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Effects of an oral low-dose estradiol-17β exposure during pregnancy on uterine physiology, embryos and offspring in pigs

Veronika Leopoldine Flöter

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Vorsitzender: Prof. Dr. Harald Luksch

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2. Prof. Dr. Martin Klingenspor

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Contents

Abbreviations

ADI	acceptable daily intake
App.	appendix
BMD	bone mineral density
BPA	bisphenol A
bw	body weight
cDNA	complementary DNA
CGI	CpG islands
CpG	cytosine guanidine nucleotide sequence
CT	computed tomography
CSA	cross-sectional area
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DEG	differentially expressed gene
DES	diethylstilbestrol
DNA	desoxyribonucleic acid
DNMT	DNA methyltransferase
E1	estrone
E1G	estrone glucuronide
E2	estradiol-17β
EDC	endocrine disrupting chemical
ER	estrogen receptor
FDR	false discovery rate
HOXA10	homeobox A10
IGF1	insulin-like growth factor 1
ISC	intracellular signaling cascade
JECFA	Joint FAO/WHO Expert Committee on Food Additives
mRNA	messenger RNA
miRNA	microRNA
MS-HRM	methylation-sensitive high resolution melting

ncRNA	non-coding RNA
NOEL	no-observed-effect level
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
pQCT	peripheral quantitative computed tomography
qPCR	quantitative PCR
RNA	ribonucleic acid
RNA-Seq	RNA sequencing
RT-qPCR	reverse transcription qPCR
SEM	standard error of the mean
SSI	strength strain index
TCDD	2,3,7,8-tertacholordibenzo-p-dioxin

Abstract

Endocrine disrupting chemicals (EDCs) with estrogenic properties are ubiquitous in the human environment. They usually occur at very low concentrations, which may not induce major physiologic effects, but may still lead to changes at the molecular level. Development is a particular sensitive time for exogeneous stimuli to also induce lasting changes through epigenetic mechanisms such as DNA methylation. EDCs include natural hormones like estradiol-17β (E2). In this thesis, effects of E2 as the most potent natural estrogen were studied regarding its direct and lasting effects during development. Selected concentrations, mimicking the daily exposure to presumably safe threshold values in the humen, namely the acceptable daily intake (ADI, 0.05 µg/kg body weight/day), a dose close to the no-observed-effect level (NOEL, 10 µg/kg body weight/day) and a high dose (1000 µg/kg body weight/day), respectively, were applied orally to pigs. As the preimplantation period is regarded as highly sensitive for the development of an organism and even embryonic losses after estrogen exposure have been described in the pig, sows were fed these distinct doses from insemination until day 10 of pregnancy. Compared to the control group of sows that were only treated with the carrier, E2 was significantly elevated in the high dose group in plasma, bile and somatic tissues. In addition, unconjugated and conjugated metabolites of E2 were also elevated in the NOEL dose group. Subsequent RNA sequencing depicted a dose-dependent effect on the endometrial mRNA expression with 14 (ADI), 17 (NOEL) and 27 (high dose) differentially expressed genes (DEGs). Endometrial microRNAs (miRNAs) as important regulatory molecules of gene expression were additionally analyzed by small RNA sequencing. Here, no effect was observed regarding the endometrial miRNA transcriptome. In addition to the sows, sexing of the blastocysts was established followed by a transcriptome analysis. Thus, for the first time the transcriptome of single preimplantation embryos was explored after estrogen exposure. In the high dose group, 982 DEGs were found in the female blastocysts, followed by 62 DEGs in the NOEL dose group. The male embryos were hardly affected with only 3 DEGs in the NOEL dose group. Comparing the gene expression pattern between the sexes and doses revealed female embryos becoming more similar to the male embryos with increasing E2 dose. In order to assess if gestational exposure may also induce lasting effects in the offspring, sows were fed the respective doses during the entire period of pregnancy, hereby mimicking continuous environmental exposition. Pre- and postpubertal offspring were analyzed. Many parameters were unaffected by the treatment; such as plasma hormone concentrations (E2, total estrogens, progesterone, testosterone, leptin, insulin-like growth factor 1), onset of puberty, weight at birth, weight at slaughter, and uterine weight. Bone was selected as a target organ, as EDC exposure during development can affect the bone phenotype later in life. Similar to data in other

large animals, few but distinct effects were observed in the low-dose groups. In the prepubertal male offspring the strength strain index was lower in the ADI dose group at the proximal tibia, as analyzed by peripheral quantitative computed tomography. In the postpubertal female offspring the cortical and total cross-sectional area at the femur midpoint was larger in the NOEL dose group, which was determined by computed tomography. In addition to bone, homeobox A10 (*HOXA10*) was selected as a known estrogen sensitive target gene in the uterus. A methylation-sensitive high resolution melting approach (MS-HRM) followed by pyrosequencing was established, thus avoiding potential amplification bias that may occur during polymerase chain reaction. As opposed to rodent studies, in the present porcine model neither effect on *HOXA10* mRNA expression nor DNA methylation was measured in the uterus after the estrogen exposure. Furthermore, various tissues exhibiting larger differences in the *HOXA10* mRNA expression were compared. For a single CpG site, an association of promoter DNA methylation with mRNA expression was detected in the prepubertal female piglets. This was not observed in the postpubertal siblings. Taken together, low-dose E2 treatment led to sex-specific effects in the preimplantation embryos as well as in the offspring, including non-monotonic dose responses in the latter. This substantiates on the one hand that developing organisms are highly sensitive towards exogenous estrogens and on the other hand that the current safety thresholds for E2 need to be revised.

Zusammenfassung

In der menschlichen Umwelt sind endokrine Disruptoren mit östrogener Wirkung allgegenwärtig. Normalerweise liegen diese Substanzen in sehr geringer Konzentration vor, wodurch es in der Regel zu keinen größeren, physiologisch sichtbaren Effekten kommt. Dennoch können Veränderungen auf molekularer Ebene in den Zellen auftreten. Der Kontakt mit solchen Substanzen ist vor allem kritisch während sich der Körper im Wachstum befindet, da es hier auch zu langanhaltenden Veränderungen durch epigenetische Mechanismen wie DNA-Methylierung kommen kann. Zu den endokrinen Disruptoren gehören auch natürliche Hormone wie Östradiol-17β (E2). In dieser Doktorarbeit wurden die direkten und langfristigen Auswirkungen von E2, dem wirksamsten natürlich vorkommenden Östrogen, nach Exposition während früher Entwicklungsphasen studiert. Die Konzentrationen wurden in Anlehnung an die aktuell für Menschen gültigen und als sicher angenommenen Grenzwerte ausgewählt und oral an Schweine verabreicht. Die niedrigste Dosis entspricht der zulässigen täglichen Aufnahmemenge (acceptable daily intake (ADI), 0,05 µg/kg Körpergewicht/Tag), die zweitniedrigste Dosis ist nahe an der sogenannten Dosis ohne beobachtete Wirkung (no-observed-effect level (NOEL), 10 µg/kg Körpergewicht/Tag), hinzu kam noch eine hohe Dosis (1000 µg/kg Körpergewicht/Tag). Die Entwicklungsphase bevor der Embryo sich einnistet wird als besonders empfindlich angesehen. Beim Schwein reichen die beschriebenen Effekte nach der Gabe von Östrogenen bis zum Verlust von Embryonen. Deshalb wurden die oben genannten Dosierungen Sauen vom Tag der Besamung an bis zum Tag 10 der Trächtigkeit verfüttert. Der Vergleich zu den Kontrolltieren, welchen nur das Lösungsmittel gegeben wurde, ergab, dass die Konzentration an E2 im Plasma, in der Gallenflüssigkeit und in verschiedenen Geweben in der Gruppe mit der hohen Dosis signifikant höher lang. Darüber hinaus waren die Werte für unkonjugierte und konjugierte E2-Metablolite auch in der NOEL-Gruppe erhöht. Entsprechend zeigte auch die im Anschluss durchgeführte RNA-Sequenzierung einen dosisabhängigen Effekt auf die mRNA-Expression mit 14 (ADI), 17 (NOEL) und mit 27 (hohe Dosis) differenziell exprimierten Genen (DEGs). Endometriale mikro-RNAs, welche eine wichtige Rolle in der Regulierung der Genexpression spielen, wurden zusätzlich mittels einer hierfür spezifischen RNA-Sequenzierung untersucht. Hierbei zeigte sich kein Einfluss auf das endometriale mikro-RNA-Transkriptom. Zusätzlich zu den Muttertieren wurden auch die Embryonen untersucht. Zuerst wurde bei den Blastozysten das Geschlecht bestimmt und im Anschluss eine Transkriptomanalyse durchgeführt. Hierdurch wurde zum ersten Mal untersucht, wie sich eine Östrogenexposition auf das Transkriptom einzelner Embryonen auswirkt. In der Gruppe mit der hohen Dosis wurden 982 DEGs bei den weiblichen Blastozysten gefunden, gefolgt von 62 DEGs in der Gruppe mit der NOEL-Dosis. Die männlichen Embryonen zeigten kaum

Veränderungen mit nur 3 DEGs in der NOEL-Gruppe. Der Vergleich des Genexpressionsmusters zwischen den Geschlechtern und Dosierungen ergab, dass die weiblichen Embryonen mit steigender E2-Dosierung den männlichen Embryonen ähnlicher wurden. Um zu untersuchen, ob es auch Langzeiteffekte auf die Nachkommen gibt, wurden Muttertiere während der gesamten Trächtigkeit mit diesen Dosierungen gefüttert. Diese Gabe spiegelt die Situation einer kontinuierlichen, umweltbedingten Östrogenexposition wider. Pre- und postpubertäre Nachkommen wurden anschließend analysiert. Dies ergab, dass viele Parameter durch die Gabe nicht beeinflusst wurden, wie beispielsweise Hormonkonzentrationen im Plasma (E2, Gesamtöstrogene, Progesteron, Testosteron, Leptin, Insulinähnlicher Wachstumsfaktor 1), der Beginn der Pubertät, das Geburtsgewicht, das Gewicht zum Zeitpunkt der Schlachtung und das Gewicht des Uterus. Als nächstes wurde der Knochen als Zielorgan ausgewählt, da bekannt ist, dass endokrine Disruptoren während der Entwicklung Einfluss auf den Knochenphänotyp im späteren Leben haben können. Vergleichbar mit Daten aus Studien an anderen Großtieren wurden wenige aber dennoch bestimmte Effekte in den beiden Gruppen mit den niedrigen Dosierungen beobachtet. Bei den prepubertären männlichen Nachkommen war der Strength-Strain-Index an der proximalen Tibia in der ADI-Gruppe niedriger, was mittels peripherer quantitativer Computertomographie bestimmt wurde. Bei den postpubertären weiblichen Nachkommen wurde eine größere kortikale und gesamte Querschnittsfläche in der Mitte des Femurs durch Computertomographie in der NOEL-Gruppe festgestellt. Darüber hinaus wurde das Homöobox-Gen A10 (*HOXA10*) als bekanntes, östrogen-sensitives Zielgen im Uterus ausgewählt. Es wurde eine methylierungssensitive hochauflösende Schmelzkurvenanalyse (MS-HRM) mit anschließender Pyrosequenzierung etabliert. Hierdurch wird ein möglicher Bias während der Vervielfältigung bei der Polymerasekettenreaktion vermieden. Anders als bei Studien an Nagetieren zeigte sich jedoch in dieser Untersuchung am Schwein kein Einfluss der Östrogenexposition, weder auf die *HOXA10* mRNA Expression noch auf die DNA Methylierung. Dennoch wurde bei der Untersuchung verschiedener Gewebe, welche große Unterschiede in der *HOXA10* mRNA Expression aufwiesen, für eine einzelne CpG-Stelle bei den prepubertären Schweinen eine Assoziation der Promotor-DNA-Methylierung mit der mRNA-Expression festgestellt. Diese Korrelation war in den postpubertären Tieren nicht nachweisbar. Zusammenfassend führten niedrige Dosen an E2 sowohl zu geschlechtsspezifischen Effekten in den preimplantären Embryonen als auch in den Nachkommen, einschließlich nicht-monotoner Dosis-Wirkungen in den Letzteren. Dies erhärtet einerseits, dass Organismen während der Entwicklung sehr empfindlich auf exogene Östrogene reagieren und andererseits, dass die aktuell gültigen Grenzwerte für E2 einer Überarbeitung bedürfen.

1. Introduction

1.1. Direct and epigenetic effects of estradiol-17β exposure during pregnancy

Endocrine disrupting chemicals in the human environment

Endogenous estrogens are mainly produced by the gonads (ovary and testis, respectively), to a certain amount in the adrenals and in minor concentrations locally in further extragonadal sites such as adipose tissue [1, 2]. In the female, they are involved in various processes in the reproductive organs including uterine preparation for and maintenance of pregnancy, support of fetal growth, as well as their function as embryo recognition signal in some species including the pig [3–5]. Estrogens are also important for instance in bone development and homeostasis [6, 7], regulating immune cell activity [8], vascular functions [9], adipose tissue metabolism [10] and energy balance [11]. In addition to the endogenous hormone production, humans are exposed to various exogenous estrogens of which several are ingested via nutrition or as pharmaceuticals [12]. There are natural estrogens such as estradiol-17β (E2), which is the most potent member of this group of substances. It is found in very small concentrations in milk, meat and related products [13–15]. Next to its own properties as EDC [16–18], E2 is a common model substance in EDC research to compare to estrogenic effects [19–24]. Natural estrogens can also be of plant origin (phytoestrogens), like genistein and daidzein in soybeans, or products of fungi (mycoestrogens) such as zearalenone [25, 26]. Other than natural estrogens, synthetic estrogen-like compounds named xenoestrogens include plasticizer such as bisphenol A (BPA), pesticides like dichlorodiphenyltrichloroethane (DDT), and pharmaceuticals such as diethylstilbestrol (DES) and ethinyl estradiol. Many phytoestrogens and xenoestrogens can be detected in human body fluids [27–30]. Phytoestrogens have been found in infant urine and blood, especially upon feeding soy-based formula [27]. BPA has been detected in blood, urine and breast milk of adults, as well as in cord blood of infants [28, 29, 31].

After exposition, all of the above-mentioned substances are able to affect the endocrine system, and can thus be classified as endocrine disrupting chemicals (EDCs) [12, 25]. To date, different definitions regarding EDCs exist [12, 32]. The world health organization (WHO) defines them as “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations” [32], while the U.S. Environmental Protection Agency (EPA) has chosen a more detailed, molecular approach describing them as “an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne

hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process" [12]. In this thesis, EDCs are addressed in the context of the definition by the EPA, as low-dose effects may be found at the molecular level without direct evidence of adverse health effects.

In order to assure human safety, regulatory agencies usually perform risk assessment using the safety factor approach [33–35]. From the available data, threshold values such as the no-observed-effect level (NOEL) are derived. Differences in species, sex, age and between individuals are incorporated using safety factors, generally assuming that the dose-response relationship is monotonic. Thus, a presumably safe concentration, the acceptable daily intake (ADI), is calculated, but usually not tested in further studies [33]. In general, the focus of such investigations is on finding the highest dose that does not exert an alteration. Therefore, lower doses are rarely investigated in depth. This can be problematic, as non-monotonic dose-responses and low-dose effects are commonly known to exist, specifically in case of steroid hormones. In terms of E2, the ADI (0.05 µg/kg body weight (bw)/d) was calculated with a safety factor of 100 from the NOEL dose (5 µg/kg bw/d), derived from studies on postmenopausal women [34]. This is presumably not sufficient evidence to ensure safety during more sensitive periods such as development. For example, prepubertal children are highly susceptible to exogenous steroids because of their much lower endogenous production rates and concentrations than assumed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). In addition, estrogen receptors (ERs) are already expressed [25, 36–40]. Furthermore, the developing body undergoes large changes making them more vulnerable as exposure may not only exert direct but also lasting consequences that may impact on later phenotype and/or health outcomes. Support for low-dose *in utero* effects comes from rodent studies regarding the intrauterine position of male and female fetuses, as well as from human twin studies [33]. Moreover, differing compounds may exert additive effects [41]. Thus, there are several reasons why the current threshold values may be questioned [33, 36].

Developmental origins of health and disease

In the 1990th, Barker described the association of a low birth weight with an increased risk for developing cardiovascular diseases later on [42, 43]. Since then, much evidence has accumulated linking an altered early environment to health and disease states later in life [44]. This is known as the "developmental origins of health and disease" (DOHaD) hypothesis [45–47]. It describes that in addition to the genotype especially the prenatal environment is able to shape the developing

phenotype. This plasticity allows the embryo to adapt to its later environment as it is perceived *in utero*. If then the postnatal environment does not match the prenatal information, an increased disease risk can arise.

Before this concept was developed, there were examples linking early EDC exposure with later onset of disease and many more have been described since [12, 33, 48, 49]. In the 1940s to 1960s, the synthetic drug DES was given to pregnant mothers supposing it would prevent possible adverse outcomes such as spontaneous abortions [48]. However, DES turned out to be teratogenic and a mild carcinogen. Thus, daughters exposed *in utero* to DES were found to have an increased risk of clear cell adenocarcinoma of the vagina and the cervix. In both sexes, malformations of the genital tract occurred. Notably, the detection of adverse outcomes in the daughters such as adenosis was more frequent when DES treatment had started within the first two month of pregnancy compared to initiation within the fifth month. In general, certain time windows during development have demonstrated being particularly sensitive to environmental cues, such as the preimplantation period where widespread epigenetic remodeling occurs [12, 44, 46, 50]. Next to alterations in the number of certain cell types, which can e. g. shape the bone phenotype, an underlying molecular mechanism for lasting adaptations is the alteration of epigenetic marks [44, 46, 51–55].

Epigenetics

The term „epigenetic“ dates back to Conrad Waddington (1942), who used it in the sense of causal mechanisms that are involved in how the phenotype emerges from the genotype [56–59]. Thus, it is used to define a global view on changes that occur during development through interactions both between cells and by environmental stimuli finally resulting in an altered morphology. In the 1990s, a definition from a more molecular biological point of view emerged, omitting the need for the endpoint of a phenotypic alteration. Arthur Riggs (1996) defines epigenetics as “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in desoxyribonucleic acid (DNA) sequence” [57]. Heritability implies an alteration to be passed on even when the initial stimulus that had it brought about has disappeared. Therefore, mechanism such as DNA methylation, Polycomb and Trithorax system account as classically epigenetic. Especially DNA methylation has been considered in this regard. Its pattern is erased and reestablished during development [60, 61] and then basically maintained throughout life. This process is conducted by DNA methyltransferases (DNMT) [62, 63]. Certain changes still occur later in life such as global demethylation of repeated DNA during aging [64] and pathologic

changes as seen in cancer [65]. In mammals, DNA methylation primarily occurs at the cytosine residue of cytosine guanidine nucleotide sequences (CpG sites) [66, 67] and is crucial for various biological processes such as transcriptional regulation, cellular differentiation and transposon silencing [61, 63, 68]. Next to single CpG sites in the genome that are mainly methylated, 2 % of all CpG sites are found in clusters (about one every 10 base pairs), which are called CpG islands (CGIs) [69, 70]. CGIs are most often unmethylated and sometimes heavily methylated, and they are situated in the promoter region of 60 % to 70 % of the human protein-coding genes. Promoter CGI methylation leads to stable gene silencing and is involved in X-inactivation and genomic imprinting [61, 69, 71]. In addition, 50 % of the CGIs are found in inter- and intragenic regions. Furthermore, methylation of a single CpG site is able to affect gene transcription [72, 73] and can be associated with tissue specific messenger ribonucleic acid (mRNA) expression [74]. Overall, several mechanisms in gene expression regulation through DNA methylation are known [75, 76].

The use of the term “epigenetic” has expanded to further mechanisms with the potential of altering gene functions helping the cell to “remember” past events where the mechanisms of heritability are not always clear or the alterations are only short-lived – these include non-coding RNAs (ncRNAs) and chromatin modifications [57, 77]. In addition, it has become evident that the epigenetic regulation is often a complex interplay between different mechanisms regulating gene expression, where ncRNAs have a pivotal role [77, 78]. There are many different classes of small ncRNAs (snRNAs) [78]. PIWI-interacting RNAs (piRNAs) are involved in maintaining transposon silencing and regulate various epigenetic processes such as guiding DNA methylation in the male germline. They have only lately become the focus of many studies and still several questions remain [79]. In contrast, the mechanisms of microRNA (miRNA) biogenesis and its functions in regulating mRNA expression have been intensively studied and described in detail [78, 80, 81]. Apart from their classically known function in the regulation of about 50% of the protein-coding genes, miRNAs are also involved in epigenetic regulation like their integration in silencing complexes that alter chromatin structure leading to promoter DNA methylation and thus gene silencing.

Mechanisms of estrogen action

Estrogens impact cellular functions in multiple ways, particularly gene transcription. They mainly act through their nuclear receptors (ERα, ERβ). Classically, ER binding of estrogens leads to receptor dimerization. The dimer then directly interacts with the DNA at estrogen response elements (EREs) [82, 83]. Another way of inducing genomic actions is indirectly by the E2-ER

complex interacting with other DNA-bound transcription factors such as the stimulating protein-1 (SP1). Furthermore, estrogens can act in a non-genomic way through membrane situated receptors such as palmitoylated ERs and G-protein coupled estrogen receptors, thus initiating intracellular signaling cascades (ISCs) often mediated through protein kinases [82–84]. These ISCs can on the one hand lead to rapid modifications of cellular functions in the cytosol or regarding the plasma membrane. On the other hand, they can indirectly influence gene transcription. Similarly, cytosolic ligand bound ER can interact with other proteins inducing an ISC [82]. Genomic actions may either lead to activation or repression of gene transcription, depending on further interacting coregulators [85–87].

Through these coregulators, receptor bound estrogens also lead to modification of epigenetic marks, which is particularly known regarding histone modifications [21, 83, 85, 88, 89]. Regarding DNA methylation, there are studies showing ER-bound estrogens altering the expression of DNMTs and thus affecting local and global DNA methylation level [90–92]. In addition, one study demonstrated E2-ER dependent changes in promoter DNA methylation for an estrogen responsive gene concurrently altering its expression [93]. Further analyses indicated that other transcription factors including SP1, which may interact with the E2-ER complex, as well as DNMTs are involved in this process. Still, the exact mechanism for specific local DNA methylation changes through estrogens remains to be elucidated.

Estrogen exposure is often linked to alterations in miRNA expression. This is known for E2 particularly from *in vitro* studies using breast cancer cell lines [94, 95], but also from using other cells such as cells from human endometrial tissue [96, 97]. *In vivo*, miRNAs are differentially expressed during the menstrual cycle as well as in disease states [97–100] indicating potential hormonal regulation. Effects of an E2 treatment on uterine miRNA expression has also been shown in a mouse model [101]. Some studies have investigated the signalling mechanism using ICI 182,780, an ERα specific inhibitor, and/or Chromatin immunoprecipitation (ChIP) thereby demonstrating ERα specific miRNA regulation [97, 101–103].

In general, specific low-dose effects and non-monotonic dose-responses are well known for hormonal substances [33]. This has also been shown after early EDC exposure in differing settings, multiple species, and for various targets such as the uterus [104, 105] including uterine homeobox A10 (*Hoxa10*) expression [104], bone [106–108], body composition [16], the prepubertal luteinizing hormone (LH) surge [109], *ERα* and *ERβ* mRNA expression in the hypothalamus [110] and behavior [111]. Potential mechanisms involved are receptor down-regulation and thus desensitization, receptor selectivity and competition, and endocrine negative feedback loops [33]. Thus, dose dependently, estrogenic substances may exert diverse estrogenic

as well as anti-estrogenic effects on each cell type, depending on the dose, the expressed receptors and cofactors, and the concentrations of other competing estrogenic substances including endogenous hormones.

Another aspect that determines the response to EDCs is the route of exposure [112–115]. The pharmacokinetics of orally administered E2 have extensively been investigated in pigs. Most of the ingested E2 is metabolized by the gut wall [115–117]. Further transformation occurs in the liver. Via the bile, the estrogens then undergo enterohepatic cycling [117, 118]. Thus, no or only very little unconjugated E2, and to some extent estrone (E1), can be found in the peripheral blood [16, 115, 117]. In contrast, high amounts of conjugated estrogen metabolites reach the circulation in order to be excreted through the kidney [115–117, 119]. Estrone glucuronide (E1G) is the main metabolite in pigs, and together with lower amounts of E2-glucuronide and sulfated estrogens, they add up to 90 % of the metabolized E2 [115, 116]. Due to their polarity, unconjugated estrogens can easily diffuse from blood into the tissues [112, 120, 121]. Retention as well as accumulation also depends on the amount of expressed ERs, thus contributing to cell-specific effects [112, 120–123]. Accumulation has for example been shown for the uterus, as major target organ of estrogens. Although conjugated estrogens may not exert direct estrogenic effects, they appear in tissues and cells can actively deconjugate estrogens thereby regulating the availability of active ligand [120, 124–127]. Furthermore, conjugated estrogens such as estrone sulfate in plasma may serve as a estrogen reservoir [124, 126].

Estrogen exposure during early pregnancy

Estrogens are particularly important during early pregnancy in various species such as in mice [128, 129], pigs [4, 130] and humans [131–133]. They are involved in the preparation of the endometrium for implantation of the embryo. After conception, low concentrations of estrogens prevail in the maternal circulation [134, 135]. In mice, the following increase around implantation determines the window of receptivity [128, 129]. A mid-luteal increase is also observed in primates, although the function is less clear [128, 132, 134]. Slightly higher estrogen concentration in humans might favor implantation. In sows, the plasma estrogen concentration remains low until after implantation [135, 136]. A first local rise occurs on days 11 to 12 of pregnancy because porcine embryos secrete estrogens as pregnancy recognition signal to prevent luteolysis [4].

Exposure to exogenous estrogens during the preimplantation phase can not only affect the mother including abnormal endometrial functions and an altered intrauterine environment, but also the embryo and the embryo-maternal communication. *In vitro* studies have shown that estrogens can

directly impact on the embryo [22, 137, 138]. Evidence from assisted reproductive technologies with often higher maternal E2 concentrations indicated an association with higher implantation failure [139, 140] and increased adverse placental outcomes such as small for gestational age (SGA) babies [141, 142], as well as altered gene expression and DNA methylation in the placenta [143]. Studies in mice using a single dose [144] as well as continuous exposure [144–147] during early pregnancy led to effects ranging from endometrial gene expression changes, an altered morphology and secretory activity, fewer implantation sites to smaller litters at birth. Similar effects have been observed in pigs exposed to estrogens for few days shortly before implantation, specifically on days 9 and 10 or on days 7 to 10 of pregnancy [18, 130, 148–154]. In contrast, exposure to low concentrations of EDCs at other points in time, such as from day 2 to 6 or after day 10 did not cause such drastic effects [18, 149, 150, 155–157]. The reason that pigs, with estrogen as their maternal recognition signal, are highly sensitive during this small window before implantation is presumably due to a desynchronization of the uterine preparation with embryonic development [130].

The preimplantation phase is a particularly sensitive time for external stimuli to induce not only direct, but also lasting alterations in the offspring [23, 24, 50]. For example, mice treated only during the preimplantation period with E2 or methoxychlor depicted postnatal sex-specific effects [23, 24]. Further evidence stems from studies on assisted reproductive technologies [50]. One reason for the vulnerability is that prior to implantation huge remodeling of epigenetic marks, such as DNA methylation and histone modifications, occurs in the embryo [50, 60, 61, 158–160]. In terms of DNA methylation, at first following fertilization, most sites are demethylated [159, 160]. Only imprinted genes and a few other sites have been reported to escape this demethylation process [158, 161]. The main wave of de novo methylation starts at the late morula to early blastocyst stage [159, 160, 162], and lasts in the mouse until gastrulation [163–166]. Certain differences between species exist such as regarding the timing of DNA remethylation in general and concerning the embryoblast and the trophectoderm [159, 160, 162, 163, 167]. In the pig, implantation is delayed compared to human and mouse, and global DNA methylation of the embryo has been described until day 10 [158, 159].

DNA methylation is of particular interest concerning lasting consequences as on the one hand the mechanism of its mitotic propagation is known [62, 63]. On the other hand, there are studies linking developmental EDC exposure to DNA methylation differences later in life.

In terms of miRNA, stage-specific expression in the developing embryo has been described in multiple species [168–170]. Furthermore, important functions have been proposed for miRNAs being expressed in the endometrium [98, 100, 170–172]. MiRNAs, expressed in the endometrium,

have been associated with the regulation of genes that are crucial for differentiation, angiogenesis, immune functions, receptivity, proliferation and extracellular matrix factors, which are important in the preparation of the endometrium for implantation [100, 171, 173]. Steroid hormones are mainly responsible for regulating these processes, potentially also through the regulation of miRNA expression [98]. There are only very few studies analyzing miRNA expression after EDC exposure during pregnancy or directly postnatally [174–176], although it is known that E2 treatment can modify miRNA expression [94–96, 101].

Lasting effects of gestational estrogen exposure

As pregnancy continues, estrogen concentrations in the circulation increase and reach particularly high concentrations towards parturition [16, 136, 177]. This is more pronounced in humans, many higher primates and ungulates due to their placental estrogen secretion, as compared to rodents where the corpus luteum continues to produce estrogens [3, 178, 179]. It has been proposed that due to the higher endogenous prevailing estrogens, humans are less sensitive to exogenous estrogens during pregnancy, as most studies have been conducted using rodents [178]. In this regard, the pig is more closely resembling the women as rodents do [16, 136, 177].

Not only the preimplantation phase, but gestation as a whole has been shown as a sensitive time where external stimuli may induce lasting effects [12, 44, 49, 180]. At later stages during pregnancy, the development of specific organs can be affected [16, 180, 181]. Fürst *et al.* [16] demonstrated an altered body composition with an increase of total body fat in the male prepubertal piglets exposed to E2 during the entire gestation at a concentration close to the NOEL as well as when using a high dose. This might be due to effects on the lineage commitment of adipocyte precursor cells [16, 181]. Still, most studies have applied EDCs for a specific period during pregnancy, while only few studies have looked at the effects of continuous exposure throughout gestation [12, 154]. In ICR mice, continuous E2 exposure during the entire pregnancy led to a birth rate of almost 80% in the lower dose group [20]. A high dose of Zearalenone fed to rats during the entire pregnancy decreased the number of liveborn pups [182]. Zearalenone in the fodder at intermediate to high concentrations fed for longer periods during gestation was also able to affect the fetuses often including reduced litter size of pregnant sows [154, 183–185]. In contrast, even a high dose of E2 during the entire pregnancy did not affect the number of piglets born [16], although, similarly to zearalenone, its detrimental effect when given slightly before implantation is known [18, 130, 148–152, 154]. Overall, depending on the sensitive window during gestation, estrogens can cause various direct and lasting alterations (Fig. 1) [12, 49].

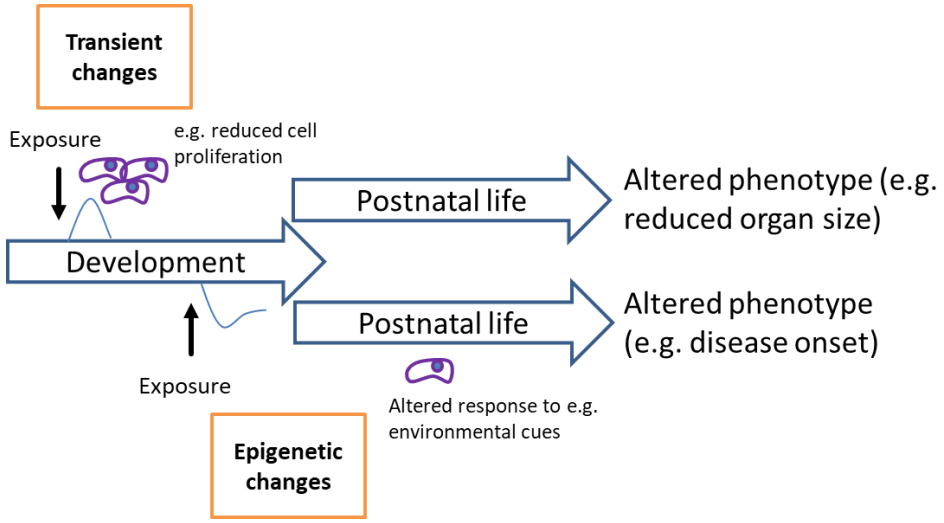

Fig.1: During development, external stimuli may induce either transient or lasting effects on cell functions and epigenetic marks. There are in general two possibilities for lasting phenotypic alterations to arise, either through an alteration in the number of certain cell types, or through heritable epigenetic changes that can be the basis for an altered response to environmental cues possibly resulting in disease onset later in life.

Uterine HOXA10 expression as potential target of developmental estrogen exposure

Classically, the reproductive organs are a known target of EDCs [12, 49]. Early exposure may affect sexual development and may even contribute to various reproductive disorders including cancer and decreased fertility [12, 17, 186–188]. In this regard, the *Hoxa10* gene in the uterus has been shown as a potential target [104, 189–191]. The evolutionary conserved *HOX* genes encode transcription factors that are essential for determining the anteroposterior body axis in the embryo and functional differentiation in the adult [192]. *Hoxa10* is involved in the morphological development of the uterus from the Müllerian duct [193]. Most of the adult female *Hoxa10* knockout mice are infertile due to embryonic death before implantation, coinciding with the time of *Hoxa10* expression in wildtype mice [193, 194]. Similarly, *HOXA10* expression increases during the periimplantation period in multiple species including human and pig [195–198]. In addition, the pattern of *HOXA10* abundance during the estrous cycle has been analyzed in women and dogs [195, 196].

Hormones including estrogens are involved in *HOX* gene regulation [192]. *In vitro* studies have shown that *HOXA10* expression can be induced through the classic ER-dependent pathway [19, 199, 200]. Furthermore, E2 treatment of human as well as porcine primary endometrial cells increased its expression [195, 201]. In a mouse model, promoter DNA methylation abrogated ERα binding to the *Hoxa10* promoter, thus preventing the respective increase in its expression *in vitro* [190]. *In vivo*, after gestational BPA exposure, the offspring showed a reduced promoter and intronic DNA methylation concomitantly with higher *Hoxa10* mRNA and protein expression. Furthermore, effects of EDC exposure on *Hoxa10* mRNA expression have been demonstrated directly after *in utero* treatment [202, 203], directly after treatment postnatally [189], as well as in adult animals that were exposed during development [190, 204]. Notably, some effects are similar in animals treated with EDCs [104, 191, 205–207] and *Hoxa10* knockout mice [193] or adult mice with modified *Hoxa10* mRNA and protein expression [208]. In exposed rodents, alterations in the uterine morphology [205], a reduced number of implantation sites [104], increased embryo resorption, and reduced pregnancy rates [206, 207] were detected. Morphologic alterations and reduced embryonic survival was also found in neonatally treated sows [191].

Bone development as potential target of developmental estrogen exposure

Sex hormones play an important role in bone development and metabolism throughout life. Starting with fetal development, ERs are expressed in various bone areas and cell types [37, 209, 210]. After birth, growth hormone (GH) and insulin-like growth factor 1 (IGF1) are highly important for bone development [6]. Although sex steroid concentrations are low, ERs are expressed [39, 211] and estrogens have been associated with bone maturation [212, 213]. During puberty, estrogens are of significance for the pubertal growth spurt and essential for epiphyseal fusion in both sexes [6, 7]. Differences in the timing and length of puberty along with differences in sex hormones and GH-IGF1 actions lead to the establishment of a sexual dimorphic bone phenotype with males exhibiting larger bones and thicker cortices than females. In males, the trabecular bone is formed by testosterone signaling through the androgen receptor (AR), while in females the signaling involves E2 through ERα [6]. For cortical bone accrual not only testosterone, but also E2 signaling through ERα is necessary in males, while in females E2 and both receptor isoforms, ERα and ERβ, are involved. Males achieve a higher peak bone mass during young adulthood than females, which is one of the reasons for the lower incidence of fractures with increasing age also in men. During adulthood, estrogens are important to maintain the balance between bone formation and resorption [7]. Decreased sex hormone concentrations later in life, especially

beginning with menopause in women, result in bone loss, leading to an increased risk of developing osteoporosis.

Previous data mainly derived from rodent models show direct and/or lasting alterations due to EDC exposure early in life on bone development and metabolism [51]. Many aspects determine the result such as the dose and time of exposure, the substance, the sex, the species, the time of analysis, the method, as well as the target which has been analyzed. As quite consistently shown in female rodents, early EDC exposure led to an increase in the bone mineral density (BMD) in adulthood [106, 214–216], contrasting more variable results from studies using large animal models [107, 217]. Similarly, conflicting effects on BMD have been found in male offspring [106, 107, 214, 216, 218]. Furthermore, some studies using rodents have described an increase in the peak load in the female offspring [214, 216], while another study showed decreased bone strength parameters [108]. In the latter study, a low dose of DES was given during gestation and lactation and a resulting increase in femur length was described. This contrasts data from Migliacchio *et al.* [219], which used the same low dose but only during gestation. They found an increase in bone mass and no alteration in femur length. Next, certain effects have also been demonstrated concerning bone area parameters [106–108, 214, 217]. Hermsen *et al.* [107] showed an increase in the total cross-sectional area (CSA) in female rhesus monkeys only in the low-dose and not in the high dose treatment group. Regarding the mechanism, additionally to effects directly on bone cells [44, 51–53], lasting alterations affecting the bone phenotype may also rely on changes in endocrine functions [17, 51, 217], the timing of puberty [17, 220], and/or changes in the amount of body fat [16, 221, 222].

The usage of large animals as model organism is an important step to transfer experimental results from rodents to humans. As porcine bone closely resembles the ones of humans, pigs are used for bone research and have been recommended by the U.S. Food and Drug Administration (FDA) for osteoporosis research [223–225]. Yet, only a couple of studies have analyzed the effects of early EDC exposure on bone development in large animals, however not in the pig [51, 107, 217, 218, 226, 227].

1.2. Aims of the study

Various estrogenic EDCs occur at low doses in the environment and may particularly impact on developing organisms. Therefore, the objective of this study was to investigate if the declared ‘safe’ threshold values for E2, the most potent endogenous estrogen, are still valid if including both a molecular and an epigenetic perspective. Thus, E2 was used as model estrogen for a low-dose estrogenic EDC exposure (Fig. 2). The preimplantation window of embryo development is highly sensitive regarding exogenous stimuli. Thus, one part of this study aimed at analyzing continuous exposure to differing doses of E2 regarding its potential to affect the mother sow as well as the male and female embryos on day 10 of pregnancy. *In vivo* effects of estrogenic EDCs on the blastocyst transcriptome had not yet been examined. We aimed at integrating analyses concerning the pharmacokinetics of the orally applied E2 doses to unravel potentially underlying mechanisms. The other part of this study focused on lasting effects in pre- and postpubertal offspring after complete gestational E2 exposure to observe if lasting effects occurred in the progeny. Next to endocrine parameters, two known target organs of steroid hormones, namely the uterus and the bone, were selected.

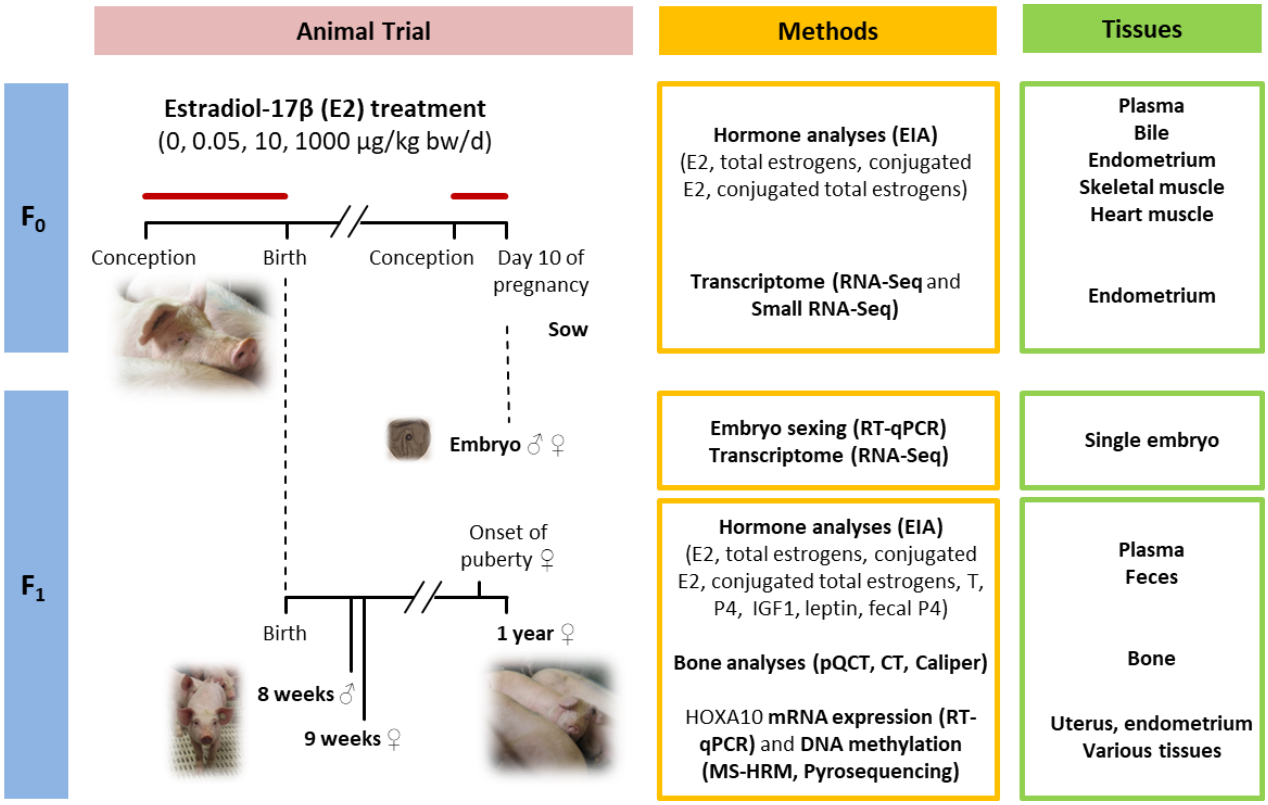

Fig. 2: Scheme representing the experimental approach of this thesis. Pregnant sows (F_0) were fed the respective E2 dose or the carrier (red bar) during pregnancy. After exposure during the entire pregnancy, the male and female offspring (F_1) were sampled prepubertally, while another group of female offspring was slaughtered after the onset of puberty at about one year of age. The sows were again treated from conception until day 10 of pregnancy. This time, sows and embryos (F_1) were sampled. ♀ = female, ♂ = male, bw = body weight, d = day, EIA = enzyme immuno assay, RNA-Seq = RNA sequencing, RT-qPCR = reverse transcription quantitative polymerase chain reaction, pQCT = peripheral quantitative computed tomography, CT = computed tomography, HOXA10 = homeobox A10, MS-HRM = methylation-sensitive high resolution melting, P4 = progesterone, T = testosterone*, IGF1 = insulin-like growth factor 1.*

2. Material and methods

Animal trials and sampling

In order to analyze direct and epigenetic effects of E2, an animal trial was conducted by the research group of Dr. Susanne Ulbrich (Physiologie Weihenstephan). The selected single doses were 0, 0.025, 5 and 500 µg/kg body weight (bw). These doses were fed in the main trial twice per day with a 12 hours interval. Thus, as intended, a daily amount for the two low doses was achieved corresponding to the ADI and close to the NOEL as announced for humans by the JECFA with 0.05 and 5 µg/kg bw/d, respectively [34]. The control group was fed the ethanol carrier only and a high dose group received 1000 µg/kg bw/d as an effect dose as positive control. In an initial trial, plasma pharmacokinetics of estrogens after a single oral dose were investigated in male castrated pigs [16]. In brief, animals were catheterized (jugular vein; n = 2-3/treatment group). They were fed the E2 on the following day before normal feeding. The first blood samples were taken two and one hour before feeding the E2, then sampling was conducted every 15 minutes for four hours, continuing with one sample every hour until 24 hours with a break between hour 13 and 20. The main study consisted of two parts (Fig. 3). In the first part, sows (n = 6-7/treatment group) were treated with E2 throughout gestation in order to analyze epigenetic consequences in the offspring [16] (Flöter *et al.* 2016, appendix (App.) II). After the gestational E2 exposure, male (8 weeks old) and female (9 weeks old) offspring were analyzed prepubertally (n = 10-12/treatment group and sex), and a second group of females were assessed postpubertally (about one-year old, n= 8-14/ treatment group). In the latter, estrous cycle signals and behavior were observed. After at least three reproductive cycles per sow and daily monitoring to determine the day of the cycle, they were slaughtered between days 10 to 13 post estrus (luteal phase). In the second part, the experiment was continued with the sows in a subsequent pregnancy in order to analyze direct E2 effects on mothers and embryos at the time of preimplantation (Flöter *et al.* 2019, App. IV). In brief, after feeding the respective E2 dose from insemination (day 0) onwards, sows (n = 4-6/treatment) were slaughtered on day 10 of pregnancy, one hour after obtaining the last dose. Regarding sample collection, pieces of the selected tissues were immediately frozen in liquid nitrogen and stored at -80 °C. Tibia and femur of the right hind leg, as well as ethylenediaminetetraacetic acid (EDTA) stabilized plasma and bile samples were stored at -20 °C. In addition, from 23 weeks on, a fresh rectal feces sample was taken weekly from female offspring, which was stored at -20 °C. In order to obtain the embryos, uterine horns were flushed with phosphate-buffered saline (PBS, autoclaved, pH 7.4), at first using 10 ml, which was kept for later analyses, and subsequently with 50 ml to obtain all embryos. Pictures were kindly taken by

Myriam Reichenbach (Chair for Molecular Animal Breeding and Biotechnology, Gene Center of the Ludwig-Maximilians-Universität (LMU) Munich) of the embryos before they were frozen in liquid nitrogen and stored at -80 °C. Uterine flushings were stored at 20 °C. Animals with embryos at their blastocyst stage were included in the RNA sequencing (RNA-Seq) experiments. The animal trial was approved by the local authorities (Ref# 55.2-1-54-2531-68-09; District Government of Upper Bavaria), the experiments were performed with permission from the local veterinary authorities and conducted following accepted standards of humane animal care.

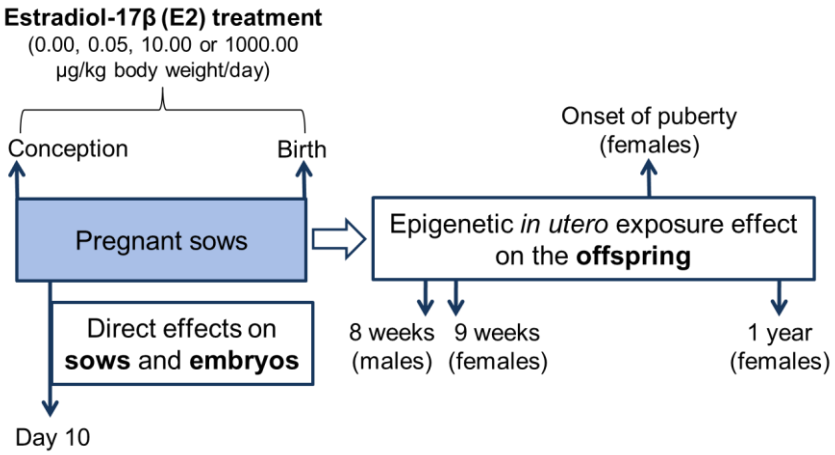

Fig. 3: Study design of the main animal trial. At first, E2 was fed during the entire pregnancy and the offspring were subsequently analyzed to detect epigenetic effects. Secondly, sows were fed E2 in a subsequent pregnancy and were slaughtered on day 10 of pregnancy to analyze direct effects on both sows and embryos.

RNA from endometrial tissue (isolated by A. Klanner, A. Samborski, and S. Gebhardt) and respective plasma samples from two additional studies, the so called “estrous cycle study” and “preimplantation study” [228–230](Pistek *et al.* 2013, App. I, Fig. 1), were kindly provided by Dr. S. Bauersachs (Laboratory for Functional Genome Analysis (LAFUGA), Gene Center, LMU Munich).

Hormone analyses

Enzyme immuno assays (EIAs) were used to analyze plasma, bile and tissue hormone concentrations. In plasma samples, E2 and total estrogens (E1, E2, estradiol-17α; while estradiol-17α is absent in the pig) [231, 232], testosterone [233], progesterone [234], as well as IGF1 and leptin [235] were measured as described in detail (Flöter *et al.* 2016, App. II; Flöter *et al.* 2019, App. IV). In order to analyze conjugated steroids, the frozen phase obtained during the ether extraction process was further used instead of the supernatant. After digestion with an enzyme mixture containing glucuronidase and arylsulfatase (Merck KGaA, Darmstadt, Germany), samples were comparably processed as for unconjugated steroid measurements (Flöter *et al.* 2019, App. IV). The protocol slightly differed between plasma, tissues (endometrium, skeletal muscle, heart muscle) and bile. Tissues were homogenized, and the extraction was slightly intensified with longer incubation times and for bile also with more concentrated digestion buffer in terms of conjugates steroids. The first corpus luteum formation, as a marker for puberty, was detected assessing fecal progesterone levels. The analyses were conducted as described earlier [236] with slight modifications (Flöter *et al.* 2016, App. II). All hormone analyses were kindly performed by Brigitte Dötterböck, Waltraud Schmid and Stefanie Berthold (Physiology Weihenstephan), respectively.

Bone measurements

Femur and tibia were analyzed in pre- and postpubertal offspring. In the latter, peripheral quantitative computed tomography (pQCT, STRATEC XCT 2000 (SA); Stratec, Pforzheim, Germany) could not be used due to size limitations; therefore, computed tomography (CT) measurement was applied. Measurement settings were kindly established by Dr. D. Seidlova-Wuttke (University of Göttingen, Department of Endocrinology) (Flöter *et al.* 2016, App. II). In brief, tibia and femur were separated and femur length was determined with an electronic caliper. Three bone areas (proximal femur, distal femur, proximal tibia), close to the epiphyseal growth plate were assessed with pQCT. Three consecutive slices (1 mm apart) were taken after positioning the scanner using a coronal computed radiograph (scout view). Subsequently, data processing was done to obtain trabecular and cortical-subcortical BMD, as well as CSA and the polar strength strain index (SSI), as described in detail by Flöter *et al.* 2016, App. II.

The CT (Aquilion CX, Toshiba Medical Systems Cooperation, Tochigi, Japan) measurements including the analyses of the CSA at the femoral midpoint, as well as femoral volume, length and

CT number in Hounsfield Units were kindly performed by Dr. Gabriela Galateanu (Leibniz Institute for Zoo and Wildlife Research (IZW) Berlin) (Flöter *et al.* 2016, App. II).

Extraction of RNA

Total RNA from tissue samples of the female pre- and postpubertal offspring was extracted with the NucleoSpin RNAII Kit (Macherey Nagel, Düren, Germany) with slight modifications. A frozen tissue peace (~3mm³) was placed into 600 µl buffer RA1 and complemented with 6 µl β-mercaptoethanol. A MagnaLyser (Roche, Mannheim, Germany) in combination with Matrix-Green beads (MP Biomedicals, Illkirch, France) was used for homogenization. After filtration of the lysate, 600 µl ethanol (70 %) were added. Furthermore, the second washing step was repeated in order to improve RNA purity. Regarding the extraction of total RNA from embryos, the AllPrep RNA/DNA Micro Kit (Qiagen, Hilden, Germany) was utilized to account for the small sample amount. The manufacturer's protocol for cells was applied with some modifications to increase sample purity and concentration. In brief, frozen embryos were put into a mixture of 700 µl buffer RTL and 7 µl β-mercaptoethanol. Disruption was accomplished by pipetting up and down, followed by single short vortexing. A syringe and needle approach was followed to homogenize the samples. In terms of the RNA purification, the manufacturer's protocol "purification of total RNA containing small RNA from cells" was followed. The incubation steps D3 and D4 using buffer RPE were extended to 4 min and 2 min, respectively. Elution of the RNA was conducted twice, reusing the first flow-through in the second elution step. In order to determine its quantity and purity, the NanoDrop 1000 (Peqlab, Erlangen, Germany) was used at 260/280 nm and 230/280 nm, except for embryonic samples which were additionally measured with the Qubit™ (Invitrogen) using the RNA BR Assay for more precise quantification. Its integrity was assessed with the Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany) utilizing the RNA 6000 Nano Kit (Agilent). Most RNA Integrity Number (RIN) values were between 8 and 10 (Pistek *et al.* 2013, App. I; Flöter *et al.* 2018, App. III; Flöter *et al.* 2019, App. IV), samples were only included in the study if the RIN was above 6 in order to ensure good performance in the following reverse transcription quantitative polymerase chain reaction (RT-qPCR) [237, 238]. RNA samples were stored at -80 °C.

Gene expression analyses with RT-qPCR

In order to analyze mRNA and small RNA expression, two-step approaches were chosen with reverse transcription followed by quantitative real-time PCR. These experiments were conducted in accordance with the MIQE guidelines [239]. For mRNA analyses, 1 µg of total RNA was reverse

transcribed into complementary DNA (cDNA) as described earlier [240]. The subsequent qPCR reactions were conducted on the CFX384™ Real-Time PCR Detection System (Bio-Rad, München, Germany). Cycling was performed with a final volume of 10 µl including 1µl of cDNA. For the analyses of the tissues of the pre- and postpubertal female offspring as well as from the endometrium of the “preimplantation study” and the “estrous cycle study”, the MESA Blue qPCR MasterMix Plus for SYBR® Assay No ROX (Eurogentec, Köln, Germany) was used. Therefore, 5 µl MESA Blue MasterMix, 0.15 µl forward primer [20 µM], 0.15 µl reverse primer [20 µM], 3.7 µl nuclease free water and 1 µl of cDNA sample or water as negative control were merged. In order to perform the technical validation of the RNA-Seq using the endometrial samples of the sows on day 10 of pregnancy, the SsoFast™ EvaGreen® Supermix (Bio-Rad) was utilized. Each well was composed of 5 µl SsoFast™ EvaGreen® Supermix, 0.2 µl forward primer [20 µM], 0.2 µl reverse primer [20 µM], 0.07 µl Visi-Blue™ (TATAA Biocenter AB, Goteborg, Sweden), 3.53 µl nuclease free water, and 1 µl of cDNA sample. Commercially synthesized primers (Sigma-Aldrich, Taufkirchen, Germany) were applied, and of each primer pair, a PCR product was sequenced (4baselab, Reutlingen, Germany) to verify product identity. Subsequently, melting curve analysis was used to monitor amplification of the respective product. The primer sequences, gene accession numbers, product length, and annealing temperature can be found in the respective appendices (Pistek *et al.* 2013 App. I; Flöter *et al.* 2019, App. IV, Suppl. Tab. S1). In terms of analyzing small RNAs, reverse transcription was performed with the miScript II RT kit (Qiagen). The subsequent qPCR reaction was conducted on the Rotor-Gene (Qiagen) with the QuantiTect SYBR Green PCR kit (Qiagen) and a final volume of 10 µl, which was composed of 5 µl QuantiTect SYBR Green PCR Master Mix, 1 µl miScript Primer Assay, 1 µl miScript Universal Primer, 2.25 µl nuclease free water and 0.75 µl cDNA sample. Commercial target gene (miScript Primer Assay, Qiagen; Flöter *et al.* 2018, App. III, Tab.1) and potential reference gene (miScript Control Assays, Qiagen; Flöter *et al.* 2018, App. III)) forward primers were bought. The cycle of quantification (Cq) data obtained for each qPCR experiment were analyzed by relative quantification and calculation of fold changes as recommended by Livak and Schmittgen [241]. The appropriate reference genes were selected using the NormFinder and/or GeNorm algorithm (GenEx Pro Ver 4.3.4 software multiD Analyses AB, Gothenburg, Sweden), while normalization was performed following the BestKeeper method [242].

mRNA expression analyses with RNA-Seq

RNA-Seq of the endometrial samples from the sows on day 10 of pregnancy was kindly carried out by A. Klanner and S. Krebs (AG Blum, Gene Center, LMU Munich) as described in Flöter *et*

al. 2019, App. IV (n = 4/treatment group; with the exception that in the ADI dose group (n = 3) from one sow RNA was extracted twice). RNA-Seq from single embryos was kindly conducted by Dr. J. Kühn Georgijevic (Functional Genomics Center, Zurich, Switzerland) as outlined in Flöter *et al.* 2019, App. IV (supposed n = 6/sex and treatment group (control, NOEL, high dose) from 4 sows/treatment group with at least one embryo/sex; overall n = 36; however, due to one wrongly assigned embryo in the NOEL dose group, there were 5 male and 7 female embryos from 3 and 4 sows, respectively). RNA-Seq data from endometrial samples as well as from the embryos was processed by means of the Genomatix software (Genomatix Software GmbH, Munich, Germany). The obtained reads were mapped on the porcine genome sequence using the Genomatix Mining Station with the Sus Scrofa Genome Library NCBI build 4, the ElDorado Version 12.2012, "fast" as mapping type, and a minimum alignment quality of 92 %. Then, statistical analysis for differential expression was performed. The resulting endometrial data were further modified on a locally installed version of Galaxy [243]. A cut-off value for a transcript to be regarded as transcribed or otherwise as turned off was set to at least 10 reads. In order to assume its expression in a treatment group, a minimum of samples was needed with at least 10 reads. This was defined regarding the endometrial samples to be at least 3 out of 4 samples. The ArrayExpress database (EMBL-EBI) was used to deposit the RNA-Seq data from both experiments, the endometrium (accession number E-MTAB-6242 (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6242)) and the blastocysts (accession number E-MTAB-6263 (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6263)).

The database for annotation, visualization and integrated discovery (DAVID 6.8) (https://david.ncifcrf.gov) [244] was used to perform a functional annotation clustering analysis of the differentially expressed genes (DEGs). Therefore, the gene ontology FAT terms of cellular component, biological process, and molecular function were selected. Further details can be found in Flöter *et al.* 2019, App. IV.

miRNA expression analysis using small RNA-Seq

For the small RNA-Seq, the same endometrial samples were used from the sows at day 10 of pregnancy as in the RNA-Seq approach to analyze mRNAs. This was kindly executed by the Genomics Core Facility of the European Molecular Biology Laboratory Heidelberg (EMBL Heidelberg, Genomics Core Facility, Heidelberg, Germany) as detailed in Flöter *et al.* 2018, App. III. With the aim of analyzing miRNA expression, the small RNA-Seq data was further processed using Galaxy [243], installed at the Gene Center (AG Blum). This approach had been developed

by S. Bauersachs (Gene Center, LMU Munich). Trimming of the reads from both ends was conducted using the “Fastq quality trimmer” [245] with a window size of 3 bases, a step size of 1 base, and a mean quality score of at least 30. Clipping of the adapter sequences was performed with settings for sequence retrieval only if they were at least 17 bases in length, and sequences with unknown bases (N) were discarded. Next, using “filter by quality” only sequences were kept where all bases had a phred score of at least 25. The quality of the remaining reads was evaluated using FastQC. Then, read counts were derived for all samples by determining the number for each identical sequence. Alike for the mRNA data analyses of the endometrium, sequences defined as not expressed, which did not have at least 10 reads in at least 3 of the 4 samples in at least one treatment group, were removed. The remaining sequences were compared with miRNAs from differing species (pig, mouse, human, cow) as retrieved from miRBase (www.miRBase.org, release 20.0). Therefore, databases were created for these four species using “make blast database” (http://www.ncbi.nlm.nih.gov/books/NBK1763/), and mapped against the obtained sequences from the endometrial samples by means of “blastn-short” (expectation value: 1.0, word size: 5) [246]. The output was filtered and only miRNAs that matched a sequence from the database to 100 % regarding its sequence and length were kept for the subsequent statistical analyses. The RNA-Seq data have been deposited in the database repository Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo; accession number GSE89343).

Extraction of genomic DNA

DNA extraction from tissues of the pre- and postpubertal female offspring was performed with the peqGOLD Tissue DNA Mini Kit (Peqlab) as described earlier [247]. Purity and quantity were measured spectrophotometrically with the NanoDrop 1000 (Peqlab). Purity was assumed with 260/280 nm ratios above 1.8 and 260/230 nm ratios larger than 1.6. As DNA from muscle and liver samples tented to have the latter ratio below the cut off, adequate values were obtained after purification with the genomic DNA Clean & Concentrator Kit (Zymo Research, Irvine, CA, USA) (Pistek *et al.* 2013, App. I). The extraction of DNA from single embryos was performed in combination with the RNA extraction using the AllPrep RNA/DNA Micro Kit (Qiagen) as described above (Flöter *et al.* 2019, App. IV). DNA was extracted according to manufacturer’s protocol. Its quality was similarly assessed using the NanoDrop 1000 (Peqlab). Although sample quality was generally low with a mean 260/230 nm ratios of 0.2 ± 0.16 (±SD), it was assumed to be sufficient for determining embryonic sex by qPCR.

DNA methylation analysis

For gene specific DNA methylation analysis, an integrated approach with bisulfite conversion, methylation-sensitive high resolution melting (MS-HRM) followed by pyrosequencing was chosen and conducted as described earlier [74]. In brief, an artificially methylated and unmethylated DNA standard was created. Genomic DNA from the samples and the standard were bisulfite converted by using the EZ DNA Methylation-Gold Kit (Zymo Research). Thus, the information of DNA methylation is turned into sequence information by producing artificial single nucleotide polymorphisms (SNPs). The standard was mixed to obtain 0 %, 25%, 50 %, 75 %, and 100% degree of methylation, which were amplified alongside the samples in a MS-HRM PCR approach. This step is especially crucial in establishing adequate primers, as biases towards originally methylated or unmethylated sequences are common [247–251]. Altogether, a gold-standard for the detection of single CpG site methylation was applied [252] and by using the HRM approach there was a control regarding a potential PCR amplification bias [247, 250, 253]. The MS-HRM PCR primers as well as the sequencing primers for the subsequent pyrosequencing run were designed using the PyroMark Assay Design Software 2.0 (Qiagen) and were commercially synthesized (Sigma-Aldrich). They are shown in the respective appendix (Pistek *et al.* 2013, App. I). The underlying porcine *HOXA10* promoter sequence was retrieved by using the multiple species alignment tool at the Ensemble homepage (http://www.ensembl.org/; comparative genomic functions, genome assembly Sscrofa10.2), looking for the homologous promoter region as compared to the sequence in mice [190] and humans [254] where DNA methylation changes have been demonstrated together with alterations in *HOXA10* expression. The primer sequences, including the underlying DNA sequence, as well as product length, number of included CpG sites and the annealing temperature is depicted in Pistek *et al.* 2013, App. I. The MS-HRM PCR was conducted on a Rotor-Gene Q instrument (Qiagen), and using its software for normalizing the melting curves. Thus, a qualitative impression on the degree of methylation was obtained. In the subsequent pyrosequencing run, which was conducted with a Pyromark Q24 system (Qiagen), site specific DNA methylation data was obtained for 10 CpG sites (Fig 4) and analyzed with the respective software (version 2.0.6, Qiagen).

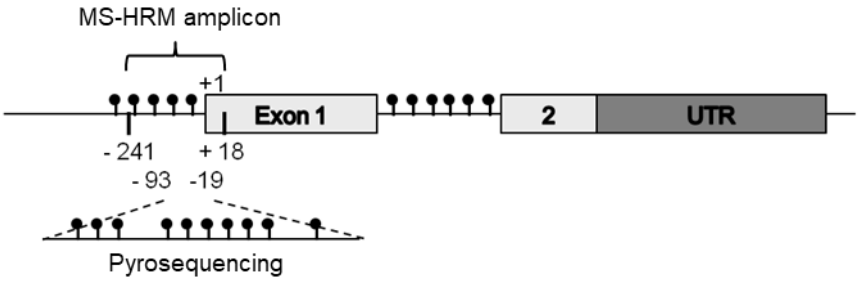

Fig. 4: Scheme of the proposed porcine HOXA10 gene. The area under investigation is in the promoter region as part of a CpG island. CpG sites = black dots, +1 = transcription start site, UTR = untranslated region.

Embryo sexing

The qPCR approach for identifying the sex of the embryos was established. Primers that fit mRNA as well as DNA for the Y-chromosomal specific gene sex determining regain Y (*SRY*) and the autosomal gene histone (*H3F3A*) were designed and validated (Flöter *et al.* 2019, App. IV). The detailed primer information can be found in Flöter *et al.* 2019, App. IV, suppl. Tab. S1. The DNA from the embryos of the control, NOEL and high dose group (overall n = 65; ≥ 4 embryos/sow from 4 sows/treatment) was used as input for embryo sex discrimination. The qPCR was performed with the SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen) using a LightCycler 2.0 (Roche). The final volume of 10 µl contained 5 µl 2X SYBR® Green Reaction Mix, 0.2 µl forward primer [20 µM], 0.2 µl reverse primer [20 µM], 1 µl 20X Bovine Serum Albumin, 2.4 µl of nuclease free water, and 0.2 µl of SuperScript® III RT/Platinum® *Taq* Mix. All samples were run in duplicate, due to varying amounts and quality. The subsequent RNA-Seq analysis of 36 embryos showed that only one embryo of the NOEL dose group assumed to be male turned out to be female while for all other embryos the sex had been correctly assigned.

Transcription factor binding site and sequence conservation analyses

Transcription factor binding sites at the *HOXA10* gene promoter were analyzed in-silico using MatInspector (Genomatix) [255]. The analysis of the degree of conservation of the *HOXA10* promoter sequence between 12 eutherian mammals (homo sapiens, pan troglodytes, gorilla gorilla, pongo abelii, macaca mulatta, callithrix jacchus, mus musculus, rattus norvegicus, bos taurus, sus scrofa, canis familiaris, equus caballus) was conducted at the ensemble website

(http://www.ensembl.org) using the Enredo, Pecan, Ortheus (EPO) pipeline tool of genomic alignments (Pistek *et al.* 2013, App. I).

Statistics

The E2 application trial encompassed four groups, one control and three treatment groups. Therefore, one-way analysis of variance (ANOVA) was selected, with the Dunnett's post hoc test as only the comparison of the treatment groups with the control group were of interest (Pistek *et al.* 2013, App. I; Flöter *et al.* 2016, App. II; Flöter *et al.* 2018, App. III; Flöter *et al.* 2019, App. IV). Regarding the "preimplantation study" and the "estrous cycle study" the Bonferroni post hoc test was applied in order to obtain multiple comparisons (Pistek *et al.* 2013, App. I). For the technical validation of the RNA-Seq data from the day 10 endometrial samples, t-tests were performed from the qPCR results between the respective treatment group and the control animals that had shown a significant regulation in the RNA-Seq experiment (Flöter *et al.* 2019, App. IV). In case, data was not normally distributed, the natural logarithm of the data was used for the statistical analysis (Flöter *et al.* 2016, App. II; Flöter *et al.* 2018, App. III; Flöter *et al.* 2019, App. IV). In order to integrate the nested design of the main E2 trial with exposed siblings (offspring or embryos) of the same sow, a mixed model was applied using the SAS program package release 9.2 (2002; SAS Institute, Inc., Cary, NC, USA) as detailed in the respective appendices (Flöter *et al.* 2016, App. II; Flöter *et al.* 2018, App. III). Differential expression analyses of the high-throughput sequencing data, performed always between two selected groups, was conducted as follows. The endometrial reads were analyzed on the Genomatix Genome Analyzer. The EdgeR algorithm was used with an adjusted p-value of smaller than 0.05 and a cut-off fold change of log2 ≥ or ≤ 1. The embryonic reads could be analyzed with the BioConductor package EdgeR applying the „estimateGLMRobustDisp" method [256] due to the higher number of at least 5 samples per group. A false discovery rate (FDR) of 5 % was applied. Venn diagrams (webtool Venny 2.1 [257]) and hierarchical clustering pictures (MeV_4_8 v10.2 [258]) were also generated using data with $p < 0.0001$ and a fold change of at least 1.5. This was kindly conducted by Dr. S. Bauersachs as described in Flöter *et al.* 2019, App. IV. The preprocessed miRNA data from the small RNA.Seq approach was analyzed using DESeq 2.11 [259] (http://www.bioconductor.org/) in R 386.2.15.3 (Flöter *et al.* 2018, App. III). Apart from graphical presentations of high-throughput sequencing results, data was plotted using SigmaPlot program package release 11.0 (SPSS, Chicago, IL, USA). For correlation analyses, the Pearson correlation coefficient was computed. Data are shown as mean ± standard error of the mean (SEM). If not otherwise addressed in this section, with p-values smaller than 0.05 statistically significant differences were assumed.

3. Results and discussion

3.1. Oral exposure to low doses of E2 may exhibit its effects through conjugated and unconjugated metabolites

The plasma pharmacokinetic study depicts low-dose effects and the possibility of estrogen accumulation

The route of exposure determines the kinetics and which metabolites will reach the tissues, thus determining the possible effects of ECDs [112–115]. The pharmacokinetic study [16] was designed to evaluate the dose-response of the respective oral E2 doses in the plasma of male castrated piglets, thus using the pig model with the least amounts of endogenous estrogens present. Therefore, it should be possible to detect minimal changes by the exogenous E2. The male castrated piglets were fed the dose once, which was applied to the pregnant sows in the main trial twice daily. Fürst *et al.* [16] had shown that the two low doses (0.025 and 5 µg E2/kg bw respectively) did not alter plasma E2 concentrations, while the high dose (500 µg E2/kg bw) led to a fast increase and after a second peak, lower but still elevated plasma E2 concentrations persisting from 6 to 12 hours. Additionally, conjugated and unconjugated estrogen metabolites were measured in the same blood plasma samples as well as in samples from these piglets at 21 to 24 hours after the E2 dose was fed (Flöter *et al.* 2019, App. IV, Fig. 1). Total estrogens (reflecting the sum of E1 and E2), conjugated E2 and conjugated total estrogens showed a general pattern similar to E2 in the high dose with two initial peaks and lasting elevated concentrations. A similar dose-response pattern has also been shown for humans [114]. The very fast increase in unconjugated estrogens already after 15 minutes can at least in parts be explained by sublingual uptake as depicted in pigs earlier [260]. The first maximum of both, the conjugated total estrogens and the conjugated E2 was slightly later after 30 minutes in the high dose group and after 45 minutes in the NOEL dose group. This is in agreement with other studies in pigs that have also shown E2 to be rapidly converted into conjugated forms. The largest quantities will be already converted by the gut wall, mainly into E1G, further processing occurs in the liver [115–117]. In the present data, this is reflected by much higher concentrations of conjugated total estrogens (sulfated and glucuronidated E1 and E2) with 65,128.7 pg/ml as compared to much lower concentrations of conjugated E2 with 9,523.9 pg/ml at 30 minutes. The second maximum in the high dose group appeared after two to three hours, likely caused by remaining estrogens in the stomach [115] and by estrogens reabsorbed from the gut upon enterohepatic cycling [117, 118]. The single increase found in the NOEL dose group of the conjugated E2 reached basal levels at

about three hours after the dose was fed, whereas conjugated total estrogens had lasting elevated concentrations still at 24 hours. Similarly, the persisting elevated concentrations at 12 hours in the high dose group, including unconjugated E2, were still at a similar height after 21 to 24 hours. This phenomenon may be explained by enterohepatic cycling of the estrogen metabolites [117, 118]. No alterations were found after application of the ADI dose group and unconjugated estrogens were also unaffected in the NOEL dose group. This is similar to data introducing relatively low doses of E2 into the stomach of pigs showing slightly elevated E1 and E2 concentrations occurring in the portal vein, however not anymore in the jugular vein, where only conjugated metabolites were present [117].

These data indicate on the one hand that low-dose effects in the NOEL dose group may derive from conjugated estrogens as intermediates [120, 124–126]. On the other hand, as persisting elevated concentrations prevailed at 12 and still at 24 hours after the treatment, the application of these doses every 12 hours in the main trial could result in estrogen accumulation.

Strongly elevated estrogen concentrations in body fluids of sows on day 10 of pregnancy

Hormone concentrations in plasma and bile after continuous E2 exposure for 10 days were analyzed. The last dose was fed one hour before slaughter. All four assays – E2, conjugated E2, total estrogens, conjugated total estrogens – detected significantly elevated concentrations in the high dose group in both body fluids, while all but E2 showed also significantly higher concentrations in the NOEL dose group (Flöter *et al.* 2019, App. IV, Tab. 1). Fold changes of these differences compared to the control group are depicted in Flöter *et al.* 2019, App. IV, Table 2. In the bile, the amount of conjugated estrogens was higher compared to the unconjugated forms in the control animals as well as in all treatment groups. However, the relative increase with increasing doses of E2 was much more pronounced for the unconjugated estrogens. In contrast, in the plasma the relative increase was much stronger in the conjugated forms. In the high dose group, plasma E2 was 3-fold and total estrogens 17-fold higher, while in the bile they were 2489-fold and 3152-fold higher, respectively, as compared to the control. Similarly, in the NOEL dose group, plasma total estrogens were 3-fold and in the bile 49-fold higher as compared to the controls. This indicates that the unconjugated estrogens are preferentially transferred to the bile. In contrast, the increase found for conjugated total estrogens in the high dose group was much stronger in the plasma, namely 2332-fold compared to 414-fold in bile. This is in agreement with

the study by Bottoms *et al.* [115] showing that the pig excretes estrogens mainly as E1G through the kidney. In their experiment, an even higher dose compared to the study at hand was used.

In comparison with the pharmacokinetic study, the NOEL dose group of the continuously exposed sows depicted higher plasma total estrogen concentrations (25.3 ± 2.8 pg/ml (control); 30.4 ± 5.0 pg/ml (ADI); 71.9 ± 23.9* pg/ml (NOEL); 419.5 ± 80.8* pg/ml (High dose); $p < 0.001$) while these remained low in the male castrated piglets (average concentration over the entire sample period: 21 ± 5 pg/ml (control), 10 ± 1 pg/ml (ADI), and 11 ± 2 pg/ml (NOEL)) (Flöter *et al.* 2019, App. IV, Tab. 1). This presumably indicates an estrogen accumulation upon continuous treatment, particularly as the concentration in the control animals were comparable.

Fürst *et al.* [16] have shown that the sows that were exposed throughout pregnancy had elevated E2 concentrations in the high dose group on days 35, 49 and 70 of pregnancy (30.2 pg/ml, 55.8 pg/ml and 80.8 pg/ml, respectively), while in the control (12.0 pg/ml, 19.1 pg/ml and 24.4 pg/ml, respectively), the ADI and the NOEL dose groups plasma E2 concentrations remained low. This, additionally indicates an estrogen accumulation through increasing concentrations at 12 hours after feeding of the E2 and thus during the plateau phase.

Increased estrogen concentrations in tissues of sows on day 10 of pregnancy

E2 and its metabolites were significantly higher in all tissues in the high dose group, as well as in the endometrium and heart regarding total estrogens and conjugated total estrogens in the NOEL dose group (Flöter *et al.* 2018, App. III, Fig. 1; Flöter *et al.* 2019, App. IV, Tab. 1). This provides a basis for direct exposure effects in these tissues.

Unconjugated estrogen concentrations in the plasma get rapidly cleared [112], as shown above. In contrast, tissues can retain steroids for a longer time [112, 121]. Consistently, a stronger relative increase of E2 and total estrogens was found in the three tissues one hours after the last E2 exposure in comparison with the plasma (Flöter *et al.* 2019, App. IV, Tab. 2). In the high dose group, 5 to 27-fold higher E2 concentrations and 100 to 228-fold higher total estrogen concentration were measured in the heart and endometrium, respectively. Similar, a 5 and 6-fold significant relative increase of total estrogens was found in these tissues in the NOEL dose group compared to the control animals. Estrogens are mainly excreted as conjugated metabolites through the kidney, which is possible due to their polar nature, while minor amounts of the nonpolar unconjugated estrogens are excreted via the feces [115]. This is reflected in the present data through high amounts of conjugated estrogens in the plasma and a comparably small relative increase in the tissues. All three tissues depicted a similar relative increase of about 10-fold higher

conjugated E2 concentrations and about 100-fold higher conjugated total estrogens in the high dose group, compared to 361-fold and 2332-fold in the plasma, respectively. In the NOEL dose group, the endometrium depicted a 9-fold and the heart a 3-fold higher concentration of conjugated total estrogens. In the three tissues, the relative increase of the unconjugated estrogens was slightly higher than of the conjugated counterparts. Clearance of the estrogens depends on the tissue [112, 120, 121, 261], as for example the concentration of expressed ER facilitates estrogen retention [112, 120–123]. In several species the uterus contains the highest ER abundance, the skeletal muscle has intermediate and the heart even lower concentrations [74, 112, 262]. Rather small differences between the three tissues were observed in the relative increase one hour after the E2 application. Hanson *et al.* [121] have demonstrated that the ratio of target to non-target tissue is low early post application, then especially increasing from two hours on. Thus, by measuring already at one hour after the application, increasing tissue dependent differences may not yet be visible.

3.2. Preimplantation E2 exposure affects the endometrial and embryonic transcriptome

In the present study, the elevated and potentially accumulating concentrations of E2 and its unconjugated and conjugated metabolites through the continuous exposure every 12 hours until day 10 of pregnancy in the NOEL and high dose group (Flöter *et al.* 2019, App. IV) provide the possibility to exert estrogenic effects. In addition to estrogens potentially reaching the embryo, effects on the offspring may also be based on indirect actions of the estrogens such as on the endometrium altering its secretion. Environmental low-dose estrogen exposure would usually affect the entire gestation as analyzed below (chapter 3.3). However, we additionally focused on the very early phase, the preimplantation period, as sensitive time for potential low-dose effects.

Dose dependent effect on the endometrial mRNA but not miRNA expression

The endometrium is highly important for the preparation of pregnancy and implantation including embryo maternal communication through the histotroph, the molecules secreted by the uterine epithelia into the uterine fluid surrounding the preimplantation embryo. Earlier studies have shown that in addition to alterations in the mRNA expression [130, 148, 149], changes in protein expression, secretion and endometrial surface structures [130, 149, 151] were observed after estrogen exposure only on days 9 and 10. In the present study, the mRNA expression after

continuous E2 exposure was analyzed and alterations in all treatment groups were found with a dose-dependent increase in the number of differentially expressed genes (≥ 2-fold regulated, adj. $p < 0.05$) with 14 (ADI), 17 (NOEL) and 27 (high dose) DEGs, respectively (Flöter *et al.* 2019, App. IV, Tab. S2). Most DEGs were upregulated through the E2 exposure compared to the control animals and only 6 DEGs were regulated in more than one treatment group (Flöter *et al.* 2019, App. IV, Fig. 2). Technical validation of the result was performed for 21 genes using RT-qPCR. Most genes had similar fold changes in both analyses (Flöter *et al.* 2019, App. IV, Tab. 5) leading to a significant linear correlation of these values ($p > 0.001$, $R^2 = 0.505$). Nine of 14 DEGs (64 %) could be validated in the high dose group, while only 2 of 7 DEGs (29 %) were significant in the RT-qPCR analysis in the NOEL dose group and also 2 of 7 DEGs in the ADI dose group. In comparison, the study by Ross *et al.* [148] administering E2-cypionate on days 9 and 10 which have even led to embryonic degeneration later on also detected a relatively small number of regulated transcripts (≥ 1.8-fold, $p < 0.1$) using microarray analyses with 9 DEGs on day 10, 71 DEGs on day 13 and 21 DEGs on day 15. Four DEGs were found in the high dose treatment group in the present study that have also been shown to be regulated in the study by Ross et al. [148], namely, retinol binding protein 4 (*RBP4*) on day 10, and on day 13 solute carrier family 39 member 2 (*SLC39A2*), sulfotransferase family 2A member 1 (*SULT2A1*), and vanin 2 (*VNN2*).

From the 51 DEGs with differential abundance in total in at least one of the three treatment groups in the endometrium, 40 were used in the DAVID analysis yielding 16 functional annotation clusters. The 10 most enriched clusters (Flöter *et al.* 2019, App. IV, Tab. 4) contained some biological processes that are known to have a role in the uterine preparation during early pregnancy such as extracellular region, positive regulation of transport and of secretion, and leukocyte activation [130, 263]. Although other studies reported most effects on days 13 and 15 and only few overlapping DEGs were found, some functional categories were similarly affected, possibly indicating general target categories of genes affected by the E2 treatment [148, 149]. These include, processes related to cell death, developmental processes, cell activation, and transport.

Some general reasons that might play a role in only detecting low numbers of regulated genes may be the higher variance through using the pig as outbred animal in addition to potentially lower overall included numbers of animals/samples as compared to inbred mice and cell culture experiments. Most importantly, the complete endometrial tissue was analyzed which contains many different cell types. It has been shown for example that some changes only occur in the epithelium [130, 148, 264].

Van der Weijden *et al.* [265] used tissue samples of the study at hand and analyzed 57 target genes (cell cycle regulators, tumor suppressor genes, methylation specific enzymes) in 6 different

tissues of the pregnant sows on day 10 of pregnancy. The number of DEGs detected upon E2 exposure was 10 in the corpus luteum, 7 in the endometrium and heart, 5 in the skeletal muscle, 4 in the spleen and 3 in the liver. While in the endometrium most changes occurred in the high dose group in that study, in the corpus luteum, heart and skeletal muscle often the ADI and/or NOEL dose groups were also affected. In addition, DNA methylation of 3 of these significantly regulated genes, namely Ras Association Domain Family Member 1 (*RASSF1*), Phosphoserine Aminotransferase 1 (*PSAT1*) and Cyclin Dependent Kinase Inhibitor 2D (*CDKN2D*), was measured in the endometrium, corpus luteum and liver. Although DNA methylation changes were small, the liver showed the most prominent alterations with many CpN dinucleotide sites affected, most often in the NOEL and high dose group. There was no correlation of DNA methylation with the mRNA expression. Still, these results evidence that a more global low-dose effect through the E2 exposure took place in these sows.

In addition to the mRNA measurements, high-throughput sequencing of small ncRNAs and qPCR of selected, potentially E2 dependent miRNAs was performed. This is particularly interesting, as miRNAs are postulated to regulate about 50 % of the protein coding genes [80], while only very few studies have analyzed *in vivo* effects of EDCs on miRNA expression [101, 174–176].

Unlike human and mouse, there are so far only few porcine miRNAs annotated (n = 326 mature miRNA in miRbase 20.0). Thus, as miRNAs are quite conserved between species, the sequencing results were additionally compared to the known mature miRNA sequences of human and mouse, as well as to the cow as a second large animal to increase the number of sequences analyzed. Through these comparisons 212 porcine, 272 human, 235 murine and 205 bovine sequences of mature miRNAs were detected in the endometrial samples. Deducting the identical sequences in multiple species led to 444 different sequences of mature miRNAs (Flöter *et al.* 2018, App. III, Tab. S2). The additional 232 sequences most certainly represent so far unannotated porcine miRNAs and/or variants of miRNAs. The distribution of these sequences between the species showed that about one third of the 212 porcine sequences could be found in all four species and about another third was specific to the pig (Flöter *et al.* 2018, App. III, Fig. 4). The 20 highest expressed known porcine miRNAs accounted for 82.7 % of the mapped reads (Flöter *et al.* 2018, App. III, Fig. 3A). The expressed miRNAs were statistically analyzed for each species separately revealing no significantly differentially expressed miRNAs between the treatment groups compared to the control group (adjusted $p > 0.05$). This was substantiated by the cluster analysis of the 30 porcine miRNAs with the highest variance depicting no clustering of the treatment groups (Flöter *et al.* 2018, App. III, Fig. 3B). Furthermore, twelve miRNAs, known from the literature as

potentially estrogen regulated miRNAs and encompassing low to high expressed miRNAs, were validated by RT-qPCR [97, 266–274]. There was no significant difference in any of these miRNAs (Flöter *et al.* 2018, App. III, Tab. 2, Fig. S2).

This result contrasts existing *in vitro* and *in vivo* data depicting estrogenic substances regulating miRNA expression [95, 101, 174, 176]. In the present study, the concentration one hour after the last exposure was 3.1 ng E2/g in the endometrial tissue of the high dose group, while the control animals had 0.1 ng E2/g endometrial tissue. In many cell culture experiments depicting miRNA expression changes, a treatment dose of 10 nM (2.7 ng E2/ml) was used [95]. Still, the context (tissue versus cultured cells) and exposure time, and most importantly the biological background differs. For example, in the study by Klinge *et al.* [95], MCF7 human breast cancer cells were used, which contain very high amounts of ERα. This might explain to a certain extend the higher E2 responsiveness compared to the present study. Earlier *in vivo* studies using microarray or similar analyses found some regulated miRNAs [101, 174, 176]. However, even using human placentas of malformed fetuses that had been exposed *in utero* to BPA revealed only 18 significantly dysregulated miRNAs out of 1349 analyzed miRNAs [174]. Still, it is surprising to find no effect on miRNA regulation in the present study (Flöter *et al.* 2018, App. IV). Additionally, an in-depth miRNA expression analyses, including miRNA variants, of male as well as female embryos from the present study (n = 6 per treatment group and sex), where a quite large number of mRNA transcripts were regulated in the female embryos, as outlined in the next section below, also revealed no differentially expressed miRNAs [275], indicating a more general lack of effect of the applied E2 doses on miRNA transcript levels. This could be due to the period of exposure, the route of exposure, the timing of the application with feeding twice daily the half dose and slaughtering one hours after the last regular administration of E2, or a habituation towards E2 because of the continuous treatment. The observed mRNA changes may be caused by miRNA independent gene regulatory mechanisms.

E2 particularly affects the transcriptome of female embryos by leveling sex-specific differences

The mechanism behind mRNA alterations in the ADI dose group remains to be elucidated, as no alterations in estrogen concentrations were observed in this dose group, as detailed earlier (chapter 3.1.). Furthermore, there was a dose-dependent increase in the number of genes altered in their mRNA expression in the endometrium, as described above (chapter 3.2.). Thus, the focus now was set on analyzing male and female embryos from the NOEL and the high dose group.

Microscopic pictures were taken of the embryos (n = 230), carefully flushed from the uterine horns, prior to freezing. They were all at the same stage (hatched blastocysts), contained an embryonic disc and displayed normal, stage-specific development. No differences (p = 0.80) were found between the treatment groups and the control group regarding embryo size with an average of 2.2 mm ± 0.1 mm. Similarly, the number of embryos did not differ between the groups (n = 14.4 ± 0.33 per sow; p = 0.33) (Flöter *et al.* 2018, App. III). Additionally, no significant association (p = 0.892) was found between the sex of the blastocysts and the treatment dose (Flöter *et al.* 2019, App. IV, Tab. 3). This is in accordance with the finding that litter size, sex distribution and body weight did not differ at birth after exposure of the sows to the same doses during the entire pregnancy [16]. Only very few studies have looked at complete gestational estrogen exposure. There is one study in mice analyzing continuous E2 exposure during the entire pregnancy [20]. However, they found a dose-dependent decrease in the number of pups alive at birth. This fits to other data in mice depicting significant effects also regarding litter size and related parameters during pregnancy already after continuous exposure as well as after only preimplantation estrogen exposure [144–147]. Studies in pigs have so far only investigated the effect of exposure for particular days and periods during pregnancy or included postnatal treatment. Interestingly, an exposure on days 7 to 10 [152] or days 9 and/or 10 [18, 148, 150, 151] led to embryo degeneration. The reason seems to be that in pigs, with embryonic estrogen secretion as maternal recognition signal secreted on days 11 to 12 [4], exposure only slightly before this point in time leads to a desynchronization of the uterine preparation with embryonic development [130]. In contrast, treatments from day 2 to 6 [155] or at any day between days 11 to 15 [18, 150, 155–157] did not cause such drastic effects. Similarly, the continuous exposure of the present study seems not to have been as disruptive as exposure only slightly before the estrogen secretion of the embryos [18, 130, 148, 150–152], indicating some kind of habituation effect even to the high dose [16].

The general sex-specific mRNA expression differences (FDR 5%) between the two control groups revealed 50 DEGs higher expressed in female embryos and 35 DEGs higher expressed in males (Flöter *et al.* 2019, App. IV, Tab. S4). This seems to be in contrast to Bermejo-Alvarez *et al.* [276] who found in their microarray analysis (FDR $P < 0.05$) one third of their transcripts to be differentially expressed between male and female bovine embryos (day 7). A closer look revealed that most differences in the latter study were rather small. Thus, when selecting a cut-off fold change of 2, they had only 55 differentially regulated transcripts. Despite general differences such as the species and the day of analysis, this rather small number of transcripts is in line with the data from the present study, where a cut-off fold change of 2 led to 60 remaining DEGs. In addition, Heras *et al.* [277] who used RNA-Seq and the EdgeR algorithm for the statistical analyses (FDR

corrected p-value < 0.05) but also analyzed bovine blastocysts on day 7 similarly found only several DEGs regulated between the sexes. In *in vivo* produced embryos they depicted 225 DEGs regulated and with a cut-off fold change of 2 there were 119 remaining DEGs. In the *in vitro* produced embryos, they even had lower numbers.

To my knowledge, the present study is the first describing *in vivo* effects of an estrogenic substance on the whole transcriptome of male and female preimplantation embryos. The analysis of the RNA-Seq data using an FDR of 5% showed 982 and 62 DEGs in the high dose and NOEL dose group of the female embryos, respectively, compared to the control group (Flöter *et al.* 2019, App. IV, Tab. S3). In contrast, none and only 3 DEGs were detected in the male embryos of the high dose and NOEL dose group, respectively. Similar to the endometrium, there was a dose dependent effect in the female embryos, and in the high dose group clearly more up- than downregulated genes. The predominant effect on the female embryos may be due to sex-specific differences in the transcriptome, proteome, methylome and/or metabolome prevailing during early embryo development [276, 278–280]. Similarly, it has been shown that alterations of the embryonic environment through diet and nutrients led to sex-specific changes during the preimplantation phase [277, 279].

A second analysis of the data was performed to avoid the bias from the algorithm calculating the correction for multiple testing. Therefore, a cut-off was set for the p-value with $p < 0.0001$ and for the fold change with ≥ 1.5. This led to 73 and 32 DEGs in the female embryos of the high dose and NOEL dose group, respectively. Similar to the analysis using an FDR of 5%, only few regulated genes were detected in the male embryos with 5 and 9 DEGs in the high dose and NOEL dose group, respectively. The resulting DEGs (Flöter *et al.* 2019, App. IV, Tab. S5, Tab. S6) were used for the Venn diagram (Flöter *et al.* 2019, App. IV, Fig. 3) and the hierarchical clustering analysis (Flöter *et al.* 2019, App. IV, Fig. 4a, Fig. 4b). The Venn diagram revealed only few genes regulated in more than one group. Two hierarchical clustering pictures were generated, one only for genes where the samples are shown grouped for their sex and dose (Flöter *et al.* 2019, App. IV, Fig. 4a), and another where samples and genes were clustered (Flöter *et al.* 2019, App. IV, Fig. 4b). Both figures showed female embryos dose dependently becoming more similar to the male embryos.

In general, the masculinization may be transient and/or lasting. One argument for a transient effect is that after exposure of sows during the entire gestation, none of the observed postnatal effects pointed towards a masculinization of the female offspring [16, 265, 281](Pistek *et al.* 2013, App. I; Flöter *et al.* 2016, App II). In contrast, a study in mice analyzing the offspring after preimplantation exposure to E2, depicted changes in the anogenital distance indicating a masculinization in

females as well as a demasculinization in the males [23, 24]. The latter aspect could also be observed in the male piglets exposed during the entire pregnancy having an increase in body fat percentage [16, 282]. A transient masculinization at this point in time preimplantationally may be due to the E2 minimizing potential sex-specific differences in the velocity of embryo development [279]. However, there is scarce data regarding this effect *in vivo* and no data regarding estrogenic substances. Most studies on embryos have been performed *in vitro* investigating different culture conditions such as modifications in the energy substrate [283]. They predominantly resulted in a faster development of the male embryos [279, 283].

The underlying mechanism of the observation of the E2 leveling sex-specific differences in the mRNA expression presumably involves changes in the uterine fluid. This may either be directly through increasing E2 metabolites that have at least been shown to reach the endometrial tissue (Flöter *et al.* 2018, App. III, Fig. 1; Flöter *et al.* 2019, App. IV, Tab. 1) or through effects on the endometrium altering its secretion. An indication for the latter are the above-mentioned mRNA expression changes (chapter 3.2.). Overall, a molecular fingerprint of low-dose effects was observed at concentrations currently presumed to have no effect. Although it is not known if these effects may have lasting consequences for health later on, sensitive points in time and molecular analyses should be included in the risk assessment of hormonal substances.

As during early development many epigenetic changes occur that may be affected by external stimuli [50, 60], the question arises if there are epigenetic changes in the day 10 embryos and if they may even last into postnatal life. In the analyzed mRNA-Seq data, DNA methyltransferases and other genes associated with direct impact on the epigenome were not found to be altered and also gene ontology terms involving epigenetics did not appear in the DAVID analysis (Flöter *et al.* 2019, App. IV, Tab. 6). However, in a separate analysis, three genes were analyzed regarding their DNA methylation patter in the embryos [265]. As in all three genes small but significant hypomethylation were detected, this may hint towards a more global effect. Interestingly, two genes were also altered in the liver of the one-year-old sows that had been exposed to E2 during the entire pregnancy [265]. In this case, a hypermethylation was observed, potentially indicating lasting epigenetic effects through the preimplantation exposure.

3.3. Gestational E2 exposure does not affect uterine HOXA10, but certain bone parameters in pre- and postpubertal offspring

Assuming that an environmental exposition to low doses of estrogenic substances usually can prevail through the entire gestation, lasting consequences in pre- and postpubertal offspring of sows fed the same E2 doses (ADI, NOEL, high dose) daily from insemination until parturition were subsequently investigated.

Low-dose effects on bone parameters in male and female offspring

In the prepubertal offspring, next to bone length, differing parameters were measured at three epiphyseal bone sites (proximal tibia, proximal and distal femur) using pQCT. These included total and trabecular BMD and CSA, respectively, as well as the SSI. In the male piglets, out of all parameters at all sites measured, only one finding was significantly affected. The SSI, a surrogate parameter of fracture strength in torsion, was significantly reduced in the ADI dose group ($p = 0.002$) at the proximal tibia (96.3 ± 11.6 mm^3 (control), 45.0 ± 10.5 mm^3 (ADI); 71.9 ± 11.0 mm^3 (NOEL); 55.1 ± 10.5 mm^3 (high dose); $p = 0.008$) (Flöter *et al.* 2016, App. II, Fig. 1). Although not significant, a similar pattern with lowest SSI values in the animals of the ADI and the high dose group was observed at the distal femur ($p = 0.129$; Flöter *et al.* 2016, App. II, Fig. 1) and the proximal femur ($p = 0.169$; Flöter *et al.* 2016, App. II, Suppl. Tab. A.2), indicating a more general effect. However, biomechanical testing would be necessary for a more conclusive interpretation. Still, this result is in accordance with other studies in male animals where differing parameters were altered indicating weaker bones [107, 108, 218]. Particularly, a study that treated rhesus monkeys with 2,3,7,8-tertacholordibenzo-p-dioxin (TCDD) had shown such an effect to specifically occur in the low-dose group [107].

None of the parameters was affected in the female prepubertal offspring, although there was a tendency of a lower total ($p = 0.060$) and trabecular BMD ($p = 0.080$) at the distal femur, most obvious in the high dose group (Flöter *et al.* 2016, App. II, Tab. 2). Although not significant, similarly to the tendency for BMD in the prepubertal piglets, the postpubertal offspring of the high dose group depicted a slightly later onset of puberty as indicated by the first corpus luteum formation ($p = 0.180$) (Flöter *et al.* 2016, App. II, Fig. 3). This occurred around the age of 8 months, while the difference of the high dose group to the control group was on average 22 days. In contrast to these results, studies on EDC exposure in rodents have often shown effects on the timing of puberty [17, 284], only few studies in large animals have analyzed puberty parameters

after early estrogen exposure [109, 285–287]. Some showed a lack of a significant effect on the timing of puberty [109, 286, 287]. In contrast, Lyche *et al.* [285] demonstrated that gestational and lactational exposure to polychlorinated biphenyl (PCB)153 led to a 9-day delay in the onset of puberty in goats at about 7 months of age. As there was quite a large variation regarding the age at the first cycle in the present study, it may be necessary to increase the numbers of pigs in future studies to unravel if there is an effect or not through gestational E2 exposure on the onset of puberty in the pig. In humans, data in girls revealed that those who were older at menarche exhibited a lower BMD already before the onset of puberty [220]. This interrelation and the influence on it through developmental EDC exposure would also be an interesting aspect for analysis.

The CT analyses at the mid-femoral diaphysis (shaft of the bone) of the one-year-old female offspring depicted an overall significant difference for total CSA (683.1 ± 18.8 mm² (control), 709.8 ± 17.8 mm² (ADI), 770.5 ± 23.5 mm² (NOEL), 736.9 ± 13.9 mm² (high dose); $p = 0.03$), and the pairwise comparison with the control animals showed that the NOEL dose group had a larger total CSA ($p = 0.02$) (Flöter *et al.* 2016, App. II, Fig 2). This was mainly due to the increase found for the cortical CSA ($p < 0.05$), where again the NOEL dose group depicted higher values compared to the control group (444.6 ± 14.0 mm² and 503.1 ±17.1 mm², respectively; $p = 0.03$). The medullar CSA was unaltered. The other parameters, including the femur length, volume and CT number were unaffected (Flöter *et al.* 2016, App. II, Tab. 3). Focusing on data from studies using large animals, in adults [107, 217] as well as at a fetal stage of development [226], most effects concerned changes at the diaphysis. This is similar to the results in the one-year-old offspring, while no conclusion can be drawn regarding the prepubertal offspring as no diaphyseal measuring point was assessed. Particularly, Hermsen *et al.* [107] analyzing rhesus monkeys exposed to TCDD during gestation and lactation also depicted an inverted U-shaped response with a larger total CSA at the mid-diaphysis of the femur in the low-dose group. In contrast, the mid-diaphysis was unaffected in goats treated with PCB 153; however, a smaller total CSA at the diaphysis at 18 % of the total bone length was found [217]. As PCB 126, also applied during gestation and lactation in the afore mentioned study, did not affect bone development, these differences between the studies may be substance specific. In addition to the CSA, many more bone parameters are important for overall bone strength [107, 288, 289]. Thus, without mechanical testing, a final conclusion cannot be drawn on whether the observed increase in cortical CSA may increase bone stability or not.

The finding that most of the analyzed parameters, including plasma hormone concentrations of IGF1 and Leptin, were unaffected in the offspring (Flöter *et al.* 2016, App. II, Tab. 1, Tab. 2, Tab.

3, Tab. 5, Suppl. Tab. A.2) and only certain parameters were affected, is similar to other studies in this field [106–108, 214, 216–218]. Several studies have demonstrated long-term effects of early EDC exposure on bone parameters in female adult offspring [106–108, 214, 216, 217, 219]. Similarly, two parameters were significantly affected in the postpubertal female animals in the present study, while no effect was observed in the prepubertal female offspring. The time of analysis is highly important. More globally, this was also shown by Wuttke *et al.* [290] analyzing a life-long estrogen exposure in rats. They found that estrogen exposure led to an increased trabecular BMD before puberty, no alterations during puberty, a reduced BMD in the adult, while again higher values appeared in the aged animals. In humans, evidence on bone development shows that acquired properties during childhood and adolescence are associated with bone health later on [220]. In addition, already small differences in the obtained peak bone mass might strongly affect the risk of developing osteoporosis. However, alike most studies focusing on bone effects from early EDC exposure [51], the present study also did not target aged animals. Overall, distinct but rather minor changes occurred at the selected points during development; still, it would be fascinating to see if effects would be observable in the aged sow.

Non-monotonic dose-responses are common for steroids [33] and early EDC exposure has also been shown to exert effects on bone specifically occurring at low doses [106–108]. Concerning the observed effects in the one-year-old female offspring, the E2 exposure may have affected bone cells directly. Plausible mechanisms of action may include a reduced expression of ERβ through the alteration of its promoter DNA methylation [291]. Signaling through ERβ limits female cortical bone growth [6], which seems to be increased in these animals. Furthermore, male ERβ knock-out mice depict normal bone growth, while in the female animals an increase in radial bone growth with larger cortical CSA was found [6, 292]. The second possibility is the alteration of the numbers of certain bone cell types with an increase in osteoblasts and a decrease in osteoclasts [52, 219], as it has been observed in directly exposed prepubertal piglets [52]. Indirect effects on bone cannot be ruled out, although, so far, no effects on other parameters, such as hormone concentrations, were detected in the NOEL dose group [16] (Pistek *et al.* 2013, App. I). Similarly, no correlation of cortical CSA and total CSA, respectively, with plasma hormone concentrations was detected (Flöter *et al.* 2016, App. II, Tab. 4).

The here presented results demonstrate alterations in low-dose groups, at concentrations that for humans are claimed to be at the ADI or close to the NOEL, which is in accordance with previous data on body composition [16] and DNA methylation [265] in these animals. Many of the before mentioned studies in rodents [106, 108, 215, 293] or large animals [107, 217, 226, 227] have also used concentrations at or close to human exposure levels [106–108, 215, 293] or close to

environmental concentrations [226, 227]. Although humans are usually exposed to lower E2 concentrations than used in the present study, there are diverse environmental estrogens [14, 25], potentially exerting additive estrogenic effects [41]. Thus, these results substantiate the high sensitivity of developing organisms to exogenous estrogens and highlight the need to reevaluate the current threshold values.

Analyses in female offspring focusing on HOXA10 reveals most parameters unaltered

At slaughter, absolute and relative uterine weight did not differ between the E2 treatment groups and the control group in the prepubertal female offspring (Pistek *et al.* 2013, App. I, Suppl. Tab. A1). Similarly, in the one-year-old female offspring, body weight, age at slaughter, number of corpora lutea and plasma hormone concentrations (E2, total estrogens, testosterone, progesterone, IGF1, leptin) were unaltered by the treatment (Pistek *et al.* 2013, App. I, Suppl. Tab. A1; Flöter *et al.* 2016, App. II, Tab. 5). This is in line with data published by Fürst *et al.* [16] showing that plasma E2 and testosterone concentrations, total fat and body weight at slaughter was unaffected in the female prepubertal animals. Only at weaning, the female animals of the ADI dose group depicted a significantly lower body weight compared to the control group.

As reproductive tissues are a major target of estrogens and several studies had shown uterine *HOXA10* as potential target of estrogenic EDCs during pregnancy or neonatally [104, 189, 190, 199, 202–204, 294, 295] this was consequently selected as major target for analyses in the female offspring.

HOXA10 mRNA expression and promoter DNA methylation depicted two minor alterations in the uterine tissue of prepubertal offspring (Pistek *et al.* 2013, App. I, Fig. 2a, Fig. 2b). The mRNA expression showed an overall significant difference ($p=0.02$) whereas the comparison of each treatment group with the control group was not significant. In addition, the analyses of 10 CpG sites in the promoter region depicted slight but significant alterations at CpG site 4, which however did not correlate significantly with the mRNA expression. The one-year-old sows were completely unaffected regarding *HOXA10* mRNA expression and promoter DNA methylation in the endometrium (Pistek *et al.* 2013, App. I, Fig. 2c, Fig. 2d). Finding no effect is in contrast to other studies and may potentially be explained by one of the following three reasons.

The most obvious difference is that previous studies were mainly performed in rodents. On the one hand, there are some conserved findings regarding *HOXA10* such as an increased uterine or endometrial expression in mice, humans, pigs, canine and bonnet monkey at the time of

implantation [194–198]. On the other hand, species specific differences prevail. In rodents, estrogens have been shown to downregulate *Hoxa10* mRNA and protein expression [104, 189] while in pigs an upregulation was reported [201, 295]. In the present study in pigs, an average promoter DNA methylation in the prepubertal uterus of 2.1 ± 0.1% and in the caudal endometrium of the one-year-old sows of 4.1 ± 0.1% (Pistek *et al.* 2013, App. I, Fig. 2b, Fig. 2d), as well as low values in all other tissues analyzed were found (Pistek *et al.* 2013, App. I, Fig. 4c, Fig. 4d). Similarly, low values prevail in tissues including the endometrium of adult humans and baboons [254, 296, 297]. This is in contrast to prepubertal mice, where the promoter DNA methylation has been shown to be at an average of 70% [190]. This indicates severe species differences in the epigenetic regulation of *HOXA10* potentially explaining differences in the outcome to EDC treatment. Furthermore, the endogenous estrogen concentrations during pregnancy, particularly towards the end, are higher in pigs and humans due to placental estrogen synthesis, which is absent in rodents [16, 178, 298]. This may be another reason for the pig being less sensitive to gestational estrogenic treatment compared to mice and rats, where *in utero* effects on *Hoxa10* have mainly been described.

Secondly, mainly other EDCs had been used such as BPA [104, 189, 190, 199], DES [202, 203, 294, 299] and methoxychlor [204], while one study applied estradiol-valerate [295]. Although *in vitro*, using the human endometrial adenocarcinoma cell line Ishikawa, all these substances were able to increase *HOXA10* mRNA expression [199, 204], *in vivo* studies depict diverging results. For example, early postnatal low-dose BPA exposure led to a decrease of *Hoxa10* mRNA and protein expression in pregnant adult rats [104]. Likewise, promoter DNA methylation in murine offspring was higher when treated with DES and reduced when BPA was applied *in utero* [190, 294]. In addition to the substance, the dose is of importance as non-monotonic dose-responses are known for hormonal substances [33]. In the present study, a wide range of E2 concentrations was used with two low doses and one high dose. Still, no substantial effect on *HOXA10* mRNA expression and promoter DNA methylation was detected.

Thirdly, the time of exposure may impact on the result as it was shown in rodents that *in utero* exposure to BPA on days 9 to 16 reduced Hoxa10 promoter DNA methylation in the offspring [190], whereas neonatal treatment did not exert this effect [189]. Studies in pigs had mainly focused on exposure early postnatally as sensitive time window, linked to the lactocrine hypothesis, as after birth differentiation processes in the uterus are mediated by ERα while having low endogenous estrogen concentrations as its synthesis by the ovaries has not yet started [187]. Suitably, Chen *et al.* [295] demonstrated *HOXA10 mRNA* expression changes in piglets on postnatal day 14 directly following estradiol-valerate exposure starting at birth. However, analyses

in sows after a similar exposure neonatally did not impact on *HOXA10* mRNA expression. This demonstrates the importance of the time of analysis.

In summary, differences in the species, the substance and dose, the time of exposure and the time of target analyses may underlie the findings of a lack of lasting effects on *HOXA10* through the E2 treatment during the entire pregnancy in the pig.

The results in the male and female offspring after complete gestational E2 exposure depicted more effects on the males [16](Pistek *et al.* 2013, App. I; Flöter *et al.* 2016, App. II). Consistent with this result, a high-throughput sequencing approach by Kradolfer *et al.* [281] analyzing the uteri of the prepubertal piglets showed that only one single gene in the ADI dose group, Integrin alpha E (*ITGAE*), was differentially expressed. In contrast to this finding in females, 130 DEGs were measured in the prepubertal male prostate and 3 DEGs in the testis. In addition, three genes of the 130 DEGs were analyzed regarding DNA methylation depicting differential methylation in the gene body of the biglycan (*BGN*) gene. Still, there was also another finding in the liver of the one-year-old female offspring [265]. Targeted DNA methylation analyses depicted slight but significant DNA hypermethylation in Cyclin Dependent Kinase Inhibitor 2D (*CDKND2*) and Phosphoserine Aminotransferase 1 (*PSA1*) but not Ras Association Domain Family Member 1 (*RASSF1*). More precisely, mainly CpN dinucleotide sites in the NOEL and/or high dose group were altered compared to the control group, namely 6 out of 12 sites in the putative promoter region of *CDKND2* and all 13 sites analyzed in the coding region of the first exon of *PSAT1*. Thus, a molecular fingerprint of the gestational E2 exposure was determined in the female offspring at the level of DNA methylation.

Promoter DNA methylation of HOXA10 is not associated with gene expression postpubertally, but may be involved in tissue specific expression prepubertally

In order to analyze the relationship of *HOXA10* mRNA expression and DNA methylation in a broader context, pigs were studied during the estrous cycle as well as during early pregnancy, and many different tissues were compared to each other. The former is particularly interesting as fast and cyclic changes of DNA methylation underlying changes in mRNA expression have been described *in vitro* [300, 301]. In addition, it has been demonstrated earlier that *HOXA10* promoter DNA methylation was inversely associated with its mRNA expression in humans [254], mice [190] and baboons [296].

The porcine estrous cycle was studied on five different days, starting with day 0 (estrus), day 3, 6, 12 and 18. *HOXA10* was significantly regulated ($p < 0.0001$) (Pistek *et al.* 2013, App. I, Fig. 3a),

whereby highest expression was measured on day 0 and thus at the time where highest E2 concentrations prevail [302]. The mRNA expression on day 0 was significantly higher than on all other days analyzed. A 2.5-fold lower transcript abundance was detected on day 3 ($p < 0.0001$), which was then 1.5-fold higher again on day 6 ($p = 0.04$). Days 12 and 18 had values in between days 3 and 6 and did not differ significantly from days 3 and 6. *HOXA10* mRNA expression changes during the estrous cycle have also been shown in humans [195, 303] and dogs [196], with humans depicting a similar range in the endometrial tissue with a difference of about 2-fold [195]. During early pregnancy, several species have shown an increase in *HOXA10* mRNA expression [194, 196–198], which is in accordance with the data of the study at hand (Pistek *et al.* 2013, App. I, Fig. 3c). In pregnant sows, a significant higher *HOXA10* expression on day 14 compared to day 12 ($p = 0.02$) was observed. On days 10 and 12, non-pregnant and pregnant animals had similar transcript abundance, while it remained at this level in the non-pregnant animals, the increase found in the pregnant animals led to a significant difference on day 14 ($p = 0.003$). In cyclic as well as pregnant and non-pregnant animals, DNA methylation analyses revealed an overall very low methylation percentage without major changes (Pistek *et al.* 2013, App. I, Fig. 3b, Fig. 3d). Only CpG site 3 was affected by the day of the estrous cycle ($p = 0.04$), but did not significantly correlate with *HOXA10* expression. Similarly, only one of 10 CpG sites depicted an effect of the day in the non-pregnant animals ($p=0.03$), which also did not correlate with the mRNA expression. These results are in contrast to data from other species [190, 254, 296]. Although *HOX* genes are conserved and important transcription factors [188], still there are species-specific differences in uterine development, morphology and function. Thus, evolutionary changes may have also affected *HOXA10* gene regulation.

Next, as tissue specific gene expression often shows much larger differences and promoter DNA methylation was shown to be associated with tissue specific transcription [304, 305], *HOXA10* was investigated in a large set of reproductive and non-reproductive tissues (Pistek *et al.* 2013, App. I, Fig. 4). The highest expression was depicted in the uterus and endometrium of pre- and postpubertal animals of the control group, respectively. These are the organs where *HOXA10* is mainly studied and where its functions are well described. Nevertheless, *HOXA10* mRNA expression was also detected at various levels in all other tissues with the heart showing lowest transcript abundance in both groups of animals (Pistek *et al.* 2013, App. I, Fig. 4a, Fig. 4b). This indicates that HOXA10 is of importance in many organs, and not only essential as transcription factor for uterine development and function. A maximum difference between tissues of 20,000-fold in the prepubertal animals and 6,000-fold in the one-year-old offspring was determined. In addition, some changes in the expression level were observed comparing the pre- to postpubertal

stage. Promoter DNA methylation at the 10 CpG sites was overall low in all tissues with an average methylation of 3% and minor differences between 1 and 13% (Pistek *et al.* 2013, App. I, Fig. 4c, Fig. 4d, Suppl. Fig. A.1). The correlation analyses between mRNA expression and promoter DNA methylation depicted a significant negative association at each of the 10 CpG sites in the prepubertal offspring (Pistek *et al.* 2013, App. I, Tab. 1), which could not be observed in the postpubertal animals. The correlation was strongest at CpG site 3 ($p < 0.001$, $R^2 = 0.551$; Pistek *et al.* 2013, App. I, Fig. 4e). DNA sequence analyses showed this CpG site to be a potential binding site for a transcriptional activator and that it is conserved between humans, pigs, rats, mice and many other mammals (Pistek *et al.* 2013, App. I, Suppl. Fig. B1). This is interesting, as it has been shown that DNA methylation at a single CpG site could abrogate transcription factor binding [72]. In addition, Fürst *et al.* [74] found DNA methylation at a single CpG site as potential underlying cause for tissue specific mRNA expression. Thus, these findings indicate differences in the possibility of gene expression regulation through promoter DNA methylation being present only during prepubertal development but not in the postpubertal offspring. Thereby, this thesis contributes information about potential developmental tissue specific gene regulation.

4. Conclusions and outlook

Development is regarded as a highly sensitive time for environmental stimuli to induce lasting impacts. This may be partially due to the occurrence of large changes of epigenetic marks, particularly during the preimplantation period, and higher cell proliferation. The study presented here, together with further investigations using the animal trials undertaken here, substantiates gestation as a sensitive time window for EDC exposure (Fig. 5) [16, 265, 281]. It was demonstrated that even a low-dose E2 exposure during early development induced certain sex-specific mRNA expression, DNA methylation and /or morphologic alterations at different time points during development, namely preimplantationally, prepubertally and postpubertally, respectively. Thus, potentially indicating a priming during development for disease onset later in life [306]. The pig as animal model was selected due to its placental estrogen synthesis that, unlike in rodents, better resembles the human during pregnancy with particularly high estrogen concentrations towards the end of gestation. These higher concentrations could indicate more resistance to exogenous estrogens [178]. Indeed, the herein presented data has shown much less and less pronounced effects as compared to rodent data. As data from large animal models bridging the gap to humans are scarce, the current study adds a substantial amount of new results to the field. Significant findings were made, such as that orally applied estrogens induced elevated plasma concentrations of conjugated and unconjugated metabolites as well as a marked increase in tissue estrogen concentrations, also present in the NOEL dose group. These estrogens, reaching the endometrium during the preimplantation period, could provide the basis for the direct dose dependent effects on the endometrial and embryonic transcriptome on day 10 of pregnancy, and possibly also for the observed lasting effects. Next to species specific differences, the presented data show that the timing of the estrogenic stimulus is highly important. In the pig, strong effects including abortion are described through short term estrogen application slightly before implantation [18, 130, 151]. In order to more closely mimicking the environmental exposure situation to low doses of EDCs such as for example E2 in foods and BPA in plastic bottles of beverages, this study at hand focused on continuous estrogen exposure throughout the entire pregnancy. Less drastic effects were detected pointing towards a habituation effect. This might also explain the lack of changes in the endometrial miRNA transcriptome.

Although environmental concentrations of E2 are usually below the ADI, additive effects of the numerous estrogenic substances are possible [41]. The detection of multiple low-dose effects in this study substantiates the need for a careful revision of the proposed threshold level for E2. These threshold values, set in 1999 [34] relying on human data in postmenopausal women, have

been questioned earlier [33, 36], particularly regarding developmental exposure with a much more sensitive part of the population. This applies not only to children with their low endogenous sex hormone concentrations, but also to the embryonic phase as depicted herein with the leveling of the sex-specific gene expression profile in blastocysts. This molecular fingerprint in the embryos may imply a functional perturbation and/or an effect on their developmental velocity.

In this regard, it remains to be investigated whether the observed molecular effects on day 10 of pregnancy in the embryos are only transient or if they mirror fingerprints that lead to changes later in time or may even be connected to the observed phenotypic outcomes in the postnatal animals. Importantly, DNA methylation analyses need to be integrated, at best using a genome-wide high-resolution DNA methylome approach, as first results showed gene-specific methylation differences even at low doses in both embryos and the offspring postnatally [265]. Such findings could help establishing epigenetics as a new and sensitive parameter for the risk assessment of EDCs. Furthermore, in a follow-up study to confirm and increase the knowledge of the observed gestational effects of E2, the integration of the following foci stands to reason: (a) including biomechanical testing of the bones, (b) another sampling point in time for both a follow up of the prepubertal bone phenotype in adult boars and the aged animals, (c) the further analyses of the uterine flushings particularly concerning their amount of the various estrogens, as well as (d) the therein contained extracellular vesicles (EV) loaded with small RNAs.

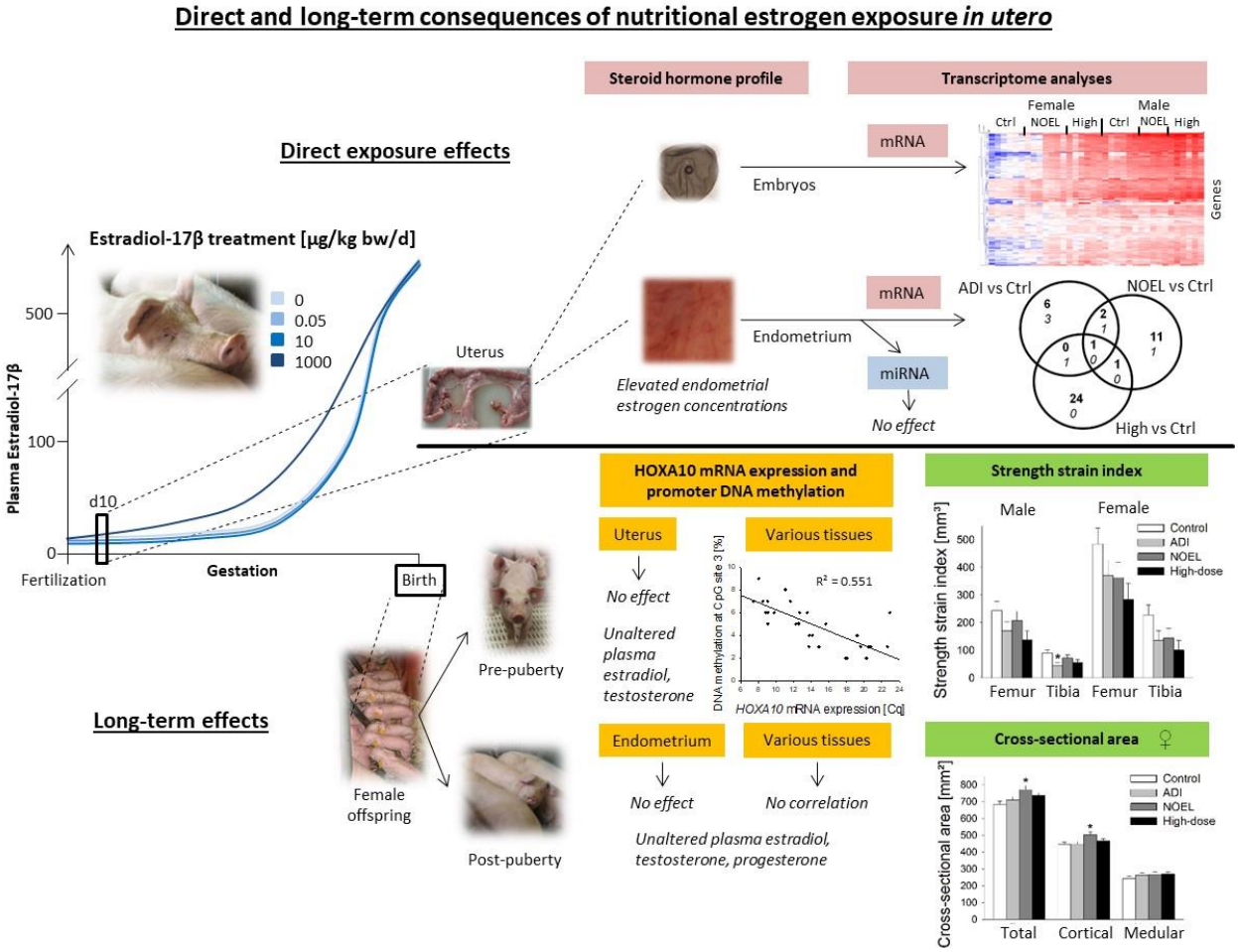

Fig. 5: Overview of the observed findings through direct and gestational oral estradiol-17β (E2) exposure. E2 was fed in concentrations corresponding to the "acceptable daily intake level" (ADI), close to the "no observed effect level" (NOEL) and a high dose (0.05, 10 and 1000 µg/kg body weight (bw)/day (d), respectively), as well as a carrier only as control group. The plasma E2 concentrations during pregnancy are depicted according to Fürst et al. 2012 [16]. The main results of the four manuscripts, appendix I (orange), II (green), III (blue) and IV (red) are depicted including the following original figures (Pistek et al. 2013, App.I, Fig. 4e), (Flöter et al. 2016, App.II, Fig.1 and Fig. 2), and (Flöter et al. 2019, App.IV, Fig. 2 and 4a), respectively. IGF1 = insulin-like growth factor 1.

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Scientific communications

Original peer reviewed scientific publications as first author

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Appendix

Appendix I

VL Pistek, RW Fürst, HS Kliem, S Bauersachs, HHD Meyer, SE Ulbrich „HOXA10 mRNA expression and promoter DNA methylation in female pig offspring after in utero estradiol-17β exposure", *J Steroid Biochem Mol Biol.* **2013**;138C:435-444

Appendix II

VL Flöter, G Galateanu, RW Fürst, D Seidlová-Wuttke, W Wuttke, E Möstl, TB Hildebrandt, SE Ulbrich „Sex-specific effects of low-dose gestational estradiol-17β exposure on bone development in porcine offspring", *Toxicology.* **2016**;366-367:60-7

Appendix III

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Appendix IV

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- Main contribution to execution of the animal trial
- Conception and execution of experiments
- Analysis and interpretation of data
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- Writing the original draft of the manuscript

Veronika Flöter

V. Flöter

Susanne E. Ulbrich

Susanne E. Ulbrich

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HOXA10 mRNA expression and promoter DNA methylation in female pig offspring after *in utero* estradiol-17β exposure

Veronika L. Pistek[a,b], Rainer W. Fürst[a], Heike Kliem[a], Stefan Bauersachs[c], Heinrich H.D. Meyer[a,1], Susanne E. Ulbrich[a,*]

[a] *Physiology Weihenstephan, Technische Universität München, Weihenstephaner Berg 3, 85354 Freising, Germany*
[b] *Z I E L PhD Graduate School 'Nutritional Adaptation and Epigenetic Mechanisms', Technische Universität München, Freising, Germany*
[c] *Molecular Animal Breeding & Biotechnology and Laboratory for Functional Genome Analysis (LAFUGA), Gene Center, Ludwig-Maximilians-Universität München, Feodor-Lynen-Strasse 25, 81377 Munich, Germany*

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ABSTRACT

Early exposure to environmental estrogens may exert lasting impacts on health. In rodents, homeobox A10 (HOXA10) was demonstrated to be a target of early endocrine disruption, as indicated by persistent changes in uterine *HOXA10* expression and promoter DNA methylation in the offspring. This study aimed at analyzing long-term effects of estradiol-17β on porcine uterine HOXA10. Therefore, offspring were exposed *in utero* to low (0.05 and 10 μg/kg body weight/day) and high (1000 μg/kg body weight/day) doses, respectively. We, furthermore, investigated whether promoter DNA methylation was generally involved in regulating *HOXA10* expression. Unexpectedly, the maternal estrogen exposure did not distinctly impact *HOXA10* expression and promoter DNA methylation in either pre- or postpubertal offspring. Although differential *HOXA10* expression was observed in endometrial tissue during the estrous cycle and the pre-implantation period, no concurrent substantial changes occurred regarding promoter DNA methylation. However, by comparing several tissues displaying larger differences in transcriptional abundance, *HOXA10* expression correlated with promoter DNA methylation in prepubertal, but not postpubertal, gilts. Thus, promoter DNA methylation could affect gene expression in pigs, depending on their stage of development. Clearly, early estrogen exposure exerted other effects in pigs as known from studies in rodents. This may be due to endocrine differences as well as to species-specific peculiarities of tissue sensitivity to estradiol-17β during critical windows of development.

1. Introduction

Humans are exposed to various exogenous substances that interfere with the endogenous hormone system [1]. Among these compounds, which are summarized as endocrine disrupting chemicals (EDC), there are many ubiquitous xenoestrogens like some plasticizers, pesticides, or pharmaceuticals. In addition, natural hormones present in food, such as phytoestrogens in plant products, contribute to the pool of estrogenic substances to which humans are exposed. Nowadays, it is widely accepted that disruptive stimuli during pre- and perinatal development are of concern regarding health consequences later in life [2]. This is known as the hypothesis of developmental origin of health and disease (DOHaD). EDC may contribute to the observed adverse effects, as developing organisms are particularly sensitive to exogenous hormonal influences [3,4]. This has been described in numerous studies on experimental and wildlife animals, with epigenetic changes in DNA methylation as a potential underlying mechanism reasoning observed outcomes later in life [5,6].

In this regard, the female reproductive tract has been shown to be sensitive to early EDC exposure, which affects fertility and reproductive health later in life [1,7,8]. Uterine homeobox A10 (*Hoxa10*) expression and promoter DNA methylation are possible targets of early estrogen exposure that can be involved in reduced uterine receptivity in adult animals [9–12]. HOX genes are highly evolutionary conserved transcription factors that are essential for regulating the axial patterning of the body during embryogenesis [13]. They are additionally involved in differentiation processes in the adult. Hoxa10 is best known for its role in the uterus regarding morphologic development and tissue patterning [14]. In the adult organism, Hoxa10 is essential for embryo survival and implantation as demonstrated by knockout mice, which are infertile [15]. Differentially expression of endometrial *HOXA10* during the estrous cycle

Abbreviations: Bp, base pairs; DES, diethylstilbestrol; BPA, bisphenol A; E2, estradiol-17β; EDC, endocrine disrupting chemicals; MS-HRM, methylation-sensitive high resolution melting; P4, progesterone; T, testosterone.

* Corresponding author. Tel.: +49 8161 714429; fax: +49 8161 714204.
E-mail addresses: pistek@tum.de (V.L. Pistek), fuerst@wzw.tum.de (R.W. Fürst), kliem@wzw.tum.de (H. Kliem), bsachs@genzentrum.lmu.de (S. Bauersachs), ulbrich@wzw.tum.de (S.E. Ulbrich).

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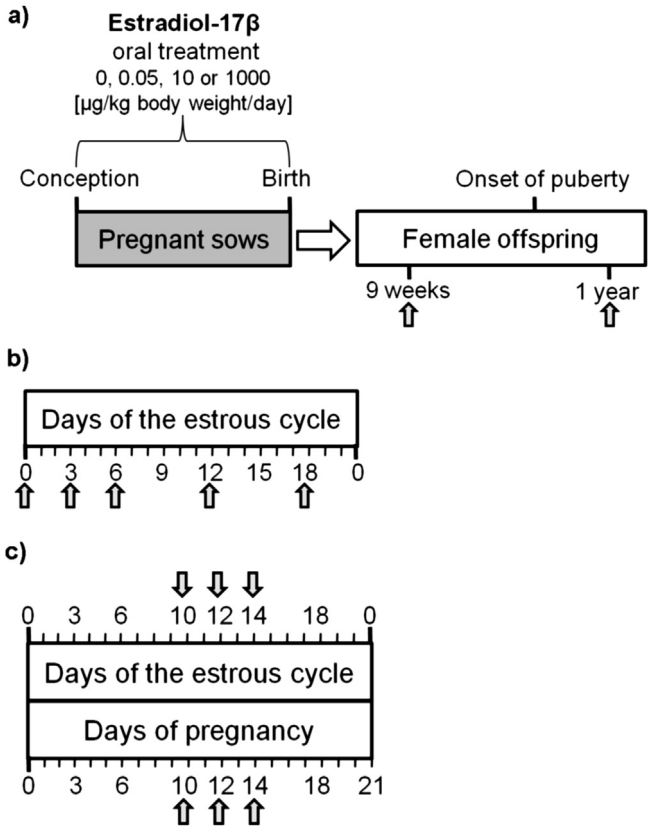

Fig. 1. Design of the 'E2 exposure study' (a), 'estrous cycle study' (b), and 'pre-implantation study' (c), respectively. Arrows (⇑) indicate sampling points and day 0 represents the estrus.

has been shown in humans, with highest abundance at the time of implantation during the mid-secretory phase [16]. Additionally, *HOXA10* expression increases during early pregnancy in the peri-implantation period in mice [15], pigs [17], canine [18], and bonnet monkey [19]. Some of the changes found in Hoxa10 knockout mice [14] and in mice with altered *Hoxa10* expression using gene transfection [20], can also be found in mice, rats and pigs treated with xenoestrogens. Mice and rats showed altered uterine morphology [21], fewer implantation sites [9], reduced pregnancy rates [22], and increased embryo mortality [23]. A reduced embryo survival was also demonstrated in pigs [12]. Furthermore, EDC exposure altered *HOXA10* expression directly after *in utero* [24,25] or early postnatal exposure [10,26], and in adulthood after developmental exposure [11,27], respectively. The pre-implantation phase in pigs has been shown to be a sensitive time phase during pregnancy with respect to disruption through exogenous estrogenic compounds, specifically prior to the naturally occurring alterations in estrogen concentrations [28–30]. These treatments have shown to induce embryonic losses.

In vitro studies using the human Ishikawa cell-line have depicted a mechanism of how estrogens bound to the estrogen receptor activated *HOXA10* expression through binding to estrogen response elements in the promoter region [31–33]. In addition, porcine and human primary endometrial cells treated with estradiol-17β (E2) have shown an increase in *HOXA10* expression [16,34]. Furthermore, *in vitro* studies of Bromer et al. [11] demonstrated that promoter DNA methylation inhibited binding of estrogen receptor alpha to the estrogen response element and prevented an E2 mediated increase of *Hoxa10* promoter activity. As shown in mice, this is a potential mechanism explaining developmental long-term consequences of early estrogen exposure [11].

Similar to endogenous hormones, EDC are able to exert effects at very low, environmental relevant doses and do not always act in a linear dose dependent manner [35]. It has been shown in rodents, pigs, sheep and humans that even low dose exposure to estrogenic substances can have lasting consequences [9,36–39]. In female ovine offspring, *in utero* exposure to methoxychlor and bisphenol A (BPA) impaired the timing and the amplitude of the prepubertal luteinizing hormone (LH) surge, respectively [38]. In addition, the hypothalamic mRNA expression of estrogen receptor alpha and beta were altered, possibly being related to changes in the LH surge [39]. Furthermore, early exposure to E2, also at low doses, affected postnatal development. Altered body composition after gestational oral supplementation in male porcine offspring [37], and behavioral alterations in female ovine offspring after gestational and lactational exposure [40] were demonstrated.

In the present study, distinct concentrations of E2, with two low doses representing the acceptable daily intake (ADI) and the no observed effect level (NOEL), as recommended for humans [41], as well as a high dose, were applied in a pig animal model. The high dose has already been shown to influence body composition in the male offspring [37]. In contrast to other studies using rodents, which is a common animal model, the pig was chosen due to its similarities with the human regarding placental estrogen synthesis [37,42]. We tested the hypothesis that *in utero* estrogen treatment affects uterine *HOXA10* expression and promoter DNA methylation in pre- and postpubertal female offspring. Additionally, to deepen the understanding of *HOXA10* expression in the pig and its regulatory mechanisms, we analyzed if promoter DNA methylation was involved in *HOXA10* expression. The endometrium during the estrous cycle and the pre-implantation period as well as several reproductive and non-reproductive tissues were under specific investigation.

2. Materials and methods

2.1. Animal trials and sampling

An 'E2 exposure study' (Fig. 1a) was conducted as described earlier [37]. In brief, German Landrace sows inseminated with Pietrain semen were fed distinct amounts of E2 during the whole length of gestation (n = 6–7/treatment). There were two low dose treatment groups corresponding to the ADI (0.05 µg/kg bw/d) and approximately the NOEL (10 µg/kg bw/d) as recommended for humans [41], and a high dose (1000 µg/kg bw/d) as well as a control group (ethanol carrier only) [37]. The E2 was dissolved in ethanol and half of the daily dose (in 2 ml ethanol) was fed manually within a bread roll (20 g) in the morning and evening, respectively, just before feeding the animals. Female offspring were slaughtered at 9 weeks of age (prepubertal, n = 12/treatment) and at about 1 year of age (postpubertal, n = 8–14/treatment), respectively. Adult female offspring had at least three estrous cycles after onset of puberty and were slaughtered during the luteal phase (day 10–13 post-estrus following frequent observation of estrus behavior). Samples of prepubertal uterus and postpubertal caudal endometrium were immediately shock-frozen in liquid nitrogen and stored at −80 °C. Blood samples collected at slaughter were stabilized with EDTA, and plasma was separated by centrifugation at 4 °C and stored at −20 °C until further analysis.

In a second study, the porcine endometrium was sampled during the estrous cycle ('estrous cycle study', Fig. 1b). Cross-bred prepubertal gilts of German Landrace and Pietrain were synchronized at the age of 6 month by rehousing in the morning of day −4 (with day 0 being the day of estrus), injection of 750 iU Intergonan (equine chorionic gonadotropin) at day −4, and injection of 750 iU Ovogest (human chorionic gonadotropin) at day −1. Gilts

were slaughtered at day 0, 3, 6, 12 and 18 ($n = 6\ day^{-1}$), respectively. Endometrial samples were taken from the cranial, intermediate and caudal part of the uterine horn. The samples were immediately placed in RNAlater (Ambion, Huntingdon, Cambridgeshire, UK) and incubated at 4 °C overnight, followed by the removal of RNAlater and storage at −80 °C until further analysis. Plasma samples were collected at slaughter as described above.

The third study targeted the pre-implantation period ('pre-implantation study', Fig. 1c) and was partially described earlier [43]. In brief, prepubertal German Landrace gilts were synchronized similar to the 'estrous cycle study' and inseminated at estrus either with Duroc semen or with seminal plasma from the same boar for the control groups to detect embryo-induced alterations. The gilts were slaughtered at day 10, 12, and 14 post-estrus ($n = 7–10\ day^{-1}$ and status), respectively. Endometrium and plasma was sampled as described above, and tissue samples were additionally taken from implantation sites at day 14 as described earlier [43]. Only pregnant animals where embryos were detected were considered for further analyses.

In a fourth 'tissue and development study', additional samples were collected from the control animals of the 'E2 exposure study' including uterus, cervix, kidney, vagina, muscle, thymus, breast tissue, ovary, ileum, oviduct isthmus, oviduct ampulla, adrenal gland, mesenterial lymphnode (mes. LN), lung, heart, and liver, from prepubertal animals (9 weeks old), as well as caudal endometrium, cranial endometrium, cranial cervix, oviduct isthmus, oviduct ampulla, ovary, corpus luteum (CL), heart, spleen, and liver, from postpubertal animals (1 year old). Samples were collected and treated as described above.

All animal treatments were approved by the local authorities (Ref# 55.2-1-54-2531-68-09; District Government of Upper Bavaria). All experiments were performed according to accepted standards of humane animal care.

2.2. Hormone analyses

Our own competitive enzyme immuno assays (EIA) were performed using plasma samples. Estradiol-17β (E2) was measured after ether extraction as described earlier [44]. The protocol of Blottner et al. was applied for quantification of testosterone (T) [45]. Progesterone (P4) was determined according to Prakash et al. [46]. All samples were quantified in duplicates. The lower detection limit for T, P4, and E2 was 0.02 ng/ml, 0.35 ng/ml, and 2.0 pg/ml, respectively, and all intra- and interassay CVs were <10%.

2.3. Extraction of RNA

Total RNA from the 'E2 exposure study' and the 'tissue and development study' was isolated using the NucleoSpin RNAII Kit (Macherey Nagel, Düren, Germany). Extraction was performed according to the manufacturer's protocol with minor changes. Namely, the amount of RA1 buffer was increased to 600 μl plus 6 μl β-mercaptoethanol, homogenization was achieved by means of Matrix-Green beads (MP Biomedicals, Illkirch, France) and a MagnaLyser (Roche, Basel, Switzerland). In order to adjust the binding conditions, 600 μl of 70% ethanol was added. In addition, the second washing step was performed twice to increase RNA purity. The samples were immediately put on ice and then stored at −80 °C. Total RNA extraction from endometrial tissues of the 'estrous cycle study' and the 'pre-implantation study' was performed by means of TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. RNA quality and quantity was determined using the NanoDrop 1000 (peqLab, Erlangen, Germany). For RNA integrity assessment the Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany) was applied with the RNA 6000 Nano Kit (Agilent) and handled according to the manufacturer's protocol. Only RNA samples with RIN numbers larger than 6 were used for the analyses to ascertain good performance in qRT-PCR [47,48]. The mean RIN value was 8.8 ± 0.9 (±SD).

2.4. Two step quantitative real time PCR

Reverse transcription of RNA samples was performed as described earlier [49], using the following cycling protocol: annealing with 20 min at 21 °C, elongation with 120 min at 48 °C, denaturation of the enzyme for 2 min at 90 °C. The resulting cDNA was stored at −20 °C. The CFX384™ Real-Time PCR Detection System (Bio-Rad, München, Germany) with the MESA Blue qPCR MasterMix Plus for SYBR® Assay No ROX (Eurogentec, Köln, Germany) was used to conduct real time qPCR measurements. The qPCR reaction was performed with a final volume of 10 μl, consisting of 5 μl MESA Blue MaserMix, 0.15 μl of forward (20 μM) and reverse primer (20 μM), respectively, as well as 0.7 μl RNase free water and 4 μl of 1:4 diluted cDNA. Nuclease free water instead of cDNA served as a negative control. In order to amplify specific gene fragments, the following primers were used (60 °C annealing temperature): HOXA10 (ref: AF281156; 120 bp product) (for 5′-AAAGAGCGGCCGGAAGAA-3′, rev 5′-ACGCTGCGGCTGATCTCTAG-3′ [34], histone (H3F3A; ref: BT020962; 233 bp product) (for 5′-ACTGGCTACAAAAGCCGCTC-3′, rev 5′-ACTTGCCTCCTGCAAAGCAC-3′), ubiquitin (UBK3; ref: Z18245, 198 bp product) (for 5′-AGATCCAGGATAAGGAAGGCAT-3′, rev 5′-GCTCCACCTCCAGGGTGAT-3′), tyrosine 3-monooxygenase (YWHAZ; ref: XM_001927228, 141 bp product) (for 5′- AGGCTGAGCGATATGATGAC-3′, rev 5′-GACCCTCCAAGATGACCTAC-3′). An amplified PCR product from each primer pair was sequenced to assure product identity. Subsequently, the specific melting point served to verify the product. The cycle of quantification (C_q) was calculated after baseline subtracted curve fitting using the single threshold method (Bio-Rad CFX Manager V1.5.534.0511 software). Relative quantification of the qPCR products was performed as recommended by Livak and Schmittgen [50]. Reference genes were selected using the NormFinder from the GenEx software (GenEx Pro Ver 4.3.4 software multiD Analyses AB, Gothenburg, Sweden). *HOXA10* C_q values were normalized (ΔC_q) using the geometric mean of the three reference genes (H3F3A, UBK3, YWHAZ) according to the BestKeeper method [51]. For graphical presentation, fold changes were calculated [50].

2.5. DNA extraction and bisulfite conversion

The extraction of DNA from tissues samples and the following bisulfite conversion were conducted as described earlier [52]. DNA purity and quantity were assessed using the NanoDrop 1000. Muscle and liver DNA were purified before conducting the bisulfite conversion using the genomic DNA Clean & Concentrator Kit (Zymo Research, Irvine, CA, USA), because the 260/230 ratio was below 1.6.

2.6. Combined methylation-sensitive high resolution melt (MS-HRM) and pyrosequencing

HOXA10 promoter DNA methylation analyses were performed using PCR amplification on bisulfite converted DNA with high resolution melt (HRM) followed by pyrosequencing as reported recently [53]. The HOXA10 sequence of interest was derived using the comparative genomic function with the genomic alignment tool at the ensemble website (http://www.ensembl.org/; genome assembly Sscrofa10.2). Here, different species were aligned to retrieve the sequence of interest, namely the homologous promoter region of porcine HOXA10, where DNA

methylation changes have been associated with alterations in *HOXA10* expression in humans [54] and rodents [11]. The HOXA10 primer sequences used for the MS-HRM analysis were 5′-GGTAGTTTTTGTAGTTTTTGGTTTTTGGGTTGAGGTA-3′ and 5′-Bio-ACCCTTTCTAACTAACATTTCTTATACAAAACATACT-3′ (58 °C annealing temperature). The primer sequence for the subsequent pyrosequencing reaction was 5′-GTTTAAGAAATTAAATTGGGAGT-3′. The MS-HRM primers are situated in the CpG island of the 5′ promoter region, located at −204 bp to −19 bp, with +1 bp being the transcription start site (TSS). The pyrosequencing analysis included all 10 CpG sites from −93 bp to −19 bp.

2.7. Transcription factor binding site and sequence analysis

The retrieval of transcription factor binding sites was performed by means of the MatInspector (Genomatix, Munich, Germany) software algorithm [55]. The sequence analysis was conducted using the ensemble genome browser (http://www.ensembl.org). The comparative genomics with the genomic alignment functions was conducted with the 12 eutherian mammals EPO alignment to assess the degree of conservation of the sequence that was used to conduct the DNA methylation analysis.

2.8. Statistical analyses

The SAS program package release 9.2 (2002; SAS Institute, Inc., Cary, NC, USA) was used for the statistical analyses. An ANOVA mixed model was applied in order to assess differences between groups. In the 'E2 exposure study', the Dunnett's *post hoc* test was carried out to evaluate the possible difference between treated and control groups. In the 'estrous cycle study', we first analyzed whether there were differences in *HOXA10* expression between the three uterine areas from which endometrial tissues were taken at each individual day of the estrous cycle. As no differences were apparent, the data were integrated into the mixed model as repeated measurements, considering them as tissue replicates. In order to compare all days of the estrous cycle that were under investigation, the Bonferroni *post hoc* test was selected. For the 'pre-implantation study', the measurements from the three uterine areas were also compared first, separated by the day and the status. No differences were found and thus, the data were integrated into the mixed model as repeated measurements, considering them as tissue replicates. Then, a mixed model was applied to analyze the effect of the day, separately for the control and pregnant animals, as well as to compare the status separately at each of the three days analyzed. The Bonferroni *post hoc* test was applied. Although major changes in transcriptional abundance were apparent in the 'tissue and development study', a statistical analysis was not performed due to the limited number of different animals under investigation ($n = 2$). The correlation analyses were conducted by computing the Pearson correlation coefficient with SAS, and the linear regression analysis was performed using SigmaPlot program package release 11.0 (SPSS, Chicago, IL, USA). All analyses using DNA methylation data were conducted for each single CpG site. Differences with a p-value of <0.05 were considered significant. Graphs were plotted using SigmaPlot. Data are shown as mean ± SE.

3. Results

3.1. E2 exposure study

Neither the data presented for the prepubertal animals by Fürst et al. [37] nor absolute and relative uterine weight (uterine weight/body weight) differed between all groups analyzed (Suppl. Table A1). In the postpubertal offspring, neither body weight, age at slaughter nor number of corpora lutea showed significant differences between the treatment groups and the control group (Suppl. Table A1). Furthermore, free plasma E2, T, and P4 were similar in all groups analyzed (Suppl. Table A1). The average concentration was 15.8 ± 0.8 pg E2/ml, 92.3 ± 6.0 pg T/ml, and 30.0 ± 1.6 ng P4/ml.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2013.09.006.

Although there was an overall significant treatment effect on uterine *HOXA10* mRNA expression in the prepubertal piglets ($p = 0.02$), neither group was significantly different from the control group (Fig. 2a). *HOXA10* promoter DNA methylation was generally low (2.1 ± 0.1% on average), and there were no treatment effects in the prepubertal piglets, except at CpG site 4 between the control group and the highest dose group ($p = 0.02$) (Fig. 2b). However, promoter DNA methylation at site 4 did not correlate with *HOXA10* mRNA expression (data not shown). In the postpubertal sows, *HOXA10* mRNA expression (Fig. 2c) and promoter DNA methylation (Fig. 2d) were unaffected.

Prepubertal piglets had significantly higher *HOXA10* mRNA expression compared to the postpubertal sows in all treatment groups ($p < 0.0001$) with a difference of 2.6-fold on average (Fig. 2a and c). Regarding promoter DNA methylation, the largest differences between pre- and postpubertal animals were observed at CpG site 3 with mean values in the prepubertal animals of 3.7 ± 0.3 and 9.7 ± 0.6 in the adult sows ($p < 0.0001$) (Fig. 2b and d).

3.2. Estrous cycle study

Plasma P4 concentration significantly changed throughout the estrous cycle ($p < 0.0001$). It was lowest at day 0 (0.3 ± 0.1 ng/ml) and highest at day 12 (31.2 ± 4.9 ng/ml). Intermediate levels were found at day 3 (7.9 ± 0.9 ng/ml), day 6 (13.4 ± 2.2 ng/ml) and at the end of the luteal phase, at day 18 (14.5 ± 6.10 ng/ml).

Endometrial *HOXA10* expression significantly varied during the estrous cycle ($p < 0.0001$) (Fig. 3a). The transcript abundance was highest at day 0, 2.5-fold lower at day 3 ($p < 0.0001$), and 1.5-fold higher again at day 6 ($p = 0.04$). The day of the cycle significantly affected *HOXA10* promoter DNA methylation only at CpG site 3 ($p = 0.04$) (Fig. 3b). However, this did not correlate with *HOXA10* expression (data not shown).

3.3. Pre-implantation study

The plasma concentrations of P4, as well as the number of corpora lutea (CL), did not vary between the control and the pregnant group at day 10, 12 and 14 post-estrus, respectively (Suppl. Table B1). However, day 10 control animals had significantly higher P4 concentrations compared to controls at day 12 ($p = 0.0171$). Analog to these findings, the number of CL was significantly higher in control animals at day 10 compared to day 12 ($p = 0.0438$). There was no significant difference in either P4 concentration or number of CL in the pregnant animals at days 10, 12 and 14 post-estrus, respectively. In addition, the number of CL significantly correlated with P4 concentration ($p < 0.0001$). In contrast, HOXA10 mRNA expression neither correlated with P4 concentration ($p = 0.1386$) nor with the number of CL ($p = 0.3786$).

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Early pregnant endometrial *HOXA10* transcript abundance was significantly affected by the day of the cycle ($p = 0.02$) (Fig. 3c), with day 12 exhibiting a lower expression compared to day 14 ($p = 0.02$). In the non-pregnant control animals, there was a significant overall effect of the day of the cycle on *HOXA10* expression ($p = 0.04$).

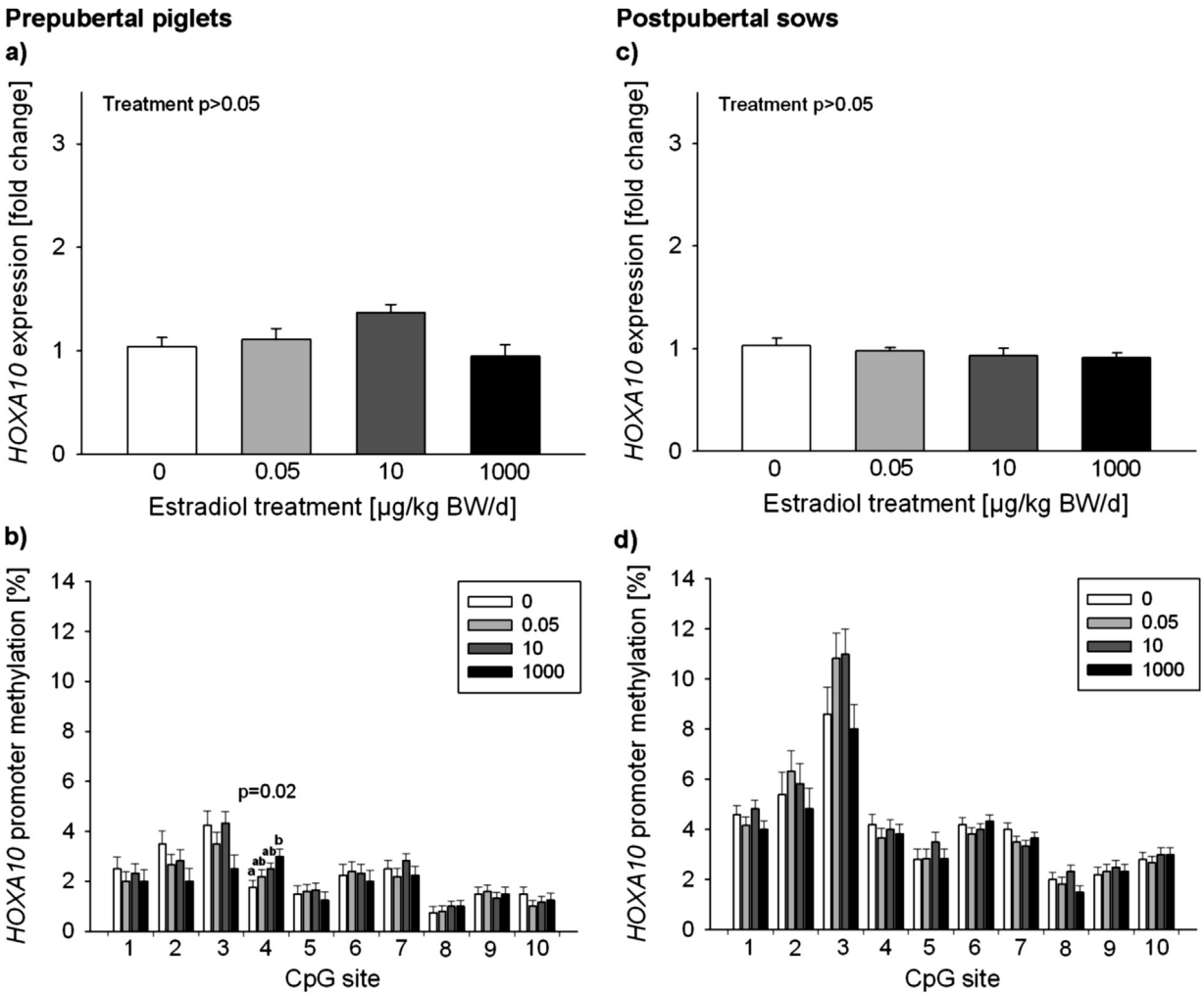

Fig. 2. *HOXA10* mRNA expression and promoter DNA methylation in offspring from E2 treated sows. *HOXA10* transcript abundance was unaltered compared to the control group in the uterus of prepubertal piglets ($n = 12$/group) (a) and in the endometrium of postpubertal sows ($n = 8$–14/group) (c), respectively. Similar results were found for promoter DNA methylation at almost all CpG sites analyzed in prepubertal piglets ($n = 4$–6/group) (b) and postpubertal sows ($n = 5$–6/group) (d), respectively. Values are depicted as mean ± SE. Different superscript letters indicate significant differences ($p < 0.05$).

The pair-wise comparison at each day revealed significantly higher transcript levels in the pregnant animals at day 14 ($p = 0.03$). This difference was even more pronounced at implantation sites (data not shown). The *HOXA10* promoter DNA methylation analysis did not reveal any changes at nine out of the ten CpG sites. There was a significant effect of the day of the cycle only at CpG site 2 in the non-pregnant animals ($p = 0.03$) (Fig. 3d). However, there was no significant correlation of promoter DNA methylation with mRNA expression (data not shown).

3.4. Tissue and development study

The uterus and endometrium of pre- (Fig. 4a) and postpubertal (Fig. 4b) pigs, respectively, showed a higher expression of HOXA10 compared to the other tissues. When comparing the highest with the lowest expressing organs in pre- and postpubertal animals, the differences in *HOXA10* expression were up to 20,000-fold and 6000-fold, respectively. Reproductive organs showed either high (uterus, cervix, oviduct isthmus) or low (oviduct ampulla) *HOXA10* transcript abundance. However, there were some alterations between the two developmental groups, *e.g.* *HOXA10* expression was lower in the isthmus but higher in the ovary of prepubertal compared to postpubertal animals. Similar findings were also observed regarding *HOXA10* promoter DNA methylation in prepubertal piglets (Fig. 4c) and postpubertal sows (Fig. 4d), such as higher methylation in the isthmus of the latter. Only CpG site 3 is shown in these figures, a graph containing all CpG sites can be found in the supplements (Suppl. Fig. A1).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2013.09.006.

A correlation analysis of *HOXA10* gene expression and promoter DNA methylation revealed a significant negative correlation at all 10 CpG sites in the prepubertal piglets, ranging from $p = 0.04$ to $p < 0.0001$ (Table 1). As this effect was strongest at CpG site 3, a linear regression was performed and is depicted in Fig. 4e and f for pre- and postpubertal animals, respectively. In contrast to prepubertal animals, no CpG site analyzed in postpubertal animals showed a significant correlation. In addition, if only taking into account the tissues that were analyzed in both developmental groups, the regression at site 3 in prepubertal animals was still significant ($p = 0.02$, $R^2 = 0.417$).

The analysis of the DNA sequence at CpG site 3 showed that it is part of the binding site for a transcription factor named “activator-, mediator- and TBP-dependent core promoter element for RNA polymerase II transcription from TATA-less promoters”. This site was found to be conserved between humans, pigs and many other mammals, however not in mice and rats (Suppl. Fig. B1).

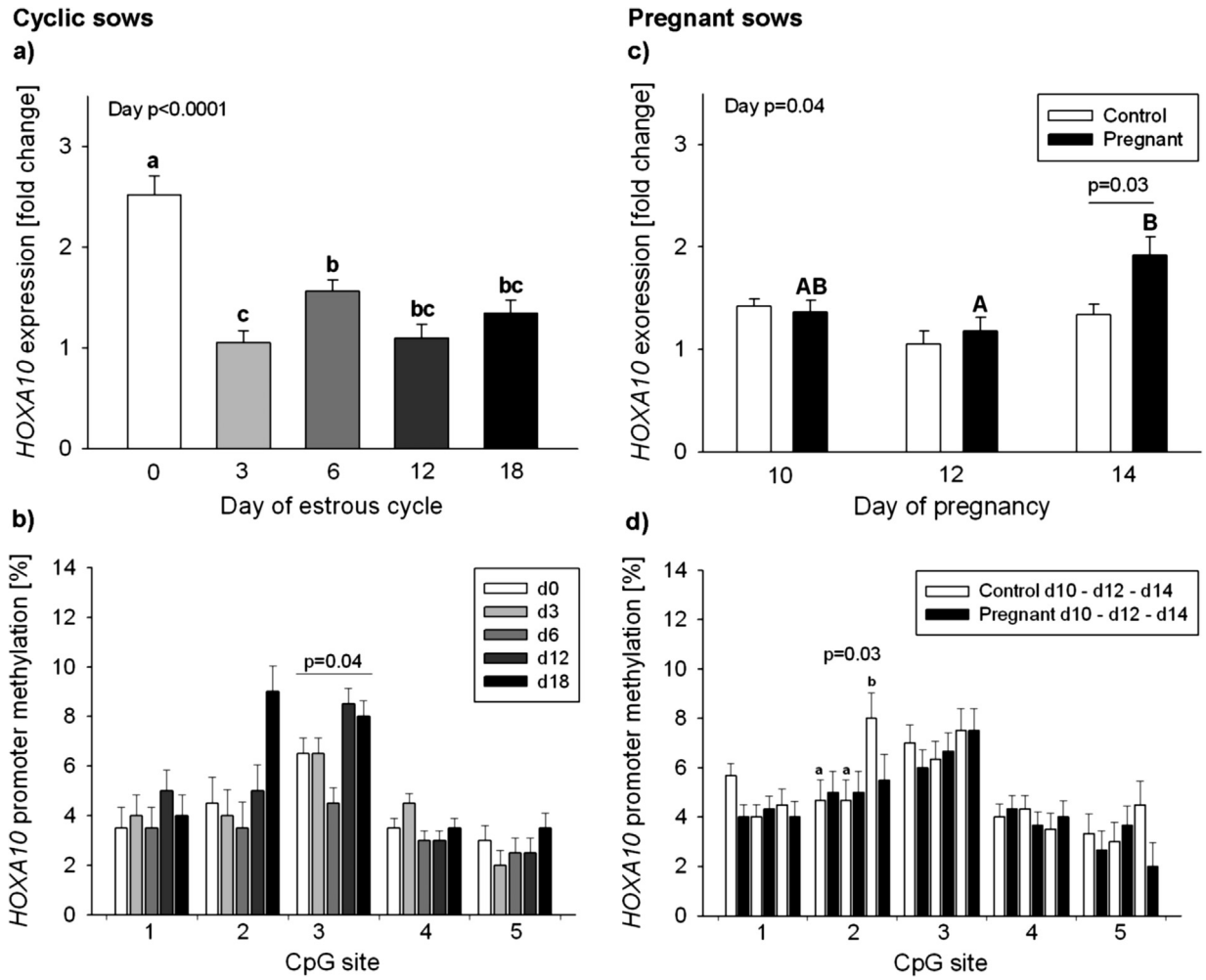

Fig. 3. Endometrial *HOXA10* mRNA expression and promoter DNA methylation in cyclic and early pregnant gilts. *HOXA10* transcript abundance was highest at estrus ($n = 6$/group) (a), and significantly different between early pregnant (black bars) and control (white bars) animals at day 14 ($n = 4$/group) (c). *HOXA10* promoter DNA methylation at CpG sites 1–5 is shown. It was similar between the time points analyzed except for CpG sites 3 and 2, respectively, in the mentioned groups [($n = 2$/group) (b) and ($n = 2$–3/group) (d), respectively]. Different superscript letters indicate significant differences between groups ($p < 0.05$). Control and pregnant animals were compared separately regarding effects of the day of the cycle.

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4. Discussion

Exogenous E2 possesses endocrine disrupting properties [28,29,37,40,56]. However, in contrast to our expectation, we did not find profound effects of orally administered E2 during pregnancy on *HOXA10* expression in neither pre- nor postpubertal offspring. Concerning *HOXA10* promoter DNA methylation, only one CpG site was significantly altered in the prepubertal uterus, but did not correlate with gene expression. This result is contradictory to previous studies, which may be due to the following reasons.

At first, reported effects of EDC may be substance- and dose specific. In contrast to the present study where E2 was applied, *Hoxa10* mRNA and protein expression changes were mainly demonstrated using BPA [9–11,32] or DES [24,25,57,58]. Only few studies have used other estrogenic substances such as methoxychlor [27] or estradiol valerate [26]. The named estrogenic substances are known to activate *HOXA10* mRNA and protein expression *in vitro* as shown in human Ishikawa cells [27,32]. However, *in vivo* during early development they may act in diverging ways. For instance in mice, DES was shown to exert a long-term increase in *Hoxa10* promoter DNA methylation, while BPA led to a decrease [11,58]. Long-term decrease of neonatal low-dose BPA exposure on *Hoxa10* gene expression has been demonstrated in rats [9]. As non-monotonic dose responses are well known to occur [35], a wide range of E2 doses were applied in the present study. These doses were

Table 1
Correlation analysis of HOXA10 mRNA expression with promoter DNA methylation at each single CpG site in the different tissues from prepubertal animals.

	HOXA10 [C_q]	CpG 1	CpG 2	CpG 3	CpG 4	CpG 5	CpG 6	CpG 7	CpG 8	CpG 9	CpG 10
HOXA10 [C_q]	1	0.481	0.540	0.724	0.609	0.579	0.630	0.530	0.403	0.385	0.444
p-Value		0.007	0.003	<0.0001	0.0004	0.0008	0.0002	0.003	0.03	0.04	0.02

The Pearsons correlation coefficient and the *p*-value of each CpG site are shown.

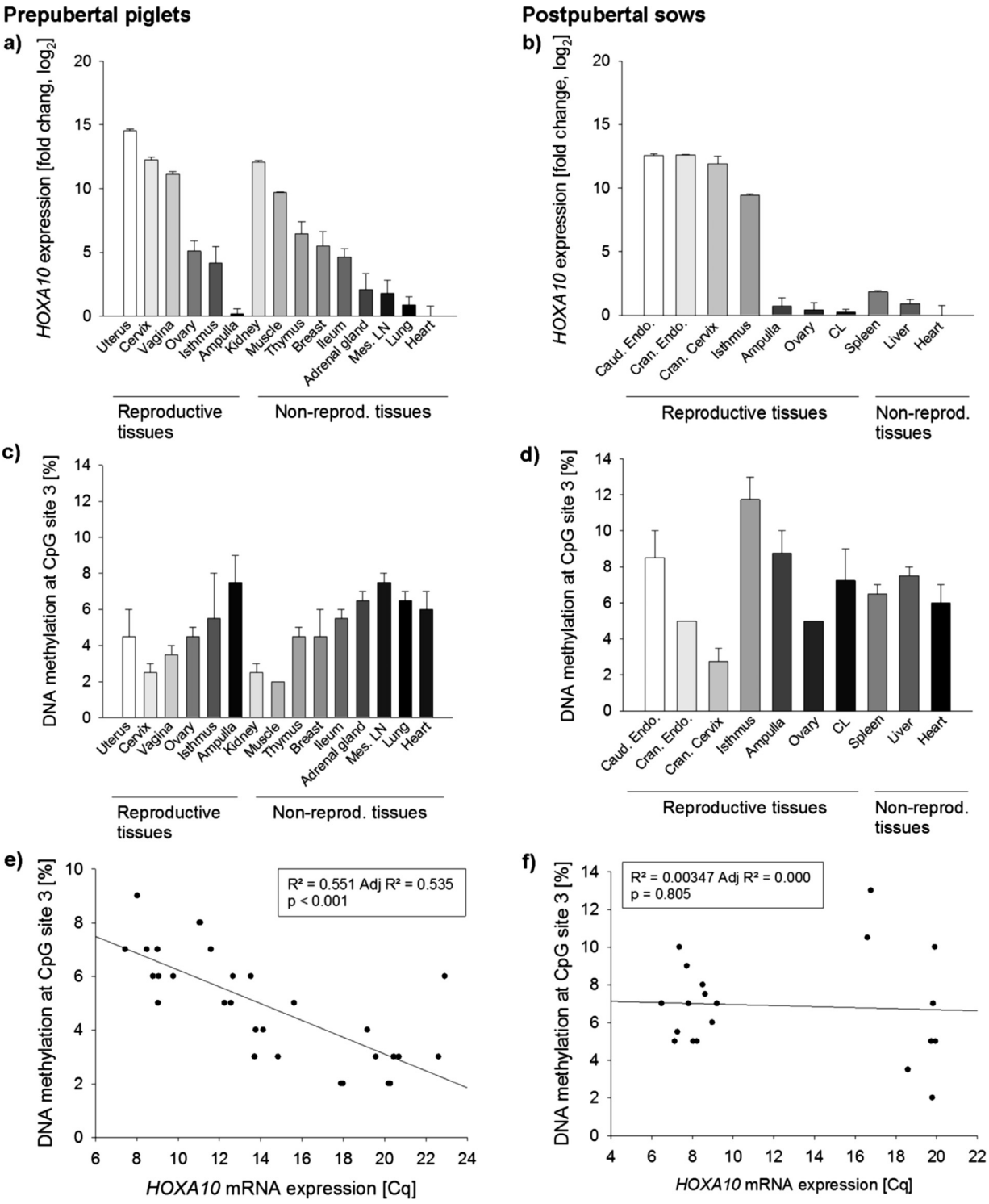

Fig. 4. Developmental *HOXA10* expression and promoter DNA methylation. *HOXA10* transcript abundance displays large differences within reproductive and non-reproductive tissues ($n = 2$/tissue) of prepubertal piglets (a) and postpubertal sows (b), respectively. The corresponding *HOXA10* promoter DNA methylation at CpG site 3 in the different tissues is depicted in (c) and (d), respectively, showing a much smaller extend of variation than the mRNA expression. The correlation of *HOXA10* expression and DNA methylation of all tissues analyzed at CpG site 3 is significant in prepubertal piglets (e) but not in postpubertal sows (f).

assumed to cover most of the possible effects of environmental E2 exposure. However, as none of the doses showed substantial effects, *in utero* application of E2 might generally not lead to changes in porcine *HOXA10* expression and promoter DNA methylation.

Second, it is well known that reported effects of EDC depend on the time of exposure and the time of target analysis. Regarding the former, Bromer et al. [11] showed that *in utero* BPA exposure led to a decrease in *Hoxa10* promoter DNA methylation in mice, while postnatal exposure of BPA in rats did not affect promoter DNA methylation [9]. Most of the porcine studies applying exogenous estrogens and focusing on uterine development have been performed in the context of the lactocrine hypothesis [7]. This hypothesis assumes that the early postnatal phase is a sensitive

window. Postnatally, uterine differentiation occurs under control of estrogen receptor alpha, but without endogenous ovarian estrogen synthesis. In respect of the time of target analysis, an increase of *HOXA10* expression was shown in piglets directly after neonatal exposure to estradiol valerate for 14 days [26]. However, a long-term effect on *HOXA10* expression in the adult animals was not observed. In the present study pre- and postpubertal female offspring were under investigation, but neither porcine *HOXA10* expression nor DNA methylation was substantially affected by the oral application of E2 during the entire length of pregnancy. Thus, the reported long-term effects on *HOXA10* may be due to specificities of sensitive windows of application and the time of target analysis.

This leads to the third possible reason, namely species-specific differences. Unlike in rodents, the pig is exposed to high endogenous concentrations of estrogens during the prenatal period due to the placental estrogen synthesis [37,59]. This as well holds true for women and substantiates the hypothesis that an *in utero* exposure to environmental relevant doses of estrogens is unlikely to be effective [60]. However, there are studies in humans indicating effects due to low dose exposures, such as during gestation through hormones from a twin [35]. In the present study, *in utero* estrogen exposure did not affect body weight in the female offspring, which is in contrast to results in mice [61,62]. Concerning *HOXA10*, differences as well as similarities between species appear. It seems to be conserved that uterine *HOXA10* expression increases during implantation [15,17–19]. This finding was further strengthened by our result that *HOXA10* expression was higher in pregnant sows compared to cyclic animals at day 14. However, *in situ* and *in vivo* studies indicate that estrogens are able to downregulate *Hoxa10* mRNA and protein expression in rodents [9,10], whereas they lead to an increase of *HOXA10* in pigs [26,34]. Furthermore, the murine *Hoxa10* promoter was shown to possess an average of 70% DNA methylation prepubertally [11]. We found the HOXA10 promoter to be very low methylated in all tissues analyzed, similar to adult humans and baboons [54,63,64]. Thus, species-specific peculiarities need to be taken into account when extrapolating epigenetic effects on *Hoxa10* from rodents to other species.

Next to developmental long-term consequences of early estrogen exposure, we analyzed *HOXA10* expression and DNA methylation in pre- and postpubertal animals as HOXA10 is known to be important for female reproductive health [15,65,66].

HOX genes are important conserved regulators [67]. However, as uterine development, morphology and function differ between species, the regulation of *HOX* genes may have equally undergone evolutionary changes. The role of *HOXA10* promoter DNA methylation regulating mRNA expression is particularly interesting, as two recent studies have indicated the possibility of cyclic changes in DNA methylation as an underlying regulatory mechanism of gene expression [68,69]. An inverse relationship between *HOXA10* expression and promoter DNA methylation has been observed in mice [11], baboons [63], and humans [54]. Thus, a more general possible involvement of DNA methylation causing transcriptional changes of *HOXA10* was studied in pigs. *HOXA10* mRNA abundance undergoes changes during the estrous cycle in human [16,70] and dogs [18]. We demonstrated here that *HOXA10* was significantly higher at estrus than at any other day analyzed, thus at the time where high E2 concentrations prevail [71]. In addition, *HOXA10* mRNA expression increased during early pregnancy, similar to what was shown earlier in several species [15,17–19]. Furthermore, we observed only few changes of promoter DNA methylation and no association with mRNA expression neither during the estrous cycle nor during early pregnancy in the pig. The observed changes in Hoxa10 expression in rodents after early estrogen exposure were found most pronounced in the subepithelial stroma [9,11], representing a large subset of cells. Concordant, HOXA10 protein is widely expressed in the uterine epithelial and stromal cells in the pig as well as in other species [9,11,63,72]. Thus, it may be assumed that changes in *HOXA10* promoter methylation would be detected in DNA from endometrial homogenates, and not only from specific cell types.

The regulation of tissue specific gene expression by DNA methylation is generally acknowledged [73,74]. The magnitude of difference in *HOXA10* expression in endometrial tissue during the estrous cycle was about 2-fold in the present study, which is similar to what has been observed in humans [16]. To study more enhanced expression differences, we analyzed different porcine tissues. To date, *HOXA10* expression has mainly been investigated in the uterus of several species, while also being found in some other tissues. Thus it seems that HOX-genes are not only essential transcription factors for tissue development, but are also of importance in differentiated organs. We found large differences in gene expression, and although HOXA10 promoter DNA methylation was low, there were still some changes from pre- to postpubertal stage. This is in accordance with the observation that DNA methylation still undergoes changes after birth and later in life, reflecting developmental changes in the composition of cell types and/or cellular maturation processes with changes in DNA methylation occurring [75–78]. Interestingly, there was a significant correlation of *HOXA10* gene expression and DNA methylation in prepubertal piglets. This effect was most pronounced at CpG 3, a site that was shown to be a conserved sequence in many mammalian species as well as a potential target for a transcriptional activator to bind. The idea that methylation at a single CpG sites influences transcriptional regulation was depicted by Tierney and colleagues as it abrogated transcription factor binding [79]. While Fürst et al. demonstrated that methylation at a single CpG site can be associated with tissue specific gene expression [53]. A similar association could not be depicted in the postpubertal animals, indicating potential differences in gene regulation during development compared to the postpubertal state. The large mRNA expression differences observed between multiple tissues during adulthood may be due to epigenetic or non-epigenetic mechanisms. For example, it may be related to different histone modifications [80] or to tissue specific expression of transcription factors and their co-factors.

In summary, neither *HOXA10* mRNA expression nor promoter DNA methylation was substantially affected by the *in utero* E2 exposure, independent of a low or high dose treatment. The analyses were performed in the uterus and endometrium of pre- and postpubertal porcine offspring, respectively. This finding is in contrast to published data in rodents where Hoxa10 has been demonstrated as a target gene of early EDC exposure regarding mRNA and protein expression, as well as promoter DNA methylation [9,11]. However, this result does not exclude other tissue-specific transcriptome and/or methylome effects in the offspring targeted by the E2 exposure. Rather, it emphasizes the well-known observation that effects of EDC are substance, dose, target, window of exposition and, most importantly, species-specific. Thus, it may point toward different effects on HOXA10 in species of high estrogen sensitivity, due to low endogenous estrogen concentration during pregnancy, such as in rodents [59]. Notably, this is neither the case in pigs nor in humans [37,60]. The absence of a pronounced association between *HOXA10* expression and DNA methylation highlights that DNA methylation at this genomic loci is not generally involved in *HOXA10* expression. However, *HOXA10* mRNA expression correlating to promoter DNA methylation in prepubertal piglets, but not in adult sows, is a peculiar finding that contributes to broadening the knowledge of developmental tissue specific gene regulation.

Conflict of interest

The authors state that there is no conflict of interest that would interfere with the impartiality of this scientific work.

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Appendix II

VL Flöter, G Galateanu, RW Fürst, D Seidlová-Wuttke, W Wuttke, E Möstl, TB Hildebrandt, SE Ulbrich „Sex-specific effects of low-dose gestational estradiol-17β exposure on bone development in porcine offspring", *Toxicology*. **2016**;366-367:60-7

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- Main contribution to execution of the animal trial
- Execution of experiments
- Analysis and interpretation of data
- Drafting of tables and figures
- Writing the original draft of the manuscript

Veronika Flöter

V. Flöter

Susanne E. Ulbrich

Susanne E. Ulbrich

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Sex-specific effects of low-dose gestational estradiol-17β exposure on bone development in porcine offspring

Veronika L. Flöter[a,b], Gabriela Galateanu[c], Rainer W. Fürst[a], Dana Seidlová-Wuttke[d], Wolfgang Wuttke[d], Erich Möstl[e], Thomas B. Hildebrandt[c], Susanne E. Ulbrich[a,*]

[a] Physiology Weihenstephan, Technische Universität München, Weihenstephaner Berg 3, 85354 Freising, Germany
[b] Z I E L PhD Graduate School 'Nutritional Adaptation and Epigenetic Mechanisms', Technische Universität München, Freising, Germany
[c] Department of Reproduction Management, Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Straße 17, 10315 Berlin, Germany
[d] Department of Endocrinology, University Medicine Goettingen, Robert-Koch-Str. 40, 37099 Goettingen, Germany
[e] Department of Biomedical Sciences, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria

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ABSTRACT

Estrogens are important for the bone development and health. Exposure to endocrine disrupting chemicals during the early development has been shown to affect the bone phenotype later in life. Several studies have been performed in rodents, while in larger animals that are important to bridge the gap to humans there is a paucity of data. To this end, the pig as large animal model was used in the present study to assess the influence of gestational estradiol-17β (E2) exposure on the bone development of the prepubertal and adult offspring. Two low doses (0.05 and 10 μg E2/kg body weight) referring to the 'acceptable daily intake' (ADI) and the 'no observed effect level' (NOEL) as stated for humans, and a high-dose (1000 μg E2/kg body weight), respectively, were fed to the sows every day from insemination until delivery. In the male prepubertal offspring, the ADI dose group had a lower strength strain index ($p = 0.002$) at the proximal tibia compared to controls, which was determined by peripheral quantitative computed tomography. Prepubertal females were not significantly affected. However, there was a higher cortical cross-sectional area (CSA) ($p = 0.03$) and total CSA ($p = 0.02$) at the femur midpoint in the adult female offspring of the NOEL dose group as measured by computed tomography. These effects were independent from plasma hormone concentrations (leptin, IGF1, estrogens), which remained unaltered. Overall, sex-specific effects on bone development and non-monotonic dose responses were observed. These results substantiate the high sensitivity of developing organisms to exogenous estrogens.

1. Introduction

Humans and animals are ubiquitously exposed to natural and synthetic substances with estrogenic activity potentially acting as endocrine disrupting chemicals (EDCs) on various organs and body systems (Diamanti-Kandarakis et al., 2009; McLachlan, 2001). According to the Developmental Origin of Health and Disease hypothesis (DOHaD), developmental plasticity allows early environmental changes to result in epigenetic adaptations possibly affecting the onset of diseases during adult life (Hochberg et al., 2011). In this regard, prenatal and early postnatal phases have been demonstrated as sensitive to exogenous influences. Developing organisms can strongly respond to even very low doses of estrogenic EDCs, as endogenous hormone levels are low while their receptors are already in place (Aksglaede et al., 2006; Barle et al., 2008; Knapczyk et al., 2008; McLachlan, 2001; Nilsson et al., 2002). Such effects have also been shown for natural substances such as estradiol-17β (E2) (Fürst et al., 2012; Rasier et al., 2006).

Consequently, studies analyzing early EDC exposure on bone development and metabolism, which have mainly been conducted in rodents, demonstrated various direct and/or lasting effects (Agas

Abbreviations: ADI, acceptable daily intake; BMD, bone mineral density; CT, computed tomography; CL, corpus luteum; CSA, cross-sectional area; DES, diethylstilbestrol; EDC, endocrine disrupting chemical; EIA, enzyme immunoassay; E2, estradiol-17β; ER, estrogen receptor; HU, Hounsfield units; NOEL, no observed effect level; pQCT, peripheral quantitative computed tomography; SSI, strength strain index.

* Corresponding author. Current address: ETH Zurich, Animal Physiology, Institute of Agricultural Sciences, Universitätstr. 2, 8092 Zurich, Switzerland.
E-mail addresses: pistek@wzw.tum.de (V.L. Flöter), galateanu@izw-berlin.de (G. Galateanu), rainer.fuerst@helmholtz-muenchen.de (R.W. Fürst), d.swuttke@verdevital.de (D. Seidlová-Wuttke), w.wuttke@verdevital.de (W. Wuttke), Erich.Moestl@vetmeduni.ac.at (E. Möstl), hildebrandt@izw-berlin.de (T.B. Hildebrandt), seu@ethz.ch (S.E. Ulbrich).

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et al., 2013). The outcome can depend on the time of exposure, the substance and dosage, the species or strain, the gender, the time of analysis, as well as the bone and bone area under investigation. For example, *in utero* and neonatal EDC exposure have often shown to result in an increased bone mineral density (BMD) in adult female rodent offspring (Kaludjerovic and Ward, 2008, 2009; Piekarz and Ward, 2007; Rowas et al., 2012), while the results are more dissimilar in large animals (Hermsen et al., 2008; Lundberg et al., 2006). Conflicting results have also been described regarding BMD in male offspring (Hermsen et al., 2008; Kaludjerovic and Ward, 2008; Lind et al., 2009; Piekarz and Ward, 2007; Rowas et al., 2012 Lind et al., 2009; Piekarz and Ward, 2007; Rowas et al., 2012). Furthermore, studies on female rodent offspring depicted an increase in the peak load (Kaludjerovic and Ward, 2008; Piekarz and Ward, 2007 Piekarz and Ward, 2007), whereas a reduction in bone strength parameters and an increased femur length have also been shown (Pelch et al., 2012). The latter study by Pelch and colleagues (Pelch et al., 2012) specifically used a low-dose diethylstilbestrol (DES) treatment, which was applied during gestation and lactation. In contrast, Migliacchio and colleagues (Migliaccio et al., 1996) showed that the same low-dose given only during gestation did not alter femur length and led to an increase in bone mass. Similar to the effects on the bone length, strength and density, alterations have also been described concerning several bone area parameters (Hermsen et al., 2008; Kaludjerovic and Ward, 2008; Lundberg et al., 2006; Pelch et al., 2012 Lundberg et al., 2006; Pelch et al., 2012; Rowas et al., 2012). In female rhesus monkeys, an increased total cross-sectional area (CSA) at the femur midpoint was demonstrated in a low-dose but not in a high-dose treatment group (Hermsen et al., 2008).

Lasting alterations in the bone phenotype may be due to direct influences of EDCs on bone cell number and activity (Agas et al., 2013; Chen et al., 2009; Hochberg et al., 2011; Javaid and Cooper, 2002). In addition, EDCs could indirectly affect the bone development through alterations of endocrine functions (Agas et al., 2013; Lundberg et al., 2006; Rasier et al., 2006), the onset of puberty (Bonjour and Chevalley, 2014; Rasier et al., 2006 Rasier et al., 2006) and/or body fat content (Csakvary et al., 2012; Fürst et al., 2012; Zhuo et al., 2014). One mechanism causing lasting changes is the alteration of epigenetic marks such as DNA methylation (Hochberg et al., 2011; McLachlan, 2001; Nilsson and Skinner, 2015). In line with this, steroid hormone receptor complexes are able to change histone modifications (Wierman, 2007).

In addition to rodents, the use of large animal models is important to transfer experimental results to humans. The pig has been used in bone research, since human bones are closely resembled (FDA, 1994; Litten-Brown et al., 2010; Pearce et al., 2007 Litten-Brown et al., 2010; Pearce et al., 2007). Consistently, the U.S. Food and Drug Administration (FDA) has recommended to use larger animals such as the pig next to ovariectomized rats as second species for preclinical drug evaluation for treating postmenopausal osteoporosis (FDA, 1994). However, there are only a few studies using large animals to analyze the influences of EDC exposure during gestation and/or neonatal on the bone development (Agas et al., 2013; Gutleb et al., 2010; Hermsen et al., 2008; Lind et al., 2009, 2010; Lundberg et al., 2006). The published results indicate some effects, although in parts deviating from data using rodents. A reason could be that – similar to humans – in large animal models higher estrogen concentrations prevail during pregnancy compared to rodents, due to placental estrogen synthesis, which is absent in the latter (Challis and Linzell, 1971; Lange et al., 2002; Robertson and King, 1974; Witorsch, 2002).

In this study, effects of gestational E2 exposure on the bone development and associated endocrine parameters were analyzed in porcine offspring. Since hormones can show non-monotonic dose responses with some effects specifically occurring at low doses (Vandenberg et al., 2012), the focus was laid on two low doses corresponding to the safety thresholds for humans—the acceptable daily intake (ADI) and the no observed effect level (NOEL) (JECFA, 1999). To our knowledge, this is the first study addressing early estrogen exposure on long term bone outcome in pigs.

2. Materials and methods

2.1. Animal experiment

The animal trial was conducted as described earlier (Fürst et al., 2012; Pistek et al., 2013). In brief, sows (n = 6–7/group) were orally exposed to E2 (1, 3, 5(10)-ESTRATRIEN-3, 17β-DIOL, Steraloids, Newport. USA) twice daily (0, 0.05, 10 and 1000 μg E2 per kg body weight (BW) per day (d), respectively) from insemination until delivery. At birth, no significant differences of the analyzed parameters, including the numbers of piglets, their weight and gender distribution, were detected (Fürst et al., 2012). Male and female offspring (n = 10–12 per group, overall n = 42 and 46, respectively) were slaughtered prepubertally at the age of 8 weeks (d 56) and 9 weeks (d 63), respectively. A second group of females (n = 7–13/group; overall n = 41) was kept until the age of about one year. Siblings were included in the experiments; on average two per sow. Adult boars were not assessed due to housing limitations. Starting when these gilts were 23 weeks old, a fresh rectal feces sample was taken each week to detect the first corpus luteum (CL) formation as a marker of puberty. These samples were immediately put on ice and stored at −20 °C. Prior to slaughtering, estrous cycle behavior was monitored at least once a day and the animals were slaughtered during the luteal phase (d 10 to d 13 post estrus) after at least three estrous cycles.

The femur and tibia from the right hind leg of all animals were stored at −20 °C, after they were separated from most of the surrounding tissue. Plasma was obtained from EDTA (AppliChem, Darmstadt, Germany) supplemented blood after centrifugation at 4 °C and was then stored at −20 °C.

The animal trial was approved by the District Government of Upper Bavaria and performed in accordance with accepted standards of humane animal care.

2.2. Bone measurements

Both the femur and the tibia of the prepubertal male and female offspring were analyzed using peripheral quantitative computed tomography (pQCT, STRATEC XCT 2000 (SA); Stratec, Pforzheim, Germany). The remaining flesh was removed from thawed bones and both the tibia and the femur were separated. Subsequently, pQCT measurements were taken at three bone areas, namely directly below the epiphyseal plate at the proximal tibia and the distal femur, as well as directly above the epiphyseal plate at the proximal femur. By means of a coronal computed radiography (scout view) the scanner was positioned at the site of measurement where three consecutive slices with 1 mm thickness were scanned. Further processing of the data was performed using the software version 5.40 with contour mode 1 and peel mode 2. A lower threshold of 280 mg/cm^3 and an upper threshold of 400 mg/cm^3 were set for the detection of trabecular bone and in order to separate it from the cortical/subcortical region. The threshold for the strength strain index (SSI) was set to 380 mg/cm^3. A voxel size of 0.200 mm was used. Thus, total and trabecular BMD and CSA, as well as the polar SSI were obtained. Cortical bone was still scarce at the time of analysis and was therefore not analyzed. In addition,

the length of the femur and tibia were measured using an electronic caliper.

In the one year old female offspring, the pQCT could not be used due to size limitations. Thus, computed tomographic data was acquired from all bone specimens using a whole body high-resolution, 128-slice CT scanner (Aquilion CX, Toshiba Medical Systems Cooperation, Tochigi, Japan). The settings for the CT helical scans were: 100 kVp; 300 mA; 1.0 s rotation time; field-of-view (D-FOV) extra-large (LL); pitch factor 0.64; all scans were performed at constant table height; soft tissue reconstruction kernel (FC 03); 0.5 mm scan slice thickness. The reconstruction algorithm "Body-standard Protocol" was used with 0.5/0.25 mm slice thickness/slice interval for three-dimensional (3D) imaging and two dimensional (2D) measurements. A dedicated multi-software workstation (ViTREA Version 6.2 medical diagnostic software, Vital Images Inc., Minnetonka, USA) provided a wide variety of clinical viewing protocols for 2D and 3D processing and analysis of the CT images.

Imaging analysis comprised multiple consecutive steps:

1) Each femuro-tibial specimen ("bone specimen") was identified and virtually isolated using both "Trim" and "Sculpt and Exclude" functions to avoid interference of other specimens in the same CT image during measurements.
2) Tri-dimensional (3D) rendering was performed for each bone using the same 3D protocol ("Bone CT, Window/Level Bone: 3500/400"). Four 3D projections were then generated: antero-posterior (AP), postero-anterior (PA), medio-lateral (ML), and latero-medial (LM). These 3D images were produced to show the next femoral aspects: anterior, posterior, medial and lateral for further identification, if necessary, and for the morphologic and pathologic, if any, assessment.
3) Sectional planes. Oblique multi-planar reconstructions (MPRs) were established at a constant W/L: 1300/325, as follows: (i) the mid-sagittal plane passes through the femoral head and greater trochanter, proximally, through the dorsal vascular nutrient foramen and intercondylar (lateral and medial femoral condyles), distally, (ii) the mid-coronal plane is established parallel to the long axis of the femur passing through the caudal part of the femoral head proximally and the intercondylar groove, distally. Consequently, the axial plane was perpendicular to the femoral long axis. These planes were carefully checked both in 3D and MPR images, for accuracy.
4) Femoral length. In order to determine the femoral length, MPR images were generated for each bone specimen by means of the "Oblique 100 mm maximum intensity projection (MIP) Segmented" algorithm, (W/L: 1300/325). Femoral length was measured as the distance between two horizontal lines drawn at the femoral extremities. These parameters were gauged in two orthogonal projections (coronal and sagittal planes).
5) Femoral mid-shaft distance. Half of the femoral length was established as the true mid-diaphysis point and the axial plane was positioned at this level.
6) Mid-femoral diaphysis CSAs. Cortical, medular and total CSA were quantified morphometrically (mm^2) at exactly the mid-shaft femoral diaphysis (named femur midpoint) (Kaludjerovic and Ward, 2008). The external and internal bone cortical contours were traced manually (MIP projections, oblique MPRs, mid-axial images, 0.5 mm slice thickness; W/L: 1300/325) and the areas of the respective surfaces were calculated automatically by the ViTREA's software. Consequently, total femoral CSA and medular CSA were measured while cortical CSA was calculated by subtracting medular CSA from total CSA.
7) Volumetric measurements. Femoral volume (mL) and femoral mean CT number, in Hounsfield units (HU), were automatically generated at a slice thickness of 0.5 mm by the Vital Images 6.2 Software ("Muscoskeletal CT, 3D Analysis, Segment Anatomy, Bone" Protocol). Three consecutive measurements of the femoral volume (V1, V2, V3) were performed and recorded; subsequently the mean femoral volume was calculated.

2.3. Hormone measurements

Plasma concentrations of insulin-like growth factor 1 (IGF1) and leptin were measured using competitive enzyme immunoassays (EIA) (Velazquez et al., 2011). Total estrogens (estrone, E2 and estradiol-17α) were determined by EIA as described earlier (Meyer et al., 1990). This value reflects the sum of E2 and estrone, as the amounts of estradiol-17α are negligible in the pig (Robertson and King, 1974). Extraction of estrogens from plasma was performed according to Hageleit et al., 2000 (Hageleit et al., 2000) with slight modifications. In brief, 0.5 ml plasma and 6 ml *tert*-butylmethylether/petrolether 30/70 v/v (AppliChem) were agitated for two hours and were then allowed to stand for 0.5 h. After being stored over night at −60 °C, the supernatant was decanted and the residues were dried in a speedvac and then dissolved in 500 μl assay buffer.

The antibody for measuring immunoreactive progesterone metabolites in feces was raised in rabbits against 5α-Pregnan-3ß-ol-20-on hemisuccinate: bovine serum albumin (BSA; Serva, Amstetten, Austria). Cross-reactivities of the assay are shown in Supplementary Table A1. EIAs were performed as described earlier (Schwarzenberger et al., 1996) with minor modifications. Five ml of 80% methanol (J.T. Baker, VWR, Ismaning, Germany) was added to 0.5 g wet feces, mixed for 10 min and centrifuged at 4 °C at 2340 × *g* for 15 min. 10 μl of the supernatant were added to 1.99 ml assay buffer (1:200 dilution). Microtiter plates coated with own goat-anti-rabbit IgG and saturated with 0.1% BSA were used. After two washing steps using cold Tween 20 (Sigma Aldrich, Munich, Germany), 50 μl from the prepared samples were added to each well, as well as 0.25 pg–250 pg per well of progesterone standard (Sigma Aldrich). Next, the biotinylated label (1:2 Mio), and the antibody (1:200 000) were added and incubated overnight on a rocking plate at 4–6 °C. After four washing steps, streptavidin-peroxidase reaction (1:20,000) was performed at 4–8 °C for 45 min. After another four washing steps the substrate was added and incubated for 45 min at 4–8 °C. The enzymatic reaction was then stopped and the optical density was measured.

2.4. Statistics

The statistical evaluation was performed using SAS 9.2 (SAS Institute, Inc.,Cary, NC, USA). The mean values of the three subsequent slices from the pQCT data were used, as well as the mean values of CT numbers (Hounsfield units) and femural volume. In order to account for the use of siblings, a random intercept model was applied using the mixed procedure with the repeated measurement function as described by Kiernan and colleagues (Kiernan et al., 2012). The denominator degrees of freedom were calculated using the residual method (Bell et al., 2013). Differences between groups were assessed using the Dunnett's post hoc test. In case of data being not normally distributed, this data were logarithmized for the statistical analysis. Correlation analyses were conducted with the corr procedure in SAS. For linear regression analyses and graphical presentation SigmaPlot 11.0 (SPSS, Chicago, IL, USA) was applied. Data are displayed as mean ± SE. Statistical significant differences were assumed with p-values < 0.05.

Table 1
Bone length, BMD and CSA data of the male prepubertal offspring.

Treatment [μg/kg BW/d]	Control (0)		ADI (0.05)		NOEL (10)		High-dose (1000)		Overall p-Value
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
Femur length (mm)	104.7	2.1	101.0	1.9	104.1	2.0	98.5	1.9	0.115
Tibia length (mm)	105.7	3.5	103.0	2.4	106.4	2.3	101.4	2.3	0.455
Distal femur									
Total BMD (mg/cm^3)	297.8	7.1	281.2	7.0	287.9	7.1	282.1	7.0	0.311
Trabecular BMD (mg/cm^3)	282.9	6.2	268.7	6.1	275.7	6.2	271.3	6.1	0.428
Total CSA (mm^2)	731.3	29.2	696.9	27.5	745.3	29.2	651.4	27.5	0.105
Trabecular CSA (mm^2)	638.4	26.7	614.6	25.0	660.5	26.7	585.0	25.0	0.210
Proximal tibia									
Total BMD (mg/cm^3)	280.5	5.8	263.9	5.4	270.6	5.6	271.7	5.4	0.243
Trabecular BMD (mg/cm^3)	277.7	5.7	262.0	5.4	267.9	5.5	269.0	5.4	0.276
Total CSA (mm^2)	587.0	27.6	544.9	25.0	586.2	26.2	523.4	25.0	0.234
Trabecular CSA (mm^2)	531.9	28.8	488.0	26.2	525.6	27.4	474.9	26.2	0.388

ADI = acceptable daily intake. BMD = bone mineral density. CSA = cross-sectional area. NOEL = no observed effect level. SE = standard error.

3. Results

3.1. Bone parameters

3.1.1. Prepubertal male offspring

Bone length, BMD and CSA measurements at the distal femur and the proximal tibia of the prepubertal male animals (n = 10–11/group) are depicted in Table 1, and for the proximal femur in Supplementary Table A2. There were no significant alterations concerning these parameters.

The SSI was significantly different in male prepubertal offspring at the proximal tibia (96.3 ± 11.6 mm^3, 45.0 ± 10.5 mm^3; 71.9 ± 11.0 mm^3; 55.1 ± 10.5 mm^3; p = 0.008). The animals of the ADI dose group depicted a lower SSI compared to the control group (p = 0.002) as shown in Fig. 1. Although depicting a similar pattern, the SSI was not significantly affected at the distal femur (243.8 ± 32.8 mm^3, 169.9 ± 32.3 mm^3, 208.0 ± 32.8 mm^3, 137.3 ± 32.3 mm^3; p = 0.129; Fig. 1).

3.1.2. Prepubertal female offspring

Female prepubertal offspring (n = 11–12/group) did not display significant differences in bone parameters (Table 2 – distal femur and proximal tibia; Supplementary Table A2 – proximal femur). Still, there was a tendency of a lower total BMD (p = 0.060) and trabecular BMD (p = 0.080) at the distal femur, which although less pronounced was also observable at the proximal tibia. A similar pattern was observed for the SSI at the distal femur (484.2 ± 57.6 mm^3, 370.6 ± 55.0 mm^3, 362.4 ± 55.1 mm^3, 283.8 ± 57.6 mm^3; p = 0.121) and the proximal tibia (226.9 ± 35.6 mm^3, 118.3 ± 35.8 mm^3, 145.1 ± 34.2 mm^3, 100.7 ± 35.6 mm^3; p = 0.123) that are shown in Fig. 1.

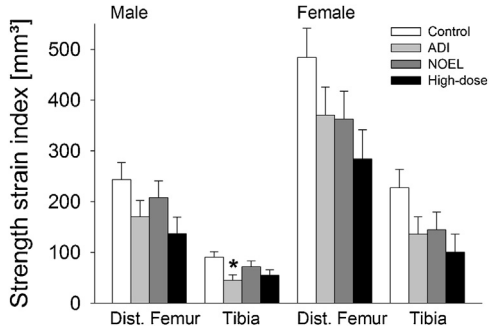

Fig. 1. Strength strain index (SSI) in prepubertal offspring after *in utero* E2 exposure. Results in male (n = 10–11/group) and female (n = 11–12/group) offspring at the distal femur and proximal tibia are depicted. The oral E2 doses during gestation had been 0 (Control), 0.05 (ADI), 10 (NOEL) and 1000 (high-dose) μg E2/kg BW/d, respectively. The SSI was lower in the ADI dose group compared to the control group in the male offspring. Values are depicted as mean ± SE. Asterisks indicate significant differences (p < 0.05).

3.1.3. Adult female offspring

Total CSA (683.1 ± 18.8 mm^2, 709.8 ± 17.8 mm^2, 770.5 ± 23.5 mm^2, 736.9 ± 13.9 mm^2; p = 0.03) and cortical CSA (444.6 ± 14.0 mm^2, 447.6 ± 13.3 mm^2, 503.1 ± 17.1 mm^2, 467.0 ± 11.3 mm^2; p < 0.05) measured at the femural midpoint were significantly affected by the treatment in the adult female offspring (Fig. 2). The NOEL dose group revealed a significantly larger total CSA (p = 0.02) and cortical CSA (p = 0.03), whereas the medular CSA was unaffected (242.1 ± 13.5 mm^2, 262.5 ± 12.9 mm^2, 267.0 ± 16.1 mm^2, 270.5 ± 11.8 mm^2; p = 0.44). There were no significant differences regarding femur length, volume and CT number (Table 3). In addition, cortical CSA was not correlated with plasma hormone concentrations (Table 4).

3.2. Hormone concentrations

There were no significant differences in plasma concentrations of IGF1 and leptin between the treatment groups and the control group in neither male nor female prepubertal offspring (Table 5).

Similar results were found for the adult female offspring (Table 5), with no significant differences between the treatment groups regarding plasma IGF1, leptin and total estrogens (reflecting estrone and E2 concentrations). Although not statistically significant, the high-dose group had the first CL formation on average three weeks later than the control animals (32.1 ± 1.3 weeks, 31.6 ± 1.3 weeks, 33.6 ± 1.6 weeks, 35.4 ± 1.3 weeks; p = 0.180; Fig. 3).

4. Discussion

In this study, we analyzed the effect of gestational oral E2 exposure on specific bone parameters and potentially related endocrine parameters in the offspring. While no significant alterations were detected in the high-dose group, a low-dose effect was demonstrated in male piglets as well as in female sows.

Studies in male offspring after early EDC exposure have shown certain alterations in bone geometry and density (Kaludjerovic and Ward, 2008; Lind et al., 2009; Rowas et al., 2012 Lind et al., 2009; Rowas et al., 2012) as well as in bone strength and/or respective surrogate parameters (Hermsen et al., 2008; Lind et al., 2009; Pelch et al., 2012). In male rhesus monkeys, an early 2,3,7,8-tetrachlorodibenzop-dioxin (TCDD) treatment led to a change of two biomechanical parameters in the low-dose group potentially indicating a more fragile bone (Hermsen et al., 2008). Male mice

Table 2
Bone length, BMD and CSA data of the female prepubertal offspring.

Treatment [μg/kg BW/d]	Control (0)		ADI (0.05)		NOEL (10)		High-dose (1000)		Overall p-Value
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
Femur length (mm)	110.7	1.8	111.1	1.7	113.5	1.7	113.2	1.8	0.568
Tibia length (mm)	114.3	1.8	112.9	2.0	115.1	1.8	114.4	2.0	0.860
Distal femur									
Total BMD (mg/cm^3)	320.4	7.5	303.3	7.1	302.7	7.2	290.6	7.5	0.060
Trabecular BMD (mg/cm^3)	291.9	5.1	281.6	4.7	284.3	4.8	272.9	5.1	0.080
Total CSA (mm^2)	826.4	30.3	830.2	29.6	848.4	29.3	832.0	30.3	0.955
Trabecular CSA (mm^2)	662.2	30.2	697.2	29.4	724.9	29.1	719.4	30.2	0.448
Proximal tibia									
Total BMD (mg/cm^3)	288.7	6.7	275.5	6.8	281.6	6.5	268.5	6.7	0.194
Trabecular BMD (mg/cm^3)	282.1	6.0	271.7	6.0	277.8	5.8	264.9	6.0	0.207
Total CSA (mm^2)	695.1	26.4	695.4	26.7	699.7	25.6	665.8	26.4	0.788
Trabecular CSA (mm^2)	623.3	27.1	636.9	27.5	638.5	26.4	604.0	27.1	0.789

ADI = acceptable daily intake. BMD = bone mineral density. CSA = cross-sectional area. NOEL = no observed effect level. SE = standard error.

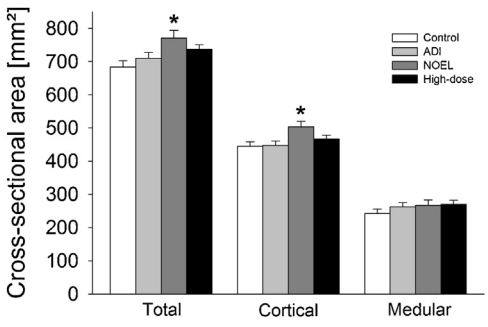

Fig. 2. Cross-sectional area (CSA) in adult female offspring after *in utero* E2 exposure.
The CSA was measured at the femur midpoint in the adult female offspring (n = 7–13/group). The oral E2 doses during gestation had been 0 (Control), 0.05 (ADI), 10 (NOEL) and 1000 (high-dose) μg E2/kg BW/d, respectively. Cortical and total CSA were significantly higher ($p < 0.05$) in the NOEL dose group compared to controls. Values are depicted as mean ± SE. Asterisks indicate significant differences.

receiving DES during early development depicted a reduction in bone strength parameters when torsional force was applied (Pelch et al., 2012). In addition, the polar moment of inertia, a surrogate parameter of the resistance to torsion, was reduced in rams exposed to a mixture of EDC (Lind et al., 2009). Overall, weaker bones were repeatedly reported in male offspring after early EDC exposure. Although in contrast to the studies above mentioned we analyzed bones of prepubertal males at the epiphysis. We found a reduction in the polar SSI as surrogate parameter of fracture strength in torsion. This result similarly suggests a negative effect on bone strength. It was not only found in the ADI dose group at the proximal tibia, but also as a similar trend of a non-monotonic dose response at the proximal and distal femur, depicting lowest values in the ADI and high-dose group, respectively. Therefore, the observed effect seems to occur more globally in these animals.

At prepuberty there was no significant change in any of the analyzed parameters in the female piglets. In contrast to the prepubertal female piglets, significant bone alterations were found in the adult offspring. This is in accordance to other studies analyzing the long-term effects from early EDC exposures (Hermsen et al., 2008; Kaludjerovic and Ward, 2008, 2009; Lind et al., 2009; Lundberg et al., 2006; Migliaccio et al., 1996; Pelch et al., 2012; Piekarz and Ward, 2007; Rowas et al., 2012). It may be explained by the large changes occurring during the pubertal growth phase in bones. Wuttke and colleagues (Seidlová-Wuttke et al., 2008) demonstrated that a life-long phytoestrogen exposure compared to a phytoestrogen free diet affected specific bone parameters differently depending on the time of the analysis. They found a higher trabecular BMD at prepuberty, no difference during puberty, a reduction during adulthood, and again a higher density in the aged rats.

In general, hormonal substances can exert low-dose effects and non-monotonic dose response on exposed animals and humans (Vandenberg et al., 2012). This has even been demonstrated for low concentrations of endogenous hormones, such as steroid concentrations depending on the intrauterine positioning in terms of the sex of the neighboring sibling(s). Studies using different EDCs early during development have depicted effects on specific bone parameters particularly occurring at low doses, while the underlying mechanisms are still unknown (Hermsen et al., 2008; Pelch et al., 2012; Rowas et al., 2012). Similarly, in the adult female offspring we found an increase in the total and cortical CSA in the NOEL dose group. All other parameters that have been analyzed earlier, including plasma steroid hormone concentrations, were not significantly altered in these animals (Fürst et al., 2012; Pistek et al., 2013). As additionally neither cortical nor total CSA correlated with plasma hormone concentrations, a potential underlying mechanism of the maternal treatment on the cortical and total CSA could involve direct effects on bone cells. The larger cortical CSA at the femur midpoint accounted for the major part of the increase found in the total CSA. It has been demonstrated that ERβ knockout-mice depict a gender specific phenotype (Callewaert et al., 2010; Windahl et al., 1999). While it had no effect on male mice, cortical bone growth and thus CSA was increased in female mice. Although speculative, as signalling through ERβ limits female cortical bone growth (Callewaert et al., 2010), the

Table 3
Femur length, volume and CT number of the female adult offspring.

Treatment [μg/kg BW/d]	Control (0)		ADI (0.05)		NOEL (10)		High-dose (1000)		Overall p-Value
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
Femur length (mm)	237.7	2.5	245.8	2.4	244.5	3.0	242.9	2.4	0.13
Mean femur volume (ml)	441.4	11.2	472.3	10.8	473.3	13.4	449.3	10.2	0.13
Mean CT number (HU)	360.1	17.1	368.6	16.5	407.0	20.3	374.8	15.9	0.35

HU = Hounsfield units. SE = standard error.

Table 4
Correlations of mid-femoral CSA values with hormone concentrations of the adult female offspring.

	E2	Total estrogens	T	P4	IGF1	Leptin
Medular CSA						
R	0.24	0.05	0.20	0.18	−0.01	0.13
p-value	0.125	0.774	0.216	0.260	0.930	0.432
Cortical CSA						
R	0.11	0.09	0.01	−0.08	0.15	0.15
p-value	0.498	0.576	0.933	0.620	0.354	0.362
Total CSA						
R	0.22	0.09	0.12	0.05	0.10	0.18
p-value	0.173	0.577	0.440	0.763	0.552	0.273

CSA = cross-sectional area. E2 = estradiol-17β. IGF1 = insulin-like growth factor 1. P4 = progesterone. T = testosterone. R = Pearsons correlation coefficient. Significance was assumed with $p < 0.05$.

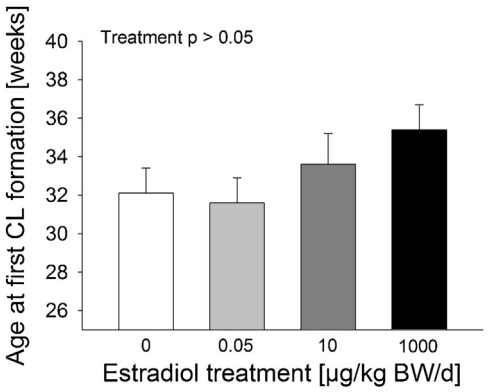

Fig. 3. Age at the first corpus luteum (CL) formation after *in utero* E2 exposure. The overall mean age of this first estrous cycle was 33.3 weeks in the female offspring. Although not significantly different ($p > 0.05$), the high-dose group had the first estrous cycle on average three weeks later than the control animals. Values are depicted as mean ± SE.

expression of ERβ might be reduced in the offspring on the NOEL dose group through promoter DNA methylation. This mechanism has been observed in adult rodent ovary after perinatal EDC treatment (Zama and Uzumcu, 2009) and might as well account for the observations made here.

Similar to the data collected from adult large animals (Hermsen et al., 2008; Lundberg et al., 2006), most alterations in exposed fetal sheep concerned diaphyseal points of measurement (Gutleb et al., 2010). Gestational and lactational exposure of goats to polychlorinated biphenyl (PCB) 153 led to a lower total CSA and marrow cavity area at the diaphysis at 18% of the total bone length, while the mid-diaphyseal part was unaffected (Lundberg et al., 2006). In contrast, after gestational and lactational exposure of rhesus monkeys to TCDD, there was a higher total CSA at the mid-diaphysis in the low-dose group but not in the high-dose group revealing an inverted U-shaped dose response (Hermsen et al., 2008). The result of the latter is similar to the presented data in the adult female offspring, where the group receiving a NOEL dose was affected. Hermsen and colleagues (Hermsen et al., 2008) additionally performed biomechanical analyses at the mid-diaphysis of the right femur by using a three-point bending test. They did not observe changes through this radial load application. In contrast, theoretically, if compression would be applied, bones with a larger CSA would be more certainly stiffer and stronger (Macintyre and Lorbergs, 2012). However, the quality of the bone is also highly important, including parameters such as microarchitecture, bone turnover and size of mineral crystals, which also affect bone strength (Licata, 2009). Therefore, bone strength cannot be predicted from the present data.

The adult female offspring of the high-dose group tended to have their first corpus luteum formation later. Studies in large animals have demonstrated differing results (Green et al., 1990; Lyche et al., 2004; Rainey et al., 1990; Savabieasfahani et al., 2006), while alterations in the timing of puberty through early exposure to EDC was repeatedly demonstrated in rodents (Buck Louiset al., 2008; Rasier et al., 2006). Bonjour and Chevalley summarized evidence in humans depicting that bone mass and maybe also biomechanical and structural properties that have been acquired until young adulthood can be associated with fracture risk later in life (Bonjour and Chevalley, 2014). Later age at menarche has been linked to a lower BMD already before puberty. In the present study, there was a tendency of a reduced BMD in the female siblings of the high-dose group slaughtered at 9 weeks of age. Due to the high variance regarding the age of the first corpus luteum formation in the present study, it would be highly interesting to increase the numbers of pigs in future studies to verify the observed trend.

Thorough investigations were performed with two different CT methods for the respective age dependent bone size strengthened by the inclusion of plasma hormone analyses and the onset of puberty. Distinct but rather small differences were detected regarding bone parameters. Thus, the biological significance is difficult to foresee. We would like to emphasize that possible long-term consequences cannot be ruled out because of the indications given at an earlier stage of development. Time point dependent effects may change during development making it hard to predict the outcome (Connelly et al., 2015; Seidlová-Wuttke et al., 2008). In addition, the effect may also depend on the time phase of the exposure (Hotchkiss et al., 2008). However, by including two distinct time points during development, namely before and at

Table 5
Plasma hormone concentrations of the male and the female animals.

Treatment [µg/kg BW/d]	Control (0)		ADI (0.05)		NOEL (10)		High-dose (1000)		Overall p-Value
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
Male prepubertal offspring									
IGF1 (ng/ml)	121.4	11.8	99.4	11.1	102.6	11.8	97.1	11.1	0.494
Leptin (ng/ml)	3.4	0.5	3.2	0.5	3.4	0.5	3.0	0.5	0.910
Female prepubertal offspring									
IGF1 (ng/ml)	170.0	18.0	128.0	18.0	118.6	18.2	148.7	18.0	0.220
Leptin (ng/ml)	3.5	0.3	3.2	0.3	3.9	0.3	3.1	0.3	0.236
Female adult offspring									
IGF1 (ng/ml)	123.4	10.2	127.4	10.0	118.5	12.2	121.9	9.8	0.952
Leptin (ng/ml)	12.4	2.7	10.2	2.7	10.4	3.3	5.2	2.6	0.251
Total estrogens (pg/ml)	47.5	7.3	37.8	7.2	43.5	8.9	40.6	7.2	0.695

ADI = acceptable daily intake. IGF1 = insulin-like growth factor 1. NOEL = no observed effect level. SE = standard error.

puberty, we aimed at focusing on important set-points of development. Similar to many other studies analyzing EDC effects on bone development (Hermsen et al., 2008; Kaludjerovic and Ward, 2008, 2009; Lind et al., 2009; Lundberg et al., 2006; Pelch et al., 2012; Piekarz and Ward, 2007; Rowas et al., 2012), we also did not analyze aged animals, which may have given further substantiation on the occurrence of a long-term imprint. In humans relatively small differences in peak bone mass are calculated to result in a substantial reduction in the risk of developing osteoporosis (Bonjour et al., 2009). Therefore, with increasing age, the observed differences might gain more significance.

To our knowledge, this is the first study using the pig as an animal model to analyze lasting consequences of low-dose early estrogen treatment on bone development. Similar to other studies in male (Hermsen et al., 2008; Kaludjerovic and Ward, 2008; Lind et al., 2009; Pelch et al., 2012; Piekarz and Ward, 2007; Rowas et al., 2012 Lind et al., 2009; Pelch et al., 2012; Piekarz and Ward, 2007; Rowas et al., 2012) and female (Hermsen et al., 2008; Lundberg et al., 2006) animals, many parameters were unaffected by the estrogen exposure, while others, namely the SSI and the CSA, showed particular alterations. The observed gender specific effects appeared at concentrations presently considered to be at the ADI and the NOEL dose. Our study therefore adds further evidence to previous studies that have demonstrated long-term effects on the bone development from concentrations at or close to human exposure levels, or close to environmental concentrations (Gutleb et al., 2010; Hermsen et al., 2008; Kaludjerovic and Ward, 2009; Lind et al., 2010; Lundberg et al., 2006; Pelch et al., 2012). Using E2 as model estrogen, our results substantiate the high sensitivity to low-doses of exogenous estrogens during embryonic and fetal development.

Conflict of interest

All authors confirm that there is no conflict of interest interfering with the impartiality of the scientific work at hand.

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Appendix A. Supplementary data

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Appendix III

VL Flöter, A-K. Lorenz, B Kirchner, M Pfaffl, S Bauersachs, SE Ulbrich „Impact of preimplantational oral low-dose estradiol-17β exposure on the endometrium: The role of miRNA", *Mol Reprod Dev.* **2018**;85(5):417-426

Substantial contributions by Veronika Flöter:

- Main contribution to execution of the animal trial
- Conceptualization of experiments
- Supervision of AKL in execution of experiments and data analysis
- Analysis and interpretation of data
- Writing of the manuscript – review and major editing

Veronika Flöter

V. Flöter

Susanne E. Ulbrich

Susanne E. Ulbrich

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RESEARCH ARTICLE

Molecular Reproduction & Development

Impact of preimplantational oral low-dose estradiol-17β exposure on the endometrium: The role of miRNA

Veronika L. Flöter[1,2] | Anne-Kathrin Lorenz[1,2] | Benedikt Kirchner[2] | Michael W. Pfaffl[2] | Stefan Bauersachs[1] | Susanne E. Ulbrich[1]

[1] ETH Zurich, Animal Physiology, Institute of Agricultural Sciences, Zürich, Switzerland

[2] Department of Animal Physiology and Immunology, School of Life Sciences, Life Science Center Weihenstephan, Technical University Munich, Freising-Weihenstephan, Germany

Correspondence
ETH Zurich, Animal Physiology, Institute of Agricultural Sciences, Universitätstrasse 2, 8092 Zürich, Switzerland.
Email: susanne.ulbrich@usys.ethz.ch

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Porcine conceptuses synthesize estrogens between Day 11 and 12 as signal for maternal recognition of pregnancy. A preimplantational estrogen exposure to pregnant gilts has been associated with embryonic losses and changes in endometrial mRNA expression. MicroRNAs (miRNAs) play a key role in the mRNA regulation by modulating the expression. Effects of estrogens on endometrial miRNAs have not been investigated in this context so far. Thus, we studied the endometrial expression profile of miRNAs in the pig at gestational Day 10 after daily estradiol-17β (E2) application starting at fertilization using either 0, 0.05 (ADI—acceptable daily intake), 10 (NOEL—no-observed-effect level) and 1,000 (high dose) µg E2/kg body weight/day, respectively. In endometrial homogenates, E2 ($p < 0.001$) and total estrogen concentrations ($p < 0.001$) were significantly increased, namely 28- and 160-fold, respectively, in the high dose group as compared to the control. Additionally, total estrogens were sixfold elevated in the NOEL group. Interestingly, high-throughput sequencing of small non-coding RNA libraries did not indicate any differentially expressed miRNAs between the treatment groups and the control group. The expression of 12 potential E2 target miRNAs investigated by RT-qPCR were equally unaffected. Thus, preimplantational E2 exposure resulted in significantly higher endometrial estrogen concentrations, but did not perturb the expression profile of endometrial miRNAs.

KEYWORDS
deep sequencing, endocrine disrupting chemicals, estrogen, pig, pregnancy

1 | INTRODUCTION

Endocrine disrupting chemicals (EDCs) are a class of exogenous substances affecting endogenous hormonal systems, reproduction and health (Diamanti-Kandarakis et al., 2009). Estradiol-17β (E2) can exhibit properties of an EDC (Geisert et al., 1991; Malcolm et al., 2006; Pope, Lawyer, Butler, Foote, & First, 1986; Rasier, Toppari, Parent, & Bourguignon, 2006). Suitably, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) announced an acceptable daily intake (ADI) of 0.05 µg E2/kg body weight (bw) for humans in 1999 (JECFA, 1999). Only recently, we demonstrated that orally administered low doses of E2 to pigs during the entire period of pregnancy affected body composition in male offspring (Fürst et al., 2012), bone parameters in male and female offspring (Flöter et al., 2016), as well as gene

Abbreviations: ADI, acceptable daily intake; EDCs, endocrine disrupting chemicals; E2, estradiol-17β; ER, estrogen receptor; miRNAs, microRNAs; ncRNAs, non-coding RNAs; NOEL, no-observed-effect level.

Current address of Stefan Bauersachs is at Department for Farm Animals, Clinic of Animal Reproduction Medicine, Genetics and Functional Genomics, University of Zurich, Lindau, Switzerland.

expression in the prostate (Kradolfer et al., 2016). Especially prenatal development, starting as early as the preimplantation phase, has been demonstrated to be a sensitive period, when disruptive stimuli may induce long-term consequences (Amstislavsky et al., 2006; Amstislavsky, Kizilova, Golubitsa, Vasilkova, & Eroschenko, 2004; Diamanti-Kandarakis et al., 2009; Hochberg et al., 2011; Ma, Song, Das, Paria, & Dey, 2003). Particularly in the pig, effects as strong as embryonic losses have been observed (Geisert et al., 1991; Pope et al., 1986). Such effects may arise through changes in the endometrial gene expression and secretion (Geisert et al., 2006). Evidence is increasing that small non-coding RNAs (ncRNAs), especially microRNAs (miRNAs), are important gene regulatory molecules in the endometrium (Bidarimath, Khalaj, Wessels, & Tayade, 2014; Chegini, 2010). In addition, the mechanisms of miRNAs altering mRNA expression are well studied and it has been predicted that miRNAs regulate about 50% of all mammalian protein-coding genes (Krol, Loedige, & Filipowicz, 2010; Roberts, 2014; Zhou, Hu, & Lai, 2010). Still, there is especially little knowledge about effects on miRNA expression through EDCs during the early stages of development (Felice et al., 2015; Meunier et al., 2012; Nothnick & Healy, 2010; Veiga-Lopez, Luense, Christenson, & Padmanabhan, 2013).

MicroRNAs are involved in the regulation of various processes such as apoptosis, cell proliferation, and development (Bartel, 2004). Expression profiling has identified a large number of miRNAs in reproductive tissues, including the ovary and the endometrium (Chegini, 2010; Su et al., 2014). Endometrial miRNAs have been associated with the regulation of genes that are important for differentiation, proliferations, receptivity, extracellular matrix factors, angiogenesis, and immunological response processes, which are crucial in preparation of the endometrium for implantation (Bidarimath et al., 2014; Krawczynski, Bauersachs, Reliszko, Graf, & Kaczmarek, 2015; Wessels et al., 2013). Steroid hormones and their respective receptors such as estrogen receptors (ER) α and β largely control these processes (Chegini, 2010). Information about estrogen regulated expression of miRNAs comes primarily from research in breast cancer cell lines treated with E2 (Cicatiello et al., 2010; Klinge, 2012). Moreover, the potential regulation of endometrial miRNAs by ovarian steroids was indicated through their different expression during the menstrual cycle (Chegini, 2010; Sha et al., 2011), as well as through treatment experiments (Nothnick & Healy, 2010). Alterations in miRNA expression were observed after E2 treatment of human endometrial stromal and glandular epithelial cells (Toloubeydokhti, Pan, Luo, Bukulmez, & Chegini, 2008). Nothnick and Healy (2010) demonstrated the involvement of the classical pathway of estrogens acting through ERα, as after pretreatment with the ERα specific inhibitor ICI 182,780, E2 did not alter the miRNA expression.

In the present study, we aimed at elucidating if continuous preimplantational E2 exposure affects the expression of endometrial miRNAs. Therefore, we analyzed the influence of three distinct concentrations of E2–the dose corresponding to the ADI, a dose close to the no-observed-effect level (NOEL) and a high dose—on the endometrial expression profile of miRNAs in sows at day 10 of pregnancy.

2 | RESULTS

2.1 | Evaluation of the embryos

All recovered embryos (n = 230) were hatched blastocysts and showed normal, stage-specific development and displayed an embryonic disc. Neither earlier stages (2-, 4-, 6-, or 8-cell-stage embryos) nor elongated embryos were observed. There was neither significant difference between the treatment groups and the control group in the number of embryos (p = 0.33) nor in the size of the embryos (p = 0.80) with 1.89 mm ± 0.48 mm (n = 61), 2.48 mm ± 0.55 mm (n = 54), 1.91 mm ± 0.43 mm (n = 61), and 2.27 mm ± 0.48 mm (n = 54) in the control, ADI, NOEL, and the high dose group, respectively.

2.2 | Abundance of estrogens in endometrial samples

The concentration of endometrial E2 was significantly affected by the oral E2 treatment (overall p < 0.001; Figure 1). E2 depicted higher amounts in the high dose group compared to the control group (3056 ± 830 pg/g and 111 ± 4 pg/g, respectively). E2 in neither the ADI (82 ± 22 pg/g) nor the NOEL group (195 ± 57 pg/g) was significantly different from the control group. The abundance of total endometrial estrogens (estrone, E2, estradiol-17α), which in the pig is the sum of E2 and estrone (E1) due to negligible amounts of estradiol-17α, was also significantly affected (overall p < 0.001; Figure 1). Total estrogens were unaltered in the ADI group (156 ± 22 pg/g), but higher in the NOEL (881 ± 252 pg/g) and the high dose group (24,657 ± 9,557 pg/g) in comparison to the control group (154 ± 12 pg/g).

2.3 | Measurement of miRNA expression with high-throughput sequencing

Small ncRNA sequencing of porcine endometrium was performed. The number of raw reads per library and the number of discarded reads as yielded in the following quality control steps are detailed in Table S1.

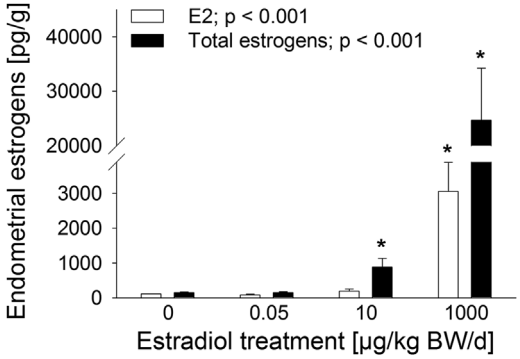

FIGURE 1 Abundance of estradiol-17β (E2) and total estrogens in the porcine endometrium after E2 exposure. The three treatment groups corresponding to the acceptable daily intake (ADI), close to the NOEL (no-observed-effect level,) and a high dose (0.05, 10, and 1,000 µg E2/kg body weight/day, respectively) were compared to the control animals. Significant differences are indicated by an asterisk (p < 0.05). Values are depicted as mean ± SEM (n = 4–6 per group)

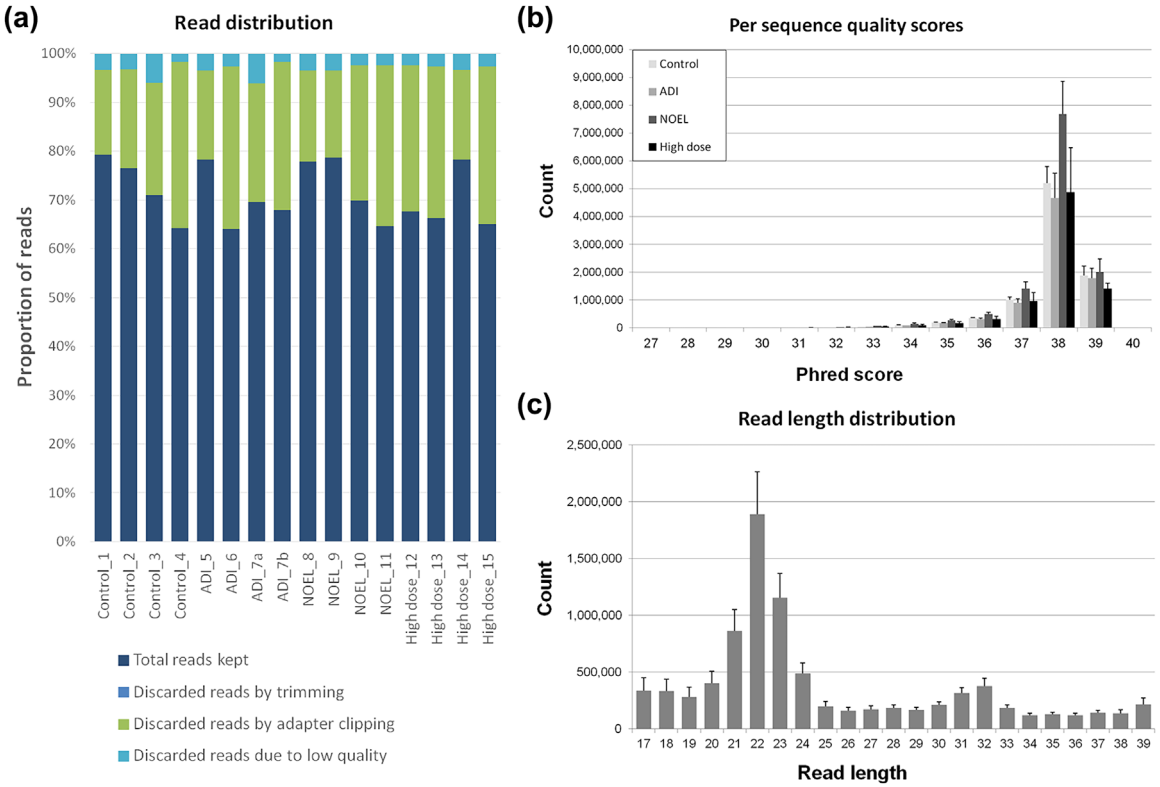

FIGURE 2 Reads during quality assessment. (a) Proportion of reads. The 16 samples ($n = 4$ per treatment group) are depicted on the x-axis. Each biological replicate is indicated by a different number following the treatment information (control, acceptable daily intake (ADI), no-observed-effect level (NOEL) and high dose corresponding to 0, 0.05, 10, and 1,000 µg E2/kg bw/d). The raw reads correspond to 100%. Reads were stepwise discarded. First, reads were discarded by trimming. Next, adaptor only reads, reads without the 3′ adaptor, reads with unknown bases (N) and sequences shorter than 17 nucleotides were discarded depicted as "discarded reads by adaptor clipping." Then, reads containing bases with a Phred score below 25 were discarded "due to low quality." (b) The mean Phred score per sequence (x-axis) of the "total reads kept" is shown for the four treatment groups with the respective number of sequences (y-axis). Most reads had a mean Phred score of 38, irrespective of the treatment group. (c) Read length distribution of the "total reads kept" with mean values of all 16 samples. A peak can be observed at 21–23 nucleotides

The relative frequencies are shown in Figure 2a. The number of raw reads as well as the number of total reads kept were similar between the treatment groups ($p > 0.05$). There was an average of 13.0 million ± 1.2 million raw reads (mean ± SEM; $n = 16$). The selection of good quality reads from the raw reads resulted in an average of 9.2 million ± 0.8 million reads (mean ± SEM; $n = 16$) corresponding to 71% of the total reads (Figure 2a and Table S1).

The quality of the kept reads was comparable in all 16 libraries as indicated by the analysis with FastQC. The good quality of the reads was shown by a mean Phred score of mainly 38 (Figure 2b). The majority of the reads, derived from the FastQC report, had a length of 21–23 nucleotides (Figure 2c). This indicates that the samples mainly contained miRNAs.

After the sequences were counted and filtered to remove very low abundant transcripts, there were in total 41,503 different sequences left, assumingly containing various kinds of small ncRNAs. In the database, there were 326 annotated mature miRNAs in the pig (miRBase 20.0). As miRNAs are conserved between species, the data were also compared to the miRNA sequences from miRBase of human (2,578 annotated miRNAs), cow (783 annotated miRNAs), and mouse (1,908 annotated miRNAs). In the endometrial samples, 212 porcine, 272 human, 205 bovine, and 235 murine known mature miRNAs were detected. These detected mature miRNAs were analyzed per species with the DESeq algorithm. The expression of these mature miRNAs did not differ significantly (adjusted $p > 0.05$) between the treatment groups and the control group in any of the species analyzed. The 20 highest expressed miRNAs representing 82.7% of all reads of the 326 porcine mature miRNAs are shown in Figure 3a. The miR-21 was highest expressed with 12.6% and the remaining 192 porcine miRNAs accounted for 17.3% of the reads. The cluster analysis for the 30 porcine miRNAs with the highest variance revealed that the individual samples did not cluster related to the treatment group (Figure 3b). The lack of clustering emphasizes that there was no effect of the treatment on the miRNA expression.

miRNAs are quite conserved across species, therefore, many miRNA sequences found in the endometrial samples are annotated in more than one species (Figure 4). As shown in Figure 4, about one third of the known 212 porcine miRNAs are only found in the pig ($n = 62$), while about another third ($n = 63$) matched to known miRNAs from all four species. Overall, the detected 212 porcine, 272 human, 205

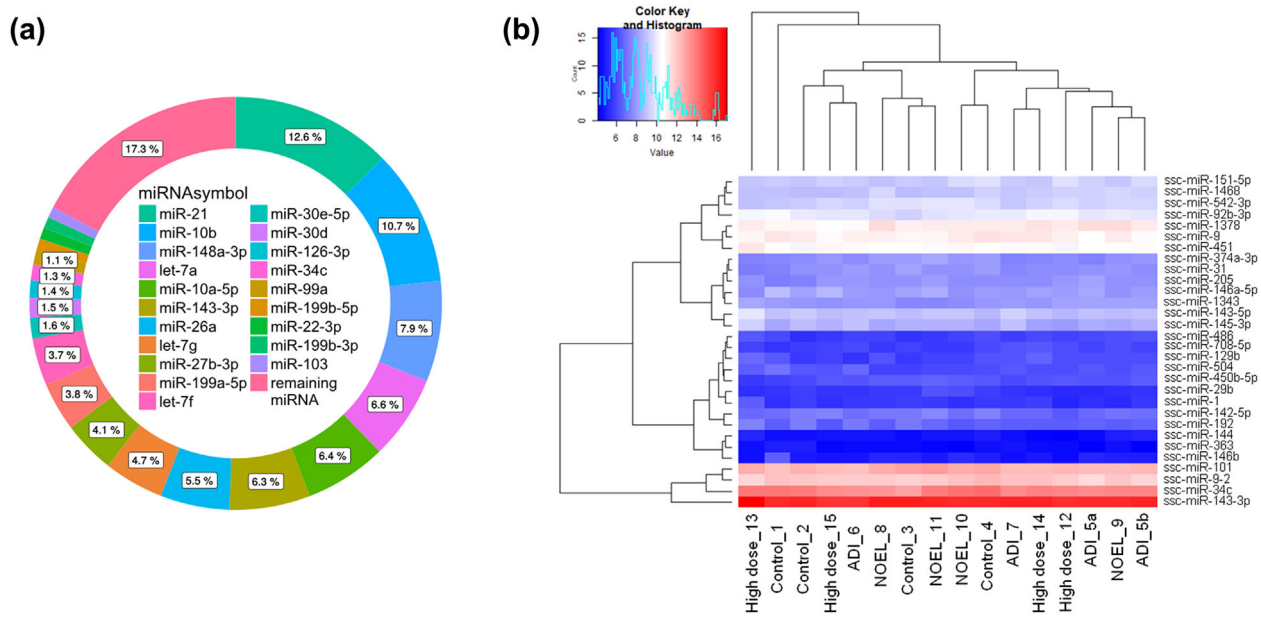

FIGURE 3 Annotated porcine endometrial miRNAs. (a) The top 20 expressed porcine miRNAs with the percentage of read counts in addition to the sum of all remaining miRNAs are depicted. (b) The heatmap of the 30 porcine miRNAs with the highest variance using rlog transformed data are shown, displaying similar expression in the samples and consequently no clustering according to the four treatment groups. Each biological replicate is indicated by a different number following the treatment information (Control, acceptable daily intake [ADI], close to the no-observed-effect level [NOEL] and a high dose group [0, 0.05, 10 and 1,000 µg/kg bw/d, respectively]). ssc, sus scrofa

bovine, and 235 murine sequences resulted without this overlap in a total of 444 expressed mature miRNA sequences in the porcine endometrium. Thus, in addition to the 212 porcine sequences (47.7%), a further 232 sequences were found matching 100% to known miRNAs from the human, cow and/or mouse (Figure S1) most likely corresponding to not yet annotated porcine miRNAs and miRNA variants. A table of these sequences including the matching miRNA information from the four species can be found in Table S2.

2.4 | Measurement of miRNA expression with RT-qPCR

Twelve potentially E2 regulated miRNAs were selected for the miRNA expression analysis using RT-qPCR. All of these miRNAs have been described in the literature in the context of altered expression due to an E2 exposition (Bhat-Nakshatri et al., 2009; Cohen et al., 2008; Di Leva et al., 2013; Katchy, Edvardsson, Aydogdu, & Williams, 2012; Klinge, 2009; Maillot et al., 2009; Pan, Luo, Toloubeydokhti, & Chegini, 2007; Wang et al., 2010; Wickramasinghe et al., 2009; Zhao et al., 2013). The miR-20a and miR-21 have been repeatedly shown to be altered upon E2 treatment (Klinge, 2009, 2012; Pan et al., 2007; Wang et al., 2010; Wickramasinghe et al., 2009). The other candidate miRNAs were selected related to different absolute expression abundance, namely from about 100 reads (miR15a, miR-29c, miR-130a), about 1,000 reads (miR-16, miR-20a, miR-146, miR-195, miR-205), about 10,000 reads (miR-27b, miR103a, miR-191) up to about 100,000 reads (miR-21). Particularly, in the breast cancer cells MCF-7 and T47D, miR-15a, miR-16, miR-20a, miR-21, miR-27b, miR-29c, miR-103, miR-146b, miR-191, and miR-195 have been shown to be influenced after E2 treatment (Bhat-Nakshatri et al., 2009; Di Leva et al., 2013; Katchy et al., 2012; Klinge, 2009). miR-20a and miR-21 have been additionally differentially expressed in human endometrial stroma and glandular cells, respectively, as well as in cancerous endometrium and endometriosis (Pan et al., 2007; Wang et al., 2010; Wickramasinghe et al., 2009). Furthermore, miR-205 has been upregulated by ex vivo E2 treatment of mouse aorta (Zhao et al., 2013) while miR-130a has been downregulated after E2 exposure in whole-body homogenates of zebrafish (Cohen et al., 2008). The expression of RNU6B, RNU5A, and SNORA73A was used for the normalization, as determined with the GeNorm and Normfinder algorithm. In all three E2 treatment groups, the endometrial expression of none of the 12 target miRNAs displayed significant difference ($p > 0.05$) to the respective control group as shown in Table 1 and Figure S2. Most often, very similar expression values were observed. Only miR-146b showed a larger variance, but similarly no significant difference ($p = 0.62$).

3 | DISCUSSION

The oral application of a high dose of E2 over the first 10 days of pregnancy lead to a 28-fold increase in endometrial E2 concentrations compared to the control group. In addition, endometrial total estrogens were not only pronouncedly elevated in the high dose group (160-fold), but also in the animals receiving the NOEL dose (6-fold). Fürst et al. (2012) demonstrated that the two low doses of E2, corresponding to the ADI and close to the NOEL, administered during

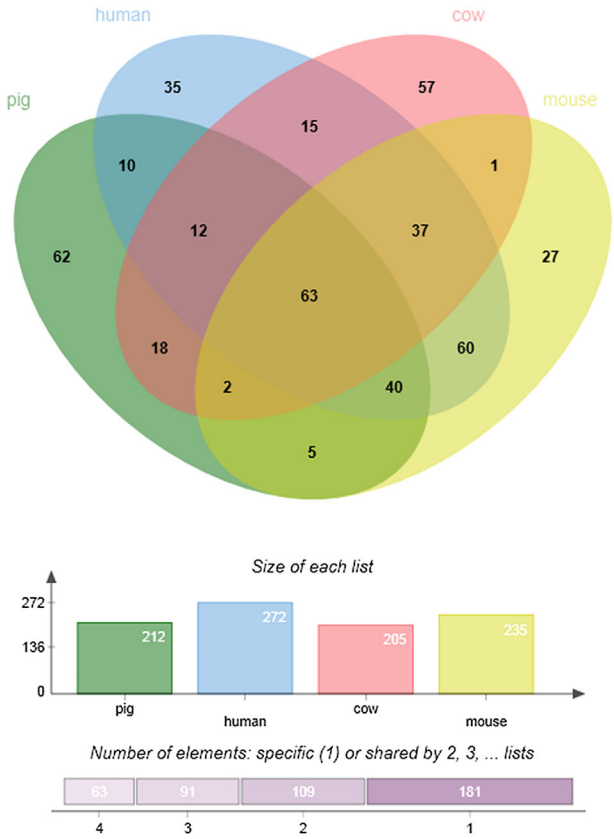

FIGURE 4 Distribution of the 444 detected sequences of mature miRNAs. The Venn diagram represents the number of detected sequences split into the species to which the sequence mapped to 100% to a known miRNA. The diagram below depicts the number of mapped sequences per species. At the bottom, the number of sequences that are shared by 1, 2, 3, or 4 species is shown

the entire length of gestation, affected body weight development and body composition, respectively, in the offspring. Further low-dose effects in the offspring were observed by analyzing the bone (Flöter et al., 2016) and prostate (Kradolfer et al., 2016). These lasting effects may be due to the altered endocrine environment during pregnancy, particularly due to exposure effects already occurring during the time of preimplantation when still low endogenous estrogen concentrations prevail. For this reason, the present in vivo study focused on analyzing Day 10 after fertilization, shortly prior to the endogenous estrogen signal secreted by the porcine embryo. The high dose group depicted a mean concentration of 3.1 ng E2/g endometrial tissue compared to 0.1 ng E2/g in the control animals, resulting from accumulated and/or remaining E2 over 10 days through feeding of 500 µg E2/kg bw twice per day (Fürst et al., 2012) and through timing of the slaughtering 1 hr after feeding the regular dose of 500 µg E2/kg bw. This is a time, where still high concentrations of E2 can be observed in the peripheral blood (Fürst et al., 2012). Using high-throughput ncRNA sequencing, 444 sequences of mature miRNAs were detected, however, not differentially expressed between the treatment groups and the control. This was further confirmed by RT-qPCR of 12 selected, potentially E2 dependent miRNAs (Bhat-Nakshatri et al., 2009; Cohen et al., 2008; Di Leva et al., 2013; Katchy et al., 2012; Klinge, 2009; Maillot et al., 2009; Pan et al., 2007; Wang et al., 2010; Wickramasinghe et al., 2009; Zhao et al., 2013). In many cell culture experiments, most often using MCF-7 human breast cancer cells, 10 nM E2 (2.7 ng E2/ml) was applied as treatment dose (Klinge, 2012). This lead to alterations in the expression of miRNAs as reviewed by Klinge (2012) including miR-15a, miR-16, miR20a, miR21, miR-27b, miR-103, miR146b, miR191, and miR-195 (Bhat-Nakshatri et al., 2009; Di Leva et al., 2013; Klinge, 2009; Maillot et al., 2009), which were also determined in the present study using RT-qPCR. The difference in E2 responsiveness in the latter studies compared to the study at hand may have its origin in the biological background, as breast cancer cell lines are out of the biological context and characterized by exceptional high concentrations of ERα (Klinge, 2012).

In contrast to miRNAs, differential endometrial gene expression has been observed on the mRNA level in the same samples in all three treatment groups (Flöter et al., under review). There were 14 (ADI), 17 (NOEL), and 27 (high dose) differentially expressed genes (DEG) in the

TABLE 1 Normalized expression of 12 endometrial miRNAs at day 10 of pregnancy after E2 exposure

miRNA	Control group (Mean 40 + ΔCq ± SEM)	ADI dose group (Mean 40 + ΔCq ± SEM)	NOEL dose group (Mean 40 + ΔCq ± SEM)	High dose group (Mean 40 + ΔCq ± SEM)	Overall *p*-value
miR-15a	29.3 ± 0.7	29.1 ± 0.6	29.7 ± 0.3	29.5 ± 0.7	0.67
miR-16	35.7 ± 0.3	35.1 ± 0.3	35.6 ± 0.1	34.8 ± 0.3	0.10
miR-20a	31.0 ± 0.5	31.0 ± 0.6	31.0 ± 0.3	31.0 ± 0.6	1.00
miR-21	38.2 ± 0.5	37.8 ± 0.5	38.6 ± 0.2	37.9 ± 0.5	0.55
miR-27b	33.7 ± 0.4	33.8 ± 0.1	34.0 ± 0.4	33.8 ± 0.4	0.96
miR-29c	34.4 ± 0.6	34.3 ± 0.4	34.3 ± 0.3	34.6 ± 0.5	0.96
miR-103	32.3 ± 0.4	32.2 ± 0.2	32.5 ± 0.2	38.3 ± 0.4	0.54
miR-130a	30.3 ± 0.3	30.4 ± 0.3	30.2 ± 0.2	30.5 ± 0.4	0.94
miR-146b	25.8 ± 1.4	25.8 ± 1.6	23.4 ± 1.8	25.2 ± 0.9	0.62
miR-191	34.2 ± 0.2	33.8 ± 0.3	33.7 ± 0.2	33.8 ± 0.3	0.53
miR-195	35.1 ± 0.3	34.2 ± 0.5	35.2 ± 0.1	34.8 ± 0.4	0.24
miR-205	31.7 ± 0.7	32.5 ± 0.6	32.1 ± 0.3	32.1 ± 0.5	0.77

endometrium with at least a twofold regulation. An earlier study by Ross et al. (2007) injected intramuscularly estradiol cypionate to sows only at days 9 and 10 of pregnancy and observed 9 (day 10), 71 (day 13) and 21 (day 15) DEG with at least 1.8-fold regulation. Interestingly, some DEG were upregulated in both studies. In the high dose group *RBP4* (retinol binding protein 4), which was also altered at Day 10 (Ross et al., 2007), and *SULT2A1* (sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone [DHEA]-preferring, member 1), *VNN2* (vanin 2) as well as *SLC39A2* (solute carrier family 39 [zinc transporter], member 2), which were altered at Day 13 (Ross et al., 2007), were elevated. Thus, it is even more surprising that miRNAs in our study were unaffected, as they are known important regulators of mRNA expression.

The most important aspect seems to be the timing of the estrogen exposure, which is different in the study at hand compared to most studies available in the pig. Not only altered endometrial expression of genes had been observed after exposure at days 9 and/or 10 of pregnancy, but also degeneration of the uterine glycocalyx (Ross et al., 2007) and even embryonic losses (Geisert et al., 1991; Pope et al., 1986). Thus, estrogen exposure shortly before the embryo starts secreting estrogens as embryonic signal for maternal pregnancy recognition which occurs at days 11–12 (Spencer, Burghardt, Johnson, & Bazer, 2004) can exert major impact. In contrast, neither estrogen exposure at days 12 and 13 (Pope et al., 1986) nor only at Day 12 (Geisert et al., 1991) induced embryonic losses. Thus, there is a strong time-point dependent component regarding effects through estrogens during the periimplantational phase. Molecular analyses indicate that exogenous estrogen may lead to a desynchronization of endometrial receptivity and embryo development resulting in embryonic loss (Geisert et al., 2006). There are scarce reports about studies which administered estrogens already starting at insemination. Evidence exists in "imprinting control region" (ICR) mice, a multipurpose research mouse model strain (Okada & Kai, 2008). Female ICR mice were implanted with an E2-containing tube prior to mating, which was maintained during pregnancy and lactation. Interestingly, most of the animals maintained their pregnancy and had a birth rate of 70%. Our continuous treatment neither lead to a change in the number of embryos at Day 10 (this study) nor in litter size (Fürst et al., 2012). Thus, we might observe a habituation or programming with subtle, but not detrimental effects toward the presence of estrogens. Certain alterations were observed in the pre- and postpubertal offspring (Flöter et al., 2016; Fürst et al., 2012; Kradolfer et al., 2016), which might also result from exposure during this very early time of development. Additionally, this leads to the possibility that administering estrogens on days 9 and 10 of pregnancy, might lead to changes in miRNA expression particularly at days 12–15 possibly involved in the drastic outcome of abortion (Geisert et al., 1991; Pope et al., 1986).

Many studies have demonstrated effects of an exposure to EDCs during embryo development directly on mothers and offspring as well as long-term effects in the latter (Amstislavsky et al., 2004, 2006; Geisert et al., 2006; Gore et al., 2015; Ma et al., 2003; Ross et al., 2007). Little is known about EDCs affecting miRNAs (Felice et al., 2015; Meunier et al., 2012; Nothnick & Healy, 2010; Veiga-Lopez et al., 2013). A former study has demonstrated direct in utero effects of bisphenol A altering fetal ovarian miRNA expression in sheep (Veiga-Lopez et al., 2013). A further study showed lasting changes after neonatal exposure to estradiol benzoate on adult rat testes including alterations in miRNA expression (Meunier et al., 2012). In contrast, there was no E2 effect on the miRNA profile in the present study, although endometrial as well as embryonic mRNA changes showed E2-dependent differentially expressed transcripts (Flöter et al., under review). Several reasons can account for this striking finding: the window of exposure, the route of administration, the substance-specific elimination kinetics, the continuous treatment that could have induced a habituation toward estrogens, as well as the timing of the analysis 1 hr after the last dose was fed. Other mechanisms of gene expression regulation than differential miRNA expression may as well account for the observed differential mRNA expression. Further functional studies need to be undertaken.

In summary, continuously applied E2 to pregnant pigs by oral ingestion increased endometrial estrogen concentrations even at a low dose that is close to the announced NOEL for humans (JECFA, 1999). Although local estrogen concentrations increased pronouncedly and lead to a perturbed mRNA expression (Flöter et al., under review), there was no E2 treatment effect on the expression profile of miRNAs in the endometrium during the blastocyst stage of pregnancy. As miRNAs can be found in extracellular vesicles (EV) from uterine flushings (Krawczynski, Najmula, Bauersachs, & Kaczmarek, 2015), the miRNA load of EV remains an important target for future analyses of periconceptional effects of EDCs.

4 | MATERIALS AND METHODS

4.1 | Animal studies and collection of tissue samples

In order to investigate effects of E2 on the expression profile of miRNAs in the pregnant endometrium, the present study was undertaken according to our previous study applying E2 during the entire pregnancy (Pistek et al., 2013). In brief, the estrous cycles of the German Landrace sows were synchronized prior to the start of the treatment. The sows were inseminated with the sperm of one Pietrain boar. For the E2 exposure, the sows were randomly assigned to a treatment group (n = 5–6 per group). One group received 0.05 μg E2/kg bw/day, corresponding to the announced ADI for humans, while another group obtained 10 μg E2/kg bw/day, related to the NOEL (JECFA, 1999). In addition, a high dose of 1,000 μg E2/kg bw/day and ethanol carrier only for the control group were used, respectively. Half of the E2 dose, dissolved in 2 ml ethanol, was fed via bread rolls (20 g) in the morning and the other half in the evening. The E2 was applied continuously for a period of 10 days, beginning with insemination until slaughter at Day 10 of pregnancy. The last dose was fed 1 hr before slaughter. The uterus was flushed with 10 ml and another 50 ml of phosphate-buffered saline (PBS, autoclaved, pH 7.4) to retrieve the embryos. All embryos were collected in a petri-dish with PBS. Subsequent, pictures of the embryos were taken. After carefully opening the uterus, endometrial samples were collected, shock frozen

in liquid nitrogen and stored at −80 °C. Plasma samples were retrieved after centrifugation of EDTA supplemented blood at 4 °C and stored at −20 °C. For the small ncRNA sequencing analyses, only animals with embryos at the blastocyst stage (n = 3–5 per group) were considered. Three sows did not conceive, as only unfertilized oocytes were found (one animal each in the control, ADI and high dose group, respectively). In addition, two animals of the ADI dose group were excluded from the study due to illnesses at slaughtering. Both animals had pus and clinical signs of inflammation in the uterus and had not been pregnant. The experiments were conducted with permission from the local veterinary authorities and were performed in accordance with the accepted standards of humane animal care.

4.2 | Hormone analyses

Analyses of estradiol-17β (E2) and total estrogen (estrone, E2, estradiol-17α) concentrations in endometrial tissue were performed using an in-house competitive enzyme immuno assays (EIA) (Hageleit, Daxenberger, Kraetzl, Kettler, & Meyer, 2000; Meyer, Sauerwein, & Mutayoba, 1990). Endometrial tissue was homogenized in liquid nitrogen using a mortar and pestle. Saline (0.5 ml) was added to 100 mg of the grounded tissue. For the extraction, the tissue was at first incubated in 6.5 ml tert. butylmethylether/petrolether 30/70 v/v overnight. After phase separation at room temperature within two days, it was frozen at −60 °C for 48 hr. The liquid ether phase was decanted and the ether was vaporized. After adding of 500 µl assay puffer, abundance of E2 and total estrogens was determined as described earlier (Hageleit et al., 2000).

4.3 | Extraction and quality of RNA

For further analyses, isolation of total endometrial RNA from the collected samples was performed by means of TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufactureŕs protocol (n = 4 per group, except for the ADI group, where a repeated tissue extraction was done because of only n = 3 available animals). For determining the purity and the quantity of the obtained RNA, the NanoDrop 1,000 (peqLab, Erlangen, Germany) was applied. The RNA integrity was measured with a Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). The mean RNA integrity number (RIN) was 9.4 ± 0.4 (±SD).

4.4 | Small ncRNA sequencing of endometrial samples

Total RNA was used for high-throughput sequencing, which was kindly performed by the Genomics Core Facility of the EMBL Heidelberg (EMBL Heidelberg, Genomics Core Facility, Heidelberg, Germany). For preparation of the small RNA-Seq libraries, the NEBNext© Small RNA Sample Prep Set 1 (New England Biolabs, Frankfurt-Höchst, Germany) was utilized according to the manufactureŕs instructions. The sequencing was conducted on an Illumina HiSeq 2000 (Illumina, San Diego, CA) with single-read mode and a read length of 50 bases.

4.5 | Analysis of the illumina sequence data with Galaxy

The Fastq files were analyzed with a locally installed version of Galaxy (Giardine et al., 2005) (www.usegalaxy.org, hosted by Gene Center Munich, AG Blum). The reads were trimmed from the 5′ and 3′ end with "Fastq quality trimmer" (Blankenberg et al., 2010) (window size: 3, step size: 1, quality score ≥30.0, aggregate action for window: mean of scores), the adapter sequence was clipped (min. sequence length: 17, discard sequences with unknown (N) bases: yes) and reads filtered with "filter by quality" (quality cut-off value: 25, percentage of bases in the sequence that must have a quality of at least the cut-off value: 100). Retrained reads were used for the quality report with FastQC. Subsequently, the abundance of each unique sequence was determined, the table of the read counts from all samples was then filtered to remove very low abundant sequences and to keep potential transcripts that are turned on or off by the E2 treatment (condition: at least 10 reads of each miRNA must be present in three to four samples of at least one group). As miRNAs are quite conserved between species, our data were also compared to the miRNA sequences of human, cow and mouse. Therefore, databases of mature miRNAs for pig (326 annotated miRNAs), human (2578 annotated miRNAs), mouse (1908 annotated miRNAs), and cow (783 annotated miRNAs) were obtained from miRBase (www.miRBase.org, release 20.0) and used to generate a BLAST database in the Galaxy platform utilizing "make blast database" (http://www.ncbi.nlm.nih.gov/books/NBK1763/) (molecule type of input: nucleotide, hash-index: true). Reads were mapped against these databases with "blastn" (Zhang, Schwartz, Wagner, & Miller, 2000) (blastn-short, expectation value: 1.0, word size: 5), followed by filtering of the blast output (condition: length of query sequence = length of target sequence = alignment length and number of identical bases within the alignment) in order to selected only miRNAs which aligned 100% with the database sequence.

NGS experiments have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) repository with accession number GSE89343.

4.6 | Quantitative real-time qPCR

The RNA samples were reverse transcribed into cDNA using miScript II RT kit (Qiagen, Hilden, Germany) according to the recommendations of the manufacturer. Defined cDNA fragments were amplified by quantitative real-time PCR (RT-qPCR) with specific forward primers (miScript Primer Assay, Qiagen) (Table 2). All primers are specific for the pig. If the porcine (ssc) miRNA sequence was identical with the mouse (mmu) or human (hsa) sequence, these existing primers were used. Twelve potential E2 regulated miRNAs were selected for RT-qPCR validation (Bhat-Nakshatri et al., 2009; Cohen et al., 2008; Di Leva et al., 2013; Katchy et al., 2012; Klinge, 2009; Maillot et al., 2009; Pan et al., 2007; Wang et al., 2010; Wickramasinghe et al., 2009; Zhao et al., 2013). As potential reference genes the human miScript Control Assays (Qiagen) for small nuclear RNAs (snRNA) RNU6B (NR 002752.2) and RNU5A (NR 002756.2), and the small nucleolar

TABLE 2 miScript primer assays for 12 selected porcine miRNAs

miRNA name	Mature miRNA sequence 5′ → 3′	MIMAT*
hsa-miR-15a-5p	UAGCAGCACAUAAUGGUUUGUG	68
hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG	69
mmu-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUAG	529
hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA	76
hsa-miR-27b-3p	UUCACAGUGGCUAAGUUCUGC	419
hsa-miR-29c-3p	UAGCACCAUUUGAAAUCGGUUA	681
hsa-miR-103a-3p	CAGUGCAAUGUUAAAAGGGCAU	425
hsa-miR-130a-3p	CAGUGCAAUGUUAAAAGGGCAU	425
ssc-miR-146b	UGAGAACUGAAUUCCAUAGGC	10 190
hsa-miR-191-5p	CAACGGAAUCCCAAAAGCAGCUG	440
hsa-miR-195-5p	UAGCAGCACAGAAAUAUUGGC	461
hsa-miR-205-5p	UCCUUCAUUCCACCGGAGUCUG	266

*Accession number (MIMAT) from miRBase.

RNAs (snoRNA) SNORA73A (NR 002907.2), SNORA25 (NR 003028.1) and SCARNA17 (NR 003003.2) were additionally determined.

The RT-qPCR reaction was performed with the Rotor-Gene (Qiagen) using the QuantiTect SYBR Green PCR kit (Qiagen). The master mix had a final volume of 10 µl consisting of 1 µl miScript Primer Assay, 1 µl miScript Universal Primer, 5 µl QuantiTect SYBR Green PCR Master Mix, and 3 µl of 1:4 diluted cDNA. For the negative control, nuclease free water instead of cDNA was used. In all assays, standard cycling conditions were as followed: 95 °C for 15 min, then 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s, and a melting curve. The cycle of quantification (Cq) was calculated by the Rotor-Gene software (Rotor-Gene® Q Series 1.7; Qiagen). The selection of appropriate reference genes was based on the calculations from the GeNorm and Normfinder algorithm (GenEx software 3.4.3 (Gothenburg, Sweden). For the normalization process RNU6B, RNU5A, SNORA73A were utilized. The resulting ΔCq-values were further analyzed with the ΔΔCq method (Livak & Schmittgen, 2001).

4.7 | Statistical analyses

For statistical analyses, regarding the RT-qPCR experiments the ΔCq-values were used. The logarithmized data of the hormone concentrations were taken for statistical analyses. The miRNA expression data, obtained by RT-qPCR, the endometrial hormone concentrations, measured by EIA, as well as the read counts and the number of embryos per sow were analyzed with one-way ANOVA followed by a Dunnettśs post hoc test to evaluate potential differences of the treatment groups compared to the control group using the SigmaPlot program 11.0 (SPSS, Chicago, IL). Embryo size was analyzed using SAS 9.2 (SAS Institute, Inc., Cary, NC) in order to account for the nested study design of multiple embryos belonging to the same exposed sow. A mixed model including the repeated measurement function for the embryos and a random intercept for the mother sows was applied as described by Kiernan et al. Kiernan, Tao, and Gibbs (2012). The residual method was used to calculate the denominator degrees of freedom (Bell, Smiley, Ene, Sherlock, & Blue, 2013). The Dunnett's post hoc test was applied. The results from SAS are presented as mean ± SE. Regarding the data from the small ncRNA sequencing experiment, analysis of differential miRNA expression in a treatment group versus the control group was performed with DESeq 2.11 (Anders & Huber, 2010) (www.bioconductor.org) in R 2.15.3. Differences were considered significant at an adjusted *p*-value from the snRNA sequencing <0.05 and in all other experiments at $p < 0.05$. Mean values ± SEM were used for graphical presentation of the statistical results.

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ORCID

Susanne E. Ulbrich http://orcid.org/0000-0003-0355-3869

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SUPPORTING INFORMATION

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Appendix IV

VL Flöter, S Bauersachs, RW. Fürst, S Krebs, H Blum, M Reichenbach, SE Ulbrich „ Exposure of pregnant sows to low doses of estradiol-17beta impacts on the transcriptome of the endometrium and the female preimplantation embryos", *Biol Reprod*. **2019**;100(3):624-640

Substantial contributions by Veronika Flöter:

- Main contribution to execution of the animal trial
- Conception and execution of experiments
- Analysis and interpretation of data with assistance
- Drafting of tables and figures
- Writing the original draft of the manuscript

Veronika Flöter

V. Flöter

Susanne E. Ulbrich

Susanne E. Ulbrich

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Research Article

Exposure of pregnant sows to low doses of estradiol-17β impacts on the transcriptome of the endometrium and the female preimplantation embryos†

Veronika L. Flöter[1,2], Stefan Bauersachs[1], Rainer W. Fürst[2], Stefan Krebs[3], Helmut Blum[3], Myriam Reichenbach[4] and Susanne E. Ulbrich[1,2,*]

[1]ETH Zurich, Animal Physiology, Institute of Agricultural Sciences, Zurich, Switzerland; [2]Physiology Weihenstephan, Technische Universität München, Freising, Germany; [3]Laboratory for Functional Genome Analysis (LAFUGA), Gene Center of the Ludwig-Maximilians-Universität (LMU) München, Munich, Germany and [4]Chair for Molecular Animal Breeding and Biotechnology, Gene Center of the Ludwig-Maximilians-Universität (LMU) München, Munich, Germany

*__Correspondence:__ ETH Zurich, Animal Physiology, Institute of Agricultural Sciences, Universitaetstr. 2, 8092 Zurich, Switzerland. E-mail: seu@ethz.ch

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Abstract

Maternal exposure to estrogens can induce long-term adverse effects in the offspring. The epigenetic programming may start as early as the period of preimplantation development. We analyzed the effects of gestational estradiol-17β (E2) exposure with two distinct low doses, corresponding to the acceptable daily intake "ADI" and close to the no-observed-effect level "NOEL", and a high dose (0.05, 10, and 1000 μg E2/kg body weight daily, respectively). The E2 doses were orally applied to sows from insemination until sampling at day 10 of pregnancy and compared to carrier-treated controls leading to a significant increase in E2 in plasma, bile and selected somatic tissues including the endometrium in the high-dose group. Conjugated and unconjugated E2 metabolites were as well elevated in the NOEL group. Although RNA-sequencing revealed a dose-dependent effect of 14, 17, and 27 differentially expressed genes (DEG) in the endometrium, single embryos were much more affected with 982 DEG in female blastocysts of the high-dose group, while none were present in the corresponding male embryos. Moreover, the NOEL treatment caused 62 and 3 DEG in female and male embryos, respectively. Thus, we detected a perturbed sex-specific gene expression profile leading to a leveling of the transcriptome profiles of female and male embryos. The preimplantation period therefore demonstrates a vulnerable time window for estrogen exposure, potentially constituting the cause for lasting consequences. The molecular fingerprint of low-dose estrogen exposure on developing embryos warrants a careful revisit of effect level thresholds.

Summary Sentence

Maternal oral low-dose estrogen exposure during the preimplantation period specifically targeted female embryos by inducing a male-like gene expression profile.

Key words: preimplantation embryo, endometrium, pig, estradiol, gene expression, endocrine disruptors.

Introduction

Estrogens are important mediators for the preparation of the uterus toward implantation [1–4]. During the early embryonic cleavages, maternal plasma estrogen concentrations are low [5, 6]. This is followed by an increase of estrogens around implantation (in mice at day 4) [4]. The role of mid-luteal estrogens is not as clear in primates, although human data indicate that slightly higher estrogen concentration might favor implantation [3–5]. Sows depict increasing peripheral plasma estrogen concentration after implantation [6, 7], whereas a first local rise through secretion from the elongating preimplantation embryo occurs at day 11 to 12 after fertilization [1]. Estrogens hereby function as maternal pregnancy recognition signal to inhibit luteolytic signals.

Humans are exposed to various estrogenic substances with the potential to affect the endogenous hormone systems [8, 9]. These may be natural as well as synthetic substances, so-called endocrine disrupting chemicals (EDC), which can adversely impact on developing organisms. As this has also been shown for estradiol-17β (E2) [10, 11], the latter is regarded as an EDC [9]. Especially prenatal development, starting as early as the preimplantation phase, has been demonstrated a sensitive period, when disruptive stimuli may induce long-term consequences [8, 12–14]. A preimplantation estrogen treatment has been shown to impact on the uterus, leading to an abnormal endometrial function, a perturbed intrauterine environment, and a disturbed embryo–maternal communication. Various effects, ranging from subtle changes in endometrial gene expression to pregnancy losses, have been described in mice [15–18] and pigs [19–25]. The observed alterations in the endometrium involved mRNA [15, 17, 19, 20] and protein [26, 27] expression changes, as well as differences in its secretory activity [23, 24, 28] and morphology [15, 17, 23]. In addition, estrogens reaching the embryo may also exert direct effects as indicated by in vitro studies [29–31].

The timing of the estrogen exposure seems to be highly important. In pigs, a short treatment on days 9–10 or 7–10 had strong disrupting capacity [19–25], whereas treatment after day 10 of pregnancy demonstrated none or only minor alterations [21, 32, 33]. Estrogen treatment only during the preimplantation period resulted in sex-specific changes on sexual development in murine offspring [13, 14]. This may be attributed to differences between the sexes prevailing during the preimplantation period including changes in the methylome and the metabolome [34–38]. Additionally, gestational low-dose E2 treatment in pigs has been shown to alter body composition in the male offspring [10], while bone development was affected in females [39].

The pharmacokinetic behavior of estrogens differs depending on the route of exposure and contributes to possible direct mechanisms involved in early EDC effects on tissues such as the uterus [40–43]. In the pig, the gut wall largely metabolizes orally ingested E2, while further rapid transformation occurs in the liver [43–45]. Estrogens are transported to the bile and subjected to an enterohepatic cycling [45, 46]. Thus, no [43, 45] or only low [10] concentrations of E2 are detected in the circulation following oral administration. In pigs, the predominant metabolite is estrone-glucuronide (E1G), while lower concentrations of E2-glucoronide, E1-sulfate, E1, and other minor metabolites prevail [43–45]. The main route of excretion of estrogens in the pig is the urine [47]. E1G and other glucuronides together with sulfates of E1 and E2 account for more than 90% of the metabolized E2 [43, 44].

In pigs, the circulating unconjugated estrogens are mainly bound to albumin [48, 49] and are distributed throughout the body and its tissues. Both retention and accumulation depend, at least in part, on the cellular status, such as the estrogen receptor content, which also determines potential cell-specific effects [40, 50–53]. The uterus is a major target organ where estrogens accumulate. In contrast to unconjugated estrogens, the conjugated forms possess little or no direct estrogenic activity; however, they also appear in tissues [54–56]. Although the liver is the main organ of estrogen metabolism, other tissues are able to conjugate and deconjugate estrogens likewise [54, 56, 57]. This has been particularly established for breast cancer cells converting sulfated estrogen, a major circulating conjugated form of estrogens in humans [54], into its free form, thus increasing their local amount of active estrogens [57].

In the present study, the main and most potent naturally occurring estrogen in females, namely E2, was used as potential EDC. As its pharmacokinetic behavior and mode of action through its classical and nonclassical receptors is thoroughly described [58, 59], it qualifies as model substance for estrogenic environmental low-dose exposures effects. Interestingly, the pig placenta produces considerable amounts of E2 during late gestation, and thus displays an environment likely comparable to the women [10, 60–62]. In rodents, circulating estrogens are lower during pregnancy.

We investigated the plasma elimination kinetics and tissue concentrations of E2 and its metabolites after oral intake of three distinct doses. We elucidated direct E2 effects on sows and sex-specific effects on blastocysts at day 10 of gestation by introducing a next-generation sequencing approach. To our knowledge, this is the first study, investigating in vivo effects of estrogens on the embryonic transcriptome.

Materials and methods

Animals and sampling

Study 1: E2 elimination kinetics

Male castrated piglets (approximately 20 weeks old) were used as most sensitive model because of lowest concentrations of endogenous E2 in order to being able to even detect small elevations in plasma estrogen concentrations. This was performed as described earlier [10]. They were fed a defined amount of E2 once to determine kinetics of plasma estrogen concentrations. In brief, the animals received a single oral E2 dose, either 0.025 (n = 3), 5 (n = 2), or 500 μg E2/kg body weight (bw) (n = 3), respectively, or an ethanol carrier only (n = 2). Blood samples were taken at 1 h or 15 min interval, centrifuged and ethylenediaminetetraacetic acid (EDTA) plasma was stored at −20°C.

Study 2: Direct maternal E2 exposure

This is the second part of a long-term large animal trial. In the first part, the sows had been exposed to E2 over the entire length of

gestation [10]. Due to management reasons, the sows underwent further breeding until this second part started, where the same sows were again allocated to the same treatment group as in the first part. All details about the complete study design have been published by van der Weijden et al. [63] in the supplementary information. The second part (here named "study 2") was conducted as follows. German Landrace sows (n = 4–6/treatment) were cycle synchronized using Altrenogest ReguMate® for 12 days, then Intergonan® (PMSG) at 750 iU was applied once the following evening, and Ovogest® (human chorion gonadotropin) at 750 iU was applied once 3.5 days later. The next day (day 0), all animals were inseminated with sperm of the same single Pietrain boar twice, in the morning and in the evening. From insemination until day 10, sows were orally exposed to different doses of E2 (1, 3, 5(10)-ESTRATRIEN-3, 17β-DIOL, Steraloids, Newport, USA), namely with 0.05, 10, and 1000 μg E2/kg bw daily, respectively, or with ethanol carrier only (control group). The E2 concentrations were selected according to reference values for humans [58] and have been reported earlier [10, 39]. The lowest dose corresponds to the "acceptable daily intake" (ADI), and the second low dose is close to the "no-observed-effect level" (NOEL). The high dose was integrated as positive control possibly reflecting e.g., a mistaken use of oral contraceptives during early pregnancy. Half the dose was fed in the morning and the other half in the evening. One hour after ingestion of the last dose, sows were slaughtered at day 10 of pregnancy. The uterus was removed and embryos were flushed from the uterus using 10 ml phosphate-buffered saline (PBS, autoclaved, pH 7.4) per horn. These first flushings were collected, centrifuged, and stored at −20°C. Each horn was again flushed using 50 ml to ensure that all embryos were recovered. All embryos were transferred into a petri dish containing PBS. Single embryos as well as tissue samples (from endometrium, skeletal muscle, and heart) were shock frozen in liquid nitrogen and stored at −80°C. As published earlier [64], all embryos were hatched spherical blastocysts according to the expected stage and contained an embryonic disc. There was neither significant difference in number (overall n = 230, $P = 0.33$) nor in size (2.2 mm ± 0.1 mm (mean ± SEM); $P = 0.08$) of the embryos. EDTA plasma was obtained from blood samples after centrifugation at 4°C. Bile were collected and stored along with the plasma samples at −20°C. Animals were only included in the RNA sequencing (RNA-Seq) analyses if embryos were at the blastocyst stage. Three sows of different treatment groups were excluded depicting only unfertilized oocytes.

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

Hormone analyses

Plasma concentrations of E2, total estrogens (estrone (E1), E2, and estradiol-17α), testosterone, and progesterone were analyzed by enzyme immunoassay (EIA) as described recently [39]. Total estrogens were measured to estimate E1, the main unconjugated metabolite in pigs, as well as to approximate the amount of conjugated estrogen metabolites, as there was no E1 antibody available at our institute. In order to analyze conjugated steroid hormones, an additional step was added to the protocol. During the steroid extraction process after phase separation, the frozen phase including the conjugated steroids was defrosted at room temperature and further processed. Two milliliter hydrolysis buffer (50 mM Na-acetate-buffer), containing 16 μl of the enzyme mixture β-glucuronidase/arylsulfatase (Merck KGaA, Darmstadt, Germany), was added. After an incubation step overnight at room temperature, 6 ml tert-butylmethylether/petrolether 30/70 v/v was added. The samples were agitated for 2 h at room temperature, rested for half an hour at room temperature, and were then frozen overnight at −60°C. The decanted supernatant was then dried in a vacuum concentrator and 500 μl assay buffer was added to the residues. Subsequently, the EIAs were performed.

In order to analyze hormone concentrations in endometrial, muscle, and heart tissue, frozen tissue aliquots were grounded using a pestle and a mortar on dry ice. One hundred milligram were transferred into an extraction tube, 500 μl of physiologic salt solution was added, and samples were stored at −20°C. Next, an ether extraction was performed. Tert-butylmethylether/petrolether 30/70 v/v (6.5 ml) was added to each sample and agitated overnight. After phase separation, they were frozen over the weekend at −60°C. The decanted supernatant was then dried in a vacuum concentrator, 500 μl assay buffer were added, and the EIAs were performed. In addition, after the phase separation, the frozen part was used to obtain the conjugated steroids as described above. Minor modifications were integrated for bile. The hydrolysis buffer was used at 500 mM and with twice the amount of enzyme mixture. After the incubation overnight at room temperature and for another 2 h at 37°C, 6.5 ml tert-butylmethylether/petrolether 30/70 v/v was added.

RNA and DNA extraction

Total RNA from endometrial samples of pregnant sows was extracted according to the manufacturer's recommendations using TRIzol (Invitrogen, Karlsruhe, Germany) (n = 4 per treatment group, except for the ADI dose group (n = 3), where a repeated tissue extraction was performed). Next, total RNA and DNA from single embryos were extracted using the AllPrep RNA/DNA Micro Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for cells with slight modifications. In brief, 700 μl of Buffer RLT Plus supplemented with 1% β-mercaptoethanol was added to the frozen embryo. Disruption was achieved by pipetting up and down and by a single brief vortexing. Homogenization was performed using a syringe and needle. After centrifugation of the lysate using a DNA spin column, the column was stored at 4°C, while the flowthrough was processed following the protocol for "purification of total RNA containing small RNAs from cells." In order to improve RNA purity, the column was incubated with Buffer RPE at step D3 and D4 before centrifugation for 4 and 2 min, respectively. RNA elution was repeated using the first eluate to increase the final concentration. The DNA was purified subsequently. Samples were immediately put on ice. RNA and DNA samples were stored at −80°C and −20°C, respectively. Purity and quantity were assessed spectrophotometrically using the NanoDrop 1000 (peqLab, Erlangen, Germany). Additionally, RNA quantity of embryos was determined using the Qubit (Invitrogen) with the Qubit™ RNA BR Assay. RNA integrity was measured by means of the Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany) with the RNA 6000 Nano Kit (Agilent). The mean RNA Integrity Number of the endometrial samples and the embryos were at 9.3 ± 0.3 (±SD) and 9.7 ± 0.3 (±SD), respectively.

Sex determination of embryos

At least four embryos per sow from four sows per treatment group (control, NOEL, high dose) were used for the concurrent RNA and DNA extraction (n = 65). Unfortunately, the number of embryos of an appropriate quality for analysis was limited in the ADI

group. Therefore, these needed to be excluded from the analysis. The sex of the embryos was determined by means of a quantitative real-time polymerase chain reaction (qPCR) using the DNA with primers specific for the y- chromosomal gene sex determining region Y (*SRY*) in addition to primers for the autosomal histone gene H3 histone family member 3A (*H3F3A*) (Supplemental Table S1). Primers were designed using NCBI primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and their specificity was checked using gel electrophoresis. The SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen) was used on the LightCycler 2.0 (Roche Diagnostics GmbH, Mannheim, Germany). One microliter of DNA was added to 9 μl of the mastermix (5 μl of 2x SYBR® Green Reaction Mix (includes 0.4 mM of each dNTP and 6 mM $MgSO_4$), 2.4 μl of nuclease free water, 1 μl of 20x Bovine Serum Albumin (ultrapure, non-acetylated) (1 mg/ml), 0.2 μl of forward primer [20 μM], 0.2 μl of reverse primer [20 μM], and 0.2 μl of SuperScript® III RT/Platinum® *Taq* Mix (includes RNaseOUT™ Ribonuclease Inhibitor)). The program—reverse transcription at 50°C for 10 min, then 95°C for 2 min, followed by 50 amplification cycles with 5 s at 95°C, 10 s at 60°C, and 15 s at 72°C; the melting curve was performed from 55 to 95°C with 0.1°C/s, then samples were cooled to 40°C—was run with each sample in duplicate. A positive control DNA was included in each run. In the beginning, each primer pair was checked for its specificity by sequencing of the amplification product. Then, the melting point was used to identify the product. The following RNA-Seq analysis (n = 36) demonstrated that with exception of one embryo the gender had been correctly assigned.

RNA-Sequencing and data analyses of endometrium and embryos

The library preparation starting from 125 ng total RNA of each endometrial sample was performed with the Encore Complete RNA-Seq Multiplex System IB (NuGEN, AC Leek, The Netherlands) following the manufacturer's protocol. Quality and quantity were assessed with Qubit (Invitrogen) and the Bioanalyzer 2100 (Agilent). The libraries were sequenced on a Genome Analyzer IIx system (Illumina). The cBot single Read Cluster Generation kit (Illumina) and 36 Cycle Sequencing Kit v4 (Illumina) were applied to generate single-end reads (74 bp). A multiplex of the 16 samples was analyzed on four lanes. Demultiplexing was conducted by using the barcode sequence consisting of four nucleotides at the beginning of each read.

Regarding the RNA-Seq of single embryos, six embryos were selected per sex and treatment group (control, NOEL, high dose; n = 36) as well as at least one male and one female embryo per sow (n = 4 per treatment group). However, as one suspected male embryo turned out to be female in the NOEL dose group, one sow had three female and no male embryo. Thus, the NOEL group consists of five male embryos from three different sows and seven female embryos from four different sows, while all other groups comprise six embryos from four different sows. Library preparation with 100 ng RNA per sample was performed using the TruSeq Stranded mRNA Sample Prep Kit (Illumina, Inc, CA, USA) according to the manufacturer's protocol. The RNA quality and quantity were assessed with Qubit and the Bioanalyzer 2100. The pooled libraries were used for cluster generation with the TruSeq SR Cluster Kit v3-cBot-HS (Illumina). Single-end 100 bp reads were produced on an Illumina HiSeq 2000 with the TruSeq SBS Kit v3-HS (Illumina).

The RNA-Seq data were analyzed using Genomatix (Genomatix Software GmbH, Munich, Germany). Mapping was performed on the Genomatix Mining Station (Sus scrofa, Genome Library, NCBI build 4, ElDorado Version 12–2012, mapping type "fast", alignment minimum quality 92%).

The mapped reads of the endometrium were analyzed for differential expression on the Genomatix Genome Analyzer using edgeR with default settings (*P*-value threshold 0.05, with adjusted *P*-value, and log2 fold change of > = or < = 1). Further handling of the significantly regulated transcripts was done with Galaxy [65] installed at the Gene Center (LMU Munich, Germany, AG Blum). The cut-off for defining a gene as being transcribed in a sample was set to having at least 10 reads. At least three out of four samples of one treatment group had to have more than nine reads for not being discarded in order to allow genes to be turned on or off by the treatment.

The mapped reads of the blastocysts were analyzed differently, as due to the higher number of samples per treatment group (n ≥ 5). Thus, analysis of differential gene expression was performed with the BioConductor package EdgeR using the "estimateGLMRobustDisp" [66]. A false discovery rate (FDR) of 5% was used as threshold for significance of differential gene expression. Venn diagrams were generated for genes from all four comparisons with *P*-values smaller than 0.0001 ($P < 0.0001$) including a fold change cut-off of 1.5 using the webtool Venny 2.1 [67]. Hierarchical clustering (HCL) was performed by the use of MeV_4_8 v10.2 [68] for the same genes used for the Venn diagrams.

RNA-sequencing data from both experiments have been deposited in the ArrayExpress database at EMBL-EBI under the accession number E-MTAB-6242 (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6242) for the endometrium and E-MTAB-6263 (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6263) for the embryos.

A functional annotation clustering was computed using the database for annotation, visualization, and integrated discovery (DAVID 6.8) (https://david.ncifcrf.gov) [69] in order to visualize biological motifs. *Homo sapiens* was used as reference species. A gene list containing the gene symbols of differentially expressed genes (DEG) were clustered based on the assignment of the genes to the gene ontology (GO) FAT terms of biological process, cellular component, and molecular function. Some of the default options were adjusted, amongst others due to the relatively small number of DEG (similarity threshold = 0.6, initial and final group membership = 2, EASE score = 0.2). Only DEG where gene symbols were available and annotated in the database could be analyzed.

Technical validation of the endometrial RNA-sequencing data

A subset of genes that were differentially expressed according to the RNA-Seq analysis was additionally technically validated by a two-step reverse transcription qPCR (RT-qPCR). In many cases, more than one transcript of a single gene was shown to be regulated in the RNA-Seq analysis. If possible, primers were designed for each transcript. However, often this was not possible. Therefore, one qPCR primer pair may fit to more than one transcript determined in the RNA-Seq analysis. This is indicated in the list of all primers (Supplemental Table S1) in a separate column containing the accession number of each transcript fitting to the respective primer pair. The identical RNA samples were used. They were reverse transcribed into cDNA as described by Klein and colleagues [70]. Quantitative real-time PCR was conducted using the SsoFast™ EvaGreen® Supermix (Bio-Rad, Munich, Germany) along with 384 well plates

and a final volume of 10 μl per sample. The master mix consisted of 5 μl SsoFast™ EvaGreen® Supermix, 0.2 μl of the forward primer [20 μM], 0.2 μl of the reverse primer [20 μM], 0.07 μl Visi-Blue™ (TATAA Biocenter AB, Goteborg, Sweden), and 3.53 μl RNase-free water. One microliter of cDNA was added into each well containing the master mix, while 1 μl of nuclease-free water and 1 μl of an endometrial cDNA mixture served as negative and positive control, respectively. Quantitative real-time PCR runs were performed on the CFX384™ Real-Time PCR Detection System (Bio-Rad) with the following settings of 30 s at 95°C, 40 cycles with 5 s at 95°C, and 10 s at 60°C to 64°C depending on the primers annealing temperature (Supplemental Table S1); the melting curve was performed from 65°C to 95°C with steps of 0.5°C and 5 s per increment; finally, the plate was cooled to 4°C. A qPCR product from each set of primers was sequenced to confirm product identity. Subsequently, the melting curve analysis with the specific melting point of each product was used. Data analysis using the obtained Cq values was performed as previously described [71]. For relative quantification, four reference genes were selected using NormFinder (GenEx Pro Ver 4.3.4 software multiD Analyses AB, Gothenburg, Sweden), namely *H3F3A*, ubiquitin B (*UBB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) (Supplemental Table S1).

Statistics

The SigmaPlot program package release 11.0 (SPSS, Chicago, IL, USA) was used for all statistical analyses and graphical presentations except for the statistic evaluation of the RNA-Seq data. The logarithmic values of steroid hormone concentrations from plasma, bile, endometrium, skeletal muscle, and heart tissue samples were analyzed using ANOVA with the Dunnett post hoc test for comparison of the three treatment groups with the control group. In order to compare the number of male and female embryos between the treatment groups, a contingency table was made and subsequently a χ^2 test was applied. The RNA-Seq data from the endometrium as well as from the embryos were statistically analyzed using the edgeR algorithm of the Genomatix Genome Analyzer. Thus, the treatment groups were compared with the control group and the male and female data sets from the same treatment group were tested for differential gene expression, respectively. For the statistical analyses of the normalized qPCR data t-tests were applied between the treatment group that had been found significantly regulated in the RNA-Seq experiment and the control group. Regarding the linear regression analysis and its graphical presentation between the fold changes of the qPCR results and the respective fold changes from the RNA-Seq experiment, if values for more than one transcript were available from the RNA-Seq data that were simultaneously amplified with one primer pair, the mean was used. Thus, for each qPCR fold change value there was one corresponding value from the RNA-Seq experiment. The data are depicted as mean ± SEM. Significant difference was assumed with $P < 0.05$.

Results

Elimination kinetics in male castrated pigs

The blood plasma concentrations before and after feeding the respective dose of E2 (0, 0.025, 5, and 500 μg/kg bw, respectively) were determined. E2 concentrations were measured and published earlier [10]. In brief, the two low doses did not lead to any notable increase in plasma E2 concentrations with average values of 5.6 ± 1.3 pg/ml (mean ± SEM) and 5.5 ± 1.9 pg/ml, respectively. The control animals depicted an average concentration of 5.3 ± 2.5 pg/ml. The high dose showed a peak after 15 min with an average of 77.3 ± 23.9 pg/ml. Plasma E2 concentrations did not decline to basic levels but remained elevated from 6 to 12 h at 23.5 ± 4.0 pg/ml. Yet unpublished data showed that even after 21 to 24 h, there was still an average plasma concentration of 22.2 ± 7.5 pg/ml.

The animals receiving the high dose showed an increase in plasma concentration of total estrogens with a first maximum at 15 min with 115.3 ± 26.6 pg/ml, and a second maximum at 2 h and 45 min with 115.8 ± 70.4 pg/ml (Figure 1A). The concentration remained elevated over 24 h with a plateau phase at about 46 pg/ml. The other treatment groups did not show an increase after feeding and revealed average concentrations of 21 ± 5 pg/ml (control), 10 ± 1 pg/ml (ADI), and 11 ± 2 pg/ml (NOEL), respectively.

The pattern for conjugated E2 is shown in Figure 1B. The high-dose group reached a first maximum of 9523.9 ± 5318.7 pg/ml at half an hour, a second maximum at 2 h and 45 min with 10 232.3 ± 7213.9 pg/ml, and decreased then to a plateau phase at about 4200 pg/ml. In NOEL gilts, there was also an increase reaching a maximum at 45 min with 335.1 ± 207.1 pg/ml, then it declined to almost basal levels at 3 h. The ADI group depicted 64 ± 28 pg/ml, and the control group had mean concentrations of 40 ± 12 pg/ml.

The conjugated total estrogens showed a similar pattern (Figure 1C). The high-dose group depicted a first maximum of 65 128.7 ± 27 963.6 pg/ml at 30 min and a second maximum at 2 h and 45 min with 91 262.3 ± 64 961.1 pg/ml. Again, the plateau phase lasted for at least 24 h at about 33 000 pg/ml. Neither the ADI nor the control animals depicted an increase. Average concentrations were 49.3 ± 8.7 and 51.6 ± 9.0 pg/ml, respectively. The NOEL animals showed a maximum of 2509.9 ± 1456.3 pg/ml after 45 min. In addition, there was a plateau phase at about 500 pg/ml.

Steroid hormones at day 10 of pregnancy

One hour after feeding one half of the daily dose, all analyzed free and conjugated estrogens showed significantly elevated estrogen concentrations in the high-dose group (Table 1). Selected ones also had significantly elevated total estrogens in the NOEL group.

The plasma concentrations of E2 were 3-fold higher in the high-dose group compared to the controls ($P = 0.003$). The respective fold change differences of significantly altered estrogen concentrations compared to the control animals are shown in Table 2. The amounts of total estrogens were also significantly altered ($P < 0.001$) with 3-fold and 17-fold higher concentrations in the NOEL dose group and the high-dose group, respectively.

Concurrently, high concentrations of E2 ($P < 0.001$) and total estrogens ($P < 0.001$) were determined in the bile after feeding the high dose (Table 1). The concentration was about 2500-fold and about 3100-fold higher for E2 and total estrogens, respectively (Table 2). Similar to plasma, total estrogens were also significantly higher in the bile from the NOEL dose group with 49-fold higher concentrations compared to the control animals (Table 2). Thus, large quantities of unconjugated estrogens appeared in the bile, either as E2 or after conversion as E1.

In the endometrium, heart, and skeletal muscle, E2 and total estrogens were significantly higher in the high-dose group, with increases of about 15-fold and about 150-fold, respectively (Table 2). Additionally, in the NOEL dose group, total estrogens

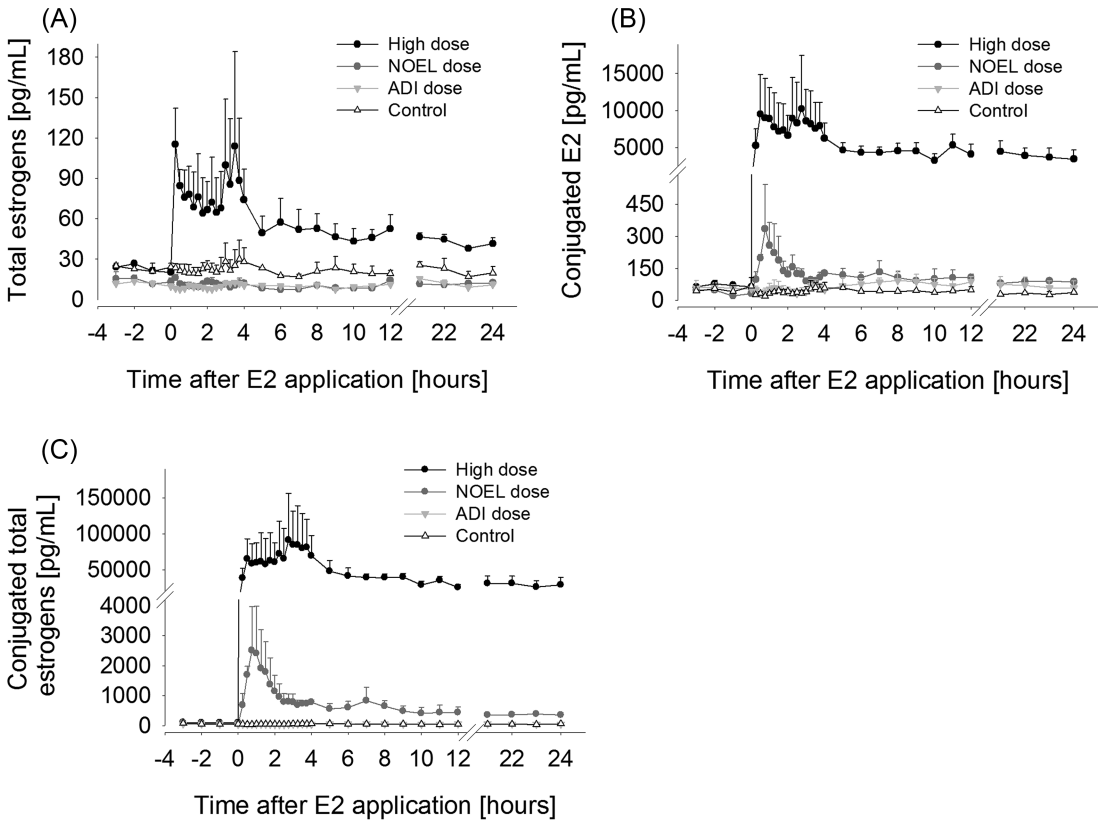

Figure 1. Plasma kinetics of distinct oral doses of E2 in male castrated pigs. There were four treatment groups (0, 0.025, 5, and 500 μg E2/kg bw, respectively); the two low E2 doses represent half of the daily dose of the ADI (acceptable daily intake) and close to the NOEL (no-observed-effect level) as announced for humans; similarly, half of the daily dose of the high-dose group as applied in the study 2 was fed. Plasma total estrogen (A), conjugated E2 (B), and conjugated total estrogen (C) concentrations are depicted as mean ± SEM (n = 2–3/treatment group).

were significantly 6- and 5- fold higher in the endometrium and the heart, respectively.

In the bile, where the concentrations of conjugated estrogens exceeded the unconjugated forms, the relative increase (Table 2) was much more pronounced for the unconjugated forms. The conjugated forms had only about 800-fold and about 400-fold higher conjugated E2 and conjugated total estrogens, respectively, in the high-dose group compared to the controls. In contrast, in the plasma, the increase of conjugated E2 and conjugated total estrogens with 361-fold and about 2300-fold, respectively, was much more pronounced compared to the unconjugated forms (Table 2).

Elevated concentrations of conjugated estrogens were detected in the tissue samples (Table 1). The increase in the high-dose group compared to the control group was in a similar range in all three tissues with about 10-fold and about 100-fold for conjugated E2 and conjugated total estrogens, respectively, thus showing a slightly lower increase compared to the unconjugated estrogens (Table 2).

Overall, there were marked increases in the high-dose group regarding all analytes and considerable changes occurred in the NOEL dose group while no effects were found in the animals fed the ADI dose.

Progesterone and testosterone were similarly analyzed. There were no significant differences of progesterone ($P > 0.5$) in plasma, bile, and tissues (endometrium, skeletal muscle, heart). The average concentrations were 14.1 ± 1.7 ng/ml in the plasma, 33 474.3 ± 13 042.4 ng/ml in the bile, 28.1 ± 1.8 ng/g in the endometrium, 46.5 ± 3.0 ng/g, in the skeletal muscle, and 94.3 ± 9.4 ng/g in the heart. Testosterone showed an overall significant difference in the plasma samples (41.1 ± 7.1 pg/g (control), 65.8 ± 7.1 pg/g (ADI), 36.6 ± 6.4 pg/g (NOEL), and 29.8 ± 4.7 pg/g (high dose); $P = 0.03$), and significantly higher values in the high-dose group compared to the control animals in skeletal muscle tissue (82.5 ± 10.3 pg/g (control), 88.6 ± 10.0 pg/g (ADI), 104.8 ± 21.1 pg/g (NOEL), and 181.5 ± 29.1 pg/g (high); $P = 0.02$). Testosterone was neither different in the bile (1805.8 ± 464.8 pg/ml, $P = 0.6$) nor in the heart (281.0 ± 86.7 pg/g, $P = 0.5$).

Embryo sexing

The number of embryos per sex and treatment group is shown in the contingency table (Table 3). The χ^2 test depicted that the proportion of male and female embryos was not statistically significantly associated with treatment dose ($P = 0.892$).

Effects on gene expression in endometrium

Differentially expressed genes ($P < 0.05$) were determined in the endometrium of all treatment groups resulting in 14, 17, and 27 DEG in the ADI, NOEL, and the high-dose group compared to the control, respectively. Thus, the highest dose revealed the highest number of regulated genes. Most of the genes were upregulated, and only few overlapping genes between the different E2 treatment groups were

Table 1. Hormone concentrations after continous E2 treatment and feeding of the last dose 1 h before slaughter at day 10 of pregnancy.

	Control	ADI	NOEL	High dose	Overall *P*-value
Plasma					
E2 [pg/ml]	19.5 ± 3.2	21.9 ± 5.4	25.5 ± 14.8	**67.2 ± 7.3***	0.003
Total E [pg/ml]	25.3 ± 2.8	30.4 ± 5.0	**71.9 ± 23.9***	**419.5 ± 80.8***	<0.001
Conj. E2 [pg/ml]	49.9 ± 5.1	51.1 ± 1.7	**367.7 ± 35.7***	**18 005.8 ± 4706.2***	<0.001
Conj. total E [pg/ml]	95.5 ± 33.5	109.6 ± 24.9	**3503.3 ± 449.2***	**222 798.4 ± 51803.5***	<0.001
Bile					
E2 [ng/ml]	0.2 ± 0.08	0.2 ± 0.1	2.6 ± 2.1	**488.6 ± 431.9***	<0.001
Total E [ng/ml]	0.7 ± 0.3	1.3 ± 0.8	**34.1 ± 30.5***	**2188.5 ± 1400.4***	<0.001
Conj. E2 [ng/ml]	2.5 ± 0.7	2.8 ± 0.7	**27.0 ± 9.9***	**1992.8 ± 1083.4***	<0.001
Conj. total E [ng/ml]	20.4 ± 8.0	17.3 ± 3.6	**453.0 ± 160.1***	**8432.0 ± 1568.0***	<0.001
Endometrium					
Conj. E2 [pg/g]	135.8 ± 16.0	98 ± 14.0	214.4 ± 53.5	**2951.2 ± 1149.2***	<0.001
Conj. total E [pg/g]	90.5 ± 14.7	73.5 ± 16.8	**834.6 ± 168.2***	**9497.6 ± 2178.1***	<0.001
Skeletal muscle					
E2 [pg/g]	14.4 ± 3.2	131.5 ± 110.6	29.8 ± 4.4	**302.6 ± 164.0***	0.007
Total E [pg/g]	14.5 ± 3.3	86.5 ± 74.0	36.1 ± 3.4	**1449.7 ± 663.7***	<0.001
Conj. E2 [pg/g]	93.5 ± 12.4	176.4 ± 31.3	158.5 ± 23.1	**849.6 ± 315.9***	<0.001
Conj. total E [pg/g]	56.4 ± 2.3	108.0 ± 24.6	122.7 ± 16.8	**5654.9 ± 2844.3***	<0.001
Heart muscle					
E2 [pg/g]	129.8 ± 93.8	24.4 ± 3.4	50.2 ± 10.3	**664.5 ± 343.7***	0.002
Total E [pg/g]	20.1 ± 9.8	28.7 ± 16.0	**100.8 ± 23.1***	**4586.7 ± 1774.2***	<0.001
Conj. E2 [pg/g]	274.4 ± 128.3	147.0 ± 51.7	294.9 ± 71.6	**1431.1 ± 475.9***	0.001
Conj. total E [pg/g]	105.9 ± 19.8	130.5 ± 36.5	**310.4 ± 56.7***	**9567.4 ± 3449.5***	<0.001

E2 = estradiol-17β, conj. = conjugated, E = estrogens (E2β, E2α, E1). Data are shown as mean ± SEM (n = 4–6). A significant difference between a treatment group and the control group is indicated by bold letters and an asterisk ($P < 0.05$).

Table 2. Fold changes of treatment groups vs. control group, where significantly higher concentrations occurred in the respective treatment group compared to control animals.

		E2 [x-fold]	Total estrogens [x-fold]	Conj. E2 [x-fold]	Conj. Total estrogens [x-fold]
Plasma	NOEL/Ctrl	–	3	7	37
	High/Ctrl	3	17	361	2332
Bile	NOEL/Ctrl	–	49	11	22
	High/Ctrl	2489	3152	796	414
Endometrium	NOEL/Ctrl	–	6	–	9
	High/Ctrl	27	161	22	105
Skeletal muscle	NOEL/Ctrl	–	–	–	–
	High/Ctrl	21	100	9	100
Heart muscle	NOEL/Ctrl	–	5	–	3
	High/Ctrl	5	228	5	90

Table 3. Distribution of male and female embryos per treatment group.

Treatment	Control	NOEL	High dose	Total
Sex				
Male	14	11	12	37
Female	12	7	9	28
Total	26	28	21	65

found (Figure 2). The respective genes are listed in Supplemental Table S2.

For the functional annotation analysis of all endometrial DEG (of the ADI, NOEL, and high-dose group comparisons in sum), there were 42 gene symbols from which 40 were found in the DAVID database. Sixteen clusters were formed. The 10 most enriched clusters are depicted in Table 4. In general, extracellular structure organization, response to stimulus, cell activation, multiple aspects of apoptosis, catalytic activities, regulation of transport, secretion, signaling and cell communication, developmental processes, metabolic processes, particularly including phosphorus metabolic processes, and regulation of immune system process are the most represented functional categories.

Validation of endometrial RNA-sequencing data

Overall, 21 genes including 23 transcripts, found to be differentially expressed in the RNA-Seq experiment, were technically validated by RT-qPCR using the identical samples. The results are shown in Table 5. The two datasets fit well, as the linear regression analysis revealed an overall significant correlation ($P < 0.001$; $R^2 = 0.505$, adj. $R^2 = 0.486$). In general, most genes differentially expressed in

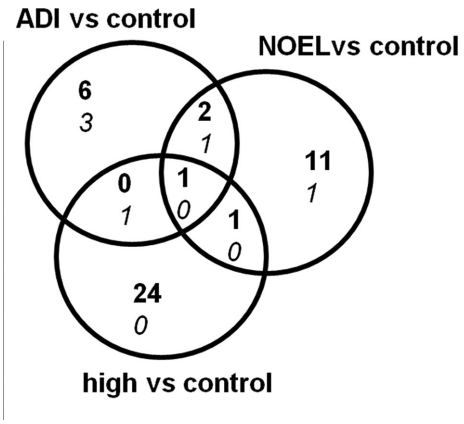

Figure 2. Venn diagram of the differentially expressed genes (DEG) in the endometrium. The number of DEG from the RNA-Seq experiment of sows treated with distinct doses of E2 until day 10 of pregnancy (n = 4 per treatment group). Bold letters indicate higher expression, italic letters indicate lower expression after E2 treatment compared to the control. ADI—acceptable daily intake, NOEL—no-observed-effect level.

the high-dose group could be validated. In the low-dose exposure groups, there were often similar fold changes between the two experimental approaches although in many cases the RT-qPCR data did not reach significance. This might also be due to the use of a *P*-value threshold of 0.05 in the RNA-Seq data analysis.

Effects on gene expression in day 10 embryos

By applying an FDR of 5%, 982 and 62 DEG were detected in the female blastocysts of the high-dose and the NOEL group compared to the controls, respectively. This included 373 down- and 609 upregulated genes in the high-dose group and 31 down- and 31 upregulated genes in the NOEL group. In the male blastocysts none and three DEG were found in the high-dose and the NOEL group compared to the controls, respectively. There were two downregulated and one upregulated transcripts. Thus, there was a more pronounced effect in the female embryos compared to males, demonstrating sex-specific effects of the E2 treatment. All regulated genes are named in Supplemental Table S3, while detailed transcript lists were deposited in the ArrayExpress database at EMBL-EBI under the accession number E-MTAB-6263 (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6263).

Concerning general sex-specific differences, an analysis with an FDR of 5% between male and female controls was performed. There were 35 DEG higher expressed in the male embryos, including 8 Y- and 7 X-chromosomal genes. In addition, 50 DEG were higher expressed in the female embryos, containing 33 X-chromosomal genes. All respective transcripts are shown in Supplemental Table S4.

In order to perform a comparison of the results from the different analyses between treatments and controls that is not biased by the algorithm calculating the correction for multiple testing, a cut-off for the nominal *P*-value of $P < 0.0001$ together with a fold change cut-off of 1.5 was used. These results are shown in Figure 3 (Supplemental Table S5). In the female embryos, there were 32 genes in the NOEL group, 18 with lower and 14 with higher expression after E2 treatment, while in the high-dose group there were 73 genes, 18 with lower and 55 with higher expression after E2 treatment. These overall 99 different DEG included only one X-chromosomal gene (Supplemental Table S6), integrator complex subunit 6 like (*INTS6L*, previously known as DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26B (*DDX26B*)), which was 2.7-fold higher expressed in the high-dose group compared to the control group. This indicates no specific treatment effect on the expression of X-chromosomal genes in the female embryos. Only few genes were detected for the male embryos with a total of nine and five genes in the NOEL and the high-dose group, respectively (Figure 3).

From the commonly regulated genes between treatment groups, there were four DEG regulated in the female NOEL and high-dose group, namely ral guanine nucleotide dissociation stimulator (*RALGDS)* and *LOC100624113* upregulated as well as vitamin D receptor *(VDR)* and selenoprotein I (*SELENOI*; previously known as ethanolaminephosphotransferase 1 (*EPT1))* downregulated. Furthermore, one differentially expressed gene (folate hydrolase 1 (*FOLH1*)) was upregulated in the female treatment groups as well as in the male NOEL group.

Hierarchical clustering of the same genes as used for the Venn diagram ($P < 0.0001$, fold change cut-off 1.5, Supplemental Tables S5 and S6), grouped according to their sex and the applied dose, is shown in Figure 4A, while in Figure 4B clustering was performed for samples as well as for genes. Both HCL indicate female embryos becoming more similar to male embryos due to E2 treatment, particularly evident in the high-dose group of female embryos.

The functional annotation clustering was performed for the female embryos, as males did not depict sufficient numbers of DEG. Again, the same genes as for the Venn diagram were used. Eighty-five DEG from the comparisons of treated female embryos (of the NOEL and high-dose group comparisons in sum) with female controls could be used having an official gene symbol. Seventy seven of these DEG were detected in the DAVID database leading to 22 clusters of which the 10 most enriched are shown in Table 6. These 10 clusters encompass biological processes involved in cell cycle, organic acid metabolism, catabolic processes particularly including organic substances, regulation of catalytic activity, multicellular organism development including embryonic organ development, signal transduction, as well as the positive regulation of biosynthetic processes and gene expression, cellular components of the endoplasmic reticulum, the cytoplasmic region and synapses, as well as molecular functions regarding nucleotide binding and hydrolase activity.

Discussion

The preimplantation phase is a sensitive time window where gestational administration of estrogens may affect dams and embryos [15, 16, 18–20, 23–25] possibly impacting on the offspring later in life [13, 14]. EDC such as certain low-dose estrogens are taken up orally through food [9, 58, 72, 73]. Therefore, we modeled the effects of a continuous oral low-dose E2 administration on the transcriptome of embryos. We delineated potential mechanisms of E2 tissue distribution and metabolism and focused on the endometrium as a major target of estrogens, which may impact on embryo development.

Fuerst and colleagues [10] have shown a fast increase in circulating plasma E2 after feeding a single high dose to male castrated pigs. The maximum concentration was measured after 15 min. This concentration declined rapidly and reached a plateau phase that still persisted with slightly elevated concentrations at 12 h. As by the definition of low dose and thus as intended by the study design, plasma E2 did not increases in the two low-dose groups. In contrast, in the present study analyzing the same animals, we observed a fast increase in plasma concentrations of conjugated estrogens after feeding not only the high dose of E2 but additionally in the NOEL group. Other studies introducing E2 into the stomach of prepubertal gilts have

Table 4. The 10 most enriched functional annotation clusters in the endometrium of sows treated with E2 (DEG of ADI, NOEL, and high dose in sum).

Annotation cluster	Enrichment score	GO terms[a]
1	1.65	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides (3); hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds (3)
2	1.45	apical part of cell (5); apical plasma membrane (3)
3	1.44	extracellular space (10); extracellular region (17); extracellular region part (14); membrane-bounded vesicle (12)
4	1.41	organ growth (4); embryonic morphogenesis (4); tube development (4); developmental growth (4)
5	1.30	oxidation-reduction process (6); reactive oxygen species metabolic process (3)
6	1.28	extracellular matrix organization (4); extracellular structure organization (4); cell activation (5); response to metal ion (3)
7	1.23	lung development (3); respiratory tube development (3); respiratory system development (3); tube development (4)
8	1.2	regulation of apoptotic process (9); regulation of programmed cell death (9); positive regulation of apoptotic process (6); positive regulation of programmed cell death (6); positive regulation of cell death (6); regulation of cell death (9); activation of cysteine-type endopeptidase activity involved in apoptotic process (3); apoptotic process (9); positive regulation of endocytosis (3); programmed cell death (9); positive regulation of cysteine-type endopeptidase activity involved in apoptotic process (3); negative regulation of signaling (7); positive regulation of secretion by cell (4); positive regulation of cysteine-type endopeptidase activity (3); positive regulation of protein phosphorylation (6); cellular response to growth factor stimulus (5); cell death (9); positive regulation of endopeptidase activity (3); positive regulation of secretion (4); positive regulation of phosphorylation (6); response to growth factor (5); positive regulation of peptidase activity (3); negative regulation of protein phosphorylation (4); regulation of intracellular signal transduction (8); negative regulation of multicellular organismal process (6); positive regulation of phosphorus metabolic process (6); positive regulation of phosphate metabolic process (6); negative regulation of phosphorylation (4); positive regulation of cellular protein metabolic process (7); regulation of cell communication (11); regulation of vesicle-mediated transport (4); negative regulation of signal transduction (6); negative regulation of intracellular signal transduction (4); regulation of cysteine-type endopeptidase activity involved in apoptotic process (3); secretion (6); regulation of signaling (11); regulation of endocytosis (3); positive regulation of protein secretion (3); positive regulation of protein metabolic process (7); positive regulation of protein modification process (6); positive regulation of MAPK cascade (4); regulation of signal transduction (10); regulation of cysteine-type endopeptidase activity (3); negative regulation of apoptotic process (5); negative regulation of programmed cell death (5); negative regulation of cell communication (6); positive regulation of cellular component organization (6); protein processing (3); MAPK cascade (5); positive regulation of establishment of protein localization (4); ERK1 and ERK2 cascade (3); response to organic substance (10); protein polymerization (3); egulation of ERK1 and ERK2 cascade (3); signal transduction by protein phosphorylation (5); negative regulation of phosphorus metabolic process (4); negative regulation of phosphate metabolic process (4); negative regulation of cell death (5); regulation of autophagy (3); cell activation (5); protein maturation (3); positive regulation of hydrolase activity (5); cellular protein complex assembly (4); positive regulation of transport (5); negative regulation of protein modification process (4); regulation of cellular response to stress (4); regulation of protein phosphorylation (6); secretion by cell (5); cellular response to organic substance (8); negative regulation of response to stimulus (6); cellular response to stress (7); regulation of secretion by cell (4); phosphate-containing compound metabolic process (10); phosphorus metabolic process (10); cellular response to chemical stimulus (9); regulation of phosphorylation (6); intracellular signal transduction (9); protein complex assembly (6); regulation of MAPK cascade (4); protein complex biogenesis (6); regulation of secretion (4); response to oxygen-containing compound (6); positive regulation of proteolysis (3); vesicle-mediated transport (6); regulation of endopeptidase activity (3); nitrogen compound transport (4); positive regulation of cellular metabolic process (9); positive regulation of macromolecule metabolic process (9); regulation of cell proliferation (6); regulation of protein secretion (3); positive regulation of cell communication (6); positive regulation of catalytic activity (6); regulation of peptidase activity (3); positive regulation of signaling (6); signal release (3); regulation of phosphate metabolic process (6); regulation of phosphorus metabolic process (6)
9	1.09	negative regulation of multicellular organismal process (6); renal system development (3); urogenital system development (3)
10	1.04	positive regulation of cell–cell adhesion (4); positive regulation of cell adhesion (4); regulation of cell–cell adhesion (4); regulation of leukocyte activation (4); positive regulation of T-cell activation (3); positive regulation of leukocyte cell–cell adhesion (3); regulation of cell activation (4); response to lipid (5); cell activation (5); positive regulation of lymphocyte activation (3); regulation of T-cell activation (3); response to lipopolysaccharide (3); positive regulation of leukocyte activation (3); regulation of leukocyte cell–cell adhesion (3); response to molecule of bacterial origin (3); regulation of cell adhesion (4); positive regulation of cell activation (3); single organismal cell-cell adhesion (4); regulation of apoptotic signaling pathway (3); leukocyte activation (4); regulation of lymphocyte activation (3); single organism cell adhesion (4)

[a]Number of genes are shown in brackets.

Table 5. Comparison of RNASeq and qPCR results for selected transcripts of the endometrial samples.

Treatment group			RNASeq results[b]						qPCR results[b]					
			High dose		NOEL		ADI		High dose		NOEL		ADI	
Ssc gene name	Ssc gene symbol[c]	Accession number[a]	Fold	*P*-value	Fold	*P*-value	Fold	*P*-value	Fold	*P*-value	Fold	*P*-value	Fold	*P*-value
Dual specificity phosphatase 6	*LOC100622968 (DUSP6)*	ENSSSCT00000032467	2.6	0.08	–	–	–	–	2.2	**0.02**	–	–	–	–
		XM_003361537	2.4	**<0.001**	–	–	–	–						
Vanin 1	*VNN1*	NM_214133	7.8	**0.01**	–	–	–	–	9.5	**0.03**	–	–	–	–
		ENSSSCT00000004620	9.0	**<0.001**	–	–	–	–	2.8	0.6				
Xanthine dehydrogenase	*XDH*	ENSSSCT00000009326	5.5	**0.02**	–	–	–	–	3.6	**0.01**	–	–	–	–
		XM_003481256	5.2	**0.02**	–	–	–	–						
Dehydrogenase/reductase 7	*DHRS7*	ENSSSCT00000005603	2.0	**0.01**	–	–	–	–	1.9	**0.02**	–	–	–	–
		NM_001244160	2.1	**0.01**	–	–	–	–						
PYD and CARD domain containing	*LOC100522011 (PYCARD)*	ENSSSCT00000008505	2.2	**0.009**	–	–	–	–	2.0	**0.02**	–	–	–	–
		XM_003124468	2.2	**0.009**	–	–	–	–						
Matrix metallopeptidase 8	*MMP8*	XM_003129816	2.6	**0.01**	–	–	–	–	2.8	**0.01**	–	–	–	–
Thiopurine S-methyltransferase	*TPMT*	NM_001243675	2.0	0.08	–	–	–	–	1.8	**0.009**	–	–	–	–
		ENSSSCT00000001168	2.1	**0.009**	–	–	–	–						
					–	–	–	–	2.1	**0.03**	–	–	–	–
	LOC100736750	ENSSSCT00000005135	4.4	**0.009**	–	–	–	–	6.5	**<0.001**	–	–	–	–
		XM_003480372	4.4	**0.009**	–	–	–	–						
Sulfotransferase family 2A member 1	*SULT2A1*	ENSSSCT00000003471	5.4	**0.009**	–	–	–	–	4.0	0.07	–	–	–	–
		NM_001037150	5.2	**0.009**	–	–	–	–			–	–	–	–
Cytochrome P450 2C49	*CYP2C49*	NM_214420	3.2	**<0.001**	–	–	–	–	2.9	0.08	–	–	–	–
T-cell activation RhoGTPase activating protein	*TAGAP*	ENSSSCT00000004481	2.3	**0.03**	–	–	–	–	1.8	0.07	–	–	–	–
Fin bud initiation factor homolog (zebrafish)	*LOC100620302 (FIBIN)*	ENSSSCT00000032511	7.2	0.7	21.7	**<0.001**	7.8	0.2	2.1	**0.02**	6.0	0.09	4.3	0.1
		XM_003360933	6.6	**0.02**	14.2	**<0.001**	7.0	**0.002**						
Vascular cell adhesion molecule 1	*VCAM1*	NM_213891	–	–	2.5	**0.002**	–	–	–	–	1.4	0.4	–	–
		ENSSSCT00000007515	–	–	2.5	**0.001**	–	–						
N-acylsphingosine amidohydrolase 1	*LOC100512932 (ASAH1)*	XM_003134187	–	–	2.2	**<0.001**	–	–	–	–	1.5	0.2	–	–
		ENSSSCT00000007660	–	–	2.2	**<0.001**	–	–						
Cyclin D binding myb-like transcription factor 1	*DMTF1*	XM_003130208	–	–	2.0	**0.02**	–	–	–	–	1.2	0.3	–	–
		ENSSSCT00000016768	–	–	2.2	**0.01**	–	–						
		XM_003357483	–	–	2.1	**0.02**	–	–						
Von Willebrand factor A domain containing 5A	*VWA5A*	ENSSSCT00000016541	–	–	2.2	0.06	1.9	0.3	–	–	2.9	**0.01**	2.8	**0.03**
		XM_003129995	–	–	2.1	**0.05**	2.2	0.06						
Proprotein convertase subtilisin/kexin type 9	*LOC100620501 (PCSK9)*	ENSSSCT00000029382	–	–	6.9	**<0.001**	5.0	**<0.001**	–	–	9.2	**0.03**	6.6	**0.03**

Table 5. *continued*

Ssc gene name	Treatment group: Ssc gene symbol[c]	Accession number[a]	RNASeq results[b]: High dose Fold	High dose P-value	NOEL Fold	NOEL P-value	ADI Fold	ADI P-value	qPCR results[b]: High dose Fold	High dose P-value	NOEL Fold	NOEL P-value	ADI Fold	ADI P-value
Ras association domain family member 4	*RASSF4*	NM_001243337	–	–	–2.8	**0.02**	–2.6	**0.04**	–	–	–1.5	0.1	–1.2	0.3
C-type lectin domain family 18, member A	*CLEC18A*	XM_003126879	–	–	–	–	–3.8	**0.004**	–	–	–	–	–3.4	0.06
Adhesion G protein-coupled receptor G2	*ADGRG2* (previously known as *GPR64*)	ENSSSCT00000029867	–	–	–	–	2.5	**0.02**	–	–	–	–	2.3	0.06
S100 calcium-binding protein G	*S100G*	ENSSSCT00000013286	–	–	–	–	4.2	**0.006**	–	–	–	–	2.9	0.3
		NM_214140	–	–	–	–	4.3	**0.006**						

[a]Different accession numbers indicate different transcripts that were regulated in the RNA-Seq analysis. Often, more than one transcript was measured with one primer pair by the qPCR approach.
[b]Significant differences between treatment groups and the control group are indicated by bold letters ($P < 0.05$).
[c]Gene symbols in brackets as retrieved using NCBI or Ensembl in June 2018.

demonstrated that E2 is rapidly converted into conjugated metabolites [43–45]. Analyses of blood from portal veins have shown that E2 had been mainly converted into E1G already by the gut wall [44, 45]. In addition to further processing in the liver, this may explain the fast and strong increase of the conjugated estrogens in the plasma found in the present study. Similar to data from Ruoff and colleagues [45], concentrations of free estrogens in peripheral plasma after exposure to the two low doses remained at basal levels. The second maximum after 2 and 3 h in the high-dose group probably resulted from both potentially remaining estrogens in the stomach [43] and from enterohepatic cycling of estrogens [45, 46]. The latter may also explain the plateau phases until 24 h after the application. Concordantly, plateau phases in the pig have been shown to prevail for more than 12 h [43, 74, 75]. A dose response with two peaks and a plateau phase after oral treatment is as well known from humans [42].

Although sows and castrated male piglets might differ in their metabolism of E2, important aspects can be drawn from the study on male piglets for the study in sows. At first, it shows that a very fast peak of E2 in plasma was observed, indicating that the amounts measured after 1 h in the sows were most probably below the maximum levels achieved after E2 intake. Similar to the results in castrated boars after a single E2 dose, all plasma estrogen concentrations were elevated in the high-dose group in continuously exposed sows at day 10 of pregnancy 1 h following the last oral exposition. Conjugated estrogens were as well elevated in the NOEL dose group. Second, E2 was continuously present at a plateau phase, still remaining elevated after 12 h. This leads to the assumption that when feeding the E2 dose to the sows every 12 h an accumulation may occur. As unconjugated total estrogens were elevated in the sows in the NOEL dose group, an accumulation due to the continuous exposure is indicated. Third, as even in this sensitive male model no low-dose effect on the plasma E2 concentration was observed—similar to the results in the sows—it is likely that the effects observed in the sows and embryos are due to E2 metabolites, possibly also the conjugated ones.

In the bile of the continually exposed sows, the relative increase with increasing doses of E2 was much more pronounced for the unconjugated compared to the conjugated estrogens. We assume that the ability of the liver to conjugate estrogens might have been saturated, so that a higher amount of remaining unconjugated estrogens was transported to the bile. The opposite was found in the plasma with a much stronger relative increase of conjugated estrogens compared to the unconjugated forms. Thus, quite some of the glucuronide and sulfate conjugates reached the plasma before being excreted by the kidney. Bottoms and colleagues have shown that by using an even higher dose as herein, the main route of excretion still remained urine with E1G as main metabolite [43].

The tissue concentrations of estrogens were determined as estrogens can hereby exert direct estrogenic effects on cellular functions. While plasma concentrations are rapidly cleared by the liver, tissues are capable of retaining the steroids for a longer time [40, 51]. Concordantly, in the sows at 1 h after the application, the relative increase in unconjugated estrogen concentrations observed in the NOEL and high dose was less pronounced in plasma compared to the increase found in the different tissues. The clearance can be tissue dependent [40, 50, 51, 76]. One main factor is the presence of estrogen receptors, as increasing amounts enable the tissue to better retain estrogens [40, 50–53]. The uterus as a major target organ for estrogens possesses particularly high amounts of estrogen receptors [40, 77]. Yet, little differences between the tissues analyzed herein were detected. One reason may be that the last E2 feeding had only

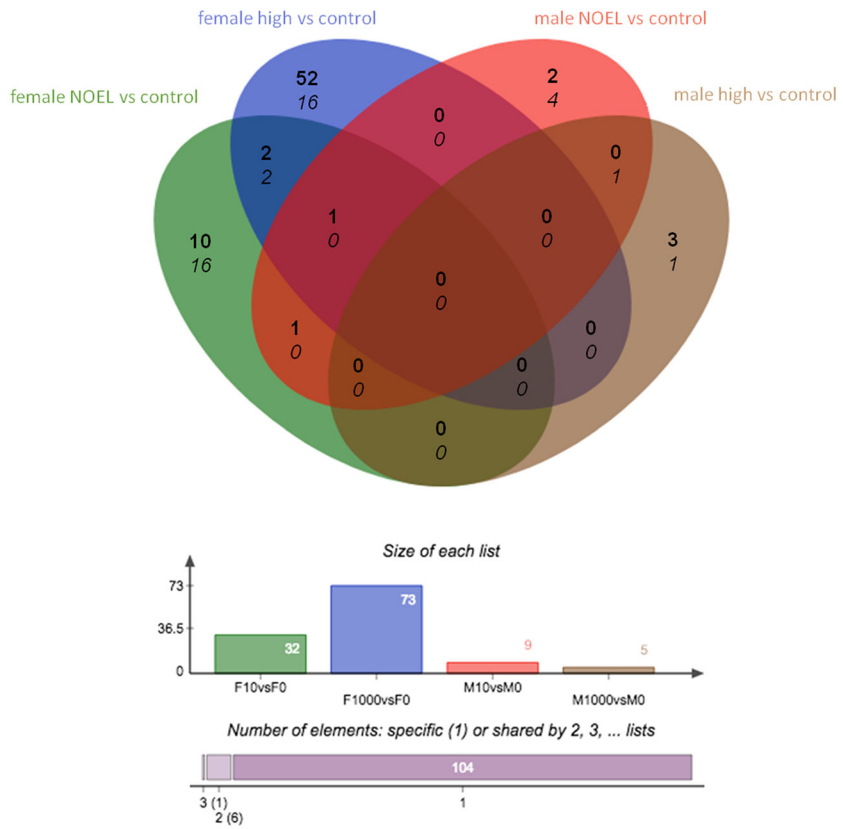

Figure 3. Venn diagram of RNA-Seq results in embryos. The number of genes with $P < 0.0001$ and a cut-off fold change of 1.5 from the RNA-Seq experiment of embryos (n = 5–7/treatment group) are shown. The sows were treated with carrier only, a dose close to the no-observed-effect level (NOEL) or a high dose of E2 (0, 10, and 1000 μg E2/kg bw/d, respectively) until day 10 of pregnancy. Bold letters indicate higher expression, italic letters indicate lower expression after E2 treatment compared to the control.

been applied 1 h before slaughter. The effect is assumed to increase with time, more pronounced from 2 h on, as indicated by other studies [51, 53].

Estrogens are important for uterine receptivity [1–3, 5, 24]. Disruption of pregnancy through estrogen exposure at days 9 and 10 has been associated with endometrial mRNA expression changes [19, 20, 24]. Using microarray analyses, Ross and colleagues [19] have found 9, 71, and 21 genes differentially expressed at day 10, 13, and 15, respectively. Thus, only relatively few alterations were detected. One reason may be the analysis of the complete endometrial tissue, as particular alterations only occur in certain cell types such as the epithelium [19, 24, 78]. Still, the E2-cypionate (E2C) treatment induced one downregulated and eight upregulated genes at day 10 [19]. This is comparable to the present study with overall 6 down- and 45 upregulated genes. Additionally, we detected a dose-dependent increase in regulated genes and even low-dose effects were demonstrated. Some identical genes were similarly upregulated in the high-dose group in the present study as compared to the study by Ross and colleagues [19]. Namely, retinol binding protein 4 (*RBP4*) was highly expressed at day 10, while vanin 2 (*VNN2*), sulfotransferase family 2A member 1 (*SULT2A1*), and solute carrier family 39 member 2 (*SLC39A2*) have been increased at day 13 but not at day 10 [19]. The genes affected by our E2 treatment were grouped into functional clusters, depicting some biological processes known to be involved in uterine preparation such as positive regulation of secretion and transport, extracellular region, and leukocyte activation [24, 79]. Some of the involved functional categories are similar to results from other studies such as processes related to cell death, transport, leukocyte activation, cell activation, and developmental processes [19, 20]. However, the comparison is difficult as the other studies had most alterations at gestational days 13 and 15 and led to embryonic losses. Thus, although we similarly found gene expression changes in the endometrium, mainly other genes were affected. In addition, the continuous E2 exposure did not lead to embryonic losses [10]. Interestingly, our preimplantation E2 exposure did not affect the endometrial miRNA expression profile [64].

The increasing concentrations of E2 and/or its metabolites reaching the uterus as depicted in this manuscript and elsewhere [64], respectively, are one possible explanation for the expression differences found in the NOEL and the high-dose group compared to the control animals. Despite a 160-fold increase in unconjugated total estrogens in the endometrium, there was no difference in the number of offspring at birth after treatment over the entire pregnancy [10]. Other studies with estrogenic treatments only at days 9– 10 or 7–10 of gestation demonstrated endometrial mRNA expression changes [19, 20, 24] and embryonic losses [22, 23, 25]. Thus, pigs, with estrogens as their maternal recognition signal, seem highly sensitive concerning treatments starting slightly before implantation, presumably due to a desynchronizing of the uterine and the embryonic development [24]. This phenomenon seemingly does not occur with continuous exposure even at a relative high dose as used in the present study and earlier [10].

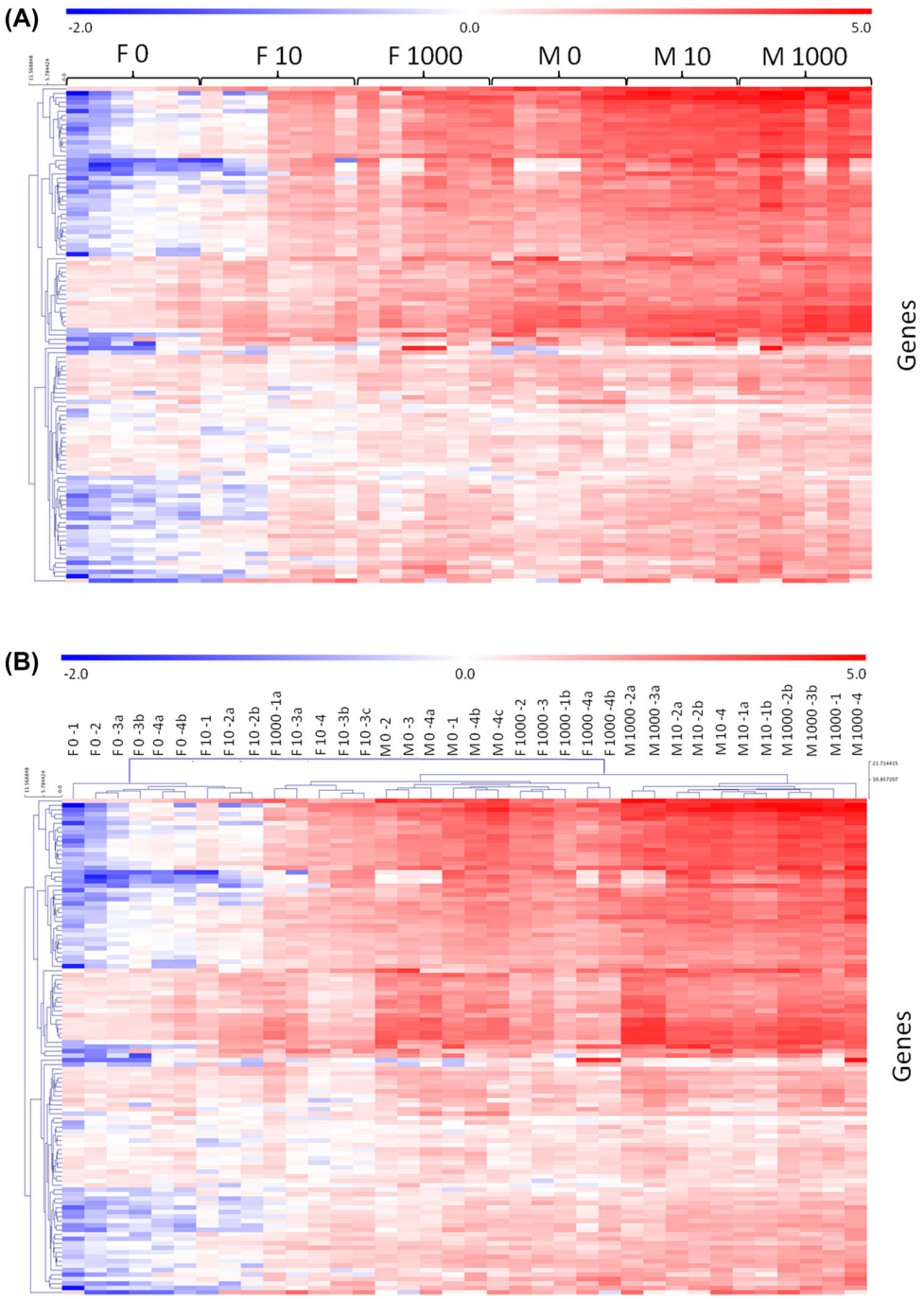

Figure 4. Hierarchical clustering of RNA-Seq results in embryos. Genes with $P < 0.0001$ and a cut-off fold change of 1.5 from the RNA-Seq experiment of embryos (n = 5–7/treatment group) are shown. The sows were treated with distinct doses of E2 until day 10 of pregnancy. Clustering of the genes only (A) and clustering of genes and samples (B) are depicted. F—female, M—male; treatment doses [μg/kg bw/d] are indicated by the letters 0, 10, and 1000; within each treatment group and sex differing mother sows are name with 1 to 4, while siblings additionally contain letters a to c.

To our knowledge, our results are the first to report sex-specific mRNA expression differences in blastocysts after in vivo estrogen exposure. There was a pronounced treatment effect on female but not male embryos. These sex-specific effects may be related to the fact that differences between the sex prevail during the preimplantation embryo development such as in their methylome, transcriptome, proteome, and metabolome [35–38]. In line, adaptations to environmental changes such as diet and nutrients have been shown to be sex-specific [35, 80].

Sex-specific analyses using microarrays revealed that in bovine in vitro produced blastocyst at day 7, one third of the genes showed sex-specific expression (FDR, $P < 0.05$) [38]. This is not reflected in our findings of 85 DEG between male and female control embryos. However, Bermejo-Alvarez et al. [38] also reported that by using a

Table 6. The 10 most enriched functional annotation clusters in female embryos of sows treated with E2 (DEG of NOEL and high dose in sum).

Annotation cluster	Enrichment score	GO terms[a]
1	1.69	organic acid metabolic process (10); carboxylic acid metabolic process (9); oxoacid metabolic process (9)
2	1.42	endoplasmic reticulum (14); endoplasmic reticulum part (11); nuclear outer membrane-endoplasmic reticulum membrane network (10); endoplasmic reticulum membrane (9)
3	1.26	cell cycle (13); cell cycle process (11)
4	1.25	excitatory synapse (5); postsynaptic specialization (4); postsynaptic density (4); postsynapse (5); synapse (7); synapse part (6)
5	1.20	cytoplasmic region (5); cell cortex (4)
6	1.13	cellular catabolic process (13); macromolecule catabolic process (9)
7	1.10	positive regulation of GTPase activity (8); regulation of GTPase activity (8); guanyl-nucleotide exchange factor activity (5); positive regulation of hydrolase activity (8); positive regulation of catalytic activity (11); GTPase regulator activity (4); small GTPase mediated signal transduction (5); nucleoside-triphosphatase regulator activity (4)
8	1.09	embryonic organ morphogenesis (5); inner ear development (4); ear development (4); inner ear morphogenesis (3); ear morphogenesis (3); sensory organ morphogenesis (4); embryonic organ development (5); renal system development (4); urogenital system development (4); sensory organ development (5)
9	1.07	positive regulation of cellular biosynthetic process (13); positive regulation of biosynthetic process (13); positive regulation of nitrogen compound metabolic process (13); positive regulation of gene expression (11)
10	1.07	organonitrogen compound catabolic process (6); small molecule catabolic process (4); carbohydrate derivative catabolic process (3)

[a]Number of genes are shown in brackets.

fold change larger than 2, only 53 transcripts were higher expressed in females and 2 in males. Next to general differences (in vitro—in vivo; bovine—porcine; day 7–day 10), in the present study, interestingly, the comparison between the control groups revealed a similar total number of transcripts with 41 higher expressed in males and 19 higher expressed in females when setting a cut-off fold change of 2 for the differential expressed transcripts. Heras et al. [80] also selected bovine blastocysts at day 7 and analyzed in vivo as well as in vitro (serum and serum-free) produced embryos after RNA-Seq with EdgeR (FDR corrected P-value < 0.05). Comparing male and female embryos of the same treatment group without a cut-off fold change or with a fold change of at least 2, they observed 225 and 119 (in vivo), 54 and 54 (in vitro with serum), and 54 and 48 (in vitro serum-free) DEG, respectively. Thus, they did not observe a large number of genes differentially regulated between the sexes, which is similar to the present study.

Strikingly, with increasing E2 dose, the gene expression profile of female embryos became more similar to the males. There are reports of sex-specific differences in the speed of embryo development [35]. Thus, there may be the possibility that the estrogen treatment led to alterations in the normal timing of the development of the female embryos making them appear more similar to the males at this point in time. Otherwise, they may have adapted a phenotype more similar to the male embryos. Unfortunately, there are only few in vivo studies regarding the sex-specific velocity of early embryo development [35]. Most studies used in vitro produced embryos depicting more often a faster development of male embryos, but this also seems to depend on the culture conditions. For example, in the pig, the energy substrate has been demonstrated to be important in this respect [81].

The disruptive potential of estrogens including E2 has been shown in vitro [29–31]. In vivo, short-term application of estrogens directly before implantation has demonstrated direct effects on the endometrial gene expression profile [19, 20, 24] as well as disruption of the gestational process later on, including embryonic losses [21–25]. In the present study, we also observed endometrial gene expression changes. However, as shown by continuously administering E2 over the entire gestation to the same sows as used in the study at hand in a previous pregnancy, neither alterations in body weight nor litter size nor sex distribution were found at birth [10]. Thus, our continuous E2 treatment starting with insemination was less disruptive as treatments only directly before implantation [21–25]. Still, lasting consequences were observed in the offspring, namely a bone density phenotype, a shift in body composition, as well as gene expression differences mainly in the prostate [10, 39, 82]. Likewise, a study in mice demonstrated that continuous estrogen exposure only during the preimplantation phase led to sex-specific alterations in the offspring [13, 14]. Both sexes were affected, including a masculinization of the female offspring [14]. Although we neither observed changes in genes involved in the process of modifying DNA methylation such as DNA methyltransferases nor obtained a GO term involving epigenetics in the DAVID analyses, a separate analysis of DNA methylation changes in the embryos and offspring showed that epigenetic marks have been affected in both [63]. Three genes were analyzed from which two were significantly affected in the embryos and offspring. These are the cell cycle regulator cyclin dependent kinase inhibitor 2D (*CDKN2D*) and the tumor suppressor gene phosphoserine aminotransferase 1 (*PSAT1*). A subtle but significant hypomethylation was observed in the embryos, while in the liver of the 1-year-old female offspring a similar small effect, but in this case a hypermethylation was determined. Although detailed underlying mechanisms remain to be explored, this indicates the possibility of lasting changes due to the preimplantation E2 exposure.

Overall, we evidence that oral maternal E2 exposure targeted the endometrium and particularly the developing blastocysts by leveling their physiologically inherent sex-specific gene expression profile. This perturbation was either induced through direct effects of E2 metabolites or through alterations in the endometrial secretion impacting on the embryo. It may imply both a functional perturbation of the embryo and/or a shift of its developmental velocity. Notably, the molecular fingerprint at a low dose currently considered as NOEL is thereby of considerable importance. The disturbed

embryonic development may likely entail sex-specific adult phenotypes increasingly described in offspring of EDC exposed mothers. Therefore, a careful revisit of effect level thresholds seems warranted.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental Table S1. List of all primers used for qPCR validation of endometrial transcripts.
Supplemental Table S2. Differentially expressed genes in the endometrial samples (RNA-Seq).
Supplemental Table S3. Differentially expressed genes (gene symbols) in the embryos (RNA-Seq).
Supplemental Table S4. Differentially expressed transcripts of the comparison between male and female control embryos.
Supplemental Table S5. Differentially expressed genes (gene symbols) of the embryonic Venn Diagram (Figure 3).
Supplemental Table S6. Differentially expressed genes in the embryonic samples ($P < 0.0001$, fold change cut-off 1.5).

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Author Contributions

SEU and VLF designed the research. VLF, RWF, MR, and SK performed the research. VLF, SB, SK, and HB analyzed the data. VLF and SEU wrote the manuscript. All authors discussed the results and commented on the manuscript.

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