Spring 2020 – Systems Biology of Reproduction
Discussion Outline – Female Reproductive Tract Development & Function
Michael K. Skinner – Biol 475/575
CUE 418, 10:35-11:50 am, Tuesdays & Thursdays
February 13, 2020
Week 5

Female Reproductive Tract Development & Function

Primary Papers:
1. Mondejar, et al. (2012) Reproduction in Domestic Animals, 47(Suppl. 3) 22-29.
2. Hongling & Taylor (2015) CSH Persp Medicine, 6:a023002.
3. Cunha, et al. (2018) Differentiation, 101:39-45.

Discussion

Student 10: Contemporary Paper-Ref #1 above
- What are the functions of the oviduct?
- What methods were used?
- Are secretions important?

Student 11: Contemporary Paper-Ref #2 above
- What are HOX genes and role in development?
- What are endocrine disruptors and mechanism?
- How do they alter female reproductive tract?

Student 12: Contemporary Paper-Ref #3 above
- What mesenchymal and epithelial cell recombinants were used?
- What endocrine impacts and genes involved?
- What is the role of mesenchyme in the induction of epithelium?

Reproduction in Domestic Animals

Reprod Dom Anim **47** (Suppl. 3), 22–29 (2012); doi: 10.1111/j.1439-0531.2012.02027.x
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The Oviduct: Functional Genomic and Proteomic Approach

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Contents

The mammalian oviduct is an anatomical part of the female reproductive tract, which plays several important roles in the events related to fertilization and embryo development. This review examines and compares several studies related to the proteomic and transcriptomic profile of the oviduct in different domestic animals. This information could be important for clarifying the role of oviductal factors in different events regulating fertilization and early embryo development, as well as for improving synthetic media for *in vitro* maturation/*in vitro* fertilization/embryo culture techniques (IVM/IVF/EC).

Introduction

The concept of the oviduct as a passive structure involved in the transport of gametes has been substituted by that of a dynamic structure actively involved in several functions. The oviduct, also called the Fallopian tube in primates, is the organ in which fertilization takes place. Moreover, numerous studies have indicated that the oviduct and, especially, oviductal secretions play a key role in aspects related to gamete maturation, sperm capacitation and the development of the preimplantation embryo (Hunter 1998; Avilés et al. 2010).

Anatomically, the oviduct consists of four regions designated infundibulum, ampulla, isthmus and uterine-tubal junction. The mucosa of the oviduct shows primary and secondary folds of different height and orientation with a typical tree branch–like structure of varying degrees of complexity. Interspersed among these mucosal projections is a complex system of crypts, pockets and grooves (Hunter et al. 1991; Yániz et al. 2000). This complex anatomical structure contributes to sperm selection and probably participates in the regulation of the number of sperm that reach the site of fertilization, thus controlling polyspermy and providing different oviduct microenvironments (Hunter 2012). The epithelium is mainly formed by two different cell types: ciliated cells and non-ciliated cells or secretory cells (Fig. 1). The distribution and morphology of both cell types changes during the oestrous cycle, the anatomical region of the oviduct and even the specific region of the mucosa fold (apical or basal regions). Thus, it has been reported that ciliated cells are more abundant along lateral walls and in the apical region of longitudinal folds than in the basal regions among the mucosa folds (Abe 1996; Yániz et al. 2000; Yániz et al. 2006). In pig, a morphometric analysis even showed differences in the epithelial cells between two breeds (Abe and Hoshi 2008). In addition to these anatomical and histological differences, there are species-specific differences in the physiology of the oviduct. For example, in some species, ovulation is restricted to one of the two oviducts. It was previously reported that the concentration of different hormones (e.g. progesterone, prostaglandins) and the gene expression pattern in the ipsilateral oviduct differs from that observed in the contralateral oviduct (Wijayagunawardane et al. 1998; Bauersachs et al. 2003).

The Oviduct Provides the Most Efficient Environment for the Success of Fertilization and Early Embryo Development

For most domestic animals, the fertility rate is generally higher than 50%. If a uniparous female animal is served during the oestrous period by a male with good sperm quality, the pregnancy rate may reach 60–70%. Among animals that deliver more than one offspring, in many cases the pregnancy rate can even exceed 90% (De Kruif 2003). However, in some cases, it is necessary to use assisted reproductive techniques (ARTs) to solve infertility and subfertility problems. For example, ARTs are linked to the protection and saving of species threatened by extinction, research and genetic improvement (Cseh and Solti 2000).

Nowadays, the production and development of embryos until the blastocyst stage in most of mammalian species can be achieved under *in vitro* conditions, with limitations that depend on the species (Table 1 and Data S1). In pig, for example, the developmental competence of *in vitro*-produced embryos is low compared with their *in vivo* counterparts (Kikuchi et al. 1999). An insufficient cytoplasmic ability for the development and polyspermy of *in vitro* matured oocytes and improper culture conditions for IVP embryos are thought to be responsible for this low efficacy (reviewed in Nagai et al. 2006). In cattle, although polyspermy is not a real problem in *in vitro* embryo production, the process is considered inefficient; while maturation and fertilization may appear to proceed normally, the proportion of embryos reaching the transferable stage is rarely over 40% and those that do reach this stage are often compromised in quality and competence. In equine species, IVF and development rates remain low (Hinrichs et al. 2002; Goudet 2011). The technology of *in vitro* oocyte maturation followed by the application of ICSI has been established to achieve fertilization *in vitro*. In this way, the rates of embryo development ranged from 5% to 10% in early studies but can reach 40% in later ones.

As mentioned above, fertilization and early embryonic development occurs in the oviduct. The quality of

*Both authors contributed equally to this work.

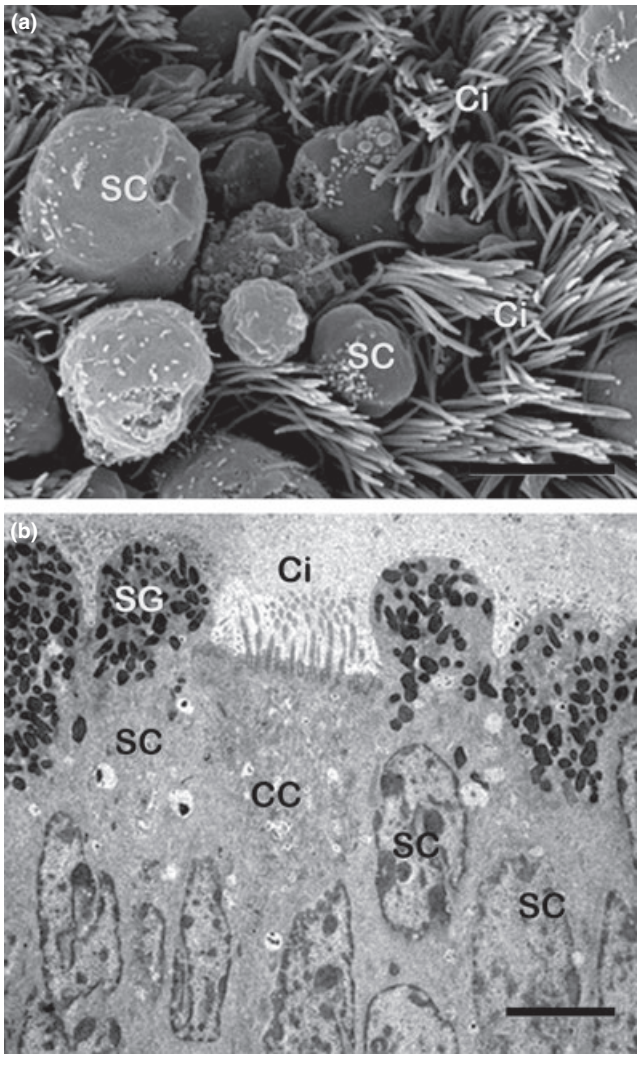

Fig. 1. Epithelial cells of the ampullary-isthmic junction (AIJ) of bovine oviduct. (a) Scanning electron micrographs of the epithelial surface of the ampulla of bovine oviduct in late follicular phase. (b) Electron micrograph of the epon-embedded AIJ. Ciliated cells (CC), secretory cells (SC), secretory granules (SG) and cilia (Ci). Bar: 5 μm

Table 1. Rate of *in vitro* fertilization (IVF), polyspermy, cleavage, morula and blastocyst formation in different domestic animals

Species[a]	*In vitro* fertilization (IVF) (%)	Polyspermy (%)	Cleavage (%)	Morula (%)	Blastocyst (%)
Cat[1]	46–80		30–80	70–97	0–46
Cow[2]	60–92	34–45.6	64–95	60–80	32–65
Dog[3]	10–78	41.2–68	10	0–4	0
Goat[4]	32–37		42–54		10–20.8
Horse[5]	0–84	0–2.4	9–80[b]		4–40[b]
Pig[6]	30–100	32–92.5	20–80		2–40
Rabbit[7]	77–85		64–92		0–85
Sheep[8]		3–21	30–80	50	3.9–57

[a]References used to prepare this table are included in the Supporting Information.
[b]Results obtained after ICSI.

the *in vitro*-produced embryos does not reflect the quality of their *in vivo* counterparts. Results of many studies suggest that culture conditions during *in vitro* embryo production may influence the developmental potential of the early embryo and its quality (Lonergan et al. 2007). Strategies developed to improve embryo development include the use of coculture media with epithelial cells, culture media supplemented with different proteins or growth factors and embryo culture in a foreign oviduct. The trans-species transfer of embryo to oviducts has been used to optimize early embryo development in different species, with the oviduct proving to be the best environment (Gandolfi and Moor 1987; Gutiérrez-Adán et al. 2004).

The Oviduct is Involved in Regulation of Sperm, Oocyte and Embryo Physiology

The efficiency of fertilization is lower *in vitro* than *in vivo* for most species. However, it is unknown whether this is the result of (i) failures in final gamete maturation, (ii) deficient sperm–oocyte interaction or (iii) the lower ability of the recently formed zygote to develop. All these steps, which probably jointly affect the final outcome of *in vitro* procedures, take place in the oviduct under physiological conditions and, consequently, a study of the factors affecting them is of great importance for further advances in the reproductive biology field.

Sperm

The role of the oviduct in male gamete capacitation mediated by binding of the spermatozoa to oviductal epithelial cells has been described in several species (Suarez 1998; Hunter 2012; Goudet 2011). In most cases, bound spermatozoa in the isthmus have been shown to decrease their movement and to prolong their survival, delaying the capacitation process (Suarez 2008; Fazeli et al. 2003). Partial identification of different proteins and carbohydrates involved in sperm binding and release from the oviduct (Suarez 2001; Talevi and Gualtieri 2010; Gualtieri et al. 2010; Talevi et al. 2010), as well as of the relationship between ovulation and the release of capacitated spermatozoa, has also been made (Gualtieri et al. 2005; Suarez 2007). However, a complete description of all the molecular pathways involved in these processes remains under research (Hunter 2012), and better understanding of these pathways will offer new tools for improving *in vitro* reproduction in domestic animals and also in humans.

Other possible roles of the oviduct as regards the male gamete have been related to the selection and guidance of spermatozoa towards the egg (Holt and Fazeli 2010). Moreover, the arrival of spermatozoa within the oviduct regulates gene expression in oviductal epithelial cells (Thomas et al. 1995; Fazeli et al. 2004; Georgiou et al. 2005, 2007). The oviduct may also be involved in a sperm selection process (Rodríguez-Martínez et al. 2005). After mating, where a large number of sperm are deposited in the vagina or uterus, very few are able to reach the site of fertilization. Severely deformed sperm cannot enter the oviduct (Styrna et al. 2002); however, sperm with a normal morphology or with few anomalies and a progressive linear movement can penetrate the uterotubal junction and enter the isthmus (Shalgi et al. 1992; Holt and Van Look 2004; Nakanishi

et al. 2004). Then, sperm with appropriate receptors on their surface may bind to the epithelial cells for a period up to 30 h and form a preovulatory sperm reservoir (Rodríguez-Martínez et al. 2005; Hunter 2012). A seminal plasma protein called, BSP1 (or PDC-109), and annexin present in the apical membrane of the epithelial cells play a key role in this process (reviewed in Hung and Suarez 2010).

It has been reported that the female genital tract has a positive effect on the fertilization potential of spermatozoa that have been genetically altered (Kawano et al. 2010; Turunen et al. 2012). In mouse, Turunen et al. pointed to an 80% decrease in *in vitro* fertilization with sperm that lack CRISP4 compared with the wild type. These data indicated that, even if the physiology of the sperm is seriously compromised, the genetically modified mice were fertile, as wild-type animals, in normal mating. In our opinion, these results provide a new view of the uterine/oviductal contribution to sperm maturation in the genital tract.

The above studies could contribute to the development of sperm treatments with uterine and/or oviductal secretions (or uterine/oviductal tissue explants) to improve the sperm quality of animals with a low seminal quality, or in the case of damaged sperm after cryopreservation. We consider that this finding in mouse is worth investigating in other domestic animals and also in humans.

Oocyte

With regard to the oocyte, the role of the oviduct on its final maturation, especially at the zona pellucida (ZP) level, has not been deeply studied, although it was suggested more than 20 years ago that oviductal glycoproteins may act to enhance the various functions of the ZP (Yang and Yanagimachi 1989). Recently, this role has been partially clarified when OVGP1 and heparin-like glycosaminoglycans from the oviductal fluid were seen to bind to the ZP and make it resilient to enzymatic digestion and to sperm binding and penetration (Coy et al. 2008). This mechanism represents a novel view of the so-called 'ZP hardening', which had been considered until now as a post-fertilization event associated with cortical granule exocytosis (cortical reaction). Now, it is known that the ZP undergoes those maturational changes in the oviduct before the arrival of spermatozoa and that these modifications may be crucial for any further oocyte response to sperm entry. As an example, polyspermy levels in the pig and cow are significantly affected by the contact of the ZP with oviductal secretions and, as a consequence, the final rate of fertilization is modified (Coy et al. 2008). A similar effect of the oviductal fluid on ZP maturation has recently been shown in the sheep and goat (Mondéjar 2011).

Embryo

Finally, it cannot be forgotten that the recently formed zygote remains in the oviduct for a variable period of time, depending on the species but never < 48 h. During this time, oviductal secretions are subjected to important changes derived from hormonal transition from an oestrogen-dependent to a progesterone-dependent environment. As we reported previously, the oviductal fluid protects the embryo against adverse impacts on mtDNA transcription/replication and apoptosis (Lloyd et al. 2009a). Moreover, a number of embryotrophic factors from the oviduct have been described (Lee and Yeung 2006; revised by Avilés et al. 2010), although functional experiments to clarify the specific role of each one and their potential use for improving *in vitro* culture systems remain to be performed. In the following paragraphs the identification and role of some of these factors will be discussed.

Functional Genomic and Proteomic Analysis of the Oviductal Cells and Secretions

Oviductal fluid is a complex fluid formed by different metabolic and macromolecular components from blood plasma and epithelial cell secretions (reviewed in Buhi et al. 2000; Aguilar and Reyley 2005; Georgiou et al. 2005; Leese et al. 2008; Avilés et al. 2010). Most studies of the oviductal fluid have identified one, or a low number of, protein(s) in the oviduct by means of conventional analytical methods (Avilés et al. 2010). Other studies have tried to identify more components using complex technologies that include two dimensional electrophoresis (Gandolfi et al. 1989; Buhi et al. 2000). However, until now, it has been possible to identify only some of the proteins detected in the 2D gel by preparing specific antibodies. Fortunately, thanks to the development of the mass spectrometry instruments and the deciphering of the genome of different species, it is nowadays possible to identify a large number of proteins contained in complex body fluids and to study gene expression patterns in different tissues. Here, we describe the results previously reported in the literature and by our group obtained by using transcriptomic and proteomic analysis.

Transcriptomic Analysis

A transcriptomic analysis of the oviduct has been performed in bovine, human and mouse species (Bauersachs et al. 2003; Fazeli et al. 2004; Bauersachs et al. 2004; Tone et al. 2008; George et al. 2011).

In cattle, the epithelial cells were obtained by scraping the mucosal epithelial layer of the complete oviduct using a glass slide from heifers in oestrous and dioestrous phases (Bauersachs et al. 2004) and in the postovulatory period (Bauersachs et al. 2003). During the postovulatory period, authors found differences for 35 genes when comparing gene expression in the ipsilateral and contralateral oviduct. Twenty-seven genes were up-regulated in the ipsilateral oviduct, and eight were down-regulated (Bauersachs et al. 2003). The comparative analysis of the gene expression between oestrous and dioestrous phase showed that 77 genes were differentially expressed; 37 and 40 genes were up-regulated in the oestrous and dioestrous phases, respectively (Bauersachs et al. 2004). These genes have been related to the immune response, protein secretion and

modification, endocytosis, signalling and the regulation of transcription.

In women, a recent study compared the gene expression profile of epithelial cells of the Fallopian tube between the follicular and luteal phases (George et al. 2011). The authors identified five genes up-regulated and 15 down-regulated in the luteal phase (supplementary file in George et al. 2011). Some of these genes are of potential interest for different aspects related to fertilization and embryo development. For example, mRNA for heparanase was detected in the human oviductal mucosa. In a previous study, our group provide strong evidence to support a role for OVGP1 and heparin in blocking polyspermy (Coy et al. 2008). It was observed that heparin contributes to the stabilization of the OVGP1 effect. Our proteomic analysis also identified the existence of heparanase in the porcine oviductal fluid, agreeing with the results mentioned previously for the human oviduct. Heparin molecules have also been related to the release of bovine sperm bound to the oviductal epithelia (Gualtieri et al. 2010). Therefore, it seems possible that the heparanase present in the oviduct contributes to the regulation of these processes. Future experiments are necessary to confirm this hypothesis.

GPX3 mRNA, a glutathione peroxidase, is another gene differentially expressed in the human oviduct. This enzyme is involved in the redox balance and could make an important contribution to the control of DNA damage that affects gametes and the embryo (Aitken and De Iuliis 2010) and also to the sperm binding to the oviduct through the reduction of SS to SH (Gualtieri et al. 2009).

We have performed a detailed analysis of human specimens from the microarray experiment stored in the Gene Expression Omnibus (GEO) accessible through GEO Series accession number GSE10971 (Tone et al. 2008; Data S2). A total of 5703 genes of the original 54 675 probes present in the microarray were detected. This number of expressed genes is in accordance with a previous study performed in other human tissues using microarray analysis (Su et al. 2004). The list of genes was analysed and classified using the DAVID Bioinformatics Resource 6.7 (DAVID). Genes were classified according to the cellular localization, and genes encoding secreted proteins were also included (Fig. 2). It can be observed that 394 (8%) correspond to genes that codify for plasma membrane proteins and 245 (5%) of the expressed genes correspond to secreted proteins that can be classified into different groups (Fig. 3).

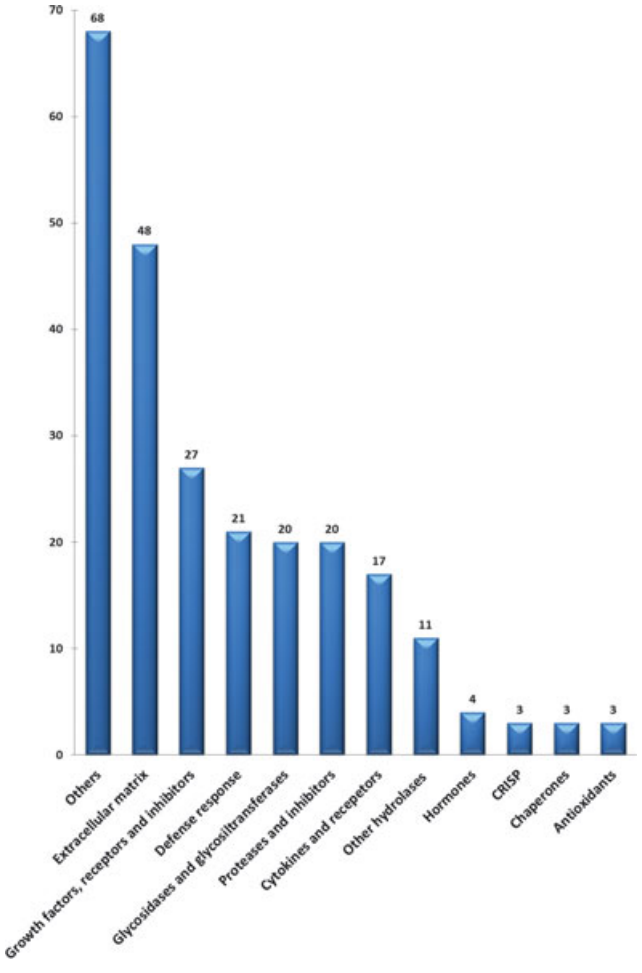

Fig. 3. Functional clustering of genes classified as 'secreted' using the DAVID bioinformatic tool using data from normal Fallopian tube reported in Tone et al. (2008)

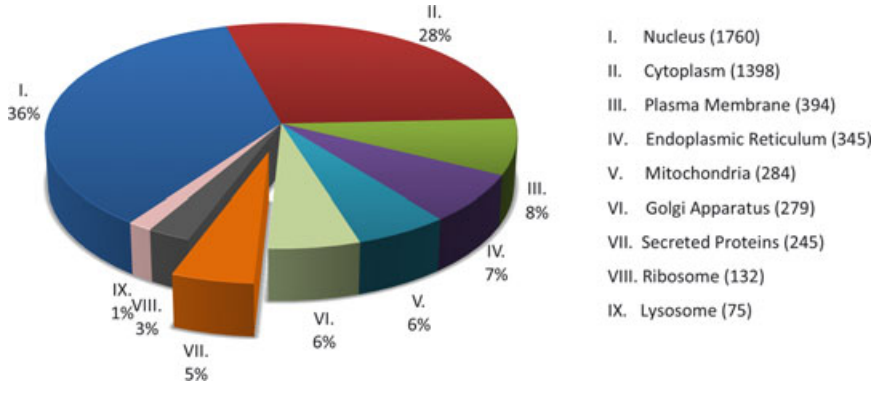

Fig. 2. Gene expression analysis of normal Fallopian tube (GEO: GSE10971). Gene clustering was performed according to the DAVID. The values represent the number and percentage of genes identified

Some of the mRNA detected in this study has previously been identified in the oviductal epithelium in other species (Avilés et al. 2010); however, future studies are needed to confirm these data. The study by Tone et al. (2008) of gene expression analysis was performed using the oviductal mucosa and laser microdissection. Therefore, other cells (fibroblast, endothelial cells, lymphocyte, mast cells, etc.) present in the laminae propia are included in this analysis, and, consequently, some of the genes detected may not correspond to the epithelial cells covering the oviductal lumen. Other techniques like immunocytochemical and *in situ* hybridization analysis can be used to demonstrate the direct relationship between the expressed genes and the oviductal epithelium.

A gene expression study of the porcine oviduct in different phases of the oestrous cycle is currently in progress in our laboratories. In this review, we will provide unpublished information about the gene expression in the oviduct in the preovulatory phase of the cycle. For the analysis, the ampullary-isthmic junction region of the oviduct was selected because fertilization takes place in this region. The hybridization was performed using a microarray of 43 803 probes (Porcine (V2) 4x44K) from Agilent (Agilent Technologies, Madrid, Spain). Further details of the methodology employed is described in Data S2. Then, a total of 2968 genes were detected; this number is low compared with the human samples owing to incomplete annotation of the porcine genome. More than 480 genes are shared between the human and the porcine oviduct. Similarities between the porcine and bovine gene expression are even lower owing to the low number of gene available for comparative purposes (Bauersachs et al. 2007) (Fig. 4). The known genes expressed in the bovine oviduct epithelial cells are reduced owing to the fact that only differentially expressed genes were analysed using a subtracted library. In mice, a comparison of the only available data (125 genes) (Fazeli et al. 2004) showed that 82 of the genes are shared with the human oviduct, 17 are shared with the pig, and only two genes are expressed in the four species studied. In general, these results indicate that some genes are expressed in all four species, suggesting that basic components play a similar function and are evolutionarily conserved. These results are in agreement with functional events observed in a heterologous situation. Thus, embryo development from bovine species can be produced in the ewe oviduct (Rizos et al. 2007). Moreover, a pre-fertilization hardening of the ZP was observed when oocytes and oviductal fluids from different species are used (Mondéjar 2011); but this process is not always produced on the same scale or in all species. This finding is probably due to the existence of expressed genes that are not shared among the species.

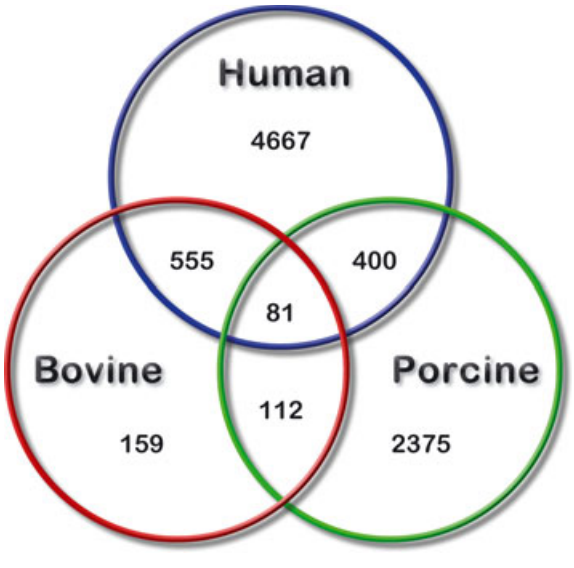

Fig. 4. Venn diagram showing overlapping and non-overlapping gene expression on human, bovine and porcine oviduct

Proteomic Analysis

More than 160 proteins have been seen to be expressed or secreted by the oviduct of different species (Avilés et al. 2010). In pig, numerous proteins have been identified in the epithelial cells (Buhi et al. 2000; Georgiou et al. 2005; Sostaric et al. 2006; Seytanoglu et al. 2008) and also in the oviductal secretions (Georgiou et al. 2005, 2007; Mondéjar et al. 2009; Mondéjar 2011). At least 32 proteins from the oviductal fluid are affected by the presence of gametes, most of which are affected by the presence of the male gamete (Georgiou et al. 2007). In our laboratory, we performed an analysis of the porcine oviductal fluid from preovulatory phase. The oviductal fluid was obtained by luminal aspiration of previously dissected oviducts as previously reported (Carrasco et al. 2008). After centrifugation (5200 × ***g*** for 10 min) to remove the mucus and cellular debris, the samples were analysed by one dimensional SDS-PAGE electrophoresis under reducing conditions. The different bands were visualized by silver staining and were cut and digested with trypsin for subsequent proteomic analysis. The data were analysed by LC/MSD Trap Data Analysis Version 3.2 (Bruker Daltonik, GmbH, Germany), and the search for matches was conducted with the Spectrum Mill MS Proteomics Workbench (Agilent Technologies, Santa Clara, CA, USA) against the most recent version of the NCBInr database.

A total of 291 proteins were identified in the porcine oviductal secretions (Mondéjar 2011); however, only 35 of these proteins were detected in our microarray analysis (Mondéjar 2011), probably due to the incomplete array annotation. The different proteins can be classified into different groups as performed previously (Avilés et al. 2010) (Fig. 5). In this analysis, only 27 secreted proteins (9.3%) were identified; however, most of the proteins correspond to cellular components (90.7%). It is striking that a low number of the proteins identified correspond to typically secreted proteins compared with the total number of proteins. In the oviductal fluid, other proteins that are not typically secreted by the epithelial cells can be detected. These proteins may be divided into two main groups: proteins that come from the transudate of blood (for example, albumin and plasminogen) and other non-secreted cellular proteins. Some of them are proteins present in cellular organelles such as the nucleus, mitochondria,

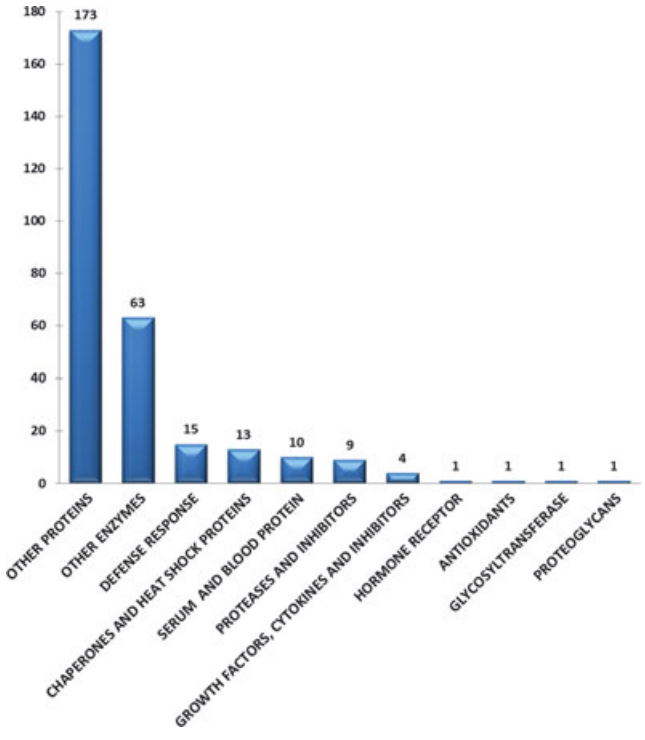

Fig. 5. Classification of the different proteins identified in the porcine oviductal fluid by proteomic analysis

endoplasmic reticulum and lysosomes. The origin of these proteins is probably related to different processes such as epithelial cell renewal or the secretory activity by apocrine and holocrine processes (Crow et al. 1994; Steffl et al. 2008); however, blood or oviductal cells contamination cannot be excluded.

The most abundant peptides identified correspond to albumin and the OVGP1 as expected owing to the fact that these proteins are abundant in oviductal fluid. Owing to space restriction, only three of the identified proteins are discussed in more detail.

Several heat shock proteins (HSP) were detected in our proteomic analysis, confirming their existence, as reported previously (Bauersachs et al. 2004; Georgiou et al. 2005). These proteins are usually considered to be intracellular proteins, although they have also been detected in the human serum and plasma (Molvarec et al. 2010). It was observed that HSP70 can be secreted by a non-classical pathway (Mambula and Calderwood 2006). A similar process, which remains to be confirmed, could exist in the oviduct. It was reported that HSPA8 and HSP60 are able to associate with spermatozoa, thus improving their survival in several species (Elliott et al. 2009; Lloyd et al. 2009b).

Another interesting protein identified in our study is annexin, which is considered a cytosolic protein; however, it was also reported that this protein can be secreted by a non-classical pathway (Christmas et al. 1991). It was previously reported that the bovine sperm interaction with the oviductal epithelium is mediated by BSP1 associated with the sperm membrane and with annexin molecules in the oviductal epithelium (Ignotz et al. 2007). Annexin in the oviductal fluid could regulate this type of interaction.

In our study, we detected, by both gene expression and proteomic analysis, the deleted in malignant brain tumours 1 (DMBT1) protein, confirming its presence at mRNA level in the bovine (Bauersachs et al. 2004) and human (Tone et al. 2008) oviduct. Very recently, it was reported that the DMBT1, previously called SPG, is expressed in the porcine oviduct (Teijeiro et al. 2012). In this study, the authors demonstrated the presence of this protein at the apical surface of the epithelial cells.

Concluding Remarks

A large body of evidence strongly supports the complexity of and the important role played by the oviduct and its secretion in different aspects of gamete maturation, fertilization and embryo development. The oviduct undergoes important changes in several aspects, including its anatomy in different regions, changes in the histology and physiology of the mucosa during the ovarian cycle and a complex gene expression pattern that is modified according to the ovarian cycle status and also to the presence of gametes and embryos. More precise information is needed about the different genes expressed and proteins synthesized and secreted by the oviduct in its different regions, hormonal and other physiological conditions to clarify the role played by the oviduct. However, for this, knowledge of the complete genome annotation of different animals is necessary. The information obtained for different animals will also contribute to understanding the mechanisms conserved in the different species and also others that are responsible for species specificity. This information will contribute to improving different aspects of the methodology used in ARTs in domestic animals, endangered wildlife species and even human beings.

Acknowledgements

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Conflict of interest

None of the authors have any conflicts of interest to declare.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Data S1. References.

Data S2. Methods.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

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The Role of Hox Genes in Female Reproductive Tract Development, Adult Function, and Fertility

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HOX genes convey positional identity that leads to the proper partitioning and adult identity of the female reproductive track. Abnormalities in reproductive tract development can be caused by *HOX* gene mutations or altered *HOX* gene expression. Diethylstilbestrol (DES) and other endocrine disruptors cause Müllerian defects by changing *HOX* gene expression. *HOX* genes are also essential regulators of adult endometrial development. Regulated *HOXA10* and *HOXA11* expression is necessary for endometrial receptivity; decreased *HOXA10* or *HOXA11* expression leads to decreased implantation rates. Alternation of *HOXA10* and *HOXA11* expression has been identified as a mechanism of the decreased implantation associated with endometriosis, polycystic ovarian syndrome, leiomyoma, polyps, adenomyosis, and hydrosalpinx. Alteration of *HOX* gene expression causes both uterine developmental abnormalities and impaired adult endometrial development that prevent implantation and lead to female infertility.

HOX genes comprise a family of regulatory molecules that encode highly conserved transcription factors. In the past several decades, molecular and genetic evidence indicates that *HOX* genes are expressed along anterior–posterior axes and control morphogenesis and cell differentiation during normal embryonic axial development; this mechanism for assigning differential identity along previously uniform axes is used in species as diverse as *Drosophila* and humans (McGinnis and Krumlauf 1992). *HOX* genes have a similar role in the specification of the developmental fate in individual regions of the female reproductive tract, where they regulate developmental axis in the embryonic period. *HOX* genes also give specific identity to the developing endometrium during the menstrual cycle in adults. The cyclic growth of endometrium is dependent on the ordered production of estrogen and progesterone. *HOX* gene expression is regulated by sex steroids, and this regulated expression plays an important role in endometrial development and endometrial receptivity (Taylor et al. 1997, 1998, 1999b). Here, we review the role of *HOX* genes, specifically the *HOXA*/*Hoxa* genes, in reproductive tract development, endometrial cyclic growth and embryo implantation, and the alterations in *HOXA*/*Hoxa* gene expression that can lead to infertility.

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HOX GENES AND THEIR ROLE IN THE BODY PLAN

HOX Genes

Homeobox genes (as known as *HOX* genes) comprise a group of highly conserved genes that are essential regulators of anterior–posterior (A–P) axial pattern development. In 1978, the relationship between the location of a homeotic gene and positional development identity was first recognized in *Drosophila* (Lewis 1978). Six years later, the *HOX* genes were cloned and sequenced in the fruit fly *Drosophila melanogaster* (McGinnis et al. 1984a,b; Scott and Weiner 1984). Since then, multiple *HOX* genes have been identified in many species, including humans. *HOX* genes encode proteins that act as transcription factors. In each of the *HOX* genes, a 183-bp highly conserved sequence was identified, which encodes a 61-amino acid region, called the homeodomain (HD). Structural analyses have shown that the HD can self-fold, and form a structural motif called a "helix-turn-helix motif." Through this motif, the HD, a DNA binding domain, recognizes a typical core DNA sequence, typically TAAT or TTAT, and regulates the expression of target genes, many of which play a role in axial development (Gehring et al. 1994; Krumlauf 1994; Gruschus et al. 1999; Passner et al. 1999).

Like all other insects, *Drosophila* has eight *HOX* genes, which are clustered into two complexes in close proximity, the antennapedia (Ant-C) complex and bithorax (Bx-C) complex. In mice and humans, *Hox*/*HOX* genes are clustered into four unlinked genomic loci, *Hox a-d* (mouse) or *HOX A-D* (human); each locus contains nine to 13 genes and all four clusters contain a total of 39 *HOX* genes. Those four paralogues, classified by sequence similarity, are located on chromosomes 6, 11, 15, and 2 in mice and chromosomes 7, 17, 12, and 2 in humans. The clustered *HOX* genes are believed to have arisen from gross duplication of a single common ancestral cluster. Presently, none of the paralogues have 13 genes, so some duplicated genes must have been lost during the course of evolution (Krumlauf 1994).

Hox Genes and Vertebrate Axial Development

In general, expression of the *HOX* genes follows a 3′ to 5′ order, which means, *HOX* genes at 3′ end are expressed earlier in development than their 5′ neighbors within the same cluster. The position in the cluster reflects both the timing and spatial position of developmental expression (Hunt and Krumlauf 1992; McGinnis and Krumlauf 1992). *HOX* genes have a well-characterized role in embryonic development, during which they determine identity along the A–P body axis. In vertebrates, gastrulation forms three germ layers: ectoderm, endoderm, and mesoderm. *HOX* genes are first expressed in the mesoderm during early gastrulation, and the 3′ genes are expressed first in anterior locations and then the 5′ genes are expressed later in the distal sacral regions. The role of mammalian *HOX* genes in regulating segmental patterns of hindbrain, skeleton axis and the limb axis is well established. In mice, gain- and loss-of-function experiments have revealed the spatiotemporal expression controlled by *Hox* genes in skeleton development (Ramirez-Solis et al. 1993; Horan et al. 1995; Fromental-Ramain et al. 1996; Favier and Dolle 1997). For instance, loss of *Hoxb4* expression leads to defects in the first and second cervical vertebrae. Targeted mutations of *Hoxa9* and *Hoxd9* result in anterior transformations of distinct lumbosacral vertebrae. There are transformations of sacral and first caudal vertebrae in *Hoxa11* knockout mice. In the vertebrate nervous system, the hindbrain or rhombencephalon develops under the regulating of such segmental patterning directed by *Hox* gene expression as well; regional expression of *Hox* genes in the hindbrain is thought to confer identity to rhombomeres (Carpenter et al. 1993; Mark et al. 1993; Goddard et al. 1996; Studer et al. 1996; Morrison et al. 1997; Manzanares et al. 1999; Ferretti et al. 2000; Yau et al. 2002). Mice harboring a *Hoxa1* mutation have alteration in hindbrain segmentation, deleting all or part of rhombomere5 (r5). The absence of *Hoxb1* function results in an apparent segmental transformation of r4 to an r2-like rhombomere identity. *Hox-* is essential for r4 development. *Hoxa3* and *Hoxb3* genes are segmentally ex-

pressed in r4 and r6. *Hoxa4*, *Hoxb4*, and *Hoxd4* have anterior limits in the hindbrain, but map to the junction between rhombomeric segments r6 and r7. Vertebrate *HOX* genes not only specify positional identity along the A–P axis of the body plan, but also provide positional values on the axis of the developing limb (Davis and Capecchi 1996; Nelson et al. 1996; Goff and Tabin 1997; Scott 1997). The most 5′ members of the *Hoxa* and *Hoxd* clusters (*Hoxa9-13* and *Hoxd9-13*) are particularly important in vertebrate limb development. *Hoxa9* to *Hoxa10* and *Hoxd9* to *Hoxa10* are expressed in the developing upper arm/leg; *Hoxa11* and *Hoxd9* to *Hoxa13* are expressed in the development of the lower part of the arm/leg. *Hoxa13* and *Hoxd10* to *Hoxd13* are expressed during specification of the hand/foot. The first identified human limb malformation related to a defective *HOX* gene was synpolydactyly, which results from mutations in the *HOXD13* gene (Muragaki et al. 1996). The role of *HOX* genes in vertebrate axial patterning is similar to but more complex than that in *Drosophila*. In the mice and humans, *Hox/HOX* gene clusters provide a considerably overlapping expression pattern, which provides for the possibility of redundancy.

THE ROLE OF HOX GENES IN FEMALE REPRODUCTION

HOX Genes and Structure of Female Reproductive Tract

The female reproductive system is derived from the paramesonephric (Müllerian) duct, which ultimately develops into the fallopian tube (oviduct), uterus, cervix, and upper part of the vagina. The developing of female reproductive tract is patterned by the differential expression of *HOX* genes in the Müllerian duct.

In the developing Müllerian duct, a number of posterior *Abdominal B (AbdB) HOX* genes were found to be expressed in partially overlapping patterns along the A–P axis. In vertebrates, *HOX* genes in paralogous groups *Hoxa9-13* develop a characteristic spatial distribution throughout the Müllerian duct (Taylor et al. 1997; Taylor 2000; Goodman 2002). *AbdB* genes are expressed according to their 3′ to 5′ order in the *HOX* gene clusters. *Hoxa9* is expressed at high levels in areas that will become the oviduct, *Hoxa10* is expressed in the development of the uterus, *Hoxa11* is found in the primordial lower uterus and cervix, and *Hoxa13* is seen in the ectocervix and upper vagina. No gene exists in the *Hoxa* cluster that is a paralogue of *Hoxd12* or *Hoxc12*; hence, there is no *Hoxa12* gene. This expression pattern is conserved between mice and humans (Fig. 1). Targeted mutagenesis of these genes results in region-specific defects along the female reproductive tract. *Hoxa10* deficiency causes the homeotic transformation of the anterior part of the uterus into an oviduct-like structure. *Hoxa13* null embryos show a hypoplastic urogenital genital sinus and agenesis of the posterior portion of the Müllerian duct. When the *Hoxa11* gene is replaced by the *Hoxa13* gene, posterior homeotic transformation occurs in the female reproductive tract: the uterus, in which *Hoxa11*, but not *Hoxa13* is normally expressed, becomes similar to the more posterior cervix and vagina, in which *Hoxa13* is normally expressed (Satokata et al. 1995; Benson et al. 1996; Warot et al. 1997).

Although *HOX* genes were once considered to be expressed only during embryonic development, persistent *HOX* gene expression was first well characterized in the adult female reproductive tract (Benson et al. 1996; Taylor et al. 1997). The adult reproductive tract undergoes a continuing developmental process during each menstrual cycle; proliferation and differentiation of endometrium coupled with angiogenesis leads to a new endometrium in each estrus or menstrual cycle. In both mice and humans, the expression of *Hoxa9-13/HOXA9-13* in the adult reproductive tract has been described as the same regions as their expression in the embryo (Dolle et al. 1991; Favier and Dolle 1997; Taylor et al. 1997; Warot et al. 1997). Specifically, *Hoxa10/HOXA10* and *Hoxa11/HOXA11* are expressed in the endometrium of the adult mice and humans. The expression of these two genes varies in an estrus/menstrual cycle-dependent manner (Fig. 2). *Hoxa10/HOXA10* and *Hoxa11/HOXA11* are expressed in the proliferative phase of the endometrium and increase during the secretory phase (Taylor et al.

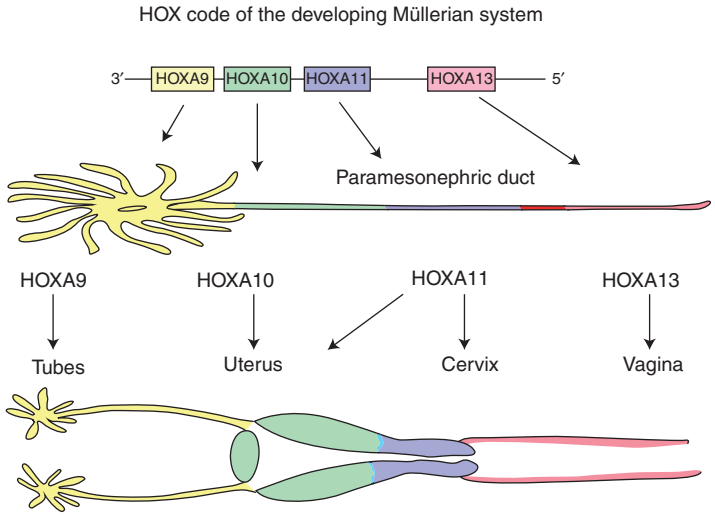

Figure 1. *HOX* code of the developing Müllerian system (adapted from Taylor 2000).

1997, 1998, 1999b). Persistent *HOX* gene expression in the adult may be a mechanism to retain developmental plasticity in the female reproductive tract.

Emx2 is a divergent Homeobox gene, which is a mammalian homolog of the *Drosophila* empty spiracles (ems) gene. The vertebrate *Emx2* gene is located outside of the *Hox* cluster, and is expressed in the developing vertebrate brain as well as the urogenital system (Simeone et al. 1992a,b). In the embryo, *Emx2* is expressed in the epithelial components of the pronephros, mesonephros, ureteric buds, and the Wolffian and Müllerian ducts. In mouse embryos, *Emx2* expression is greatly diminished in male gonad, but strong expression remains detectable throughout the female gonad. Null mutants of *Emx2* mice fail to develop kidneys, gonads or a reproductive tract (Pellegrini et al. 1997; Svingen and Koopman 2007). In adults, *EMX2* has been detected in the human uterus. The expression of *EMX2* displayed a dynamic pattern that varied with the developmental phase of the human reproductive cycle (Fig. 2) (Troy et al. 2003).

The Role of HOX Genes in Female Fertility

Female fertility is a broad term, which includes the ability to reproduce or become pregnant. Multiple factors influence female fertility, including normal aging and several disease processes. However, two processes are essential for normal female fertility: ovarian follicular maturation and embryo implantation. In vertebrates, *HOX* genes are involved in both of these processes.

Ovarian follicle development is a complex process in which many transcription factors participate. As described above, *HOX* genes containing the evolutionarily conserved HD sequence encode a family of DNA-binding transcription factors whose functions are crucial for embryonic development in vertebrates. In 1995, *HOXA4* and *HOXA7* expression was first described in the human unfertilized oocytes (Verlinsky et al. 1995). Sequence analysis of cDNA libraries generated from human unfertilized oocytes confirmed the expression of *HOXA7* (Adjaye and Monk 2000). Furthermore, in human ovarian folliculogenesis, *HOXA7* expression is nearly absent in primordial follicles but high in primary and mature follicles. During follicular maturation, the subcellular localization of *HOXA7* changes from nuclear to predominantly cytoplasmic. This differential localization indicates that *HOXA7* undergoes cell type- and stage-specific changes during the human ovarian folliculogenesis, and regulates proliferative

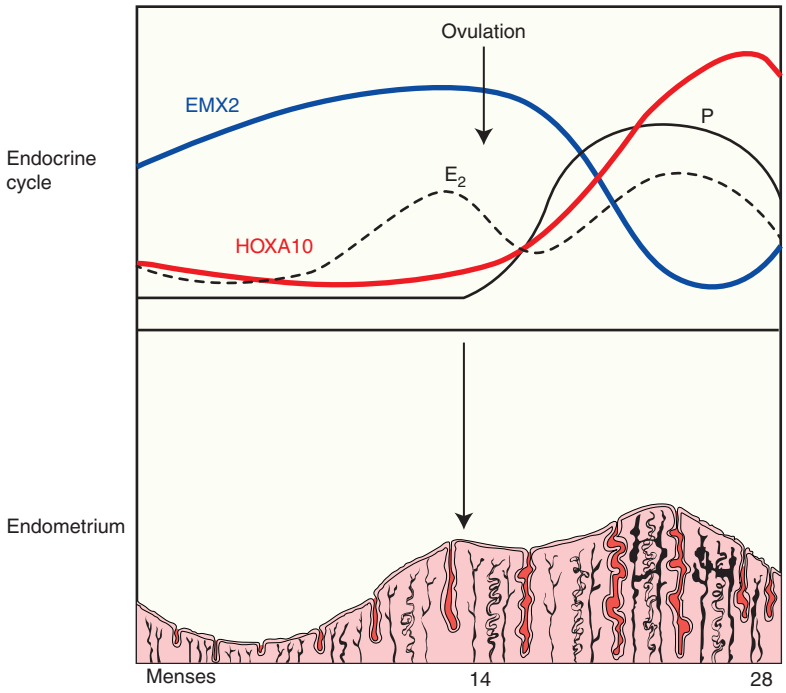

Figure 2. The pattern of *HOXA10* expression in the human endometrium through the menstrual cycle (adapted from Taylor 2000). *HOXA11* expression closely parallels that of *HOXA10*.

activities of ovarian follicles (Ota et al. 2006). Granulosa cells surround the developing oocyte, providing a critical microenvironment for follicular growth. During this process, the oocyte and the granulosa cells establish mutual interactions and their growth is regulated by coordinated paracrine mechanisms. *HOXA7* modulates granulosa cell growth and proliferation not only via the regulation of the epidermal growth factor receptor (EGFR), but also forms dimers with the *HOX* gene cofactor pre-B-cell leukemia transcription factor 2 (PBX2) to bind the specific promoter regions in the human granulosa cells. *HOXA7* plays an important role in ovarian follicular maturation (Ota et al. 2008; Zhang et al. 2010).

Embryo implantation is critical for female reproduction. This process is a complex event requiring synchronization between a developing embryo and receptive endometrium. Fundamental to this process is the dynamic and precisely ordered molecular and cellular events that drive and stabilize the interaction between the developing embryo and its host endometrium. As described above, *Hoxa10*/*HOXA10* and *Hoxa11*/*HOXA11* are expressed in endometrial glands and stroma throughout the estrus/menstrual cycle. These two *HOX* genes are essential for embryo implantation in both mice and humans (Hsieh-Li et al. 1995; Satokata et al. 1995; Benson et al. 1996; Gendron et al. 1997). Targeted mutation of either *Hoxa10* or *Hoxa11* in the mice leads to infertility related to defects in uterine receptivity. Embryos produced by *Hoxa10* deficient mice are viable and can successfully implant in wild-type surrogates. However, those embryos are not able to implant or survive in the uteri of *Hox* gene knockout mice. Although the uteri of these knockout mice appear anatomically normal, they do not support the development or implantation of their own embryos, nor of embryos from the wild-type mice. Histologic abnormalities were noted in the *Hoxa10* deficient mice, resulting in a homeotic transformation of the anterior part of the uterus into an oviduct-like structure. Similarly,

the mice with a homozygous mutation in the *Hoxa11* gene are infertile because of implantation defects. Those mice have reduced endometrial glands and decreased leukemia inhibitory factor (LIF) secretion. Targeted mutation of orthologous *Hox* genes such as both *Hoxd9* and *Hoxd10* in mice does not result in abnormalities on uterine structure or position (De La Cruz et al. 1999). Although no human females with mutations in *HOXA10* and *HOXA11* have been described, it has been reported that patients with lower implantation rates have lower *HOXA10* and *HOXA11* expression in the secretory phase, which indicates that maternal *HOX* gene expression is conserved and necessary for endometrial receptivity (Taylor et al. 1999b; Bagot et al. 2000; Taylor 2000).

Estrogens and Progesterone Regulate Hox Gene Expression in the Reproductive Tract

So far, few regulators of *HOX* gene expression have been identified. Sex steroids have been investigated in the regulation of the *HOX* genes at the 5′ end of the cluster, which determine the posterior development, including the development of female reproductive tract (Taylor et al. 1997, 1998, 1999b; Ma et al. 1998; Cermik et al. 2001; Goodman 2002). During each reproductive cycle, endometrial epithelial and stromal cells display a well-defined cyclic pattern of functional differentiation under the influence of estrogen and progesterone. Menstrual cyclicity is regulated by timed expression of estrogen and progesterone, which act both independently and in concert to up-regulate *HOXA10* and *HOXA11* expression in the endometrium. In normal cycling women, *HOXA10* and *HOXA11* levels increase, reaching maximal expression during the mid-secretory phase, and remaining elevated throughout the secretory phase. In endometrial stromal cells, 17β-estradiol and progesterone significantly increase *HOXA10* and *HOXA11* expression. *HOXA9* is under the control of both estrogen and progesterone as well. The regulation of *HOX* gene expression in the adult uterus by ovarian steroids is related to its position within the cluster and mediated by the direct action of estrogen and progesterone receptors on these genes.

Humans are exposed to a wide variety of chemicals that have estrogenic properties. Those estrogenic compounds show profound and lasting effects on essential developmental genes in female reproductive tract. They have potential to alter the expression of estrogen responsive genes, such as *HOX* genes. These changes are likely to influence reproductive competence. Diethylstilbestrol (DES) is a nonsteroidal estrogen, a well-known teratogen. This chemical alters the localization of *Hox* gene expression along the axis of the developing murine reproductive tract, and induces developmental anomalies of female reproductive tract (Ma et al. 1998; Akbas et al. 2004). DES exposure in utero shifts *Hoxa9* expression from the oviducts to the uterus and leads to decreases in both *Hoxa10* and *Hoxa11* expression in the uterus. The decreased expression of the *Hoxa* genes may cause a "T-shaped" uterus, a structure that is characterized by branching and narrowing of the uterus into a tube-like phenotype. This phenotype is likely caused by expression of the *Hox* gene that controls tubal identity (*Hoxa9*) ectopically in the uterus. Because the multiple *HOX* gene clusters provide an overlapping expression pattern in the mice and humans, the complete transformation into an oviduct is probably prevented.

Studies on xenoestrogens, such as methoxychlor (MXC) and bisphenoyl A (BPA), have shown that exposure to these chemicals also alters the *Hoxa10* expression in female reproductive tract (Block et al. 2000; Suzuki et al. 2004; Fei et al. 2005; Markey et al. 2005; Sugiura-Ogasawara et al. 2005; Daftary and Taylor 2006; Smith and Taylor 2007). MXC is a pesticide and this chemical is associated with female reproductive defects after either prenatal or postnatal exposure. MXC specifically alters *Hoxa10* gene expression, specifically the *Hoxa10* gene expression. This *HOX* gene is responsible for normal uterine development and fertility, and its expression is permanently repressed in the uterus of mice exposed to MXC in utero. This effect is mediated through the *HOXA10* estrogen response element (ERE) in a dose-dependent pattern.

BPA, another xenoestrogen, is a common component of polycarbonate plastics, epoxies used in food storage, canned goods, and dental sealants. BPA is also associated with adverse reproductive outcomes in both animal models and humans. After exposure to BPA in utero, *Hoxa10* expression is increased in female mice and this altered expression persisted in adults. The alternation of the gene expression persists long after exposure and alters the normally precise, temporal regulation of *Hoxa10* in reproductive tract development. This permanently modified expression of *Hoxa10* contributes to the decline in female reproductive potential. Despite its opposite effect on *HOX* gene expression in vivo, BPA behaves similarly to MXC in vitro by stimulating the *HOXA10* ERE. The difference seen after in utero exposure likely represents the unique molecular signals present in the embryo and underlies the increased risk of exposure to environmental chemicals during critical periods of development. Exposure to various xenoestrogens alters *Hoxa10* gene expression in the developing reproductive tract, and these exposures may lead to permanent alteration of gene expression in the adult (Fig. 3) (Taylor 2008).

HOX GENES AND INFERTILITY

HOX genes are essential for endometrial development and embryo implantation in both mice and humans. As described above, the association between alteration of *Hoxa* gene expression and fertility is evident in animal models (Fig. 4) (Paria et al. 2002). The *Hoxa10*/*HOXA10* and *Hoxa11*/*HOXA11* genes act as important transcriptional moderators that either activate or repress the downstream target genes; these targets include b3-integrin and *Emx2*/*EMX2*, which are themselves important for embryo implantation. As discussed earlier, in normal cycling women, there is a surge of *HOXA10* and *HOXA11* expression during the mid-secretory phase; diminished *HOXA10* and *HOXA11* expression in the secretory phase leads to low embryo implantation rates. Impaired uterine receptivity has been studied in several gynecological diseases that lead to infertility. These include endometriosis, polycystic ovarian syndrome, leiomyoma, and hydrosalpinx. Compared with controls, there is diminished *HOXA10* and *HOXA11* expression in woman with each of those disorders (discussed in detail below). Although differential mechanisms may lead to decreased expression, it appears that altered *HOX* gene expression is so central to the process of implantation that decrease of their expression is required to diminish implantation. Alterations in the expression of *HOX* genes cause infertility in humans primarily by endometrial receptivity defects and impaired implantation.

HOX Genes and Endometriosis

Endometriosis is an estrogen-dependent benign inflammatory disease defined by the presence of viable endometrial tissue outside the uterine cavity. The prevalence of endometriosis has been estimated as up to 10% to 15% of reproductive-age women and 30%–50% of women with endometriosis have infertility (Verkauf 1987; Olive and Pritts 2001). Multiple factors are considered to contribute to endometriosis related infertility, including altered folliculogenesis, impaired fertilization, poor oocyte quality, and defective implantation. Here, we will focus on the role of diminished implantation as it is related to diminished *HOX* gene expression. In patients with endometriosis, implantation rates are reduced during both natural and assisted reproductive technology cycles, even in patients with minimal disease (Barnhart et al. 2002). Two of the *HOXA* genes, *HOXA10* and *HOXA11*, involved in uterine embryogenesis and endometrial receptivity, have been implicated in the pathogenesis of endometriosis-associated infertility. In humans, the expression of both *HOXA10* and *HOXA11* rises dramatically during the implantation window and remains elevated throughout the secretory phase. However, patients with endometriosis do not show this rise in *HOXA10* and *HOXA11* (Taylor et al. 1999a; Kim et al. 2007; Lee et al. 2009).

HOXA10 downstream target genes are also involved in this pathologic mechanism. As discussed above, *EMX2* is a divergent Homeobox gene, cyclically expressed in the adult

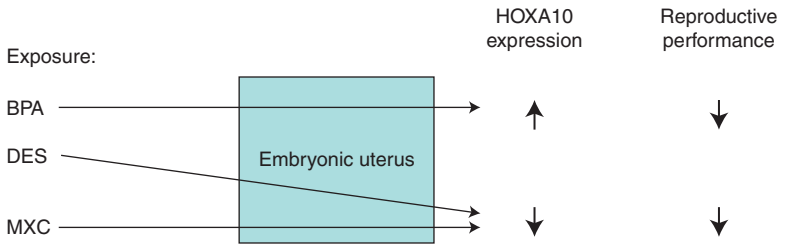

Figure 3. Exposure to various xenoestrogens alters *HOXA10* gene expression in the developing reproductive tract. BPA, bisphenol A; DES, diethylstilbestrol; and MXC, methoxychlor.

endometrium. Endometrial *EMX2* expression is directly regulated by endogenous endometrial *HOXA10*. Normally *EMX2* expression is down-regulated in the peri-implantation period; however, this regulated expression fails in women with endometriosis (Troy et al. 2003; Daftary and Taylor 2004). Further demonstrating the important role of this target gene, altering the endometrial *Emx2* levels is not only associated with defective implantation, but also reduces litter size in mice (Taylor and Fei 2005). Aberrant endometrial *EMX2* expression in women with endometriosis is mediated by altered *HOXA10* expression.

Furthermore, another biomarker of endometrial receptivity to embryonic implantation is also found to be decreased in endometriosis. Integrins are ubiquitous cell adhesion molecules that participate in cell–cell and cell–substratum interactions. These molecules undergo dynamic alterations during the normal menstrual cycle in the human endometrium. b3-integrin is expressed in endometrium at the time of implantation, and the disruption of in-

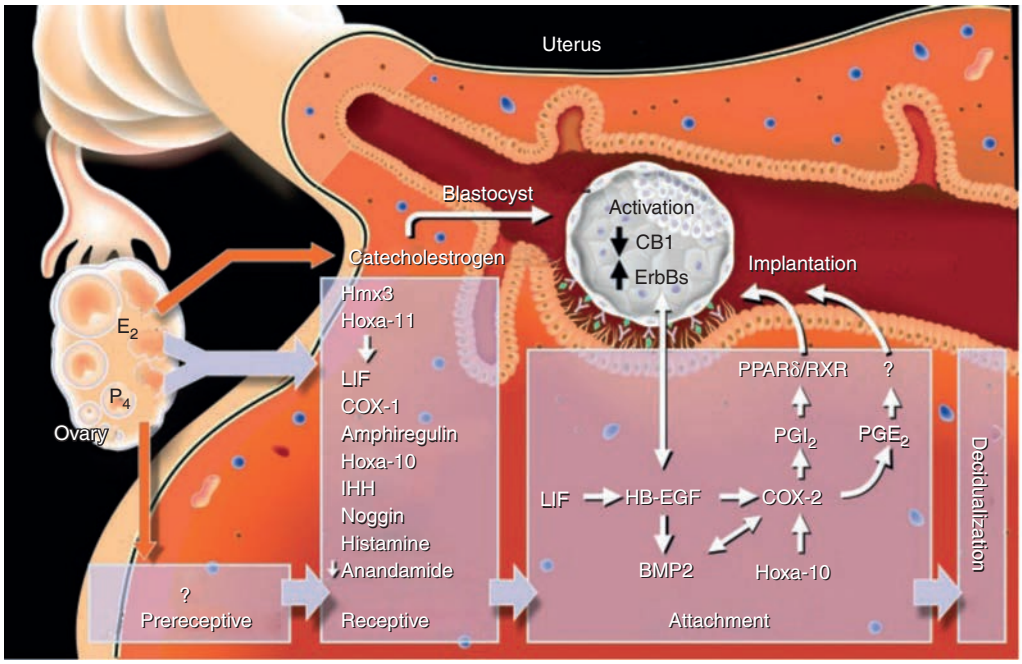

Figure 4. Molecular signaling during implantation in the mouse and human. (From Paria et al. 2002, reprinted, with permission, from The American Association for the Advancement of Science # 2002.)

tegrin expression is associated with decreased uterine receptivity and infertility (Lessey and Young 1997). Interestingly, b3-integrin subunit is a direct *Hoxa10* downstream target gene, and directly regulated by *HOXA10* in endometrial cells. Aberrant expression of both *HOXA10* and integrins have been described in the endometrium of women with endometriosis (Lessey et al. 1994; Lessey and Young 1997; Daftary et al. 2002; Klemmt et al. 2006; Cakmak and Taylor 2011).

Recent studies indicate that epigenetic modifications may play an important role in pathological process in endometriosis. Epigenetics refers to heritable alteration of DNA by long-lasting covalent methyl modification without DNA sequence changes. These epigenetic changes have been described in numerous studies including hypermethylation of *HOXA10*, progesterone receptor-b, and E-cadherin or hypomethylation of genes for estrogen receptor-b and steroidogenic factor 1 (Guo 2009; Senapati and Barnhart 2011). In both murine and baboon endometriosis models, hypermethylation of the promoter region of *Hoxa10*/*HOXA10* and decreased expression of *Hoxa10*/*HOXA10* genes were shown in eutopic endometrium (Kim et al. 2007; Lee et al. 2009). In humans, hypermethylation of *HOXA10* was identified in the endometrium of women with endometriosis (Wu et al. 2005). The DNA methyltransferase (DNMT) is a family of enzymes, which catalyze the transfer of a methyl group to DNA. DNMT 1, 3A, and 3B were found to be overexpressed in the epithelial component of endometriotic implants. However, only DNMT3A was found to be up-regulated in eutopic endometrium of women with endometriosis (Wu et al. 2007). A recently published study, using a genome-wide methylation array, shows that *HOXA10* expression was repressed and methylation of *HOXA10* gene was altered by 1.3-fold in human endometriosis (Naqvi et al. 2014). Other *HOX* genes, such as *HOXD10* and *HOXD11*, also showed significantly altered methylation in endometriosis (Naqvi et al. 2014). Epigenetic programming of *HOX* gene expression in endometriosis leads to lasting alterations in endometrial receptivity.

HOX Genes and Polycystic Ovarian Syndrome

Polycystic ovarian syndrome (PCOS) is a common endocrine disease, afflicting 5% of women of reproductive age. It is characterized by anovulation and elevated androgen action. Infertility associated with PCOS derives from chronic anovulation. Despite the ability to correct ovulatory disorders, pregnancy rates remain paradoxically low, and spontaneous pregnancy loss rates are high. In women with PCOS, between 30% and 50% of all conceptions miscarry (Giudice 2006). Some data also suggest that poor oocyte quality, implantation failure, and higher rates of miscarriage further complicate achieving and maintaining a pregnancy in women with this disorder. Women with PCOS are also at significantly higher risk of endometrial hyperplasia (Niwa et al. 2000). PCOS may have complex effects on the endometrium, contributing to the infertility. Furthermore, increasing evidence and emerging data have shown that endometrial receptivity contributes to the infertility of PCOS even in the setting of ovulation induction (Giudice 2006). An increase in the expression of *HOXA10* in the endometrium is necessary for receptivity to embryo implantation. However, endometrial biopsies obtained from women with PCOS in ovulatory cycles have shown that *HOXA10* expression is decreased compared with normal fertile women during the secretory phase (Cermik et al. 2003). In vitro, *HOXA10* expression is repressed by testosterone (Cermik et al. 2003). Testosterone also prevents the increased expression of *HOXA10* induced by estradiol or progesterone. Dihydrotestosterone produced an effect similar to that of testosterone, whereas flutamide blocked the testosterone effect. Diminished uterine *HOXA10* expression may contribute to the diminished reproduction potential of women with PCOS, illustrating a significant effect of the disease on receptivity. Elevated androgen levels may induce infertility associated with PCOS by altering *HOX* gene expression.

As discussed above, b3-integrin, a biomarker of endometrial receptivity to embryonic implantation, is a *HOX* target gene that is

directly regulated by *HOXA10* in endometrial cells. The expression of this biomarker is decreased in endometrium from women with PCOS compared with fertile controls (Apparao et al. 2002). Also, as described above, after ovulation induction treatment of infertility in PCOS, implantation rates remain low. In fertile women, when ovulation is induced with clomiphene citrate, the treatment provokes the expression of endometrial integrins at the implantation window. Interestingly, integrin is decreased in endometrial biopsy specimens from women with PCOS even after clomiphene citrate treatment (Gonzalez et al. 2001; Jakubowicz et al. 2001).

HOX Genes and Leiomyoma

Leiomyomas (fibroids) are the most common benign uterine tumor of reproductive age women. The growth of leiomyoma is strictly related to sex steroids and their receptors. Their presence is associated with menorrhagia and poor reproductive outcomes. The prevalence of uterine fibroids approaches to 33% of women of reproductive age based on clinical assessment, and up to 50% on ultrasound scans. This disorder presents in 5%–10% of women with infertility (Payson et al. 2006; Revel 2012).

The presence of a distorted uterine cavity caused by leiomyomas significantly decreases in vitro fertilization (IVF) pregnancy rates. Fortunately, myomectomy can increase the pregnancy rates in patients with leiomyoma-related infertility (Bulletti et al. 1999; Surrey et al. 2001). However, the mechanisms by which leiomyoma cause infertility are not fully known. *HOXA10* is expressed in human myometrium and its expression also has a menstrual cycle-dependent pattern. In vitro, *HOXA10* expression is induced in endometrial stromal cells by progesterone, but in the primary myometrial cells, progesterone suppresses *HOXA10* expression (Cermik et al. 2001; Matsuzaki et al. 2009; Rackow and Taylor 2010; Sinclair et al. 2011). It is clear that there are different factors involved in the regulation of *HOXA10* by progesterone in myometrium than endometrium. Further, independent of any change in progesterone concentration, endometrial *HOXA10* and *HOXA11* expression are significantly decreased in uteri with submucosal myomas compared with controls. This effect is not localized to the endometrium overlying the myoma; rather the decreased *HOXA10* expression is seen throughout the endometrium. This global effect of the myoma on endometrium suggests the presence of a diffusible factor that would influence endometrial receptivity remote from the myoma itself. Indeed, we have recently reported that TGFb secreted by myomas leads to decreased BMP receptor expression and subsequent *HOXA10* repression (Sinclair et al. 2011). Leiomyoma alter endometrial receptivity by secreting TGFb and altering genes including *HOXA10* that are required for implantation.

HOX Genes and Hydrosalpinx

Hydrosalpinx is an inflammatory disease involving the oviduct. The prevalence of hydrosalpinges in patients suffering from tubal disease is relatively common and ranges from 10% to 13% when diagnosed by ultrasound, and up to 30% when diagnosed by hysterosalpingography or at the time of surgery (Cakmak and Taylor 2011). Women with hydrosalpinges have decreased implantation rates in IVF, and their pregnancy rates can be improved with salpingectomy before IVF. The hydrosalpinx generates an inflammatory fluid that may interfere with endometrial receptivity and embryonic implantation mechanically or chemically (Zeyneloglu et al. 1998; Camus et al. 1999). Although a study has shown that culturing mice embryos in the medium containing hydrosalpinx fluid can suppress embryo maturation and promote degeneration, this toxic effect does not affect human embryos. (Mukherjee et al. 1996; Strandell et al. 1998) We performed an in vitro study demonstrating that hydrosalpinx fluid decreased endometrial *HOXA10* mRNA expression in a dose-dependent pattern. Subsequently, studies on women with hydrosalpinges show that the expression of *HOXA10* was significantly lower in women with hydrosalpinges compared with fertile controls. After salpingectomy, *HOXA10* expression in infertile women with hydrosal-

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pinges was similar to that of age-matched fertile women, indicating that salpingectomy restores *HOXA10* expression to physiological levels (Daftary and Taylor 2002; Daftary et al. 2007).

As described above, b3-integrin subunit is a well-characterized endometrial receptivity marker, directly regulated by *HOXA10* in endometrial cells. In women with the presence of hydrosalpinges, the expression of b3-integrin is also reduced. Interestingly, two thirds of patients with hydrosalpinx who underwent salpingectomy also showed return of *HOXA10* and b3-integrin back to normal levels (Bildirici et al. 2001).

SUMMARY

All metazoans use *HOX* genes to regulate embryonic patterning. *HOX* genes play a fundamental role in morphogenesis during embryonic development. Well-characterized examples include the role of *HOX* genes in the patterning of the vertebrate hindbrain, skeleton, and limbs. In reproduction, *HOX* genes determine positional identity during embryonic development of the female reproductive tract. Abnormalities in reproductive tract development are related to *HOX* gene mutations and to alterations in the normal *HOX* gene expression patterns. This has been clearly shown in mice with targeted *Hox* gene mutations as well as in mice exposed to chemicals with estrogenic properties such as DES. In the adult, the endometrium undergoes an ordered process of differentiation leading to receptivity to implantation. *HOX* genes are also essential to this process. As transcription factors, *HOX* genes control cyclical endometrial development and receptivity by activating or repressing the expression of target genes. *HOXA10* and *HOXA11* expression increases drastically in the mid-secretory phase, the time of implantation, and they remain elevated throughout the secretory phase. This increased expression is necessary for embryonic implantation; decreased *Hoxa10*/*HOXA10* and *Hoxa11*/*HOXA11* expression at this time leads to decrease implantation rates in both mice and humans. Impaired uterine receptivity has been studied in several infertility-related gynecological diseases, such as endometriosis, polycystic ovarian syndrome, leiomyoma, and hydrosalpinx. Alternation of *HOXA10* and *HOXA11* expression has been identified as a mechanism of the decreased implantation associated with these disorders. Alteration of *Hoxa* gene expression causes both uterine developmental abnormalities and impaired adult endometrial development that prevent implantation and lead to female infertility.

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Tissue interactions and estrogenic response during human female fetal reproductive tract development

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ABSTRACT

The role of tissue interactions was explored to determine whether epithelial differentiation within the developing human reproductive tract is induced and specified by mesenchyme in tissue recombinants composed of mouse vaginal mesenchyme + human uterine tubal epithelium (mVgM+hTubE). The tissue recombinants were grown in DES-treated ovariectomized athymic mice. After 2–4 weeks of in vivo growth, several vaginal specific features were expressed in the human tubal epithelium. The mesenchyme-induced effects included morphological change as well as expression of several immunohistochemical markers. Although the mesenchyme-induced shift in vaginal differentiation in the human tubal epithelium was not complete, the partial induction of vaginal markers in human tubal epithelium verifies the importance of mesenchymal-epithelial interactions in development of the human female reproductive tract.

In a separate experiment, DES-induction of uterine epithelial progesterone receptor (PGR) and estrogen receptor 1 (ESR1) was explored in tissue recombinants composed of wild-type or *Esr1*KO mouse uterine mesenchyme + human fetal uterine epithelium (wt UtM+hUtE and *Esr1*KO UtM+hUtE). The rationale of this experiment was to determine whether DES-induction of PGR and ESR1 is mediated directly via epithelial ESR1 or indirectly (paracrine mechanism) via mesenchymal ESR1. DES-induction of uterine epithelial ESR1 and PGR in *Esr1*KO UtM+hUtE tissue recombinants (devoid of mesenchymal ESR1) formally eliminates the paracrine mechanism and demonstrates that DES induction of human uterine epithelial ESR1 and PGR is directly mediated via epithelial ESR1.

1. Introduction

The tacit, but usually unproven, assumption inherent in animal models is that they are reflective of human biology. This approach is generally useful, even though substantial differences exist between human and animal anatomy, development and pathology. One field for which animal/human pathology is particularly congruent is the effects of exogenous estrogens on the developing female reproductive tract. Administration of the potent synthetic estrogen, diethylstilbestrol (DES), to pregnant women from the 1940s to the 1970s resulted in a broad spectrum of estrogen-induced malformations of the uterine tubes, uterine corpus, cervix and vagina that include T-shaped uterotubal junctions, malformed incompetent cervix, abnormally shaped endometrial cavity, vaginal adenosis as well as clear cell vaginal adenocarcinoma (Jefferies et al., 1984; Rennell, 1979; Stillman, 1982; Titus-Ernstoff et al., 2010; Herbst et al., 1971, 1975; Robboy et al., 1977, 1984, 2018; Hoover et al., 2011). An immense animal literature preceded/confirmed the effects of exogenous estrogens on female reproductive tract development. In addition, animal studies have provided a molecular underpinning for the teratogenic effects of exogenous estrogens on urogenital development (Herbst and Bern, 1981; Bern and Talamantes, 1981; Bern et al., 1984; McLachlan et al., 1975, 2001; McLachlan, 1981; Newbold et al., 1983; Newbold and MaLachlan, 1985; Newbold, 1995, 2004, 2008; McLachlan and Newbold, 1996; Kurita et al., 2004; Kurita, 2011; Laronda et al., 2012, 2013). The animal literature on this topic is replete with

Abbreviations: ESR1, Estrogen receptor alpha; DES, diethylstilbestrol; PGR, progesterone receptor

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estrogen-induce vaginal, cervical, uterine and tubal (oviductal) anomalies including cervicovaginal adenosis. While animal models are useful and relevant to many aspects of human biology/pathology, they are frequently the basis of governmental policy designed to protect human health. Accordingly, whenever possible it is important to establish by experimental means the relevance and predictability of animal studies to human biology/pathology.

Even though mouse-human similarities are now appreciated in estrogen-induced anomalies in the female reproductive tract, the molecular mechanisms that lead to human malformations remain enigmatic, despite some clues from animal studies (McLachlan and Newbold, 1996; Kurita et al., 2004; Kurita, 2011; Laronda et al., 2012, 2013; Terakawa et al., 2016). Direct experimentation on xenografts of human fetal female reproductive tracts treated with DES have provided important insights into the genesis of human malformations and have provided essential bio-endpoints of estrogenic endocrine disruptors. In this regard, we pioneered xenograft methods in 1982 in which human fetal female reproductive tracts were grown in athymic mouse hosts treated with DES and other hormonally active agents (Cunha et al., 1987b, 1987a; Robboy et al., 1982; Taguchi et al., 1983). Unfortunately molecular and immunohistochemical advances, not yet discovered, prevented exploration of biological mechanisms in our earlier studies. More recently, we have revisited human female reproductive tract development in a series of three papers that included a compendium of differentiation markers and how DES administration affects them in vivo (Cunha et al., 2017a, 2017b; Robboy et al., 2017).

Studies carried out over 40 years ago established that uterine and vaginal mesenchyme induces and specifies epithelial differentiation (Cunha, 1976; Kurita, 2010, 2011; Kurita et al., 2001, 2005). Accordingly, vaginal mesenchyme instructively induces uterine epithelium to undergo vaginal epithelial differentiation (VgM+UtE⇝vaginal differentiation), and uterine mesenchyme instructively induces vaginal epithelium to undergo uterine epithelial differentiation (UtM +VgE⇝uterine differentiation). These inductive effects involve both morphological as well as molecular effects on the target epithelium. During vaginal development in mice, inductive cues from vaginal mesenchyme elicit epithelial expression of ΔN p63 (an isoform of p63) in Müllerian epithelium, which specifies vaginal squamous epithelial differentiation (Kurita and Cunha, 2001; Kurita et al., 2004, 2005; Terakawa et al., 2016). p63 is a member of the p53 family of transcription factors. Likewise, immunohistochemical detection of ΔNp63 in fetal and adult human vaginal epithelium suggests a similar role of ΔNp63 in human vaginal epithelial differentiation (Kurita et al., 2005; Fritsch et al., 2012, 2013; Cunha et al., 2017a). The current paper explores the role of mesenchymal-epithelial interactions in a tissue recombinant model consisting of mouse vaginal mesenchyme + human fetal uterine tube epithelium (mVgM+hTubE). The rationale for this particular experimental model is that expression of several differentiation markers is vastly different in vaginal versus tubal epithelium.

In both human and mouse female reproductive organs, estrogen receptor 1 (ESR1; also known as estrogen receptor α) is the dominant receptor for estrogen (Matsuzaki et al., 1999; Dupont et al., 2000). Earlier, we detailed the ontogeny of ESR1 during human fetal uterine development (Cunha et al., 2017a), demonstrating that ESR1 is first expressed in mesenchymal cells of human uterine corpus. Indeed, ESR1-immunoreactivity in uterine epithelial cells is rarely seen before the 21st gestational week, when endogenous estrogen levels are elevated (Oakey, 1970), thus suggesting that uterine epithelial ESR1 may be estrogen induced. Analysis of human fetal uterine xenografts treated with DES has verified this prediction (Cunha et al., 2017b). However, given that prior to DES treatment, ESR1 was detected in uterine mesenchyme and not epithelium, there are two potential mechanisms of DES induction of uterine epithelial ESR1: (a) DES may induce epithelial ESR1 directly via epithelial ESR1 whose expression is below the sensitivity of immunohistochemistry. (b) Alternatively, DES may induce epithelial ESR1 indirectly via mesenchymal ESR1 (paracrine mechanism). The same question is relevant to DES induction of epithelial progesterone receptor (PGR) in the developing human female reproductive tract (Cunha et al., 2017b).

The goal of the current paper based on our prior work (Cunha et al., 2017a; Robboy et al., 2017) is to use xenograft models (a) to determine the role of mesenchymal-epithelial interactions in epithelial differentiation during human female reproductive tract development, and (b) to determine whether estrogen regulates human uterine epithelial ESR1 and PGR via direct or paracrine mechanisms using tissue recombinants composed of human fetal uterine epithelium combined with mouse uterine mesenchyme derived from wild-type or *Esr1* knockout (*Esr1*KO) mice.

2. Materials and methods

2.1. General comments

The Committee on Human Research at UCSF (IRB# 12–08813) approved the collection of human fetal specimens devoid of patient identifiers after elective termination of pregnancy. Fetal age was estimated using heel-toe length (Drey et al., 2005). Gender was determined by Wolffian and Müllerian duct morphology as previously described (Robboy et al., 2017). Female internal genitalia were identified and isolated from the abortus specimen using a dissecting microscope. For this study 10 human fetal specimens were used at 8, 9, 10, 12, 13, 14, and 18 weeks of gestation.

2.2. Response of human fetal grafts of uterine corpus to DES in vivo

Intact human fetal reproductive tracts containing the uterine tube, uterine corpus, uterine cervix and vagina were grown for 4 weeks under the renal capsule of untreated and DES-treated (20 mg DES subcutaneous pellet) of ovariectomized female athymic mice as described previously (Cunha et al., 2017b). Histology and immunohistochemistry for ESR1 and PGR were performed on tissue sections as described below.

2.3. Preparation of heterotypic tissue recombinants

Tissue recombinant studies included: (a) mouse vaginal mesenchyme + human uterine tubal epithelium (mVgM+hTubE) and (b) wild-type or *Esr1*KO mouse uterine mesenchyme + human fetal uterine epithelium (wt UtM+hUtE and *Esr1*KO UtM+hUtE). For mVgM +hTubE tissue recombinants, mouse vaginal mesenchyme was isolated from 3-day-old neonatal mice and the tube epithelium was derived from 12 to 13 week specimens. To explore regulation of human uterine epithelial ESR1 and PGR, uterine mesenchyme was isolated from 5-day-old wild-type and *Esr1*KO neonatal mice and the human uterine epithelium was derived from 10 to 12 week specimens. Heterozygous male and female *Esr1*KO mice, a gift from Drs. Pierre Chambon and Andrée Krust, were bred to produce the *Esr1*KO neonatal mice, which were genotyped as described previously (Dupont et al., 2000). Tissue recombinant and xenografting methods have been described previously (Cunha, 1976; Cunha and Baskin, 2016). For mVgM+hTubE, wt UtM +hUtE and *Esr1*KO UtM+hUtE tissue recombinants, all hosts were ovariectomized at the time of grafting and were treated with a 20 mg DES pellet or were untreated (sham). For hosts bearing mVgM+hTubE tissue recombinants, the rationale for treating the hosts with DES was to promote stratified squamous vaginal differentiation. In response to DES grafts of human fetal uterine tube remain simple columnar, while grafts of human fetal vagina differentiate a thick glycogenated stratified epithelium (Cunha et al., 2017b). Thus, should vaginal differentiation be elicited in human tubal epithelium by mouse vaginal mesenchyme, epithelial differentiation should be distinctive. After 2 or 4 weeks of growth under the renal capsules, the tissue recombinants were

Table 1
Antibodies used in this study.

Antibody #	Source	Catalogue #	Dilution
AR (Androgen receptor)	GeneTax	EPR1535(2)	1/100
ESR1 (Estrogen receptor 1)	Dako	Ab16660	1/100
KRT14 (Keratin 14)	BioGenex	LL002	1/100
KRT19 (Keratin 19)	EB Lane	LP2K	1/100
PGR (Progesterone receptor)	Abcam	Ab16661	1/100
RUNX1 (runt-related transcription factor 1)	Abcam	Ab92336	1/100
TP63 (Tumor protein 63)	Santa Cruz Biotechnology	sc-8343	1/100

harvested, fixed in 10% buffered formalin, embedded in paraffin and serially sectioned at 7 μm. Every twentieth section was stained with hematoxylin and eosin. Remaining paraffin sections were utilized for immunohistochemical staining with the antibodies indicated (1) (Table 1).

Immunostaining was detected using horseradish peroxidase-based Vectastain kits (Vector Laboratories, Burlingame, CA). Negative controls lacked the primary antibody. Tissue recombinants composed of mouse mesenchyme and human epithelium were stained with Hoechst dye 33,258 to verify the species origin of the tissues as described (Cunha and Vanderslice, 1984). This study is based upon the analysis of 18 mVgM+hTubE, 6 wt UtM+hUtE and 5 *Esr1*KO UtM+hUtE tissue recombinants (Table 2).

3. Results

3.1. Epithelial and mesenchymal differentiation in grafts of human fetal female reproductive tracts grown in DES-treated ovariectomized female hosts

3.1.1. General comments

Age is an important factor affecting expression of epithelial differentiation markers (Cunha et al., 2017a). All human fetal specimens used for tissue recombinant studies were 12–13 weeks of gestation old at which time epithelial differentiation markers are beginning to be expressed, well before terminal epithelial differentiation.

3.1.2. Tissue recombinants composed of mouse vaginal mesenchyme plus human uterine tube epithelium (mVgM+hTubE)

Previous studies in mice have shown that differentiation of Müllerian epithelium within the female reproductive tract of the mouse is specified by inductive cues from the mesenchyme (Cunha, 1976; Kurita et al., 2001; Kurita, 2011). To determine whether mesenchymal induction plays a role in differentiation of human Müllerian epithelium, tissue recombinants were prepared with 3-day postnatal mouse vaginal mesenchyme (known to be a vaginal inductor) and human uterine tubal epithelium from 12- to 13-week fetuses. Sections of all six mVgM+hTubE tissue recombinants were screened with Hoechst dye staining to verify that the mesenchyme was mouse and the epithelium was human (Fig. 1), thus eliminating the potential artifact of mouse vaginal mesenchyme contaminated with its own homotypic mouse epithelium.

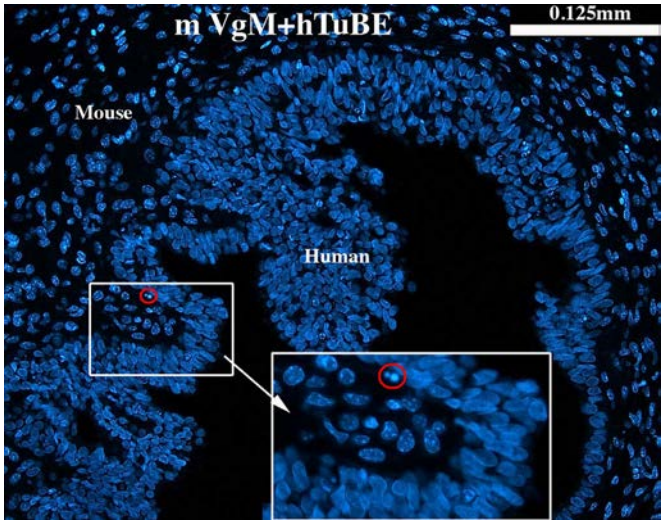

Fig. 1. Section of a tissue recombinant composed of neonatal mouse vaginal mesenchyme plus 13 week human fetal uterine tube epithelium (mVgM+hTubE) stained with Hoechst dye 33258 to verify the species origin of the mesenchyme and epithelium. Mouse nuclei contain many bright chromatin bodies, whereas human nuclei lack such intranuclear bodies (Cunha and Vanderslice, 1984). The red-circled pycnotic nucleus in the lower magnification images can be seen at higher magnification.

Expression of several epithelial differentiation markers (AR, KRT6, TP63 and RUNX1) is distinctly different in the human tubal versus vaginal epithelium (Table 2). Fig. 2 depicts mVgM+hTubE recombinants grown in DES-treated female athymic mouse hosts and indicates that mouse vaginal mesenchyme elicited a partial shift in epithelial histo-differentiation and differentiation marker expression from uterine tubal epithelial differentiation to vaginal epithelial differentiation (Table 2). Expression of some tubal epithelial markers (KRT14 and

Table 2
Epithelial differentiation of human uterine tube, human vagina and mVgM+hTubE tissue recombinants.

Feature	Uterine tube	Vagina	mVgM+hTubE
Simple columnar epithelium	Yes	No	No
Stratified squamous epithelium	No	Yes	Yes
KRT6	No	Yes	Yes
TP63	No	Yes	Yes
Androgen receptor	Yes	No	No
RUNX1	No	Yes	Yes
KRT14	No	Yes	No
KRT19	Yes	No	Yes

Uterine tube epithelial features are shaded red, vaginal epithelial feature are shaded green.

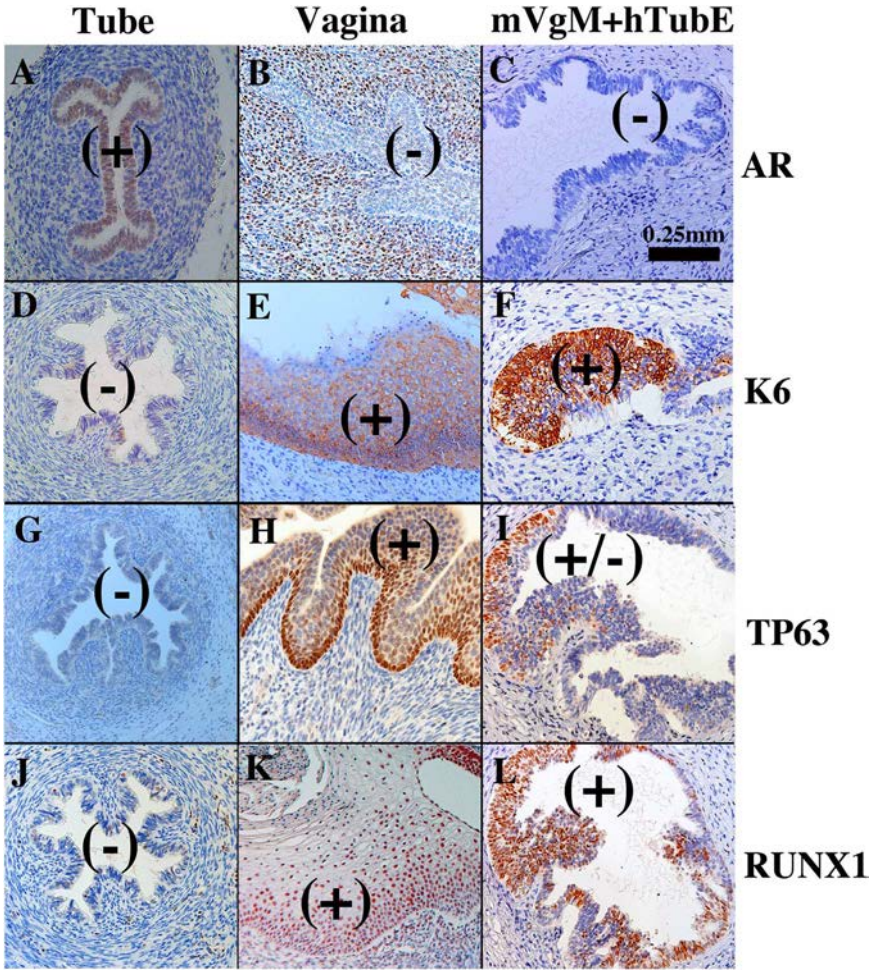

Fig. 2. Tissue recombinants composed of neonatal mouse vaginal mesenchyme plus 13 week human fetal uterine tube epithelium (mVgM+hTubE) grown for 4 weeks in DES-treated hosts and immunostained for various vaginal epithelial markers as indicated. Human uterine tube (A, D, G, J) and vagina (B, E, H, K) at 16–18 weeks of gestation serve as controls. Note induction of KRT6, TP63 and RUNX1 and down regulation of AR in epithelium of the mVgM+hTubE recombinants, indicative of an effect of mouse vaginal mesenchyme on expression of differentiation markers in human tubal epithelium. (+) and (-) indicate epithelial marker expression.

KRT19) were unchanged in mVgM+hTubE recombinants, suggesting that mouse vaginal mesenchyme was able to elicit only a subset of vaginal differentiation markers.

3.1.3. Tissue recombinants composed of epithelium of the human uterine corpus plus wild-type or Esr1KO uterine mesenchyme (wt UtM+hUtE and Esr1KO UtM+hUtE)

The background for this experiment is based upon the ontogeny of ESR1 in the human fetal uterine corpus. From 8 to 13 weeks ESR1 is undetectable in both the epithelium and mesenchyme of the human fetal uterine corpus (Fig. 3A-C). Subsequently, ~14 weeks to 18 weeks the mesenchyme of the human fetal uterine corpus become ESR1-positive (Fig. 3D), while the epithelium remains mostly ESR1-negative with the exception of rare ESR1-positive epithelial cells interspersed within the ESR1-negative uterine epithelial cells (Cunha et al., 2017a). When 10- and 13-week human fetal female reproductive tracts are grown for 4 weeks in DES-treated ovariectomized female hosts, ESR1 was induced in epithelium of the uterine corpus (Fig. 3E & F) (Cunha et al., 2017b). To determine whether DES induction of human fetal uterine epithelial ESR1 is mediated indirectly via mesenchymal ESR1 (paracrine effect) or directly via epithelial ESR1, we prepared tissue recombinants composed of epithelium of the human uterine corpus (hUtE) and uterine mesenchyme from either *Esr1*-positive wild-type mice or *Esr1*-negative *Esr1*KO mice (wt UtM+hUtE and *Esr1*KO UtM +hUtE). For this experiment we used hUtE from 10- and 12-week fetuses, an age before epithelial ESR1 was detectable (see Fig. 3A-C).

wt UtM+hUtE and *Esr1*KO UtM+hUtE tissue recombinants were grown under the renal capsule of female athymic mice that were ovariectomized at the time of grafting and implanted subcutaneously with a 20 mg DES pellet. After 1 month of growth, sections of all tissue recombinants were stained with Hoechst dye 332,598 to verify that the stroma of the harvested grafts was mouse and the epithelium was human (Fig. 4E) as described previously (Cunha and Vanderslice, 1984). ESR1 immunstaining of all tissue recombinants also verified the mesenchymal genotype (Fig. 4A & C): wt UtM+hUtE tissue recombinants contained ESR1-positive mesenchymal cells (Fig. 4A), whereas *Esr1*KO UtM+hUtE tissue recombinants were devoid of ESR1-positive mesenchymal cells (Fig. 4C). wt UtM+hUtE tissue recombinants consistently (6/6) contained an ESR1-positive human uterine epithelium (Fig. 4A). *Esr1*KO UtM+hUtE tissue recombinants also (5/5) contained an ESR1-positive human uterine epithelium even though the surrounding stromal cells were ESR1-negative (Fig. 4C).

To further explore the cellular mechanism of uterine epithelial steroid receptor expression (direct versus paracrine), we examined regulation of the PGR in wt UtM+hUtE and *Esr1*KO UtM+hUtE tissue recombinants. During the time frame of 8–14 weeks of gestation, PGR is undetectable (Fig. 5A-B) in both epithelium and mesenchyme of all organs of the developing human female reproductive tract (Cunha et al., 2017a). However, when human fetal female reproductive tracts were grown in DES-treated ovariectomized female hosts, PGR was induced globally within epithelial and stromal cells throughout the female reproductive tract, especially in the uterine corpus (Fig. 5C)

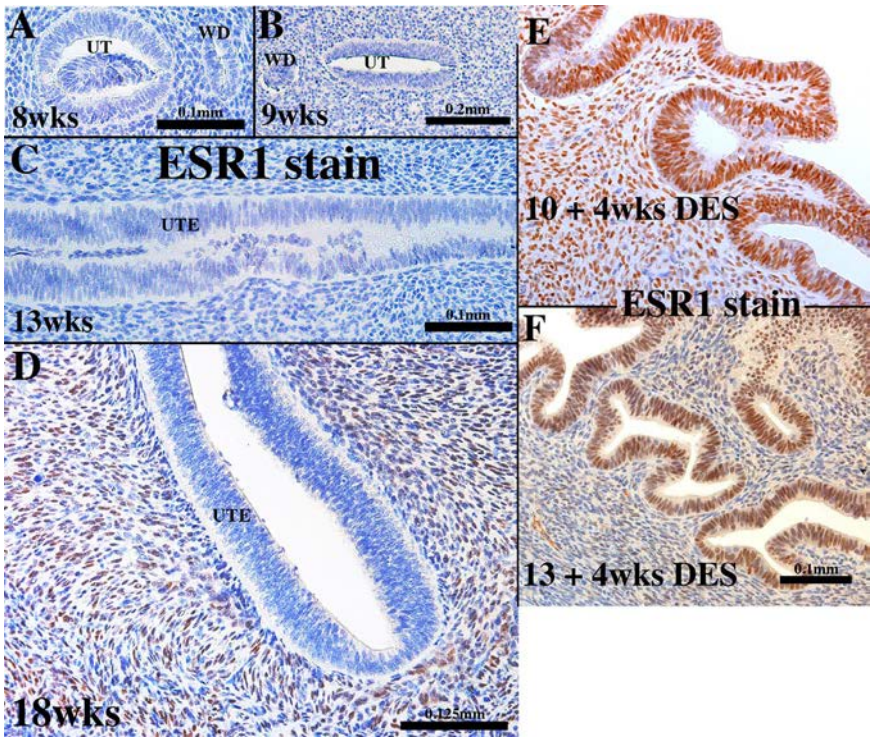

Fig. 3. Sections of developing human fetal uterine corpus immunostained for ESR1 at the ages indicated (A-D). At all stages (8–18 weeks) ESR1 is undetectable in the uterine epithelium. At 18 weeks, the stroma is ESR1-positive. (E & F) are sections of grafts of 10 week (E) and 13 week (F) human fetal uterine corpus grown for 4 weeks in DES-treated ovariectomized hosts and immunostained for ESR1. Note induction of epithelial ESR1.

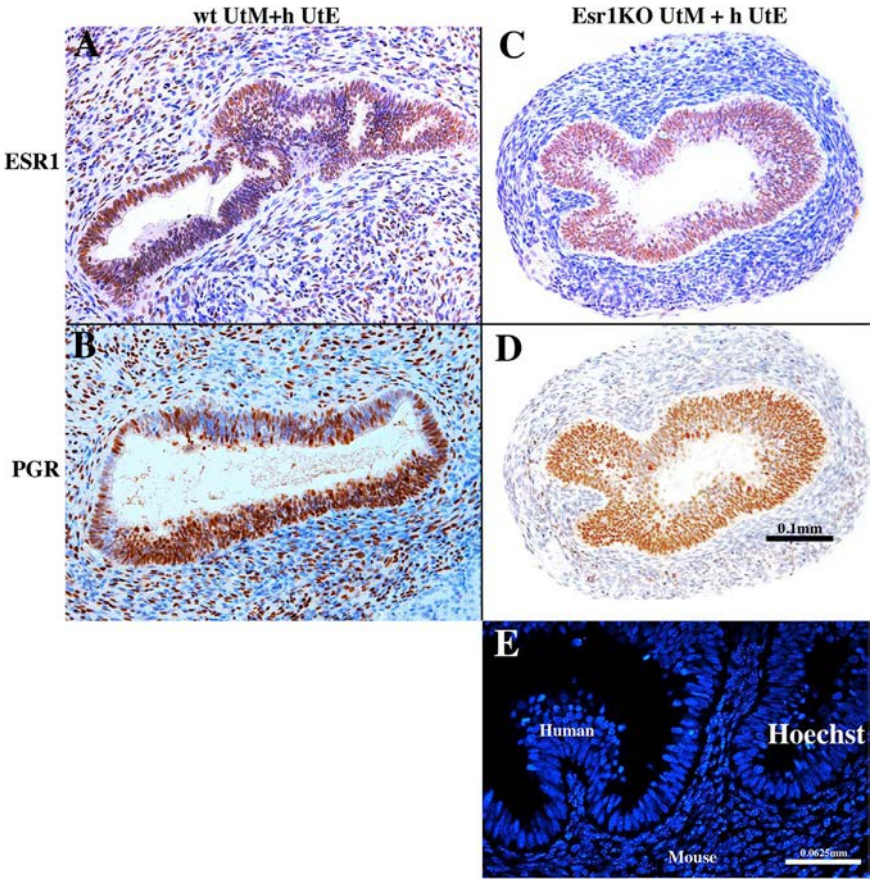

Fig. 4. Tissue recombinants composed of neonatal mouse wild-type uterine mesenchyme plus human fetal uterine tube epithelium (wt UtM+hUtE) (A & B) and αERKO uterine mesenchyme plus human uterine tube epithelium *Esr1*KO UtM+ h UtE) (C & D) grown in DES-treated hosts and immunostained for ESR1 (A & C) and PGR (B & D). DES induced ESR1 and PGR even when the mesenchyme was genetically devoid of ESR1. Sections (C) and (D) are adjacent sections stained for ESR1 (C) and PGR (D). (E) is a section of a graft of *Esr1*KO UtM+hUtE tissue recombinants stained with Hoechst dye 33,258 to verify the tissue origin.

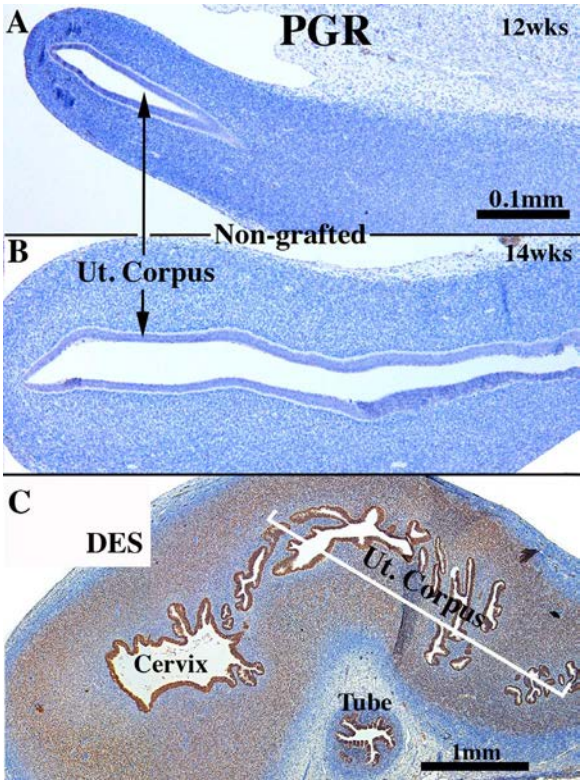

Fig. 5. Sections of non-grafted human female fetal reproductive tracts (A and B) at the ages specified immunostained for PGR. (C) A 13 week human fetal uterine corpus grown for 4 weeks in DES-treated ovariectomized hosts and immunostained for PGR. Note DES induction of epithelial and stromal PGR.

(Cunha et al., 2017b). This observation is in keeping with the established idea that PGR is an estrogen inducible protein (Janne et al., 1975; Horwitz and McGuire, 1979). Epithelial PGR was detected in both wt UtM+ human UtE (Fig. 4B, 6/6) and *Esr1*KO UtM+ human UtE (Fig. 4D, 5/5) tissue recombinants following growth for 1 month in DES-treated hosts, demonstrating the PGR is induced by DES directly via epithelial ESR1.

4. Discussion

Since epithelial differentiation within the female mouse reproductive tract is induced by cues from the mesenchyme (Cunha, 1976), we examined whether this concept also applies to the developing human female fetal reproductive tract through analysis of tissue recombinants composed of mouse vaginal mesenchyme and human tubal epithelium (mVgM+hTubE). The rationale of this experiment was to focus on two organs whose epithelial differentiation is markedly different (uterine tube=simple columnar epithelium versus vagina=stratified squamous epithelium) and whose profile of differentiation markers is also substantially different (see Table 2).

Our current studies extend prior works indicating that mesenchyme of various female reproductive organs induces and specifies the developmental fate of epithelium in laboratory animals. In our current studies neonatal mouse vaginal mesenchyme was grown in association with human fetal epithelium of uterine tubal origin, and the resulting epithelium expressed vaginal epithelial differentiation both morphologically and with vaginal immunohistochemical markers. Thus, mVgM +hTubE tissue recombinants formed a stratified epithelium that expressed KRT6, TP63, RUNX1, while concurrently abolishing expression of AR, an epithelial differentiation marker normally present in human fetal uterine tube epithelium, but not in human vaginal epithelium (Cunha et al., 2017a). However, the epithelium that formed was not perfectly vaginal as a few tubal pattern markers (absence of KRT14 and presence of KRT19) were retained in the partially induced epithelium. There are several reasons for this partial mesenchyme-induced shift in epithelial differentiation: (a) The mVgM+hTubE tissue recombinants were grown for either 2 or 4 weeks in host mice, and this may be insufficient to achieve full vaginal differentiation. Studies of longer duration, e.g., 1–2 months, may be required to achieve full vaginal epithelial differentiation. (b) Use of uterine tubal epithelium younger in age might result in a more complete vaginal epithelial differentiation as age can affect epithelial responsiveness (Cunha, 1976). Nonetheless, simultaneous induction of partial vaginal differentiation coupled with vaginal mesenchyme-induced loss of at least one tubal differentiation marker formally validates the concept that mesenchymal-epithelial interactions play an important role in differentiation within the human female fetal reproductive tract, perhaps via molecular mechanisms similar to those revealed in mouse studies. In any case, the use of mouse/human heterospecific tissue interaction studies, particularly those employing mutant mouse tissues, could be used in the future to dissect molecular mechanisms of human female (and male) reproductive tract development.

The second experiment in this paper deals with the mechanism of estrogenic induction of ESR1 and PGR in human fetal uterine epithelium, that is, whether DES elicits uterine epithelial ESR1 and PGR (a) directly via epithelial ESR1 or (b) indirectly (paracrine mechanism) via ESR1 in adjacent uterine mesenchyme. This question becomes even more interesting in so far as ESR1 was undetectable by immunohistochemistry in the human fetal uterine corpus at the age of the human uterine epithelium used to prepare the tissue recombinant (Fig. 3).

The background and rationale for this experiment is that ESR1 and PGR, whose expression is normally undetectable in second trimester human fetal uterine epithelium (Cunha et al., 2017a), were induced by DES treatment (Cunha et al., 2017b). Estrogen is known to up-regulate ESR1 mRNA and protein (Read et al., 1989; Saceda et al., 1988) and PGR (Janne et al., 1975; Horwitz and McGuire, 1979) via binding of liganded ESR1 to estrogen-response elements associated in the genes encoding ESR1 and PGR (Liu et al., 2003; Mehta et al., 2016).

We found that uterine epithelial ESR1 and PGR were induced by DES in *Esr1*KO UtM+hUtE tissue recombinants (devoid of mesenchymal ESR1), which formally eliminates the paracrine mechanism and demonstrates that DES induction of human uterine epithelial ESR1 and PGR is directly mediated via epithelial ESR1. With respect to estrogen-induced epithelial PGR in human uterine epithelium, we have previously addressed this question with heterospecific tissue recombinants consisting of adult human uterine epithelium and *Esr1*KO UtM. However, our earlier study was inconclusive as the *Esr1*KO mouse strain utilized for the previous study expressed a truncated form of *Esr1* in UtM (Kurita et al., 2005b). Since the *Esr1*KO mice utilized in the current study are truly *Esr1* null (Dupont et al., 2000), regulation of PGR in human UtE unquestionably does not require mesenchymal *Esr1*. Thus, epithelial PGR was prominently expressed *Esr1*KO UtM+ human UtE tissue recombinants (5/5) having *Esr1*-negative mesenchyme even though *Esr1*was undetectable in the human fetal uterine epithelium used to construct the *Esr1*KO UtM+ human UtE tissue recombinants. DES-induction of ESR1 in human fetal uterine epithelium was also observed in *Esr1*KO UtM+ human UtE tissue recombinants suggesting that estrogenic induction of ESR1 is a direct effect mediated by epithelial *Esr1*. It is perhaps worth noting that the design of this experiment does not exclude a possible role of mesenchymal ESR2.

It is perhaps worth noting that the mechanism of regulating uterine epithelial PGR in mice is vastly different than that in human. In mice uterine epithelial PGR is strongly expressed in untreated ovariectomized mice and is profoundly down regulated upon administration of estrogen, and effect that is mediated indirectly via stromal ESR1 (paracrine mechanism) (Kurita et al., 2000). This finding in mice contrasts strikingly with the direct estrogenic induction of PGR in human cells and tissues.

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