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Maternal L-Carnitine supplementation ameliorates renal underdevelopment and epigenetic changes in male mice offspring due to maternal smoking.

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Abstract

Epidemiological and animal studies showed that L-carnitine (LC) supplementation can ameliorate oxidative stress-induced tissues damage. We have previously shown that maternal cigarette smoke exposure (SE) can increase renal oxidative stress in newborn offspring with postnatal kidney underdevelopment and renal dysfunction in adulthood, which were normalised by LC administration in the SE dams during pregnancy. Exposure to an adverse intrauterine environment may lead to alteration in the epigenome, a mechanism by which adverse prenatal conditions increase the susceptibility to chronic disease later in life. The current study aimed to determine whether maternal SE induces epigenetic changes in the offspring's kidney are associated with renal underdevelopment, and the protective effect of maternal LC supplementation.

Method: Female Balb/c mice (7 weeks) were exposed to cigarette smoke (SE) or air (Sham) for 6 weeks prior to mating, during gestation and lactation. A subgroup of the SE dams received LC via drinking water (SE+LC, 1.5mmol/l) throughout gestation and lactation. Male offspring were studied at postnatal day (P)1, P20, and 13 weeks.

Results: Maternal SE altered the expression of renal development markers glial-cell line-derived neurotrophic factor and fibroblast growth factor 2, which were associated with increased renal global DNA methylation and DNA methyltransferase 1 mRNA expression at birth. These disorders were reversed by maternal L-carnitine administration.

Conclusion: The effect of maternal SE on renal underdevelopment involves global epigenetic alterations from birth, which can be prevented by maternal LC supplementation.

Highlights:

- Maternal smoking changes developmental markers
- Maternal smoking induces renal DNA hypermethylation
- Maternal intake of L-carnitine can normalised DNA methylation and mitigate the adverse impact on kidney developmental markers

Key words: maternal smoking, antioxidant, kidney development, global methylation.

1. Introduction

Epigenetics refers to mitotically heritable changes in gene expression and phenotype that are not mediated by alterations in the underlying DNA sequence. DNA methylation is the most studied epigenetic modification and describes the covalent addition of a methyl group to the 5th carbon of a cytosine (5-mC) residue that is located adjacent to a guanine nucleotide (CpG dinucleotide). Methylation of DNA can modulate gene transcription by influencing binding of proteins to DNA that initiates transcription. DNA hypermethylation is therefore considered an inhibitor of transcription while hypomethylation activates transcription.

A specific epigenetic code is necessary for regulated development of the growing foetus. The establishment of the epigenetic program starts soon after fertilization when all DNA methylation marks are erased in the preimplantation embryo with subsequent resetting of the genome-wide methylation pattern in a cell-type specific manner in order to dictate somatic development. The theory of foetal programming, which links chronic disease in adulthood to adverse early life conditions, is increasingly recognised as an important determinant of individual's health. The pathophysiology underpinning foetal programming may partly be explained by epigenetic modification. Prenatal smoke exposure has been associated with reduced kidney volume in the offspring [1,2]. Using an animal model of maternal smoking, we have previously demonstrated that maternal smoking induces renal underdevelopment and oxidative stress in the offspring from birth and this was associated with renal dysfunction at adulthood [3,4].

DNA methylation is an important regulator of gene expression and increasingly recognised as a major mechanism of disease pathogenesis, including Chronic Kidney Disease (CKD). DNA methylation occurs primarily on cytosine residues in CpG dinucleotides [5] and may promote the development of renal disease and its comorbidities either due to environmental factors or inheritance from the mothers [6]. Epigenetic changes caused by maternal smoking during gestation have been reported in human foetal liver [7] and in the blood and buccal epithelial cells of newborns and adolescents [8,9], but have not been investigated in the kidneys.

Dietary supplementation with antioxidants, has been shown to have a beneficial role in decreasing the effect of oxidative stress-caused damage in the lymphocyte DNA due to cigarette smoking [10]. The amino acid supplement L-carnitine (LC) has been shown to have anti-inflammatory [11], anti-apoptotic [12] and anti-oxidative [13] effects. In rodents, LC supplementation has been shown to improve atherosclerosis [14] and chronic renal failure induced by partial nephrectomy [15]. In addition, LC has been shown to be essential in foetal development and intrauterine maturation of the brain and lung [16];

We have recently demonstrated that maternal LC supplementation during gestation and lactation reversed small body weight and kidney weight at birth, as well as normalising renal oxidative stress and renal dysfunction due to maternal SE in the male offspring [4]. However, the effect of LC on epigenetic signatures and renal developmental markers is not known. This study therefore aimed to investigate the link between poor kidney development induced by maternal smoke exposure, which we have previously confirmed and renal epigenetic changes at various ages. We then determined whether the effect of maternal smoking on renal development and associated epigenetic changes in the offspring's kidney can be reversed by maternal LC supplement.

2. Results

Maternal LC supplementation reversed the effect of maternal SE on the offspring development

As shown in Table 2, SE offspring have smaller body weight than the sham offspring ($P < 0.05$) with reduced kidney mass ($P < 0.05$), which was not persistent until adulthood. Such growth delay was significantly reversed by maternal LC supplementation ($P < 0.05$ and $P < 0.01$ respectively).

We have previously published that maternal SE significantly reduced glomerular development in the offspring at birth and this was demonstrated to be reversed by maternal LC administration during gestation and lactation [4]. Maternal LC also reversed the changes in glomerular size and number due to maternal SE in the offspring [4]. Here we reported the potential molecular mechanism underlying renal development and disorders.

Effect of maternal SE and LC supplementation on renal GDNF level in the offspring

GDNF signalling is believed to be the most critical factor for early-stage nephrogenesis during foetal life [24,25]. Maternal SE increased renal mRNA expression of GDNF in the offspring at birth ($P < 0.05$, Figure 2A). Similarly, renal GDNF protein level was increased in SE offspring at P1 ($P < 0.05$), which was nearly doubled at P20, although without statistical significance (Figure 2B). Maternal LC supplementation normalised the trend at P20 (Figure 2B).

Effect of maternal SE and LC supplementation on renal FGF2 level in the offspring

Several members of the fibroblast growth factor (FGF) family can induce renal development [26]. FGF2 is a proliferating and differentiating signalling factor that promotes metanephric mesenchymal survival [21]. Maternal smoking increases renal FGF2 mRNA and protein levels in the offspring at birth and weaning ($P < 0.05$, Figure 3A). Although maternal LC administration had no effect on FGF2 at P1, FGF2 mRNA and protein levels in the offspring due to maternal smoking were reversed by maternal LC administration ($P < 0.05$, Figure 3A and 3B).

Effect of maternal SE and LC supplementation on renal Pax2 level in the offspring

Pax2 is known to regulate mesenchyme condensation around the tips of the ureteric bud and metanephric mesenchymal transdifferentiation into epithelial cells [27]. Maternal smoking increases renal Pax2 mRNA and protein levels in the offspring at birth ($P < 0.05$, Figure 4A). Maternal LC administration significantly reversed the increased renal Pax2 mRNA and protein expression in the offspring due to maternal SE ($P < 0.05$ compared to SE respectively, Figure 4A and 4B).

Effect of maternal SE and LC supplementation on renal global methylation in the offspring

The foetal environment is considered crucial in the regulation of epigenetic markers and their resulting influence on differentiation of cells and organs. We aimed to assess the effect of maternal SE on global methylation in the offspring renal tissue and this was measured at three key time points from birth to adulthood. Global methylation in the kidney was significantly increased at day 1 in the offspring of SE mothers ($p<0.01$ vs. Sham) and this effect was completely abrogated by maternal LC administration during gestation and lactation ($p<0.01$ vs SE, Figure 5A). The differential change in renal DNA methylation of SE offspring was lost by P20 (weaning age) and was not seen during adulthood at week 13 (Figures 5B and C). Consistently with the change of global DNA methylation at P1, the mRNA expression of DNA methyltransferase (Dnmt) 1 was significantly upregulated by maternal SE (Figure 6, $P < 0.05$). Dnmt3a and 3b showed similar trends of upregulation (Figure 6). LC administration significantly reversed the expression of Dnmt1 ($P < 0.05$).

4. Discussion

The main finding of this study is that maternal SE induces renal global methylation in the offspring at birth in association with kidney underdevelopment and upregulated renal development markers. Maternal LC supplementation during gestation and lactation significantly restored normal kidney development and epigenetic changes. This in addition to our previous report, which demonstrated the LC ameliorated renal injury markers and kidney function at adulthood [18], provides a proof of concept for the potential therapeutic role of LC to reduce the risk of renal pathology in the offspring of smoking mothers.

We have previously reported renal underdevelopment in early life and albuminuria in adulthood in offspring of smoke exposed mothers in a mouse model [3]. Using the same mouse model of maternal SE, we also demonstrated the restoration of normal kidney development and normalisation of albuminuria when SE dams received dietary LC supplementation during gestation and lactation [18]. Here, we investigated the underlying mechanisms of the beneficial effect of maternal LC treatment

in the offspring. This study demonstrated the aberrant expression of several renal developmental factors in SE offspring, which was abrogated if mothers received dietary LC. There was an adaptive upregulation of FGF2, GDNF and Pax2 in underdeveloped kidneys from the SE offspring, each of which is critical for normal renal development in different stages of nephrogenesis. FGF2 for example promotes metanephric mesenchyme maintenance and increase in stromal cells population within the metanephric mesenchyme [21], the very first steps of nephrogenesis. GDNF on the other hand induce ureteric bud branching [23], and Pax2 is involved in nephron differentiation [22]. The increased expression of these factors at birth may reflect a necessary adaptive response to renal underdevelopment caused by an adverse in utero environment, namely maternal SE in this study. Importantly, the mRNA expression of those development genes was normalised if LC was administered during gestation in the SE mothers in association with normalisation of nephrogenesis.

The fact that FGF2 mRNA and protein levels as well as GDNF protein level remained significantly increased until weaning age (P20) supports the notion that in utero cigarette smoke exposure has long lasting effects on the offspring and may predispose them to foetal programming resulting in susceptibility to kidney disease in the long term. The upregulation of these growth factors was not seen in offspring of the SE+LC dams, highlighting again the potentially beneficial role of L-carnitine. Despite their important role in early life, if these growth factors remain elevated after renal development is complete, they may exert detrimental effects on renal health. FGF2 for example is considered as a strong mitogen for cortical renal fibroblasts and may promote autocrine fibroblast growth. Increased levels of FGF2 mRNA and protein expression have been shown to be associated with tubulointerstitial fibrosis [28]. Our results may suggest that LC mediated reduction of FGF2 at weaning may potentially prevent fibrogenesis in the long term. Indeed in our previous study, the renal expression of the inflammatory marker MCP-1 in adulthood was significantly reduced in SE offspring if the mothers had LC supplementation during gestation and lactation compared to those from the SE mothers without intervention [4]. The long term anti-inflammatory effect of maternal LC supplementation in the kidneys of the adult offspring is consistent with a previous study [29].

Although the maternal effect is still the major focus of fetal programming research, there has been emerging evidence regarding the paternal contribution to this process [30,31]. In comparison to maternal-fetal programming, paternal-fetal programming seems to be less potent [32]. Particularly in regard to the effects of parental smoking on kidney development, it has been demonstrated that while maternal smoking during pregnancy is associated with both smaller combined kidney volume and impaired kidney function in childhood, paternal smoking only affects kidney volume [33].

Although newborn babies from the smokers seem to have similar blood carnitine levels as the non-smokers [34], the newborns do not have the capacity to biosynthesise sufficient LC for their needs. Supplementation in the mother allows them to source additional LC from the breast milk to fulfil metabolic requirements [35]. The inability of neonates to synthesize sufficient carnitine is due to immature hepatic gamma-butyrobetaine-hydroxlase activity. However, as pregnancy proceeds, blood carnitine concentration in the pregnant mother becomes lower than in the non-pregnant condition which may affect foetal exposure and hence levels [36]. Although no study has been carried out to quantify optimal LC concentrations in breast milk, it has been reported that another antioxidant vitamin E concentration is much lower in smokers' breastmilk [37], while in an animal study, breast milk protein concentration is also somewhat lower in the mothers exposed to cigarette smoke during lactation [38]. In humans, a previous study showed no specific effect of maternal LC supplementation (10 mg/kg/d) on the growth and development of premature neonates [39]. However, the limitation of this study is that only body weight gain was used to assess growth and development, which can't reflect the development of individual organs. This dose is also relatively lower than the regular dose of 2g/dose in healthy people [40,41]. Moreover, another animal study showed that dietary LC during pregnancy and lactation did not improve the growth of the offspring [42]. This may be because that the study was carried out in normal animals without growth abnormalities. As such, when LC was used in the mothers who had premature neonates, it significantly improved nutrition level and metabolism in the infants [43]. LC has also been shown to have a beneficial role in embryogenesis in mice embryos incubated in harsh environment, such as in the presence of pro-inflammatory tumour necrosis factor α and H2O2 [44] .

Carnitine deficiency normally correlates with nutritional inadequacies and can be observed in low-birth weight newborns due to reasons such as maternal smoking, patients with renal tubular disorders, and patients with chronic renal failure undergoing haemodialysis [35,45]. Our results suggest that if mothers continue to smoke during gestation and lactation, LC supplementation during these two critical developmental periods can improve renal development in offspring and ameliorate renal dysfunction at adulthood [18].

In this study we demonstrated global hypermethylation in the SE offspring which was completely abrogated by maternal LC supplementation during gestation and lactation. The gene expression of Dnmt1 was upregulated in the kidney of P1 offspring born to smoke-exposed dams and normalised by LC administration. The result is consistent with the changes of global DNA methylation levels in these offspring, suggesting the effects of LC may be partially mediated via epigenetic modification events. However, the specific mechanism requires further investigation.

Cigarette smoke is considered a powerful inducer of oxidative stress and DNA methylation modification [46]. The effect of prenatal smoke exposure on global DNA methylation has been investigated in previous studies. While some studies observed global hypomethylation in cigarette smoke exposed individuals [47,48], the findings by Terry et al. which reported DNA hypermethylation in response to prenatal cigarette smoke exposure are consistent with our observations here [49]. However, methylation profiles can be tissue and species dependent. Most studies used DNA from human peripheral leucocytes, cord blood or buccal cells as these are easy to obtain. To our knowledge, we are the first to report cigarette smoke-induced alterations in DNA methylation in offspring's kidneys. We also found that the global methylation changes in the SE offspring did not last during the development. At weaning age, the difference in methylation between the SE offspring and Sham offspring were not seen. The reason for such age related epigenetic change is not clear although environmental factors are known to alter gene methylation [50]. Nevertheless, aberrant DNA methylation profiles in the early period of life can potentially influence organ development and predispose individuals to disease susceptibility at later stages of life. Further investigations to screen gene specific methylation patterns are required. Indeed epigenome-wide association studies interrogating more than 450.000 CpG sites have become available in recent years. The largest study to date was a metaanalysis of 13 cohorts of smokers' offspring whose DNA was processed with a Human Methylation 450k Bead Chip assay. A total of 3000 differentially methylated CpG sites corresponding to 2000 genes not previously related to smoking were discovered. Pathway and functional analysis revealed that many of the genes are involved in embyogenesis and developmental pathways [51]. The authors discussed a number of differentially methylated genes in detail of their association with orofacial clefts; however we note that some of these genes such as BMP4, BMP6 and TIMP2 also play key roles in kidney development [52-54], which were not changed in P1 offspring's kidney in this study (data not shown). CpG methylation analysis of other key genes in renal development, such as the differentially expressed growth factors in this study, is needed in future studies.

This study hereby demonstrated the ability of maternal LC supplementation to normalise hypermethylated DNA and the expression of Dnmt1 in the SE offspring. Such de-methylation function of LC has also been observed in a previous in vitro study using human cells [55]. As L-carnitine reduces oxidative stress in the offspring [4], and differential DNA methylation of oxidative stress pathways is dominant in the maternal smoking cohort [56], L-carnitine may indirectly regulate the global DNA methylation level in the offspring kidney by improving antioxidant/oxidative balance. Indeed, previous evidence has suggested a "Free radical theory of development", which links the

redox buffer system and the metabolism of methyl group, and ultimately DNA methylation changes during development [57]. Further gene-specific investigation is required to fully justify the hypothesis. As LC is a readily available over-the-counter supplement with excellent safety profile, its repurposed application in mothers who continue to smoke during pregnancy is worthy of consideration.

3. Materials and methods

Animal model and tobacco cigarette smoke exposure protocol

The animal experiment was approved by the Animal Care and Ethics Committee at the University of Technology Sydney (ACEC#2011-313A). Briefly, female Balb/c mice (7 weeks old, Animal Resources Centre, Perth, Australia) were housed at 20 ± 2ºC and maintained on a 12:12 hour light/dark cycle (lights on 06:00h). After the acclimatization period, the mice were divided into Sham exposure group (Sham, n=12), and SE group (SE, n=24). SE was performed as previously described (2 cigarettes, twice daily for 6 weeks prior to mating, during gestation and lactation [3]). Neither the male breeders nor the offspring were exposed to cigarette smoke. A subgroup of the SE dams was treated with LC (SE+LC, n=12) via drinking water (1.5mmol/l [17]) starting at mating until pup weaning at postnatal day (P) 20 as previously described [4].

Tissue collection

Only male offspring were studied as we have shown that renal developmental and functional disorders caused by maternal smoking were only prominent in the male [3] not female offspring [19], reflected by changes in glomerular size and number as well as urinary albumin creatinine ratio. Male offspring have also been shown to be more susceptible to fetal programming of kidney disorders by maternal obesity [20]. Male offspring were sacrificed at three different time points; postnatal (P)1 (birth), P20 (weaning), and at week (W)13 (adulthood). Briefly, the pups were weighed and anaesthetized with sodium thiopental (0.1ml/g, i.p., Abbott Australasia PTY. LTD, NSW, Australia). The right kidney was harvested, weighed, snap frozen in liquid nitrogen and then stored in -80ºC. The left kidney was fixed in 10% formalin (Sigma Aldrich, VIC, Australia).

Real-time PCR

Total RNA was extracted from kidney tissue using RNeasy plus mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The single strand cDNA was synthesised using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). quantitative real-time PCR was performed using pre-optimized SYBR Green primers (Table 1, Sigma Aldrich, VIC, Australia) and SensiFAST™ SYBR Hi-ROX Kit (Bioline, Toronto Canada) in an ABI7900HT Sequence Detection System (SDS 2.4, Life Technologies, CA, USA). The target genes

were selected due to the previous evidence of their relevance to kidney development [21-23], and methylation regulation. The results were analysed by relative quantitation by RQ Manager Software (RQ 1.2.1, Applied Biosystems) using ΔΔCt. The average mRNA expression of the Sham (Control) group was used as the calibrator and 18S rRNA was used as the housekeeping gene.

Kidney immunohistochemistry (IHC) staining

For IHC staining, 4μm kidney sections were de-waxed, dehydrated, and endogenous peroxidase was blocked. The sections were incubated with rabbit anti-mouse primary antibodies against proteins known to be associated with renal development and which we have previously demonstrated to have dysregulated expression in offspring of SE mothers , including glial-cell line-derived neurotrophic factor (GDNF, 1:100, Santa Cruz Biotechnology, CA, USA), fibroblast growth factor 2 (FGF2, 1:250, Santa Cruz Biotechnology, CA, USA), and paired box transcription factor (Pax2, 1:1750, Santa Cruz Biotechnology, CA, USA) at 4^{o}C overnight. Negative controls were prepared by replacing the primary antibodies with rabbit IgG. The sections were exposed to Envision & system-HRP labelled polymer secondary anti-rabbit antibodies (Dako, CA, USA). On average, 6 different non-overlapping fields of the same kidney section were captured and 6-8 mice were used from each group. Quantitation of the positive signals in the images was performed using Image J software (Image J, NIH, USA).

Global DNA methylation

Genomic DNA was extracted from mouse kidneys using the DNeasy blood and tissue kit (Quiagen, Hilden, Germany). Offspring samples from control, smoke-exposed and smoke-exposed + L-carnitine treated mice were used. For the detection of global methylation the MethylFlash Methylated DNA quantification kit (Epigentek, Farmingdale, NY, USA), a colorimetric antibody based kit was used that detects 5-mC, thus quantifying the relative amount of methylation in the total DNA sample.

Statistical analysis

The results are expressed as mean ± SEM. The differences between the groups were analysed using one-way ANOVA followed by LSD *post hoc* tests (Prism 6, Graphpad CA, USA). $P<0.05$ was considered significant.

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Figure legends

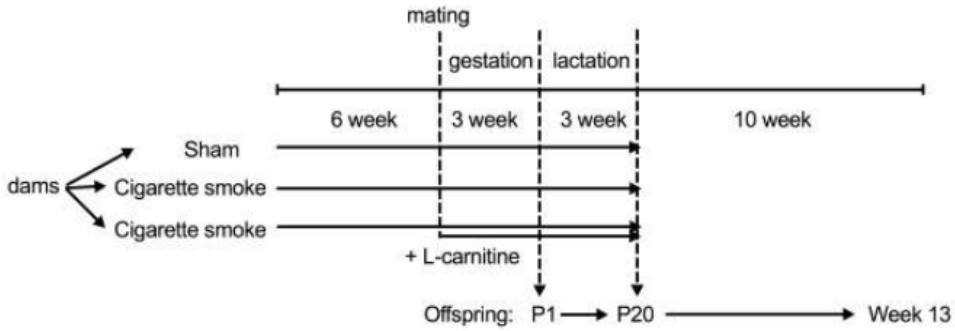

Figure 1: Animal model and treatment scheme

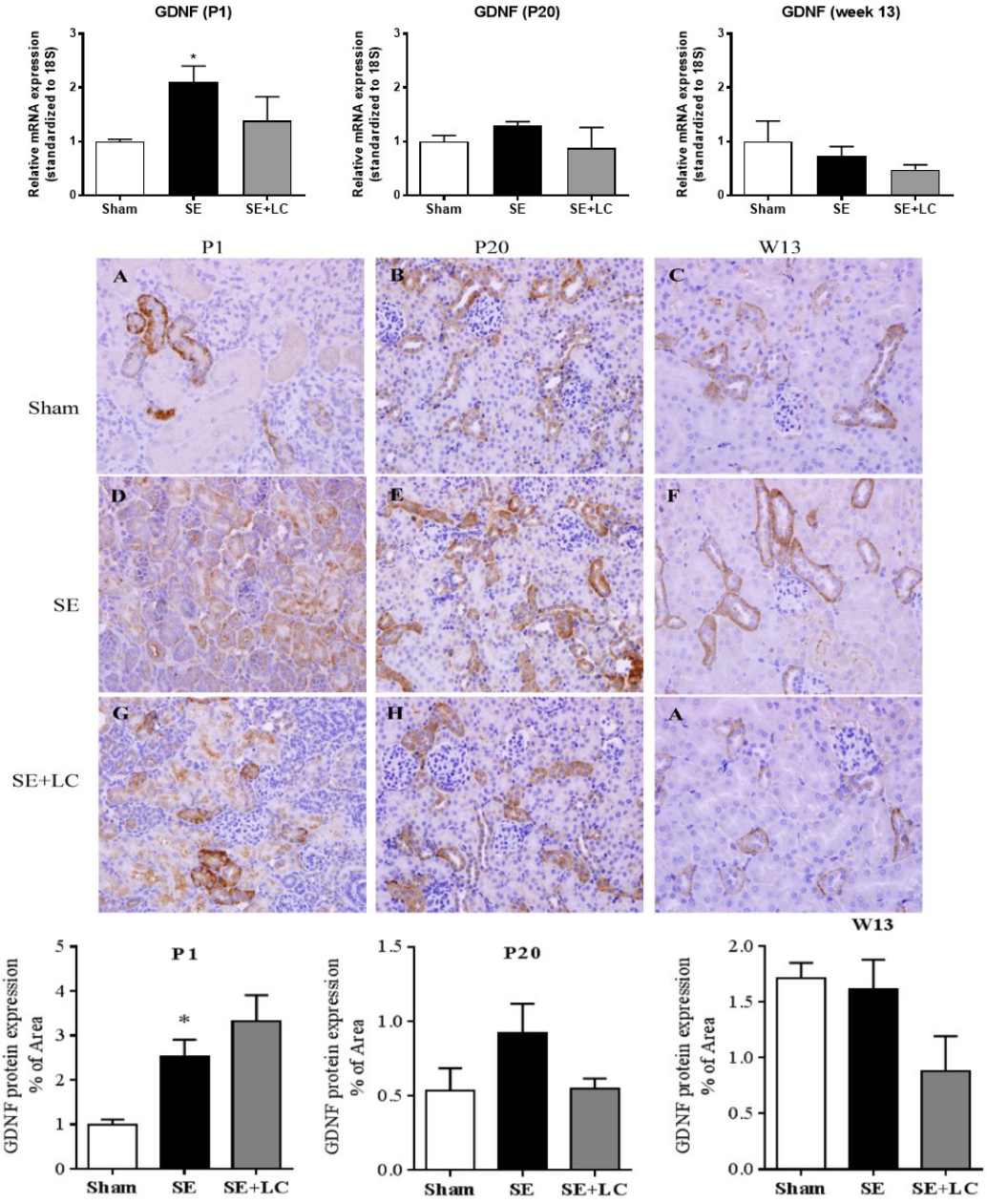

Figure 2: Effect of maternal smoking and LC administration on renal mRNA and protein expression of GDNF in the male offspring at P1, P20 and week 13. A) GDNF mRNA expression in the renal offspring from dams exposed to Sham, SE and SE+LC. B) Representative immunostaining images and quantitative images for GDNF proteins in the 3 groups at P1, P20 and Week 13. Results are expressed as Mean ± SEM. n = 5-6. * $P < 0.05$ SE vs Sham, $^{\#}$ $P < 0.05$ SE+LC vs SE.

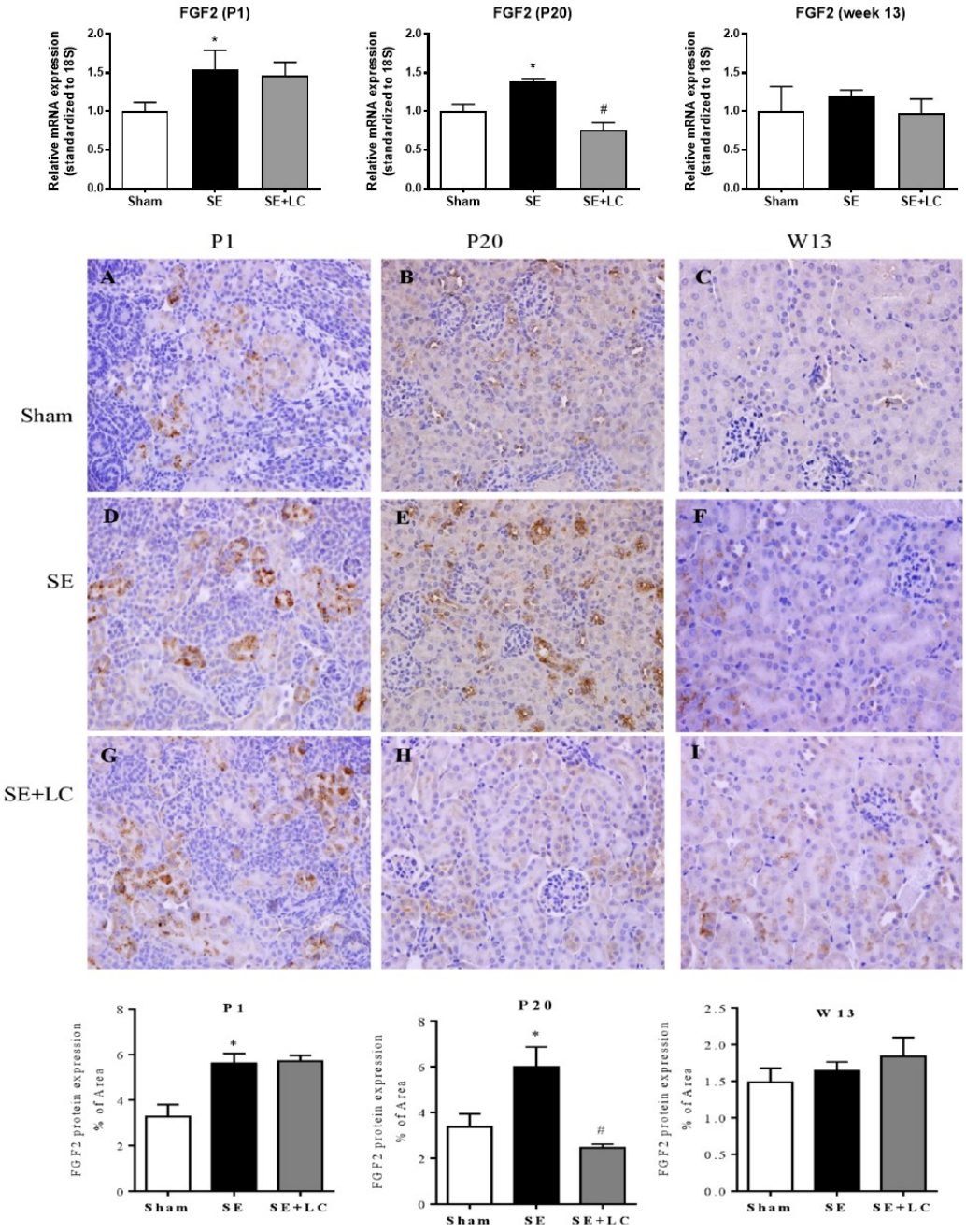

Figure 3: Effect of maternal smoking and LC administration on renal FGF2 mRNA and protein expression in the male offspring at P1, P20 and week 13. A) FGF2 mRNA expression in the renal offspring from dams exposed to Sham, SE and SE+LC. B) Representative immunostaining images and quantitative images for FGF2 proteins in the 3 groups at P1, P20 and Week 13. Results are expressed as Mean ± SEM. n = 6. Mag. 40X. * $P < 0.05$ SE vs Sham, $^{\#}$ $P < 0.05$ SE+LC vs SE.

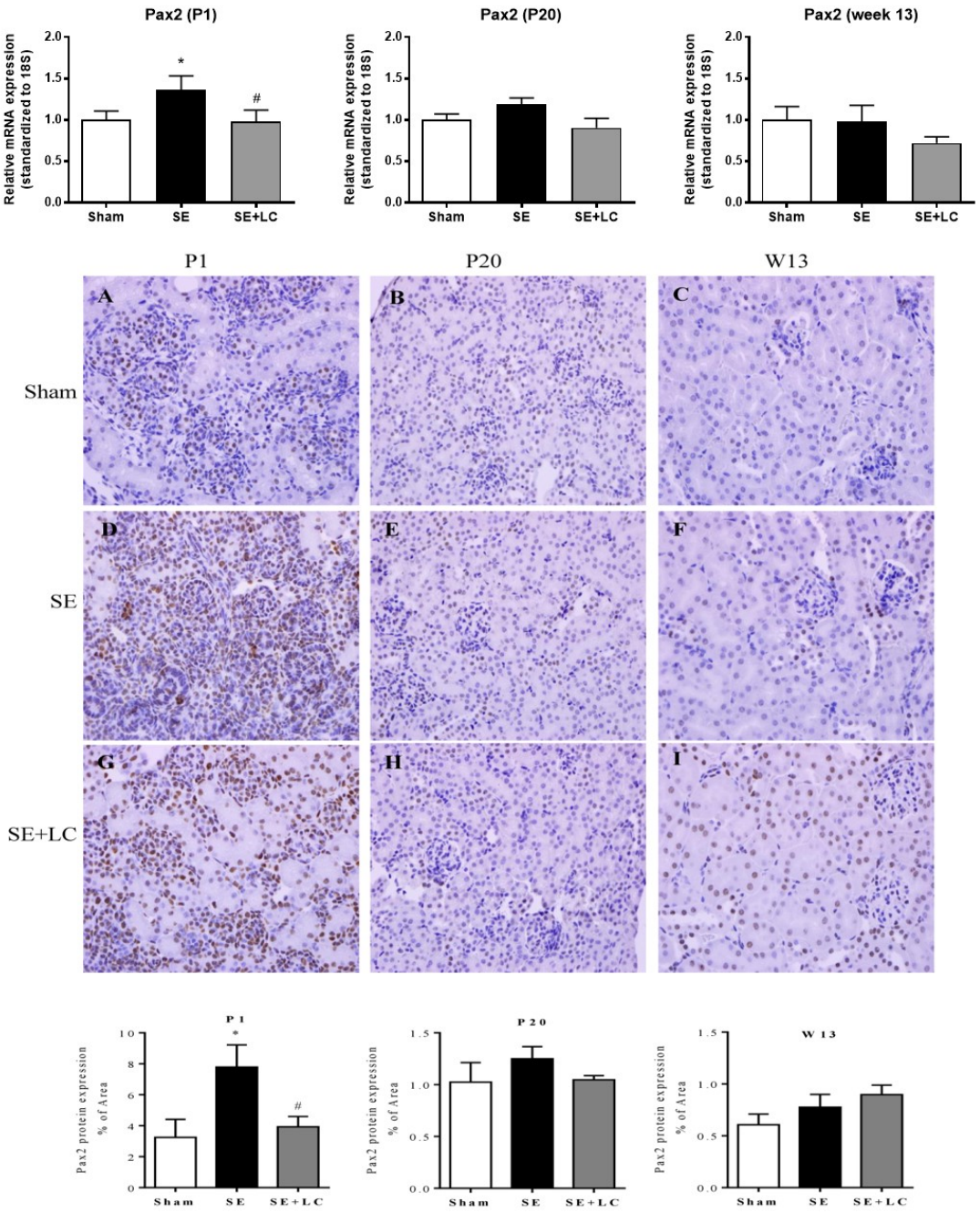

Figure 4: Effect of maternal smoking and LC administration on renal Pax2 mRNA and protein expression in the male offspring at P1, P20 and week 13. A) Pax2 mRNA expression in the renal offspring from dams exposed to Sham, SE and SE+LC. B) Representative immunostaining images and quantitative images for Pax2 proteins in the 3 groups at P1, P20 and Week 13. Results are expressed as Mean ± SEM. n = 6-8. Mag. 40X. * $P < 0.05$ SE vs Sham, $^{\#}$ $P < 0.05$ SE+LC vs SE.

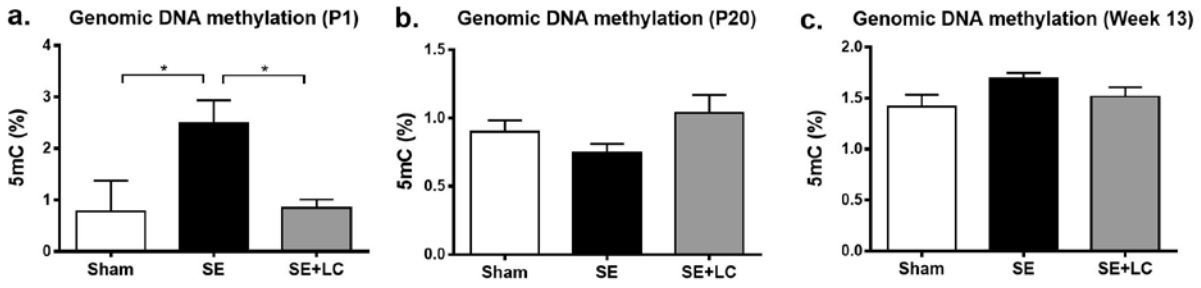

Figure 5: Renal DNA global methylation in the offspring of smoking mother administered with LC or vehicle at P1 (A), P20 (B) and Week 13 (C). Results are expressed as Mean ± SEM. * P < 0.05

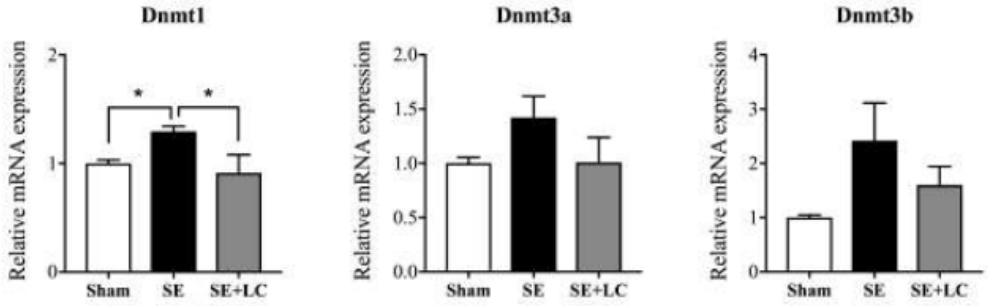

Figure 6. RNA expression of DNA methyltransferases in the offspring kidney at P1**.** Results are expressed as Mean ± SEM. * P < 0.05

Table 1: Forward and reverse sequences of the primers for rt-PCR.

Primer	**Forward 5′ -3′**	**Reveres 5′ -3′**
18S	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTTATGGTC
GDNF	ATTTTATTCAAGCCACCATTA	GATACATCCACACCGTTTAGC
FGF2	GACCCCAAGCGGCTCTACTGC	GTGCCACATACCAACTGGAGT
Pax2	CGCCGTTTCTGTGACACACAATC	TGCTTGGGACCAAACACAAGGTG
Dnmt1	GTGAACAGGAAGATGACAAC	CTGGATCCTCCTTTGATTTC
Dnmt3a	ACCAGAAGAAGAGAAGAATCC	CAATGATCTCCTTGACCTTAG
Dnmt3b	GACTTCATGGAAGAAGTGAC	TATCATCCTGATACTCTGTGC

FGF: fibroblast growth factor; GDNF: glial-cell line-derived neurotrophic factor; Pax: paired box gene; Dnmt: DNA methyltransferase.

Table 2: Effects of maternal SE on growth and development in male offspring.

P1	**Sham**	**SE**	**SE+LC**
Body weight (g)	1.63 ± 0.07	1.42 ± 0.08*	1.67 ± 0.05#
Kidney weight (g)	0.0085 ± 0.0005	0.0067 ± 0.0004*	0.0081 ± 0.0005##
Kidney/Body (%)	0.53 ± 0.03	0.50 ± 0.04	0.51 ± 0.03
P20	**Sham**	**SE**	**SE+LC**
Body weight (g)	10.0 ± 0.21	9.61 ± 0.10	10.1 ± 0.27
Kidney weight (g)	0.066 ± 0.002	0.060 ± 0.003	0.068 ± 0.002
Kidney/Body (%)	0.66 ± 0.02	0.63 ± 0.03	0.68 ± 0.01
W13	**Sham**	**SE**	**SE+LC**
Body weight (g)	25.6 ± 0.3	25.0 ± 0.4	25.7 ± 0.4
Kidney weight (g)	0.19 ± 0.01	0.19 ± 0.01	0.20 ± 0.01
Kidney/Body (%)	0.76 ± 0.02	0.76 ± 0.02	0.77 ± 0.02

Results are expressed as Mean ± SEM. n=7-11. One-way ANOVA followed by LSD *post hoc* tests were used to analyze the data among three groups at the same age. * $P < 0.05$ SE vs Sham, $^{\#}$ $P < 0.05$ and $^{\#\#}$ $P < 0.01$ SE+LC vs SE