, and received institutional approval from the University of Southampton Biomedical Research Facility Research Ethics Committee (no ethical approval number).

**Animal procedures**

Virgin female C57/BL6 mice were fed Rat and Mouse No.1 Maintenance Diet (SDS, Essex, UK.; See Supplemental Table 1) *ad libitum* from when they were weaned and maintained on a standard 12 hour light/dark cycle throughout. Female mice at 90 days of age were mated. Once pregnancy had been established by the presence of a vaginal plug, females were removed and caged separately. Age matched virgin females were used as a non-pregnant, comparison group. Pregnant females were killed on pregnancy days 7, 14 and 18 (typical duration of pregnancy in this strain is 21 days). Non-pregnant females were killed at age 90 days. All animals were fasted for 6 hours prior to killing by CO2 asphyxiation with cervical dislocation. Livers were collected, weighed, frozen immediately in liquid nitrogen and stored at -80oC.

**Histological procedures**

Snap frozen livers were fixed in Optimal Cutting Temperature compound (Tissue-Tek, CA, USA), cut into 7 μm sections and mounted onto glass slides. Analysis of morphology, cell size and number was carried by staining with haematoxylin and eosin. Frozen sections were air dried, immersed in 0.1% (w/v) Mayer’s haematoxylin for 10 minutes and washed in running water for 5 minutes. Sections were then immersed in 1% (w/v) eosin for 2 minutes and then washed under running water for 5 minutes. The sections were then dehydrated through increasing concentrations of ethanol, cleared in Clearene (Sigma-Aldrich, Dorset, UK) and mounted in Pertex (Histolab Gothenburg, Sweden). Sections were examined using an Eclipse E600 microscope (Nikon UK Ltd, Surrey, UK) with 40x objective lens. A graticule image taken at the same magnification was used to scale the images. 10 Fields were analysed per section and cell area was measured using image J software (Research Services Branch, Maryland, USA). 5 Non-overlapping fields were analysed per section to determine cell number using the ITCN Image J Pluggin (Bio-image Informatics, California, USA) and drawing an area of 90 x 90 μm. 2 sections were analysed per liver and 3 livers were analysed at each gestational age.

**RNA isolation and microarray analysis**

Whole livers from non-pregnant and pregnant (day 14 and 18) mice (n=8 /group) were ground to a fine powder under liquid nitrogen using a pestle and mortar. RNA was extracted using Trizol (Sigma-Aldrich, Dorset, UK) as described [21]. RNA Quantity and contamination with solvent and protein were assessed using a NanoDrop1000 (Labtech, East Sussex, UK). Transcriptome analysis was carried out using the Illumina MouseRef-8 v2.0 Expression BeadChip. Hybridisation and data normalisation were carried out by the Genome Centre (Barts and The London, School of Medicine and Dentistry, London, UK). Pathway analysis was carried out using Ingenuity IPA software (Qiagen, California). These data were filtered in order to eliminate false positives. Transcripts with detection p > 0.05 and/or with non-significant differences between groups, and/or less than 2-fold change were excluded from further analysis.

In order to extend the number of time points in pregnancy for analysis and to test the generalisability of our findings, we carried out secondary analysis of data derived from analysis of the liver transcriptome of FVB wild-type mice by the Affimetrix Mouse Gene 1.0 ST array held at the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) repository (accession number GSE41438). These data were derived from analysis of the effect of pregnancy on the liver transcriptome at gestational ages 7.5, 10, 15 and 19 days compared to non-pregnant animals. Findings from this experiment published previously were restricted to genes associated with drug metabolism [22]. These genes were not reported in the current analysis.

**Quantitative RTPCR**

Quantitative RT-PCR was carried out essentially as described [23] using primers listed in See Supplemental Table 2. RNA was treated with DNase before conversion to cDNA. cDNA (20μl) conversion was used for PCR amplification using SYBR Green [23]. Data were analysed using the standard curve method [24]. The housekeeping genes, Gapdh and Rpl13a, were selected using the GeNorm kit (PrimerDesign, Southampton, UK) [25].

**Methylation and hydroxymethylation dependent immunoprecipitation with next generation sequencing**

Genomic DNA was isolated [23], quantified using a NanoDrop1000, and the integrity and RNA contamination were assessed by bioanlyser. Purified DNA was sonicated to 100-500bp fragments, followed by DNA end repair and ligation of sequencing adaptors. The DNA was denatured and immunoprecipitation using a 5mC or 5hmC antibody followed by PCR amplification and library QC, prior to sequencing (6 samples per lane 50PE) on a Hiseq2000. The sonication, immunoprecipitation and sequencing were carried out by Beijing Genomics Institute (Beijing, China). The data were filtered by removing the adaptor sequences and low quality reads. The MeDip-seq data were mapped to the reference genome using the Short Oligonucleotide Analysis Package2 (SOAP2) software pipeline with only unique alignments containing no more than 2 mismatches taken forward for further analysis. Whole genome peak scanning was carried out using MACS1.4.0. For each differentially methylated region, the number of reads of each sample was calculated with probability of 0.05 or lower and a difference of read numbers more than 2-fold.

**Bisulphite pyrosequencing**

Validation of the MeDipSeq analysis was carried out by sodium bisulphite pyrosequencing essentially as described [23]. The choice of differentially methylated regions (DMRs) for validation was restricted by the ability to design sequencing assays (See Supplemental Figure 6). Hence only the *Zc3h12d* DMR was validated by pyrosequencing. Briefly, Genomic DNA was prepared and bisulphite conversion was carried out using the EZ DNA methylation kit (ZymoResearch, Irvine, CA, USA). Primers were designed to cover a 1.5 kb region encompassing 500 bp DMRs using PSQ Assay Design Software (Biotage, Uppsala, Sweden) (See Supplemental Table 3; See Supplemental Figure 1). Modified DNA was amplified using KAPA2G Robust Hot Start Taq DNA polymerase (Labtech, Ringmer, East Sussex, UK) and PCR primers listed in See Supplemental Table S2. PCR products were immobilised on streptavidin– sepharose beads (GE Healthcare UK Ltd., Amersham, Buckinghamshire, UK), washed, denatured and released into annealing buffer containing the sequencing primers (See Supplemental Table 2). Methylation analysis was carried out using the SQA kit on a PSQ 96MA pyrosequencer (Biotage), and percentage methylation was calculated using Pyro Q CpG software (Biotage) [23].

**Statistical analysis**

Comparisons between different time points in pregnancy were by 1-way ANOVA with Tukey’s *post hoc* correction for multiple comparisons using SPSS software (IBM SPSS Statistics for Apple Macintosh, Version 20.0; IBM Corporation, Armonk, NY, USA). Statistical significant was assumed at a probability of < 0.05. Statistical analysis of microarray data was through the IPA analytical software package as describes in the relevant sections.

**Results**

**Liver growth was associated with hyperplasia and hypertrophy**

As expected, pregnancy caused a statistically significant increase in liver weight at 7, 14 and 18 days gestation (ANOVA P <0.0001; Figure 1A). Hepatocyte area did not differ significantly from non-pregnant animals on day 7, but was greater on 14 (56%) and 18 (33%) days gestation (ANOVA P = 0.0016; Figure 2B). Hepatocyte density did not differ significantly from non-pregnant animals on day 7, but was significantly lower on 14 (28%) and 18 (27%) days gestation (ANOVA P = 0.043; Figure 1C). Pregnancy was associated with increased Ki67 and cyclin B1 expression which indicated activation of the mitotic cycle. Ki67 mRNA expression was greater (ANOVA P = 0.0006) at 7 (122%), 14 (131%) and 18 (78%) days gestation compared to non-pregnant animals (Figure 1C). *Cyclin B1* mRNA expression was also greater than in non-pregnant animals (ANOVA P = 0.018) at 7 (53%) and 14 (83%) days gestation, but was not significantly different from non-pregnant liver on day 18 (Figure 1D).

**Pregnancy induces gestational age-related changes in the liver transcriptome**

To determine the changes in gene expression associated with these different stages of pregnancy, RNA from D14 and D18 was analysed using the Illumina MouseRef-8 v2.0 Expression BeadChip. The expression of 115 transcripts (74 up-regulated) differed by at least 2 fold (P < 0.05) between pregnancy day 14 and non-pregnant animals (See Supplemental Table 4). The expression of 123 transcripts differed (77 up-regulated) by at least 2-fold on day 18 compared to non-pregnant animals (See Supplemental Table 4). Of these, 79 transcripts were altered at both time points, 76 in the same direction (See Supplemental Table 4). Five genes were among the ten most up-regulated genes on both day 14 and 18 gestation compared to non-pregnant animals; Kik1b4, Crybb3, Serpina6, Nedd9 and snai3 (See Supplemental Table 4). Five genes were among the ten most down-regulated genes at both 14 and 18 days gestation compared to non-pregnant animals; Cyp2b13, Abcc3, Cyp2c50, Avpr1a and Hamp (See Supplemental Table 4). The Illumina microarrays were validated by qRT-PCR for 9 most altered genes (See Supplemental Figure 2). There was good agreement between the differences in expression measured by microarray and RTPCR.

Compared to non-pregnant liver, the IPA categories enriched amongst the differentially expressed genes on day 14 were Disease and Disorders, Molecular and Cellular Function and Physiological Systems (Figure 2 A-C). The categories containing the most altered genes on day 18 were Disease and Disorders, Molecular and Cellular Function and Physiological Systems (Figure 2 D-F). The sub-categories altered relative to non-pregnant animals on day 18 were similar to those changed on day 14 with the exception of Molecular Transport which had greater representation on day 18 than on day 14 (See Supplemental Figure 3).

**Confirmation of the predicted changes in the liver transcriptome in a second mouse strain**

We sought to test the findings in C57BL/6 mice that were measured using the Illumina platform, by secondary analyses of published data of the liver transcriptome on pregnancy days 7.5, 10, 15 and 19 in FVB wild-type mice measured using the Affimetrix microarray platform [22]. 43/115 genes that were altered on pregnancy day 14 compared to non-pregnant C57BL/6 mice were also altered in FVB mice on pregnancy day 15 (total differentially expressed genes 264). 28/123 Genes that were altered compared to non-pregnant animals on pregnancy day 18 in C57BL/6 mice were also altered in FVB mice on pregnancy day 19 (total differentially expressed genes 139) (See Supplemental Tables 5 and 6).

**The effect of pregnancy on the expression of genes in the cell proliferation and organismal death pathways**

We used data from both C57BL/6 and FVB mice to investigate the contribution of cell hyperplasia and apoptosis to maternal liver growth during pregnancy by focussing on the Cell Proliferation and Organismal Death pathways. Details of differentially expressed genes in the Cell Proliferation pathway are summarised in See Supplemental Table 5. On pregnancy day 7.5, 16 genes in the Cellular Proliferation pathway were differentially expressed (11 (69%) predicted to up-regulate the pathway) compared to non-pregnant FBV mice (activation z-score 3.166). On day 10, 31 genes in the Cellular Proliferation pathway were differentially expressed (20 (65%) predicted to up-regulate the pathway) compared to non-pregnant FBV mice (activation z-score 3.031). On day 14, 22 genes associated with Cell Proliferation pathway were differentially expressed (11 (50%) predicted to upregulate the pathway) in pregnant compared to non-pregnant C57BL/6 mice (activation z-score 0.964). On pregnancy day 15, 34 genes in the Cellular Proliferation pathway were differentially expressed (17 (50%) predicted to upregulate the pathway) compared to non-pregnant FBV mice (activation Z-score 0.054). On day 18, 24 genes in the Cellular Proliferation pathway were differentially expressed (13 (54%) predicted to upregulate the pathway) compared to non-pregnant C57BL/6 mice (activation Z-score 1.595). On day 19, 27 genes were differentially expressed (16 (59%) (predicted to upregulate the pathway) compared to non-pregnant FBV mice (activation Z-score 2.112).

On day 7.5, 17 genes associated with Organismal Death pathway were differentially expressed (12 (71%) predicted to down-regulate the pathway) compared to non-pregnant FBV mice (activation z-score -2.112) (See Supplemental Table 6). On day 10, 33 genes associated with Organismal Death pathway were differentially expressed (22 (68%) predicted to down-regulate the pathway) compared to non-pregnant FBV mice (activation z-score -2.112) (See Supplemental Table 3). On day 14, 3 genes associated with the Organismal Death were differentially expressed compared to non-pregnant C57BL/6 mice which was too few to predict the net effect on the pathway. On day 15, 58 genes involved in Organismal death were differentially expressed (28 (48%) predicated to down-regulate the pathway) compared to non-pregnant FBV mice (activation z-score -0.102). On day 18, 31 genes involved in Organismal Death were differentially expressed (24 (77%) predicated to down-regulate the pathway) compared to non-pregnant C57BL/6 mice (activation z-score -1.027). On day 19, 24 Genes involved in Organismal Death were differentially expressed (13 (54%) predicted to down-regulate the pathway) compared to non-pregnant FBV mice animals, but these changes were not predicted to alter the overall activity of the pathway.

We tested the apparent suppression of apoptosis further by measuring the mRNA expression of key genes in the apoptotic pathway namely tumour protein-53 (*Trp53*), Fas receptor (*Fas*) and caspase 6 *(Casp6)* [26] in the liver of non-pregnant and pregnant C57BL/6 mice. *Trp53* mRNA expression was significantly lower at pregnancy days 7 (33%), 14 (47%) and 18 (44%) compared to non-pregnant animals (Figure 3A). *Fas* mRNA expression was significantly lower at pregnancy days 7 (50%), 14 (55%) and 18 (48%) compared to non-pregnant animals (Figure 3B). C*asp6* was significantly down-regulated only on pregnancy days 14 (51%) and 18 (54%), but did not differ significantly from non-pregnant animals on day 7 (Figure 3C).

**Pregnancy-induced changes in the DNA methylome**

Methylaiton-depenentent immunoprecipitation and hMethyl immunoprecipitation sequencing were used to identify sequences that may be differentially methylated upon pregnancy. Four regions showed significantly lower (2-fold) methylation in day 14 pregnant mice compared to non-pregnant animals (Table 1). Three regions were located within introns of ATPase Phospholipid Transporting 8A2 (*Atp8a2)*, Cell Adhesion Molecule-2 (*Cadm2)* and Adenylate Cyclase-Associated Protein-2  (*Cap2)*, and one in the coding sequence of inc finger CCCH domain-containing protein 12D (*Zc3h12d)* (Table 1). Two regions showed significantly lower (2-fold) hydroxymethylation in day 14 pregnant mice compared to non-pregnant animals (Table 1). These were located in the 5’ untranslated region of Membrane Spanning 4-Domains A1 (*Ms4a1)* and in the intron of latrophilin-3 (*Lphn3)*. Validation of the change in the *Zch12d* DMR by pyrosequencing showed that 3/6 CpG loci covered by this region had lower methylation in day 14 pregnant compared to non-pregnant liver (See Supplemental Figure 4). There were no DMRs associated with genes involved in cell proliferation or apoptosis that were differentially expressed in pregnant versus non-pregnant liver.

The level of methylation of 3 / 4 CpG loci in the LINE-1 amplicon tested was significantly lower at 14 and 18 days gestation compared to liver from non-pregnant or day 7 pregnant mice (Figure 4A). Furthermore, average methylation across the LINE-1 region measured was significantly lower (ANOVA P < 0.0001) at 14 days (9%) and 18 days (8%) of gestation compared to non-pregnant or day 7 pregnant mice (Figure 4B). The LINE-1 transcript was not detected in liver from non-pregnant or pregnant mice.

**Pregnancy-associated changes in expression of dnmts and tets**

*Dnmt3a* mRNA expression was lower compared with non-pregnant animals on pregnancy days 14 (60%) and day 18 (39%), but did not differ significantly on pregnancy day 7 (ANOVA P = 0.0046; Figure 5A). *Dnmt3b* mRNA expression was lower compared with non-pregnant animals on pregnancy days 14 (51%) and day 18 (17%), but did not differ significantly on pregnancy day 7 (ANOVA P = 0.011; Figure 5B). There was no significant effect of pregnancy on *Dnmt1* expression (Figure 5C). *Tet2* mRNA expression was increased compared to non-pregnant animals on pregnancy days 7 (122%), 14 (188%) and 18 (136%) (ANOVA P = 0.019; Figure 5E). There were no significant differences between pregnant and non-pregnant animals in the mRNA expression of *Tet1* or *3* (Figure 5 D,F).

**Discussion**

The findings of this study show that the pregnancy-associated increase in liver size was associated with hypertrophy, and with changes in the expression of the transcriptome that were consistent with increased proliferation and decreased apoptosis during specific periods in gestation. However, the findings did not support the suggestion that pregnancy-associated changes in the hepatic transcriptome involve altered gene-specific DNA methylation.

Although it is well established that the maternal liver adapts to pregnancy both in terms of size and function, there have been relatively few studies into underlying mechanisms and consequently there are conflicting observations about the relative contribution of key cellular processes, specifically hypertrophy and hyperplasia, to maternal liver growth [10, 12-15]. The present findings show that the rapid period of liver growth in mid and late gestation was accompanied by hyperplasia and/or hypertrophy in pregnant rodents [12-14]. mRNA expression of Ki67 and cyclin B1 was increased on pregnancy days 7 and 14 which suggests that maternal liver growth in early pregnancy involves primarily hypertrophy, while in mid gestation was associated with both hypertrophy and hyperplasia. Lower cell density at these time points may indicate a more rapid increase in hepatocyte size compared with increasing cell number. If so, this would be in agreement with a previous reports that the DNA content of term pregnant liver is approximately twice that of non-pregnant animals and that term pregnancy was associated with expression of cell proliferation markers [15]. Increased Ki67 expression in the liver of pregnant rodents has been reported to occur in the absence of mitotic figures [10]. One possible explanation is cell cycle arrest following the induction of mitosis leading to accumulation of DNA without an increase in cell number. Although this could explain lower liver cell density in mid and late gestation despite increased Ki67 and cyclin B1 expression, this interpretation is not consistent with the findings of the transcriptome-wide analysis. Late gestation was associated with hyperplasia, it was also associated with lower expression of mitotic markers. Together these findings suggest that hyperplasia was sufficient to support the modest increase in liver weight between non-pregnant and day 7 pregnant mice (9%), while both hyperplasia and hypertrophy were required for the increment in liver weight between pregnancy days 7 and 14 (13%). However, the substantial (36%) increase in liver weight between pregnancy days 14 and 18 required additional mechanisms.

The results of analysis of the effect of pregnancy on the expression of the liver transcriptome were validated by qRT-PCR. Combining the data sets from the current and published [22] results facilitated comprehensive analysis of the expression of the liver transcriptome during pregnancy. Comparison of pregnancy associated changes in the liver transcriptome between mouse strains at similar gestational ages showed limited agreement at the level of individual genes (37% of differentially expressed genes were altered on both day 14 in C57BL/6 and d15 FVB mice and 25% of differentially expressed genes were altered on day 18 in C57BL/6 and day 19 in FVB mice). This may reflect, at least in part, innate differences and differences in the precise gestational age during periods of dynamic change between mouse strains, and the use of different microarray platforms. Nevertheless, very similar pathways were altered in both C57BL/6 and FVB wild-type mice. This suggests that, irrespective of the gene-specific changes, there was a common drive between strains to change the activity of specific pathways which suggests that such adaptions are important for liver growth and function during pregnancy.

The combed the findings of transcriptome-wide analysis of the livers of pregnant compared to non-pregnant C57BL/6 and FVB mice predicted that cell proliferation was increased at all time points measured between day 7.5 and day 19. Increased expression of three of the five genes that were most up-regulated at gestational ages 14 and 18 days has been shown to be associated with cell proliferation. Liver cancer cells express higher levels of *Kik1b4* [27], possibly acting via hepatocyte growth factor [28], *Serpina6* [29] and *Nedd9* than non-transformed tissue. In addition, *Snai3* is involved in cell differentiation during embryogenesis [30], but has not been detected previously in adult liver. Decreased expression of four of the five genes that were most down-regulated at gestational ages 14 and 18 days has been shown to be associated with cell proliferation. RNAi knockdown of the Cyp2b family of cytochrome P450 has been shown to induce increased liver weight [31]. Decreased expression of *Abcc3* has been shown to be involved in liver cancer progression [32] and hepatocarcinoma cells express lower levels of *Hamp* than untransformed cells [33]. These findings are in agreement with previous studies that have shown up-regulation of cell proliferation-associated genes in the liver of pregnant rodents [10, 15] and with the immunohistochemical analyses reported here.

Analysis of the predicted effects of the changes in expression of genes in the Organismal Death pathway in pregnant compared to non-pregnant mice indicated that apoptosis was down-regulated between days 7.5 and 18, but not on day 19 when the expression of this pathway did not differ significantly from non-pregnant mice. These findings were confirmed by measurement of the mRNA expression of key genes in the apoptosis pathway; *Trp53*, *Fas* and *Casp6* [34]. This showed that *Trp53* and *Fas* were down-regulated by up to 50% between days 7 and 18, while *Casp6* was only down-regulated, albeit to a similar extent on days 14 and 18. The precise effect on apoptosis of such differential changes in expression cannot be deduced from these findings. However, since *Trp53* and *Fas* act upstream of *Casp6* [34], it is likely that the net activity of the apoptosis pathway was down-regulated at all time points that were measured. Furthermore, it is well established that down regulation of *Trp53*, acting via down regulation of apoptosis, can lead to cell proliferation associated with cancer [35].

Together these findings show for the first time that the mRNA expression of genes involved in apoptosis are down-regulated in during pregnancy in mice. This suggests that in addition to increased cell proliferation and hyperplasia, liver growth in pregnancy also involves a reduction in cell death. This may be of particular importance in late gestation when the expression of genes involved cell proliferation was decreased.

DNA methylation is an important epigenetic mechanism that regulates transcription [17] and which can be modified by a range of environmental inputs including hormones [36]. Reproductive tissues have been shown to exhibit function-related changes in DNA methylation. For example, lactation has been shown to be associated with hypomethylation and increased expression of genes encoding casein *Csn2* and *Csn3* in the mammary glands of mice [37]. The methylation status of the prostaglandin E receptor 2 gene has been shown to be associated positivity, while methylation of long interspersed nuclear element (LINE)-1 was associated negatively with gestational age in women [38]. The present findings show lower average and CpG locus-specific methylation of LINE-1 at 14 and 18 days gestation, but not on day 7. The magnitude of the decrease was comparable to that reported in leukocytes of subjects exposed to air pollution [39], in multiple sclerosis patients [40] and in early gastric cancer [41]. LINE-1 sequences account for over 20% of the mouse genome [42] and the level of LINE-1 methylation has been shown to correlate with global DNA methylation [43, 44]. Hypomethylation of LINE-1 sequences has been reported to be associated with cell proliferation [45], consistent with the findings here. Using MeDip and hMeDip-seq, we also report pregnancy induced methylation changes, although the number of changes was very limited. Importantly, none of the altered regions were associated with differentially expressed genes. Thus these findings do not support the suggestion that altered transcription in pregnancy involved changes in DNA methylation. However, this may reflect the fact that MeDip and hMeDIp-seq are relatively low resolution techniques that detect methylated regions of about 200bp in length rather than individual CpG sites and so changes at individual CpG sites may be missed, as well as smaller changes in methylation such as those detected by pyrosequencing at LINE-1 sequences.

DNA methylation status has been suggested to reflect the balance between DNA methyltransferase and demethylase activities [19], the latter catalysed putatively by Tet enzymes [46]. The present findings are consistent with a decrease in LINE-1 methylation in pregnancy such that lower DNA methylation in early pregnancy may be due to increased demethylase activity, but that in mid and late gestation both decreased methylation *de novo* and increased demethylation may be involved. If so, the involvement of Dnmt 3a and 3b may indicate that hypomethylation may be in proliferating rather than post-mitotic cells.

The findings of this study show that the increase in maternal liver weight in mice during pregnancy involves integrated changes in cell size, proliferation and apoptosis that differ between time points in gestation. We suggest the following model of liver growth in pregnancy (Figure 6). In early pregnancy (days 7 and 7.5), activation of cell proliferation appears to underlie liver growth, rather than hypertrophy and down-regulation of apoptosis. In mid pregnancy (days 14 and 15), the significant liver growth appears to involve hepatocyte hypertrophy, leading to lower cell density, increased cell proliferation and decreased apoptosis. These changes persist into late gestation (days 18 and 19), although the level of cell proliferation may decrease and apoptosis did not appear to be suppressed. Reactivation of apoptosis may contribute to *postpartum* reduction in liver size. One additional implication of these findings is that reduction in apoptosis may facilitate rapid increase in liver size while limiting nutritional demands on the mother for synthesis of new liver tissue.

**Conflict of interest statement:** none declared

**AUTHOR CONTRIBUTIONS:** G.C. Burdge, K.A. Lillycrop, and M.A. Hanson designed the study; L.R. Priceperformedmost of the experiments**,** analysed the results and contributed to the design of the study and interpretation of the data; N.A. Irvine performed the LINE-1 DNA methylation experiments; G.C. Burdge wrote the first draft of the manuscript; and all authors revised and approved the final version.

**References**

1. Herrera E. Metabolic adaptations in pregnancy and their implications for the availability of substrates to the fetus. *Eur J Clin Nutr* 2000; **54 Suppl 1**:S47-S51.

2. Bustamante JJ, Dai G, Soares MJ. Pregnancy and lactation modulate maternal splenic growth and development of the erythroid lineage in the rat and mouse. *Reprod Fertil* Dev 2008; **20**:303-310.

3. Nielsen JH, Svensson C, Galsgaard ED, Moldrup A, Billestrup N. Beta cell proliferation and growth factors. *J Mol Med (Berl)* 1999; **77**:62-66.

4. Smith RW. The effects of pregnancy and lactation on the activities in rat liver of some enzymes associated with glucose metabolism. *Biochim Biophys Acta* 1975; **411**:22-29.

5. Dickmann LJ, Tay S, Senn TD, Zhang H, Visone A, Unadkat JD, Hebert MF, Isoherranen N. Changes in maternal liver Cyp2c and Cyp2d expression and activity during rat pregnancy. *Biochem Pharmacol* 2008; **75**:1677-1687.

6. Mesbah MM, Baldwin RL. Effects of diet, pregnancy, and lactation on enzyme activities and gluconeogenesis in ruminant liver. *J Dairy Sci* 1983; **66**:783-788.

7. Dai G, Bustamante JJ, Zou Y, Myronovych A, Bao Q, Kumar S, Soares MJ. Maternal hepatic growth response to pregnancy in the mouse. *Exp Biol Med (Maywood)* 2011; **236**:1322-1332.

8. Reyes H, Kern F, Jr. Effect of pregnancy on bile flow and biliary lipids in the hamster. *Gastroenterol* 1979; **76**:144-150.

9. Olausson H, Sohlstrom A. Effects of food restriction and pregnancy on the expression of insulin-like growth factors-I and -II in tIssues from guinea pigs. *J Endocrinol* 2003; **179**:437-445.

10. Milona A, Owen BM, van Mil S, Dormann D, Mataki C, Boudjelal M, Cairns W, Schoonjans K, Milligan S, Parker M, White R, Williamson C. The normal mechanisms of pregnancy-induced liver growth are not maintained in mice lacking the bile acid sensor Fxr. *Am J Physiol Gastrointest Liver Physiol* 2010; **298**:G151-158.

11. Shekhar S, Diddi G. Liver disease in pregnancy. *Taiwan J Obstet Gynecol* 2015; **54**:475-482.

12. Orlova, II. [Changes in the dimensions of the nuclei and cells of rat livers during pregnancy]. *Biull Eksp Biol Med* 1969; **67**:117-119.

13. Hollister A, Okubara P, Watson JG, Chaykin S. Reproduction in mice: liver enlargement in mice during pregnancy and lactation. *Life Sci* 1987; **40**:11-18.

14. Gielchinsky Y, Laufer N, Weitman E, Abramovitch R, Granot Z, Bergman Y, Pikarsky E. Pregnancy restores the regenerative capacity of the aged liver via activation of an mTORC1-controlled hyperplasia/hypertrophy switch. *Genes Dev* 2010; **24**:543-548.

15. Bustamante JJ, Copple BL, Soares MJ, Dai G. Gene profiling of maternal hepatic adaptations to pregnancy. *Liver Int* 2010; **30**:406-415.

16. Bird AP. Functions for DNA methylation in vertebrates. *Cold Spring Harb Symp Quant Biol*. 1993; **58**:281-285.

17. Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev* 2002; **16**:6-21.

18. Arnold AP, Chen X, Itoh Y. What a difference an X or Y makes: sex chromosomes, gene dose, and epigenetics in sexual differentiation. *Handbook Exp Pharmacol* 2012; **214**:67-88.

19. Szyf M. The dynamic epigenome and its implications in toxicology. *Toxicol Sci* 2007; **100**:7-23.

20. Tan L, Shi YG. Tet family proteins and 5-hydroxymethylcytosine in development and disease. *Devel* 2012; **139**:1895-1902.

21. Hoile SP, Lillycrop KA, Thomas NA, Hanson MA, Burdge GC. Dietary protein restriction during F0 pregnancy in rats induces transgenerational changes in the hepatic transcriptome in female offspring. *PloS ONE* 2011; **6**:e21668.

22. Shuster DL, Bammler TK, Beyer RP, Macdonald JW, Tsai JM, Farin FM, Hebert MF, Thummel KE, Mao Q. Gestational age-dependent changes in gene expression of metabolic enzymes and transporters in pregnant mice. *Drug Metab Dispos* 2013; **41**:332-342.

23. Hoile SP, Irvine NA, Kelsall CJ, Sibbons C, Feunteun A, Collister A, Torrens C, Calder PC, Hanson MA, Lillycrop KA, Burdge GC. Maternal fat intake in rats alters 20:4n-6 and 22:6n-3 status and the epigenetic regulation of Fads2 in offspring liver. *J Nutr Biochem* 2013; **24**:1213-1220.

24. Cikos S, Bukovska A, Koppel J. Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis. *BMC Mol Biol* 2007; **8**:113.

25. Vandesompele J, De PK, Pattyn F, Poppe B, Van RN, De PA, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; **3**:RESEARCH0034.

26. Zuckerman V, Wolyniec K, Sionov RV, Haupt S, Haupt Y. Tumour suppression by p53: the importance of apoptosis and cellular senescence. *J Pathol* 2009; **219**:3-15.

27. Kawaguchi M, Kataoka H. Mechanisms of hepatocyte growth factor activation in cancer tissues. *Cancers (Basel)* 2014; **6**:1890-1904.

28. Shagisultanova E, Gaponova AV, Gabbasov R, Nicolas E, Golemis EA. Preclinical and clinical studies of the NEDD9 scaffold protein in cancer and other diseases. *Gene* 2015; **567**:1-11.

29. Shen N, Gong J, Wang Y, Tian J, Qian J, Zou L, Chen W, Zhu B, Lu X, Zhong R, Guo A, Wang L, et al. Integrative genomic analysis identifies that SERPINA6-rs1998056 regulated by FOXA/ERalpha is associated with female hepatocellular carcinoma. *PLoS One* 2014; **9**:e107246.

30. Zhuge X, Kataoka H, Tanaka M, Murayama T, Kawamoto T, Sano H, Togi K, Yamauchi R, Ueda Y, Xu Y, Nishikawa S, Kita T, et al. Expression of the novel Snai-related zinc-finger transcription factor gene Smuc during mouse development. *Int J Mol Med* 2005; **15**:945-948.

31. Damiri B, Holle E, Yu X, Baldwin WS. Lentiviral-mediated RNAi knockdown yields a novel mouse model for studying Cyp2b function. Toxicol Sci 2012; 125:368-381.

32. Carrasco-Torres G, Fattel-Fazenda S, Lopez-Alvarez GS, Garcia-Roman R, Villa-Trevino S, Vasquez-Garzon VR. The transmembrane transporter ABCC3 participates in liver cancer progression and is a potential biomarker. *Tumour Biol* 2016; **37**:2007-2014.

33. Udali S, Guarini P, Ruzzenente A, Ferrarini A, Guglielmi A, Lotto V, Tononi P, Pattini P, Moruzzi S, Campagnaro T, Conci S, Olivieri O, et al. DNA methylation and gene expression profiles show novel regulatory pathways in hepatocellular carcinoma. *Clin Epigenetics* 2015; **7**:43.

34. Strasser A, O'Connor L, Dixit VM. Apoptosis signaling. *Annu Rev Biochem* 2000; **69**:217-245.

35. Liu Y, Chen C, Xu Z, Scuoppo C, Rillahan CD, Gao J, Spitzer B, Bosbach B, Kastenhuber ER, Baslan T, Ackermann S, Cheng L, et al. Deletions linked to TP53 loss drive cancer through p53-independent mechanisms. *Nature* 2016; **531**:471-475.

36. Lillycrop KA, Burdge GC. Environmental challenge, epigenetic plasticity and the induction of altered phenotypes in mammals. *Epigenomics* 2014; **6**:623-636.

37. Rijnkels M, Freeman-Zadrowski C, Hernandez J, Potluri V, Wang L, Li W, Lemay DG. Epigenetic modifications unlock the milk protein gene loci during mouse mammary gland development and differentiation. *PLoS One* 2013; **8**:e53270.

38. Burris HH, Baccarelli AA, Motta V, Byun HM, Just AC, Mercado-Garcia A, Schwartz J, Svensson K, Tellez-Rojo MM, Wright RO. Association between length of gestation and cervical DNA methylation of PTGER2 and LINE 1-HS. *Epigenetics* 2014; **9**:1083-1091.

39. Chen R, Meng X, Zhao A, Wang C, Yang C, Li H, Cai J, Zhao Z, Kan H. DNA hypomethylation and its mediation in the effects of fine particulate air pollution on cardiovascular biomarkers: A randomized crossover trial. *Environ Int* 2016; **94**:614-9.

40. Neven KY, Piola M, Angelici L, Cortini F, Fenoglio C, Galimberti D, Pesatori AC, Scarpini E, Bollati V. Repetitive element hypermethylation in multiple sclerosis patients. *BMC Genet* 2016; **17**:84.

41. Kim EJ, Chung WC, Kim DB, Kim YJ, Lee JM, Jung JH, Lee YK. Long interspersed nuclear element (LINE)-1 methylation level as a molecular marker of early gastric cancer. *Dig Liver Dis* 2016; **48**:1093-1097.

42. Mouse Genome Sequencing Cconsortium, Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, Agarwala R, Ainscough R, Alexandersson M, An P, Antonarakis SE, *et al*. Initial sequencing and comparative analysis of the mouse genome. *Nature* 2002; **420**:520-562.

43. Weisenberger DJ, Campan M, Long TI, Kim M, Woods C, Fiala E, Ehrlich M, Laird PW. Analysis of repetitive element DNA methylation by MethyLight. *Nucleic Acids Res* 2005; **33**:6823-6836.

44. Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res* 2004; **32**:e38.

45. Oricchio E, Sciamanna I, Beraldi R, Tolstonog GV, Schumann GG, Spadafora C. Distinct roles for LINE-1 and HERV-K retroelements in cell proliferation, differentiation and tumor progression. *Oncogene* 2007; **26**:4226-4233.

46. Shen L, Song CX, He C, Zhang Y. Mechanism and function of oxidative reversal of DNA and RNA methylation. *Annu Rev Biochem* 2014; **83**:585-614.

**Figure 1.** Effect of pregnancy on liver weight, histology and expression of cell cycle-associated genes. (A) Liver weight; (B) liver cell area; (C) liver cell density; (D) Ki67 mRNA expression; (E) cyclin B1 mRNA expression. Values are mean ± SEM. (A) n = 20, non-pregnant, n = 15 at day 7, n = 10 at day 14 and n = 9 at day 18. (B) n = 3 livers from which 2 sections were taken from each, and 10 cells were counted from each section. (C) As (B), but 5 fixed fields were counted per section. (D) n = 5 for non-pregnant, n = 10 at day 7, n = 8 at day14 and n = 8 at day 18. (E) n = 5 for non-pregnant, n = 10 at day 7, n = 9 at 9 day14 and n = 9 at day 18. Means indicated by different letters differed significantly (P < 0.01).

**Figure 2.** Top five most altered pathways in A – C day 14 and D – F day 18 pregnant liver compared to non-pregnant mice (n=12 mice per group). (A,D) Diseases and Disorders category; (B, E) Molecular and Cell Function (C,F) Physiological systems.

**Figure 3.** Effect of pregnancy on expression of genes involved in apoptosis. Values are mean ± SEM for n = 12 mice per gestational age. Means with different letters differed significantly (P < 0.05).

**Figure 4.** Values are mean ± SEM for n = 10 samples / gestational age. DNA Methylation of individual CpG loci in LINE-1 sequences. Means with different letters were significantly different (P < 0.05). (A) Methylation status of individual CpG loci and (B) average methylation across 4 CpG loci. Comparisons between gestational ages for each locus by ANOVA were CpG 1 P = 0.027; CpG 2 P = 0.0031 and CpG 3 P < 0.0001.

**Figure 5.** The effect of pregnancy expression of DNA methyl transferases (Dnmts) and Ten-Eleven translocases (Tets) in liver. Values are mean ± SEM for n = 12 mice per gestational age. Means with different letters were significantly different (P < 0.05).

**Figure 6.** Schematic summary of the changes in gene expression and liver size and histology during pregnancy in mice. Early pregnancy, days 7 and 7.5; mid pregnancy, days 10, 14 and 15; late pregnancy, days 18 and 19.

**Table 1**

**Differentially methylated and hydroxymethylated regions at day 14 of gestation compared to non-pregnant mice**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Gene Name | Name | Location of DMR | Chromosome | Chromosomal location (bp) | Fold  | P |
| Differentially methylated regions |
| *Atp8a2* | ATPase, aminophospholipid transporter-like, class 1, type 8A member 2 | Intron | chr14 | 60469858 - 60470357 | -2 | 0.001 |
| *Cadm2* | Cell adhesion molecule 2 | Intron | chr16 | 67391428 - 67391852 | -2 | 0.0009 |
| *Cap2* | Adenylate cyclase-associated protein 2 | Intron | chr13 | 46697785 - 46698284 | -2 | 0.008 |
| *Zc3h12d* | Zinc finger CCCH-type containing 12D | CDS | chr10 | 7586931 - 7587480 | -2 | 0.004 |
|  | Differentially hydroxymethylated regions |
| *Ms4a10* | Membrane-spanning 4-domains, subfamily A, member 10 | 5’ UTR/ intron | chr19 | 11043152 - 11043547 | -2 | 0.001 |
| *Lphn3* | Latrophilin 3 | Intron | chr5 | 81854947 - 81855374 | -2 | 0.01 |

DMR, differentially methylated region