Epigenetic effects of prenatal estradiol-17β exposure on the reproductive system of pigs

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ABSTRACT

There is growing evidence that early life exposure to endocrine disrupting chemicals might increase the risk for certain adult onset diseases, in particular reproductive health problems and hormone dependent cancers. Studies in rodents suggest that perinatal exposure to even low doses of estrogenic substances can cause adverse effects, including epigenetic reprogramming of the prostate and increased formation of precancerous lesions. We analyzed the effects of an *in utero* exposure to the strongest natural estrogen, estradiol-17β, in a pig model. Two different low and one high dose of estradiol-17β (0.05, 10 and 1000 μg/kg body weight/day) were orally applied to gilts during pregnancy and potential effects on the reproductive system of the offspring were analyzed. No significant effects on sperm vitality parameters and testes size were observed in adult boars. However, prenatal exposure to the high dose decreased absolute, but not relative weight of the testes in prepubertal piglets. RNA sequencing revealed significantly regulated genes of the prepubertal prostate, while testes and uteri were not affected. Notably, we found an increased prostate expression of *CCDC80* and a decreased *ADH1C* expression in the low dose treatment groups. *BGN* and *SPARC*, two genes associated with prostate tumor progression, were as well more abundant in exposed animals. Strikingly, the gene body DNA methylation level of *BGN* was accordingly increased in the high dose group. Thus, while only prenatal exposure to a high dose of estrogen altered testes development and local DNA methylation of the prostate, even low dose exposure had significant effects on gene expression in the prostate of prepubertal piglet offspring. The relevance of these distinct, but subtle transcriptional changes following low dose treatment lacking a clear phenotype calls for further long-term investigations. An epigenetic reprogramming of the pig prostate due to prenatal estrogen cannot be neglected.

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1. Introduction

Humans and animals are exposed to a number of exogenous chemicals that have the potential to influence their endocrine system. Those substances which exert adverse effects on an organism or its offspring are termed endocrine disrupting chemicals (EDCs) (WHO-UNEP, 2012). The "developmental origins of health and disease" (DOHaD) concept proposes that diseases in adult life can potentially originate from environmental factors that affect development during early life, with the embryonic phase being the most sensitive stage of life (Barker, 2004; Skakkebaek et al., 2011). The biological mechanisms underlying this programming are poorly understood, but epigenetic changes are likely to play a major role (Waterland and Michels, 2007). One of the best documented

Abbreviations

ADH1C	alcohol dehydrogenase 1C
ADI	acceptable daily intake
BGN	biglycan
BPA	bisphenol A
CCDC80	coiled-coil domain containing 80
CpG	cytosine-phosphate-guanine
DEG	differentially expressed gene
DES	diethylstilbestrol
E2	estradiol-17β
EDC	endocrine disrupting chemical
ESR	estrogen receptor
NOEL	no observed effect level
PCa	prostate cancer
Sc	subcutaneous injection
SPARC	secreted protein acidic and rich in cysteine
TSS	transcriptional start site
UTR	untranslated region

examples of EDC exposure in humans is diethylstilbestrol (DES), which was prescribed from the 1940s till the 1970s to an estimated 2 to 8 million pregnant woman, in the wrong belief of preventing miscarriages (Newbold, 2008). While treated mothers had an increased risk for breast cancer, exposed sons and daughters were predisposed to various health problems (Reed and Fenton, 2013). Most frequently found were reproductive tract abnormalities, such as subfertility or infertility in both genders and a rare form of vaginal cancer (Reed and Fenton, 2013). Importantly, many of these effects only became evident in adult life, decades after the original exposure.

The reproductive tract is a major target of EDCs with estrogenic activity. On the male side, most concern focuses on low semen quality, cryptorchidism, hypospadias and testicular cancer. While the incidence of the latter has clearly been rising over the last decades, it is not clear whether the same holds true for the first three illnesses (Bay et al., 2006; Thorup et al., 2010). It has been hypothesized that these four abnormalities are symptoms of a common underlying cause, called testicular dysgenesis syndrome (TDS), and that EDCs might contribute to TDS (Bay et al., 2006; Skakkebaek et al., 2001). In support of this hypothesis, a study reported that men who were exposed to dioxin *in utero* or during lactation after the Seveso accident in Italy had a 40% decrease in sperm counts (Mocarelli et al., 2011). Dioxin has an antiestrogenic effect that is primarily mediated through interaction with the aryl hydrocarbon receptor (AhR). Swan et al. found that sperm concentrations were decreased in men of mothers that consumed high amounts of red meat during pregnancy, possibly due to the uptake of steroids used in meat production (Swan et al., 2007). However, it remains unclear if low doses that are relevant for human every-day exposure can have an adverse effect. It has been shown that strong EDCs like DES can impair testes size and function in neonatal rats at a dose of 5 µg/day sc. (subcutaneous injection) (McKinnell et al., 2001; Sharpe et al., 1998) or 100 µg/L in drinking water (Sharpe et al., 1995). In sheep, twice weekly sc. injection of pregnant ewes with 0.5 µg DES/kg bw significantly reduced testis weight and the number of sertoli cells in male offspring (Sweeney et al., 2000). And in a rat model it was demonstrated that estrogen (1 µg E2/day sc.) has an effect on testicular gonocyte maturation as it changed the migration capacity of this germ cell population from the lumen towards the basal lamina of the seminiferous cords (Vigueras-Villasenor et al., 2006). It also has to be considered that the effects of estrogen are time-dependent, as there exist estrogen-sensitive periods for gametogenesis. Testicular exposure to estrogen during neonatal stages for example may be more critical since final gonocyte maturation takes place here giving rise to spermatogonia (Sharpe and Skakkebaek, 1993; Vigueras-Villasenor et al., 2006).

Another putative target of endocrine disruption in males is the prostate gland, which is highly dependent on steroid hormones during embryonic development (Prins et al., 2007). Rates of prostate cancer (PCa), which is the second most common cancer in men, have been rising over the last decades in most parts of the world (Center et al., 2012). One notable finding is that PCa is more frequent in African-American (AA) than in Caucasian-American (CA) men (Farrell et al., 2013). Since circulating estrogen concentrations during pregnancy are higher in AA than in CA women (Henderson and Feigelson, 2000; Potischman et al., 2005), it has been hypothesized that this could contribute to the increased incidence of PCa among AA men (Ho et al., 2011). The developmental sensitivity of the prostate to estrogens is supported by a human fetal prostate model, which demonstrated that estrogen exposure can alter the differentiation and epigenetic programming of the embryonic prostate (Saffarini et al., 2015). Further evidence comes from studies in rats, showing that perinatal exposure to 17β-estradiol-3-benzoate (EB, 1250 µg/day sc.) or bisphenol A (BPA, 5 µg/day sc. and 50 µg/kg bw/day oral) increases the formation of precancerous prostate lesions (Ho et al., 2006; Yean Wong et al., 2015). This was associated with changes in gene expression, local DNA methylation and H3K9 acetylation in the prostate (Ho et al., 2006; Tang et al., 2012; Yean Wong et al., 2015). In particular, the putative oncogene *Hmgn5* was overexpressed and hypomethylated, while the putative tumor suppressor gene *Hpcal1* was silenced and hypermethylated. Importantly, epigenetic alterations were observed prior to histopathologic changes, implying that prostate cancer might be associated with epigenetic changes that are measurable long before disease outbreak. Possibly, epigenetic markers could thus be used to assess the risk of prostate cancer as a result of endocrine disruption.

It was shown that also the female reproductive tract is sensitive to early endocrine disruption, possibly leading to reduced fertility and reproductive tract abnormalities in later life (Crain et al., 2008; Reed and Fenton, 2013). This is exemplified in the uterotrophic assay, which measures the potential of chemicals to stimulate uterine growth and is frequently used to assess the estrogenic potential of chemicals (Reel et al., 1996). Recently, the effect of BPA exposure on the fetal uterus of rhesus macaques was investigated. This study found that morphological development was not affected, but the expression of key developmental genes was altered in the BPA (400 µg/kg bw/day oral) exposed uteri, possibly influencing its function in later life (Calhoun et al., 2014).

In the present study, two low (0.05 and 10 µg/kg body weight/day) and a high (1000 µg/kg bw/day) concentration of estradiol-17β (E2) were orally applied to gilts during the whole pregnancy. The low doses are in the range of the human "acceptable daily intake" (ADI) of 0.05 µg/kg bw/d and the "no observed effect level" (NOEL) of 5 µg/kg bw/d (JECFA, 1999). The pig was chosen as a model system due to its similarities to humans in placental estrogen synthesis (Simpson and MacDonald, 1981). Unlike in humans and pigs, placental estrogen levels remain low during pregnancy in rodents (Strauss et al., 1996). Furthermore, the embryonic development of pigs is more similar to humans than that of other common model organisms. In humans and pigs, the prostate is already developed *in utero*. In contrast, the rodent prostate is only rudimentary developed at birth and undergoes development in the first two weeks postnatally (Prins et al., 2006). Previously, we found that E2 exposure increased body fat percentage in male piglets

(Furst et al., 2012b) and slightly perturbed bone parameters in female offspring (submitted manuscript). However, uterine expression and promoter DNA methylation of *HOXA10* were not affected in female piglets (Pistek et al., 2013). Also sex-ratio, litter size and the endocrine status of both genders were not affected by the E2 treatment (Furst et al., 2012b). In this report, we investigated the effects of *in utero* E2 exposure on testis, prostate and uterine development. We particularly focused on epigenetic changes that are measurable by alterations in transcription levels and DNA methylation. With this approach, we identified several target genes of estrogenic developmental reprogramming in the prostate.

2. Materials and methods

2.1. Animal studies

The animal trial was performed with German Landrace sows inseminated with Pietrain semen, as described previously in detail (Furst et al., 2012b). An initial pharmacokinetic study was performed to measure the elimination kinetics of different doses of E2. In the main trial, E2 was applied at concentrations of 0, 0.05, 10 and 1000 μg/kg body weight/day to sows orally (n = 6—7 per treatment) during the whole pregnancy. Prior to normal food ratios, half of the dose was fed in the morning, the other half in the evening. E2, including 2 mL of ethanol carrier, was fed within a bread roll (20 g), the control group received only carrier. In sows of the high dose group, plasma E2 concentrations were elevated about 2—3 fold during early and middle pregnancy, but not during late pregnancy (due to naturally increasing E2 levels). No significant changes in free circulating plasma E2 concentrations were measured in sows of the two low dose groups. The main group of male offspring was slaughtered prepubertally at 8 weeks of age (n = 15—17 per treatment), the second group at about one year of age (n = 3—6 per treatment). Due to handling reasons, female progeny was slaughtered at 9 weeks of age (n = 12 per treatment).

All experiments and sampling were conducted in accordance with accepted standards of humane animal care and were approved by the District Government of Upper Bavaria, reference # 55.2-1-54-2531-68-09.

2.2. HE staining and quantification of centrally located gonocytes

The testes were removed from the epididymis and the vaginal tunic, sectioned into tissue samples sized about $1 \times 1 \times 1$ cm using a microtome blade and fixed in Bouin's solution (10% formaldehyde, 4% picric acid, 5% acetic acid) for hematoxylin eosin (HE) staining. After 24 h in Bouin's solution the tissue samples were transferred to 70% alcohol, followed by paraffin embedding.

In order to assess testicular morphology, in particular the localization of gonocytes within the seminiferous cords, 4 μm sections of Bouin-fixed paraffin-embedded samples mounted on glass slides were stained with HE. Morphological evaluation was performed via light microscopy using a Zeiss Axioskop (Carl Zeiss Microscopy GmbH, Jena, Germany) with an Olympus DP 70 camera and the Olympus DP Soft software (Olympus Deutschland GmbH, Hamburg, Germany).

To determine the portion of centrally located gonocytes within the seminiferous cords, 300 gonocytes per animal were counted in randomly chosen visual fields. It was distinguished between gonocytes in contact to the basal lamina (basal gonocytes) and gonocytes without contact to the basal lamina (centrally located gonocytes) according to (Vigueras-Villasenor et al., 2006). Thus, for each group a total of 900 gonocytes (basal and centrally located) were analyzed and subsequently, the percentage of centrally located gonocytes was determined.

2.3. Anti-DDX4 (VASA)-Immunohistochemistry

After deparaffinization and inhibition of the endogenous peroxidase activity with 3% H_2O_2 in 80% ethanol for 30 min, immunohistochemical sections were pretreated with sodium citrate buffer (pH 6.0) for 20 min at 96—99 °C on a heating plate. Slides were then cooled down for 30 min at room temperature, blocked with 20% normal goat serum for 20 min and incubated with the primary antibody (anti-DDX4 (VASA), Abcam, UK, Catalog-No.: ab13840, dilution 1:200) over night at 4 °C. VASA is known to play a role in germ cell development and represents a germ cell maturation marker (Hancock, 1957; Henning et al., 2012). The sections were then exposed for 30 min at room temperature with EnVision™ + Kit HRP Rabbit DAB+ (Dako, Hamburg, Germany, Catalog-No. K4011). After visualization with DAB, some sections were either counterstained with hematoxylin for 2 s, or no counterstaining was performed, and rinsed with running water. Finally, all slides were mounted with Eukitt™ (O. Kindler GmbH, Freiburg, Germany). Negative controls were performed by substitution of the primary antibody by buffer.

2.4. Semen analysis

Three ejaculates of each of four offspring boars from sows treated with 1000 μg/kg bw/d E2 and of five offspring boars from control sows were collected by the gloved-hand technique. Ejaculate volumes were determined and aliquots were diluted 1:10 with pre-warmed Beltsville Thawing Solution (BTS, Minitüb, Tiefenbach, Germany) to a total volume of 100 ml. Aliquots of raw semen and extended semen portions were transported temperature-controlled overnight to the University of Veterinary Medicine Hannover for further analysis. Semen was stored at +17 °C in darkness until analysis.

Sperm concentration in raw ejaculates was determined using a hemocytometer and total sperm numbers were calculated. Further sperm parameters were assessed in extended semen samples. At 24 h, sperm morphology was evaluated from a semen sample fixed in formol citrate using phase contrast microscopy (Hancock, 1957) and the integrity of acrosomal and plasma membranes was determined by flow cytometry after double staining with Propidium iodide (PI) and FITC-Peanut Agglutinin (Henning et al., 2012). After 24, 48 and 72 h of storage, sperm kinematics were assessed using the computer-assisted semen analysis (CASA) system SpermVision® (Minitüb, Tiefenbach, Germany) as described previously (Henning et al., 2012). The responsiveness to the capacitation inducer bicarbonate was assessed in semen stored for 24 h as described (Henning et al., 2012, 2015).

2.5. DNA and RNA extraction

Tissue samples for nucleic acid extraction were collected after slaughter, immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. Total RNA from the uterus of piglets was extracted using the NucleoSpin RNAII Kit (Macherey Nagel, Düren, Germany), as described previously (Pistek et al., 2013). Total DNA and RNA from the prostate (corpus prostatae), testes, liver, spleen, heart and adrenal gland of male piglets and boars was extracted with the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. For homogenization of tissues, the Qiagen TissueLyser II and 5 mm stainless steel beads (Qiagen) were used. RNA concentrations were quantified using the NanoDrop 2000 (peqLab, Erlangen, Germany). The integrity of the RNA was assessed using the Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany) and the Agilent RNA 6000 Nano Kit. RNA integrity numbers (RIN) were between 7 and

10 for all samples.

2.6. RNA sequencing

For each treatment and gender, samples from 6 piglets were further processed for RNA sequencing (RNA-seq). Female piglets were derived from 6 different sows, male piglets from 5 different sows. The same animals were used for the sequencing of prostate and testes. Sequencing libraries were constructed with 100 ng of total RNA using the TruSeq Stranded mRNA Sample Prep Kit (Illumina, San Diego, CA, USA). Sequencing was performed on an Illumina HiSeq 2500 machine using single end 100bp sequencing.

The obtained sequence reads (Fastq files) from porcine prostate, uterus and testis tissue samples were analyzed with several tools on a locally installed version of Galaxy (Giardine et al., 2005). First, the sequence reads were checked for Illumina adapter contamination with the tool FastqMcf and clipped if necessary. Afterwards, the resulting sequences were trimmed on the 5 prime end and also quality filtered with a sliding window approach with a tool called Trimmomatic (v 0.33) (LEADING: 3 nt., SLIDINGWINDOW: 5 nt., QUALTIYCUTOFF: 28 and MINLEN: 50 nt.). All Fastq files were quality checked before and after trimming with FastQC (v0.11.2) to ensure the correct quality procedure and double check the result. Reads were mapped with Tophat2 (v2.0.11) (Kim et al., 2013) to the porcine genome sequence assembly (Sscrofa10.2, August 2011) and with the corresponding GFF annotation file from the National Center for Biotechnology Information (NCBI) (ftp://ftp.ncbi. nih.gov/genomes/ Sus_scrofa/GFF). To count all mapped reads per gene we used the BioConductor package QuasR (v1.8.2) (Gaidatzis et al., 2015) within a modified R script. The resulting count table was filtered to remove genes that didn't have a minimum of 20 counts in 5 out of 6 samples in at least one treatment group. Analysis of differential gene and isoform expression was performed with the BioConductor DESeq v1.8.1 (Anders and Huber, 2010). An adjusted p-Value of 0.01 was used as threshold for significance of differentially expressed genes.

2.7. cDNA synthesis and qPCR

500 ng of each RNA sample was reverse transcribed with the GoScript Reverse Trancription System (Promega, Madison, WI, USA), using the following reaction mix: 10 μl RNA/H_2O, 0.5 μl Oligo(dT)$_{15}$ primer, 0.5 μl random primer, 4 μl reaction buffer, 2.5 μl $MgCl_2$, 1 μl dNTPs, 0.5 μl RNasin, 1 μl reverse transcriptase. The reaction mix was incubated in a PCR cycler with the following conditions: 5 min at 25 °C, 60 min at 42 °C and 15 min at 70 °C.

Messenger RNA (mRNA) levels were determined by quantitative real-time PCR (qPCR) on a CFX384 Real-Time PCR Detection System (Bio-Rad, Munich, Germany) with the KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Wilmington, MA, USA). The qPCR was performed in a reaction volume of 10 μl, consisting of 5 μl 2× Master Mix, 0.4 μl of each primer (10 μM), 0.07 μl VisiBlue (TATAA Biocenter, Göteborg, Sweden), 3.13 μl water and 1 μl cDNA. The primers that were used are listed in Table S1. Cq values were obtained using a single threshold and relative quantification of the mRNA levels was performed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). For normalization, the geometrical mean of the three reference genes H3F3A, UBB and YWHAZ was used.

2.8. Design of methylation assays

We searched for CpG islands in the promoter (until 10 kb upstream of the TSS) and the coding region of genes of interest. A publically available CpG island searcher (Takai and Jones, 2003) (http://cpgislands.usc.edu/) was used to identify CpG islands with the following lower limit values: 55% GC, 0.65 ObsCpG/ExpCpG, 200

bp lengths, 100 bp gap between islands. No CpG islands were identified in the *SPARC* gene body or promoter. The porcine *ADH1C* gene contains two small CpG islands, 1 kb and 3.5 kb downstream of the transcriptional start site (TSS). We designed assays for both regions, however, only the assay for the second region covering 8 CpG sites was implemented. Analysis of the first region was not possible, presumably due to self-binding of the PCR product, which interfered with the sequencing process. *CCDC80* contains two small CpG islands 1.7 kb and 2.6 kb downstream of the TSS, for both of which we successfully designed assays spanning 17 CpG sites in total. The *BGN* gene has a very high CG content of 67% (*ADH1C*: 38%, *CCDC80*: 42%) and contains numerous CpG islands. We selected two islands 0.6 kb and 11.7 kb downstream of the TSS, covering 15 CpG sites in total. The primers for the PyroMark assays were designed using the PyroMark Assay Design Software 2.0 (Qiagen, Hilden, Germany) and are listed in Table S2. In order to confirm their accuracy, the assays were established with control DNA of known methylation levels (0, 50 and 100%). These methylation standards were generated by whole genome amplification and *in vitro* methylation of genomic DNA, as previously described (Furst et al., 2012a).

2.9. Bisulfite pyrosequencing

One μg of genomic DNA was bisulfite converted using the EpiTect Fast Bisulfite Conversion kit (Qiagen) according to the manufacturer's instructions. The concentration of bisulfite converted DNA was measured using the Quantus fluorometer and the QuantiFluor® ssDNA System (Promega, Madison, WI, USA). 20 ng of DNA was used as a template for each of the subsequent PCRs. PCR amplification was performed on a Labcycler (Sensoquest, Göttingen, Germany) using the PyroMark PCR kit (Qiagen). Gel electrophoresis was applied to confirm the presence of a single PCR band of the expected size. CpG methylation was quantified using the PyroMark Q48 Autoprep System (Qiagen) and the PyroMark Q48 Advanced CpG Reagents (Qiagen). Methylation values were calculated with the PyroMark Q48 Autoprep 2.4.1 Software (Qiagen).

2.10. Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics Software version 22 (IBM, Böblingen, Germany). To test for differences in weight parameters, gonocyte localization, qPCR expression levels and DNA methylation, ANOVA and a Dunnett's post hoc test were performed, using the non-treated group as control. P-Values < 0.05 were considered significant. For analysis of the qPCR data, the relative expression values were log transformed. No statistical analysis was performed of the expression data of the different tissues, due to the small sample number (n = 2). Analysis of DNA methylation was conducted individually for each CpG site, mean methylation values were used for linear regression statistics. All data are presented as mean ± standard error of the mean (SEM).

For statistical analysis of the sperm parameters, average values of the three samples per boar were used. To test for statistically significant differences, the student's *t*-test was used. For 3 of the 22 parameters analyzed, the normality test failed. In these cases, a Mann-Whitney test was performed.

3. Results

3.1. Relative testes weight and testes morphology of piglets is not affected by in utero E2 exposure

In all the male offspring of the experiment, both testes were descended at birth, and no gross abnormalities of the reproductive organs could be observed. As shown previously, neither litter size

nor birth weight of the piglets were affected by the treatment (Furst et al., 2012b). However, at the age of 8 weeks, male piglets from the high dose group (1000 μg E2/kg bw/day) were by trend lighter than those of the control group (13.7 ± 0.84 kg vs. 16.4 ± 0.51 kg, p = 0.07) (Fig. 1A). At the same age, weight of the testes and epididymis was also reduced in the high dose group (16.8 ± 1.1 g vs. 20.6 ± 0.6 g, p = 0.04) (Fig. 1B). No effect was observed in the ADI (0.05 μg E2/kg bw/day) and NOEL (10 μg E2/kg bw/day) dose groups. Comparison of the ratio between testes and body weight resulted in very similar values in all four groups (Fig. 1C). As shown previously, blood plasma concentrations of neither E2 nor testosterone differed significantly in either of the sexes at slaughter (Furst et al., 2012b). In 9 week old female piglets, body weight, uterine weight and relative uterine weight was not altered in any of the treatment groups (Pistek et al., 2013).

To analyze if maternal estrogen exposure caused changes in testes morphology, testicular development or germ cell maturation, histological sections of a subset of randomly chosen 8 week old animals were examined (n = 3 animals per group). Using hematoxylin eosin (HE) staining, as well as well as immunohistochemistry of the germ cell lineage specific marker VASA, no significant phenotypic differences were apparent (Fig. S1). In order to quantify the developmental stage of germ cells, we determined the percentage of gonocytes that were located centrally, meaning they had not yet reached the seminiferous tubule basal lamina. The percentages [±SEM] of centrally located gonocytes in the four groups were 66.8 ± 4.1% (Control), 69.6 ± 8.0% (ADI), 70.9 ± 1.1% (NOEL) and 74.3 ± 2.6% (high dose) (n = 3 animals per group, 300 gonocytes quantified per animal). The differences between the groups were not statistically significant (p > 0.05).

3.2. Sperm parameters are normal in boars prenatally exposed to high E2 levels

Randomly chosen piglets from each group were raised to adulthood and analyzed at one year of age (n = 3–6 animals per group). In these animals, no effect of the E2 exposure on body and testicular weight was observed (Fig. 1D–F). Furthermore, sperm quality of boars from the control and high dose groups was assessed (Table 1). In all 27 ejaculates, quantitative parameters, i.e. volume, sperm concentration and total sperm counts, fulfilled minimum requirements for boar ejaculates according to the guidelines of the umbrella organization of organized pig production in Germany (ZDS, 2005). Eight boars (four from the high dose and four from the control sows) consistently showed normospermia, whereas one boar from the control group revealed dysspermia in all three ejaculates. Dysspermia was apparent in motility and morphology values below minimum requirements for boar sperm quality (ZDS, 2005). Membrane integrity, sperm kinematics including total motility, progressive motility, curvalinear velocity, amplitude of lateral head displacement and beat cross frequency did not differ between the treatment groups. In addition, the specific responsiveness to the capacitation inducer bicarbonate did not show significant differences.

3.3. Prenatal E2 exposure induces transcriptional changes in the prostate

We performed RNA sequencing (RNA-seq) on uterine, testis and prostate whole tissue samples of 8 week (male) and 9 week (female) old prepubertal piglets (n = 6 per group). The transcriptome of each treatment group was analyzed in comparison with the respective control group. Overall, we identified only one differentially expressed gene (DEG) in the uterus and only 3 DEGs in the
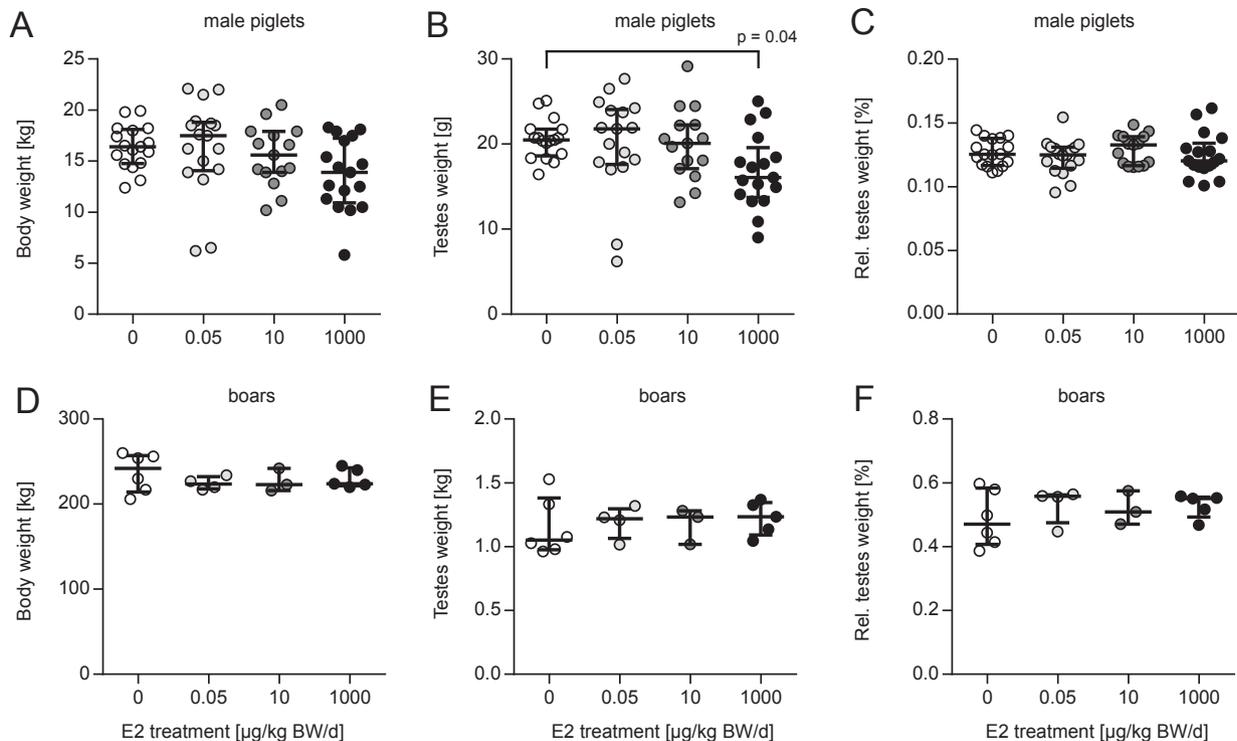
Fig. 1. Body parameters of pigs exposed to different concentrations of estradiol-17β *in utero*. Body weight, testicular weight (including the epididymis) and relative testicular weight are shown for individual 8 week old piglets (**A–C**) and one year old boars (**D–F**). Bars represent the 25th, 50th and 75th percentile, the number of animals for the 0, 0.05, 10 and 1000 μg groups is 17, 17, 15 and 17 for piglets and 6, 4, 3 and 5 for boars.

Table 1
Sperm parameters (mean ± SEM) of boars exposed prenatally to a high estrogen dose (1000 μg E2/kg bw/day, n = 4) and control boars (n = 5). Samples were either native semen or extended semen at the indicated time points (24, 48 or 72 hours after collection).

Parameter	Semen sample	High dose	Control	P Value
Volume [mL]	native	234 ± 22	237 ± 37	>0.1
Sperm concentration [× 10^6/mL]	native	412 ± 32	392 ± 35	>0.1
Total sperm count [× 10^9]	native	94.3 ± 8.1	87.2 ± 7.9	>0.1
Morphological abnormal sperm, all defects [%]	24 h	15.9 ± 2.0	23.0 ± 8.0	>0.1
Morphological abnormal sperm, primary defects [%]	24 h	1.0 ± 0.2	2.0 ± 1.2	>0.1
Membrane defect [%]	24 h	11.8 ± 1.4	17.6 ± 6.2	>0.1
Specific response to bicarbonate [%]	24 h	48.9 ± 1.0	37.7 ± 5.6	>0.1
Total motility [%]	24 h	89.9 ± 2.0	86.0 ± 2.9	>0.1
Total motility [%]	48 h	89.7 ± 2.8	84.9 ± 3.3	>0.1
Total motility [%]	72 h	87.6 ± 3.6	82.7 ± 3.3	>0.1
Progressive motility [%]	24 h	81.8 ± 2.9	73.2 ± 3.7	>0.1
Progressive motility [%]	48 h	79.7 ± 4.3	71.8 ± 4.3	>0.1
Progressive motility [%]	72 h	77.2 ± 6.1	67.8 ± 5.2	>0.1
Velocity curve line, VCL [μm/sec]	24 h	119 ± 13.8	109.0 ± 4.6	>0.1
Velocity curve line, VCL [μm/sec]	48 h	115 ± 12.4	98.4 ± 7.1	>0.1
Velocity curve line, VCL [μm/sec]	72 h	107 ± 15.4	93.7 ± 10.5	>0.1
Amplitude lateral head displacement, ALH [μm]	24 h	2.93 ± 0.24	2.82 ± 0.14	>0.1
Amplitude lateral head displacement, ALH [μm]	48 h	2.84 ± 0.18	2.61 ± 0.15	>0.1
Amplitude lateral head displacement, ALH [μm]	72 h	2.66 ± 0.16	2.48 ± 0.20	>0.1
Beat cross frequency, BCF [Hz]	24 h	36.4 ± 1.3	35.4 ± 0.8	>0.1
Beat cross frequency, BCF [Hz]	48 h	36.7 ± 2.1	34.4 ± 1.1	>0.1
Beat cross frequency, BCF [Hz]	72 h	36.0 ± 2.6	33.6 ± 1.9	>0.1

testis. In the uterus of piglets from the ADI group, Integrin alpha E (*ITGAE*) was up regulated 1.6-fold. In the testis, NIMA-related kinase 3 (NEK3) was up regulated 1.5-fold in the ADI group. In the high dose group, calponin 1 (CNN1) was down regulated −1.7-fold and LOC100739603 was up regulated 1.8-fold.

Analysis of the prostate samples revealed 2 DEGs in the ADI group, one DEG in the high dose group and 128 DEGs in the NOEL group (Table S3). Coiled-coil domain containing 80 (*CCDC80)* was the only gene that was significantly regulated in two groups (NOEL and high dose). Of the total 130 DEGs, 120 were up regulated and 10 were down regulated. Laeverin (*LVRN*, +3.0-fold) and Alcohol dehydrogenase 1C (*ADH1C*, -3.3-fold) were the two most strongly deregulated genes.

To confirm our findings, we selected 16 up and 3 down-regulated genes and measured their expression by qPCR. We performed a technical validation using the same RNA as in the sequencing, as well as a biological validation using samples of 6 additional animals of each group (Fig. S2). Linear regression analysis of the qPCR and RNA-seq data revealed a highly significant correlation between the two data sets (R^2 = 0.785, p < 0.001). However, when the additional biological replicates were included, we only found significant expression differences in 4 of the genes (Fig. 2). *ADH1C* expression relative to the control was reduced −4.8-fold (ADI) and −8.3-fold (NOEL). Expression of *CCDC80* was increased 1.5-fold (ADI) and 1.7-fold (NOEL). Biglycan (*BGN*) expression was increased 1.6-fold (ADI) and 1.4-fold (high dose) and secreted protein acidic and rich in cysteine (*SPARC*) 1.3-fold in the NOEL group.

Using all 12 biological replicates per treatment, we also measured by qPCR the mRNA levels of *HMGN5*, *PDE4D4*, *HPCAL1*, *DNMT1*, *DNMT3A*, *DNMT3B*, *ESR1* and *AR*, which are candidate genes for endocrine disruption in the prostate (Tang et al., 2012). In agreement with our RNA-seq data, the expression of these genes remained unchanged in all treatment groups (Fig. S3). *ESR2* expression was either absent or extremely low expressed in all samples and could not be quantified by RNA-seq or qPCR.

Furthermore, we quantified the expression of the above mentioned candidate genes in the prostate of adult boars (Fig. S4). There were no significant expression differences in any of these genes between the groups. We also measured expression levels of

ADH1C, *BGN* and *CCDC80*, which were differentially regulated in prepubertal piglets. *ADH1C* expression was too low to be quantified accurately, while expression of the other two genes was not affected by the E2 treatment (Fig. S4).

We were also interested in whether the expression differences caused by the E2 exposure could be found in a tissue different than the prostate. We first measured expression of the 4 DEGs in different tissues of untreated animals (Fig. S5). We chose to further analyze the liver, where *ADH1C* is expressed about 200-fold higher than in the prostate of piglets. We found that there were no significant differences in *ADH1C* mRNA expression between the E2 treatment groups (n = 6, Fig. S6). Also the expression levels of *BGN*, *CCDC80* and *SPARC* were not affected in the liver (Fig. S6).

3.4. Prenatal E2 exposure alters prostate DNA methylation of BGN

We next asked whether the transcriptional changes in the prostate of E2 exposed piglets could be associated with epigenetic modifications. Therefore, assays to measure local CpG methylation of the genes *ADH1C*, *CCDC80* and *BGN* by bisulfite pyrosequencing were designed as described in section 2.8. Methylation of a CpG island within the gene body of *ADH1C* was around 80% and none of the CpG sites were significantly altered by maternal exposure to any of the three E2 doses in piglets (Fig. 3A,B) or boars (Fig. S7). There was no correlation between *ADH1C* methylation and mRNA expression in prostate tissue (R^2 = 0.011, adj. R^2 = −0.005, p = 0.41). We also measured *ADH1C* methylation in liver samples of untreated piglets and boars. Interestingly, methylation in the liver dropped from 76% in prepubertal to 35% in postpubertal animals, associated with a 1.9-fold increase in mRNA levels (Fig. 3C). This indicates that *ADH1C* methylation of this CpG island might be associated with transcription in the liver, but not in the prostate.

Methylation of two CpG islands in the first exon of *CCDC80* did not differ between the treatment groups in piglets (Fig. 4A,B) or boars (Fig. S7). However in piglets, all three treatment groups were by trend lower methylated at CpG 8 to 17 (Fig. 4B). For both CpG islands, there was a negative correlation between mRNA expression and DNA methylation in the prostate, which was more pronounced for CpG 1 to 7 (R^2 = 0.651, adj. R^2 = 0.423, p = 5.9E-09) than for CpG 8 to 17 (R^2 = 0.261, adj. R^2 = 0.249, p = 1.6E-05) (Fig. 4C,D). These
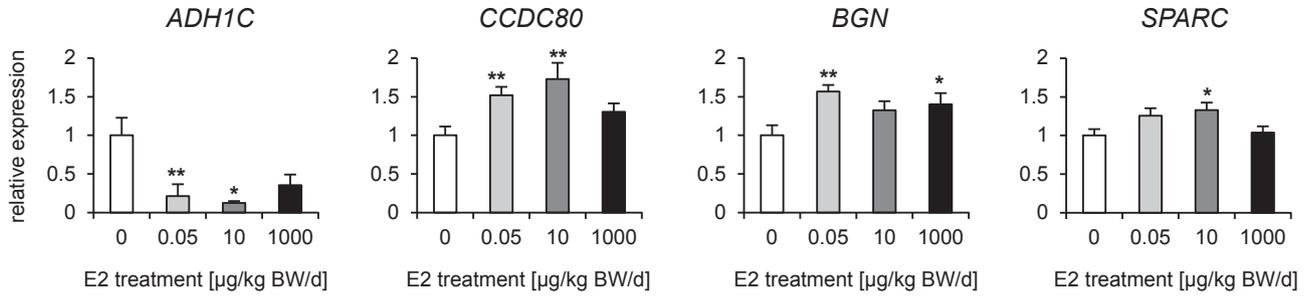
Fig. 2. qPCR expression analysis shows that *ADH1C*, *CCDC80*, *BGN* and *SPARC* are differentially expressed upon prenatal estrogen treatment in the prostate. Data are presented as mean ± SEM, n = 12, *p < 0.05, **p < 0.01.
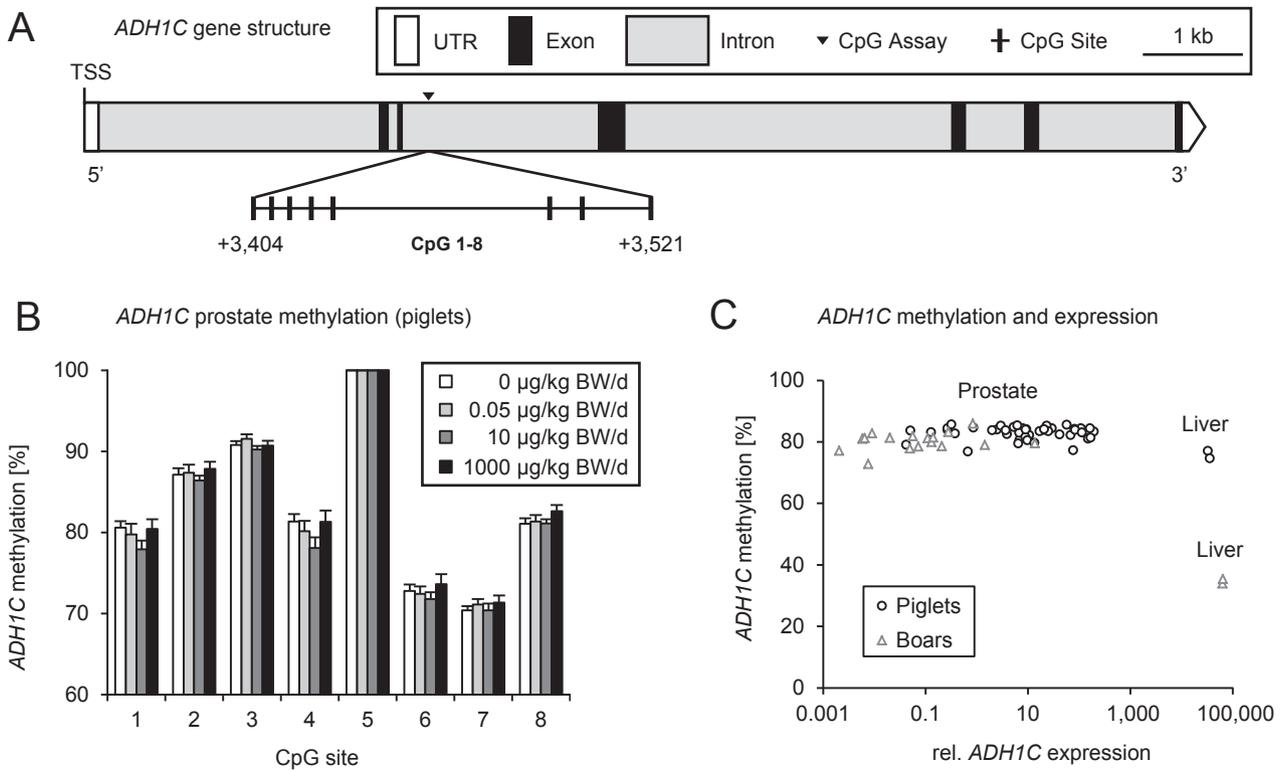

Fig. 3. Methylation of *ADH1C* is not altered in prenatally estrogen exposed animals. (**A**) Gene structure of *ADH1C* and the location of the analyzed CpG island. (**B**) Prostate *ADH1C* methylation at individual CpG sites of prepubertal piglets. Data are presented as mean ± SEM, n = 12. (**C**) Mean CpG methylation in relation to *ADH1C* mRNA expression in the liver and prostate.

correlations were mainly due to the fact that in boars, *CCDC80* expression was lower and methylation generally higher than in piglets.

For a CpG island near the TSS of *BGN*, there were no significant methylation differences between the piglet treatment groups, although methylation was by trend smaller in the ADI group at 7 of the 8 CpG sites (Fig. 5A,B). At the second locus within the gene body, methylation at CpG sites 9 to 15 was significantly increased in the high dose group (p < 0.05) (Fig. 5B). In adult boars, none of the CpG sites was affected by the E2 treatment (Fig. S7). Average methylation at CpG 1 to 8 was negatively correlated with mRNA expression among all prostate samples ($R^2 = 0.411$, adj. $R^2 = 0.401$, p = 1.5E-08) (Fig. 5C). Methylation at the second *BGN* site from CpG 9 to 15 was positively correlated with mRNA expression ($R^2 = 0.272$, adj. $R^2 = 0.260$, p = 1.2E-05) (Fig. 5D). Boars had lower *BGN* expression levels than piglets, and higher or lower methylation, depending on the CpG islands analyzed.

4. Discussion

In the present study, we analyzed the effects of prenatal estrogen exposure on the reproductive tract in a pig model system. As in many other mammals, in adult boars germ cell number, efficacy of spermatogenesis and testes size are primarily determined by the number of somatic Sertoli cells, which can only support a limited number of germ cells (Franca et al., 2000). In the pig, there are two waves of Sertoli cell proliferation: approximately a six fold increase occurs in the first month after birth, a two fold increase between 3 and 4 months of age (Franca et al., 2000). After puberty is reached with 4–5 months, the Sertoli cell population remains constant. It was shown that the early postnatal period is pivotal for the formation of the Sertoli cell population and adult testicular size (McCoard et al., 2001). Testes size and sperm numbers correlate in humans (Condorelli et al., 2013) and pigs (Wilson et al., 1977) and were shown to be sensitive to endocrine disruption in rats (Sharpe
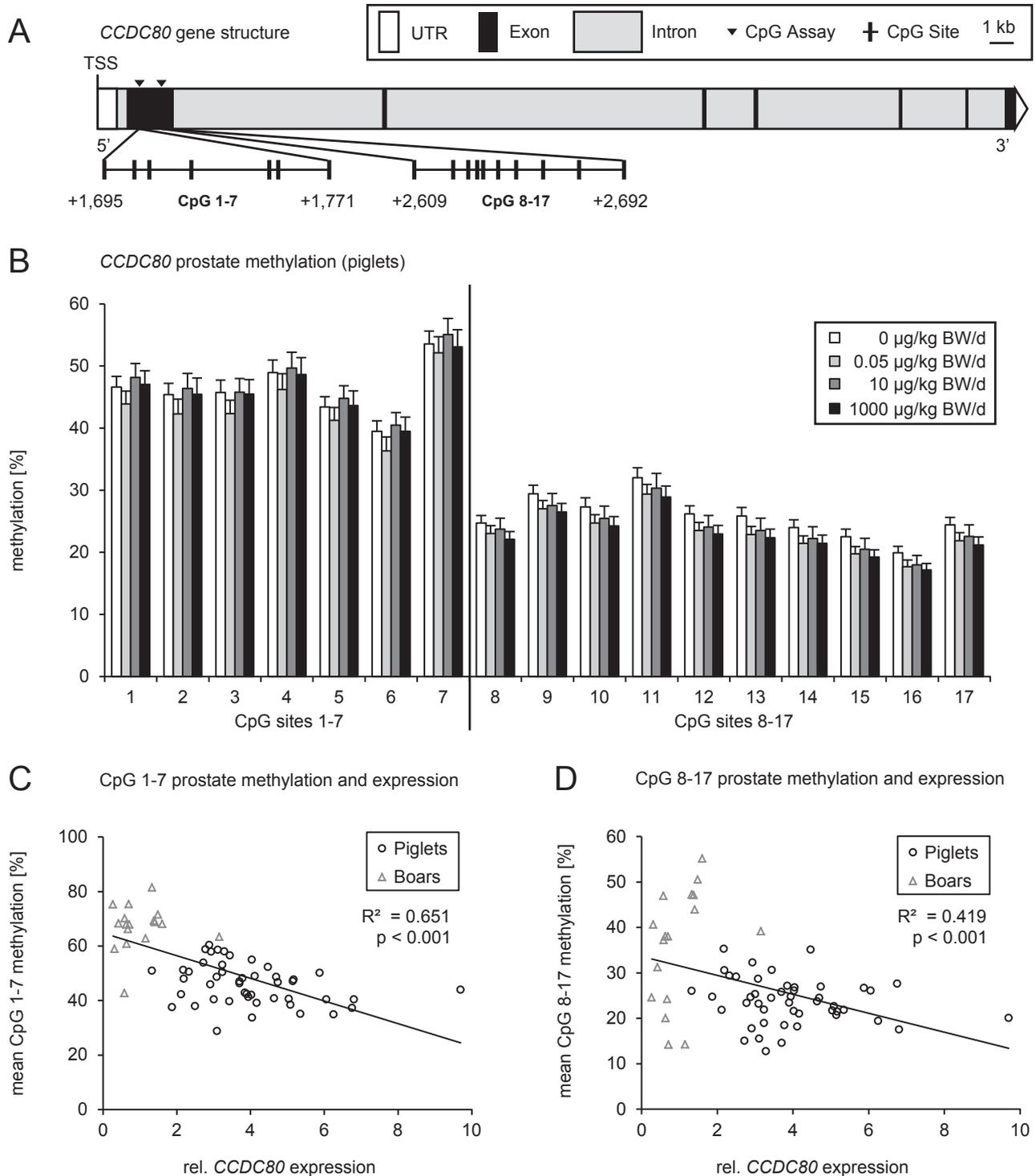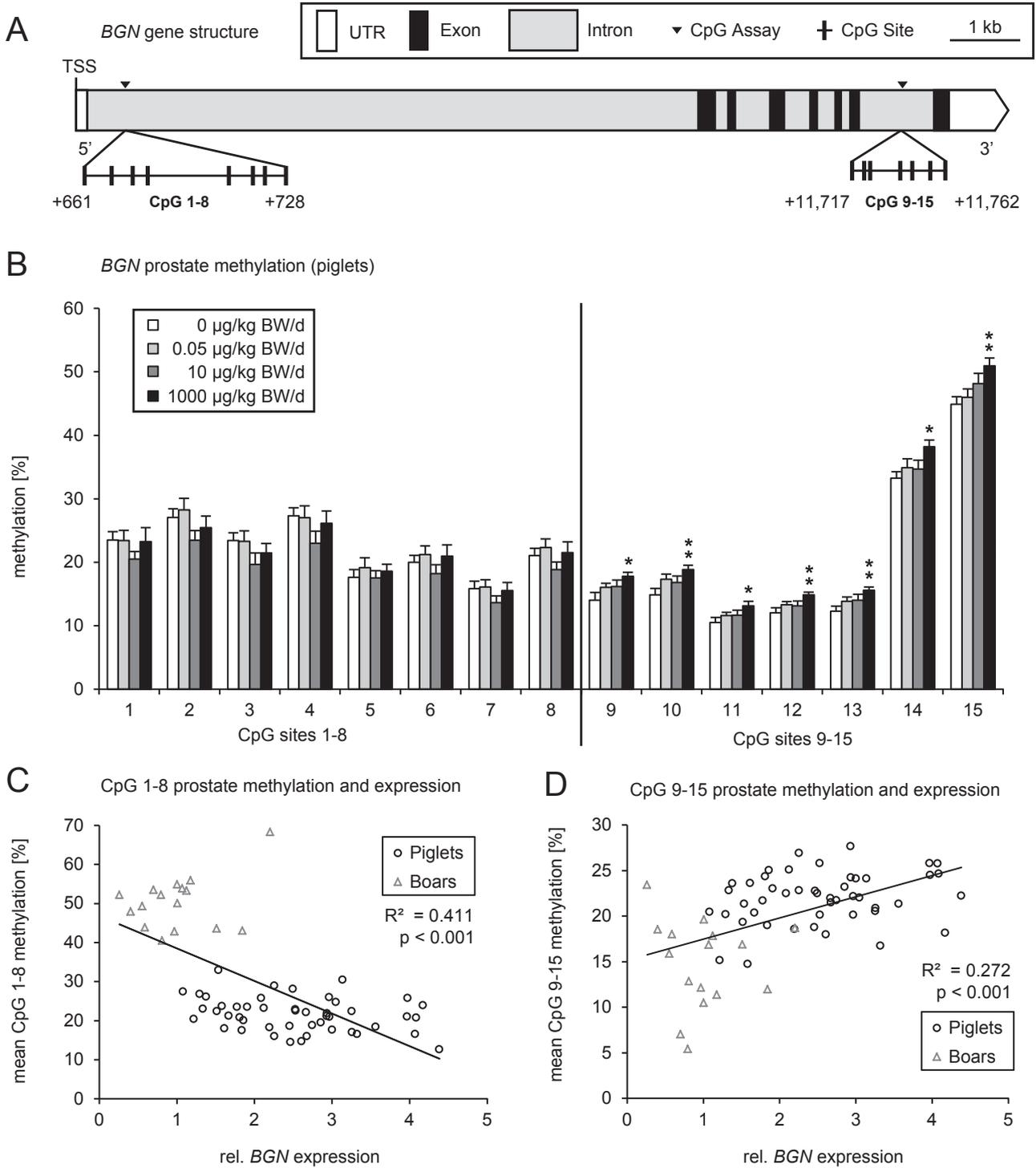
Fig. 4. *CCDC80* methylation correlates with expression and might be regulated by estrogen. (**A**) Gene structure of *CCDC80* and the location of the analyzed CpG islands. (**B**) Prostate *CCDC80* methylation at individual CpG sites of prepubertal piglets. Data are presented as mean ± SEM, n = 12. (**C, D**) Mean CpG methylation at position 1–7 and 8–17 in relation to *CCDC80* mRNA levels in the prostate.

et al., 1995). We measured testes and body weight at 8 weeks, when the first wave of Sertoli cell proliferation is finished. No effects on these parameters were found in the low dose groups (ADI and NOEL), while body and testes weight were both reduced in the high dose group. The ratio between testes and body weight remained constant in all of the groups.

As a further indicator of testicular development, we analyzed

the localization of germ cells, specifically the percentage of centrally located gonocytes in the testes of prepubertal piglets. The ratio of centrally located to basally located gonocytes represents a marker of germ cell maturation, as gonocytes have to migrate from the center to the basal lamina of the seminiferous tubules during development (Payne, 2013; Vigueras-Villasenor et al., 2006). Comparing animals from the treatment groups with the control,

Fig. 5. *BGN* methylation correlates with expression and is increased in the prostate of piglets exposed *in utero* to a high dose of estrogen. (**A**) Gene structure of *BGN* and the location of the analyzed CpG islands. (**B**) Prostate *BGN* methylation at individual CpG sites of prepubertal piglets. Data are presented as mean ± SEM, n = 12, *p < 0.05, **p < 0.01. (**C, D**) Mean CpG methylation at position 1–8 and 9–15 in relation to *BGN* mRNA levels in the prostate.

the percentage of centrally located gonocytes did not differ significantly. In contrast to male piglets, the prenatal estrogen exposure did not affect body and uterine weight of female piglets.

No effect of the treatment on body parameters or testes weight was observed in adult animals. This suggests that weight reduction in the piglets of the high dose group was only temporary. The semen samples collected from adult boars of the high dose group

did not show significant differences in sperm quality compared to the control group and both quantitative and qualitative semen traits were on a high level. Noteworthy, sperm function as tested by an extended parameter set of sperm kinematics and an *in vitro* capacitation assay was not affected by the maternal treatment. This indicates that maternal exposure to a high dose of E2 does not lead to a reduced fertility of boars. One limitation of our study is the

relatively small number of adult boars analyzed. It is possible that effects would be observable in individuals of a larger group of animals. A study with sheep found that only one fourth of animals exposed to sewage sludge chemicals showed a strong reduction in testes size and sperm production, while most animals were not affected (Bellingham et al., 2012). Thus, there seems to be a high individual variability in the vulnerability to external hormones, especially among outbred animals.

Since we could not detect any significant phenotypic differences in the testes and uteri of treated animals, we next asked if estrogen exposure could have caused a molecular phenotype on the level of DNA and RNA. To this end, we first investigated estrogen induced transcriptional changes by RNA-seq and qPCR and then analyzed local DNA methylation at selected target genes by bisulfite pyrosequencing. In rodents, studies found strong effects on gene expression by estrogen exposure in the testis (Lopez-Casas et al., 2012; Naciff et al., 2005), uterus (Diel et al., 2000; Naciff et al., 2003) and prostate (Ho et al., 2006; Tang et al., 2012; Yean Wong et al., 2015). In our study, E2 exposure only caused significant changes in the expression of 3 genes in the testis and one gene in the uterus. In contrast, 130 genes were differentially expressed in the prostate, most interestingly, almost all of them in the NOEL dose group. A technical replication of the RNA-seq data by qPCR gave very similar results, but increasing the sample size from 6 to 12 animals per treatment group only resulted in significant expression differences for 4 out of 19 tested genes. From the subset of genes validated by qPCR, we confirmed deregulation of *ADH1C*, *CCDC80*, *BGN* and *SPARC* in the prepubertal prostate. Thus, only a fraction of the 130 DEGs are likely to be differentially regulated, illustrating the importance of high biological sample numbers for transcriptional analysis.

ADH1C expression was affected most strongly and reduced approximately -5 to -8-fold in piglets of the ADI and NOEL groups. In the prostate of adult boars, *ADH1C* expression was either very low or not detectable. This might indicate that the normal decline of *ADH1C* expression in the prostate occurred earlier in treated animals. A potential role of DNA methylation and histone deacetylation in the transcriptional silencing of *ADH1C* has been reported (Dannenberg et al., 2006). When comparing *ADH1C* DNA methylation in the prostate of animals from the treatment and control groups, no effect of E2 on DNA methylation could be observed, as well as no correlation between mRNA levels and methylation. It remains possible that methylation at a second CpG island (about 1 kb downstream of the TSS) were affected by E2, but this was not possible to be assessed. ADH1C is best known for its role in oxidative ethanol metabolism in the liver, but is also expressed at lower levels in other tissues. To our knowledge, there are no studies addressing the relevance of ADH1C in prostate development. We also measured expression levels of *ADH1C* in the liver of piglets and found no difference between E2 treatment groups and the control. Thus, the observed down regulation of *ADH1C* in the prostate of prenatally E2 exposed animals is most probably tissue specific.

Expression of *CCDC80* was significantly increased in the piglet prostate of the low dose treatment groups. This correlated with slightly decreased DNA methylation levels of treated animals at a CpG island in the first exon, but the methylation differences were not significant. The secreted protein CCDC80 (also known as CL2/DRO1/SSG1/URB) was shown to be highly expressed and regulated by androgens in the prostatic smooth muscle of rats (Marcantonio et al., 2001b). *CCDC80* contains several putative estrogen response elements and is regulated by estrogen in the ovary and mammary gland of rats (Marcantonio et al., 2001a). *CCDC80* was found to become hypomethylated and up regulated in a human breast epithelial cell line treated with BPA (Fernandez et al., 2012). Several functions of CCDC80 have been proposed, including the regulation of adipogenesis (Tremblay et al., 2009) and a role as a tumor suppressor in the colon (Grill et al., 2014), ovary and thyroid (Ferraro et al., 2013; Leone et al., 2015). For technical reasons we analyzed gene expression and methylation in whole prostate tissue. As *CCDC80* has been shown to be predominantly expressed in smooth muscle cells, which only constitute a small part of the prostate, a tissue-specific analysis would be of future interest to dissect cell specific methylation of the prostate.

In the prostate of *in utero* E2 exposed piglets, we also found increased expression levels of *BGN* and *SPARC*, two ubiquitously expressed proteins associated with the extracellular matrix (ECM). We further investigated DNA methylation levels of *BGN*, which, in contrast to *SPARC*, contains a high number of CpG islands. This revealed that *BGN* methylation at a CpG island within the gene body was significantly increased in the prostate of piglets exposed to the high dose of E2. Methylation at this site was positively correlated to gene expression, while a CpG island near the TSS was negatively correlated with gene expression. This is in agreement with other studies that found methylation as a repressive mark near the TSS, but an activating mark within the gene body (Ball et al., 2009; Hellman and Chess, 2007; Jjingo et al., 2012). BGN, a member of the class I family of small leucine-rich proteoglycans (SLRPs), is a structural component of the ECM that presumably also acts as a signaling molecule in processes such as innate immunity, bone formation and muscle integrity (Nastase et al., 2012). In contrast to BGN, the multifunctional secreted glycoprotein SPARC belongs to the family of matricellular proteins, which do not serve structural roles in the ECM (Bradshaw, 2012). Two independent studies found that high expression of *BGN* and *SPARC* were associated with Gleason-sum score of prostate tumors (Lapointe et al., 2004; Singh et al., 2002) and two other studies showed this for *SPARC* only (Best et al., 2005; Thomas et al., 2000). BGN and SPARC have been suggested to increase cancer cell invasion and bone metastasis, the most frequently found metastases of prostate cancer (Arnold and Brekken, 2009; Chen et al., 2007; Edwards, 2012).

The prostate is a heterogeneous gland, raising the question in which cell types *ADH1C*, *CCDC80*, *BGN* and *SPARC* are expressed. The transcriptome for basal, luminal secretory, stromal fibromuscular and endothelial human prostate cells has previously been published (Oudes et al., 2006). Based on these data, all of the four genes are predominantly expressed in stromal cells, suggesting that the stroma might be the primary target of endocrine disruption by estradiol in our pig model. The stromal compartment was also found to be the main target of disruption in human fetal prostate xenografts exposed to estrogen (Saffarini et al., 2015). Analysis of genome wide methylation changes in this model showed that estrogen exposure predominantly altered DNA methylation of the stroma, while no significant changes were detected in whole tissues (Saffarini et al., 2015). This might explain why we only observed minor changes in DNA methylation in whole prostate tissue. Possibly, much higher differences could be observed in microdissected stromal cells. It has been proposed that the stroma plays an essential role in the development of prostate cancer. Though prostate tumors originate from epithelial cells (Goldstein et al., 2010), it was shown that malignant transformation of human prostatic epithelial cells could be induced by alteration in the stromal microenvironment (Cunha et al., 2002).

It should be noted that unlike in humans and rodents, *ESR2* is not expressed in the porcine prostate. In agreement with a previous report (Gunawan et al., 2012), we found *ESR2* expression to be absent or extremely low in all prostates of piglets and boars. We detected moderate expression of *ESR2* in the testes, but not in liver, spleen, heart or adrenal gland. In the endometrium of adult sows, *ESR2* was highly expressed (1′000-fold higher than in testes, data not shown).

We also analyzed gene expression and DNA methylation of *ADH1C*, *CCDC80* and *BGN* in the prostate of adult animals. The changes that we found in prepubertal piglets were not observed in adult boars, which could be due to different reasons. During puberty, the prostate undergoes maturation which is associated with a strong increase in epithelial cells, while the relative stromal cell mass decreases (De Klerk and Lombard, 1986). Since all three genes are predominantly expressed in the stroma, expression and DNA methylation changes might become undetectable in whole adult prostate tissue. Alternatively, the estrogen induced changes could be specific to prepubertal animals. Even in this case, the transcriptional changes during the maturation of the prostate might be of relevance for the function later in life.

In conclusion, our results demonstrate that prenatal estrogen exposure slightly altered prostate gene expression and DNA methylation of *BGN*. For *BGN* and *CCDC80*, we found strong associations between DNA methylation, gene expression and developmental stage, suggesting that methylation is involved in the regulation of these genes in the prostate. Effects of the estrogen treatment appeared tissue specific, as genes deregulated by E2 in the prostate were not affected in liver and testes tissues. Most notably, changes in gene expression were also observed at two low doses corresponding to the NOEL and ADI level of E2 in humans. Thus, the maternal oral low dose treatment during pregnancy resulted in a detectable molecular phenotype in prepubertal male offspring. Functional consequences of these molecular changes were not detected in the ejaculate of the adult male offspring in this study. Indeed, it remains uncertain how these results translate to humans. While the pig displays many physiological aspects similar to humans, such as the presence of placental estrogen synthesis in females, which is absent in rodents, the missing ESR2 expression in porcine prostate present in men is a notable difference. Overall, in testes and uterus, where ESR2 is clearly expressed, we evidence either none or only minor transcriptional effects of E2. Even at a high dose of E2 exposure, the molecular fingerprint of the maternal treatment during pregnancy in offspring is relatively small. Compared with rodents, the observed effects are distinct, but weakly pronounced. This finding suggests that the pig may be much less sensitive to prenatal estrogen exposure than rodents. Nevertheless, it remains to be determined if the subtle changes are dispensable. The determination of a molecular fingerprint of an E2 exposure on male offspring in a model where the offspring develops in a uterine environment flooded with estrogens calls for further investigations. Moreover, finding genes associated with a role in prostate cancer among those differentially expressed and methylated assigns the latter an interesting role as possible targets of endocrine disruption.

Declaration of interest

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.04.005.

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