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Epigenetic effects of endogenous and exogenous estradiol-17β on male pig development

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Abbreviations

ADI	acceptable daily intake
App	appendix
AR	androgen receptor
ATP	adenosine triphosphate
bcDNA	bisulfite-converted DNA
BPA	bishphenol A
cAMP	cyclic adenosine monophosphate
CAPs	chromatin-associated proteins
cDNA	complementary DNA
ChIP	chromatin immuno precipitation
CpG	cytosine guanidine nucleotide sequence
Cq	cycle of quantification
CRM	chromatin-remodelling complex
DES	diethylstilbestrol
DEXA	dual-energy X-ray absorptiometry scanning
DNA	desoxyribonucleic acid
DOHaD	developmental origin of health and disease
E2	estradiol-17β
EDC	endocrine disrupting chemical
EE	ethinylestradiol
EIA	enzyme immuno assay
ESC	embryonic stem cell
ESR1	estrogen receptor alpha
ESR2	estrogen receptor beta
gDNA	genomic DNA
GPER	G-protein coupled estrogen receptor
H3F3A	histone H3
HDAC	histone deacetylase
HRE	hormone response elements
HRM	high resolution melting
IGF2	insulin-like growth factor 2
IPS	induced pluripotent stem cells
IUGR	intrauterine growth retardation
JECFA	joint FAO/WHO expert committee on food additives
LBD	ligand binding domain
LC-MS	liquid chromatography - mass spectrometry
LUMA	Luminometric methylation assay
MBD	methyl-CpG-binding domain
mRNA	messenger RNA
MS-HRM	methylation-sensitive high resolution melting
NOEL	no observed effect level
p/CAF	p300/CBP-associated factor
PCR	polymerase chain reaction
RITS	RNAi induced transcriptional silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
rpm	rounds per minute
rRNA	ribosomal RNA
RT-qPCR	reverse transcription quantitative PCR
SAM	S-adenosylmethionine
SCNT	somatic cell nuclear transfer
SEM	standard error of means
SRC	steroid receptor complex
T	testosterone
TF	transcription factor
TGIF	TG-interacting factor
TGS	Transcriptional gene silencing
UBQ3	ubiquitin 3
WAT	white adipose tissue
WBC	white blood cell
WGA	whole genome amplification
ZON	zearalenone

Abstract

Estrogens regulate requirements essential in reproduction of both female and male vertebrates, but also most metabolic and morphological features can be affected by this group of steroidal hormones. Estradiol-17β (E2) acts as the most potent naturally occurring estrogenic substance within this class of signaling molecules that mainly accomplish their tasks by modifying transcriptional expression of distinct genes via binding to their specific receptors. This regulation process involves epigenetic mechanisms driving chromatin accessibility of underlying target genes but has been connected to the estrogen receptors' abundance as well. Consequently, a first approach was to establish a suitable methodology for the quantification of DNA methylation levels as one of the most prominent epigenetic mechanisms. Employing artificially generated distinctly methylated genomic DNA standards, the suitability of available individual methods was investigated. Methylation-sensitive high resolution melting (MS-HRM) with successive differential fluorescence curve analysis evolved unable of granting reliable quantitative data while uncontrolled pyrosequencing might suffer from enormous PCR bias prior to analysis. By combining MS-HRM and pyrosequencing, inherent limitations of either method were successfully resolved thereby consequently providing an effectively verified basis for discussing results on DNA methylation differences. Thus equipped, further investigations aimed at broadening the limited understanding of the most important mediator of estrogenic action, namely the estrogen receptor α (ESR1) in terms of epigenetic regulation. Three genetic regions of ESR1 were analyzed in tissues of male growing piglets exhibiting distinct transcriptional abundance. In contrast to human or rodent examples of methylation differences over adjacent sites, only one single CpG in the +1kb intragenic region of ESR1 was strikingly differently methylated according to the underlying expressional status. Identified as a putative binding site for the transcriptional repressor TG-interacting factor 1 which can recruit the chromatin-compacting histone deacetylase 1, chromatin immunoprecipitation confirmed a reduced histone H3 presence at the specific *ESR1* location in case of higher DNA methylation. In consequence, manifestation of *ESR1* abundance via a single CpG-site-based methylation difference impairing transcription factor binding might display a formerly unknown way of the gene's transcriptional regulation. In terms of the receptor's regulation by endogenous E2, male growing piglets with distinct E2 blood plasma concentrations over development were employed. Unexpectedly, major E2 concentration differences were only marginally associated with slight variations in mRNA abundance and DNA methylation levels within the investigated tissues. It thus evolves that endogenous E2 concentrations do not necessarily impact on *ESR1* expressional abundance and epigenetic mechanisms. In contrast, exposure to exogenous estrogenic substances acting as endocrine disrupting chemicals (EDC) has been found to adversely impact on fertility and obesity. Endocrine

disruption has been understood as a disturbance of the naturally occurring developmental programming process in early life where epigenetic mechanisms set the basis for regulation cascades and physiological processes later in life. Since studies investigating E2 as a potential EDC are lacking, endocrine and physiological parameters were investigated in sows and their offspring receiving distinct concentrations during the entire period of pregnancy. Applied concentrations mimicked daily consumption at the recommended ADI level (0.05 µg/kg body weight/day), at the NOEL (10 µg/kg body weight/day) and at a high dose (1,000 µg/kg body weight/day). Treated gilts receiving 1,000 µgE2/kg body weight/day were found to exhibit increased weight gain and elevated plasma E2 levels during pregnancy. Offspring displayed similar weight at birth but were reduced in weight at weaning even under *in-utero* exposure to ADI levels only. Utilizing dual-energy X-ray absorptiometry scanning, specifically males showed a significant increase in overall body fat percentage at eight weeks of age. Together with the piglets' steady endogenous hormonal status subject to E2 treatment, these data suggest an epigenetic programming effect during pregnancy for E2, causative for the depicted phenotypes. E2 can thus be considered an EDC in terms of developmental application, even at doses formerly proposed to be safe. Taken together, the herein presented findings underline that potential effects due to endogenous E2 concentrations or exogenous E2 application in male pigs seem subject to the susceptibility of the underlying developmental epigenetic mechanisms.

Zusammenfassung

Östrogene regulieren notwendige Vorraussetzungen für das Reproduktionsgeschehen sowohl in weiblichen als auch männlichen Vertebraten. Auch der Großteil metabolischer und morphologischer Eigenschaften kann durch diese Gruppe steroidaler Hormone beeinflusst werden. Innerhalb dieser Klasse von Signalmolekülen stellt Östradiol-17β (E2) das wirksamste natürlich vorkommende Östrogen dar, wobei deren Wirkung auf die transkriptionelle Expression hauptsächlich über die Bindung an spezifischen Rezeptoren vermittelt wird. In diesem Regulationsprozess spielen unter anderem epigenetische Mechanismen bei der Vermittlung der Chromatin-Zugänglichkeit der Zielgene eine wichtige Rolle, nämliches wurde aber auch für die Regulation der Östrogen-Rezeptoren selbst beschrieben. Als erster Schritt der vorliegenden Arbeit sollte demzufolge eine geeignete Methode zur quantitativen Bestimmung der DNS Methylierung, stellvertretend als einer der wichtigsten epigenetischen Mechanismen, etabliert werden. Mithilfe von künstlich erstellen DNS Standards unterschiedlicher Methylierung wurde zunächst die Tauglichkeit möglicher Methoden untersucht. Methylierungssensitive hochauflösende Schmelzkurvenanalyse (MS-HRM) und gekoppelte differentielle Fluoreszenzkurvenanalyse erwiesen sich als ungeeignet verlässliche quantitative Daten zu generieren, gleichzeitig kann unkontrolliertes Pyrosequencing durch mögliche Verschiebungen in der vorhergehenden PCR falsche Werte liefern. Durch die Kombination beider Methoden konnten jedoch die individuellen Nachteile erfolgreich umgangen und somit eine wirkungsvoll kontrollierte Basis für Auswertung von DNS Methylierungen geschaffen werden. Darauf aufbauend zielten weitere Untersuchungen drauf ab, das eingeschränkte Verständnis bezüglich der epigenetischen Regulation des wichtigsten Vermittlers östrogener Wirkung, nämlich des Östrogen Rezeptors α (ESR1), zu erweitern. Hierzu wurden drei genetischen Regionen des ESR1 in Geweben männlicher heranwachsender Ferkel untersucht welche unterschiedliche transkriptionelle Abundanz für das Gen aufwiesen. Während in Nagern und beim Menschen Methylierungsunterschiede über mehrer anliegende CpG-Stellen beschrieben sind, zeigte sich hier nur eine einzige CpG-Stelle in der ersten intragenischen Region des ESR1 in Abhängigkeit der zugrundeliegenden Expression deutlich unterschiedlich methyliert. Diese wurde als mögliche Bindungsstelle für den transkriptionellen Repressor TGIF identifiziert welcher die Chromatin-kompaktierende Histon Deacetylase 1 rekrutieren kann. Mittels Immunopräzipitation wurde auch tatsächlich ein vermindertes Auftreten des Histons H3 an der spezifischen ESR1 Stelle detektiert, wenn dort gleichzeitig ein hoher Grad and DNS Methylierung vorlag. Die Regulierung der ESR1 Transkripthäufigkeit über die Methylierung einer einzelnen CpG-site, welche dadurch das Anbinden eines Transkriptionsfaktors verhindert, erscheint somit als eine mögliche Form der Regulierung, welche für dieses Gen bisher noch nicht beschrieben wurde. Untersuchungen zur Frage der Regulation des

Rezeptors durch endogenes E2 wurden mithilfe von männlichen heranwachsenden Ferkeln durchgeführt welche unterschiedliche E2 Blutplasma Konzentrationen aufwiesen. Erstaunlicherweise gab es nur minimale Assoziationen zwischen der E2 Konzentration und der Menge an mRNS sowie des Grads der DNS Methylierung. Hieraus folgt, dass endogene E2 Konzentrationen nicht immer zwingend auf die Expression des ESR1 und die damit verbundenen epigenetischen Mechanismen einwirken. Im Gegensatz dazu wurde häufig beobachtet, dass eine Exposition gegenüber östrogenen Subtanzen welche als sog. Endokrine Disruptoren (EDC) fungieren, Fertilität and Adipositas negativ beeinflussen kann. Dieser Prozess der endokrinen Störung wurde mittlerweile als eine Beeinflussung natürlicherweise auftretender entwicklungsbasierter Programmierung verstanden welche die Grundlage für spätere Signalskaskaden und physiologische Regulationsprozesse darstellt. Da jedoch Untersuchungen fehlen welche E2 als einen möglichen EDC aufgreifen wurden endokrine und physiologische Parameter in Sauen und deren Nachkommen untersucht welche über den gesamten Zeitraum der Trächtigkeit verschiedenen Konzentrationen ausgesetzt waren. Die verwendeten Konzentrationen entsprachen dabei einer täglichen Aufnahme im Rahmen des empfohlenen ADI Levels (0.05 µg/kg Körpergewicht/Tag), am NOEL (10 µg/kg Körpergewicht/Tag) und einer weiteren hohen Dosierung (1,000 µg/kg Körpergewicht/Tag). Sauen behandelt mit 1,000 µg E2/kg Körpergewicht/Tag zeigten eine verstärkte Gewichtszunahme und erhöhte E2 Plasmaspiegel im Verlauf der Trächtigkeit. Während die Nachkommen bei der Geburt noch ein vergleichbares Gewicht aufwiesen erschien dieses beim Absetzen ausgehend von der *in-utero* Exposition, selbst bei ADI Dosen, reduziert. Mithilfe von Dual-Energie Röntgen-Absorptiometrie Untersuchungen konnte gezeigt werden, dass spezifisch männliche Nachkommen im Alter von acht Wochen einen signifikant erhöhten Körperfettanteil aufwiesen. Da beide Geschlechter zudem keine Veränderungen im endogenen hormonellen Status aufwiesen, lassen die hier dargestellten Daten den Schluss zu, dass die gezeigten Phänotypen durch einen epigenetisch bedingten programmierenden Effekt seitens E2 verursacht wurden. E2 kann somit bei Applikation in der frühren Entwicklung als ein potentieller EDC eingestuft werden, und dies zudem bei Dosen welche bisher als sicher galten. Zusammenfassend zeigen die in dieser Arbeit dargelegten Daten, dass mögliche Effekte durch endogen auftretende oder exogen applizierte E2 Mengen in männlichen Schweinen abhängig sind von der Zugänglichkeit der dem jeweiligen Entwicklungsstand zugrunde liegenden epigenetischen Mechanismen.

1 Introduction

1.1 *Epigenetics - a fundamental controlling mechanism of gene-expression due to endogenous and exogenous estrogenic signals*

Introduction into epigenetics – History and definition

Historically, „epigenetics“ has been attributed to seemingly abnormal events of inheritance, noticed to occur contradictory to the mendelian laws of classic genetics (Goldberg et al. 2007). In the latter, research mainly focuses on genetic variance in terms of nucleotide polymorphisms or mutations and their influence on subsequent gene-product (i.e. protein) function. In this way, classic genetics contributes to the fundamental understanding of inheritable diseases or, subject to personal living conditions, susceptibility to certain disorders provoked by a nucleotide-sequence based context. Furthermore, genetics delivers a basic insight into gene transcription mechanisms through the knowledge on functional units of gene structure like promoter regions encompassing transcription factor (TF) binding sites or docking sites for polymerases. Solely on the basis of nucleotide sequence however, coordinated RNA expression can not adequately be explained given that virtually every single cell in a eukaryote system contains an identical setup of genetic information. Thus, even after “deciphering” a genome, fundamental questions remain covering aspects at molecular level as well as systemic effects. Examples for these are e.g. the differentiation of multipotent cells into distinct functional units or prominent differences between monozygotic twins. Consequently, the question arises how endogenous or exogenous factors might, in addition to classic genetics, regulate developmental processes without changing the underlying nucleotide sequence. This topic is currently investigated in the field of epigenetic research.

Close to the early thoughts of Conrad Waddington, epigenetics is nowadays understood as a phenomenon modulating gene expression in a temporal, spatial and quantitative context thus serving as a kind of bridge between genotype and phenotype (Goldberg et al. 2007). The most contemporary definition of epigenetics is “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence” (Riggs et al. 1996).

Epigenetic mechanism regulating gene expression

During the last decade, much of the effort of epigenetic research has covered the mechanisms modulating gene expression. Of special interest were investigations focusing on modifications to DNA nucleotides and histone octamers round which the DNA is wrapped (Fig. 1). These units termed nucleosomes enable a significant compaction of genetic information thereby constituting an elegant solution to the demand of storing approximately 2 m of DNA in a cell's nucleus. A repeating polymer of nucleosomes is designated chromatin. In order to grant expression regardless of the compacted state, chromatin appears in two distinct states. Tightly condensed areas (heterochromatin) hinder access to the DNA whereas open conformations (euchromatin) enable the docking of specific proteins of the transcriptional machinery and thus allow gene expression (Grewal and Elgin 2007). Consequently, chromatin state has been encountered as a main target of epigenetic mechanism regulating gene expression.
Amongst these mechanisms, especially DNA methylation has been intensively investigated. In mammals, DNA methylation is encountered at cytosine nucleotides which are followed by a guanidine (CpG-context), areas of high CpG density are termed CpG-islands. DNA methylation of these islands has frequently been associated with transcriptional silencing if these islands are part of a gene's promoter region with its regulatory entities (Goll and Bestor 2005). Two possible mechanism are proposed how DNA methylation can impact on transcriptional expression: On the one hand, examples state that methylation of a single CpG-site can be sufficient to prevent both promoting and repressive transcription factors from docking to their respective binding sites thus modifying transcription (Choy et al. 2010; Tierney et al. 2000). On the other hand, so called methyl-CpG-binding domain (MBD) proteins are recognized to function as readers of methylated DNA and were identified to establish, maintain or facilitate transcriptional repression by the recruitment of other chromatin modulating proteins or modifications of higher order chromatin structure (Dhasarathy and Wade 2008). Besides the specific regulation of distinct genes, general functions of DNA methylation include X-chromosome inactivation in female mammals or genomic imprinting (Yang and Kuroda 2007). In the latter, genes underlying this phenomenon are inherited subject to their origin. DNA methylations hereby inactivate one of the two parental alleles thus achieving monoallelic gene expression. Moreover, DNA methylation is considered necessary to maintain genome integrity and stability by silencing transposable elements (Bird 2002).
Modifications of histone proteins encompass amongst others methylations, acetylations or phosphorylations to the easily accessible amino acid residues referred to as histone tails.

These modifications can exert both cis- and trans-effects on chromatin ultimately leading to changes in gene expression. Proximate effects are achieved through changing the physical properties of histone-tails subject to the attached residue, thus leading to a localized expansion of neighboring nucleosomes. Indirectly, histone modifications alter chromatin structure by recruiting chromatin-associated proteins (CAPs) that serve as binding partner for larger chromatin-modifying protein complexes (Kouzarides 2007).

Other important epigenetic mechanism that contribute to transcriptional regulation are RNAi-mediated transcriptional gene silencing (TGS) involving the RNAi induced transcriptional silencing complex (RITS) (Grewal and Elgin 2007; Verdel et al. 2004) or polycomp and trithorax proteins, the latter being an essential part of early developmental regulation (Schuettengruber et al. 2007). The group of ATP-dependent chromatin-remodeling complexes (CRMs) is another example of the major contributors to the topic of epigenetic regulation (Johnson et al. 2005; Saha et al. 2006). This large group of multi-protein complexes regulates access to the DNA by events such as DNA-sliding, histone exchange or eviction as well as altered nucleosome structure (looping). Through these mechanisms, CRMs can distinctly reorganize chromatin structure thus enabling either activation or repression of underlying DNA. These multimeric complexes are part of the protein machinery that exerts the trans-effects of DNA methylation and histone modifications via MBDs and CAPs as mentioned above. Also the RNAi mediated silencing is achieved with the help of CRMs plus additional influence of DNA methylation and histone modification. This has lead to widely agreed believe that epigenetic mechanism are connected in a broad network of co-regulation in order to corporately achieve targeted variation of chromatin and subsequently alter a gene’s expression status.

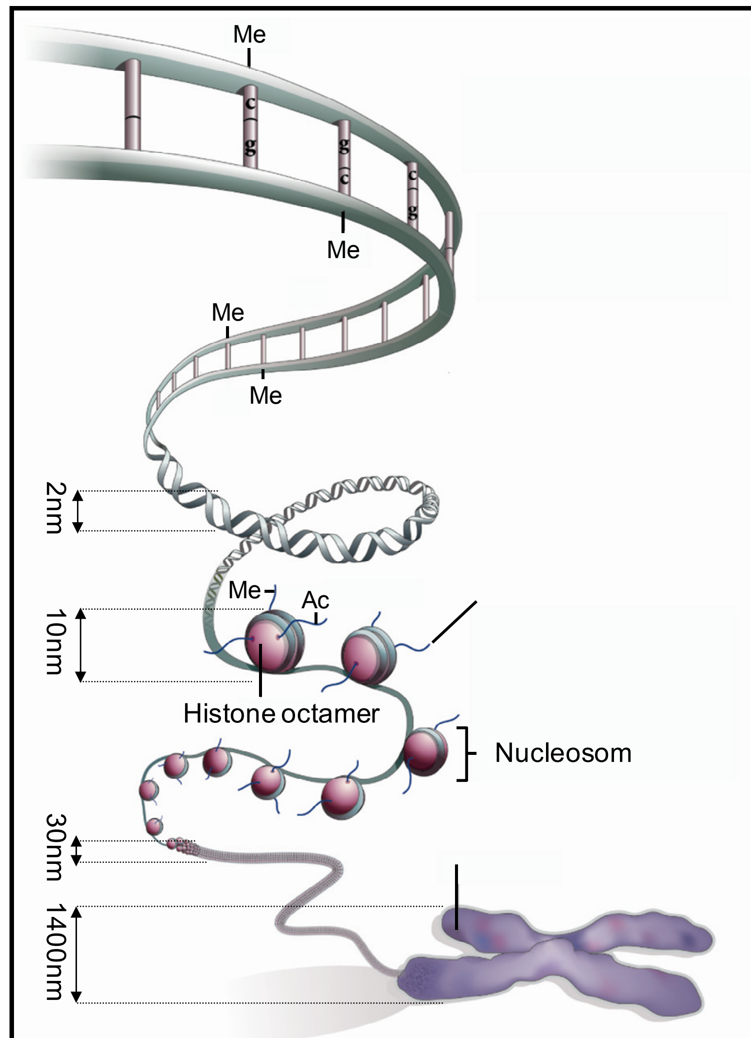

Fig. 1. Schematic depiction of assorted epigenetic modifications. DNA is subjected to methylation at cytosine residues whereas amino-terminal tails of histone proteins can be altered by methyl (Me), acetyl (Ac) or various other functional groups impacting on chromatin structure thus guiding transcriptional expression. Modified following Qiu (Qiu 2006).

Development – epigenetic by principle

The combination of epigenetic mechanism as depicted above drives programs of gene expression without changing the underlying sequence of DNA. Thus, development characterized by the generation of different organs and tissues with distinct expression patterns from the same source of DNA is epigenetic by principle (Reik 2007). In theory, the subsets of TFs defining a muscle cell's gene expression via epigenetic mechanisms would consequently turn a bone cell in a similar muscle cell if all other bone-specific TFs were removed. This concept was indeed impressively supported recently by the generation of

induced pluripotent stem cells (IPS). By expressing four transcriptional regulators (Oct3/4, Sox2, Klf4 and c-Myc) in fibroblast, these were reprogrammed to become embryonic stem cell (ESC)-like (Takahashi and Yamanaka 2006; Wernig et al. 2007). In the same consequence, transplantation of an individual's somatic cell into an oocyte without nucleus (somatic cell nuclear transfer, SCNT) grants the development of a cloned animal (Reik 2007). The case that most cloned animals and also the in-vitro derived IPS cells suffer from incorrect epigenetic marks (Morgan et al. 2005) highlights, that the orchestrated interplay of TF signaling, epigenetic mechanisms and associated development is far from being fully understood. The epigenetic mechanistic actions driving the early developmental processes are under enormous investigation. It is meanwhile elucidated, that especially DNA methylation plays an important role in early preimplantation embryos (Abdalla et al. 2009; Reik 2007; Weaver et al. 2009). While mature sperm and eggs are comparably methylated to somatic cells (Bestor 2000), both parental genomes are demethylated after fertilization. Whereas demethylation of the maternal genome requires DNA replication for this mechanism, the paternal genome in contrast is actively demethylated by a so far unidentified mechanism (Mayer et al. 2000). Several reasons are discussed why the paternal genome can not „escape“ active demethylation amongst these are the protamine to histone exchange process or specific complexes either favoring or hindering docking of demethylase complexes (Abdalla et al. 2009). It is assumed that this wave of demethylation serves to replace gametic methylation marks while methylated or unmethylated imprinted gens are not affected. At the time of implantation, both genomes are remethylated for to set early embryonic marks and thus allow gene transcription important for further embryo development. Later on, basic somatic methylation patterns follow, allowing for full differentiation and cell specificity (Reik 2007). It is interesting to mention that also primordial germ cells (including imprinted regions) are demethylated at early stages of embryonic development. Few days later, remethylation takes places and cells pause at this level before their entry into the generation process of eggs or sperm (Reik et al. 2001) (Fig. 2).

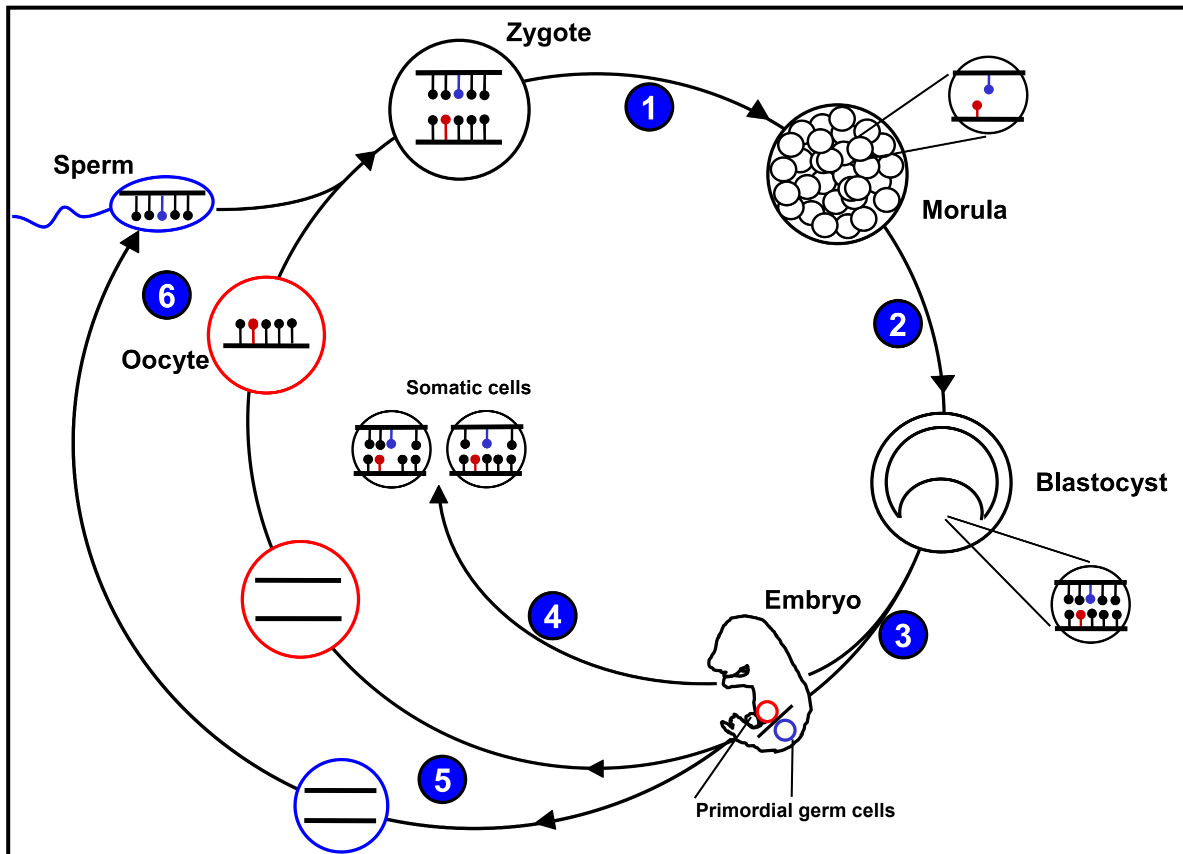

Fig. 2: Developmental dynamics of DNA methylation. A fertilized zygote is subjected to massive loss of DNA methylation (black pins) while imprinted marks (maternal origin: red pins, paternal origin: blue pins) are kept (1). After morula stage, epigenetic marks necessary for embryo development are established (2). During further development (3), distinct gene expression patterns define specific somatic cell types (4). Early in embryo development, methylation information including imprints is completely erased in primordial germ cells (5) before a re-establishment prior to sperm and oocyte maturation (6).

Estrogenic action, receptors and epigenetics

Of major importance amongst the above mentioned TFs serving as cellular signals that mediate developmental processes are steroid hormones. In particular, estrogens are acknowledged to regulate developmental processes affecting most metabolic, behavioral and morphological features later in life. Tissues regulated in function are amongst others bone and cartlidge (Karimian and Savendahl), fat (Cooke and Naaz 2004), brain (Beyer 1999), bone (Cutler 1997) and muscles (Ihemelandu 1981). Furthermore, estrogens regulate primary sexual processes essential for reproduction in both genders (Abney 1999; Couse

and Korach 1999; Meyer 1999). These hormones accomplish their tasks mainly by modifying the expression patterns of specific target genes, mediated via their well characterized receptors. Membrane-bound subpopulations of these "classic" estrogen receptors ERα (ESR1) and ERβ (ESR2) and related receptors like G-protein coupled estrogen receptor (GPER) (Meyer et al. 2011; Prossnitz and Barton 2011) have been shown to employ non-genomic actions of estrogens (Wierman 2007). These non-genomic actions consist of the activation of e.g. the ERK/MAPK pathway (Nadal et al. 2001; Segars and Driggers 2002) which allows a rapid expressional regulation of downstream target genes involved in reproductive processes (Fig. 3). Other signaling targets include cytoplasmic modifications like ion concentrations which can in turn mediate cellular trafficking (Bjornstrom and Sjoberg 2005; Levin 2005; Zhang and Trudeau 2006).

In their role as classic transcription factors, estrogen receptors of cytoplasmic or nuclear localization have been established to be of major importance. Classical regulation involves the diffusion of the ligand (e.g. estradiol-17β, E2) into cells, binding to the receptor which induces its conformational change and dimerization. This complex then recognizes palindromic hormone response elements (HRE) in genes and acts on transcription in a direct way. An indirect alternative has also been described to involve protein-protein interactions of the ligand-bound receptor modifying the function of other TFs (Wierman 2007) (Fig. 3). Interestingly, differences in steroidal actions are based on how various ligands fit into the ligand binding domain (LBD) of the receptor. According to the binding partner, this determines a distinct conformational change that enables specific types of adaptors/cofactors to be recruited to the complex thus modifying subsequent gene transcription (McDonnell et al. 1995; Shiau et al. 1998). A well-investigated member of these cofactors is the p160 coactivator family which consists e.g. of the steroid receptor complex (SRC) family, cAMP response enhancer binding protein (CBP)/p300) and the p300/CBP-associated factor (p/CAF). These coactivators have been found to be modular in structure and contain binding sites for the binding to ATP chromatin remodeling complexes and factors with histone acetylase and histone methylase activity, thus enabling their transcriptional regulation via epigenetic mechanism (Hall et al. 2001; McKenna and O'Malley 2002; Nilsson et al. 2001; Smith and O'Malley 2004). Accordingly, recruited corepressors have been described to activate histone deacetylases (HDACs) which can hinder RNA expression by chromatin compaction (Wierman 2007) (Fig. 3).

Besides the regulation of downstream target genes, estrogens are also able to modulate their own receptor transcription. This grants the ability to adjust the sensitivity of cells towards the E2 signal. The curiosity that depending on the cell type E2 can trigger either an enhancement or a decrease in *ESR1* expression (Donaghue et al. 1999; Ing and Tornesi

1997) is still under investigation. So far, rapid signaling cascades seem to be interesting candidates since the individual subsets of signal transduction molecules within different cell types presumably vary dramatically (Marino et al. 2006). Consequently, *ESR1* expression is variable in different tissues and moreover in a time-specific manner subject to the circulating and local E2 concentrations.

The mechanistic establishment of these tissue specific *ESR1* patterns has been described to be mediated by DNA methylation and was associated with further chromatin modifying factors such as histone deacetylases, similar to what is known from other genes (Shiota et al. 2002; Vesuna et al. 2011; Wei et al. 2011; Yang et al. 2000). For example, hypermethylation of the ESR1 promoter CpG-island in breast cancer has been found to correlate with the loss of *ESR1* expression in these tumors (Flanagan et al. 2010; Yoshida et al. 2000). A similar pattern has also been found in the non-pathological state of the neonatal mouse brain where increasing ESR1 promoter DNA methylation decreased the associated *ESR1* expression (Westberry et al. 2010). In contrast, an early investigation comparing *ESR1* expression in human tissues found the promoter CpG island demethylated while areas up- and downstream were methylated subject to the underlying expression status (Piva et al. 1989). A consequent investigation and thus understanding of *ESR1* epigenetic regulation in terms of DNA methylation patterns is currently still missing.

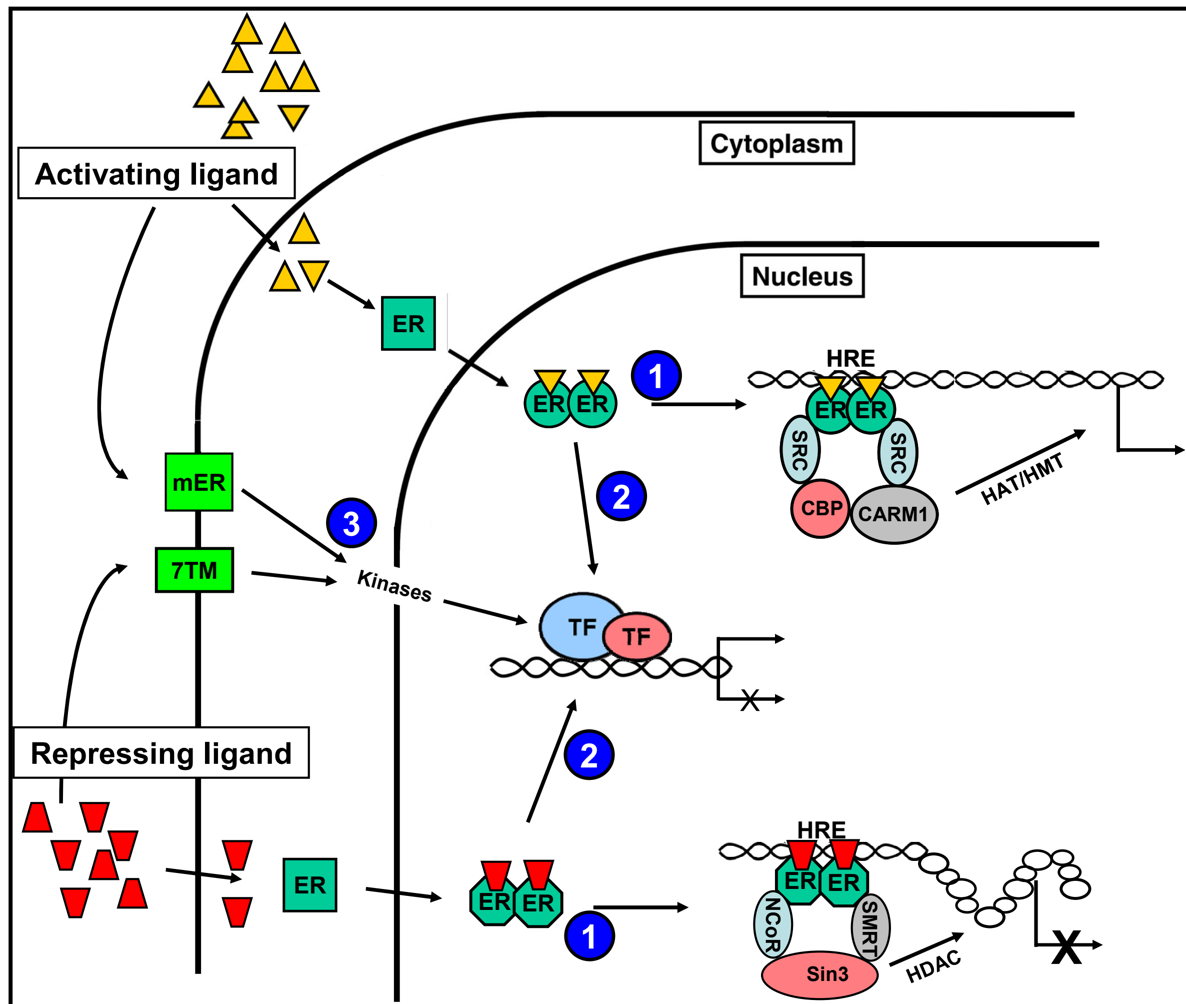

Fig. 3. Transcriptional regulation via estrogen-receptors. Lipophylic ligands are able to dissociate through the cytoplasm membrane and dock to their appropriate steroid receptor. Depending on the type of ligand, this induces a distinct conformational change. After localization into the nucleus, the ligand-receptor complex can exert its effects in two ways. On the one hand, dimerized receptors can bind to hormone response elements (HRE) (1) and enable transcription via the recruitment of either activating or repressing cofactors and their effect on chromatin structure. On the other hand, the steroid-receptor complex can induce or decrease transcriptional expression via direct interactions with other transcription factors (2). A third possibility of transcriptional regulation involves membrane-bound estrogen receptors (mER) or seven-transmembrane receptors (7TM) which enable a rapid impact on transcription factors via the activation of kinase pathways (3) (expanded following Wierman 2007).

Signals from the outer world – developmental programming, DOHaD and EDCs

Epigenetic mechanisms play an important role in the establishment of endocrine signaling during development. In addition to changing transcriptional programs subject to an immediate endogenous stimulus, epigenetics is also suggested to mediate developmental plasticity (Feinberg 2007; Jaenisch and Bird 2003; Jirtle and Skinner 2007; Zhang and Ho 2011). This concept involves long-lasting adjustments of the cellular physiology including endocrinologic signaling and metabolism in early (embryonic and prepubertal) development in response to an environmental, i.e. exogenous, stimulus. The processes by which environmental conditions permanently alter the structure and function of mammalian tissues is referred to as developmental programming (Barker 2001; Fowden and Forhead 2009). This adaption mainly occurring during critical developmental periods is actually supposed to be beneficial for the organism in terms of preparing it for the later demands in life (Zhang and Ho 2011). Disadvantages however occur, when developmental adaption is confronted with a contradictory environment. An intensely studied field in this topic is fetal nutrition and disease later in life. For example, an epidemiologic study with children suffering from war-time famine states that mothers' limited nutritional supply during early stages of pregnancy correlated with the offspring's increased risk for adiposity and cardiovascular diseases (Kyle and Pichard 2006). Based on this epidemiological observation, Barker und Hales set up their hypothesis of the „thrifty phenotype" stating that early life undernourishment probably lead to the consequence of a permanent modification in insulin-dependent metabolic pathways causing the depicted effect (Hales and Barker 2001). The thrifty principle would adapt the fetus in a way, that metabolic rate, hormone production and sensitivity match the expected limited amount of food. Other investigations added, that also the neuroendocrine system regulating nutritional uptake and metabolism might have undergone missprogramming (Martin-Gronert and Ozanne 2006). In consequence, if the postnatal nutritional availability does not match the programmed situation, this can lead to the depicted failures of an overstrained metabolism (Hales and Barker 2001). Similar to the human situation, animal studies mimicking intrauterine growth retardation (IUGR) have found declining beta-cell function in the pancreas and a risk for type 2 diabetes when a high availability of nutritional energy predominates later in life (Zhang and Ho 2011). Investigation in the context of war-time famine found persistent methylation differences of the imprinted IGF2 gene and the appetite-regulator leptin correlating to the later adverse outcomes (Gluckman et al. 2009), thus supporting the above stated concept of epigenetic mechanism driving developmental programming/plasticity in the sensitive window of fetal life. The phenomenon that adverse environmental signals might evoke disease later in life subject to

developmental programming has been summarized in the concept of “developmental origin of health and disease” (DOHaD) (Gluckman and Hanson 2004).
Aside from undernourishment, developmental exposition to a class of exogenic substances termed “endocrine disrupting chemicals” (EDCs) is increasingly associated with the DOHaD concept. EDCs, deriving e.g. from contraceptives, plasticizers, pesticides or industry by-products have been summarized as chemicals that can interfere with endocrine signaling thus potentially affecting all biological systems of the body (Colborn et al. 1993). This is reasoned in the fact, that EDCs most often posses the ability to interact with steroid hormone receptors in the body, consequently commending themselves for the above depicted concept of developmental programming. Correspondingly, reproductive abnormalities, prostate cancer or neurological dysfunctions have been connected with fetal/neonatal exposure to e.g. estrogenic compounds like bisphenol A (BPA), Diethylstilbestrol (DES), ethinylestradiol (EE) or E2 even at low environmentally relevant doses (Gioiosa et al. 2007; Ho et al. 2006; Kortenkamp 2007; Skakkebaek et al. 2001; Timms et al. 2005; vom Saal et al. 1997). The context of low doses at which adverse programming might occur has raised awareness already in earlier investigations on EDCs (Andersson and Skakkebaek 1999; Bay et al. 2004) and ads up in the view that programming as part of a normal developmental process usually occurs at very low physiological hormone concentrations. Consequently, developmental programming might be especially error-prone if the surrounding endocrine environment is disturbed even only marginally (vom Saal et al. 1997).

Environmental estrogens and obesity

The relevance of steroidal hormones in particular those with estrogenic action as a potential endocrine disruptor in humans arises mainly from the usage of estrogen-containing contraceptives and the treatment of postmenopausal symptoms (Fürst et al. *under review*). Another important aspect in this topic is the consumption of meat which was commonly produced with steroidal growth promoters in the US and other countries (Schiffer et al. 2001). Important to this point is that varying doses of exposure could occur under good (implants discarded-low dose) or under bad (misplaced implants, non-discarded implants, abuse- high dose) veterinary practice (Daxenberger et al. 2000). In contrast to countries like the US, Canada or South Africa, governmental authorities of the EU are meanwhile aware of the problematic that EDCs, particular those with estrogenic action, might constitute a jet unclear thread to the health of humans, particularly children. Thus, the EU banned

production and importation of meat derived from animals treated with growth promoting hormones in 1985, leading to one of the most insistently led trade controversies between the US and the EU, the so-called “Beef Hormone Dispute”. In May 2011 this debate came to an end with the US lifting import duties on European food in return for the EU opening their markets to the import of hormone-free beef. While economic concerns seem bargained (Johnson 2010), the scientific dispute about the safety of anabolic steroids remains. This is especially the case for the group of estrogens with its most potent natural offset E2, the representative substance the trade dispute had mainly focused on. The latest evaluation report of the Expert Committee on Food Additives of the JECFA (JECFA 1999) whose decisions the US mostly relied on during the trade dispute, could not draw a final conclusion about the hazard potential of E2 at that time. However, numerous studies meanwhile clearly implicate an important role for estrogens e.g. in breast cancer development (Cavalieri and Rogan 2011; Howell and Evans 2011; Okoh et al. 2011; Zwart et al. 2011). It seems obvious, that estrogens like E2 do not only stimulate cell proliferation but can also cause DNA damage and thus probably initiate tumors via their oxidation products (Crooke et al. 2006; Yager and Davidson 2006). The JECFA’s risk assessment for E2 in 1999 resulted in an up to nowadays announced acceptable daily intake (ADI) of 0.05 µg/kg body weight (bw) based on the no observed effect level (NOEL) in postmenopausal women (JECFA 1999).

Besides the evident influence of ÉDCs on reproductive processes, recent investigations indicate that an exposure to estrogenic substances such as BPA, DES and genistein can also impair adipocyte development and obesity (Heindel and vom Saal 2009; Janesick and Blumberg 2011; Newbold et al. 2009). For example, an increase in postnatal growth and obesity in adulthood was discovered due to the exposition of low doses of the plasticizer BPA during fetal or early neonatal life in rodents (Akingbemi et al. 2004; Miyawaki et al. 2007; Rubin et al. 2001). Mechanistic investigations for this phenomenon point toward BPA impacting on the differentiation of fibroblasts to adipocyte precursors and affecting (adipocyte) glucose transport (Masuno et al. 2002; Sakurai et al. 2004) as well as inhibition of adiponectin release or Glucocorticoid receptor (GR) activation (Ben-Jonathan et al. 2009; Sargis et al.). Similar mechanisms have been found with other EDCs (Dieudonne et al. 2000; Feige et al. 2007; Grun et al. 2006; Kirchner et al. 2010; Sargis et al. 2010). Similar effects to that of BPA were encountered for DES (Newbold et al. 2008; Newbold et al. 2005, 2007). There, body fat percentage was increased even at doses down to 1µg/kg/day. Rodent models have also been utilized investigating an exposure to phytoestrogens such as genistein, which has been acknowledged to both increase body weight and obesity (Newbold et al. 2007; Ruhlen et al. 2008) and decrease adiposity as well (Cederroth et al.

2007). Differences in the reported phenotypes are supposed to occur due to way of application, time window of exposure and supplied amount, highlighting them as elementary factors in evaluating the possibility of programming influences. As a surrogate trying to explain increasing obesity in humans, nearly exclusively rodents have been utilized in the investigations on estrogenic obesogens so far. Specifically in terms of adipose tissue however, mature adipocytes are formed from beginning of the second trimester in humans while in rodents solely the conversion of mesenchymal cells to preadipocytes takes place during fetal life (Ailhaud et al. 1992). Thus, EDC might not display comparable effects in rodents as expected in humans subject to the time window of exposure, which underlines the need to enhance the number of different animal models. In terms of investigated substances, the relevance of BPA investigations seems appropriate given that the human population is widely exposed to it (Somm et al. 2009), phytoestrogens are part of normal food and DES a drug given to mothers from the 1940s until the 1970s. Studies analyzing the effect of the most potent natural estrogen E2 in this field of research are missing up to date. This however seems mandatory given that E2 has an approximately 2,000-fold higher estrogen receptor binding affinity than BPA (Krishnan et al. 1993; Nagel et al. 1997) and phytoestrogens which are also thought to possess only week estrogenic potential still exert obesogenic effects. Since E2 is also endogenously produced during pregnancy in the human, an animal model like the pig sharing similar placental estrogen production (Simpson and MacDonald 1981) in contrast to rodents would be needed when investigating the possible effects of this hormone given as an additional exogenous stimulus.

1.2 Aims of the study

Epigenetic mechanisms significantly contribute to the mechanistic realization of a gene's distinct expression program during development thus enabling the appropriate systematic constitution of living organisms. Steroidal hormones have been acknowledged as central and powerful regulators in this interplay with the potential for adverse outcomes in case of inadequate signaling. The work at hand aimed at highlighting the contribution of estradiol-17β as the most potent estrogenic steroidal hormone in these processes. By initially developing a controlled high-resolution methodology for investigating DNA methylation as a representative epigenetic modification, a deepened understanding of the transcriptional regulation of the most important mediator of estrogenic actions was to be acquired. The potential of naturally occurring E2 concentrations in piglets on the receptor's expression status were of subsequent interest. In terms of exogenous estrogenic signaling, an animal trial mimicking oral ingestion of E2 during pregnancy was to be accomplished and potential outcomes assessed that would link *in-utero* exposition to developmental programming impacting on progeny. By incorporating low exposure levels, this investigation foremost aimed to clarify whether established 'safe' doses for E2 can be asserted as a reasonable recommendation with respect to developmental epigenetic effects.

2 Material and methods

Tissue sampling for the establishment of epigenetic methodology and investigation of endogenous E2 effects

For the establishment of an unbiased DNA methylation analysis at single CpG-site resolution, blood and tissues of cycle heifers and pregnant cows were analyzed as described in detail recently (Fürst et al. 2012b). Animal trial, slaughtering and tissues preparation were kindly conducted by the research group of Dr. Susanne Ulbrich (Physiology Weihenstephan). Investigations of distinct E2 plasma concentrations and their effect on male piglets during postnatal development utilized tissue and blood samples collected at Versuchsstation Thalhausen (Fürst et al. 2012a). In brief, one male sibling of a randomly selected sow each was slaughtered at <1 h, 11 d or 56 d after birth, representing states of high (birth), intermediate (before weaning) and low (prepubertal) E2 occurrence, respectively. Sampling of animal tissues was conducted with permission from the regional veterinary authority, following accepted standards of humane animal care.

Animal trial of E2 application during pregnancy

In order to determine a possible influence of exogenous E2 exposure during pregnancy, distinct amounts of E2 were orally fed to sows during the whole period of pregnancy (Fürst et al. *under review*). Applied amounts were chosen to mimic situations of daily E2 exposure at the human acceptable daily intake (ADI) level (0.05 µg/kg bw/d), the human NOEL level (10 µg/kg bw/d) and a high dose (1000 µg/kg bw/d), respectively. The main trial was preceded by an initial pharmacokinetics study where castrated male pigs, noted for their lowest concentrations of endogenous E2, were used to guarantee the detection of slightest elevations of E2 in plasma. In brief, pigs were catherized at the *vena jugularis* and then recovered from surgery for one day. The day afterwards, pigs received a bread roll, filled with 2 ml of the carrier (ethanol) or the carrier plus the respective amount of E2. In order to resemble a continuous daily exposure through food consumption, the desired amount of total E2 ingestion for each day was allocated into two identical portions. Pigs in the pharmacokinetics study were thus fed with the same ration sows in the main trial correspondingly received twice daily. Blood samples were taken hourly before E2 application and every 15 min after application, followed by hourly sampling for additional

eight times.In the main application study, randomly selected German landrace sows were inseminated with the sperm from one Pietrain boar twice at estrus (first insemination = day 0) and twice daily received a bread roll prepared with 500 µgE2/kg bw, 5 µgE2/kg bw and 0.025 µgE2/kg bw or ethanol only, respectively. Standard food rations were weekly monitored using standardized LC-MS (Schneweis et al. 2002) for possible contamination with the estrogenic mycotoxin Zearalenon (ZON), kindly conducted by Carsten Meyer, Chair of animal hygiene, Weihenstephan. As depicted in Fig. 4, maternal hormone concentrations during pregnancy were investigated in blood samples taken at days -7, 35, 49, 70 and 97 around conception, respectively. Throughout pregnancy, weight development of sows was determined. Pig progeny were investigated for number, gender, weight and potential disorder at birth and weight development was followed over weaning (21 d after birth) until slaughter (56 d and 63 d after birth for males and females, respectively). For an endocrinological assessment, blood plasma samples were additionally collected at slaughter.

Pharmacokinetic and application study implementation and sampling were both approved by the local governmental authority, reference # 55.2-1-54-2531-68-09.

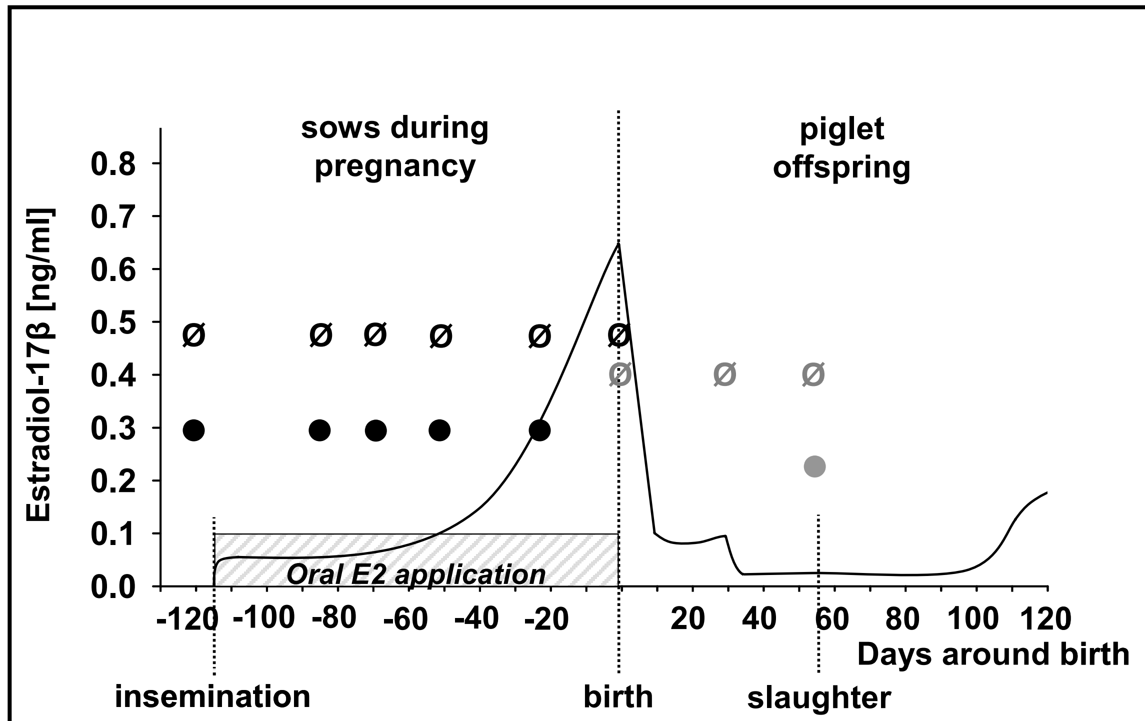

Fig. 4. Schematic depiction of peripheral plasma E2 concentrations naturally occurring in sows during pregnancy and piglet offspring before puberty. Low levels prevail in at the beginning of pregnancy but continuously rise towards birth. Offspring E2 concentrations at birth are accordingly high due to the only just interrupted hormonal exchange. The end of hormone transduction via maternal milk causing an intermediate level is reached at weaning. Sparse endogenous E2 production in piglets until the beginning of puberty (~100 d) is reflected by low plasma concentrations. In the investigated animal trial, sows orally received distinct doses of E2 starting with the day of insemination until the end of pregnancy. Blood sampling (●) and weight determination (Ø) were conducted as indicated.

Extraction of total RNA

Total RNA from peripheral white blood cells (WBC) and bovine endometrial samples was isolated using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA) as described earlier (Hammerle-Fickinger et al. 2010; Ulbrich et al. 2009a; Ulbrich et al. 2009b). Porcine RNA from investigated tissue samples was extracted utilizing the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) with modifications to the manufacturer’s instructions allowing for the nature of the samples. These included a homogenization of 20 mg tissue in 450 µl buffer RA1 plus 4.5 µl ß-mercaptoethanol using the MagnaLyser instrument (Roche, Basel, Switzerland) with Matrix-Green beads (MP Biomedicals, Illkirch, France) in three

successive pulses of 7000 rpm for 30 s. Furthermore, the second wash step with 600 µl buffer RA3 was conducted twice to grant sufficient RNA purity. After extraction procedures, quantity and purity was determined spectroscopically at 260/280 nm and 260/230 nm by the Nanodrop 1000 (peqlab Biotechnologie GmbH, Erlangen, Germany). Integrity of the acquired RNA was assed with the Agilent 2100 Bioanalyzer plus the RNA 6000 Nano Assay Kit (Agilent Technologies, Santa Clara, CA, USA) and ranged from RNA Integrity Number (RIN) 7 to 10 (0 indicating total degradation, 10 indicating intact RNA).

Gene expression analysis with RT-qPCR

Two-step quantitative real-time PCR experiments served to monitor RNA expression abundance in the respective tissues. These were conducted as described earlier (Ulbrich et al. 2009b), in accordance with the MIQE guidelines to guarantee best performance and interpretation of experiments (Bustin et al. 2009). In short, 1 µg of total RNA was reverse transcribed to complementary DNA (cDNA) with random hexamer primers (Life Technologies Ltd, Paisley, UK) using the M-MLV-Superscript Reverse Transcriptase system (Promega Corp., Madison, WI, USA). In the following PCR reaction, 1 µl cDNA and commercially synthesized oligonucleotid primers (Eurofins MWG Operon, Ebersberg, Germany) served to quantitatively amplify part of the respective target gene with the use of the Light Cycler DNA Master SYBR Green I kit (Roche, Basel, Switzerland). Applied primer sequences, gene references, amplicon length and required PCR annealing temperature are depicted in the respective appendices (Fürst et al. 2012b, App II.; Fürst et al. 2012a, App III. Tab. 1). Sanger-sequencing of the obtained PCR products was performed to verify amplification of the desired sequence fragments (4baselab, Reutlingen, Germany). The second derivative maximum method (LC software 4.05) served to obtain the respective cycle number (Cq) a defined fluorescence signal was reached in the PCR reaction. As such, Cq inversely correlates with the logarithm of the initial template concentration. For the comparison of gene expression between different tissues, means of raw Cq-values ± SEM subtracted from the arbitrary value 40 (ΔCq) are presented. This grants a high transcript abundance being indicated by a high ΔCq, a two-fold higher mRNA abundance is accordingly represented by an increase of 1 ΔCq (Livak and Schmittgen 2001). Within the same tissue, mRNA abundance was assessed normalizing the geometric mean of the three reference genes ubiquitin 3 (UBQ3), 18S rRNA and histone H3 subfamily H3A3 (H3F3A) to the target gene Cq values following the bestkeeper method by Pfaffl and colleagues (Pfaffl

et al. 2004). Results are presented as means ± SEM plus the arbitrary value 20 (ΔCq) in order to accordingly resemble high transcript abundance by a high ΔCq.

SiteFinding PCR

Originally described as a method for gene or chromosome walking (Tan et al. 2005), Site-Finding PCR was used to determine the promoter sequence of the porcine ESR1 gene. The principle of SiteFinding PCR involves an initial DNA double-strand denaturation, followed by a low-temperature priming with a SiteFinder oligonucleotide. Taq DNA polymerase subsequently extends according to the underlying sequence, the randomly attached SiteFinder thus generating double stranded DNA. In two nested PCRs, primers on the initially acquainted sequence plus primers matching the incorporated Sitefinder serve to exponentially amplify the specific target lying adjacent to the original sequence. The method was conducted as recommended (Tan et al. 2005), the resulting sequence was submitted to GenBank under the accession number FR682141 and served as basis for further analyses (Fürst et al. 2012a).

Genomic DNA extraction

Genomic DNA (gDNA) was isolated from various tissues for global and local DNA methylation analysis. While frozen tissue samples were directly subjected to the extraction procedure, WBC had to be acquired from whole blood first employing alkaline lysis of erythrocytes as described earlier (Hammerle-Fickinger et al. 2010). Isolation of gDNA was conducted using WBC pellets from 5 ml whole blood or 30 mg tissue in the peqGOLD Tissue DNA Mini Kit (peqlab, Erlangen, Germany) with appropriate modifications to the manufacturer's protocol. Briefly, WBC pellet and tissue samples were digested with standard amounts of proteinase K and DNA lysis Buffer T for 1.5 h and 3 h at 50 °C, respectively. After mechanical homogenization with ceramic beads in the MagNA Lyzer system for 30 s at 7000 rpm (Roche, Basel, Switzerland), additional 15 µl RNase A and proteinase K were added to the reaction for 30 min. Loading and binding steps were performed as recommended, spin columns were subsequently washed three times with the provided wash buffer to guarantee sufficient purity. Bound gDNA was eluted in 200 µl ultrapure water and spectroscopically assessed for quantity and purity with the NanoDrop 1000 (Peqlab, Erlangen, Germany).

Bisulfite conversion of genomic DNA

Bisulfate treatment is an essential prerequisite for PCR-based investigations on local DNA methylation. With this method, site-specific methylation information is transformed into a nucleotide-based context. Bisulfite converts unmethylated cytosines into uracil leaving methylated cytosines unchanged during the treatment. Once converted, the original methylation profile of the DNA can subsequently be determined by sequencing-based methods or high-resolution melting (HRM) after a previous PCR amplification. For the bisulfite conversion step (which greatly degrades DNA into smaller single-strands monomers, Fig. 5), the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA) was used according to the manufacturer's suggestions. In brief, 500 ng genomic DNA per sample were mixed with 130 µl CT Conversion Reagent and bisulfite-treated at 64 °C for 2.5 h. Afterwards, the DNA was desulphonated for 20 min and washed twice with the appropriate M-Wash buffer. Finally, the readily bisulfite-converted DNA (bcDNA) was eluted in 12 µl M-Elution buffer and quantity was spectroscopically determined with the NanoDrop 1000 (Peqlab, Erlangen, Germany) adjusting parameters for single stranded nucleic acids.

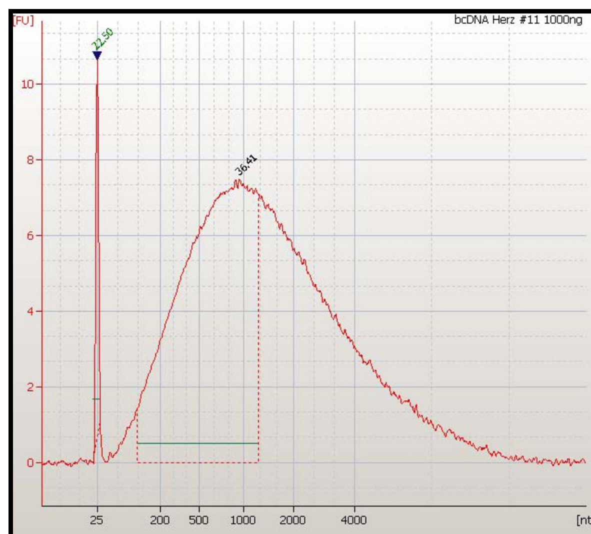

Fig. 5. Exemplary distribution of DNA fragment length after bisulfite conversion. Due to the treatment process, bcDNA is greatly degraded with most fragments consisting of not more than 500-2000 nucleotides. Primer design on bcDNA has to allow for this situation by limiting target PCR product length to less than 500bp.

Generation of bisulfite-converted DNA methylation standards

PCR on bcDNA is sensitive to amplification bias (Warnecke et al. 1997; Wojdacz and Hansen 2006). bcDNA input sample comprising distinct nucleotide sequences according to their former methylation status can get non-linearly amplified if inappropriate primers are utilized. Both a negative (i.e. unmethylated) control and a positive (i.e. methylated) control DNA of bovine and porcine origin thus had to be created to allow primer/PCR evaluation in subsequent steps of methylation analysis. Whole genome amplification (WGA) of a 25 ng gDNA template using the REPLI-g Mini Kit (Qiagen, Hilden, Germany) served to generate 5-10 µg gDNA subsequently considered as the 0 % or unmethylated control. The WGA treatment was conducted based on a 2.5 µl template scale and included all steps of DNA denaturation, neutralization and linear amplification for 10 h according to the manufacturer's instructions. The obtained WGA-DNA was purified utilizing the Genomic DNA Clean & Concentrator Kit (Zymo Research, Irvine, CA, USA) under the recommended conditions in order to remove salts and enzymes potentially disturbing successive treatment steps. This pure WGA-DNA was then used to generate fully methylated control DNA by *in-vitro* methylation using the M.SssI CpG Methylase (Zymo Research, Irvine, CA, USA). Briefly, 500 ng WGA-DNA as spectroscopically determined with the NanoDrop 1000 (Peqlab, Erlangen, Germany) were incubated in 16 µl ultrapure water with 2 µl 10x CpG reaction buffer, 1 µl 20x SAM and 1 µl CpG Methylase for 4 h at 30 °C. In order to receive the highest methylation degree possible, a subsequent incubation step for another 4 h at 30 °C with additional 0.75 µl Methylase and 1 µl SAM was added to the manufacturer's protocol before the reaction's inactivation. After bisulfite-conversion (see above), control bcDNA samples were finally mixed in 0, 25, 50, 75 and 100% unmethylated to methylated template ratios and applied in successive PCR amplification steps.

Local DNA methylation analysis using combined MS-HRM and Pyrosequencing

For the analysis of local DNA methylation patterns, bcDNA requires prior amplification in a PCR reaction. The received product can then be analyzed by high resolution melting analysis (HRM) and subsequent pyrosequencing. While the first method serves to evaluate potential PCR bias and allows a quick estimation of major methylation differences, the later grants quantitative determination of DNA methylation at single-CpG-site resolution. PCR amplification and HRM were performed sequentially on a Rotor-Gene Q (Qiagen) with the EpiTect HRM PCR Kit (Qiagen) following the manufacturer's protocol. In brief, template

bcDNA was diluted to a concentration of 4 ng/µl and 3.5 µl to 8.7 µl (depending on the reaction scale) were mixed with gene-specific primers and the required amount of HRM-mastermix for one reaction. Depending on the applied instrument for later pyrosequencing analysis, PCR was carried out in a 25 µl reaction scale for the later use in a PSQ 96MA Pyrosequencer (Biotage) or a 10 µl reaction scale for pyrosequencing in the PyroMark Q24 system (Qiagen). For the latter, the reduced amount of input product proved to be sufficient due to the superior quality of signal detection in the Q24 instrument. Primers for bovine ESR1 analysis were designed on in-silico bisulfite-converted DNA (Methyl Primer Express software, Version 1.0, Applied Biosystems) with Assay Design Software Version 1.0.6 (Biotage). The Pyromark Assay Design Software 2.0 (Qiagen) was applied for primer design to be used in porcine ESR1 investigations. Primers, underlying DNA sequences and annealing temperatures for bovine ESR1 and investigations on the porcine ESR1 are listed in the respective appendices (Fürst et al. 2012b, App II., Fürst et al. 2012a, App III. Tab. , respectively). HRM analysis was conducted on the fluorescence data acquisition channel as recommended by the manufacturer with 0.05 °C/2 s increments. By considering two regions before and after the major fluorescence (indicative for the melting of the generated PCR product), melting curves were normalized with the Rotor-Gene Q software to enable the comparison of samples with different starting fluorescence levels but distinct melting behavior. After HRM, PCR products were subsequently purified with the Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions and used as template for the pyrosequencing reaction. The amplicons were immobilized on streptavidin-sepharose beads (GE Healthcare), washed, denatured and the biotinylated strands released into annealing buffer containing the sequencing primer (described in Fürst et al. 2012b, App II.; Fürst et al. 2012a, App III. Tab. 1 for the respective applications). Each CpG-site was analyzed individually for its methylation status using the Pyro Q-CpG software (version 1.0.9, Biotage) in the PSQ 96MA Pyrosequencer (Biotage) or the PyroMark Q24 software (version 2.0.6, Qiagen) in the PyroMark Q24 system (Qiagen).

Global DNA methylation analysis using Luminometric Methylation Assay (LUMA)

On a global scale, DNA methylation was assessed using LUMA as previously described (Karimi et al. 2006a; Karimi et al. 2006b). In this method, isoschizomeric restrictions enzymes are used to digest gDNA according to their methylation status at CCGG-sites. The resulting overhangs are filled up in a pyrosequencing reaction and can thus be quantitatively assessed as total percentage of methylation at this site. While LUMA allows methylation

analysis over the whole genome, information readout is limited to a CCGG-context which is usually overrepresented in repetitive genomic areas. According to the applied instrumentation, 500 ng gDNA in the Pyromark Q24 system (Qiagen) or1 µg genomic DNA in the PSQ96 MA system (Biotage) were cleaved with FastDigest isoschizomere restriction enzymes *Hpa*II + *Eco*RI or *Msp*I + *Eco*RI (Fermentas, St. Leon-Rot, Germany) in two separate reactions for 20 min. Peak luminometric heights were assessed with the PyroMark Q24 (Qiagen) or the PSQ96™ MA (Biotage) software, respectively. The *Hpa*II/*Eco*RI and *Msp*I/*Eco*RI ratios were calculated as (dGTP + dCTP)/dATP for the respective reactions and presented DNA methylation levels were determined from the inversely proportional (*Hpa*II/*Msp*I) ratio.

Chromatin immuno precipitation (ChIP) from frozen tissue samples

ChIP assays were employed to identify binding of transcription factors and chromatin status at a specific gene location subject to the underlying DNA methylation level. Using the SimpleChIP Enzymatic Chromatin IP Kit with Magnetic Beads (Cell Signaling Technology, Danvers, MA, USA), ChIP was performed on heart and epididymis of 56 d old male piglets. For the available frozen tissue material, the manufacturer's protocol suitable for chromatin preparation from fresh single cell pellets had to be accommodated (Fürst et al. 2012a). Briefly, 1 g of frozen tissue was ground with a mortar and pestle in liquid nitrogen before proteins were cross-linked to the DNA with formaldehyde. After two wash steps and resuspension, the tissue was further homogenized by gently passing the tissue suspension 20 times through needles of different size (18 G and 20 G, successively). The suspension was subsequently centrifuged, the obtained pellet resuspended and washed. Using micrococcal nuclease, chromatin was then digested into 150-900 bp fragments before ultrasound sonication using an homogenizer Sonopuls HD70 (Bandelin electronic, Berlin, Germany) in order to break the nuclear membrane and thus release the chromatin. The prepared chromatin was stored in the appropriate ChIP-buffer at -80°C until immunoprecipitation (IP). For this procedure, antibodies specific to histone H3, transcription factors or negative control normal rabbit IgG were added to one sample of chromatin each according to the manufacturer's suggestions. The co-precipitated complex was subsequently captured by Protein G magnetic beads, crosslinking reversed and remaining DNA purified. Together with a 2 % input sample, the precipitated amount of DNA was finally quantified in a qPCR reaction on the Rotor-Gene Q (Qiagen) using the LightCycler DNA Master SYBR Green I kit (Roche). The received data represent the percentage of

immunoprecipitation for each antibody, respectively serum, at a specific site investigated. Within one tissue, the amount of a protein's association to the DNA can thus be easily assessed and compared between different genomic areas. To account for possible differences of ChIP efficiency between various tissues, occurrence of histone H3 at a distinctly methylated ESR1 CpG-site was investigated relative to the stable reference gene locus RPL30. Chromatin preparations used pooled tissues of two animals, and at least three independent experiments were performed per investigated protein. Antibodies used in the IP and primers applied for the quantification of the precipitated protein at the site of interest versus a reference locus are depicted in Fürst et al. 2012a, App III. Tab. 1.

Immunohistochemical localization of ESR1

Immunohistochemical detection was conducted to enable a localization of ESR1 in distinct structures of porcine tissues as well as a semi-quantitative estimation of overall protein abundance (Fürst et al. 2012a). In brief, porcine tissues at slaughter were fixed in formalin, dehydrated by an increasing ethanol-gradient and embedded in paraplast (Histo-Comp, Vogel, Giessen, Germany). For immunohistochemical analysis, tissue sections were dehydrated and subjected to a subsequently antigen retrieval using hot citric acid buffer. Endogenous peroxidase activity was blocked by H_2O_2, followed by incubation in normal serum to reduce nonspecific background signal. Sections were then incubated overnight at 4 °C with a normal serum or a rabbit polyclonal antibody raised against ESR1 of human origin (sc-7207, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The primary antibody was detected using the Vectastain Universal Elite ABC Kit (Vector Laboratories) in order to grant visualization even of little amounts of ESR1 in male tissues. For this procedure, slides were incubated with a biotinylated universal antibody followed by linking an avidin-biotin-peroxidase-complex. Subsequently, peroxidase activity was visualized, slides dehydrated and finally mounted in DPX (Fluka Chemie, Buchs, Germany). Images of exemplary samples were taken utilizing a digital camera system (DP72, Olympus, Hamburg, Germany) on a microscope with differential interference contrast (Leica, Wetzlar, Germany).

Dual-energy X-ray absorptiometry scanning (DEXA)

Male and female offspring of sows orally receiving estradiol-17β during *in-utero* development were investigated for their percentage of fat mass versus lean mass by Dual Energy X-ray Absorptiometry (DEXA) scanning as recently described (Fürst et al. *under review*). In brief, head and inner organs excluding abdominal fat were removed after slaughter in a standardized manner. Body composition was then assessed using QDR Series Explorer™-Bone Densitometer (Hologic Inc., Bedford, USA) at whole body scan mode with the scan length adjusted according to the individual animal (Fig. 6).

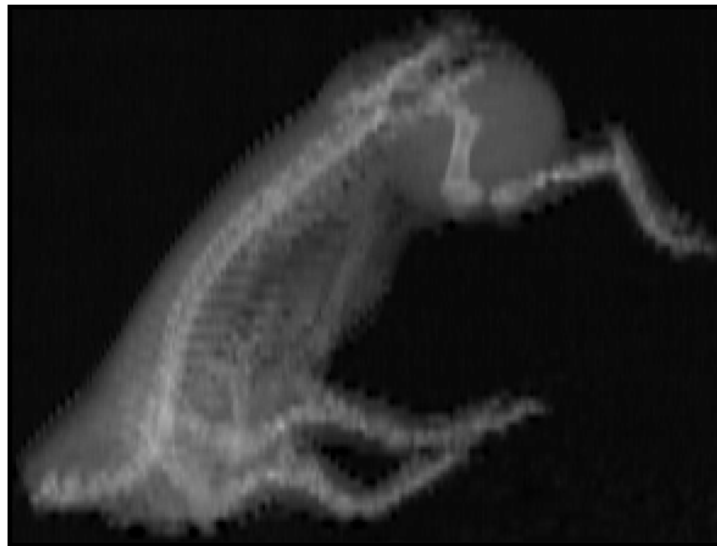

Fig. 6. Representative DEXA-scan of a slaughtered male piglet at eight weeks of age.

Plasma hormone concentration determination using competitive enzyme immuno assays

Competitive enzyme immune assays (EIA) were conducted to investigate steroid hormone concentrations in plasma of both sows and piglets as described earlier (Fürst et al. 2012a; Fürst et al. *under review*). After ether extraction, unbound E2 and T were determined in duplicates following protocols depicted by Meyer (Meyer et al. 1990), respectively Blottner (Blottner et al. 1995). This work was kindly provided by Waltraud Schmid and Brigitte Dötterböck at the chair of Physiology, Weihenstephan.

Transcription factor binding site analysis

Promoter and first intron of the porcine ESR1 gene were *in-silico* investigated for possible transcription factor binding sites in order to link DNA methylation differences with the gene's RNA expression. Utilizing the MatInspector (Genomatix, Munich, Germany) and TESS (University of Pennsylvania, Philadelphia, PA, USA) software algorithms (Cartharius et al. 2005; Quandt et al. 1995; Schug 2002) sites of distinctly methylated CpGs were examined as described (Fürst et al. 2012a).

Statistical analysis

All available data are presented as means ± SEM. T-test were performed to compare group effects between two, one way analyses of variance to determine effects of three and more treatments. For the latter, significant differences were localized by all pair wise multiple comparison procedures following the Holm-Sidak (Fürst et al. 2012b) or the Tukey method (Fürst et al. 2012a). If necessary, effects were calculated on the natural logarithm of data to ensure normal distribution. For statistical analysis in the E2 application study, models and adjusted effects accounting for the nested design of the study in terms of piglet siblings were commenced as described in detail (Fürst et al. *under review*). All depicted graphs were plotted using SigmaPlot, version 8.0 or 11.0 (SPSS Software GmbH, Munich, Germany).

3 Results and discussion

3.1 A convenient combination of MS-HRM and pyrosequencing provides bias-controlled DNA methylation analysis at single CpG-site resolution

Individual drawbacks of MS-HRM and pyrosequencing hinder proper DNA methylation analysis

For the detailed methylation analysis of a distinct gene like the estrogen receptor *ESR1* and its developmental regulation under endogenous ligand influence, methods providing quantitative results on DNA methylation patterns need to be employed. In commonly used sequencing-based analytical methods, methylation information is translated by bisulfite conversion and subsequently determined by PCR-based approaches like methylation-sensitive high resolution melting (MS-HRM) or pyrosequencing (Fürst et al. 2012b). HRM analysis utilizes the different fluorescence profiles of melting PCR products differing in their base composition subject to their former methylation status (Wojdacz and Dobrovic 2007, 2009; Wojdacz et al. 2008a). Initially developed for genotyping studies, the procedure is usually conducted as a sequence of PCR on bisulfite-converted DNA (bcDNA) followed by high-resolution thermal denaturation. As such, MS-HRM is a high-throughput platform for cost- and labor-efficient screening of methylation changes (Fürst et al. 2012b). Pyrosequencing technology as a non-electrophoretic method for DNA sequencing is based on the sequencing-by-synthesis principle utilizing the real-time detection of released pyrophosphate for accurate and quantitative analysis of DNA sequences (Ronaghi et al. 1998). With this capability, it has become the gold-standard platform for analysis of single nucleotide polymorphisms (SNPs), short repeats, RNA allelic imbalance, gene copy number and especially DNA methylation status at single CpG-site resolution (Colella et al. 2003; Fakhrai-Rad et al. 2002; King and Scott-Horton 2008; Marsh 2007; Ronaghi et al. 2007).
Both depicted methods applied separately for DNA methylation analysis suffer from their respective limitations. On the one hand, a fundamental limitation of MS-HRM is characterized by the impossibility to attain detailed information on the methylation status of single CpG-sites. Results are limited to qualitative estimations by comparing melting behavior of distinct PCR products comprising a varying number of CpG-sites, and a recent approach to obtain quantitative results on HRM data using differential fluorescence curves remains to be challenged (Liu et al. 2010). Another suggestion to this shortcoming involves

Sanger-sequencing following HRM (Wojdacz et al. 2010), but still, provided information will be limited to a qualitative readout. In pyrosequencing on the other hand, surveillance of preceding PCR on bcDNA is usually limited to agarose-gel based methods, capable of only indicating the product generation of expected size from both completely methylated and unmethylated templates. Further evaluations of the generated product are barely considered a major problem in pyroseqencing approaches although they were highly recommended (Shen et al. 2007; Warnecke et al. 1997). This necessity of PCR control arises from the specialty of the template - on bcDNA, the phenomenon of PCR bias has been recognized as differing amplification efficiencies of methylated vs. unmethylated DNA molecules and has been connected to the primes applied. As a consequence, inappropriate primer design or annealing temperature might tremendously affect the composition of the amplicon and thus later findings on site-specific methylation (Shen et al. 2007). Interestingly, MS-HRM approaches utilizing the same template have clearly addressed these limitations of PCR on bcDNA and established guidelines for primer design to control PCR bias (Wojdacz and Dobrovic 2007, 2009; Wojdacz et al. 2008a). As a consequence, the work at hand combined MS-HRM and pyrosequencing with the aim to overcome limitations of PCR control prior to pyrosequencing and MS-HRM resolution (Candiloro et al. 2011; Fürst et al. 2012b).

WGA and methylase allow the versatile generation of bcDNA standards which help to conduct unbiased MS-HRM as a first rough visual estimation of DNA methylation levels

Among the above mentioned guidelines for controlling PCR bias in MS-HRM, the incorporation of gradually methylated DNA samples as a standard to determine non-linear amplification efficiencies is of major importance. However, these standards were commercially only available for the mouse and human so far. In the work at hand, a method for the generation of gradually methylated DNA was introduced that allows standard generation from any species of interest by applying the principle of whole-genome amplification (WGA) to a mixture of genomic DNA (gDNA) (Fürst et al. 2012b; Kristensen et al. 2008). Since methylation marks are not translated to the newly synthesized strands with Taq-polymerases (used in WGA), the methylated CpG-site of one allele is subject to the underlying amplification diluted approximately 200 - 500 fold with unmethylated alleles. As a result, a template DNA with a CpG-site theoretically up to 100% methylated across all enclosed alleles is reduced down to 0.5% to 0.2% methylation at the respective site. The amplified product can thus be considered a virtually unmethylated standard. Subsequent methylase treatment in turn grants DNA completely methylated at all CpG-sites. After

bisulfite conversion, PCR on these samples revealed nearly identical set off points of exponential fluorescence increase and similar amplification efficiencies within different primer pairs, confirming that no additional bias or modification had been introduced to the samples (Fürst et al. 2012b, App. II, Fig. 4A).

For further methodological investigations, methylation levels of the bovine ESR1 gene in both white blood cells and endometrial samples from cyclic and pregnant cows served as an exemplary biological background to asses the suitability of combined MS-HRM and pyrosequencing (Fürst et al. 2012b, App. II, Fig. 3). Primers for MS-HRM were created according to previous suggestions on annealing temperature (Shen et al. 2007; Wojdacz et al. 2008b). The incorporation of a limited number of CpG dinucleotides in the primer sequence (Wojdacz et al. 2008a; Wojdacz et al. 2008b) however was avoided. This practice of introducing artificial bias by favoring methylated template amplification was proposed to grant improved resolution of MS-HRM by enabling the HRM-based discrimination of samples with overall 0.1 % and 1 % methylation. In the current approach of applying pyrosequencing to the MS-HRM product, this is not a requirement since detailed information will be acquired through the subsequent quantitative analysis of single CpG-sites. On the contrary, the identification of similar small variations in medium or highly methylated backgrounds (e.g. 75.1 % to 76 %) would be limited due to non-linear amplification. Furthermore, the distinct bias could likewise lead to overestimation of methylation differences in low-level backgrounds causing misinterpretations on biological significance when e.g. 2 % vs. 3 % turn into 4 % vs. 9 % methylation. The aim of preferably even amplification for each primer pair created under the above mentioned conditions was assessed by monitoring the gradational melting curves of the methylation dilution series. This proved excellent to exclude inadequate primers with strong bias as exemplary shown in Fürst et al. 2012b, App. II, Fig. 4B. Templates between 25 % and 75 % methylation were only weakly resolved by this discarded primer pair while DNA with 75 % methylation could not be distinguished from fully methylated samples any more after PCR. It is important to mention here that in standard PCRs with subsequent gel electrophoresis, this massive bias would not have been detectable. The primer-pairs employed for the investigation of methylation levels in the bovine ESR1 provide an example of adequate amplification revealing only a slight tendency of favoring the methylated allele while still allowing good discrimination between 0 % and 75 % methylation and separating 75% and 100% methylated DNA (Fürst et al. 2012b, App. II, Fig. 4C,D). Besides this necessary evaluation of potential PCR bias, similarities derived from normalized HRM profiles and their position relative to the methylation standards then allow a first rough estimation of the methylation content in the applied biological background (Fürst et al. 2012b, App. II, Fig. 5).

MS-HRM products can easily be introduced into subsequent pyrosequencing which is superior to quantitative differential fluorescence peak determination

While most studies utilizing MS-HRM stop at the point of visual DNA methylation evaluation, a new method was recently proposed that should allow additional quantitative determination (Liu et al. 2010). Following the authors' suggestions, differential graphs of the normalized melting curves were generated from the exemplary bovine primer pairs by standardizing the HRM profiles against the unmethylated control DNA (Fürst et al. 2012b, App. II, Fig. 6A). A linear regression curve was then established on the peak values of the methylation control standards (Fürst et al. 2012b, App. II, Fig. 4B). Employing the determined regression curves, methylation levels of two exemplary endometrial and WBC samples were calculated based on their respective differential fluorescence curves within one primer assay (Fürst et al. 2012b, App. II, Fig. 6C,D).
For the main goal of expanding MS-HRM with single CpG-site resolution, PCR products were purified and subsequently subjected to pyrosequencing. This introduction performed excellently - the rough visual estimations of average methylation from MS-HRM were approved and distinctly methylated sites were noticed to account for the differential melting behavior of the biological samples in contrast to the artificially created standards (Fürst et al. 2012b, App. II, Fig. 7C). In comparison with average methylation levels gained from pyrosequencing-derived single CpG-site data, regression curve based calculations from exactly the same amplicon proposed substantially varying data, e.g. 44.7 % vs. 22.6 % or 5.2 % vs. -1.1 % methylation (Fürst et al. 2012b, App. II, Tab. 1). Since even negative values were calculated, it evolves that the newly introduced method by Liu and colleagues suffers from serious shortcomings; Variations in PCR bias at different levels of methylation can induce inappropriate regression curves and thus imprecise data. Moreover, distinctly varying methylation levels at individual CpG-sites cannot be represented in the peak values of differential fluorescence. Thus, the non-paralleling curve shapes of biological samples within the artificial reference set are hardly comparable. Additionally, the arbitrary setting of fluorescence normalization regions in the HRM software allows a relatively strong uncontrolled influence on curve-shapes and thus on the differential fluorescence peak values.
With these drawbacks, quantitative differential fluorescence peak determination evolves as a source of misleading results and leaves MS-HRM analysis at the state of only qualitative data generation. In contrast, the concept of MS-HRM for efficiently bias-controlled screening appears a profound method if it is combined with pyrosequencing granting supplemental single CpG-site resolution as depicted (Fig. 7) (Fürst et al. 2012b).

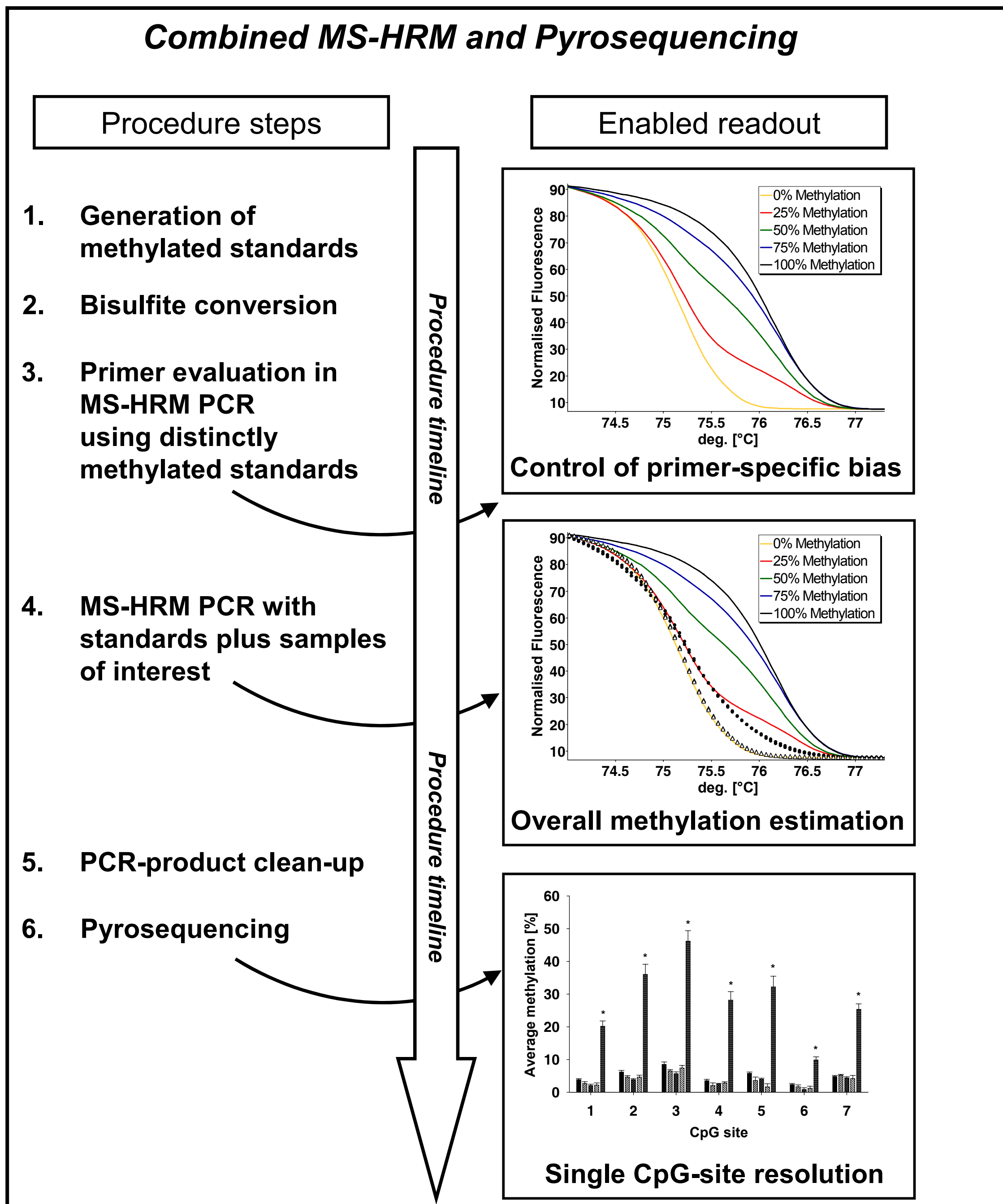

Fig. 7. Schematic depiction of necessary procedure steps in combined MS-HRM and pyrosequencing that enable bias-controlled quantitative single CpG-site DNA methylation analysis.

3.2 ESR1 expression in prepubertal piglets is linked to a formerly not described way of epigenetic regulation

Equipped with the developed methodology as described above, further interest was laid on investigating the contribution of epigenetic mechanisms to the transcriptional regulation of the most important mediator of estrogenic action, the estrogen receptor ESR1. Neither in humans nor rodents nor porcine, a deeper understanding of DNA methylation patterns guiding transcriptional regulation under physiological conditions has been achieved so far. Based on former investigations in women using low-resolution methodology (Piva et al. 1989), three functionally distinct areas in the 5' region of the *ESR1* gene evolved interesting for detailed investigations (Fürst et al. 2012a, App. III, Fig. 1). These represent an upstream promoter region (-1kb), a promoter CpG-island and an intragenic (+1kb) region. For the pig used in this work at hand as a model system of similar estrogenic endocrinology compared to the human, the promoter region was sequenced first (GenBank # FR682141) and than aligned against the human ESR1. High sequence homologies especially in essential areas (splice site borders, splice acceptance site, Tataa-boxes, GC-boxes) encouraged to propose the genetic structures as presented (Fürst et al. 2012a, App. III, Fig. 1). While the promoter region appears an obvious target where epigenetic modifications might impact on transcription, the concept of a major role in transcriptional regulation via DNA methylation also at intragenic regions has been recently proposed (Maunakea et al. 2010; Verdel et al. 2004; Zaratiegui et al. 2007). In this view, own findings in the female bovine of *ESR1* regulation via adjacent CpG-sites in the first intron (Fürst et al. 2012b) and the paralleling results of the initial report on human ESR1 methylation (Piva et al. 1989) seem to perfectly approve this concept.

ESR1 expression is not established via methylation over adjacent CpG-sites

Two exemplary tissues of male growing piglets representing an approximately 250-fold difference in naturally occurring high (epididymis) and low (heart) *ESR1* transcriptional abundance (Fürst et al. 2012a) were used to clarify underlying DNA methylation patterns. In the -1kb site, methylation levels at each side varied significantly between both tissues with higher average methylation at five of six sites in epididymis as compared to heart but only with differences of less than 8 % methylation. Overall DNA methylation did not correspond in similar magnitudes to the transcriptional abundance of the individual tissues as disclosed

for the human uterus and WBC sharing comparable differences in *ESR1* expression. The investigated area residing in the core of the *ESR1* promoter CpG-island showed DNA methylation of around 5 % at each individual site with no statistical significance occurring between tissues (Fürst et al. 2012a, App. III, Fig. 6B). This parallels observations in the human (Piva et al. 1989) and supports the theory of CpG-island methylation being rather implicated in pathophysiological events like breast cancer (Giacinti et al. 2006) than in non-pathophysiological situations (Fürst et al. 2012a). For the intragenic region at +1 kb, mRNA expression data indicating high mRNA expression in the uterus (Fürst et al. 2012b), intermediate in female WBCs and male growing piglet epididymis as well as lowest mRNA abundance in piglet heart (Fürst et al. 2012a, App. III, Fig. 3A) had expected even higher methylation levels at this pronounced area in terms of the heart muscle. However, this area neither revealed appreciable differences over all investigated CpG-sites (Fürst et al. 2012a, App. III, Fig. 6A) as seen between tissues in bovine or human, thus indicating that this region together with the -1kb area does not equally contribute to *ESR1* expression in male growing piglets as given in other depicted examples (Fürst et al. 2012b; Piva et al. 1989). Still, one site in contrast to every other showed a 2-fold higher methylation of 42 % in epididymis compared to 21 % heart ($p < 0.001$). Investigating other tissues, we accordingly found distinct methylation levels for this CpG-site corresponding to the underlying *ESR1* expression such as 35 % in testis and 27 % average methylation in kidney displaying high and intermediate transcript abundance, respectively. Regression analysis underlined that a major part of gene expression differences between piglet tissues (83 %) can indeed be explained by the associated DNA methylation level at the specific single site (Fürst et al. 2012a, App. III, Fig. 6D) (Fürst et al. 2012a).

DNA methylation at a transcription factor binding site is connected to the underlying chromatin status

Since higher DNA methylation is usually associated with reduced transcriptional expression, *in-silico* analyses were subsequently employed. At the distinctly methylated CpG-site algorithms consistently postulated a conserved binding site for the transcriptional repressor TG-interacting factor 1 (TGIF1). This member of the TALE superfamily of homeodomain proteins contains a domain for the interaction with histone deacetylase 1 (HDAC1) whereby it mediates its repressive effects by acting on the chromatin status (Gehring et al. 1994; Wotton et al. 1999). Importantly, DNA methylation of a transcription factor (TF) binding site is in theory an effective mechanism preventing transcription factors from binding to the DNA

(Choy et al. 2010; Tate and Bird 1993; Tierney et al. 2000; Watt and Molloy 1988). ChIP experiments were thus conducted in order to elucidate whether an augmented methylation at the respective site could impair the presence of TGIF and HDAC1 thus ultimately increasing transcriptional expression by impacting on chromatin density. While the adjusted protocol for chromatin preparation out of frozen tissue generated sufficient amounts at desired fractionation (Fürst et al. 2012a, App. III, Fig. 7A), commercially available antibodies directed against human or rodent TGIF and HDAC1 performed poorly. This was probably due the missing formulation suitable for ChIP experiments as well as species specificity. In contrast, histone H3 protein was perfectly determined at the differentially methylated *ESR1* site with a ChIP-validated antibody. A significantly lower appearance relative to a reference locus was found in epididymis compared to heart (101.9 ± 4.5% vs. 131.6 ± 2.8%, respectively; $p = 0.005$) (Fürst et al. 2012a, App. III, Fig. 7C), suggesting indeed a more condensed chromatin structure in the latter tissues causative for the observed lower transcriptional abundance. Apart from the necessity to complete the missing link of distinct TGIF and HDAC1 levels, the depicted method of impeding TF binding sites by single-CpG-site methylation emerges as yet undescribed way of *ESR1* transcriptional regulation. With regard to the similar ranges of DNA methylation in female bovine WBCs (15-50 %) and epididymis in male developing piglets (15-55 %) at comparable transcriptional abundance of *ESR1* (Fürst et al. 2012a; Fürst et al. 2012b), this newly discovered mechanisms could be indicative for a two-step model of mRNA expression regulation. Hypothetically, low to intermediate expression levels as given for the male situation might use single-CpG-site based methylation differences disturbing TF (activators or repressors) binding sites, while intermediated to high expression levels (as seen from female human and bovine) get manifested by DNA methylation differences over various adjacent CpG-sites (Fig. 8) (Fürst et al. 2012a).

Still, differences in the usage of DNA methylation could equally appear due to gender or developmental status given its dynamic nature (Metivier et al. 2008). Furthermore, species-specific regulation mechanisms might be causative as well since especially promoter region sequences might vary tremendously amongst species as seen e.g. in rats and humans (Kos et al. 2001). In this view, distinct methylation patterns creating smaller expressional differences within tissues might be found in certain species' promoter regions as highlighted for *ESR1* expression in the rodent brain (Westberry et al. 2010) but not necessarily in others. For the epididymis in developing piglets, 2-fold higher transcriptional abundance ($p < 0.05$) (Fürst et al. 2012a, App. III, Fig. 3C) was accompanied by several distinctly methylated CpG-sites in the proposed promoter area ($p < 0.001$, Fürst et al. 2012a, App. III, Fig. 6A),

but given their oppositional character, a final conclusion on the importance of the area for regulating transcriptional expression within tissues is unfeasible.

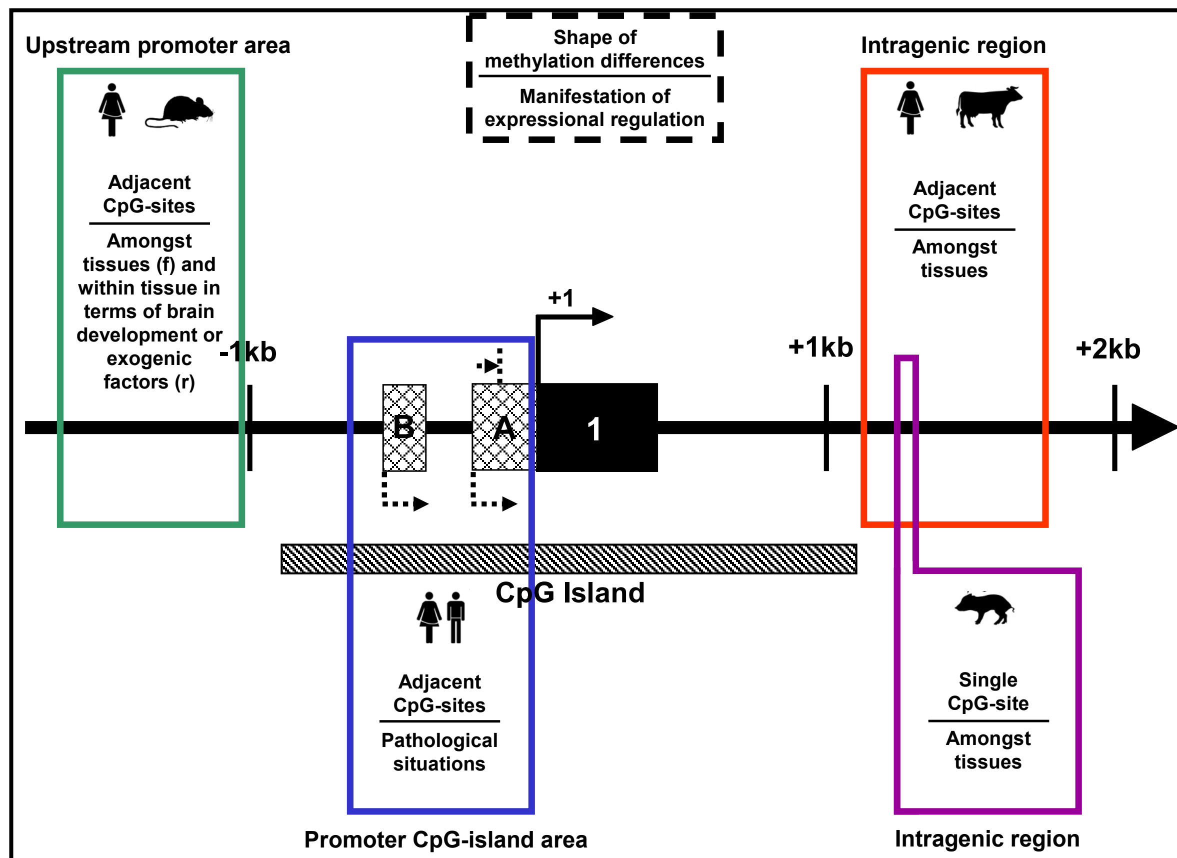

Fig. 8. Summary of areas in the ESR1 *gene established to reveal a distinct DNA methylation pattern subject to the gene's underlying expressional abundance. The use of specific sites according to the biological background (f = human female, r = rodents) highlights the complexity of this epigenetic mechanism in driving transcriptional expression. (A, B = transcriptional splice variants, +1 = translational start site, 1 = first exon)*

3.3 Piglet developing tissues are not immediately influenced by naturally occurring distinct E2 concentrations in terms of ESR1 mRNA expression

For the investigated ESR1 as described above, expressional regulation has been clearly linked to influences of its ligands, particular by the most potent naturally occurring estrogen E2. This important role for E2 was recently strengthened in the rat in terms of brain masculinization/feminization where a naturally occurring hormone surge shortly after birth could be correlated with ESR1 DNA methylation and an associated diminished mRNA expression (Kurian et al. 2010). The postnatal decline in *ESR1* mRNA abundance of the

developing mouse brain has accordingly been linked to estrogen receptor promoter DNA methylation (Westberry et al. 2010). This raised the question whether circulating steroid hormone concentrations changing dynamically during development might play an important role in regulating *ESR1* in various other different cell types and tissues as well (Fürst et al. 2012a). For this investigation, growing male piglets as model were employed since developmentally changing plasma E2 concentrations were reported in a former study (Wagner and Claus 2008). These findings were confirmed with newborns exhibiting E2 plasma concentrations of 153.5 ± 29.9 pg/ml, piglets at day 11 32.1 ± 7.2 pg/ml and eight week olds showing 4.8 ± 0.8 pg/ml (means ± SEM; ($p < 0.001$)(Fig. 2). These distinct levels presumably originate on the one hand from the instantly interrupted feto-maternal interchange at birth were sows are acknowledged to exhibit up to 400 pg/ml plasma E2 (Robertson and King 1974). On the other hand, the intake of E2 via maternal milk during suckling most likely contributes to the intermediate concentration of E2 at the age of 11 d whereas low endogenous production of E2 in male piglets weeks before the onset of puberty is reflected in the low concentrations at 56 d. 12 tissues were investigated for *ESR1* abundance over these time points but pronounced variation in mRNA expression was mainly found amongst tissues not at distinct E2 concentrations represented by the developmental days. Tissues with highest expression like epididymis varied in their transcriptional abundance up to 250-fold from tissues of low ESR1 expression such as heart (Fürst et al. 2012a, App. III, Fig. 3A). Immunohistochemical analysis confirmed this trend (Fürst et al. 2012a, App. III, Fig. 4) underlining that reproductive tissues of prepubertal males naturally exhibit relatively high *ESR1* expression occurring in concert with very low endogenous levels of E2. This might explain why these males seem especially prone to disturbance as stated in several reports of exogenous stimulation/perturbation by estrogenic substances (Delbes et al. 2006; Prins et al. 2006; Prins et al. 2008; Sato et al. 2006; Vandenberg et al. 2009). The naturally occurring E2 concentrations as presented in male developing piglet however did not relate to transcript abundance within tissues. No general decline of *ESR1* contrary to findings in the mouse brain (Kurian et al. 2010) was detected while some selected tissues rather displayed a slight increase in *ESR1* expression, e.g. kidney, adrenal gland, prostate, liver or muscle (Fürst et al. 2012a).

3.4 Distinct doses of oral E2 applied over pregnancy impact on sows and selectively program piglet offspring

Oral E2 exposition affects weight gain in sows but omits piglet birth parameters

In contrast to the obviously sparse immediate effects of endogenous E2 concentrations on the developing offspring as depicted above, exogenous estrogens acting as endocrine disruptors have been widely accepted to impact on physiological systems such as reproduction and adipogenesis. This is especially the case when these EDC are subjected to particularly sensitive targets towards hormonal disturbance, namely unborn fetuses, neonates and prepubertal children given their low endogenous hormone production but available receptors as stated above. While BPA and DES draw main attention in this field, E2 lacks the awareness as a potential EDC especially in terms of epigenetics-based developmental programming capabilities. This was aimed to be analyzed in the work at hand utilizing the pig as an animal model of placental estrogen production and prenatal fat tissue development (Ailhaud et al. 1992) likewise in human. A possible oral consumption of a high (1000 µg E2/kg bw/d) and of lower doses resembling incorporation at the NOEL and ADI level (10 µg E2/kg bw/d and 0.05 µg E2/kg bw/d, respectively) during pregnancy should be mimicked. Targeted doses were initially investigated for their pharmacokinetics in castrated male piglets. The highest amount of orally administered E2 led to a rise in average blood plasma concentrations with a peak of 77.3 ± 23.9 pg/ml (n = 3, mean ± SEM) already 15 min after application followed by a fast decline (Fürst et al. *under review*, App. IV, Fig. 2). This evolves indicative for a rapid appearance and fast clearance as seen in a sublingual application (Claus et al. 2007). Since plasma concentrations on an average level of 23.5 ± 4.0 pg/ml (mean ± SEM) were determined for this group remaining from 6 h to 12 h, enterohepatic recycling seems to play an important role in this metabolizing process as well. Compared to the control group with an average of 5.3 ± 2.5 pg E2/ml (n = 2), no major rise was measureable for the lower doses applied given the limited number of individuals and accuracy of the EIA test. However, due to the depicted persistence and low endogenous plasma hormone levels in sows following conception, these might still be effective (Fürst et al. *under review*).

During application, pregnant sows received their daily amount of E2 distributed into two bread rolls over the day plus their normal feed ration. The amount of ZON detected in feed was under 2.6 µg/kg dry mass resulting in a daily intake of less than 0.1 µg/kg bw/d. This exposition does not reach levels described to mediate estrogenic signaling via the estrogen

receptor alpha (10 µg/kg bw/d) (Bauer et al. 1987) and assures no further contributing or masking effects to our E2 supplementation, particularly at the lowest dose. Endogenous E2 plasma concentrations in all sows before application of E2 (-7 d) were expectedly low whereas during pregnancy the applied E2 was significantly displayed in the highest treatment group. Plasma E2 concentrations reached 30.2 ± 3.8 pg/ml (mean ± SEM) compared to the control group with 12.0 ± 2.4 pg/ml on day 35 ($p = 0.005$), 55.8 ± 7.7 pg/ml vs. 19.1 ± 4.0 pg/ml on day 49 ($p < 0.001$) and 80.8 ± 27.9 pg/ml vs. 24.4 ± 6.9 pg/ml on day 70 ($p = 0.023$) (Fürst et al. *under review*, App. IV, Fig. 3A). This effect was not determined under the high endogenous background levels prevailing at the end of pregnancy. In contrast to E2, testosterone (T) levels in pregnant sows remained unchanged subject to the E2 application with the exception of day 97 where animals treated with 10 µgE2/kg bw/d exhibited slightly higher concentrations of T compared to the control group ($p = 0.04$) (Fürst et al. *under review*, App. IV, Fig. 3C). Importantly, no immediate adverse effects on sows were ascertained during their exposure to E2. However, sows receiving 1000 µg E2/kg bw/d during pregnancy gained more weight than the control group (29.2 ± 1.8 % vs. 19.0 ± 0.9 %, $p = 0.006$) and a trend of augmented weight gain paralleling the ascending amounts of E2 was also seen under lower E2 exposition (Fürst et al. *under review*, App. IV, Fig. 4A). Since estrogens in female adults are reported to rather protect from obesity (Demir et al. 2008; Misso et al. 2003; Stubbins et al. 2011), simple fattening of sows seems an improbable reasoning for this observation but remains to be investigated. Weight gain did not origin from number or total mass of piglets since these did not differ at birth (Fürst et al. *under review*, App. IV, supplemental material). Likewise, no birth defects were encountered in offspring and gender was similarly distributed as well. Observations from the estrogenic EDC BPA parallel these findings since a gestational low exposure (1 mg/l drinking water) did accordingly not modify the sex ratio or litter size at birth (Somm et al. 2009).

Male piglets only appear developmentally programmed in terms of body composition

In contrast to the absent effects on piglets at birth, distinct phenotypes subject to the *in-utero* exposition with E2 occurred at weaning. Piglets exhibited a significant reduction in body weight when treated with 1.000 µg E2/kg bw/d (7.68 ± 0.19 kg, $p = 0.0071$) and by trend, this was also given in the group receiving 10 µg E2/kg bw/d (8.06 ± 0.28 kg, $p = 0.0851$) compared to the control group (8.69 ± 0.26 kg) (mean ± SEM) (Fürst et al. *under review*, App. IV, Fig. 4B). Most importantly, the conducted study marks to best of

knowledge, the first reported *in vivo* effect caused by parental application of E2 at the proclaimed ADI level (Fürst et al. *under review*). Here, 0.05 µg E2/kg bw/d decreased offspring weight to 8.15 ± 0.27 kg (p = 0.0002). Later in life, male and female animals were slaughtered with a difference of seven days at the age of eight, respectively nine weeks. Thus separated by gender, mean body weight of males still appeared to be lower subject to E2 administration similar to weaning whereas the effect of reduced weight in females was less pronounced (Fürst et al. *under review*, App. IV, supplemental material). These observations seem to partly parallel findings from DES treated mice where a catch-up growth later in life followed the initially depressed weight subject to a neonatal high dose exposure (1 mg/kg bw/d) (Newbold et al. 2007). The same effect was caused by low doses of DES (1 µg/kg bw/d), but remained exclusive for males which appear, as depicted above, probably particularly sensitive due to their endocrinological status (Newbold et al. 2008). While in contrast to our piglets prenatal high exposure DES applications detected a reduced weight already at birth, a similar compensatory growth was observed before puberty (Newbold et al. 2005). Increased offspring body weight has also been reported in numerous studies investigating developmental BPA exposure, but growth-alleviating effects or no influence were stated as well (vom Saal et al. 2012). Paradoxically, a recent study on soy-deprivation in the rodent mother's food even found increased estradiol serum concentration in fetuses appearing due to the reduced phytoestrogen concentration (Ruhlen et al. 2008) causing slighter offspring at birth but heavier adults. These depicted results highlight that distinct phenotypes can evolve according to the individual substance applied with time point as well as dose of administration contributing to a substantial degree (Fürst et al. *under review*).

Besides their depicted weight, offspring were analyzed in terms of body composition utilizing DEXA-scanning. This was conducted since DES or phytoestrogenic treatment-associated weight gain in adult life has been reported to be supported by obesity in both sexes (Newbold et al. 2007; Ruhlen et al. 2008). In piglet females, overall body fat percentage (13.8 ± 0.8 %) (mean ± SEM) did not show a statistically significant change due to the E2 treatment of their mothers (Fürst et al. *under review*, App. IV, Fig. 5B) thereby contrasting perinataly applied low doses of BPA causing an increase of parametrial WAT in female rats only (Somm et al. 2009). The tendency of reduced body weight in piglet males at slaughter however was accompanied by a significantly modified body composition favoring fat against lean mass. While control males exhibited similar levels than their female counterparts (13.1 ± 0.4 % fat, mean ± SEM), progeny of sows treated with 1000 µg E2/kg bw/d showed 16.5 ± 0.8 % body fat percentage (p = 0.0024) and 10 µg E2/kg bw/d revealed 15.5 ± 0.7 % (p = 0.0327) (Fürst et al. *under review*, App. IV, Fig.

5A). These observations of piglets exhibiting a modified body composition in preadolescent age strikingly relate to observations in humans. There, obesity is reported to not only increase in adults but similarly in children (Catenacci et al. 2009). Especially for the latter group this has drawn major concerns since the risk of severe obesity and cardiovascular diseases later in life has been associated with this spreading childhood disease (Raghuveer 2010; The et al. 2010).

While weight and body composition of piglet offspring appeared modified subject to the preceding *in-utero* treatment, both E2 and T in male and female offspring did not display significantly different plasma concentrations at slaughter (Fürst et al. *under review*, App. IV, Fig. 3B,D). This supports deliberations that instead of an immediate influence through an altered hormonal status, piglets experienced adverse developmental programming during pregnancy. In order to cause the depicted effects, E2 treatment might have shifted the commitment process of mesenchymal stem cell (MSC) towards the preadipocyte lineage instead of bone or muscle generation (Janesick and Blumberg 2011; Rosen and MacDougald 2006). In consequence, higher amounts of mature adipocytes (and thus obesity) might be generated from these enriched precursors and persist into adulthood (Janesick and Blumberg 2011) (Fig. 9). Experience with the influence of EDCs on this commitment however is sparse. So far, only tributyltin and rosiglitazone treatment has been depicted to enrich preadipocytes from a WAT-derived source of MSCs (Kirchner et al. 2010), the role of estrogenic compounds in this developmental process remains indistinct. In contrast, overexpression of the androgen receptor (AR) in males has been recently reported to inhibit adipocyte commitment (Semirale et al. 2011), and low testosterone (plus high estradiol) levels were found associated with obesity in Japanese men (Akishita et al. 2010). T via the AR has been furthermore described to mediate obesity-protecting effects in males, whereas the same is primarily conducted by E2 and ERs in females (Bjorntorp 1997). The possible connection to the depicted effects of modified body composition solely in male comes from observations that E2 is actually able to decrease AR expression in fat likewise to what has been described earlier in other tissues (Cardone et al. 1998). *In-utero* exposition to E2 might thus have diminished the necessary AR expression protecting from immoderate adipocyte recruitment especially in males. Since T via AR furthermore decreases essential co-regulators such as cEBP/α or PPARγ in the development of preadipocytes to adipocytes (Dieudonne et al. 2000; Janesick and Blumberg 2011; Singh et al. 2003) (Fig. 9), AR down-regulation via E2 signaling evolves as a reasonable form of developmental programming causing the modified body composition as observed (Fürst et al. *under review*).

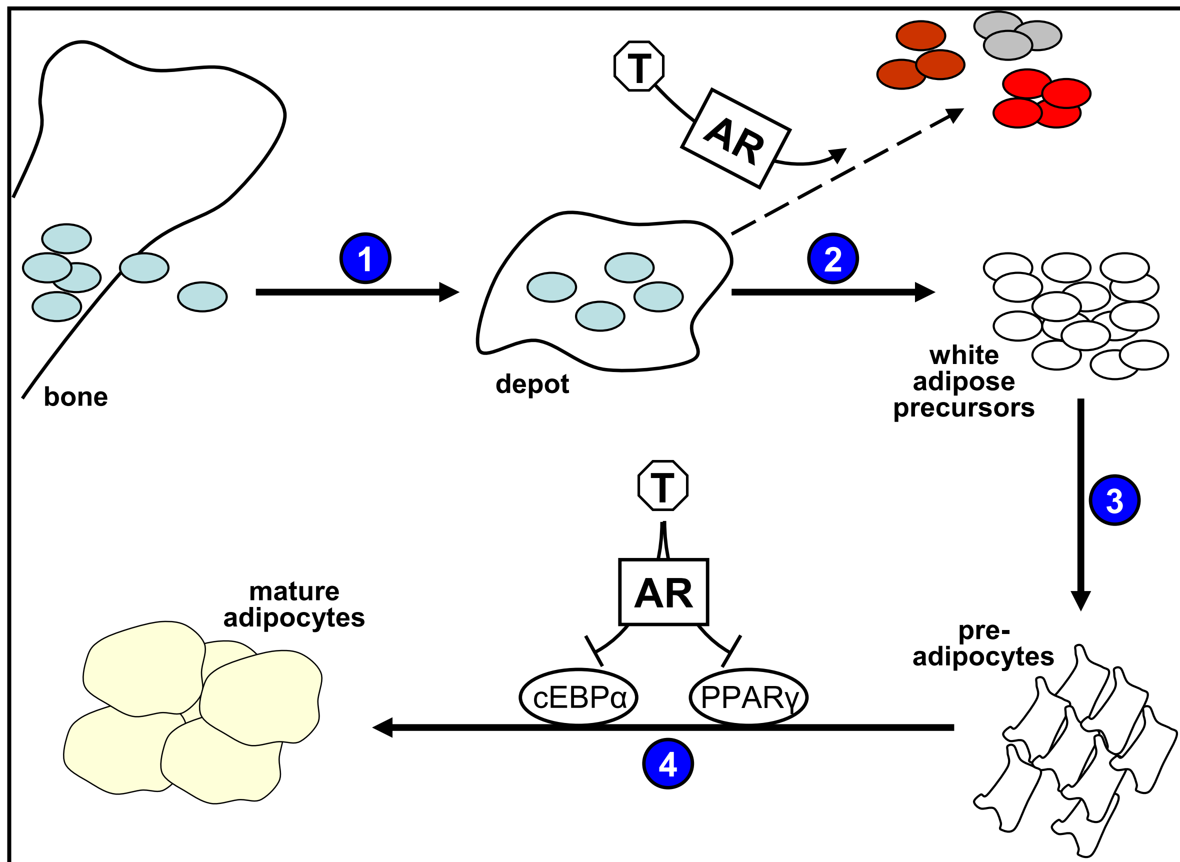

Fig. 9. Schematic depiction of T and AR contribution to adipose tissue development. MSCs (light blue) are recruited from bone marrow to later tissue depots (1). Under a diminished influence of T due to a possible AR downregulation, the recruitment of MSCs to the adipose lineage would be favored over brown fat, cartilage or muscle tissue generation (2). After the commitment of precursors to pre-adipocytes (3), specific TFs might exceptionally promote mature adipocyte differentiation due to a reduced inactivation via AR (4).

4 Conclusions and outlook

Understanding DNA methylation as an epigenetic mechanism regulating gene expression is a key prerequisite to comprehend the role of endogenous and exogenous compounds in their role of developmental programming. However, as highlighted in the work at hand, research on DNA methylation levels lack a corporately acquired standard of data generation as established e.g. in the field of RT-qPCR with the MIQE-guidelines (Bustin et al. 2009). Applying inadequate methods, plenty of results might consequently be generated reaching statistical significance but lacking biological truth. On the contrary, the newly established combination of MS-HRM and pyrosequencing provides an excellent opportunity for controlled investigations of local DNA methylation levels at CpG-site resolution. With this methodology, a previous extensive research on the gene of interest as conducted here for the mediator of estrogenic action ESR1 emerges as an absolute necessity if developmental regulatory signals such as E2 want to be connected to epigenetic patterns in later investigations. This need is based on the current finding of a single-CpG-site based regulation via impairment of a transcriptional repressor binding sites which emerges as a formerly unknown way of *ESR1* regulation. Due to the potential multiplicity of epigenetic regulations that manifest according to mRNA abundance, developmental status or maybe even species, programming events might be missed if regulatory sites are not acknowledged and fail to be investigated. In the work at hand, the depicted methodology allowed the appraisal that postnatally occurring distinct levels of E2 do not necessarily modify immediate expression levels of its mediator in male piglet tissues or impact on DNA methylation levels. Sexual differentiation as seen in the male mouse brain guided by the programming effect of E2 thus emerges as an influence on *ESR1* expression which seems highly tissue-specific and orchestrated together with the distinctly available cellular cofactors. In contrast, prenatal E2 exposition at very low doses was encountered to have long-term programming effects in piglet progeny. Strikingly paralleling the current public health concern of growing childhood obesity (Raj and Kumar 2010; Reilly 2005), male piglets displayed a shift in body composition towards fat mass at preadolescent age. Although endogenously produced, E2 thus emerges as an endocrine disruptor when applied during a critical window of development. Special attention arises from the fact that significantly modified phenotypes occurred even at doses regarded to have no adverse effects. This result facilitates former concerns that the hazard potential of exogenous hormonal compounds on health might not fully be assessed by methods evaluating immediate effects in an adult organism (Andersson and Skakkebaek 1999; Bay et al. 2004). Proposed 'no-

effect' or 'ADI' levels, in case of E2 e.g. based upon the absent relieve of menopause symptoms, may not assess the special susceptibility of the fetus/infant for adverse epigenetic developmental programming under EDC influence. In summary, these concerns are considerably substantiated by the work at hand. Based on the presented results for oral E2 application (Fürst et al. *under review*), the hazard potential of hormonally active substances such as E2 appears to implicitly require a reassessment accounting for possible long-term effects due to epigenetic regulation. As presented herein, detailed investigations especially need to allow for the specific substance of interest, potential target tissues (given the specific susceptibility due to distinct receptor abundance (Fürst et al. 2012a)) and acquaintance of involved regulatory mechanisms (Fürst et al. 2012a; Fürst et al. 2012b).

In this view, it remains to be investigated in the context of the conducted animal trial if both male and female piglets have furthermore experienced modifications of the reproductive system similar to the observations in rodents under estrogenic compound exposure. Moreover, it is important to mention that in the topic of hormonal growth promotion, anabolic steroids are most often used in a combination of an estrogen plus an androgen. It thus seems worth further attention whether the depicted effects on males in terms of body composition might be similarly caused by androgens. These could eventually impact on the estrogenic system in females likewise estrogens in males reducing the protective effects on obesity and might explain to some extend why not only boys but also girls exhibit obesity already at preadolescent age. In a follow-up study, further details such as the occurrence of hormone levels in fetuses and body composition during determination over all developmental stages consequently need to be determined, together with the epigenetic mechanism guiding the expression of relevant genes in this context.

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7 Scientific communications

Original peer reviewed scientific publication

Fürst RW*, Dahlhoff C, K. Ruhlig K, Sedlmeier E-M, Bader BL (2008). Epigenetik und Ernährung. Ernährung, 2:116–124. *all authors contributed equally

Fürst RW, Meyer HHD, Schweizer G, Ulbrich SE: Is DNA methylation an epigenetic contribution to transcriptional regulation of the bovine endometrium during the estrous cycle and early pregnancy? Molecular and Cellular Endocrinology 327 (2012) 67-77

Fürst RW, Kliem H, Meyer HHD, Ulbrich SE: A differentially methylated singe CpG-site is correlated with estrogen receptor alpha transcription. The Journal of Steroid Biochemistry and Molecular Biology (2012) doi:10.1016/j.jsbmb.2012.01.009

Fürst RW, Pistek VL, Kliem H, Skurk T, Hauner H, Meyer HHD, Ulbrich SE: Maternal low-dose estradiol-17β exposure during pregnancy impairs postnatal progeny weight development and body composition. *Under review in Endocrinology*

Dahlhoff C, Desmarchelier C, Sailer M, **Fürst RW**, Ulbrich SE, Hummel BA, Obeid R, Geisel J, Bader BL, Daniel H. Hepatic methionine homeostasis is conserved in C57BL/6N mice on high-fat diet despite major changes in hepatic one-carbon metabolism and phospholipid status. *Under review in The Journal of Biological Chemistry*

Oral scientific presentations

Fürst RW, Kliem H, Schweizer G, Meyer HHD, Ulbrich SE (2010). Analysis of estrogen receptor expression and methylation status in male growing piglets. At: 43rd Annual Conference of Physiology and Pathology of Reproduction and 35th Mutual Conference on Veterinary and Human Reproductive Medicine, München, 25.02.2010

Peer reviewed abstracts

Fürst RW, Kliem H, Bader BL, Meyer HHD, Ulbrich SE (2008). Distinct endogenous Estradiol-17β concentrations in male growing piglets evoke differential expression of estrogen receptor alpha in testis, epididymis and prostate. In: 18th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, Seefeld, Österreich 18.09.-21.09.2008, Abstract No. 202-P

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Fürst RW, Kliem H, Meyer HHD and Ulbrich SE (2011). Combining MS-HRM and Pyrosequencing for Methylation Status Analysis of the Estrogen Receptor Alpha Gene in Male Growing Piglets. In: Congress on Steroid Research, Chicago, Il, USA, 27.03.-29.03.2011

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Fürst RW, Pistek VL, Kliem H, Skurk T, Hauner H, Meyer HHD, Ulbrich SE (2012). Distinct doses of estradiol-17β orally applied during pregnancy impact on gonads, weight and body composition in pig progeny. In: Abstractband 55. Symposium der Deutschen Gesellschaft für Endokrinologie, Congress Center Mannheim, 07.-10.03.2012, No. P2 6-6

8 Curriculum vitae

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9 Appendix

Appendix I.

Fürst RW*, Dahlhoff C, Ruhlig K, Sedlmeier E-M, Bader BL: Epigenetik und Ernährung. *Ernährung 2 (2008) 116-124.* *all authors contributed equally

Appendix II.

Fürst RW, Meyer HHD, Schweizer G, Ulbrich SE: Is DNA methylation an epigenetic contribution to transcriptional regulation of the bovine endometrium during the estrous cycle and early pregnancy? *Molecular and Cellular Endocrinology 327 (2012) 67-77.*

Appendix III.

Fürst RW, Kliem H, Meyer HHD, Ulbrich SE: A differentially methylated singe CpG-site is correlated with estrogen receptor alpha transcription. *The Journal of Steroid Biochemistry and Molecular Biology (2012) doi:10.1016/j.jsbmb.2012.01.009*

Appendix IV.

Fürst RW, Pistek VL, Kliem H, Skurk T, Hauner H, Meyer HHD, Ulbrich SE: Maternal low-dose estradiol-17β exposure during pregnancy impairs postnatal progeny weight development and body composition. *Under review in Endocrinology.*

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Epigenetik und Ernährung

Nach der erfolgreichen Sequenzierung des humanen Genoms verbleiben noch fundamentale Fragen, die Genetik und Genomik bisher nur unzureichend geklärt haben. Der neue Forschungsbereich der Epigenetik versucht nun herauszufinden, welche Ursachen und Mechanismen dafür verantwortlich sind, dass z. B. Merkmale vererbt werden können, die nicht in der DNS-Sequenz festgeschrieben sind. Da es zunehmend Hinweise dafür gibt, dass die Ernährung in epigenetische Mechanismen eingreifen kann, wird im Folgenden eine Übersicht über den Zusammenhang zwischen Ernährung, metabolischer Programmierung und Epigenetik gegeben.

Die erfolgreiche Sequenzierung des menschlichen Genoms hat die Lebenswissenschaften revolutioniert. Im Zentrum der genetischen Forschung steht heute vor allem die Analyse der Varianz, im Besonderen durch SNP-Analysen (SNP: „single nucleotide polymorphisms"), in kodierenden und regulatorischen Genabschnitten sowie deren Assoziation mit dem Phänotyp bzw. individuellen Suszeptibilitäten für Erkrankungen. Eine Vielzahl neuer Methoden resultiert ebenso aus der Genomforschung. Mittlerweile ist es möglich, mittels DNS-Chips die Genexpression des gesamten Genoms (Transkriptom) – auch in Antwort auf Veränderungen der Ernährung – z. B. in Blutzellen bzw. Gewebeproben des Menschen zu analysieren.

Trotz enormer wissenschaftlicher und technischer Fortschritte verbleiben denoch fundamentale Fragen, welche die Genetik und Genomik bisher nur unzureichend geklärt haben. Wie entwickeln sich bei gleicher genetischer Ausstattung von Zellen unterschiedliche Genexpressionsmuster, die bei der Zellteilung stabil vererbt werden können? Hierfür scheint eine Art Zellgedächtnis notwendig zu sein, das in unterschiedlichen Zellentwicklungslinien weitergegeben wird und so die Differenzierung zu verschiedenen Zelltypen ermöglicht. Was bedingt die phänotypischen Unterschiede von Organismen bei scheinbar identischem Genom? Studien an eineiigen Zwillingen belegen beispielsweise Unterschiede in der Stoffwechselantwort und bei Krankheitsrisiken, obwohl beide Individuen ein identisches Genom haben sollten. Die Ursachen und Mechanismen für diese vererbbaren Merkmale, die offensichtlich nicht von der DNS-Sequenz abhängen, versucht der Forschungsbereich der Epigenetik aufzuklären.

Epigenetik und epigenetische Mechanismen

Der Ursprung des Begriffs Epigenetik („Epi" = griech. „daneben, obenauf"), welcher bereits in den 40er-Jahren durch Waddington [52] geprägt wurde, liegt in den Beobachtungen scheinbar anomaler Vererbungsmuster, die sich nicht durch die Mendelschen Gesetze bzw. die klassische Genetik erklären ließen. Die heutige Definition der Epigenetik als das „Studium mitotisch und meiotisch vererbbarer Veränderungen der Genfunktion, welche nicht durch Veränderungen der DNS-Sequenz erklärt werden können" [40], sieht diesen Forschungsbereich eher als eine Brücke zwischen Genotyp und Phänotyp. Epigenetik wird also als Phänomen verstanden, welches nachhaltig die zeitliche, räumliche und quantitative Expression eines Gens ohne eine direkte Veränderung der zugrunde liegenden DNS-Sequenz bestimmt.

Die epigenetische Forschung der letzten Jahre untersuchte vor allem die molekularen Mechanismen dieser Form der Genregulation und deren Weitergabe an die nächsten Zellgenerationen. Dabei konzentrierte man sich besonders auf die Basen der DNS und die Histonprotein-Oktamere, um welche die DNS wie um Spulen gewunden ist. Diese als Nukleosomen bezeichneten Einheiten ermöglichen es, die insgesamt etwa 2 m lange DNS einer Zelle auf ungefähr 1/10.000 dieser Länge zu verdichten und im Zellkern einzulagern. Das so gebildete Polymer aus Einheiten von Nukleosomen wird als Chromatin bezeichnet (**Abb. 1**).

Chromatin kann prinzipiell in zwei verschiedenen Zuständen auftreten. Dicht gepackte Chromatinbereiche (Heterochromatin) erschweren den Zugang zur DNS; hier spricht man von einer „geschlossenen" Chromatinkonformation. Eine „offene" Chromatinkonformation hingegen liegt vor, wenn das Chromatin weniger dicht gepackte Bereiche (Euchromatin) aufweist und somit den Zugang von spezifischen Proteinen und Enzymen ermöglicht, um die DNS in die mRNS zu transkribieren (Genexpression). Der Zu-

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stand des Chromatins bzw. die Expression der enthaltenen DNS-Abschnitte kann dabei über epigenetische Mechanismen verändert sowie stabil vererbt werden. Zu diesen Mechanismen zählen DNS-Methylierungen, Histonmodifikationen, Einbau von Histonvarianten, Polycomb-Trithorax-Genregulation, Chromatinumstrukturierung sowie die RNS-Interferenz (**Abb. 2**). Im folgenden Abschnitt werden diese epigenetischen Mechanismen näher erläutert.

DNS-Methylierungen erfolgen bei Säugetieren fast ausschließlich am Cytosin von CpG-Dinukleotid-Motiven (Abfolge der Basen Cytosin und Guanin) und führen zu 5-Methyl-CpGs mit dualer Symmetrie (**Abb. 3a**). Bereiche des Genoms, welche eine hohe Dichte von CpGs aufweisen, werden als CpG-Inseln bezeichnet. Befinden sich solche CpG-Inseln in genregulatorischen Bereichen, korrelieren deren Methylierungen häufig mit der Unterdrückung der transkriptionellen Aktivität. Die Aufrechterhaltung und Weitergabe von bestehenden DNS-Methylierungsmustern in somatischen Zellen und die De-novo-Methylierung der DNS in Keimzellen oder somatischen Zelllinien werden durch verschiedene DNS-Methyltransferasen (DNMTs) vermittelt [20, 22]. DNS-Methylierungen spielen z. B. eine wichtige Rolle bei der genomischen Prägung („genomic imprinting") und der Aufrechterhaltung der Inaktivierung des X-Chromosoms in weiblichen Säugetieren [60]. Die Expression der Gene, die der Prägung („imprinting") unterliegen, wird abhängig von ihrer elterlichen Herkunft vererbt. Durch DNS-Methylierung wird eines der zwei elterlichen Allele eines Gens inaktiviert; daher kommt es entweder zur Expression des paternalen oder maternalen Allels (monoallelische Expression). Auch während der frühen Embryogenese spielen Methylierungsreaktionen eine wichtige Rolle. Vor der Implantationsphase findet eine drastische Demethylierung der DNS statt, nach der Implantation kommt es zu einer umfangreichen De-novo-Methylierung [25].

Modifikationen von Histonproteinen finden vorwiegend an spezifischen Aminosäuren (z. B. Lysin, Arginin, Threonin, Serin) ihrer aminoterminalen Schwanzdomänen statt, die für molekulare Wech-

Zusammenfassung · Abstract

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Epigenetik und Ernährung

Zusammenfassung

Das Forschungsgebiet der Epigenetik befasst sich mit verschiedenen molekularen Mechanismen wie der DNS-Methylierung und der Modifikation von Histonen, die mitotisch und/oder meiotisch vererbbare Genregulation ermöglichen, ohne die entsprechende DNS-Sequenz zu beeinflussen. Frühe Erkenntnisse über diese Mechanismen stammen aus der Grundlagenforschung an Tumoren, die mittlerweile neue Therapieansätze und ernährungsabhängige tumorpräventive Maßnahmen eröffnen. Durch das klassische Tiermodell der *Agouti viable yellow*-Maus gelang es zu zeigen, dass die Ernährung tatsächlich epigenetisch durch DNS-Methylierung einen transgenerationellen Einfluss auf den Phänotyp ausüben kann. So führt die Supplementierung von trächtigen Mäusen mit Substraten des C1-Metabolismus zur Änderung der Fellfarbe und Suszeptibilität für die Entwicklung von Adipositas der Nachkommen. Aus vielen Humanstudien wird deutlich, dass die Ernährung das Risiko z. B. für Adipositas und Herz-Kreislauf-Erkrankungen über eine prä- und postnatale metabolische Programmierung während kritischer Zeitfenster beeinflussen kann. Besonders interessant sind hier Studien mit eineiigen Zwillingen aufgrund ihrer genetischen Identität. Diese unterstützen z. B. den Zusammenhang zwischen einem geringen Geburtsgewicht und einem erhöhten Risiko für metabolische Erkrankungen und geben gute Hinweise auf die Beteiligung epigenetischer Mechanismen bei einer nachhaltigen metabolischen Programmierung des Fetus oder Säuglings, die bis ins Erwachsenenalter reichen kann. Nahrungsfaktoren können zu Veränderungen des Epigenoms führen und damit nimmt die Ernährung als Umweltfaktor über die Epigenetik Einfluss auf die Ausprägung des Phänotyps. Der Forschungsbereich der Nutriepigenetik kann somit zu einem wichtigen Teilbereich im humanen Epigenomprojekt werden, das sich weltweit organisiert.

Schlüsselwörter

Ernährung · Epigenetik · DNS- und Histonmodifikationen · Metabolische Programmierung · Karzinogenese · Adipositas

Epigenetics and Nutrition

Abstract

Epigenetic research investigates various molecular mechanisms such as DNA methylation and histone modifications. These can establish gene expression patterns which are mitotically and/or meiotically heritable without changes in DNA-sequences. Initial insights in these mechanisms originate from experiments with tumors and have become the basis for new therapeutic strategies and nutrition-dependent tumor prevention. Using the classic animal model of the *Agouti viable yellow*-mouse, it was demonstrated that nutrition can epigenetically affect transgenerationally the expression of distinct phenotypes by DNA methylation. In this experimental approach the supplementation of pregnant mice with substrates of C1-metabolism resulted in changed coat color and susceptibility of their offspring to obesity. Various human studies show clearly that nutrition affects the risk of obesity and cardiovascular diseases and is mediated by pre- and/or postnatal metabolic programming during critical time periods. In this respect studies of monozygotic twins are of particular interest, since these subjects are genetically identical and support the correlation of low birth weight for gestational age with a higher risk of metabolic disease. These studies further indicate that epigenetic mechanisms apply during the metabolic programming of the fetus or newborn which can last until adulthood. Nutritional factors can alter the epigenome and as environmental factor thus shape the phenotype. Nutriepigenetics will therefore play an important role in the human epigenome project being organized worldwide.

Keywords

Nutrition · Epigenetics · DNA and histone modifications · Metabolic Programming · Carcinogenesis · Obesity

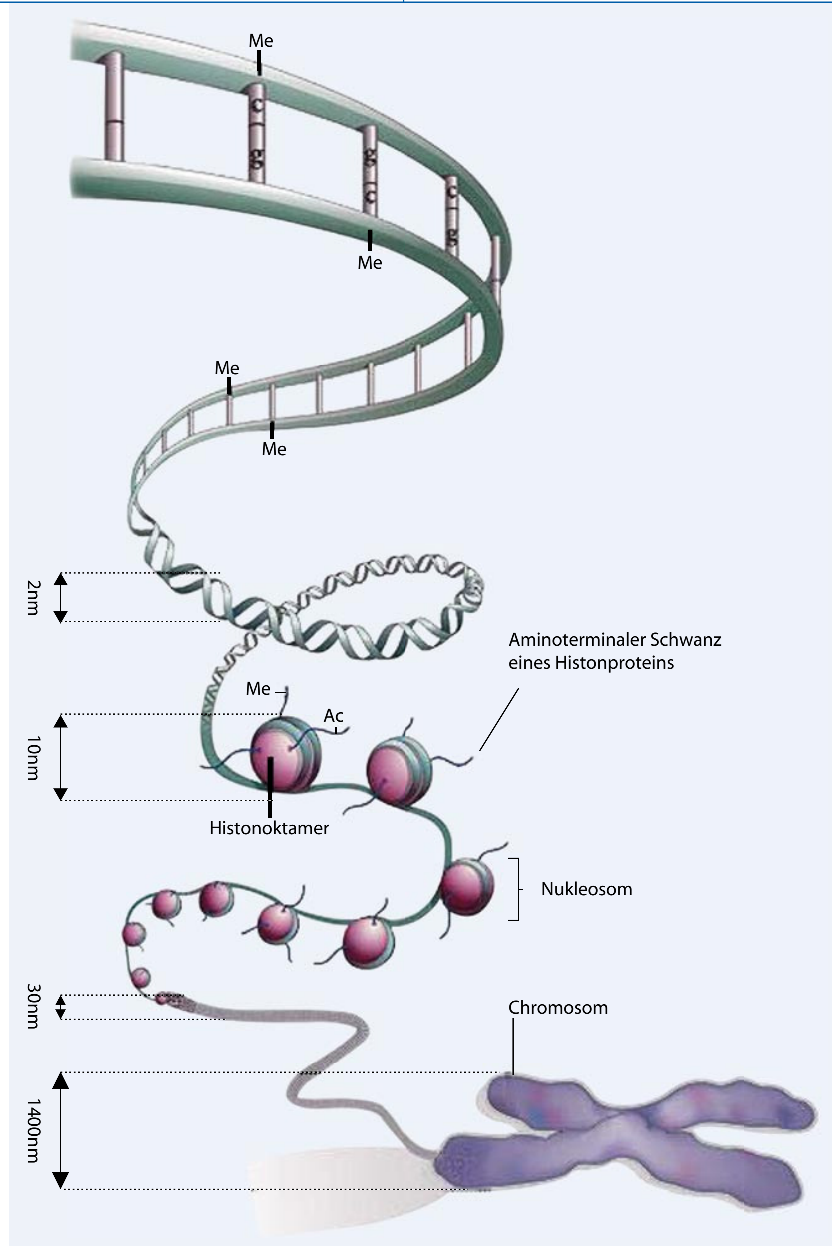

Abb. 1 ▲ Darstellung der DNS in verschiedenen Chromatinverpackungsstufen sowie der chemischen Modifikationen an der DNS und den aminoterminalen Schwänzen der Histonproteine. *Me* Methylgruppe, *Ac* Acetylgruppe. (Mod. nach [39], mit freundlicher Genehmigung © Nature Publishing Group)

selwirkungen besonders zugänglich sind (◘ **Abb. 3b**). So findet man dort Methylierungen, ladungsverändernde Acetylierungen und Phosphorylierungen sowie Ubiquitinierungen. Methylierungen an Lysinresten beispielsweise werden durch Histon-Methyltransferasen (HMTs) vermittelt, Acetylierungen durch Histon-Acetyltransferasen (HATs) bzw. Deacetylierungen durch Histon-Deacetylasen (HDACs). Hierbei dient S-Adenosyl-Methionin (SAM) als Substrat für die HMTs und Acetyl-CoA für die HATs. Die erwähnten Modifikationen verändern die Eigenschaften der Chromatinfaser und dadurch die übergeordneten Strukturen des Chromatins. Auch die Rekrutierung von wichtigen Chromatinbindungspartnern, die zur Veränderung der Chromatinstruktur benötigt werden, kann durch Histonmodifikationen beeinflusst werden. Meist lässt sich der Modifikation definierter Aminosäuren der Histonproteine ein spezifischer Effekt auf die Transkriptionsaktivität zuweisen, weshalb die unterschiedlichen und sehr komplexen Modifikationsmuster auch als „Histon-Code" bezeichnet werden [46]. Beispielsweise führt eine Acetylierung des Lysins an Position neun der Aminosäuresequenz des Histons H3 (H3K9ac) zu transkriptioneller Aktivität, während eine Methylierung an derselben Stelle (H3K9me) mit transkriptioneller Repression einhergeht. Erfolgt eine Methylierung jedoch an bestimmten Argininresten der Schwanzdomäne des Histons H4 (z. B. H4R3me), kommt es zur Aktivierung der Transkription [59].

Neuere Forschungsergebnisse zeigen darüber hinaus, dass es verschieden lange nichtproteinkodierende RNS-Moleküle gibt, die über epigenetische Mechanismen Einfluss auf die Expression nehmen können. Sehr kurze doppelsträngige RNS-Sequenzen (21–24 Nukleotide) vermitteln vermutlich das sequenzspezifische Wirken von histonmodifizierenden Effektoren wie Methyltransferasen und die damit einhergehende Veränderung der Chromatinkonformation [21, 50, 60]. Aber auch sehr lange Transkripte wie beispielsweise die 17kb-RNS des *Xist* (*X inactive specific transcript*) können zur Überführung eines spezifischen Bereichs des Genoms in Heterochromatin genutzt werden, etwa zur Stilllegung des X-Chromosoms [60].

Weitere epigenetische Mechanismen können die Struktur und Anordnung der Nukleosomen bzw. die Chromatinkonformation verändern. So kann beispielsweise durch den Austausch einzelner klassischer Histonproteine (2A, 2B, 3 und 4) durch spezifische Histonvarianten (z. B. H3.3, H2AZ) oder die Aktivität von ATP-abhängigen Chromatinumstrukturierungskomplexen die Zugänglichkeit der Enzyme des Transkriptionsapparats zu den Genen beeinflusst werden [29, 49]. Die Polycomb-Proteingruppe von Repressoren und die Trithorax-Proteingruppe von Aktivatoren stabilisieren die korrekte Genexpression einiger Entwicklungsregulatoren durch Veränderungen der Chromatinstrukturen und gelten so als Schlüsselregulatoren der Zellproliferation und Zellidentität in multizellulären Organismen [32, 42].

Aus vielen Studien wird mittlerweile deutlich, dass eine enge Vernetzung der

oben genannten Mechanismen existiert. Durch diese Interaktionen können gezielt und nachhaltig epigenetische Markierungen und Variationen in das Chromatin eingebracht werden. Die Gesamtheit dieser epigenetischen Merkmale des Genoms einer Zelle oder eines Zelltyps wird als Epigenom bezeichnet. Dieses kann eine Art vererbbares Zellgedächtnis oder Steuerprogramm in Hinblick auf die Regulation der Genexpression des Genoms aufbauen.

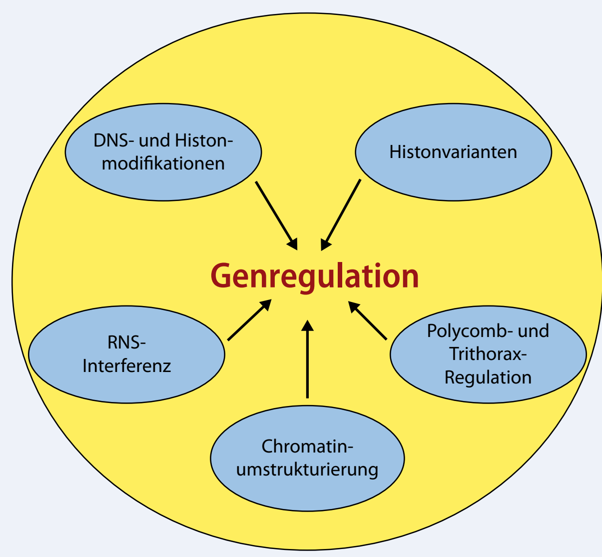

Abb. 2 ▶ Epigenetische Genregulation kann durch ein Zusammenspiel aus DNS- und Histonmodifikationen, Einbau von Histonvarianten, Polycomb-Trithorax-Proteinen, Chromatinumstrukturierung sowie RNS-Interferenz entstehen

Epigenetik und Nahrungsinhaltsstoffe in der Tumorbiologie

Zu dem bisherigen Wissensstand epigenetischer Regulationsmechanismen hat die Grundlagenforschung an Tumoren schon sehr früh einen großen Beitrag geleistet. Bereits Anfang der 80er-Jahre entdeckten Wissenschaftler den Verlust von DNS-Methylierungen in Tumorzellen [17]. Mittlerweile sind weitere epigenetische Veränderungen bei Tumoren bekannt, wie genspezifische DNS-Hypermethylierungen und -Hypomethylierungen, eine globale DNS-Hypomethylierung, veränderte Histonmodifikationen sowie der Verlust von genomischer Prägung [15]. Dadurch wurde die klassische Vorstellung der Krebsentstehung als Folge einer stetigen Akkumulation von Mutationen in der DNS der Zelle grundlegend verändert.

In der Tumorforschung wird der genspezifischen DNS-Hypermethylierung in Promotorbereichen sowie der globalen DNS-Hypomethylierung heute eine sehr große Bedeutung beigemessen. Beide Phänomene werden bei nahezu jedem Tumor gleichzeitig beobachtet. Hypermethylierungen in Promotorbereichen der Gene führen zu deren Inaktivierung. Sind davon Tumorsuppressorgene (z. B. *p16*) oder Missmatchreparaturgene (z. B. *hMLH1*) betroffen, führt dies zu unkontrolliertem Wachstum sowie einer erhöhten Mutationsrate in der Zelle. Diese Veränderungen stellen vermutlich ein sehr frühes Ereignis in der Krebsentstehung dar. Globale DNS-Hypomethylierungen in der Tumorzelle können dagegen die Freisetzung transposabler Elemente zur Folge haben. Dies macht die Chromosomen anfälliger für Strangbrüche und kann somit zur Genominstabilität führen [28, 58]. Die DNS-Hypomethylierungen werden nach neuestem Kenntnisstand auch mit der Metastasierung von Tumorzellen in Verbindung gebracht und können somit als ein späteres Ereignis bei der Krebsentstehung eingeordnet werden [45].

Da epigenetische Veränderungen schon in initialen Phasen der Krebsentstehung beobachtet werden können, ließe sich mit Hilfe der Epigenetik die Krebsdiagnostik erheblich verbessern und entsprechend die Heilungschance der Patienten deutlich erhöhen. Es gibt bereits Ansätze anomale DNS-Methylierungsmuster als Biomarker für die Früherkennung zu nutzen [19]. Beispielsweise sind für einen noch in der Entwicklung befindlichen Bluttest zur Darmkrebsfrüherkennung erste Patente angemeldet. Da im Gegensatz zu den genetischen Veränderungen epigenetische prinzipiell umkehrbar sind, bietet die Epigenetik auch Chancen für neue Therapieansätze. Daher wurden bereits in zahlreichen klinischen Studien epigenetische Therapeutika für den Einsatz zur Krebsbehandlung geprüft. In den USA ist das Medikament Vidaza® mit dem Wirkstoff 5-Azacytidin zugelassen, welches zur Therapie des myelodysplastischen Syndroms – einer lebensbedrohlichen Blutkrankheit – und Leukämie eingesetzt wird. Durch seine antagonistische Interaktion mit DNMTs führt 5-Azacytidin zur Reaktivierung von hypermethylierten Tumorsuppressorgenen [19, 38]. Weitere Substanzen bzw. Wirkstoffe werden gegenwärtig in klinischen Studien untersucht. Hierzu zählt Zebularin, welches ebenfalls auf Basis von DNS-Demethylierungen wirkt. Trichostatin A, ein Histon-Deacetylase-Inhibitor, befindet sich bereits in der Anwendung und scheint bei der Behandlung von Leukämie sehr vielversprechend zu sein. Auch synergistische Effekte beider Substanzen werden gegenwärtig geprüft [16].

Ein noch ungelöstes Problem besteht jedoch darin, dass diese Stoffe nicht nur die epigenetischen Modifikationen einer Tumorzelle, sondern auch die einer gesunden Zelle nachteilig verändern können. Hierdurch wird die Entwicklung maßgeschneiderter epigenetisch wirksamer Medikamente deutlich erschwert.

Neben sicherer Diagnostik und effektiver Therapie sind jedoch Maßnahmen zur Tumorprävention von wesentlicher Bedeutung. Hier scheint insbesondere die Ernährung ein großes Potenzial zu haben. Einige sekundäre Pflanzenstoffe könnten im Hinblick auf die Entstehung von Tumoren über epigenetische Mechanismen möglicherweise präventiv wirken. Das im grünen Tee enthaltene Hauptpolyphenol Epigallocatechin-3-gallat konnte in vitro z. B. durch Methylierung abgeschaltete Tumorsuppressorgene wieder reaktivieren. Eine ähnliche Wirkung wird bei dem Phytoöstrogen Genistein aus der Sojabohne vermutet [14]. Ob jedoch physiologische Konzentrationen dieser sekundären Pflanzenstoffe in vivo diese Wirkungen erzielen können, wird noch kontrovers diskutiert. Präklinische Studien

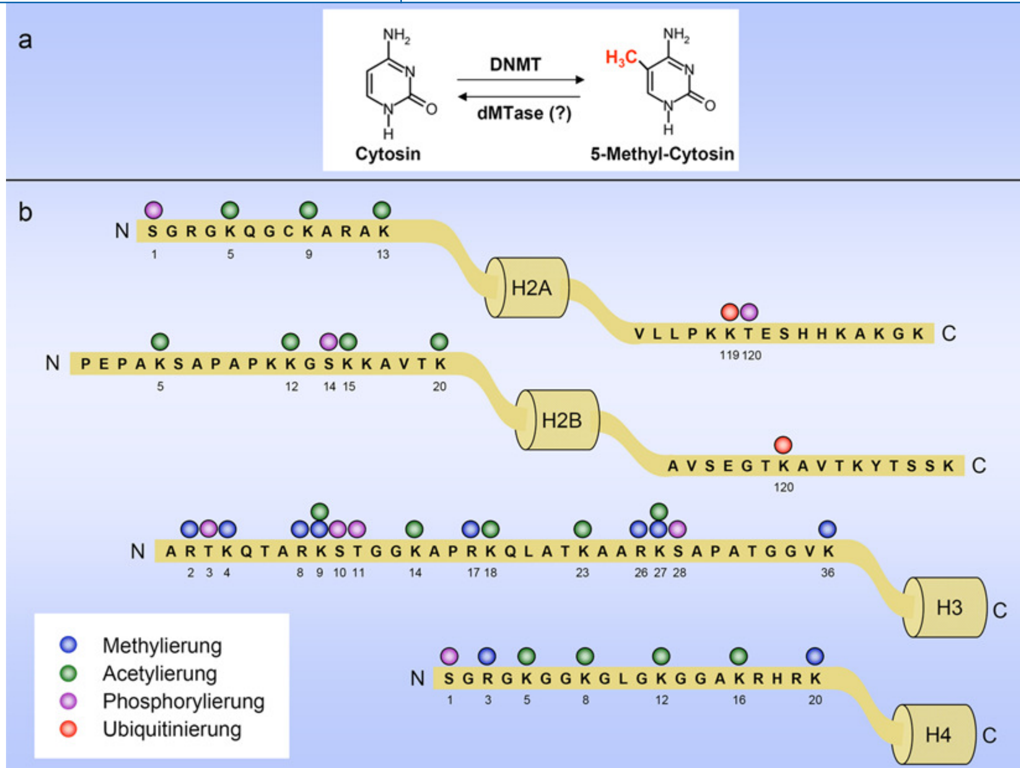

Abb. 3 ► **a** 5-Methyl-Cytosin als DNS-Modifikation, **b** bekannte Histonmodifikationen an amino- und carboxyterminalen Domänen der Histone 2A, 2B, 3 und 4, die in die Genexpression eingreifen können. (Mod. nach [43], mit freundlicher Genehmigung © Nature Publishing Group) *DNMT* DNS-Methyltransferase; *dMTase* putative DNS-Demethylase.

geben allerdings Hinweise darauf, dass ein Teil der tumorprotektiven Effekte, die mit diversen Nahrungsinhaltsstoffen in Verbindung gebracht werden, auf die Modulierung der DNS-Methylierung zurückzuführen sind. Die beste Evidenz dafür zeigen Folat, Vitamin B_{12} und B_6, Methionin sowie Cholin [9, 34]. Ihnen ist eine Rolle im C1-Metabolismus gemein, dessen Aufgabe die Bereitstellung von Methylgruppen für Methylierungsreaktionen in der Zelle ist.

Der direkte Träger dieser Methylgruppen für z. B. epigenetisch-regulatorische Prozesse ist S-Adenosyl-Methionin (SAM). Als Folge der Übertragung einer Methylgruppe auf ein Substrat (z. B. DNS) entsteht aus SAM das S-Adenosyl-Homocystein (SAH). Durch Stoffwechselprozesse, an welchen das Tetrahydrofolat – die biologisch aktive Form des Folats – als zwischenzeitlicher Methylgruppenträger beteiligt ist, wird aus SAH erneut SAM gebildet. Somit kann der Pool an Methylgruppen wieder aufgefüllt werden (**Abb. 4**). Es wird vermutet, dass die Verfügbarkeit einzelner Komponenten des C1-Metabolismus, speziell das Konzentrationsverhältnis [SAM]:[SAH], sowie katalysierende Enzyme die Methylierungsreaktion steuern und entsprechend Einfluss auf epigenetische Prozesse ausüben.

Neben möglichen epigenetischen Einflüssen von Nahrungsfaktoren auf die Tumorprävention stellt sich zunehmend die Frage, inwieweit die Ernährung über epigenetische Mechanismen auch an der Entstehung und Vermeidung anderer komplexer Erkrankungen beteiligt ist.

Einfluss nutritiver Faktoren auf epigenetische Modifikationen

Viele Studien belegen eine bedeutende Rolle der Ernährung für die Gesundheit und die Lebenserwartung sowie bei der Entstehung von Krankheiten. Im Fadenwurm *(C. elegans)*, in Hefen oder Nagern wurde z. B. gezeigt, dass eine 20- bis 40%ige Kalorienrestriktion auch über epigenetische Mechanismen die Lebensspanne verlängern und die Gesundheit positiv beeinflussen kann. Dabei spielt das *SIR2*-Gen eine zentrale Rolle. *SIR2* kodiert für ein Protein der Sirtuine, das eine Histon-Deacetylase-Funktion aufweist und z. B. in Hefen über eine Chromatinkondensation zur Stilllegung bestimmter Gene führt. Durch ihre NAD^+-Abhängigkeit ist die Histon-Deacetylase auch an den Energiestatus der Zelle gekoppelt. In Hefen kann eine Kalorienrestriktion die Zellatmung erhöhen und das Verhältnis NADH zu NAD^+ zugunsten des NAD^+ verschieben. Dies führt zu einer höheren Aktivität von SIR2 und wirkt in der Folge lebensverlängernd für die Hefe. Ebenso kann eine Kalorienrestriktion die Aktivität von SIR2 erhöhen, indem sie die Genexpression von *PNC1* (Pyrazinamidase/Nikotinamidase-1) erhöht, welches zum Abbau des *SIR2*-Inhibitors Nikotinamid führt. In Säugetieren und im Menschen existiert ein homologes Gen, *SIRT1*, dessen Protein nicht nur die Histone H1, H3 und H4, sondern noch weitere Proteine, insbesondere viele Transkriptionsfaktoren z. B. der DNS-Reparatur, der Apoptose oder Zellatmung deacetylieren kann. Somit kontrolliert *SIRT1* eine große Bandbreite wichtiger Zellfunktionen. Da die Funktion von *SIR2* hoch konserviert ist, könnte *SIRT1* ebenso beim Menschen einen lebensverlängernden Effekt der Kalorienrestriktion vermitteln. Die Signalwege des SIRT1 sind beim Menschen jedoch wesentlich komplexer [4]. Auch bei kardiovaskulären Erkrankungen wird seit kurzem die Beteiligung epigenetischer Mechanismen diskutiert. Es wird vermutet, dass durch Ernährungsfaktoren und über einen erhöhten Homocysteinspie-

gel (■ **Abb. 4**) anomale DNS-Methylierungsmuster entstehen können, welche im Tiermodell in der frühen Atherogenese bereits nachgewiesen wurden [31, 55].

Ein Tiermodell, welches einen direkten Einfluss von Nährstoffen – speziell von Faktoren des C1-Metabolismus auf epigenetische Prozesse – zeigt, ist das *Agouti viable yellow* (A^{vy}/a)-Mausmodell (agouti = wildfarben). Das A^{vy}-Allel dieser Mäuse ist eine Mutation des murinen *Agouti*-Allels (*A*), das für ein parakrines Signalmolekül kodiert, welches spezifisch Zellen des Haarfollikels dazu anregt, zu bestimmten Zeitpunkten des Haarwachstums das gelbe Pigment Phäomelanin zu synthetisieren. Aufgrund der Mutation kommt es zu einer Fehlregulation des *Agouti*-Gens, die zu einer dauerhaften Expression des A^{vy}-Allels führt. Somit wird bei A^{vy}/a-Mäusen konstitutiv Phäomelanin gebildet, woraus eine gelbe Fellfarbe der Mäuse resultiert, die im Gegensatz zu den agoutifarbenen *A/a*-Mäusen steht [12, 54]. Zusätzlich führt die Fehlregulation der Expression des *Agouti*-Gens zu weiteren phänotypischen Veränderungen. Das Agouti-Protein kann auch als Antagonist des im Hypothalamus aktiven Melanokortinrezeptors 4 (Mc4r) wirken, der an der Vermittlung des Sättigungsgefühls beteiligt ist. Die ektopische Expression des *Agouti*-Gens im Hypothalamus führt durch die Blockierung des Sättigungssignals am Mc4r zu einer Fehlregulation der Nährstoffaufnahme [51, 56, 61], sodass die A^{vy}/a-Mäuse Adipositas und Diabetes entwickeln [35].

Interessanterweise unterliegt der Expressionsstatus des A^{vy}-Allels ernährungsbedingten Einflüssen. Dieser Zusammenhang konnte in verschiedenen Fütterungsstudien gezeigt werden [6, 54, 57]. Durch die Supplementierung weiblicher Mäuse während der Trächtigkeit mit Metaboliten bzw. Kofaktoren des C1-Stoffwechsels (Folat, Vitamin B_{12}, Cholin und Betain) verlagerte sich die Fellfarbe der Nachkommenschaft mit dem A^{vy}/a-Genotyp hin zum wildfarbenen (*pseudoagouti*) Phänotyp (■ **Abb. 5**). Zusätzlich war auch die Ausbildung von Adipositas und Diabetes bei den Nachkommen reduziert. Die A^{vy}/a-Nachkommen einer Kontrollgruppe, die keine maternale Supplementierung während der Trächtigkeit bekamen, zeigten hingegen vermehrt den gelben Phänotyp.

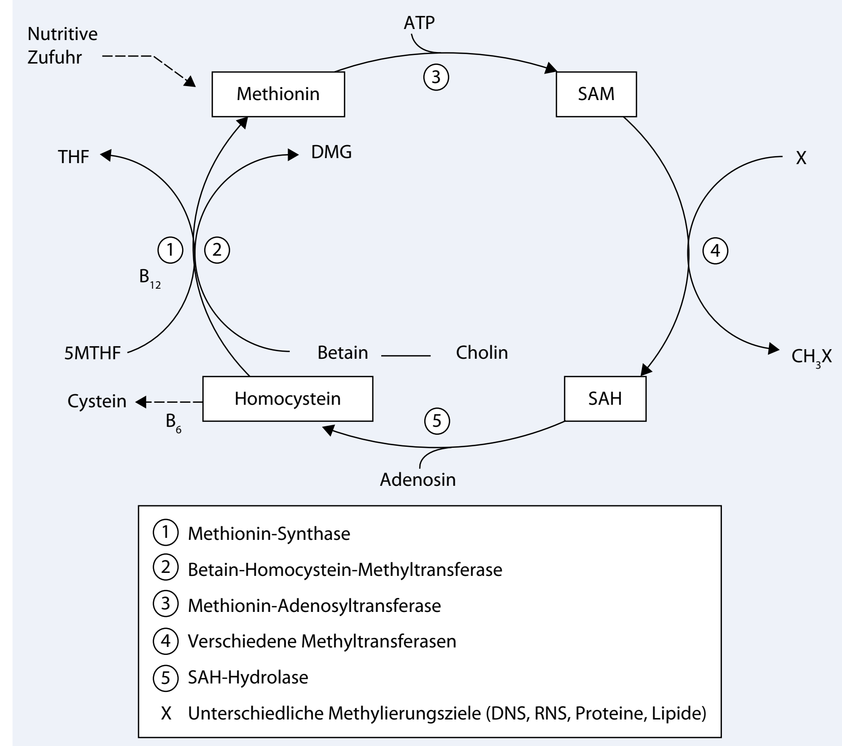

Abb. 4 ▲ C1-Metabolismus zur Aufrechterhaltung des Methylierungspools. *5MTHF* 5-Methyltetrahydrofolat, *THF* Tetrahydrofolat, *DMG* Dimethylglycin, B_{12} Vitamin B_{12} (Cobalamin), B_6 Vitamin B_6 (Pyridoxin)

Aufgrund dieser Supplementierungsstudien lag die Vermutung nahe, dass Stoffwechselkomponenten des C1-Metabolismus Einfluss auf die Regulation der Expression des A^{vy}-Allels haben. DNS-Analysen der Allele der Pseudoagouti-Nachkommen ergaben, dass durch die Supplementierung während der Trächtigkeit CpGs in einem genregulatorisch wichtigen Element des A^{vy}-Allels methyliert werden, welche die Regulation der Expression des *Agouti*-Gens beeinflussen. Diese nährstoffabhängige epigenetische DNS-Methylierung im *Agouti*-Gen scheint dabei während einer kritischen frühen Phase des Embryonalstadiums zu erfolgen [54]. Dies führt nicht nur zu einer Veränderung des Epigenotyps des *Agouti*-Gens in somatischen Zellen, sondern auch zu einer nachhaltigen Veränderung in der Keimbahn der Nachkommen. Daher kommt es zu einem transgenerationellen Effekt, wodurch die Diät des weiblichen F_0-Tieres während der Trächtigkeit nicht nur Einfluss auf die F_1-Generation, sondern auch auf die F_2-Generation ausübt. Somit ist die *Pseudoagouti*-Fellfarbe auch bei F_2-Nachkommen häufiger, deren „Großmütter" supplementiert wurden [7]. Die Entdeckung, dass der Epigenotyp des A^{vy}-Allels ernährungsbedingten Einflüssen unterliegt, führte zu weiteren Studien zum Einfluss von Nahrungsinhaltsstoffen. Eine Supplementierung mit Genistein während der Trächtigkeit übt z. B. den gleichen Effekt wie die C1-Metaboliten auf die Fellfarbe der A^{vy}/a-Nachkommen aus. Dies konnte ebenfalls auf eine Methylierung von CpGs im A^{vy}-Allel zurückgeführt werden, doch scheint dieses Phänomen nicht direkt mit dem C1-Metabolismus in Verbindung zu stehen [10].

Epigenetische Phänomene in Humanstudien

Auch für den Menschen gibt es Hinweise, dass Ernährungsfaktoren im Kontext von epigenetischen und transgenerationellen Ausprägungen eine Rolle spielen. Dies bedeutet, dass die Ernährung der Mutter nachhaltige Auswirkungen auf die Entwicklung und den Gesundheitsstaus der Nachkommen hat. In diesem Zusam-

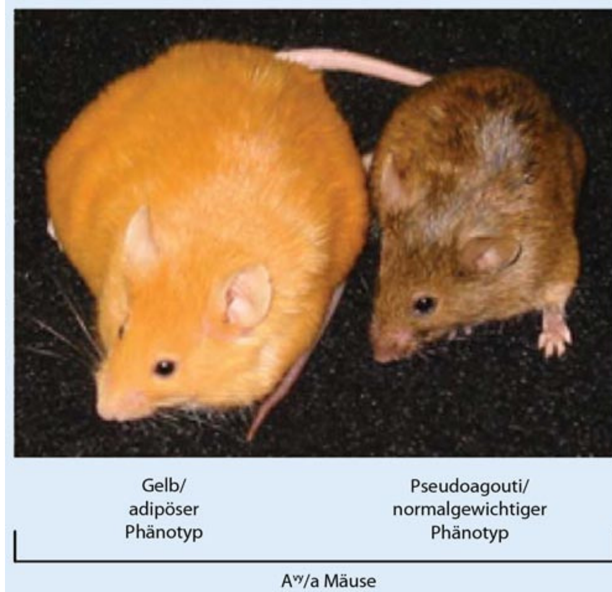

Abb. 5 ◀ Phänotypen der A^{vy}/a-Mäuse. (Mod. nach [53], mit freundlicher Genehmigung © Elsevier Inc.)

menhang sind epidemiologische Studien an Kindern holländischer Frauen oft zitiert, deren fetale Entwicklung in eine klar abgegrenzte fünfmonatige extreme Hungerperiode während des 2. Weltkriegs fiel. In diesen Studien, die momentan jedoch zunehmend kontrovers interpretiert werden, wurde gezeigt, dass eine Mangelernährung der Mutter während der frühen Schwangerschaft mit erhöhten Risiken des Kindes für die Entwicklung von Adipositas, atherogenen Blutfettwerten und Herz-Kreislauf-Erkrankungen in späteren Lebensabschnitten einhergehen. Waren die Mütter dagegen in der mittleren und späteren Schwangerschaft von der kalorischen Unterversorgung betroffen, wiesen ihre Kinder – im Vergleich zu Kindern einer Kontrollgruppe – ein vermindertes Geburtsgewicht auf. Bei ihnen konnte ein geringeres Risiko für Adipositas, aber ein höheres Risiko für eine gestörte Glukosetoleranz in späteren Lebensabschnitten festgestellt werden [30]. Basierend auf diesen Erkenntnissen verwenden viele Studien unter anderem ein geringes Geburtsgewicht – bezogen auf das entsprechende Gestationsalter – als Surrogatmarker für eine fetale Unterversorgung [41]. Hier ist auch die interessante Arbeit von Bo et al. [3] zu beachten, die postuliert, dass ein durch fetale Unterernährung verursachtes geringes Geburtsgewicht nicht per se zur Entstehung eines metabolischen Syndroms führt, sondern die Abweichung vom genetisch programmierten Geburtsgewicht ein wichtiger Faktor ist.

Hales u. Barker [23] formulierten, ausgehend von diesen epidemiologischen Beobachtungen, die Hypothese vom „thrifty phenotype“ (sparsamer Phänotyp). Sie besagt, dass die Assoziation zwischen einem geringen Fetal- bzw. Säuglingswachstum und der späteren Entwicklung des metabolischen Syndroms sowie Typ-2-Diabetes auf den Auswirkungen einer Mangelernährung im frühen Leben beruht, der dauerhafte Änderungen in den insulinabhängigen Stoffwechselwegen hervorruft. Entsprechend soll sich der Fetus über das intrauterine Milieu bereits an die Umwelt nach der Geburt anpassen, indem er die metabolische Rate und die Hormonproduktion bzw. -sensitivität dauerhaft einstellt. Dies führt zu einem geringeren fetalen Wachstum und Geburtsgewicht. Gleichzeitig könnte dadurch auch das neuroendokrine System „fehlprogrammiert“ werden, das Nahrungsaufnahme, Körpergewicht und Stoffwechsel reguliert [33, 36]. Diese These wurde in ihrer ursprünglichen Form bereits 1975 durch Arbeiten von Dörner et al. [11] über die perinatale Programmierung der Gehirnorganisation formuliert. Passt die metabolische Programmierung des Fetus nicht zur tatsächlichen Nahrungssituation nach der Geburt, kann sich dies nachteilig auswirken, indem es meist zu einem kompensatorischen Aufholwachstum im Säuglings- und Kindesalter kommt, das seinerseits das Risiko, beispielsweise an Typ-2-Diabetes zu erkranken, weiter erhöht [2, 23].

Mehrere Studien haben zwischenzeitlich gezeigt, dass nicht nur eine fetale Mangelernährung, sondern auch eine fetale Überernährung mit einem erhöhten Risiko für Adipositas und metabolischen Erkrankungen einhergeht [41].

In tierexperimentellen Studien wurde belegt, dass der Fetus durch einen mütterlichen Diabetes während der Trächtigkeit höheren Glukosekonzentrationen ausgesetzt ist und hier vor allem gegen Ende der Trächtigkeit, wenn der Fetus seine eigene Glukosehomöostase entwickelt, eine hohe Glukosekonzentration des mütterlichen Bluts einen besonders starken Einfluss ausübt. Sowohl ein milder, als auch ein schwerer Diabetes der Mutter führen beim Fetus zunächst zu einer Hyperinsulinämie. Bei mildem Diabetes werden dadurch makrosome Nachkommen geboren. Beim schweren Diabetes der Mutter werden durch die starke Hyperinsulinämie die β-Zellen des fetalen Pankreas schon vor der Geburt erschöpft und der Insulinspiegel des Fetus sinkt. Dies bedingt häufig, dass der Nachwuchs bei der Geburt sehr klein ist und eine gestörte Glukosetoleranz aufweist. Bei einer Trächtigkeit der weiblichen Nachkommen wird die gestörte Glukosetoleranz möglicherweise über epigenetische Mechanismen auch an die nachfolgende Generation weitergegeben [1]. Dabelea et al. [8] zeigten, dass Kinder diabetischer Mütter einen höheren BMI und ein dreifach höheres Risiko für Typ-2-Diabetes aufwiesen als deren Geschwister, die vor dem Auftreten des maternalen Diabetes geboren wurden. Kinder adipöser oder diabetischer Frauen wiesen auch in anderen Studien ein erhöhtes Risiko für Übergewicht, Adipositas und Störungen des Glukosestoffwechsels auf [36, 37].

Diese Ergebnisse belegen, dass die mütterliche Ernährung während kritischer Zeitfenster der Schwangerschaft die Gesundheit der Kinder über eine fetale und bzw. oder postnatale metabolische Programmierung stark und nachhaltig beeinflusst. Einige Studien berichten auch von einem möglichen Einfluss der väterlichen und sogar der großelterlichen Ernährung auf die Langlebig-

keit (Sterberaten) und metabolische Erkrankungen der Nachkommen [5, 27]. So konnten Kaati et al. [27] zeigen, dass sich durch einen Überfluss an Nahrung während der „slow-growth"-Phase des Großvaters väterlicherseits das Risiko des Enkels für Typ-2-Diabetes um das Vierfache erhöhte. Die genauen molekularen Mechanismen für diesen ernährungsabhängigen transgenerationellen Effekt über die männliche Linie sind noch unbekannt, jedoch spricht einiges dafür, dass dieser direkt durch epigenetische Modifikationen vermittelt wird [5, 27]. In tierexperimentellen Untersuchungen dauerte der negative Effekt einer Proteinmangelernährung einer Generation auf das Geburtsgewicht sogar über drei normal ernährte Generationen hinweg an, bis sich das Geburtsgewicht wieder normalisiert hatte [44].

Zwillinge sind aufgrund ihres identischen Genoms besonders gut geeignet, um den Einfluss nichtgenetischer Parameter, wie z. B. Ernährung, Umweltfaktoren und Lebensstil, zu untersuchen. So konnte bei eineiigen Zwillingen gezeigt werden, dass die phänotypischen Unterschiede, die im Laufe der Zeit deutlicher werden, von zunehmenden Veränderungen im Epigenom begleitet werden [18]. Viele Zwillingsstudien belegen, dass zu einem gewissen Anteil ein geringes Geburtsgewicht das Risiko für metabolische Erkrankungen wie Bluthochdruck, Typ-2-Diabetes und Herz-Kreislauf-Erkrankungen unabhängig von der genetischen Ausstattung erhöht [3, 24, 48], was eine Beteiligung epigenetischer Mechanismen vermuten ließe. Hinweise zur Aufklärung, welche molekularen Mechanismen für die Ausprägung unterschiedlicher Phänotypen verantwortlich sein könnten, gibt die Arbeit von Ukkola u. Bouchard [47]. Ergebnisse dieser Untersuchung von eineiigen Zwillingen im Erwachsenenalter, die 100 Tage unter standardisierten Bedingungen mit 1000 kcal über ihren Bedarf ernährt wurden, weisen darauf hin, dass einige SNPs, unter anderem im Leptinrezeptor- und Adipsin-Gen, einen deutlichen Einfluss auf die individuellen Unterschiede durch die Überernährung haben könnten. Künftige molekulare Analysen werden zeigen, ob sowohl SNPs als auch epigenetische Mechanismen, oder wahrscheinlich sogar eine Kombination von beiden, an den unterschiedlichen Auswirkungen der Ernährung auf die Physiologie und Pathologie beteiligt sind.

Ausblick

Die Epigenetik hat über die Grundlagenforschung in der Tumorbiologie mit der Einführung epigenetischer Medikamente schon die ersten Erfolge erzielt. Darüber hinaus zeigen epidemiologische Studien, dass die nutritive Programmierung des Embryos und Säuglings, neben der Anatomie und Physiologie, einen nachhaltigen Einfluss auf die Gesundheit der Nachkommen bis ins Erwachsenenalter haben kann. Eine Vielzahl von Nahrungsfaktoren, die zu Veränderungen des Epigenoms führen können und damit die Genexpression langfristig modulieren oder auch entgleisen lassen, beeinflussen nicht nur die Entwicklung von Zellen in der embryonalen bzw. fetalen Phase, sondern auch die adulten Stammzellen. Antworten auf die Fragen zu finden, welche Auswirkungen Nahrungsinhaltsstoffe auf die Ausprägung von Gestalt, Physiologie und Pathologie haben und welche Möglichkeiten es gibt, die Modifikationsmechanismen zu steuern oder reversibel zu machen, wird eine große Herausforderung für die Forschung der nächsten Jahrzehnte sein. Aus diesen Gründen wird die epigenetische Forschung nicht nur weitreichende Auswirkungen auf die menschliche Biologie und die Behandlung von Krankheiten – einschließlich der Erkenntnisse über Stammzellen, Adipositas, Diabetes, Krebs und Alterungsprozesse – haben, sondern auch auf die Landwirtschaft.

Zukünftig wird die Ernährungsforschung methodisch nach der Nutrigenomik durch die Nutriepigenomik erweitert und sich weltweit als ein wichtiger Forschungsbereich in den postgenomischen Großforschungsinitiativen wie dem Human Epigenome Project (HEP) [13, 26] einreihen. Der Anfang in diesem noch relativ jungen Teilbereich der epigenetischen Forschung – Nutriepigenetik – ist jedenfalls gemacht.

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Is DNA methylation an epigenetic contribution to transcriptional regulation of the bovine endometrium during the estrous cycle and early pregnancy?

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ABSTRACT

Epigenetic events controlling the transcriptional regulation of genes involved in endometrial function during the estrous cycle and early pregnancy have only sparsely been investigated. We analyzed the gene expression of DNA methyltransferases and the most prominent endocrine transcriptional mediator estrogen receptor alpha (*ESR1*) in the bovine endometrium of heifers at 0, 12 and 18 days following estrous and at day 18 after insemination. The luminometric methylation assay for the investigation of global DNA methylation and an elegant combination of methylation-sensitive high resolution melting and pyrosequencing for local methylation levels of *ESR1* were deployed. In spite of differential gene expression of *ESR1* among groups, no differences in endometrial *ESR1* DNA methylation during neither estrous cycle nor early pregnancy were determined. Global DNA methylation prevailed at similar low levels in endometrium, likely controlled by the observed moderate *DNMT3b* expression. Thus, the epigenetic contribution of DNA methylation influencing endometrial function seems rather limited. However, because a control tissue expressing only minute amounts of *ESR1* transcripts was locally significantly higher methylated, DNA methylation might contribute to an appropriate tissue-specific expression status underlying further specific control mechanisms of gene transcription.

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1. Introduction

Among the reproductive tissues, the endometrium undergoes functional changes during the estrous cycle which are mainly regulated by the ovarian hormones estradiol-17β (E2) and progesterone (P4) via their respective receptors. In ruminants, the timed regulation of luteolysis by uterine Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) secretion is currently attributed to distinct functions of oxytocin (OT) and its receptor (OTR) with a central role of endometrial estrogen receptor alpha (*ESR1*) expression (for a review see Spencer et al., 2004). While ESR1 abundance during the early and mid-luteal phase is probably suppressed by an inhibitory action of increasing P4 concentrations (Evans et al., 1980; Wathes and Hamon, 1993) a following decrease in endometrial P4 receptor (PGR) causes a marked increase of *ESR1* expression (Meyer et al., 1988). Plasma E2 levels during the late luteal phase are then hypothesized to serve, via ESR1, as a signal for enhanced OTR expression. In concert with high OT levels, this leads to secretion of $PGF_{2\alpha}$ from the uterus and can subsequently induce luteolytic events. This proposed mechanism of timing of luteolysis has been recently further strengthened in heifers (Araujo et al., 2009).

The morphological and functional changes in the endometrium due to the action of hormones and their receptors during the bovine cycle have been identified on gene expression level using candidate gene approaches (Groebner et al., 2010; Ulbrich et al., 2009a, 2011) as well as custom-made cDNA microarrays (Bauersachs et al., 2007). How this gene expression is mechanistically established within the estrous cycle or connected to the cyclic regulation of steroid hormones released from either follicle or corpus luteum has only recently drawn attention, supporting an epigenetic involvement in regulating central endometrial transcription factors like *ESR1*. For example, estrogenic action in rats has been linked to histone acetylation (Gunin et al., 2005; Guo and Gorski, 1989), suggesting that in target tissues like the endometrium ovarian steroid hormones may act through chromatin alterations. Furthermore, ubiquitous cellular processes occurring in the human during the menstrual cycle such as proliferation (Stein et al., 2009; Timp et al., 2009), angiogenesis (Buysschaert et al., 2008; Cao, 2009) or differentiation (Cheong and Lufkin, 2010; Hemberger, 2010) have also been clearly connected to epigenetic regulation. Besides this, there is evidence for altered epigenetic control in human endometrium in

Abbreviations: Bp, base pairs; E2, estradiol-17β; ESR1, estrogen receptor alpha; LUMA, luminometric methylation assay; MS-HRM, methylation-sensitive high resolution melting; WBC, white blood cell; WGA, whole genome amplification.

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pathological conditions (Guo, 2009; Widschwendter et al., 2009; Wu et al., 2005). The impact of estrogens on DNA methyltransferases (DNMTs) (Yamagata et al., 2009) and the evidence for crosstalk between elements of the DNA methylation machinery and molecules involved in histone modification (Fuks, 2005) explain why a most recent report showed endometrial global acetylation changes during the menstrual cycle (Munro et al., 2010). Reports on global DNA methylation status of the non-pathological endometrium during the cycle however are to our knowledge still limited to a human study where a marked reduction in cytosine methylation based on immunostaining was found in glandular epithelial cells during the secretory compared to the proliferative phase (Ghabreau et al., 2004). But while overall methylation levels can only point towards varying functions of certain tissues, the correlation of a specific gene's expression with its underlying DNA methylation levels is not supported. Local DNA methylation analyses are therefore indispensable. In the endometrium, *ESR1* has been shown to be differentially expressed during the cycle and early pregnancy (Fleming et al., 2001; Robinson et al., 2001) and is thus an interesting target to investigate an epigenetic involvement in the context of estrous cycle regulation.

In order to determine the methylation status of CpG-sites by sequencing-based procedures, methylation information needs to be translated by bisulfite conversion and subsequently determined by PCR-based approaches like methylation-sensitive high resolution melting (MS-HRM) or pyrosequencing. Initially developed for genotyping studies, HRM analysis is conducted utilizing the different fluorescence profiles of melting PCR products differing in their base composition. In methylation sensitive applications, these base differences derive from the former methylation status (Wojdacz and Dobrovic, 2007, 2009; Wojdacz et al., 2008a). Pyrosequencing as a non-electrophoretic method for DNA sequencing has emerged as a valuable platform for the analysis of single nucleotide polymorphisms (SNPs) and as well allows the analysis of DNA methylation status at single CpG-site resolution (Colella et al., 2003; Fakhrai-Rad et al., 2002; King and Scott-Horton, 2008; Marsh, 2007; Ronaghi et al., 2007). The technology is based on the sequencing-by synthesis principle involving the real-time detection of released pyrophosphate for accurate and quantitative analysis of DNA sequences (Ronaghi et al., 1998). So far, both methods have their respective limitations that may hinder proper estimation of DNA methylation. In case of MS-HRM, detailed information on the methylation status of single CpG sites cannot be acquired and results are restricted to qualitative estimations (Wojdacz et al., 2010). The phenomenon of PCR bias characterized by differing amplification efficiencies of methylated and unmethylated DNA molecules has been recognized and discussed in MS-HRM (Wojdacz et al., 2009, 2008b) but has not been considered a major problem in pyrosequencing approaches although it might tremendously affect findings on site-specific methylation (Shen et al., 2007).

In the work at hand, we investigated DNA methylation in the bovine endometrium during the estrous cycle trying to explain differential gene expression from an epigenetic perspective. Therefore, global DNA methylation levels were analyzed and aligned to the associated mRNA abundance of DNMTs. Local methylation levels of ESR1 as a gene with central importance in the estrous cycle were monitored employing the unique properties of MS-HRM and pyrosequencing combined in one convenient approach.

2. Material and methods

2.1. Animals and tissue sampling

Cyclic heifers (*Bos Taurus*, Deutsches Fleckvieh, Simmental) between 17 and 31 months old were synchronized by injecting intramuscularly a single dose of 500 μg Cloprostenol (Estrumate; Essex Tierarznei, Munich, Germany) at diestrous as described previously (Mitko et al., 2008). Around 60 h after Estrumate injection standing heat was identified through observation of sexual behaviour (i.e. toleration, sweating and vaginal mucus). Animals were slaughtered and samples collected at three representative stages of the bovine estrous cycle with its average length of 21 days. These were day 0 (estrous phase, ovulation), day 12 (diestrus phase, high progesterone) and day 18 of the estrous cycle (late diestrous phase, high progesterone) or pregnancy, respectively. The latter stage was analysed as appropriate control for early pregnancy when the implantation of the bovine embryo is initiated and requires an adequate preparation of the endometrium (Bauersachs et al., 2006). The pregnant group of animals was inseminated with cryo-conserved sperm at estrous (AI) and slaughtered at day 18 of gestation ($n = 6$) (Ulbrich et al., 2009b). Cyclic groups ($n = 6$ per group) were inseminated with the supernatant of centrifuged sperm derived from the same bull. The obtained uteri were flushed with 100 mL PBS (~21 °C). Animals were excluded from the study if no intact conceptus was detected in case of AI. The uteri were opened longitudinally and from the middle part of the ipsilateral uterine horn intercaruncular endometrium was directly frozen in liquid nitrogen and stored at −80 °C until further investigation. All animal trials were conducted with permission from the regional veterinary authority and were in accord with accepted standards of humane animal care.

2.2. Extraction of total RNA

Total RNA from endometrial samples and peripheral white blood cells (WBC) was isolated as described earlier (Hammerle-Fickinger et al., 2010; Ulbrich et al., 2009a,b). Quantity and purity was determined spectroscopically by the Peqlab NanoDrop 1000 (Peqlab, Erlangen, Germany). RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA) ranging from 7 to 10 RIN.

2.3. Two step quantitative RT-PCR

Quantitative real-time PCR (qRT-PCR) experiments were conducted using the LightCycler DNA Master SYBR Green I protocol (Roche, Basel, Switzerland) as described earlier (Ulbrich et al., 2009b). The cycle number (Cq) was calculated by the second derivative maximum method (LC software 4.05). The Cq values from the target gene were normalized against the reference gene *UBQ3*. The primers used for amplification of *UBQ3* were 5′-AGATCC AGGATAAGGAAGGCAT (forward) and 5′-GCTCCACCTCCAGGGTGAT (reverse) at 60 °C annealing-temperature (AT) and 83 °C fluorescence acquisition (FA) and for *ESR1* were 5′-AGGGAAGCTCC-TATTTGCTCC (forward) and 5′- CGGTGGATGTGGTCCTTCTCT (reverse) at 60 °C AT, 81 °C FA. *DNMT1* abundance was measured using primers 5′- CGCATGGGCTACCAGTGCACCTT (forward) and 5′-GGGCTCCCCGTTGTATGAAATCT (reverse) at 60 °C AT, 87 °C FA, primers for *DNMT3a* quantification were 5′-GAATCGCTACA GGGCTTCTG (forward) and 5′-CTGGATATGCTTCTGCGTGA (reverse) at 61 °C AT, 85 °C FA, 5′-GCAAGTTCTCCGAGATACCA (forward) and 5′-CGTATCCTGGCTTTCTCCAG (reverse) for *DNMT3b* at 60 °C AT, 80 °C FA. In order to avoid negative data, results are presented as means ± SEM plus the arbitrary value 20 (ΔCq). Accordingly, a high ΔCq resembles a generally high transcript abundance (Livak and Schmittgen, 2001).

For statistical analysis the SigmaStat program package release 3.00 (SPSS, Somers, USA) was used. The data comparing endometria from cyclic and pregnant endometrium of specific days as well as the WBC were subjected to one way analysis of variance to determine effects of the group. Significant different days within each status as well as significant different groups at each time points were localized by all pair wise multiple comparison

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procedures (Holm-Sidak method). Graphs were plotted using Sigma-Plot 8.0 (SPSS, Somers, USA).

2.4. Isolation of genomic DNA

WBC from 5 ml whole blood were isolated after alkaline lysis of erythrocytes as described earlier (Hammerle-Fickinger et al., 2010). Genomic DNA of the WBC pellet or 30 mg tissue was isolated using the peqGOLD Tissue DNA Mini Kit (Peqlab, Erlangen, Germany) according to the manufacturer's protocol with minor variations. Briefly, tissue or cell pellet was incubated in 400 µl DNA lysis Buffer T and 20 µl Proteinase K at 50 °C for 3 h or 1.5 h, respectively. The reaction was then subjected to mechanical homogenization with ceramic beads in the MagNA Lyzer (Roche, Basel, Switzerland) for 30 s at 7000 rpm followed by an incubation with 15 µl Proteinase K and 15 µl RNase A (Peqlab, Erlangen, Germany) for 30 min. After loading and binding, spin columns were washed three times with 650 µl DNA wash buffer. The extraction was completed by eluting the genomic DNA in 200 µl ultrapure water. Purity and quantity of the genomic DNA were determined spectroscopically by the NanoDrop 1000 (Peqlab, Erlangen, Germany).

2.5. Generation of differentially methylated genomic DNA and bisulfite conversion

For the evaluation of a possible HRM-PCR primer bias (Warnecke et al., 1997; Wojdacz and Hansen, 2006), both a negative/unmethylated control and a positive/methylated control DNA was created. The REPLI-g Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's protocol for whole genome amplification (WGA) of a 25 ng bovine DNA template up to approximately 5–10 µg, subsequently considered as the 0% or unmethylated control. Prior to further applications, WGA DNA was purified using the Genomic DNA Clean & Concentrator Kit (Zymo Research, Irvine, CA, USA) as recommended by the manufacturer. DNA quantity and purity were monitored spectroscopically by the Nanodrop 1000.

In order to obtain a 100% methylated control DNA, purified WGA DNA was *in vitro* methylated by the M.SssI CpG Methylase (Zymo Research, Irvine, CA, USA) with minor variations from the manufacturer's protocol. Briefly, 500 ng WGA DNA in 16 µl ultrapure water were incubated with 2 µl 10× CpG reaction buffer, 1 µl 20× SAM and 1 µl CpG Methylase for 4 h at 30 °C. 0.75 µl Methylase and 1 µl SAM were added and the mixture was incubated for another 4 h at 30 °C before its inactivation by 65 °C for 20 min. For downstream applications, the range of methylated and unmethylated allele dilutions was created after bisulfite-conversion (see below). Both control genomic DNA samples were subjected to this chemical treatment and subsequently mixed in 0%, 25%, 50%, 75% and 100% methylated to unmethylated template ratios. Bisulfite-conversion was conducted using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol on 500 ng genomic DNA per sample. Quantity of the eluted bisulfite-converted DNA (bcDNA) was determined spectroscopically by the NanoDrop 1000.

2.6. Luminometric methylation assay (LUMA)

Global DNA methylation was quantified using LUMA as previously described (Karimi et al., 2006a,b) with minor variations. Briefly, 1 µg genomic DNA was cleaved with FastDigest isochizomere restriction enzymes *Hpa*II + *Eco*RI or *Msp*I + *Eco*RI (Fermentas, St. Leon-Rot, Germany) in two separate reactions for 20 min. The digestions were performed in a 96-well format using a PSQ96 MA system (Qiagen, Hilden, Germany) and peak luminometric heights were calculated with the PSQ96™ MA software. The *Hpa*II/*Eco*RI and *Msp*I/*Eco*RI ratios were calculated as (dGTP + dCTP)/dATP for the respective reactions. DNA methylation was calculated from the (*Hpa*II/*Msp*I) ratio, whereby a ratio of 1 indicates 0% methylation and a ratio approaching 0 corresponds to 100% DNA methylation at the investigated CCGG-sites.

2.7. Methylation-sensitive high resolution Melt (MS-HRM)

PCR amplification and high-resolution melting (HRM) analysis of bcDNA were performed sequentially on a Rotor-Gene Q (Qiagen, Hilden, Germany). Using the EpiTect HRM PCR Kit (Qiagen, Hilden, Carlsbad, Germany), PCR was carried out in a 25 µl reaction scale according to the manufacturer's protocol. Primers were designed with Pyrosequencing Assay Design Software (Version 1.0.6, Biotage, Uppsala, Sweden) on in silico bisulfite-converted DNA (Methyl Primer Express software, Version 1.0, Applied Biosystems, Carlsbad, CA, USA) of ENSBTAT00000009422 (Ensemble, Sept. 2010). Forward primers were 5′-TTTGAGAATGGTGATTATGGTGGTGGTAA AG (P1f) and 5′-AGATTTTAGAGTTGGATGGGTTTTAAGGAG (P2f), reverse biotinylated primers were 5′-BIO-TACCCCTTAAACCAA AAATAAAACTTAACTAAC (P1r) and 5′-BIO-ATTTACCTCTAAATCACT AAAATATCCC (P2r). The analyzed area, PCR primer binding sites and investigated CpGs are depicted in Fig. 3. Template bcDNA was diluted to a concentration of 4.0 ng/µl and 8.7 µl were used for one reaction. The cycling protocol consisted of an initial PCR activation step of 5 min at 95 °C, followed by 50 cycles of 10 s 95 °C, 30 s 58 °C and 30 s at 72 °C. HRM analysis was conducted on the fluorescence data acquisition channel proposed by the manufacturer with temperature ramping from 68 to 72 °C in 0.05 °C/2 s increments. Melting curves were normalized with the Rotor-Gene Q software by considering two regions before and after the major fluorescence decrease caused by the melting of the PCR product to enable the comparison of samples with different starting fluorescence levels but distinct melting behaviour.

2.8. Local DNA methylation analysis using pyrosequencing

In order to determine the methylation status of individual CpG-sites within the analyzed intronic sequence of the *ESR1* gene, pyrosequencing was performed on a PSQ 96MA Pyrosequencer (Biotage, Uppsala, Sweden). PCR products of preceding MS-HRM were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) following the manufacturer's instructions and used as template for the pyrosequencing reaction. The amplicons were immobilized on streptavidin-Sepharose beads (GE Healthcare, Waukesha, WI, USA), washed, denatured and the biotinylated strands released into annealing buffer containing the sequencing primer. These were 5′-GTGGTAAAGGTAATTTTTT, 5′-GATTTTAGAGTTGGATGGG and 5′-AAGTGTTTTGTGAGTTTGA for the PCR primer pair Pf1 and Pr1 and 5′-TAAGTTTTATTTTTGGTTTA for Pf2 and Pr2. All pyrosequencing reagents were prepared and procedures performed according to the manufacturer's suggestions (Biotage, Uppsala, Sweden). The methylation status at each CpG locus was analyzed individually using the Pyro Q-CpG software (version 1.0.9, Biotage, Uppsala, Sweden).

3. Results

3.1. Expression levels of ESR1

The mRNA abundance of *ESR1* during the estrous cycle was 1.7- and 1.9-fold higher in endometrium of day 0 compared to days 12 and 18 ($p < 0.03$ and $p < 0.01$, respectively) (Fig. 1). The pregnant day 18 endometrium displayed a 1.8-fold lower expression than the respective cyclic day ($p < 0.004$). White blood cells (WBC)

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showed a significantly lower gene expression compared to all endometrial tissue groups (24.1-fold and 78.2-fold compared to pregnant days 18 and 0, respectively) ($p < 0.001$).

3.2. Global DNA methylation levels

Global DNA methylation, inversely proportional to the *Hpa*II/*Msp*I ratio, was significantly lower in endometrial samples at all investigated time points compared to WBC ($p < 0.001$) (Fig. 2B). WBC were 82.1 ± 0.7 % (mean ± SEM) methylated in contrast to 73.6 ± 0.7, 75.8 ± 0.6, 75.8 ± 1.3 and 75.8 ± 0.9 % methylation for cyclic endometrium at days 0, 12, 18 and pregnant endometrium day 18, respectively. The latter endometrial samples showed a statistical tendency between cyclic day 0 and days 12 and 18 ($p = 0.10$, respectively) as well as pregnant day 18 ($p = 0.09$).

3.3. Expression levels of DNMTs

Endometrial *DNMT1* expression levels showed no statistically significant differences over cycle or early pregnancy. The mRNA abundance in WBC was 2.9- and 2.5-fold higher than in endometrium at days 12 and 18 ($p < 0.05$). In WBC, expression levels of *DNMT3a* were significantly higher than in endometrium during early pregnancy ($p = 0.027$), whereas no differences could be detected within the endometrial groups. The *DNMT3b* mRNA abundance in WBC significantly differed from all samples of endometrial tissues analyzed ($p < 0.006$), while days 0, 12, 18 and early pregnancy exhibited similar levels of *DNMT3b*.

3.4. Sensitivity of primers used for MS-HRM

The two primer pairs used for MS-HRM and pyrosequencing of *ESR1* (P1f + P1r and P2f + P2r, Fig. 3) cover an area of +864 to +1305 bp relative to the translational start codon, encompassing a total of 16 CpG sites. The amplicon of the P1 primer pair includes twelve, the P2 primer pair seven CpG sites (Fig. 3).

Primers for MS-HRM were not created according to former suggestions favouring methylated alleles (Wojdacz et al., 2008a,b), but were designed to evenly amplify both methylated and unmethylated DNA. This requirement was reached by avoiding primers with CpG-sites or introducing a false-base at an incorporated CpG-site. Sensitivity of the primers was tested by using dilutions of fully methylated control DNA into whole genome amplified (WGA) DNA that had been subjected to bisulfite conversion. As depicted in Fig. 4A, this newly introduced procedure for the generation of a 0% and 100% methylated control lead to very similar amplification efficiencies within different primer pairs for both methylated and unmethylated sequence with nearly identical set off points of exponential fluorescence increase. The dilution series were subsequently utilized in detecting PCR primer bias. As a result, a primer pair with high bias towards the methylated situation was excluded from the analysis of *ESR1* methylation (Fig. 4B). The discarded primer pair revealed only a very limited resolution between 25% and 75% while not allowing discrimination between a 75% and a 100% methylated sample (Fig. 4B). At an annealing temperature of 58 °C, both primer-pairs P1 and P2 exhibited only a slight tendency to favour the methylated allele within a mixture of differentially methylated samples and allowed good discrimination between 0% and 75% methylation while still separating 75% and 100% methylated DNA (Fig. 4C,D).

3.5. Evaluating methylation content by MS-HRM

The normalized melting profiles of PCR products achieved from the dilution series of samples with different ratios of methylation

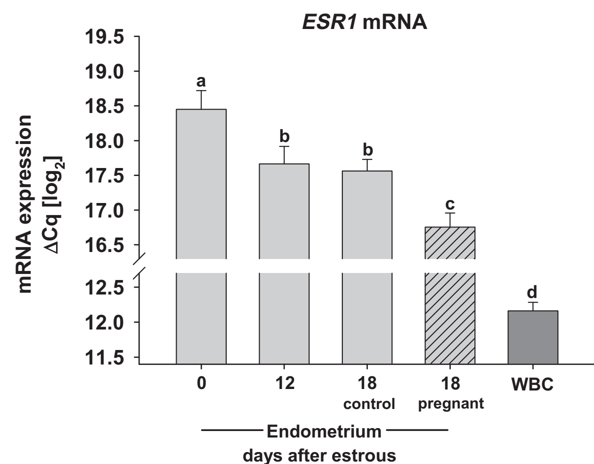

Fig. 1. Messenger RNA expression of *ESR1* in bovine endometrium during the estrous cycle and early pregnancy and in WBC. Data are presented as means ± SEM (ΔCq). Statistical analyses indicate that mRNA abundance of *ESR1* during the estrous cycle was higher in endometrium of day 0 compared to days 12 and 18 ($p < 0.03$ and $p < 0.01$, respectively) while pregnant day 18 endometrium displayed a lower expression than the respective cyclic day ($p < 0.004$). White blood cells (WBC) showed a significantly lower gene expression compared to all endometrial tissue groups ($p < 0.001$).

exhibited high consistency between different runs and replicates (data not shown). Methylation levels of endometrial samples were close to zero for all samples analyzed with both primer pairs P1 and P2, while WBC were 25–50% methylated (P1) and around 25% (P2), respectively (Fig. 5).

Next to a first visual estimation, MS-HRM data were evaluated according to a recently proposed method (Liu et al., 2010). Differential graphs of the normalized melting curves were generated with the Rotor-Gene Q software by standardizing the HRM profiles against the unmethylated control DNA. Values for the differential fluorescence peaks of the methylated template dilution series (Fig. 6A) were then plotted against the theoretical percentage of methylation within the template in order to generate a linear regression curve. This was done for both primer pairs employed in this work (Fig. 6B). With the use of the determined regression curves, methylation levels of two exemplary endometrial and WBC samples were calculated based on their respective differential fluorescence curves within one primer assay (Fig. 6C and D). For the endometrial samples, the average methylation level of the P1 assay was calculated to 0.9% and 5.2%, respectively, while the P2 assay proposed −1.2% and −1.1% methylation for the amplified products. Methylation levels of WBC ranged from 44.7% and 43.9% methylation for assay P1 to 22.6% and 19.0% methylation, respectively, for primer pair P2 (Table 1).

3.6. Verification and extension of MS-HRM results with pyrosequencing assays

Following HRM, PCR products were purified and subsequently subjected to pyrosequencing. This, on the one hand, allowed a deepened investigation of the analyzed site beyond the first screening line of MS-HRM on the identical amplicon. On the other hand, average methylation levels proposed by regression curve based calculations were compared to the methylation levels derived from single CpG-site methylation analysis using pyrosequencing.

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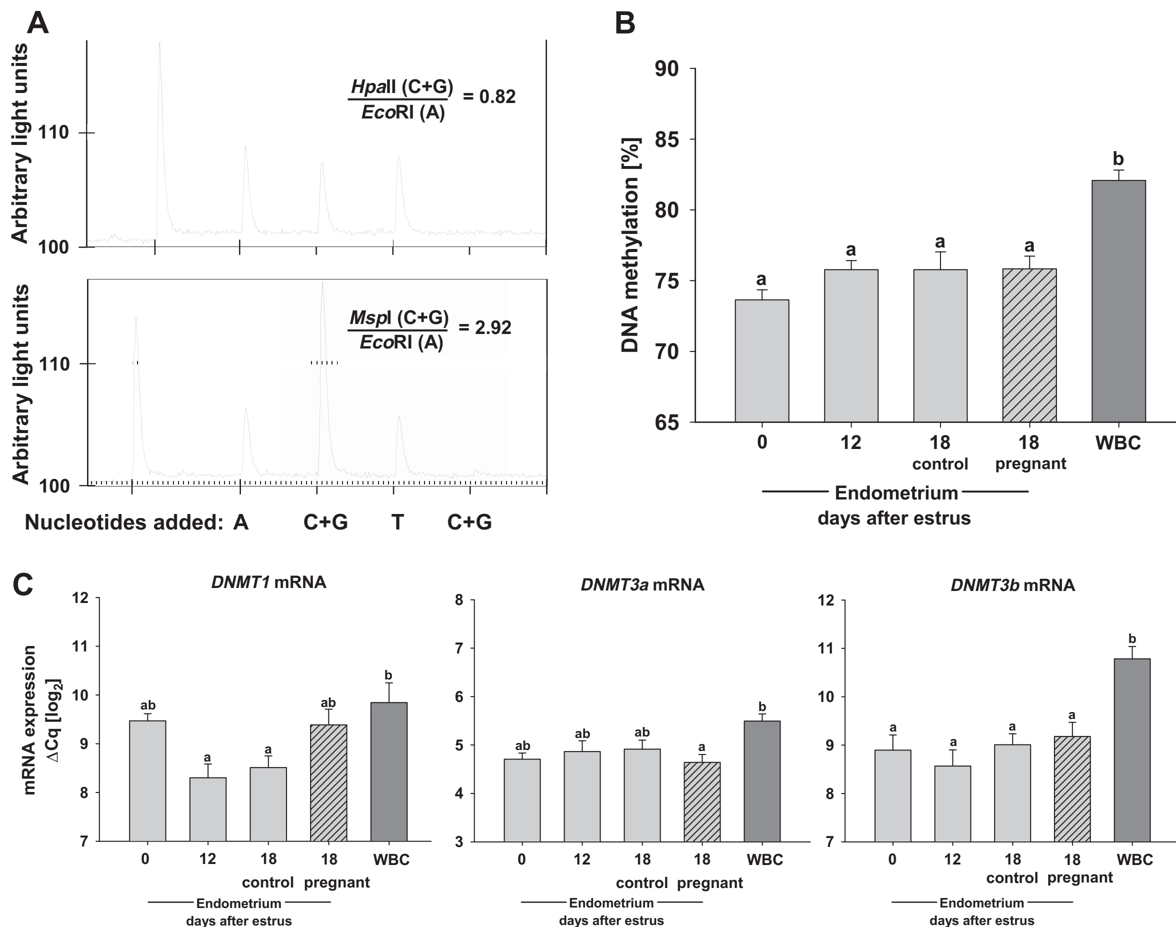

Fig. 2. Global DNA methylation analysis and DNMT expression levels. (A) A LUMA readout of an exemplary endometrial sample digested with the methylation sensitve enzyme *Hpa*II (above) and the methylation insensitive isoschizomer *Msp*I (below). Overhangs created due to digestion are complemented with the appropriate nucleotides during pyrosequencing. The resulting light emission indicates the percentage of successfully digested CCpGG sites in comparison to nonmethylation-sensitive digestions with EcoR1 creating AT-overhangs, e.g. the resulting (HpaII/EcoRI)/(MspI/EcoRI) ratio of 0.28 indicates a global DNA methylation level of 72%. (B) Global DNA methylation in endometrium and WBC according to LUMA. WBC were higher methylated at the investigated CCpGG-sites than endometrial samples ($p < 0.001$). (C) DNMT mRNA expression in endometrium and WBC. Marked differences between the two tissues were found for the *de novo* DNA methyltransferase *DNMT3b* corresponding well with the associated global DNA methylation levels. Bars not sharing identical letters differ significantly from each other ($p \leqslant 0.05$).

Average methylation levels of all 16 CpG sites were 5.9 ± 2.5% (SD) for day 0, 4.9 ± 2.4% for day 12 and 4.4 ± 2.3% for day 18 control endometrium. Pregnant day 18 animals exhibited an average methylation of 4.4 ± 2.6%, while WBC showed 27.6 ± 11.1% average methylation. Statistic analysis rated the difference of all endometrial samples to WBC highly significant, while there were no significant differences among the endometrial sampling groups. Each individual CpG-site revealed a highly significant difference between WBC and all endometrial samples ($p < 0.001$), but no significant differences among the endometrial sample groups (Fig. 7).

The average methylation levels of exemplary endometrial and WBC samples that had been subjected to MS-HRM regression curve-based methylation analysis (Table 1) were also calculated based on single CpG site data. In contrast to the proposed methylation for the amplicons of assay P1, 34.5% and 28.3% methylation were determined with pyrosequencing for the two WBC examples, respectively. The P2 assay for WBC showed methylation levels similar to HRM-derived values, 24.9% and 22.6%, respectively. This homology could not be determined for the P2 assay in the exemplary endometrial samples, which revealed 5.4% and 4.3% methylation, respectively. P1 assays of endometrium showed alike low methylation levels as compared to P2 (5.3% and 4.3%, respectively).

4. Discussion

Epigenetic events have sparely been investigated as possible mechanisms influencing the transcript abundance of genes involved in the regulation of the estrous cycle. Because former observations have revealed that genes can actually be differentially methylated within very short periodicities (Kangaspeska et al., 2008; Metivier et al., 2008), an epigenetic contribution due to the influence of endogenous hormones seems reasonable. Together with numerous discoveries of epigenetic modulations taking part in physiological events also occurring in the cyclic endometrium (Timp et al., 2009; Cao, 2009; Hemberger, 2010) and first hints indicating a role for epigenetic regulation during the menstrual cycle (Munro et al., 2010; Yamagata et al., 2009), this encouraged us to investigate the role of DNA methylation in the cyclic endometrium. We deployed LUMA for an analysis of global methylation levels and an elegant combination of MS-HRM plus pyrosequencing for a detailed description utilizing the most prominent endocrine transcriptional mediator ESR1.

Our mRNA expression data of distinct *ESR1* levels in the endometrium are in accordance with earlier findings (Fleming et al., 2001; Robinson et al., 2001) and thus provide the necessary basis

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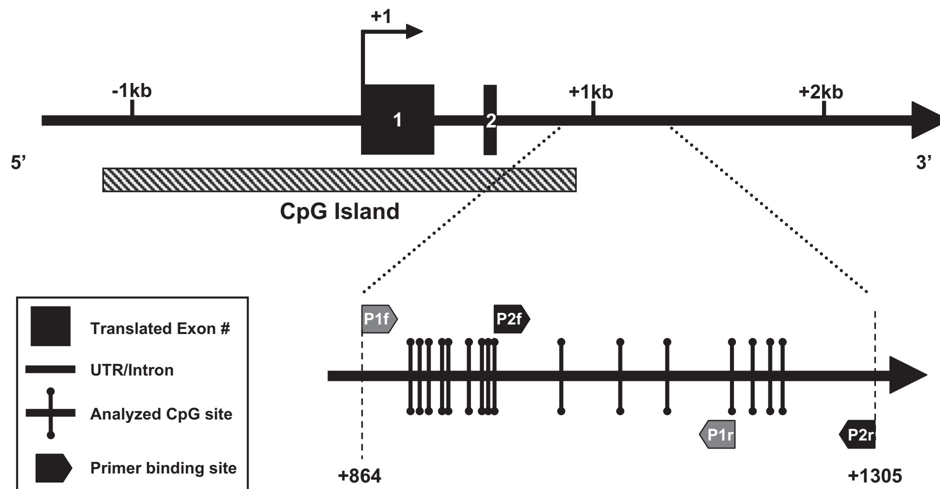

Fig. 3. Organization of the 5′ end of the bovine *ESR1* gene. A CpG island (position visualized by shaded gray bar) is proposed in the 5′ UTR covering the first two exons (black filled boxes) and introns. Regions of methylation analysis along the *ESR1* gene are shown as magnification in the lower part. 16 CpG sites, indicated by vertical bars with dotted ends, around the +1 kb region in relation the translational start codon (+1) were sequentially investigated using both MS-HRM and pyrosequencing. Block arrows display the location of the primers utilized in assays P1 and P2.

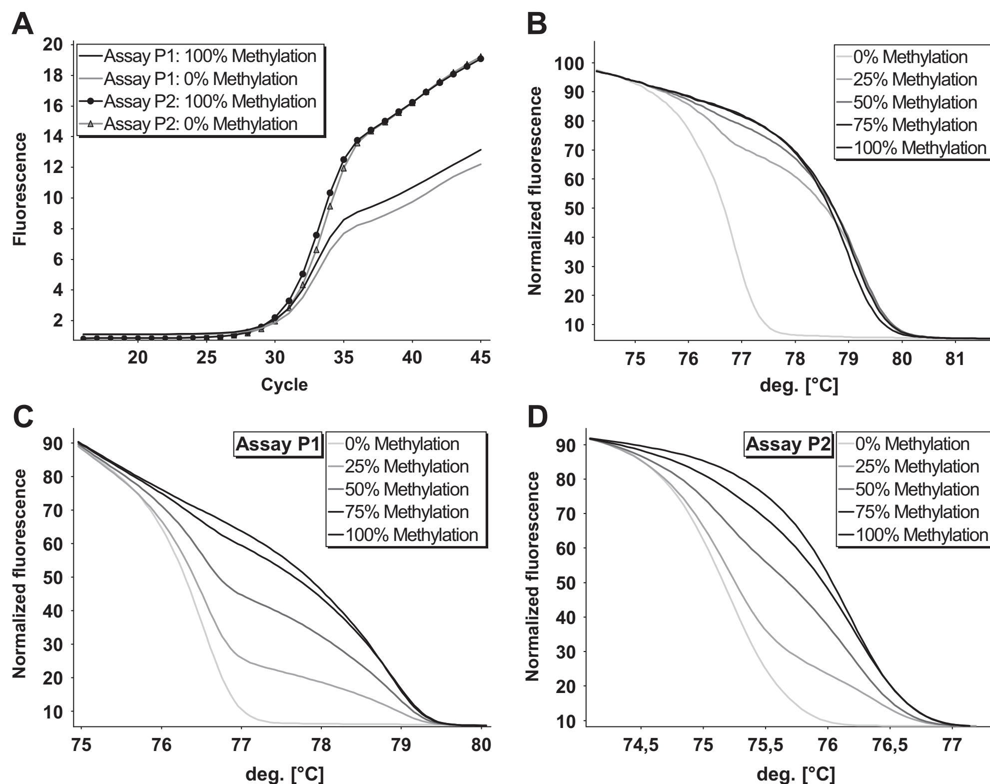

Fig. 4. Evaluation of PCR primers using MS-HRM. PCR amplification and high-resolution melting (HRM) analysis of bisulfite-converted DNA standards with discrete methylation percentage were performed sequentially on a real-time cycler. Melting curves were normalized by considering two regions before and after the major fluorescence decrease caused by the melting of the PCR product to enable the comparison of samples with different starting fluorescence levels but distinct melting behavior. (A) Validation of self-made DNA standards. The fluorescence raw channel in MS-HRM PCR shows similar amplification efficiencies and highly identical points of quantification for fully methylated and completely unmethylated samples within one primer set, respectively. (B) Normalized melting profile of a discarded primer pair. The usage of a dilution series of unmethylated and methylated templates revealed massive PCR bias towards the methylated situation. This results in an insufficient discrimination of samples with more than 25% methylation. (C) Normalized melting profile of primer pair P1. At an annealing temperature of 58 °C, PCR primer bias is reduced to a minimum facilitating good discrimination of differentially methylated template mixtures from 0% to 75% while still separating 75% and 100% methylated material. (D) Normalized melting profile of primer pair P2. Differentially methylated templates are evenly amplified at 58 °C annealing temperature offering clear separation in the range of 0% methylation to 100% methylation.

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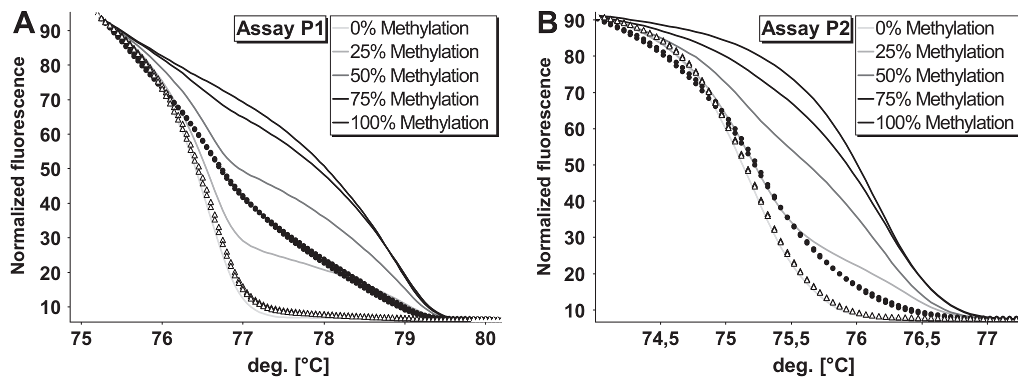
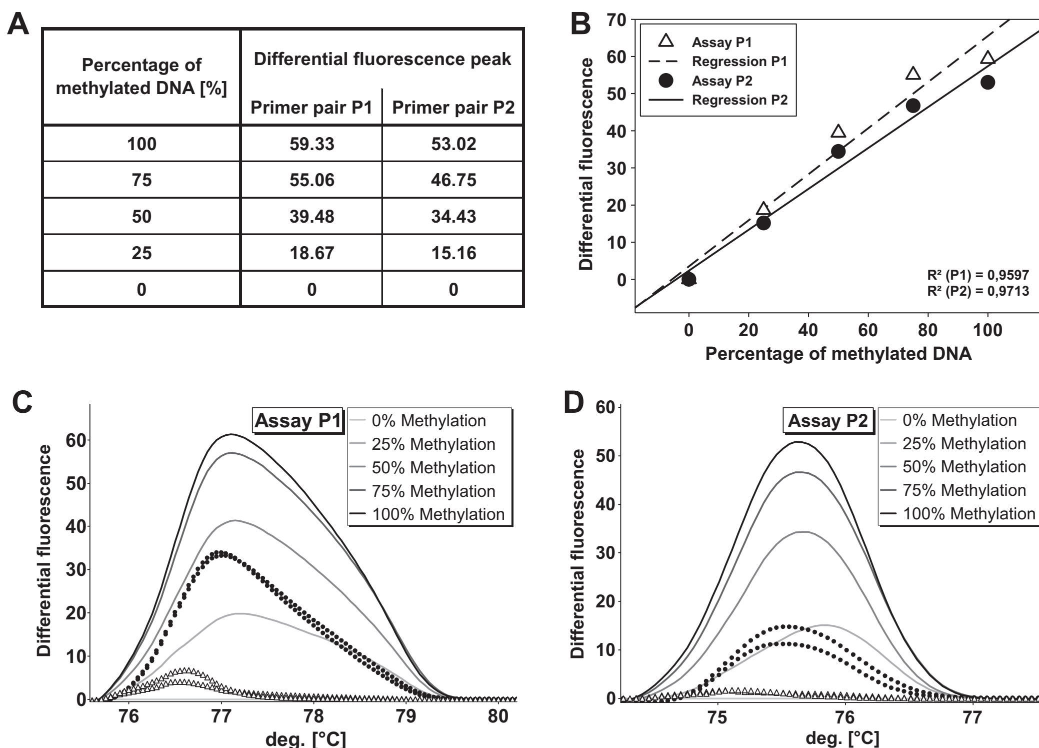


Fig. 5. Relative methylation of the investigated *ESR1* gene using MS-HRM analysis. The melting profiles of bisulfite-converted DNA from endometrium and WBC are accompanied by a dilution series of methylated DNA serving as a reference for qualitative methylation level assessment in the samples to be analyzed. (A) With primer pair P1, two exemplary depicted endometrial samples show methylation levels close to 0% while WBC according to their melting profile exhibit methylation levels between 25% and 50%. (B) Using primer pair P2, the two exemplary endometrial samples display 0% methylation, while WBC show methylation levels around 25%. For both assays, no obvious differences in methylation levels within all endometrial samples analyzed were detectable.



Percentage of methylated DNA [%]	Differential fluorescence peak	
	Primer pair P1	Primer pair P2
100	59.33	53.02
75	55.06	46.75
50	39.48	34.43
25	18.67	15.16
0	0	0

Fig. 6. Methylation analysis of MS-HRM data with regression curve-based quantification following Liu and colleagues (Liu et al., 2010). (A) Differential fluorescence values for the serial dilution of methylated DNA. Values represent the peak height of the differential fluorescence curves of the respective samples against the completely unmethylated control. (B) Regression curves for the serial dilutions of methylated DNA in assays P1 and P2. Differential fluorescence peak heights were plotted against the theoretical percentage of methylation within the template. Both assays display high values for their respective coefficient of determination ($R^2 > 0.95$). (C,D) Differential fluorescence curves of serial dilutions of methylated DNA and two exemplary samples of endometrium and WBC in assay P1 (C) and assay P2 (D). After the arbitrary setting of normalization borders, individual samples within one tissue show different curve shapes and peak heights. Using the assay-appropriate regression function, this leads to different methylation levels of the whole amplicon for the individual sample (Table 1).

for investigating gene expression regulations potentially based on DNA methylation levels. In WBC, enclosed as a non-endometrial reference tissue for further comparisons on the local and also global DNA methylation scale, the expression of *ESR1* clearly resembled the proposed situation of low receptor abundance. In the present study, we did not find significant differences in global DNA methylation in the bovine endometrium during the estrous cycle. However, the tendency of reduced methylation at estrus might be indicative for an epigenetic involvement in the regulation of the genes with high transcript abundance (Bauersachs et al., 2007). The fact that global epigenetic changes as observed in human endometrium are less pronounced in bovine may be attributed to the extent of endometrial remodelling. The reduced length of the proliferative phase during follicular development in ruminants as compared to humans does neither result in an equivalent massive endometrial growth nor subsequent shedding in the

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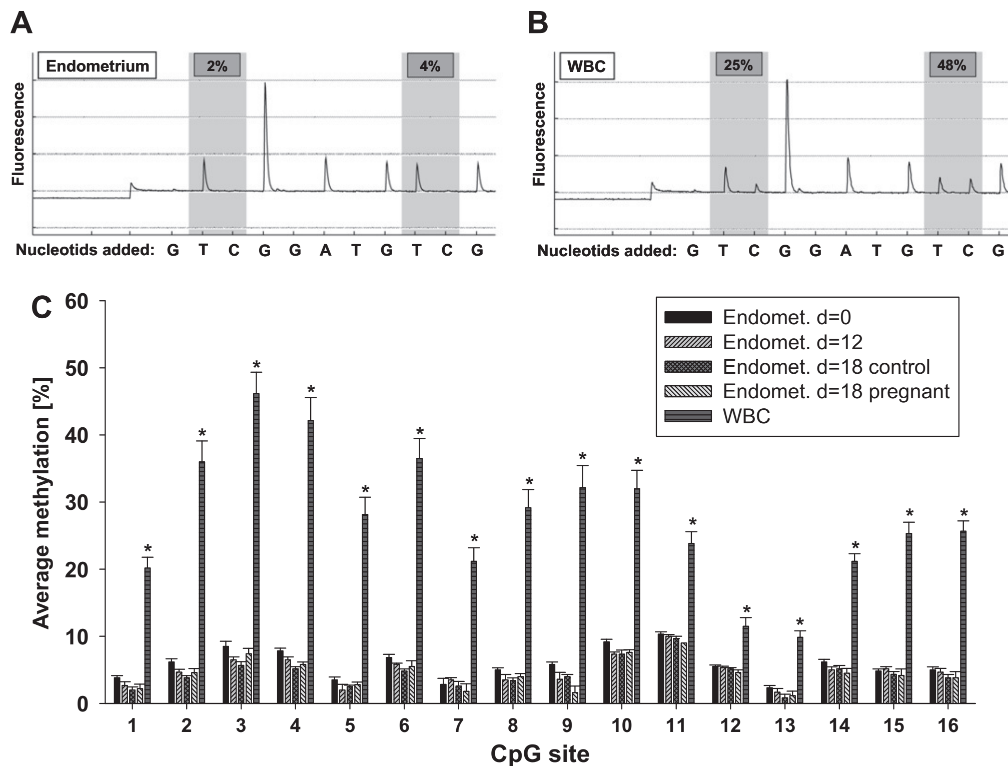

Fig. 7. Methylation analysis of the bovine *ESR1* gene by pyrosequencing on a preceding MS-HRM PCR product. (A,B) Representative pyrosequencing runs of an (A) endometrial and a (B) WBC sample are shown. The depicted CpG-sites (gray) were quantified to 2% and 4% methylation, respectively and 25% and 48% methylation, respectively. (C) Percentage methylation of CpG sites in the *ESR1* intron 2–3 in bovine endometrium and WBC. Highly significant differences (p < 0001, ∗) were found at every individual site analyzed between WBC and endometrial samples, but not among endometrial sample groups within each CpG-site.

Table 1
MS-HRM regression curve-based calculations vs. combined MS-HRM/Pyrosequencing methylation analysis. Data for two representative samples of WBCs and endometrium within the two assays P1 and P2 were compared. Calculated average methylation levels based on differential peak heights in MS-HRM differed considerably from average methylation levels based on single CpG-site methylation status analysis.

Sample	Regression curve based MS-HRM		Combined MS-HRM/Pyrosequencing	
	Calculated average methylation P1	Calculated average methylation P2	Measured average methylation P1	Measured average methylation P2
WBC #1	44.7	22.6	34.5	24.9
WBC #2	43.9	19.0	28.3	22.6
Endometrium #1	0.9	−1.2	5.3	5.4
Endometrium #2	5.2	−1.1	4.3	4.3

absence of an embryo. Global DNA methylation levels in endometrial samples from day 18 of pregnancy vs. non-pregnant heifers did not differ, although transcriptome profiles had shown differential gene expression already prior to implantation (Bauersachs et al., 2006). For this context it might be speculated that either gene-specific hypo- and hypermethylation are contrarily impacting on global DNA methylation or that transcriptional changes before implantation are only marginal compared to the amount of non-regulated genes and thus unobservable with the LUMA method. Clearly, the endometrium displayed a significant lower global methylation than WBC. This is in accordance with a recent observation in the human (Novakovic et al., 2010), where low global DNA methylation was speculated to be due to a placenta-specific switch to utilize alternative regulatory elements underlying usually highly-methylated repetitive areas. When analyzing the mediators of DNA methylation, the DNA methyltransferases (for a review see Szyf, 2010), we did not find any DNMTs significantly regulated within the endometrium. This coincides with our LUMA data that did not depict differences in global DNA methylation at the different time points of the endometrial cycle or early pregnancy. Rather than impacting on temporal local events, a specific DNMT seems to play a role in mediating basal transcriptional activities between tissues: Expression of the *de novo* DNA methyltransferase *DNMT3b* is significantly reduced in endometrial samples as compared to WBC which display relatively higher global methylation in WBC. Neither the hemi-methylase *DNMT1* nor *DNMT3a* seem to play a major role in this context.

Thus, our further goal was to additionally investigate whether specific local methylation takes part in the regulation of central endocrine mediators during the estrous cycle, thereby focusing to explain the observed differences in *ESR1* mRNA expression. The CpG methylation of *ESR1* has been encountered to be

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tissue-specific and related to the level of gene expression in humans (Piva et al., 1989a). There, the CpG-island around the *ESR1* translational start site was shown to be unmethylated in all analyzed tissues, while areas up- and downstream of the latter revealed CpG-methylation inversely correlated to the level of *ESR1* expression. Whether mammalian gene expression is actually regulated by DNA methylation mainly through its effects at CpG islands of 5′promoters has been questioned recently (Maunakea et al., 2010). Interestingly, only 2% of all human CpG-islands were found to be methylated at 5′promoters while much more frequently methylated in gene bodies. This supports a major role for intragenic methylation in regulating alternative promoters or ncRNAs involved in transcriptional regulation (Verdel et al., 2004; Zaratiegui et al., 2007). Correspondingly, a comparison of normal endometrial and carcinoma tissue showed demethylation for the 5′ region of the *ESR1* gene in both tissues while specific sites in the internal part of the gene were modified in carcinoma tissue only (Piva et al., 1989b). We thus decided to investigate an area around 1 kb downstream of the known bovine translational start site. Our hypothesis of a potential regulative site similar to human was supported by finding a CpG-island similar to the human size, coverage and location in bovine DNA due to high sequence homology. For local methylation analysis we employed the established methods MS-HRM and pyrosequencing. Investigations using MS-HRM have clearly addressed limitations of PCR on bisulfite-converted DNA (Wojdacz and Dobrovic, 2007, 2009; Wojdacz et al., 2008a), and guidelines for primer design to control PCR bias have been published recently (Warnecke et al., 1997; Wojdacz et al., 2009, 2008b). Although the method can be influenced by appropriate primer design to a high overall (i.e. over the whole amplicon) sensitivity, methylation information of single CpG sites requires further investigation. This can be conducted e.g. by subsequent sequencing following Sanger, but still, provided information will be limited to a qualitative readout (Wojdacz et al., 2010). Pyrosequencing is, accordingly to MS-HRM, also based on PCR amplification of bcDNA. The surveillance of a successful PCR on bcDNA however is usually limited to agarose-gel based methods indicating the product generation of expected size from (commercially available) both completely methylated and unmethylated templates only. In contrast to MS-HRM, PCR bias is only rarely rendered a problem when applying pyrosequencing
(Shen et al., 2007). In fact, combining methylated and unmethylated samples with subsequent annealing temperature calibration to overcome preferential amplification within the PCR has been highly suggested (Shen et al., 2007; Warnecke et al., 1997), but the proposed primer evaluation is time and cost intensive. Thus, we combined MS-HRM and pyrosequencing to overcome the limitations of PCR control prior to pyrosequencing and minimize the limitations of MS-HRM resolution (Candiloro et al., 2011).

Because samples of methylated and unmethylated DNA for PCR bias control were not commercially available for the species of interest, we easily created a standard of gradually methylated DNA by applying the principle of whole-genome amplification (WGA) to a mixture of bovine genomic DNA (gDNA) (Kristensen et al., 2008) with subsequent methylase treatment. Since methylation marks are not translated to the newly synthesized strands with WGA, the methylated CpG-site of one allele is diluted approximately 200–500 fold (data not shown) with unmethylated alleles. As a result, a template DNA with a CpG-site 100% methylated across all enclosed alleles is reduced down to 0.5–0.2% methylation at the respective site. Similar amplification efficiencies within different primer pairs for both the methylated and the unmethylated DNA with nearly identical set off points of exponential fluorescence increase indicated that no additional bias or modification had been introduced to the samples.

Primer design was conducted with respect to previous suggestions on annealing temperature to alleviate PCR bias (Shen et al., 2007; Wojdacz et al., 2008b). The introduction of artificial bias through the incorporation of a limited number of CpG dinucleotides in the primer sequence was avoided (Wojdacz et al., 2009). This would enable the HRM-based discrimination of samples with overall 0.1% and 1% methylation, although the identification of similar small variations in a medium methylated background might be limited. When applying pyrosequencing to the MS-HRM product, this improved resolution is not a requirement since detailed information is acquired through the subsequent quantitative single CpG-site analysis at a standard deviation of approximately 1% methylation (Tost et al., 2003). On the contrary, distinct bias could lead to overestimation of methylation differences and might cause misinterpretations on biological significance. By monitoring the gradational melting curves of our methylation dilution series, we were able to assess whether our aim of preferably even amplification was met by the primers to be validated. As shown for a discarded primer pair, the strong bias due to inappropriate primer design would not have been detectable using gel electrophoresis following standard PCR on only fully methylated or unmethylated samples (Fig. 4B). Both primer-pairs used in this study provided good discrimination of differentially methylated samples, allowing the application of MS-HRM for a first estimation of the methylation content of unknown samples on the basis of similarities derived from normalized HRM profiles (Wojdacz and Dobrovic, 2007). Based on this visual rating methylation content of the cyclic and pregnant endometrial samples as well as WBC revealed no difference for the estrous cycle or pregnancy but clearly stated higher methylation for the investigated area of *ESR1* in WBC compared to endometrium. On closer inspection, it is obvious that the curves of the biological samples did not completely parallel the methylation dilution series. This aspect was a first indicator for differentially methylated sites within the biological samples. While most studies on MS-HRM stick to the visual evaluation of MS-HRM results a new method has been described recently allowing a quantitative methylation determination of biological samples (Liu et al., 2010). This approach is based on generating a linear regression of differential fluorescence peaks with a dilution series of methylated DNA. We conducted the proposed method for our two primer assays, however, negative levels of DNA methylation already indicated that this proposed method suffers from serious limitations.

The following introduction of purified MS-HRM products into pyrosequencing to receive single CpG-site resolution and quantitative data performed excellently. We approved the rough visual estimation of average methylation from MS-HRM and the hint for differentially methylated sites. Statistical analysis confirmed the difference in WBC and endometrial methylation and the degree of methylation corresponding to the mRNA expression data of *ESR1* for the two tissues. Hence, the site investigated in bovine seems similarly important for some kind of "basal", tissue-related expression as in humans. However, the differential expression of *ESR1* within the endometrium during the cycle or pregnancy appeared not to be targeted by differential methylation of the investigated area since no clear interrelation between methylation status and gene expression was determined. It may be speculated that either other regions take part in the regulation of this gene within the endometrium or that expression of the *ESR1* in cycle and pregnancy is regulated by various transcription factor binding events. Comparing the quantitative analysis approach based on MS-HRM with the data received from pyrosequencing of the exactly same amplicon, our data clearly indicate that the regression-curve based average methylation levels vary from the single CpG-site based calculations. This is not astonishing since variations in PCR bias at dif-

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ferent levels of methylation dilutions lead to inappropriate regression curves and thus imprecise data. Furthermore, peak values of differential fluorescence cannot represent distinctly varying methylation levels at individual CpG-sites causing non-paralleling curve shapes of biological samples within the artificial reference set. A third aspect that has to be considered is the relatively strong influence on curve-shapes and thus differential fluorescence peak values by the arbitrary setting of fluorescence normalization regions in the HRM software. As shown for our exemplary samples, calculated methylation differences were artifacts not certified by pyrosequencing. Considering these drawbacks, this approach might cause misleading results for quantitative data generation on MS-HRM analysis. On the contrary, the use of MS-HRM as a cost, labour and time efficient screening method for differential methylation analysis is appropriate if combined with efficient sequencing procedures. As direct bisulfite sequencing might be problematic since derived electropherograms only grant a qualitative readout of difficult MS-HRM results (Wojdacz et al., 2010), the present investigations support the concept of coupling MS-HRM with pyrosequencing (Candiloro et al., 2011). The depicted way of effectively controlled quantitative data evaluation is a necessary basis for discussing results on DNA methylation differences in any field of research and their physiological or pathophysiological relevance. Applying methods not compensating for bias or incorporating artificial bias through non-linear PCR amplification might lead to results reaching statistical significance but lacking biological truth.

In conclusion, the depicted combination of MS-HRM and pyrosequencing emerged excellent for the unbiased investigation of local DNA methylation levels at CpG-site resolution. Employing this method, we found that endometrial DNA methylation of *ESR1* during the estrous cycle and early pregnancy did not correspond to its transcriptional expression status but suggests alternative expression regulation e.g. via transcription factor binding events or posttranscriptional modifications. A general epigenetic contribution of global DNA methylation influencing the bovine cycle or early pregnancy could not be assessed. Prevailing at low levels in endometrium, global DNA methylation may likely be affected by the observed reduced *DNMT3b* expression. Thus, DNA methylation may contribute to providing an appropriate expression status underlying further transcriptional regulation which distinguishes the endometrium from other somatic tissues.

Declaration of interest

The authors state to have no personal financial interests.

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A differentially methylated single CpG-site is correlated with estrogen receptor alpha transcription

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ABSTRACT

DNA methylation of the promoter region of estrogen receptor alpha (*ESR1*) is recognized as an epigenetic mechanism that regulates its mRNA abundance. We questioned whether tissues in male growing piglets were influenced in terms of DNA methylation by the developmentally occurring distinct plasma estradiol-17β (E2) concentrations. Additionally, we aimed at broadening the currently limited understanding of the epigenetic regulation of *ESR1* in physiological settings. Three distinct genetic regions of *ESR1* were analyzed using a combination of methylation-sensitive high resolution melting (MS-HRM) and pyrosequencing. Unexpectedly, major E2 concentration differences were only marginally associated with minor variations in DNA methylation and mRNA abundance. However, by analyzing two tissues showing the greatest differences in transcript abundance, we were able to find one single CpG site in the +1 kb intragenic region of *ESR1* strikingly differently methylated between heart vs. epididymis. Interestingly, this single CpG-site was identified as a putative binding site for the transcriptional repressor TG-interacting factor 1 (TGIF) which can recruit histone deacetylase 1 (HDAC1) leading to chromatin condensation. Indeed, chromatin immunoprecipitation confirmed a reduced histone H3 presence at the specific *ESR1* location in case of higher DNA methylation. We therefore hypothesize that *ESR1* expression may be manifested by a single-CpG-site based methylation difference impairing transcription factor binding.

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1. Introduction

Estrogens regulate factors essential for reproduction of female as well as male vertebrates, including fish, amphibians, reptiles, birds and mammals [1,2]. Estrogenic action, mediated in particular by the primary biologically active form estradiol-17β (E2), evokes its effects mainly by changing the expression patterns of specific target genes. Mediators of these genomic actions are the well characterized estrogen receptors ERα (ESR1) and ERβ (ESR2) that operate in combination with other co-regulators as transcription factors [3–6]. In addition to their classical nuclear localization, membrane-bound subpopulations have been shown to utilize non-genomic actions of estrogens that, among others, activate the ERK/MAPK pathway [7,8]. These can lead to rapid expression variations of downstream target genes involved in reproductive processes and cytoplasmic modifications such as ion concentrations mediating cellular trafficking [9–11]. Estrogen signaling can also target its own receptor transcription and thus modulate the sensitivity of cells towards the E2 signal. In this context, E2 has been recognized for its ability to either enhance or decrease *ESR1* expression, widely differing according to the investigated cell type [12,13]. The mechanisms behind this contradicting action on the cellular level have not been fully understood to date. However, as cascades of signal transduction molecules presumably vary within different cell types, the response of *ESR1* to E2 signaling can consequently be diverse for rapid signaling cascades that involve e.g. phospholipase C (PLC9/protein kinase C (PKCs) or phosphatidyl inositol 3 kinase (PI3K)/AKT [14].

Regulatory mechanisms for the individual cell to manifest a gene's expression difference include the selective disposition of specific transcriptional start sites with diverging transcriptional strength. In case of *ESR1*, several transcriptional start sites and their tissue-specific utilization have been discovered in human and in other species (for overviews see [15,16]). In addition, a study using breast cancer cells suggested that overall levels of *ESR1* expression may not only be influenced by specific transcriptional start sites, but also by the total number of promoters in use [12]. Irrespectively of a regulation via transcriptional initiation sites, a third possibility involves other gene positions like splice acceptor sites or intragenic regions where modifications to the DNA can influence

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the efficiency of the transcriptional process. This kind of expression regulation has been described to be mediated by DNA methylation and associated chromatin modifying factors and applies to both *ESR1* and a large number of other genes and their promoters showing tissue- or cell-dependent patterns of DNA methylation [17,18]. A common example for this is the hypermethylation of the *ESR1* promoter CpG-island in breast cancer, leading to the loss of *ESR1* expression in these tumors [19,20]. In a non-pathological state, a correlation between *ESR1* promoter DNA methylation increase and the corresponding decrease in *ESR1* expression during postnatal development has been found in mice brain [21]. Moreover, E2 exposure and DNA methylation of the *ESR1* promoter in rat brain have clearly been associated [22]. We have recently shown tissue-specific methylation of the bovine *ESR1* gene downstream of the translation start site. This is in accordance with findings in the human endometrium showing this area as responsible for tissue-specific *ESR1* expression [23]. The listed references indicate that DNA methylation changes driving physiological *ESR1* expression might occur in a time- and/or tissue-specific manner due to dynamic changes in circulating and local steroid hormone concentrations that appear over development and life.

In newborn male piglets, E2 concentrations are very high due to placental E2 synthesis [24]. Only a few weeks later, the lowest concentrations in pig lifetime predominate. The present study used this model of male growing piglets and their naturally prevailing declining E2 after birth to question whether distinct plasma concentrations may affect tissue specific *ESR1* expression by means of distinct methylation patterns of the *ESR1* gene. Coevally, we aimed at broadening the current understanding of *ESR1* epigenetic regulation by assessing three distinct genetic segments, in particular promoter, CpG-island and intron with high-resolution DNA methylation analysis to elucidate whether region-specific methylation differences correlate to expression abundances of *ESR1* during development.

2. Materials and methods

2.1. Animals and tissue sampling

German Landrace sows ($n = 6$) were randomly selected and inseminated with Pietrain sperm twice at estrus. From each sow, one male sibling was slaughtered at <1 h, 11 d or 56 d after birth, respectively. Blood samples were collected, plasma separated and stored at −20 °C. Tissue samples of heart, ileum, spleen, brain, kidney, muscle, liver, lung, adrenal gland, prostate, testis and epididymis were directly frozen in liquid nitrogen and stored at −80 °C until further investigation. Sampling was conducted in accordance with accepted standards of humane animal care and approved by the institutional vet authority.

2.2. Competitive enzyme immuno assay

The concentrations of E2 in piglet plasma were determined after ether extraction using a competitive enzyme immuno assay (EIA) as described earlier [25]. Extraction and quantification were conducted in duplicates for each sample. Results ($n = 9$ samples) are shown as means [pg/ml plasma] ± SEM. The effect of age was determined on the natural logarithm of EIA data to ensure normal distribution with a one way analysis of variance followed by all pairwise multiple comparison procedures (Tukey method) using the SigmaPlot program package release 11.0 (SPSS, Chicago, IL, USA).

2.3. Extraction of total RNA

Total RNA from tissue samples was isolated using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions with minor modifications. In brief, 20 mg of tissue was mixed with 450 μl buffer RA1 plus 4.5 μl β-mercaptoethanol and homogenized in a MagnaLyser (Roche, Basel, Switzerland) with Matrix-Green beads (MP Biomedicals, Illkirch, France) in three successive pulses of 7000 rpm for 30 s. For improved RNA purity, the second wash step with 600 μl buffer RA3 was conducted twice, resulting RNA quantity and purity was spectroscopically checked using the Peqlab NanoDrop 1000 (Peqlab, Germany). The Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) served to asses RNA integrity which ranged from RNA Integrity Number (RIN) 7–10.

2.4. Two step quantitative RT-PCR

Following the LightCycler DNA Master SYBR Green I protocol (Roche), quantitative reverse transcription PCR (qRT-PCR) experiments were performed as described earlier [26]. Cycle numbers (Cq) were determined by using the second derivative maximum method (LC software 4.05). For the comparison of *ESR1* expression between different tissues, data are presented as means of raw Cq-values ± SEM subtracted from the arbitrary value 40 (ΔCq) to achieve a high transcript abundance indicated by a high ΔCq [27]. Due to potential tissue-specific effects on the mRNA quantification procedure, no statistical analyses were employed at this stage. Transcript abundance of *ESR1* within the same tissue was assessed using the geometric mean of the three reference genes histone H3 subfamily H3A3 (H3F3A), ubiquitin 3 (UBQ3) and 18S rRNA (Table 1) to normalize target gene Cq values according to the bestkeeper method [28]. Data were normalized and relatively compared as recommended by Livak and Schmittgen [27] and are presented as means ± SEM. Data comparing *ESR1* expression within epididymis or heart were subjected to a one-way analysis of variance to reveal any group effects. Differences between time points were determined with one-way ANOVA followed by pairwise multiple comparison procedures (Tukey method). Statistical analyses were employed using the SigmaPlot program package release 11.0 (SPSS).

2.5. Immunohistochemistry

Tissues of 56 days old piglets were fixed in 3.7% formalin, dehydrated in an increasing gradient of ethanol and finally embedded in paraplast (Histo-Comp, Vogel, Giessen, Germany). For immunohistochemical analysis, sections of 5 μm were dehydrated and subsequently subjected to antigen retrieval using 0.01 M citric acid buffer (pH 6.0). Slices were microwaved for 5 min at 800 W, followed by 15 min at 400 W and adjacent cooling in phosphate-buffered saline (PBS), pH 7.4. Endogenous peroxidase activity was blocked by incubation with 1.0% H_2O_2 in PBS, and successive incubation in 1% normal horse serum (part of Vectastain Universal Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) was applied to reduce nonspecific background signal. The sections were incubated overnight at 4 °C with a rabbit polyclonal antibody raised against ESR1 of human origin (sc-7207, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:300 for epididymis and heart and 1:800 for the endometrial positive control. Negative controls received 1% normal horse serum instead of the primary antibody. Visualization of the primary antibody was conducted using the Vectastain Universal Elite ABC Kit (Vector Laboratories) according to the manufacturer's instructions. In brief, sections were incubated 1 h at room temperature with a biotinylated universal antibody diluted 1:1600 in PBS for epididymis and heart or 1:3200 for endometrium. This was followed by linking the AB-complex for 30 min at room temperature. 3,3′-diaminobenzidine served as substrate to visualize peroxidase activity. Slides were then dehydrated and mounted in DPX (Fluka Chemie, Buchs, Germany). Exemplary

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Table 1
Primers used in their respective applications.

Application	Gene/area	Reference	Orientation	Sequence	Fragment size (bp)	Annealing temp. (°C)
qRT-PCR						
	UBQ3	Z18245	Forward	AGA TCC AGG ATA AGG AAG GCA T	198	60
			Reverse	GCT CCA CCT CCA GGG TGA T		
	H3F3A	BT020962	Forward	ACT GGC TAC AAA AGC CGC TC	233	60
			Reverse	ACT TGC CTC CTG CAA AGC AC		
	18S rRNA	AF176811	Forward	AAG TCT TTG GGT TCC GGG	488	60
			Reverse	GGA CAT CTA AGG GCA TCA CA		
	ESR1	NM_214220	Forward	CCA TCA TTT TGC TTA ATT CTG GAG	194	57
			Reverse	ATG CCT TTG TTA CTC ATG TGC CTG		
MS-HRM						
	−1 kb	FR682141	Forward	AAT GTG TTT GGT GAG ATT AAT AGT GTA GA	287	58
			Reverse	Bio-AAA ATT CTC CCA AAA TCA TCC CC		
	CpG island	FR682141	Forward	AGT TGA GTT TGG AGT GAT GTT TAA GTT AA	222	58
			Reverse	Bio-ACT CTA ACC CTA CCC CTA AAA CC		
	+1 kb	FR682141	Forward	TTT TAT TTT AGA GGG AGG TTG TAG GAT TG	124	58
			Reverse	Bio-ACT ATC AAA AAT CTT CTA CCA ACA AAC TAC TCT		
	+1 kb	FR682141	Forward	AGT AAG GGT TGA ATG GGT TTT AAG GAG	320	58
			Reverse	Bio-CTC TTA AAT CAC AAA AAT ATT CCT ATC TAT CC		
Pyrosequencing						
	−1 kb	FR682141		TGT TTG GTG AGA TTA ATA GTG		
	−1 kb	FR682141		ATT TGT GGA AGG TTT GAA T		
	CpG island	FR682141		AGT ATT TTT GTA ATG TAT ATG AG		
	+1 kb	FR682141		AGG GAG GTT GTA GGA T		
	+1 kb	FR682141		AAT GGG TTT TAA GGA GT		
	+1 kb	FR682141		TGT TTT GTG AGT TAA AAA T		
ChIP qPCR						
	RPL30	NM_001190178.1	Forward	GGA TCC AGT TTT GAG CGG TA	130	60
			Reverse	TAC CCC AGC ACG TAC TTT CC		
	ESR1	FR682141	Forward	AGT CCG TGT CAG AGC AGC TT	142	60
			Reverse	GCA GGC CAG AAA TGA AAC TT		

samples ($n = 3$) were investigated und images taken using a Leica microscope with a digital camera system (DP72, Olympus, Hamburg, Germany) and corresponding software at the very same adjustments using differential interference contrast.

2.6. Isolation of genomic DNA

Genomic DNA was isolated and quality-checked as reported earlier [23]. For global DNA methylation analysis, genomic DNA was used directly while investigations on local DNA methylation required bisulfite-conversion with the EZ DNA Methylation-Gold Kit (Zymo Research, CA, USA) following the manufacturer's suggestions on 500 ng genomic DNA per sample.

2.7. Luminometric methylation assay (LUMA)

Global DNA methylation was assessed as previously described [23] with slight variations. In short, 500 ng genomic DNA was cleaved in two separate reactions with the isoschizomere FastDigest restriction enzymes *Hpa*II + *Eco*RI or *Msp*I + *Eco*RI (Fermentas, St. Leon-Rot, Germany) for 20 min. Peak luminometric heights were obtained with the PyroMark Q24 software (Qiagen, Hilden, Germany) using a Pyromark Q24 system (Qiagen) and its appropriate run setup. Figures of LUMA display the percentage of DNA methylation which is inversely proportional to the calculated (*Hpa*II/*Msp*I) ratio at the investigated CCGG-sites. Using the SigmaPlot program package release 11.0 (SPSS), pairwise multiple comparison procedures (Tukey method) were employed to determine differences within heart and epididymis ($n = 6$), while *t*-tests were performed to compare percentage of DNA methylation between both tissues at the respective days.

2.8. Combined MS-HRM and pyrosequencing

Local DNA methylation analyses using MS-HRM and pyrosequencing were executed as described recently [23] with slight variations to accommodate the Pyromark Q24 system (Qiagen). In brief, a 10 μl reaction in the preceding HRM PCR proved to be sufficient for later analysis in the Pyromark Q24 system (Qiagen). MS-HRM PCR primers and subsequent pyrosequencing sequencing primers were created on in-silico bisulfite converted DNA using the PyroMark Assay Design Software 2.0 (Qiagen). Primers are listed in Table 1, and areas covered by these are depicted in Fig. 1. Information on pig promoter and 5′ *ESR1* gene sequences were acquired by aligning uncharted pig sequences against the human and bovine ESR1 genes in addition to own DNA sequencing using the sitefinding-PCR method [29]. The resulting sequence which served as basis for further analyses was submitted to GenBank under the accession number FR682141. Annotations in Fig. 1 are proposed based on the high sequence homology to the human annotated sequence. Within one tissue, effects of age on DNA methylation per individual site were raised with one way analysis of variance followed by pair wise multiple comparison procedure (Tukey method). *t*-Tests were performed to evaluate tissue-based differences at individual CpG sites by comparing mean % methylation of all tissue samples at each time point.

2.9. Transcription factor binding site analysis

The search for possible transcription factor binding sites was conducted *in-silico* using the MatInspector (Genomatix, Munich, Germany) and TESS (University of Pennsylvania, Philadelphia, PA, USA) software algorithms [30–32].

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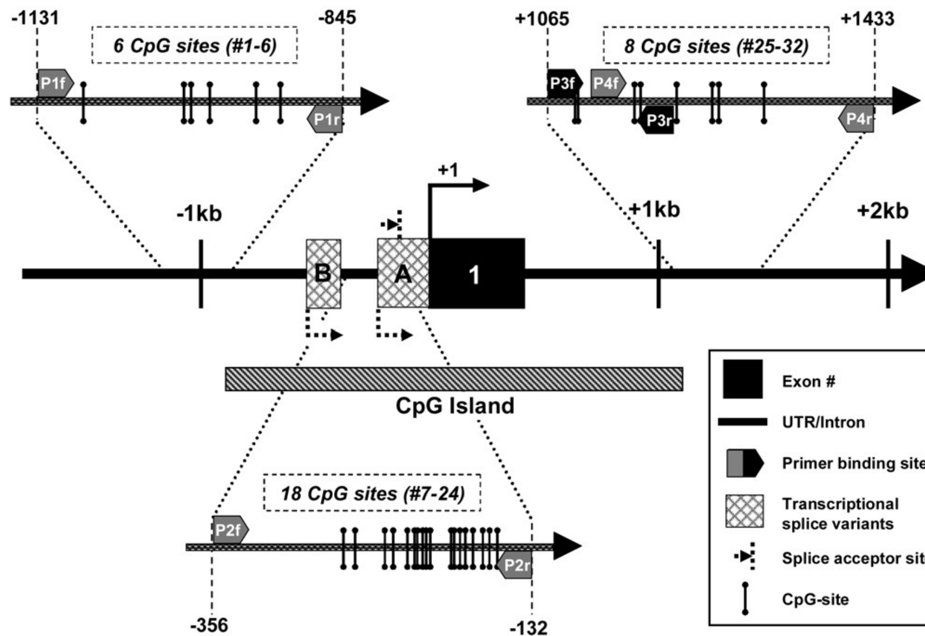

Fig. 1. Overview of the *ESR1* genomic region analyzed. The pig promoter region was sequenced (GenBank accession number FR682141) and annotations were proposed based on alignments to the human and bovine sequences. Areas analyzed for DNA methylation by a combination of MS-HRM and pyrosequencing are depicted together with the respective primers used and the number and location of CpG-sites investigated.

2.10. Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation (ChIP) was performed on heart and epididymis of 56 days old male piglets using the SimpleChIP Enzymatic Chromatin IP Kit, Magnetic Beads (Cell Signaling Technology, Danvers, MA, USA). Chromatin preparation from tissue was conducted following the manufacturer's protocol with minor variations. In brief, 1 g of frozen tissue was ground with a mortar and pestle in liquid nitrogen and afterwards crosslinked in 10 ml 1.5% formaldehyde for 10 min. Crosslinking was stopped by incubation with 1 ml 10× glycine solution for 5 min, the suspension was then centrifuged at 4 °C for 5 min at 3000 rpm, washed with 10 ml PBS solution, centrifuged and resuspended in another 10 ml PBS. Using a syringe and two needles of different size (18 G and 20 G) successively, the tissue was further homogenized by gently passing the tissue suspension 20 times through the needle. The suspension was then centrifuged at 3000 rpm for 10 min, the obtained pellet resuspended in buffer A and washed with buffer B according to the manufacturer's recommendations. After digestion with micrococcal nuclease, the suspension was divided into samples of 500 μl and each tube sonicated using an ultrasound homogenizer Sonopuls HD70 (Bandelin electronic, Berlin, Germany) with several pulses to break the nuclear membrane. The lysates were clarified by centrifugation at 13,000 rpm, the supernatants containing the digested chromatin merged and incubated for 10 min 1:5 in 1× ChIP-buffer before the final storage at −80 °C. Immunoprecipitation (IP) procedures followed the manufacturer's recommendations employing normal rabbit IgG as a negative control (Cell Signaling Technology #2729) and a ChIP-formulated anti-Histone H3 Rabbit monoclonal antibody (Cell Signaling Technology #4620) as a positive control. For each IP, a 2% input sample and the specific immunoprecipitated DNA was quantified using the LightCycler DNA Master SYBR Green I kit (Roche) for qPCR. Primers for the reference gene locus of the ribosomal protein L30 (RPL30) were adapted to the pig sequence and are listed in Table 1 together with the primers designed to cover the possible binding site of the transcriptional repressor TGIF on the *ESR1* gene. The degree of immunoprecipitation for each antibody, respectively serum, is represented as signal relative to input material for one exemplary precipitation and relative to the reference gene locus RPL30 for all three independent experiments, each performed using pooled tissues of two animals, thus representing tissue material from six male piglets. Statistically significant differences between heart and epididymis were investigated using a *t*-test in the SigmaPlot program package release 11.0 (SPSS).

3. Results

3.1. Plasma estradiol-17β in male growing piglets

Blood plasma levels of E2 in male growing piglets ($n = 9$ per time point) significantly declined from <1 h after birth until day 56 ($p < 0.001$). E2 concentration was 153.5 ± 29.9 pg/ml shortly after birth, 32.1 ± 7.2 pg/ml at day 11 and 4.8 ± 0.8 pg/ml at day 56, respectively (Fig. 2).

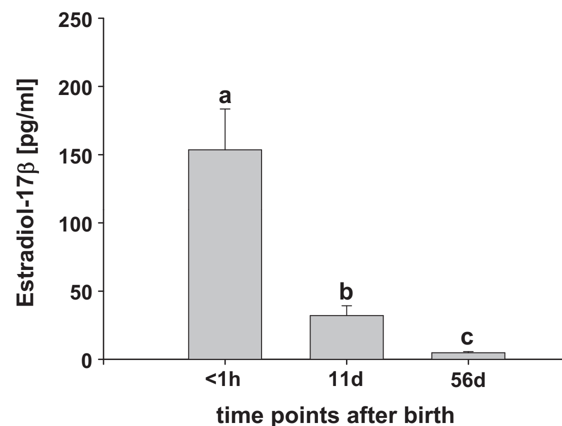

Fig. 2. Estradiol-17β plasma concentrations developmentally decline after birth in male prepubertal piglets. Data are presented as mean ± SEM. Bars not sharing identical superscript letters indicate significant differences ($p < 0.05$).

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3.2. Gene expression of ESR1 in male piglet tissues

The mRNA abundance of *ESR1* in male growing piglets varied between individual tissues. At eight weeks after birth, male reproductive tissues showed distinctly higher mRNA transcript abundance compared to non-reproductive tissues (Fig. 3A). For epididymis, the ΔCq was 15.2 ± 0.3 (mean ± SEM) whereas lowest mRNA expression was detected in heart with a ΔCq of 7.1 ± 0.4, indicating an approximately more than 250-fold difference. Within each of these two representative tissues of high (epididymis) and low (heart) *ESR1* mRNA abundance, expression was closer inspected over the investigated time points. No significant difference between time points was detected in heart (Fig. 3B), while epididymal *ESR1* mRNA abundance displayed a significant ($p = 0.018$) increase on day 11 relative to <1 h (207.9 ± 25.9% versus 100.0 ± 18.3% mRNA expression) (Fig. 3C) followed by an intermediate expression at day 56 (167.1 ± 28.2% relative expression).

3.3. Immunohistochemical localization of ESR1 in heart and epididymis

Exemplary tissue samples from time point 56 d after birth revealed faint cytoplasmic ESR1 staining in heart cardiac type muscle cells of the myocardium, while nuclei were devoid of staining (Fig. 4A). Nuclei of cells in the connective tissue as well as the cytoplasma of epithelial cells were positively labeled in the epididymis while smooth muscle cells surrounding the epithelial lumen showed no staining (Fig. 4B). The positive control uterus exhibited intensely labeled nuclei of glandular epithelial cells of the endometrium as well as nuclei of smooth muscle cells in the myometrium. Furthermore, a slight staining of the cytoplasma in the myometrium was detected (Fig. 4C).

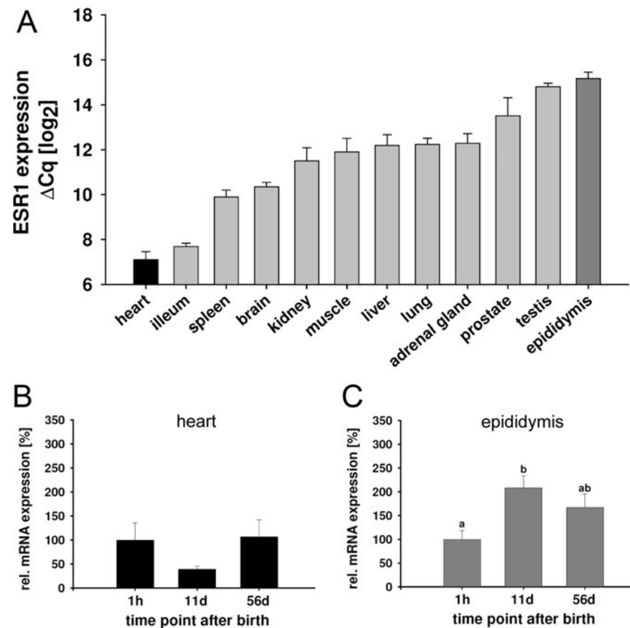

Fig. 3. Expression of *ESR1* in male piglet tissues varies considerably among tissues but not over development. (A) Abundance of *ESR1* mRNA in various piglet tissues at eight weeks of age showing an approximately 250-fold higher expression in epididymis than in heart. (B) No significant expression differences were found in heart tissue at three sampling time points during development, whereas epididymis (C) showed slight variations between 11 days and shortly after birth. All data are presented as mean ± SEM, different superscript letters indicate significant differences between groups ($p < 0.05$).

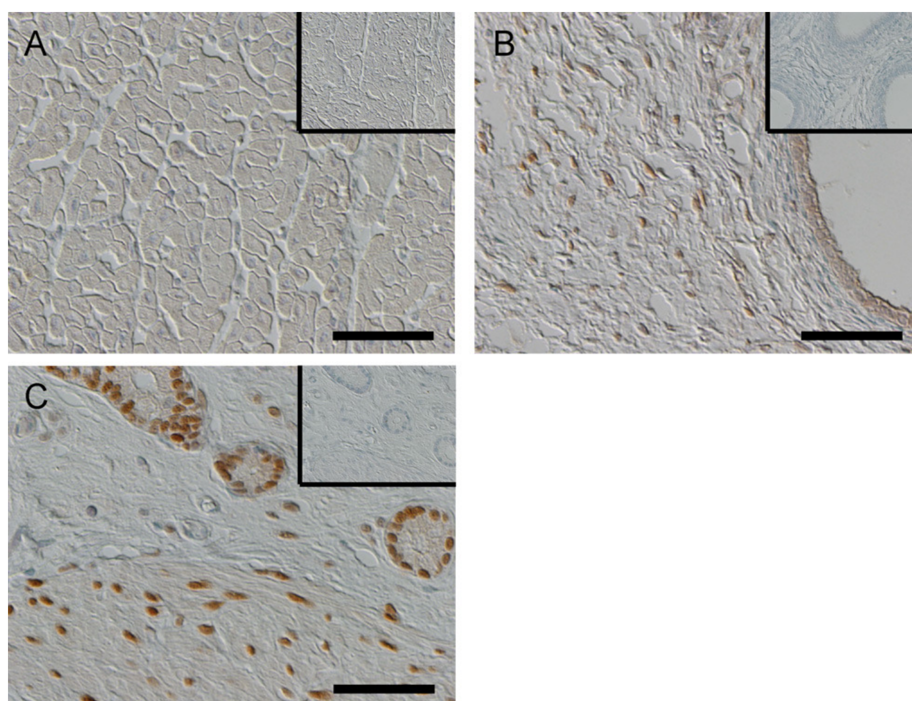

Fig. 4. *ESR1* transcript abundance is confirmed on a protein level by immunohistochemical localization. (A) Heart muscle showed only faint cytoplasmic staining, and nuclei were devoid of ESR1 signaling. (B) In contrast, epididymal nuclei of cells in the connective tissue as well as the cytoplasma of epithelial cells were found positively labeled. (C) Uterus serving as a positive control exhibited an intense staining in glandular cells and smooth muscle cells in addition to a faint signal in myometrial cytoplasma. Negative controls using the appropriate IgG are shown as inset in each picture; a black bar indicates 50 μm.

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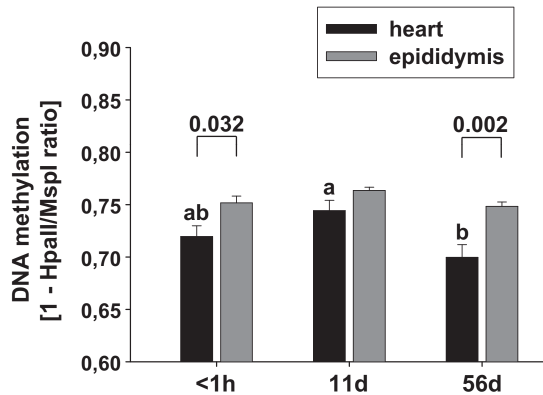

Fig. 5. Global DNA methylation in heart and epididymis is rather stable during development. While 11 d and 56 d old male piglets showed a significant slight difference in heart, no variation was detected in epididymis. Different superscript letters indicate significant differences ($p<0.05$). Newborn and 56 d old piglets exhibited higher global DNA methylation in epididymis compared to heart. Data are presented as mean ± SEM.

3.4. Global DNA methylation

Global DNA methylation was significantly lower in heart at 56 d showing 70 ± 1% (mean ± SEM) methylation compared to 74 ± 1% methylation at 11 d (Fig. 5). No significant difference was detected between tissue sampled at 56 d or 11 d and those shortly after birth displaying 72 ± 1% methylation (Fig. 5). Epididymal samples showed no significant change in global DNA methylation between time points <1 h, 11 d and 56 d with 75 ± 1%, 76 ± 1% and 75 ± 1% methylation, respectively (Fig. 5). Statistical analysis comparing heart and epididymis at the respective sampling time points highlighted a higher methylation of epididymis at <1 h ($p=0.032$) and 56 d ($p=0.002$) (Fig. 5).

3.5. Local DNA methylation at three distinct regions of the ESR1 gene

Local DNA methylation was determined at three distinct areas in the 5′ region of the *ESR1* gene (Fig. 1). Approximately 1 kb upstream of the translational start site, six CpG sites were analyzed in both heart and epididymis at the designated time points (Fig. 6A). Variations of DNA methylation in heart at different stages of development were significantly different for sites #2, #5 and #6 ($p<0.05$, respectively). In epididymis, sites #2, #3 and #6 displayed significant changes over time ($p<0.05$, respectively). When summarized across the three time points, means of both tissues varied significantly at each site with differences of less than 8% methylation. Epididymis displayed a higher average methylation at five of six sites compared to heart (Fig. 6A). The investigated area residing in the core of the *ESR1* promoter CpG island showed DNA methylation of around 5% at each individual site and every time point. No statistically significant differences between different time points or between heart and epididymis were observed (Fig. 6B). The first intronic region approximately 1 kb downstream of the translational start site revealed significantly different DNA methylation in heart over time at site #32 and at sites #29 and #32 in epididymis

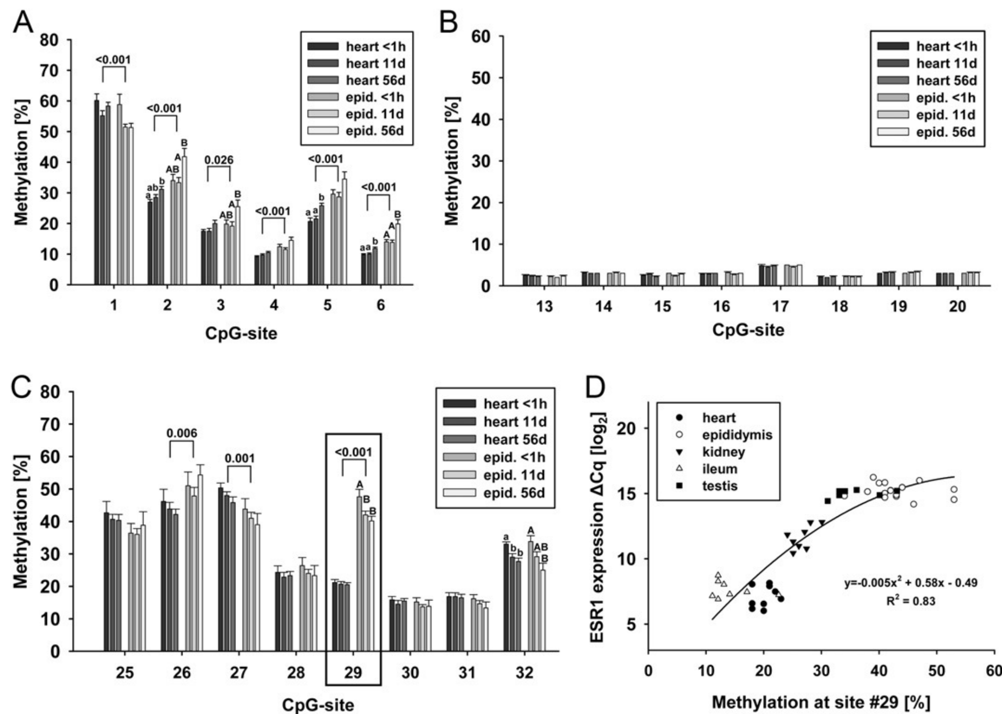

Fig. 6. Local DNA methylation analyses at three regions of the ESR1 gene reveal distinct methylation profiles. (A) At 1 kb upstream of the putative translational start site, individual CpG-sites exhibited slight variations in both heart (lowercase superscript letters) and epididymis (uppercase superscript letters) at 1 h, 11 d and 56 d after birth. The DNA methylation varying significantly between heart and epididymis over all time points is indicated (with the respective *p*-value). (B) Eight of the eighteen investigated CpG-sites are shown exemplified. The promoter CpG-island contained only minimally methylated CpG-sites with no detectable variations over time or between tissues. (C) Variations in DNA methylation within and between heart and epididymis were less abundant in the +1 kb intragenic region than in the −1 kb promoter region. Notably, the encircled CpG-site #29 showed 200% higher methylation in epididymis than in heart irrespective of the time point. Transcription factor binding site detection software predicted the transcriptional repressor TGIF bound to the sequence underlying this strikingly differentially methylated CpG-site. All data are presented as mean ± SEM, different superscript letters indicate significant differences within one specific tissue ($p<0.05$). (D) Regression analysis of epididymis, testis, kidney, heart and ileum displaying high, intermediate and low abundance of *ESR1* mRNA confirmed that a major part ($R^2=0.83$) of the depicted gene expression differences in piglet tissues can be explained by the DNA methylation level at site #29.

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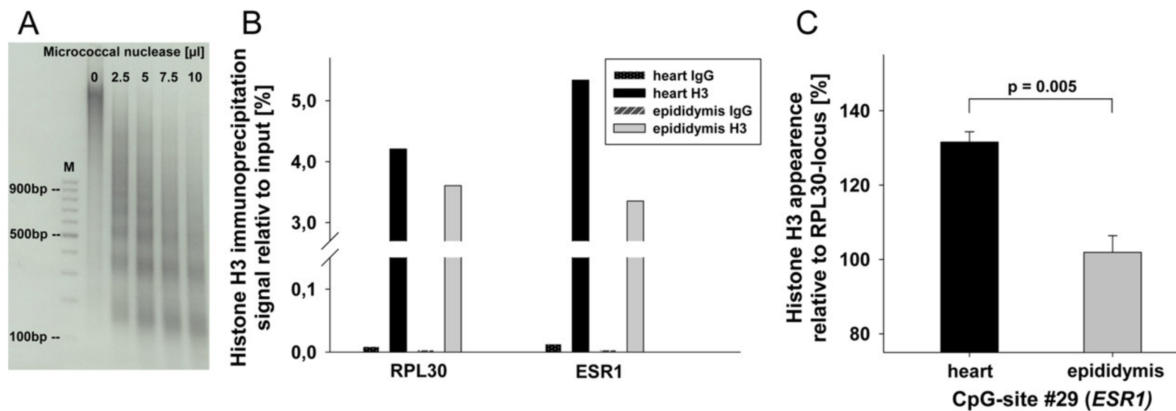

Fig. 7. Histone H3 is more abundant at the differentially methylated CpG site #29 in the low-expressing tissue. (A) Exemplary chromatin preparation of epididymis with our adapted protocol for frozen tissue, separated on a 1.8% agarose gel. Depending on the amount of employed micrococcal nuclease, chromatin is not (0 µl) or is (2.5–10 µl) digested to 1–6 nucleosomes in length (150–1050 bp) (M = molecular weight marker). (B) An exemplary ChIP-assay readout of histone H3 immunoprecipitations in heart and epididymis chromatin of 56 d old male piglets revealed distinct differences of precipitated DNA for the *RPL30* control locus and the investigated *ESR1* locus. (C) For three independent ChIP experiments, the histone H3 appearance at the differentially methylated CpG-site #29 relative to the reference locus of *RPL30* revealed a statistically significant difference of approximately 30% between the two tissues.

($p < 0.05$, respectively). Means comprising the DNA methylation of the three time points within one tissue indicated significant differences between heart and epididymis at sites #26, #27 and #29 ($p < 0.05$, respectively) (Fig. 6C). While #26 and #27 showed methylation differences of less than 7%, DNA methylation in epididymis at site #29 was 2-fold higher (42% methylation) compared to heart (21% methylation) ($p < 0.001$). For site #29, both transcription factor binding sites software programs used predicted a conserved binding site for the transcriptional repressor TGIF, whereas no stringent binding sites were postulated covering all other analyzed CpG sites that exhibited differential methylation. DNA methylation of site #29 was additionally analyzed in tissues of high (testis), intermediate (kidney) and low (ileum) ESR1 expression. Testis showed high DNA methylation (35%) whereas 27% average methylation was detected in kidney and 14% in ileum. A linear regression analysis of all investigated tissues at CpG-site #29 revealed a coefficient of determination of 0.83 (Fig. 6D).

3.6. DNA-associated proteins at a differentially methylated CpG site

The adjusted protocol for chromatin preparation out of frozen tissue granted suitable amounts at desired fractionation (Fig. 7A). Chromatin immunoprecipitations using IgG displayed recovery rates for the *RPL30* and *ESR1* locus below 0.1%, while immunoprecipitations of histone H3 displayed enrichment levels between 3% and 8% for individual assays (exemplary shown for one out of three precipitations in heart and epididymis in Fig. 7B). The histone H3 protein was determined in heart showing 131.6% ± 2.8% appearance relative to the reference locus of RPL30, epididymis displayed 101.9% ± 4.5% ($p = 0.005$) (Fig. 7C).

4. Discussion

The regulation of *ESR1* expression in mammals is influenced by its ligands, in particular by the most potent naturally occurring estrogen E2. In terms of brain masculinization/feminization, the important role for E2 was recently emphasized in the rat where a physiological E2 surge shortly after parturition was correlated with a diminished *ESR1* mRNA expression [22]. In mice, a similar phenomenon of postnatal decline in *ESR1* mRNA abundance of the developing brain has been linked to estrogen receptor promoter DNA methylation [21]. This leads us to the question whether circulating steroid hormone concentrations changing dynamically during development might play an important role in regulating *ESR1* in various other different cell types and tissues as well. In this context, we were particularly interested in investigating epigenetic mechanisms participating in this regulation. A high-resolution based view on several potentially functional sites within the *ESR1* gene that might differentially contribute to the degree of mRNA expression according to their DNA methylation patterns was of special interest.

Using male growing piglets as model, developmentally changing plasma E2 concentrations were confirmed in our study according to earlier findings [24]. Newborns exhibited high concentrations of circulating E2 that are presumably remains of the instantly interrupted feto-maternal interchange with sows that exhibit up to 400 pg/ml plasma E2 right before parturition [33]. Reduced E2 at the age of 11 d most likely was due to the intake of E2 via maternal milk during suckling, whereas marginal levels after weaning at eight weeks of age (56 d) represent the low endogenous production of E2 in male piglets weeks before the onset of puberty. We investigated 12 tissues for *ESR1* abundance over these time points and found mRNA expression varying up to 250 fold between heart showing lowest expression and epididymis with highest expression. Despite older investigations [34], our immunohistochemical staining procedure enabled localization of ESR1 in stroma and epithelial cells of the corpus epididymis, whereas the absence of nuclear staining and only faint cytoplasmatic localization asserts that the heart muscle is a tissue with minimal ESR1 abundance. The presence of ESR1 in the depicted areas is in accordance with the current understanding of epididymal function accounting for the reabsorption of testicular fluid, controlled by E2 via ESR1 [35–37]. Overall, the male reproductive tissues epididymis, testis and prostate appear to bear the highest amount of *ESR1* transcript abundance, a fact that coincides with several reports stating significant disturbances of prepubertal males encountering exogenous stimulation/perturbation by estrogenic substances (for reviews see e.g. [38–42]). As a resume, prepubertal male reproductive tissues seem especially prone to estrogenic disturbance due to the relatively high *ESR1* expression naturally occurring in concert with very low endogenous levels of E2 prior to puberty.

For the tissues under investigation, transcript abundance during development did not show a general decline of *ESR1* contrary to findings in the mouse brain [22]. While some tissues exhibited hardly any variation over time, selected tissues like kidney, adrenal gland, prostate, liver or muscle displayed a slight increase in *ESR1* expression (data not shown). In this view, the programming effect

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of E2 in the male mouse brain leading to its sexual differentiation [43] emerges as an influence on *ESR1* expression which is highly tissue-specific.

While distinct E2 plasma concentrations only had a marginal effect on *ESR1* expression, further investigations focused on the most diverging tissues in terms of naturally occurring *ESR1* expression, namely heart and epididymis. Our goal here was to link DNA methylation to mRNA expression within and between tissues. Global DNA methylation with LUMA in both heart and epididymis did not show major differences within one tissue during development. In order to connect specific local methylation with differential *ESR1* expression between various tissues in the developing piglet, we subsequently investigated DNA methylation in three functionally distinct regions of the *ESR1* gene [23]. The designated regions were chosen based on former investigations in women [44]. There, DNA methylation of the promoter (−1 kb) and an intragenic (+1 kb) region, but not the promoter CpG-island, were found associated with the prevailing *ESR1* expression status in the uterus and white blood cells displaying high expression and low methylation and vice versa, respectively [44]. For the +1 kb area, we recently confirmed these findings in the female bovine comparing endometrium and white blood cells [23], supporting the concept of a major role of DNA methylation in intragenic regions regulating transcription [45–47]. Similar to the human, our analyses of the promoter CpG-island here in the male developing piglet showed very little methylation and no difference occurred between tissues or within one tissue over time. This suggests that methylation of the CpG-island may rather be implicated in pathophysiological events like breast cancer (for a review see [48]) than during prepubertal physiological development or non-pathophysiological situations. In contrast, the −1 kb promoter area exhibited varying methylation between the individual CpG-sites. DNA methylation corresponding clearly to the expression status of the individual tissues as disclosed for the human uterus and WBC however was not observed. Similar, the inspected +1 kb intragenic region did neither display appreciable differences at individual CpG-sites. This held true for one exception: only site #29 in contrast to all other investigated sites revealed a distinctly higher methylation in epididymis compared to heart. Further tissues additionally analyzed for this CpG-site revealed a similar distinct methylation upon underlying *ESR1* expression status. Regression analysis confirmed that the major part of gene expression differences between piglet tissues seems to be linked to the DNA methylation level at site #29.

Since various examples state that the DNA methylation of a transcription factor binding sites can be a sufficient mechanism to prevent transcription factors from binding to the DNA [49–52], we included the in silico prediction of transcription factor binding sites in our analysis. While no stringent binding sites such as estrogen responsive elements (ERE) were postulated covering other minor differentially methylated CpG sites, CpG site #29 was consistently found to be located at a clear binding site for the transcriptional repressor TG-interacting factor 1 (TGIF1). TGIF1, a member of the TALE superfamily of homeodomain proteins, plays an important role in regulating various developmental processes in diverse species [53] and has been demonstrated to contain a domain which interacts with histone deacetylase 1 (HDAC1) mediating its repressive effect [54] (for a review on HDACs see e.g. [55]). Due to this result, we conducted ChIP experiments in order to investigate the chromatin state at this very specific site. Interestingly, the ChIP-validated antibody for histone H3 indeed displayed a significantly lower association of H3 to the differentially methylated *ESR1* site in epididymis compared to heart. This suggests a rather relaxed chromosomal structure which might be causative for the observed higher transcript abundance in case of higher methylation at the transcriptional repressor binding site. Still, the presence of TGIF and HDAC1 as regulation factors bound to the distinctly methylated site remains to be carefully investigated in further studies in order to clarify the mechanistic connection between local methylation, bound TGIF, recruited HDAC1 and associated chromatin status. So far, all available antibodies (directed against human or rodent proteins) performed poorly (data not shown), probably due to a lack of species specificity and absent ChIP compatibility.

In summary we hypothesize that mammals could modulate the mRNA expression via DNA methylation in at least three distinct ways. A total lack of expression as seen in tumor tissues might mainly be driven by promoter CpG-island hypermethylation or broad methylation blockades of the most abundant transcriptional start sites. Differential expression abundance as seen in white blood cells and endometrium, respectively, may become manifested by (reduced) DNA methylation over various adjacent CpG-sites. In addition, expression differences as shown here for the male piglet (e.g. heart and epididymis) could mainly be achieved by differential methylation of intragenic single-CpG-sites that impede transcription factor binding sites (either activators or repressors).

5. Conclusions

Neither mRNA abundance of *ESR1* nor DNA methylation in different tissues of male growing piglets was clearly influenced by the endogenous different E2 concentrations that impact on the developing tissues. In terms of epigenetic regulation, we present a first hint that male prepubertal piglets might regulate mRNA expression of *ESR1* between distinct tissues by differential DNA methylation of a single CpG-site causing altered binding of a transcriptional repressor.

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Maternal low-dose estradiol-17β exposure during pregnancy impairs postnatal progeny weight development and body composition

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Abstract:

Endocrine disrupting chemicals with estrogenic activity play an important role as obesogens. However, studies investigating the most potent natural estrogen estradiol-17β (E2) at low dose are lacking. We explored endocrine and physiological parameters in gilts receiving distinct concentrations of E2 during pregnancy and investigated whether adverse effects prevail in progeny due to a potential endocrine disruption. E2 was orally applied to gilts during the entire period of pregnancy at a concentration representing a daily consumption at the recommended ADI level (0.05 µg/kg body weight/day), at the NOEL (10 µg/kg body weight/day) and at a high dose (1,000 µg/kg body weight/day). Plasma hormone concentrations were determined using enzyme immuno assays and offspring body fat by dual-energy X-ray absorptiometry scanning. In treated gilts receiving 1,000 µgE2/kg body weight/day we found significantly elevated plasma E2 levels during pregnancy, paralleled by an increased weight gain. While offspring showed similar weight at birth, piglets exhibited a significant reduction in weight at weaning even when their mother's had received 0.05 µgE2/kg body weight/day only. At eight weeks of age, specifically males showed a significant increase in overall body fat percentage. In conclusion, prenatal exposition to low doses of E2 affected pig offspring development in terms of body weight and composition. In line with findings from other obesogens, our data suggest a programming effect during pregnancy for E2 causative for the depicted phenotypes. This may imply a possible contribution of environmental E2 exposure to childhood obesity.

Introduction

Endocrine disruptors have been defined as exogenic compounds with the ability to affect hormonal systems, reproduction or health – most often due to their potential to interact with steroid hormone receptors in the body cells (1). Early investigations on these substances which are mainly derived from contraceptives, plasticizers, pesticides or industry by-products, raised the awareness that the fetus and the prepubertal child are especially sensitive towards hormonal exposure (2, 3). Thus, current exposure levels regarded as safe, and the acceptable daily intake (ADI) may be considerably overestimated given the low natural steroid background in children (4, 5). Numerous reports state that fetal/neonatal exposure to not only pharmacological, but also to environmentally relevant (i.e. low) doses is related to reproductive abnormalities later in life (6-12). Swan and colleagues even reported an association between sub-fertility in males and their mothers' high consumption of red meet during pregnancy, which was commonly produced with steroidal growth promoters in the US and other countries (13). The mechanistic aspects behind these phenomena are meanwhile connected to early life epigenetic programming. This widely accepted concept implies that early exogenous signals are able to permanently modify the expression profiles of genes, thus affecting the later phenotype (14). Since programming also occurs as part of a normal developmental process and at very low physiological hormone concentrations, it might be additionally error-prone if the surrounding endocrine environment is disturbed even only marginally (12). The recognized effects of EDCs thus are thought to be caused by a disturbance of reproductive processes via epigenetic changes rather than direct DNA mutations (15). Consequently, the hazard of exogenous hormonal compounds on human health might not fully be assessed by screening for immediate effects in an adult organism like in postmenopausal women or by observing cases of tumors via massive DNA impairments directly following exposure.

Besides the evident influence of endocrine disruptors on reproductive processes, recent investigations indicate that an exposure to environmental chemicals also impairs adipocyte development (16-18). All natural or xenobiotic chemicals that have been found to impact on adipose tissue and its constituents have been referred to as "obesogens" (19). Next to organotins and heavy metals, endocrine disrupting chemicals (EDC) with estrogenic action, namely bisphenol A (BPA), Diethylstilbestrol (DES) and genistein, are of special interest in the recent investigations on EDCs and obesity. Low doses of the plasticizer BPA were found to increase postnatal growth and increase obesity in adulthood when rodents were exposed during fetal or early neonatal life (20-22). For DES, similar effects to that of BPA were encountered (23-25), even at doses down to 1µg/kg/day, resulting in an increased percentage of body fat. Exposure to phytoestrogens such as genistein has also been reported to exert effects in rodent models. These range from decreased adiposity (26) to increased body weight and obesity (25, 27). The reported phenotypes are differing due to the important aspect of way of application, time window of exposure and amount supplied.

Because phytoestrogens are thought to possess only week estrogenic potential but still exert EDC effects, we were especially interested to broaden the knowledge on the most potent natural estrogen, estradiol-17β (E2), in this field of research. E2 possesses a reduced half life compared to DES (28), but an approximately 2,000-fold higher estrogen receptor binding affinity than BPA (29, 30). The relevance of E2 as a potential endocrine disruptor in humans arises mainly from the treatment of postmenopausal symptoms and the usage of estrogen-containing contraceptives. Concerning the consumption of meat produced with anabolic steroids for growth promotion, a varying exposure dose could occur under good (implants discarded-low dose) or under bad (misplaced implants, non-discarded implants, abuse- high

dose) veterinary practice (31). Interestingly, the JECFA's latest risk assessment for E2 through meat consumption dates back to 1999 and announced an acceptable daily intake (ADI) of 0.05 µg/kg body weight (bw) based on an "no observed effect level" (NOEL) in postmenopausal women (32). While these suggestions are still status quo, the suitability of such a reference group for an appropriate estimation of a steroid's safety is more and more questioned through knowledge gained in EDC research on low doses effects during prepubertal exposure.

In the study at hand, we orally applied three distinct concentrations of E2 (ADI, close to NOEL and a high dose) to female pregnant sows in order to investigate whether adverse effects prevail in progeny due to a potential endocrine disruption. In contrast to the many rodent studies available, we chose the pig as animal model because the placental estrogen production is highly similar to the human in this species (33). When investigating external effects of a substance which is endogenously produced at distinct amounts during pregnancy, we considered this of potential importance. Likewise, humans and pigs share similar temporal patterns of organ development in contrast to laboratory rodents. Specifically in terms of adipose tissue, mature adipocytes can already be found from the beginning of the second trimester while in rodents solely the conversion of mesenchymal cells to preadipocytes takes place during fetal life (34). Thus, EDC might display comparable effects in pigs as expected in humans with respect to substance and time window of exposure.

Methods

E2 application to castrated male pigs

An initial trial aimed to clarify the pharmacokinetics of E2 after oral application in pregnant sows. Thus, the control group was fed a bread roll (20 g) with 2 ml ethanol (as carrier), while treatment groups received carrier plus the appropriate amount of E2 dissolved in ethanol. In order to mimic consumption of E2 over a day, we chose to divide the desired amount of ingestion (in both animal trials) into two identical portions of E2, namely 500 µg/kg bw, 5 µg/kg bw and 0.025 µg/kg bw, respectively.

Castrated male pigs (hybrid breeds of German Landrace sows and Pietrain boars) with 85 ± 10 kg known to exhibit lowest concentrations of endogenous E2 were used to grant the possibility of detecting even slight elevations of E2 in plasma. Eleven animals received surgery for catheterization of the *vena jugularis*. After recovery one day later, pigs received their bread roll with E2 on an empty stomach immediately followed by a normal feed ration, which was consumed rapidly. Frequent blood samples were taken hourly before E2 application and every 15 min after application. Four hours after application, samples were taken for additional eight times.

E2 application to pregnant sows

For our main goal, the investigation of effects in progeny caused by in-utero exposure to orally ingested E2, German landrace sows (n = 6 per treatment group, n = 7 in control group) were randomly selected and inseminated with the sperm from one Pietrain boar twice at estrus (first insemination = day 0). Beginning with day 0, sows twice daily received distinct amounts of E2 via ingestion of prepared bread rolls (500 µg/kg bw, 5 µg/kg bw and 0.025 µg/kg bw or ethanol only, respectively). Standard food rations for pregnant sows were given immediately after ingestion of the carrier. The feed was monitored throughout the application for possible contamination with Zearalenon (ZEA), a *fusarium* mycotoxin with known ability to bind to and signal via estrogen receptor alpha using standardized LC-MS (35). Effect levels of ZEN have been described down to 10 µg/kg bw/d (36), whereas the amount in our feed was determined to reach an exposition of even less than 0.1 µg/kg bw/d (data not presented). Blood plasma samples were taken at days -7, 35, 49, 70 and 97 around conception, respectively, to monitor maternal E2 concentrations during pregnancy. Weight gain of sows was followed throughout pregnancy. At birth, number, gender, weight and potential disorders of pig progeny were recorded and growth was followed during weaning (day 21) until slaughter at 8 weeks (males) and 9 weeks (females) of age. At slaughter, blood samples were collected, plasma separated and stored at -20°C.

In both the pharmacokinetic and the application study, sampling was conducted in accordance with accepted standards of humane animal care and approved by the local governmental authority, reference # 55.2-1-54-2531-68-09.

Competitive enzyme immuno assay

E2 concentration both in sows and in piglet plasma was determined after ether extraction using a competitive enzyme immuno assay (EIA) as described earlier (37). Testosterone (T) was determined by EIA following the protocol of Blottner et al. (38).

Dual-energy X-ray absorptiometry scanning (DEXA)

Body composition of male and female piglets was assessed by Dual Energy X-ray Absorptiometry (DEXA) using Explorer^{TM}-Bone Densitometer (Hologic Inc., Bedford, USA) from the QDR Series with a whole body scan mode. Animals were positioned in a standardized manner and scan length was adjusted accordingly to measure whole body fat content.

Statistical analysis

Data are presented as means ± SEM (n=6 gilts per treatment group, n=12-40 piglets per treatment group). For statistical analysis, the SAS program package release 9.1.3 (2002; SAS Institute, Inc., Cary, NC, USA) was used. Comparing the effect of the different treatment groups on plasma hormone concentration of the gilts, the proc mixed model was used including the treatment as fixed effect. For the analysis of the offspring parameters, the proc mixed model included the treatment as fixed effect and the gilt as random effect to account for nested design of the study (mean = 11.4 piglets per gilt). Differences between treatments were localized by ANOVA followed by a Dunnett's test against the non-treated control group as reference. Differences were considered significant at $p < 0.05$. Graphs were plotted with SigmaPlot 11.0 (SPSS Software GmbH, Munich, Germany).

Results

Pharmacokinetics of E2 in castrated male pigs

Blood plasma concentrations were monitored before and after oral administration of defined amounts of E2. In animals given 5 µg/kg bw (n = 2) and 0.025 µg/kg bw (n = 3), no notable rise in average plasma concentrations of 5.5 ± 1.9 pg/ml (mean ± SEM) and 5.6 ± 1.3 pg/ml, respectively, compared to the control group (n = 2) with 5.3 ± 2.5 pg/ml were seen. A distinct elevation of endogenous E2 was found when feeding 500 µg/kg bw (n = 3) with a peak of 77.3 ± 23.9 pg/ml (mean ± SEM) reaching maximal levels already 15 min after administration (Fig. 2). From 6 h to 12 h, plasma concentrations of this group remained on an average level of 23.5 ± 4.0 pg/ml (mean ± SEM).

Effects on sows due to E2 application

No acute adverse affects on sows such as miscarriage or toxicities were seen during pregnancy due to E2 application.

Endogenous E2 plasma concentrations before application of E2 (-7 d) were low over all groups and animals as expected. During pregnancy, blood plasma revealed significantly elevated E2 concentrations for animals in the 1,000 µg/kg bw/d group. These were 30.2 ± 3.8 pg/ml (mean ± SEM) compared to the control group with 12.0 ± 2.4 pg/ml on day 35 ($p = 0.005$), 55.8 ± 7.7 pg/ml vs. 19.1 ± 4.0 pg/ml on day 49 ($p < 0.001$) and 80.8 ± 27.9 pg/ml vs. 24.4 ± 6.9 pg/ml on day 70 ($p = 0.023$). No significantly different concentrations of E2 were found on day 97 of pregnancy where high endogenous amounts at an average of 394.4 ± 58.8 pg/ml (summed across all groups) prevailed (Fig. 3A).

Plasma T in sows reached approximately two-fold higher average concentrations during pregnancy at days 49 (40.8 ± 5.0 pg/ml), 70 (35.5 ± 3.5 pg/ml) and 97 (39.7 ± 3.8 pg/ml) compared to -7 d (21.6 ± 3.5 pg/ml). On day 35, highest plasma concentrations were determined (99.7 ± 21.8 pg/ml). Similar concentrations were found over all treatment groups at the respective sampling days with the exception of day 97. There, animals treated with 10 µgE2/kg bw/d had significantly higher concentrations of T (47.7 ± 12.1 pg/ml) compared to the control group (33.1 ± 5.3 pg/ml, $p = 0.04$).

During pregnancy (-7d to 107d), sows receiving 1,000 µg/kg bw/d significantly gained more weight than the control group (29.2 ± 1.8 % vs. 19.0 ± 0.9 %, $p = 0.006$) (Fig. 4A). At birth, no significant differences were found concerning birth defects, total weight of piglets per sow or gender distribution of progeny. Neither, number of piglets nor survival rate until weaning as indicated by the number of piglets alive was influenced by E2 treatment compared to the control (see Supplemental Material, Table 1).

Effects on piglets due to E2 application

Weight

At birth, male and female piglets revealed no significant differences in weight when sows were treated with E2 in contrast to the control group fed only the carrier (Fig. 4B). At weaning, a significant reduction in body weight was found compared to the control group (8.69 ± 0.26 kg) (mean ± SEM) when piglets were treated with 0.05 µg E2/kg bw/d (8.15 ± 0.27 kg, $p = 0.0002$) or 1,000 µg E2/kg bw/d (7.68 ± 0.19 kg, $p = 0.0071$). Treatment with 10 µg E2/kg bw/d reduced body weight to 8.06 ± 0.28 kg ($p = 0.0851$) (Fig. 4C). Due to handling reasons, male and female animals were slaughtered with a difference of seven days of age. Separated by gender, no significant differences in birth weight were found between treatment groups and the control group (Dunnett's test) although males exhibited an overall significant difference ($p = 0.011$) (Tab. 2). At weaning, female progeny of sows receiving 0.05 µg

E2/kg bw/d during pregnancy showed a significant reduction of weight compared to the control group (7.95 ± 0.30 kg vs. 8.78 ± 0.45 kg, respectively, $p = 0.0049$). Males gained significantly less weight until weaning when treated with 1,000 µg E2/kg bw/d (7.24 ± 0.34 kg) compared to the group receiving the carrier only (8.60 ± 0.25 kg; $p = 0.03$) (Tab. 2). This trend was also seen at slaughter, where male progeny of the 1,000 µg E2/kg bw/d group had a mean body weight of 83.6 % (13.7 ± 0.84 kg) compared to pigs originating from sows of the control group (16.4 ± 0.5 kg) (see Supplemental Material, Table 2).

Plasma hormone concentrations

At slaughter, E2 and testosterone (T) concentrations in blood plasma were determined in both sexes of piglets. For E2, male and female offspring did not display significantly different plasma concentrations. While females showed an average of 8.1 ± 1.5 pg E2/ml plasma (mean ± SEM) over all groups, males were determined to a mean concentration of 14.3 ± 1.5 pg E2/ml (Fig 3B). Testosterone in female piglets' plasma was not significantly changed due to their mothers' treatment conditions and reached a mean of 40.7 ± 1.5 pg T/ml. In males, individual piglets within one group exhibited markedly different testosterone concentrations independent of the E2 application *in utero*. No significant differences ($p = 0.296$) could be detected between male progeny from the control group (806.7 ± 198.3 pg/ml) (mean ± SEM) and their counterparts originating from sows treated with 0.05 µg E2/kg bw/d (563.0 ± 136.6 pg/ml), 10 µg E2/kg bw/d (531.9 ± 114.0 pg/ml) or 1000 µg E2/kg bw/d (830.4 ± 147.6 pg/ml) (Fig 3D).

Body composition

DEXA-scanning of male piglets at slaughter revealed a modified body composition in terms of fat vs. lean mass with increasing E2 treatment. While controls were determined to 13.1 ± 0.4 % fat (mean ± SEM), progeny of sows treated with 10 µg E2/kg bw/d (15.5 ± 0.7 %) ($p = 0.0327$) and 1,000 µg E2/kg bw/d (16.5 ± 0.8 %) ($p = 0.0024$) exhibited a significantly higher percentage of whole body fat (Fig. 5A). Female fat percentage (13.8 ± 0.8 %) (mean ± SEM) did not show a statistically significant change in body composition due to E2 treatment of their mothers (Fig. 5B).

Discussion

Numerous studies raised the awareness that an exposure to EDC needs to be considered as a critical mediator in the general concept of "developmental origins of health and disease" (DOHaD) (39), which focuses on chronic diseases caused by pre- and perinatal adverse influences. In the work at hand, we aimed at contributing to the current knowledge on EDC by investigating an application of estradiol-17β (E2). In our opinion, this emerged necessary since E2, the most potent naturally occurring estrogen, lacks ample attention as a potential EDC. Furthermore, to our knowledge, investigations are lacking that assess the developmental programming capabilities of E2 even at relatively low exposure levels during critical time windows. In the present study, we thus addressed the particular importance of E2 possibly causing endocrine disruption during pregnancy. In women, very low endogenous E2 concentrations of E2 prevail during early pregnancy, while very high E2 concentrations of placental origin are present during late pregnancy. By displaying a similar endocrine profile, the pig model suited best to monitor the possible outcomes of an additional E2 exposure. Furthermore, the related timeline of prenatal fat tissue development in humans and pigs supports this species as a model (34).

In our initial pharmacokinetic study, we found the highest amount of orally administered E2 leads to a distinct rise in plasma concentrations of E2. Interestingly, the maximum peak was reached already after 15 min indicating that at least part of the E2 had been incorporated similar to the rapid appearance and fast clearance seen in a sublingual application (40). The oral administration conducted here additionally led to slowly declining plasma concentrations, with a persistence of E2 up to 12 h additionally pointing towards an enterohepatic recycling. Deducing from the 500 µg/kg bw dose by linear regression, the treatment close to the NOEL (5 µg/kg bw) theoretically could generate 1/100 rise in basal E2 levels, which would be 0.75 pg/ml. However, this was not measureable under the applied number of individuals and accuracy of the test. Because of the low endogenous plasma hormone levels in sows following conception, the depicted persistence might still be effective even at very low doses.

In the main trial, the applied E2 during pregnancy was accordingly displayed in the highest treatment group until the end of pregnancy where high endogenous background levels prevailed. Interestingly, we saw a trend of augmented weight gain paralleling the ascending amounts of E2 indeed reaching significance in the group of sows receiving 1,000 µgE2/kg bw/d. Weight gain was neither caused by number nor total mass of piglets as determined at birth. Whether this effect was caused by simple fattening was not determined, but seems rather unlikely since estrogens in female adults are reported to protect females from obesity (41-43). No immediate adverse effects were detected in any sows directly exposed to E2 during the trial. Likewise, neither birth defects nor changes in gender number or birth weight due to the estrogen treatment were monitored in piglets. This is in agreement with observations of BPA administered to pregnant rats (44), where gestational low exposure (1 mg/L drinking water) did not modify the sex ratio or litter size at birth.

In addition to the direct effects of the treatment, we were especially interested to see whether the application of E2 had led to any programming effects that would create a distinct phenotype in progeny later in life. While no differences were monitored at birth, most interestingly piglets had a significantly different weight gain until weaning. This was not only given at the pharmacological dose but by trend also at the dose considered the NOEL. While the latter did not reach statistical significance, piglets originating from the group fed the ADI dose exhibited significantly lower weight than offspring from the control group. To our knowledge, this is the first report of an *in vivo* effect caused by parental application of E2 at the proclaimed ADI level.

In line with observations at weaning, mean body weight of males at slaughter appeared to be lower subject to E2 administration. The effect of reduced weight in females was less pronounced at nine weeks than at weaning which in part parallels findings from DES treated mice. There, neonatal exposure to high doses (1 mg/kg bw/d) lead to an initially depressed weight which was followed by a catch-up growth later in life (25). In males, which are probably particularly sensitive, even low doses of DES (1 µg/kg bw/d) caused this effect of reduced weight (23). The phenomenon of catch-up growth has also been found in prenatal DES application where, in contrast to our piglets, high exposure led to a reduced weight already at birth before a following compensatory growth (24). For developmental BPA exposure, a large number of studies exists that observed a subsequent increase in offspring body weight as well as reports stating none or a growth-alleviating effect (45). In terms of phytoestrogens, a recent study reported increased estradiol serum concentration in fetuses paradoxically appearing due to soy-deprivation in the mother's food. As a consequence, offspring were slighter at birth but turned out to become heavier as adults (27). These differing results show that every substance seems to exert distinct effects, leading to a variety of phenotypes. Particularly, time point and dose of administration clearly emerge to be of special importance. Reports on DES and phytoestrogens have repeatedly mentioned that high weight gain in adult life due to early estrogenic treatment was accompanied by obesity in both sexes (25, 27). Whereas perinatal exposure to low doses of BPA resulted in an increase of parametrial WAT only in female rats (44), female progeny were not affected in our piglet study. However, while specifically male piglets showed the tendency of reduced body weight, they additionally exhibited a significantly elevated total body fat percentage. Moreover, this was not only the case for the pharmacological dose, but also for the NOEL level and in line with piglets of the ADI group showing the same tendency at the preadolescent age. This strikingly parallels observations in humans, stating that obesity is not only increasing in adults but similarly in children (46). The later is of immanent concern since childhood/adolescent obesity is also associated with the risk of severe obesity in adulthood (47) and with an increased lifetime risk of developing cardiovascular diseases (48.)

Our findings on equivalent endogenous E2 and T hormone levels over all treatment groups in both sexes at slaughter suggests that processes controlling weight and body composition might have undergone indirect, probably epigenetic, programming instead of being immanently influenced by a modified hormonal status. Such a disruption might occur at the level of mesenchymal stem cell (MSC) commitment favoring the preadipocyte lineage instead of bone or muscle generation (17, 49). Studies investigating the influence of EDC on this commitment however are sparse, and only tributyltin and rosiglitazone treatment of WAT-derived MSC have been shown to lead to an enrichment of preadipocytes so far (50). Although this process is not yet fully understood, it is suggested that such an enrichment of preadipocytes may persist into adulthood with the consequence of higher amounts of mature adipocytes (and thus obesity) generated from these available precursors (17). Effects protecting from this obesity have been described to be mediated by T and the androgen receptor (AR) in males, and rather via E2 and ERs in females (51). Bjorntorp and colleagues also stated that in fat E2 is able to decrease AR expression, likewise to what has been described earlier in other tissues (52). Consistent with these findings, Semirale and colleagues only recently reported that overexpression of AR in males inhibited the commitment of precursors to the adipocytes lineage (53), and a study in Japanese men found low testosterone and high estradiol levels associated with obesity (54). Thus, we hypothesize that the in-utero exposition to E2 might have impaired normal androgen signaling important for appropriate adipogenesis in males via diminished AR expression. This would have led to an augmented allocation of MSCs to the fat cell lineage specifically in males due to a reduced protective effect. In addition, AR down-regulation might have also influenced the

preadipocyte to adipocyte conversion process (reviewed by (17)) where T usually decreases the expression of factors essential for adipose tissue development (55, 56).
In conclusion, we provide evidence that oral E2 exposition at very low doses during pregnancy in pigs has long-term programming effects in progeny. The endogenous steroid hormone E2 can thus be considered an endocrine disruptor, even if applied at a dose regarded to have no adverse effects. The depicted increase in adipose tissue of preadolescent piglets relative to their total body mass relates to the major public health concern of growing childhood obesity (57, 58), thus suggesting further studies on the long-term adverse effects of (endogenous) steroid hormone exposition on adipose tissue growth.

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Figure Legends

Fig. 1

Simplified schematic depiction of naturally occurring peripheral plasma E2 concentrations in sows during pregnancy and piglet offspring until puberty. Blood plasma E2 levels in sows are low at the beginning of pregnancy but continuously rise to approximately 650 pg/ml at birth. Offspring E2 concentrations at birth are accordingly high due to the only just interrupted hormonal exchange and decline to an intermediate level until weaning marking the end of hormone transduction via maternal milk. Until the beginning of puberty (~100 d) sparse endogenous E2 production in piglets is reflected by low plasma concentrations. In the investigated animal trial, sows orally received distinct doses of E2 starting with the day of insemination until the end of pregnancy. Blood sampling (●) and weight determination (Ø) were conducted as indicated and body composition determined at slaughter.

Fig. 2

Oral application of 500 µgE2/kg bw distinctly elevates E2 plasma concentration in male castrated piglets (85 kg). Elimination kinetics after oral application of (n = 4), 5 µg/kg bw (n = 2), 0.025 µg/kg bw (n = 3) and 0 µg/kg bw (mean ±SEM, n = 2).

Fig. 3

Oral administration of 1000 µgE2/kg bw/d is reflected through elevated E2 blood plasma concentrations in treated sows but does not impact on endogenous testosterone (T). Endogenous synthesis of both E2 and T in male and female offspring seems unaffected due to the preceding *in utero* E2 exposition. Endocrine hormones in pregnant sows and offspring determined via enzyme immuno assays: E2 (A) and T (C) plasma concentration [pg/ml] in pregnant sows under E2 application (n = 6 per group). E2 (B) and T (D) plasma concentration (mean ± SEM, [pg/ml]) in piglets at slaughter (postnatal day 56 in males, n = 17 per group except 10 µg/kg bw/d with n = 16; postnatal day 63 in females, n = 12 per group).

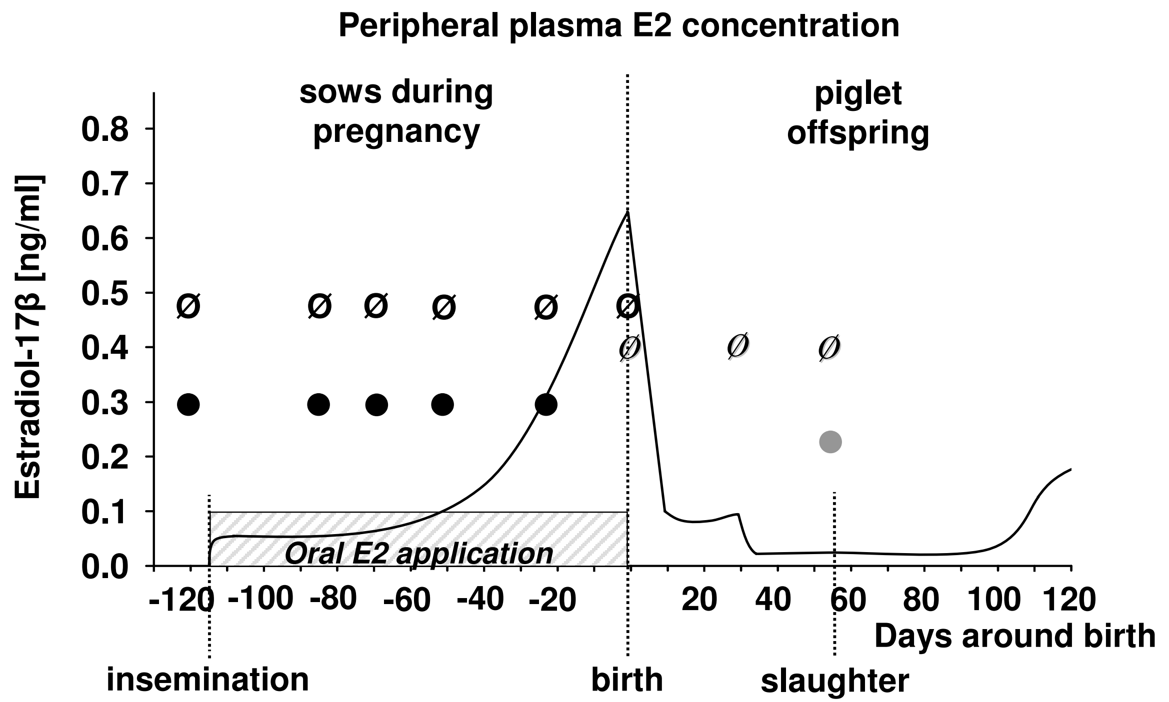
Fig. 4

Sows gain more weight under the influence of oral E2 application. Offspring are unaffected by maternal E2 exposition at birth but exhibit significantly reduced weight at weaning, even if their mothers were treated at the acceptable daily intake (ADI) level. Weight development of pregnant sows and offspring: Weight gain [%] of sows during pregnancy (n = 6 per group) (A). Weight of piglets (mean ± SEM, [kg]) at birth (B) and weaning (C) originating from treatment group 0 µg/kg bw/d (n = 70/58), 0.05 µg/kg bw/d (n = 66/55), 10 µg/kg bw/d (n = 70/52), and 1000 µg/kg bw/d (n = 65/61), respectively.

Fig. 5

Male offspring only exhibit a modified body composition favoring adipose mass subject to their mother's treatment with distinct amounts of E2 during pregnancy. Postnatal development of maternally exposed offspring: Total fat [%] of male (A, n = 5-7 per group) and female piglets (B, n = 5-10 per group) at postnatal day 56 and 63, respectively.

Fuerst_et_al_**Fig. 1**



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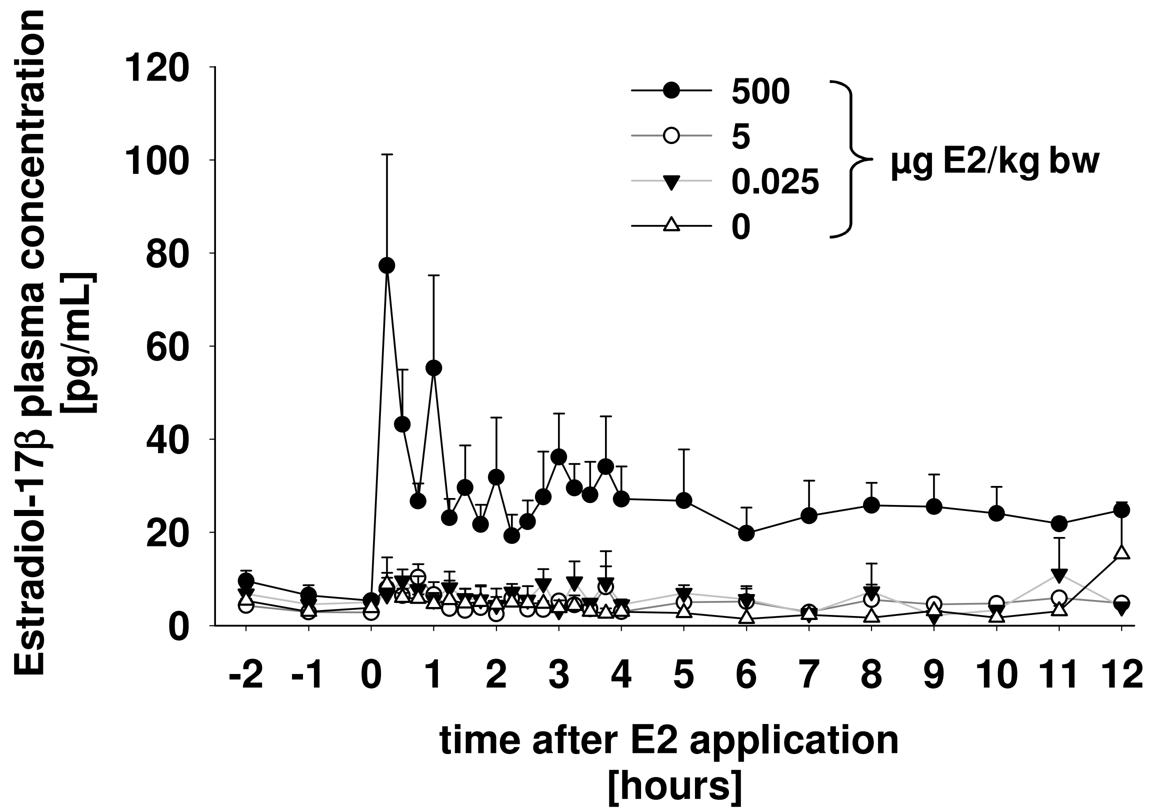

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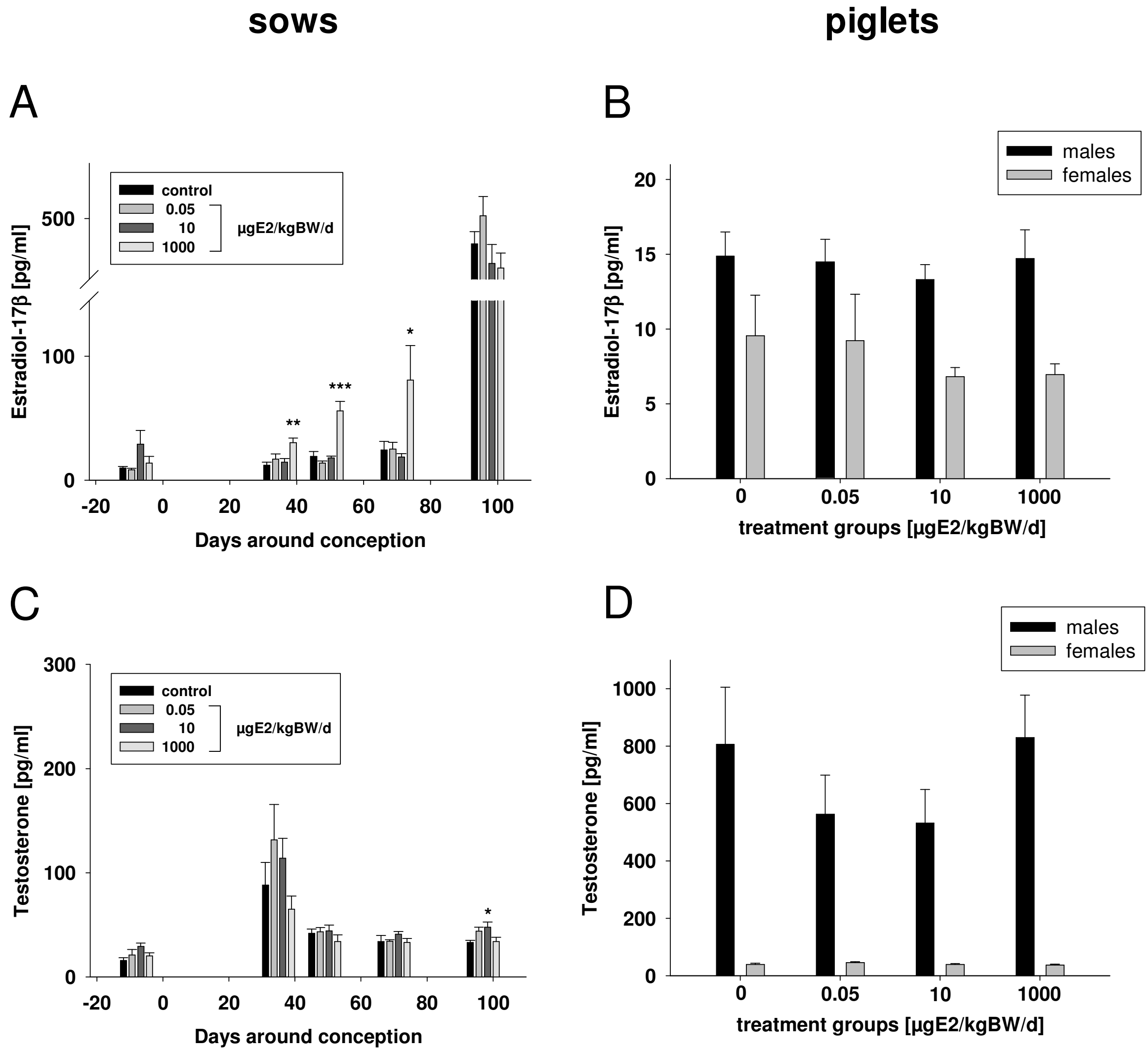

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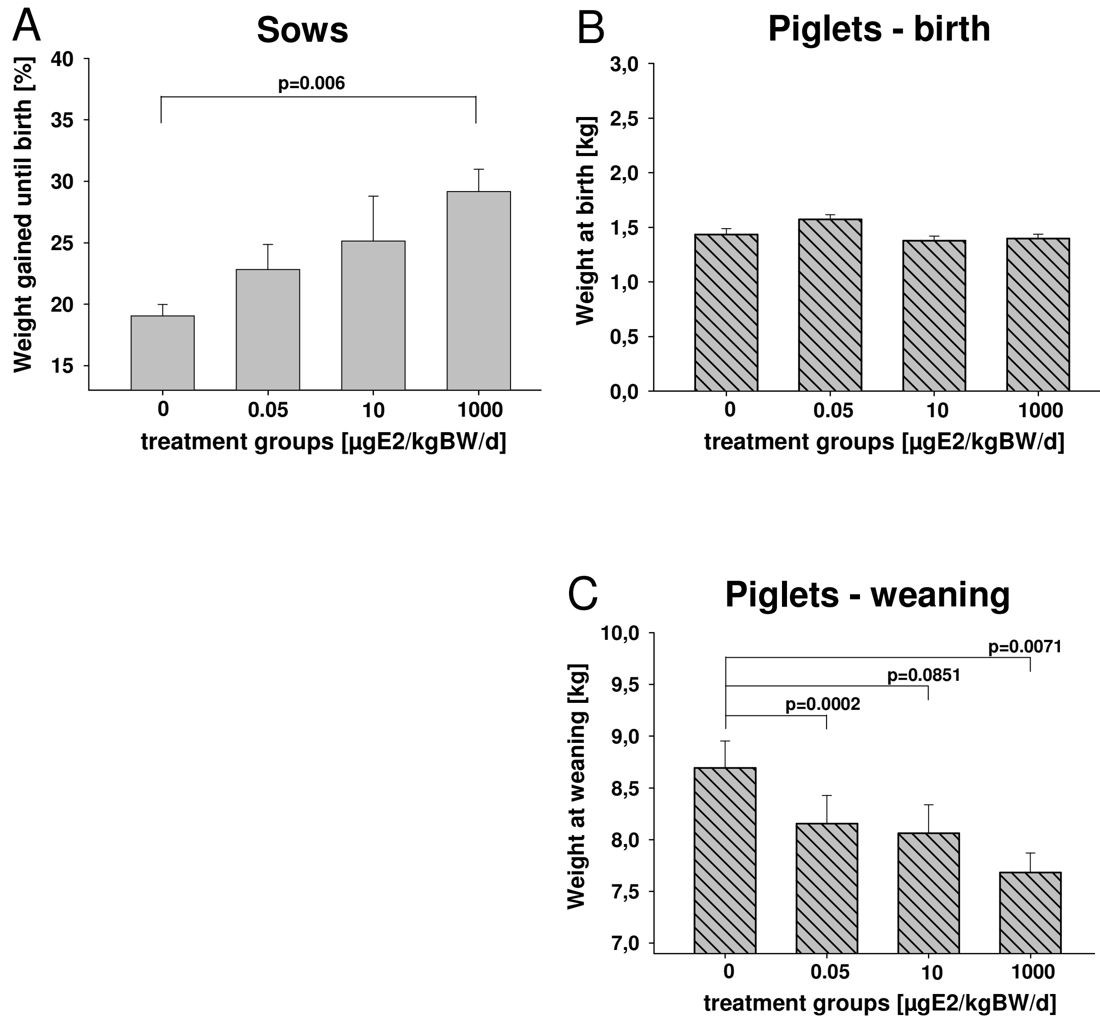

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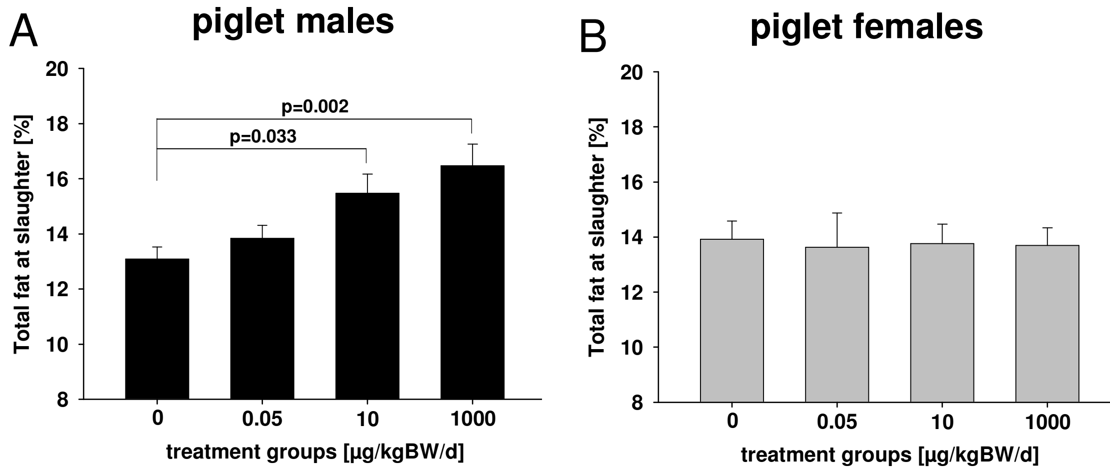

Supplemental Material, Table 1. Sow-associated piglet parameters at birth and weaning

	E2 [µg/kg bw/d]	Mean	Standard Error	Number	Minimum	Maximum	Pr > F
Total piglet weight at birth [kg]							
	0.00	15.32	2.17	7	5.46	21.99	
	0.05	19.34	1.90	6	13.91	25.66	
	10	16.27	2.24	6	7.80	22.39	
	1000	15.37	1.49	6	10.58	19.38	0.466
Piglets at birth [n]							
	0.00	10.57	1.76	7	3	15	
	0.05	12.33	1.45	6	9	18	
	10	11.83	1.96	6	6	18	
	1000	11.00	1.03	6	7	14	0.861
Male piglets at birth [n]							
	0.00	5.57	1.17	7	0	9	
	0.05	5.33	0.67	6	3	7	
	10	6.00	0.97	6	3	9	
	1000	4.33	0.92	6	0	6	0.680
Female piglets at birth [n]							
	0.00	4.86	0.67	7	2	7	
	0.05	7.00	1.32	6	3	11	
	10	5.83	1.11	6	3	10	
	1000	6.67	0.42	6	5	8	0.367
Piglets at weaning [n]							
	0.00	8.29	1.54	7	2	12	
	0.05	9.50	0.62	6	7	11	
	10	9.67	1.80	6	4	14	
	1000	10.17	1.08	6	6	16	0.779
Male piglets at weaning [n]							
	0.00	4.00	1.02	7	0	7	
	0.05	3.83	0.31	6	3	5	
	10	4.83	1.08	6	1	8	
	1000	4.00	0.86	6	0	6	0.861
Female piglets at weaning [n]							
	0.00	4.29	0.78	7	1	7	
	0.05	5.67	0.76	6	3	8	
	10	4.83	0.79	6	3	8	
	1000	6.17	0.54	6	4	8	0.286

Supplemental Material, Table 2. Piglet weight over development

	E2 [µg/kg bw/d]	Mean	Standard Error	Number	Minimum	Maximum	Pr > F
Piglet weight at birth							
male							
	0.00	1.46	0.08	39	0.61	2.52	
	0.05	1.67	0.07	32	0.76	2.45	
	10	1.44	0.06	36	0.83	2.60	
	1000	1.32	0.08	26	0.41	1.91	0.011*
female							
	0.00	1.40	0.07	34	0.38	2.32	
	0.05	1.50	0.05	40	0.77	1.97	
	10	1.31	0.06	35	0.48	1.97	
	1000	1.45	0.04	40	0.55	1.91	0.185
Piglet weight at weaning							
male							
	0.00	8.60	0.25	28	5.91	12.40	
	0.05	8.44	0.51	23	3.52	11.85	
	10	7.84	0.38	27	4.36	11.53	
	1000	7.24	0.34	24	4.16	9.94	0.067
female							
	0.00	8.78	0.45	30	4.24	14.60	
	0.05	7.95	0.30	32	4.65	11.19	
	10	8.30	0.41	25	5.10	13.27	
	1000	7.97	0.21	37	5.30	10.40	0.003
Piglet weight at slaughter							
male							
	0.00	16.36	0.51	17	12.4	19.9	
	0.05	16.28	1.11	17	6.2	22.1	
	10	15.69	0.74	16	10.2	20.5	
	1000	13.69	0.84	17	5.8	18.3	0.149
female							
	0.00	22.15	0.78	12	18.62	27.3	
	0.05	20.61	0.91	12	15.2	26.36	
	10	21.70	1.17	12	14.81	27.99	
	1000	21.65	0.70	12	16.22	24.52	0.122

* overall signficance; no significant difference of treatment groups compared to the control group