Spring 2024 – 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 8, 2024
Week 5

Female Reproductive Tract Development & Function

Primary Papers:

1. Martin, et al. (2022) J Dairy Sci, 105(10):8189-8198.
2. Du & Taylor (2015) CSH Persp Medicine, 6:a023002.
3. Major, et al. (2021) Biol Reprod, 1-15, ioab166.

Discussion

Student 10: Contemporary Paper-Ref #1 above

- What are the organs of the female reproductive tract examined?
- What methods and computational approach was used?
- What aspects of the tract were important and why?

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 1: Contemporary Paper-Ref #3 above

- What evo-devo approach for female reproductive tract was used?
- What transcription genes involved were discussed?
- What conserved processes are observed in female reproductive tract development?

J. Dairy Sci. 105:8189–8198
https://doi.org/10.3168/jds.2021-21651

Reproductive tract size and position score: Estimation of genetic parameters for a novel fertility trait in dairy cows

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ABSTRACT

The dairy industry is moving toward selecting animals with better fertility to decrease the economic losses linked to reproductive issues. The reproductive tract size and position score (SPS) was recently developed in physiological studies as an indicator of pregnancy rate and the number of services to conception. Cows are scored as SPS 1, 2, or 3 based on the size of their reproductive tract and its position in the pelvis, as determined by transrectal palpation. The objective of this study was to estimate genetic parameters for SPS to assess its potential as a novel fertility trait. Phenotypes were collected at the University of British Columbia's research herd from 2017 to 2020, consisting of 3,247 within- and across-lactation SPS records from 490 Holstein cows. A univariate animal model was used to estimate the variance components for SPS. Both threshold and linear models were fit under a Bayesian approach and the results were compared using the Spearman rank correlation (r) between the estimated breeding values. The 2 models ranked the animals very similarly (r = 0.99), and the linear model was selected for further analysis. Genetic correlations with other currently evaluated traits were estimated using a bivariate animal model. The posterior means (± posterior standard deviation) for heritability and repeatability within- and across-lactation were 0.113 (± 0.013), 0.242 (± 0.012), and 0.134 (± 0.014), respectively. The SPS showed null correlations with production traits and favorable correlations with traditional fertility traits, varying from −0.730 (nonreturn rate) to 0.931 (number of services). Although preliminary, these results are encouraging because SPS seems to be more heritable than and strongly genetically correlated with number of services, nonreturn rate, and first service to conception, indicating potential for effective indirect selection response on these traits from SPS genetic selection. Therefore, further studies with larger data sets to validate these findings are warranted.
Key words: Holstein cow, variance component estimation, genetic correlation, reproductive tract score

INTRODUCTION

Suboptimal reproductive performance is a major contributor to economic losses in the dairy industry worldwide. The main concern is decreased female fertility leading to longer lactation and an increased number of involuntary cullings (Giordano et al., 2011). In Canada, reproductive problems have been the main cause of removal from the herd in dairy cattle, representing more than 30% of involuntary cullings (Van Doormaal, 2009; OMAFRA, 2021).

Selecting cows with superior genetic merit for fertility is a long-term solution to counteract reproductive decline. Despite the efforts made toward improving reproduction, the results are still below the level needed (Miglior et al., 2017; Fleming et al., 2019). The most cited problem is the low heritability of current fertility traits (Fleming et al., 2019), often used as justification for the low weight assigned to them in breeding goals (Berry et al., 2016; Miglior et al., 2017). However, the main underlying concern is the negative genetic correlation between production and fertility leading to an economic impasse (Pryce et al., 2014; Miglior et al., 2017). Since the 1960s, this antagonistic correlation has been controversial because of the lack of corresponding evidence of a biological link between milk production and fertility (Miglior et al., 2017). The notion was that coupling intense selection for increased milk production with improvements in herd management would suffice to counterbalance the genetic deterioration of functional traits, whereas others have argued that overly intensive selection on yield was an important factor in reproduc-

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tive decline (Pryce et al., 2014; Butler and Moore, 2018). Since the negative effect of intensive selection for production traits on reproductive physiology became more commonly accepted, fertility traits have been included in national selection indices (Miglior et al., 2017). However, the underlying physiological factors affecting the reproductive system and their genetic background are still largely unknown, leading to difficulty in collecting accurate and high-quality phenotypes and preventing rapid progress with genetic selection (Fleming et al., 2019). Despite this, high-producing cows do not always exhibit poor fertility, and high milk production is not necessarily a feature of low fertility (Britt, 1992; Bello et al., 2013; LeBlanc, 2013).

Historically, fertility traits were chosen based on their simplicity and ease of measurement on a large scale (Berry et al., 2016; Fleming et al., 2019). Fertility is a complex phenotype and currently recorded traits are strongly affected by the environment and management practices. This explains why most evaluated fertility traits have low heritability but large genetic variation, which indicates good potential for selection (Miglior et al., 2017).

Recently, a novel fertility trait has been described, based on the morphology of the reproductive tract that directly relates to the fertility status of the animal (Young et al., 2017; Madureira et al., 2020). This trait consists of categorizing the female reproductive tract by transrectal palpation. As shown in Figure 1, animals are classified into 3 groups depending on the size and position score (**SPS**) of their reproductive tract, where SPS 1 describes cows with a small and compact uterus and uterine horns resting entirely on the pelvis; SPS 2 cows have a uterus of medium size with longer uterine horns resting partially outside of the pelvic cavity; and SPS 3 cows have a large reproductive tract mostly outside of the pelvic cavity.

These scores have been associated with common indicators of fertility, such as pregnancy rate and the number of services to pregnancy, in physiological studies in dairy cattle (Young et al., 2017; Madureira et al., 2020). Lower SPS scores are favorably associated with higher pregnancy rate, lower number of services per pregnancy, and lower pregnancy loss. Cows show substantial variation in the SPS score at breeding time, which is also observed across different parities (Young et al., 2017). Thus, SPS may provide a new fertility trait that has the advantage of being morphological, which is commonly accepted as being affected by fewer environmental factors and, therefore, having potentially higher heritability than current fertility traits. Our objectives were to estimate the genetic parameters of SPS using both threshold and linear models, and to estimate the genetic correlations between SPS and other economically important traits.

MATERIALS AND METHODS

Data

Phenotypes for SPS were collected at the University of British Columbia research herd (Agassiz, Canada), as part of a physiological study (Madureira et al., 2020). A total of 3,247 records within and across lactations on 490 lactating Holstein cows scored from 2017 to 2020 were included in this study. Cows were scored by transrectal palpation from calving to conception. Details on cow management and SPS measurement are provided in Madureira et al. (2020). The corresponding pedigree file was provided by Lactanet (Guelph, ON, Canada) and consisted of 23,275 animals with an average depth of 4.6 generations. The Lifetime Profit Index (**LPI**) and Pro$ index values for the cows with SPS records were also provided by Lactanet. All phenotypic and

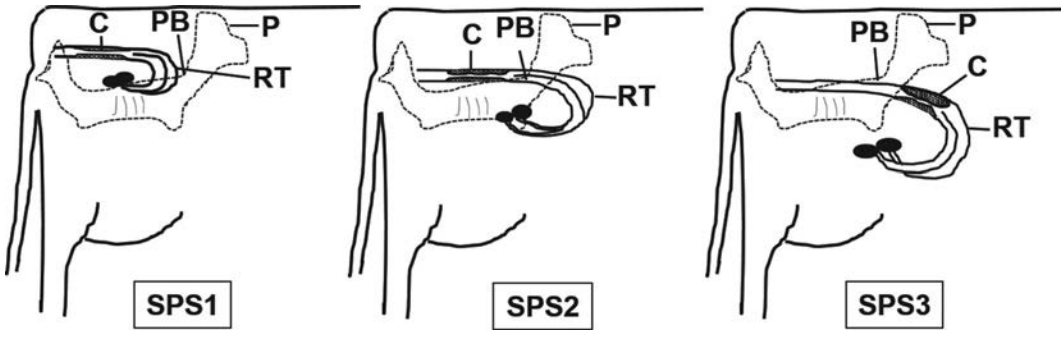

Figure 1. Assessment of the reproductive tract size and position score (SPS). Reproductive tracts positioned entirely within the pelvic cavity were designated SPS 1. Reproductive tracts in which the cervix was within the pelvic cavity, but uterine horns were outside the pelvic cavity, were designated SPS 2. Reproductive tracts in which the cervix and uterine horns lay outside the pelvic cavity were designated SPS 3 (from Young et al., 2017). C = cervix; P = pelvis; RT = reproductive tract; PB = pelvic brim; SPS = size and position score.

Table 1. Number of records of the reproductive tract size and position score (SPS)[1] by parity and stage of lactation from 490 Holstein cows over 3 yr in 1 herd

Parity	Stage of lactation[2]	Number of records			
		SPS 1	SPS 2	SPS 3	Total
1	1	10	33	22	65
	2	227	353	56	636
	3	35	116	10	161
	4	2	13	3	18
2	1	1	25	26	52
	2	109	366	51	526
	3	33	163	44	240
	4	2	21	14	37
3	1	0	15	13	28
	2	48	224	71	343
	3	16	106	33	155
	4	2	8	8	18
4	1	0	5	12	17
	2	18	149	70	237
	3	4	102	52	158
	4	0	15	10	25
5+	1	1	15	19	35
	2	16	208	72	296
	3	7	103	65	175
	4	3	15	7	25
	Total	534	2,055	658	3,247

[1]SPS 1 was attributed to cows with small reproductive tract positioned entirely within the pelvic cavity; SPS 2 designated cows with a reproductive tract in which the cervix was within the pelvic cavity, but uterine horns were outside the pelvic cavity; SPS 3 was attributed to cows with large reproductive tract lying outside the pelvic cavity.

[2]Stage of lactation (1–4) was defined as 1 to 21, 22 to 120, 121 to 240, and >240 DIM, corresponding to uterine involution, early lactation, mid-lactation, and late lactation, respectively.

pedigree data used in this study were obtained from pre-existing databases. Therefore, no animal care committee approval was necessary for the purposes of this study.

Data editing and formatting were done with R software (R Core Team, 2018) using the "nadiv" package for pedigree handling (Wolak, 2012) and the "dplyr" package for data formatting (Wickham et al., 2021). The 120-d periods for the stages of lactation were adapted for our data set by adding a uterine involution period, when the size of the reproductive tract is naturally unstable. Based on the available measurements, the uterine involution, early lactation, mid-lactation, and late lactation periods were defined as 1 to 21, 22 to 120, 121 to 240, and >240 DIM, respectively. The seasons were based on the recording date by defining October to March as the cold season and April to September as the warm season.

The distribution of the trait records by parity and stage of lactation is presented in Table 1. Of 490 cows, 213 cows (43%) had multiple records within one lactation, 5 cows (1%) had multiple single records across lactations, 243 cows (50%) had multiple records both within and across lactations, and 29 cows (6%) had a single record. On average, cows had 4 records, with a maximum of 24 records.

Genetic correlations between SPS and 13 currently evaluated traits, chosen based on their economic importance and biological relevance, were estimated. The chosen traits were categorized as follows: (1) production traits: milk, fat, and protein yields; (2) conformation traits: BCS, thurl placement, and rump angle; (3) fertility traits: age at first service, nonreturn rate at 56 d, first service to conception, calving to first service, days open, number of services; and (4) calving traits: calving ease. All trait phenotypes were provided by Lactanet and did not contain any missing records.

For conformation traits and age at first service, only one record per cow was available. For the other traits, only records from the corresponding lactation when SPS was recorded were used.

Models

Single-Trait Linear Model. The following univariate linear repeatability animal model was used to estimate variance components of SPS:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Z}_{\mathbf{a}}\mathbf{a} + \mathbf{Z}_{\mathbf{pe_w}}\mathbf{pe_w} + \mathbf{Z}_{\mathbf{pe_a}}\mathbf{pe_a} + \mathbf{e},$$

where $\mathbf{y}$ is a vector of SPS phenotypes (3 scores); $\mathbf{b}$ is a vector of fixed effects including year-season (7 levels), stage of lactation (4 levels), and linear and quadratic regression on lactation number (1, 2, 3, 4, and 5+); $\mathbf{a}$ is a vector of random animal additive genetic effects; $\mathbf{pe_w}$ is a vector of random within-lactation permanent environmental effects; $\mathbf{pe_a}$ is a vector of across-lactation permanent environmental effects; and $\mathbf{e}$ is a vector of random residual effects; $\mathbf{X}$, $\mathbf{Z}_{\mathbf{a}}$, $\mathbf{Z}_{\mathbf{pe_w}}$, and $\mathbf{Z}_{\mathbf{pe_a}}$ are corresponding incidence matrices. The random effects were assumed normally distributed as follows:

$$\begin{bmatrix} \mathbf{a} \\ \mathbf{pe_w} \\ \mathbf{pe_a} \\ \mathbf{e} \end{bmatrix} \sim \mathrm{N}\left[\begin{pmatrix} 0 \\ 0 \\ 0 \\ 0 \end{pmatrix}, \begin{pmatrix} \mathbf{A}\sigma^2_{\mathrm{a}} & 0 & 0 & 0 \\ 0 & \mathbf{I}\sigma^2_{\mathrm{pe_w}} & 0 & 0 \\ 0 & 0 & \mathbf{I}\sigma^2_{\mathrm{pe_a}} & 0 \\ 0 & 0 & 0 & \mathbf{I}\sigma^2_{\mathrm{e}} \end{pmatrix}\right],$$

where $\mathbf{A}$ is the numerator relationship matrix, σ^2_{a} is the additive genetic variance, $\mathbf{I}$ is an identity matrix, $\sigma^2_{\mathrm{pe_w}}$ is the within-lactation permanent environmental vari-

ance, $\sigma^2_{pe_a}$ is the across-lactations permanent environmental variance, and σ^2_e is the residual variance.

The final model was defined by back-selection of all fixed effects, keeping only the significant ones ($P < 0.05$). Calving score and the incidence of uterine disease were not included in the model due to the lack of this information for most cows with an SPS score, but they might contribute to the variation of SPS score and should be considered in future studies.

Single-Trait Threshold Model. Due to the novelty of the trait, threshold and linear models were both fitted for comparison. Theoretically, the threshold model is advantageous because it respects the categorical nature of the trait by fitting an appropriate non-Gaussian distribution. However, the linear model is usually preferred because it is both less complex and less computationally demanding (Meijering and Gianola, 1985). The literature also indicates that the model fit and animal ranking do not significantly differ when a linear model is used to analyze categorical data (e.g., Jamrozik et al., 2005; Negussie et al., 2008; Neuenschwander et al., 2012).

The observed phenotype is assigned to categories (1, 2, or 3) based on a latent trait called liability (l), which is assumed to be normally distributed (de Villemereuil, 2018). The following repeatability univariate threshold model was used:

$$\mathbf{l} = \mathbf{Xb} + \mathbf{Z_a a} + \mathbf{Z_{pe_w} pe_w} + \mathbf{Z_{pe_a} pe_a} + \mathbf{e},$$

where $\mathbf{l}$ is a vector of underlying liabilities corresponding to the categorical observations in $\mathbf{y}$ (1, 2, or 3), and the other terms are as previously defined.

Genetic Parameters

The (co)variance components were estimated using Gibbs sampling implemented in THRGIBBS1F90 software (Misztal et al., 2002). The analysis consisted of a single chain of 3,050,000 cycles, with the first 50,000 being discarded as a burn-in period. A long thinning interval of 3,000 cycles was used to guarantee minimization of the autocorrelation between consecutive samples. Convergence was assessed by visual inspection of the trace plots of each estimated variance component.

The Spearman's rank correlation between the EBV from linear and threshold models was used to determine whether the models would rank the animals similarly. For the threshold model, estimates from the underlying scale were used for ranking purposes.

The heritability (h^2) for SPS was calculated from the single-trait model as follows:

$$h^2 = \frac{\sigma^2_a}{\sigma^2_a + \sigma^2_{pe_w} + \sigma^2_{pe_a} + \sigma^2_e},$$

where σ^2_a, $\sigma^2_{pe_w}$, $\sigma^2_{pe_a}$, and σ^2_e were as previously defined.

Repeatability within lactation ($\mathbf{r_w}$) was calculated as follows:

$$r_w = \frac{\sigma^2_a + \sigma^2_{pe_w} + \sigma^2_{pe_a}}{\sigma^2_a + \sigma^2_{pe_w} + \sigma^2_{pe_a} + \sigma^2_e},$$

and the repeatability across lactations ($\mathbf{r_a}$) as

$$r_a = \frac{\sigma^2_a + \sigma^2_{pe_a}}{\sigma^2_a + \sigma^2_{pe_w} + \sigma^2_{pe_a} + \sigma^2_e}.$$

The expected heritability estimates $\left(h^2_n\right)$ for the average values when considering a different number of records per animal (from 2 to 10) were calculated as follows:

$$h^2_n = \frac{h^2 \times n}{1 + (n-1) \times r},$$

where n is the number of records per animal, and h^2 and r are the heritability and repeatability (either across or within lactation) estimated from the single-trait model.

Genetic Correlations

Bivariate animal models were used to estimate the genetic correlations between traits. The same previously defined linear model was used for SPS. For the other 13 chosen traits, the models were based on work by Oliveira Junior et al. (2021). The only modifications made were the removal of the herd effect and the addition of random across-lactation permanent environmental effects to fit the across-lactation repeated records of the production and reproduction traits. The model used for each trait is presented in Table 2.

A general description of the linear models used in the 2-trait analyses is as follows:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Z_a a} + \mathbf{Z_{pe_w} pe_w} + \mathbf{Z_{pe_a} pe_a} + \mathbf{e},$$

where $\mathbf{y}$ is a vector of observations; $\mathbf{b}$ is a vector of fixed effects; $\mathbf{a}$ is a vector of random animal additive genetic effects; $\mathbf{pe_w}$ is a vector of random within-lactation permanent environmental effects; $\mathbf{pe_a}$ is a vector of across-lactation permanent environmental effects; $\mathbf{e}$ is a

vector of random residual effects; and $\mathbf{X}$, $\mathbf{Z_a}$, $\mathbf{Z_{pe_w}}$, and $\mathbf{Z_{pe_a}}$ are corresponding incidence matrices.

For traits without any repeated records (conformation traits and age at first service), permanent environmental effects were not included in the model. For traits with only repeated records across lactation (production and other fertility traits), the within-lactation permanent environmental effect was not included in the model.

The variance-covariance matrices were as follows:

$$\begin{bmatrix} \mathbf{a} \\ \mathbf{pe_w} \\ \mathbf{pe_a} \\ \mathbf{e} \end{bmatrix} \sim \text{MVN}\left[\begin{pmatrix} 0 \\ 0 \\ 0 \\ 0 \end{pmatrix}, \begin{pmatrix} \mathbf{G}\otimes\mathbf{A} & 0 & 0 & 0 \\ 0 & \mathbf{P_w}\otimes\mathbf{I} & 0 & 0 \\ 0 & 0 & \mathbf{P_a}\otimes\mathbf{I} & 0 \\ 0 & 0 & 0 & \mathbf{R}\otimes\mathbf{I} \end{pmatrix}\right],$$

where $\mathbf{G}$ is the covariance matrix between traits for random additive genetic effects, $\mathbf{P_w}$ is the within-lactation permanent environmental covariance matrix, $\mathbf{P_a}$ is the across-lactation permanent environmental covariance matrix, $\mathbf{R}$ is the residual covariance matrix between traits, $\mathbf{A}$ is the additive relationship matrix, $\mathbf{I}$ is an identity matrix, and MVN indicates multivariate normal distribution.

The additive genetic correlations ($\mathbf{r_g}$) were calculated as follows:

$$r_g = \frac{cov_a(t_1, t_2)}{\sqrt{\sigma^2_{a_1} \times \sigma^2_{a_2}}},$$

where cov_a is the additive genetic covariance between trait 1 and trait 2 (t_1, t_2), and $\sigma^2_{a_1}$ and $\sigma^2_{a_2}$ are the additive genetic variances for trait 1 and trait 2, respectively.

The phenotypic correlations ($\mathbf{r_p}$) were calculated as follows:

$$r_p = r_g\sqrt{h^2_1 h^2_2} + r_{pe_a}\sqrt{pe^2_{a_1} pe^2_{a_2}} + r_e\sqrt{e^2_1 e^2_2},$$

where r_g, r_{pe_a}, and r_e are additive genetic, across-lactation permanent environmental, and residual correlations between traits, respectively. For trait i, $h^2_i = \sigma^2_{a_i}/\sigma^2_{p_i}$, $pe^2_{a_i} = \sigma^2_{pe_{a_i}}/\sigma^2_{p_i}$, and $e^2_i = \sigma^2_{e_i}/\sigma^2_{p_i}$, where $\sigma^2_{a_i}$ is the additive genetic variance, $\sigma^2_{pe_{a_i}}$ is the across-lactation permanent environmental variance, $\sigma^2_{e_i}$ is the residual variance, and $\sigma^2_{p_i}$ is the phenotypic variance, estimated as $\sigma^2_{a_i} + \sigma^2_{pe_{w_i}} + \sigma^2_{pe_{a_i}} + \sigma^2_{e_i}$. Either or both $\sigma^2_{pe_{w_i}}$ and $\sigma^2_{pe_{a_i}}$ were excluded in the calculation of $\sigma^2_{p_i}$ for traits without repeated records within or across lactation, respectively. As only SPS had repeated records

Table 2. Single-trait animal models[1] for currently selected traits used in the bivariate analyses for the estimation of the correlations between these traits and the reproductive tract size and position score

Trait[2]	Fixed effect[3]			Random effect[4]
Conformation	Round classifier	Age calving-stage lactation-round		
BCS	X	X		
Thurl placement	X	X		
Rump angle	X	X		
Production	Year-season calving	Age calving		Pe_a
Milk yield	X	X		X
Protein yield	X	X		X
Fat yield	X	X		X
Fertility	Year born-month born	Age prev calving-month first service	Age prev calving-month prev calving	Pe_a
Age at first service	X			
Nonreturn rate at 56 d	X	X		X
First service to conception	X		X	X
Calving to first service	X		X	X
Days open	X			X
Number of services	X	X		X
Calving	Year born-month born	Age curr calving-month curr calving-calf sex	Calf sire	Pe_a
Calving ease	X	X	X	X

[1]Models adapted from Oliveira Junior et al. (2021).

[2]All traits are cow-related, except age at first service, which is a heifer trait.

[3]Prev = previous; curr = current.

[4]Pe_a = across-lactation permanent environmental effect.

Table 3. Posterior mean, posterior standard deviation (PSD), and highest posterior density (HPD) interval of the genetic parameters resulting from the linear animal model for the reproductive size and position score, estimated on records from 490 Holstein cows over 3 yr in 1 herd

Parameter[1]	Mean	PSD	Low HPD	High HPD
σ^2_p	0.323	0.004	0.316	0.330
σ^2_a	0.037	0.004	0.029	0.043
σ^2_e	0.245	0.004	0.238	0.251
$\sigma^2_{pe_w}$	0.035	0.004	0.000	0.028
$\sigma^2_{pe_a}$	0.007	0.002	0.000	0.003
h^2	0.113	0.013	0.093	0.135
r_w	0.242	0.012	0.223	0.262
r_a	0.134	0.014	0.109	0.156

[1]Where σ^2_p = phenotypic variance; σ^2_a = additive genetic variance; σ^2_e = residual variance; $\sigma^2_{pe_w}$ = within-lactation permanent environmental variance; $\sigma^2_{pe_a}$ = across-lactation permanent environmental variance; h^2 = heritability; r_w = within-lactation repeatability; r_a = across-lactation repeatability.

within lactation, the within-lactation permanent environmental correlation $\left(r_{pe_w}\right)$ did not contribute to any r_p.

For all parameters, the posterior mean, posterior standard deviation (**PSD**), and 95% highest posterior density were calculated within the R software (R Core Team, 2018) based on the output of the THRGIBBS1F90 software (Misztal et al., 2002). All parameters were calculated within each of the 1,000 samples and then averaged to obtain the final estimates.

Pearson correlations between EBV for SPS and both LPI and Pro$ were used to evaluate the association of the novel trait with the current selection indices.

RESULTS AND DISCUSSION

Heritability and Repeatability Estimates

The difference between linear and threshold models is expected to increase for a combination of factors: (1) heritability is low, (2) there is a small number of phenotypic categories, and (3) there is a small number of records (Meijering and Gianola, 1985; Mrode, 2014). The Spearman rank correlation between the EBV from the 2 models was close to 1 (0.99). Based on this very similar ranking, the linear model was used for further analyses. The estimated variances, heritability, and repeatabilities from the linear model are presented in Table 3, and the same estimates from the threshold model are given in Supplemental Table S1 (https://zenodo.org/record/6925896#.YuKfsXbMJhE; Martin et al., 2022). Only estimates from the linear model will be presented and discussed hereafter. The estimated heritability, within-lactation repeatability, and across-lactation repeatability were 0.113, 0.242, and 0.134, respectively. The estimated heritability for SPS was, therefore, considerably higher than that of any fertility traits currently evaluated in dairy cattle in Canada (see Oliveira Junior et al., 2021).

These results are preliminary, as they are based on a small sample of cows from one research herd. The current data set was created to study the association of SPS with cow fertility over time and, to this end, there was no specific time window for SPS recording. The phenotypes were recorded from calving until the confirmation of conception, which could happen late in lactation for some cows. This large time window of collection, combined with the sparse repeated records, might explain the low estimated repeatability of SPS (Table 3). A more precisely defined phenotyping protocol is needed to improve SPS repeatability. In further studies, phenotypes should be assessed after uterine involution, which generally ends around 30 d after calving, and before the establishment of the next pregnancy. By doing so, the natural—but here undesirable—variation due to uterine involution could be removed.

Another point for improvement could be the definition of the trait itself. The size of the reproductive tract is a continuous trait that has been assigned to 3 ordinal categories (scores). The small number of categories may affect the repeatability of the score. A cow with a score close to the threshold between 2 categories could oscillate between 2 scores without a meaningful difference in the actual size and position of the reproductive tract. With only 3 categories, these small changes may represent a large variation between 2 records within the genetic analysis. Therefore, the repeatability of both across- and within-lactation records observed in this study may have been reduced by the long period of collection and the small number of categories for scoring SPS. Adding more categories to SPS could be beneficial to its genetic estimation. However, it would slightly increase the difficulty and possibly the precision of recording of this trait.

The repeatability of the trait indicates the upper limit of the heritability and the number of records per animal necessary to reach it (Falconer and Mackay, 1996). The expected increase in the heritability of the average from different numbers of repeated records across and within lactation for SPS is shown in Figure 2. Measuring an animal twice, either within or across lactations, would potentially double the heritability of the average SPS. With 5 records per animal, most of the potential increase would be captured but that may be unrealistic in practice.

A strategy for large-scale SPS phenotyping would be to measure SPS at the time of insemination. Insemina-

Table 4. Posterior mean, posterior standard deviation (PSD), and highest posterior density (HPD) interval of the genetic correlations resulting from the bivariate animal model between reproductive tract size and position score (SPS) and currently selected traits, estimated on records from 490 Holstein cows over 3 yr in 1 herd

	Genetic correlation		
Trait	Posterior mean (± PSD)	Low HPD	High HPD
Conformation			
BCS	0.632 (± 0.084)	0.494	0.772
Thurl placement	−0.263 (± 0.118)	−0.450	−0.072
Rump angle	−0.739 (± 0.072)	−0.866	−0.636
Production			
Milk yield	0.047 (± 0.074)	−0.079	0.167
Protein yield	−0.026 (± 0.088)	−0.171	0.115
Fat yield	0.042 (± 0.091)	−0.106	0.187
Fertility			
Age at first service	0.444 (± 0.164)	0.158	0.693
Nonreturn rate at 56 d	−0.730 (± 0.093)	−0.876	−0.582
First service to conception	0.694 (± 0.115)	0.528	0.897
Calving to first service	−0.371 (± 0.206)	−0.724	−0.066
Days open	0.435 (± 0.246)	0.035	0.805
Number of services	0.931 (± 0.029)	0.889	0.976
Calving			
Calving ease	0.061 (± 0.233)	−0.315	0.437

tion technicians could be trained to record SPS during their daily work. This would give access to reliable and large data sets without the need for large investments. However, this strategy may be biased if repeated records are used. The SPS phenotype would be repeated within lactation for animals requiring multiple breedings to reach conception, leading to a biased sample. Multiple SPS records per cow would be advantageous, but the definition of the time window for collection needs to be investigated further.

Genetic and Phenotypic Correlations

Knowledge of the genetic correlation between SPS and other economically important traits is important to assess the potential effect of selecting for SPS. These correlations are presented in Table 4. All estimates had a relatively high PSD due to the small size of the data set, but the point estimates were promising, as they were favorable in magnitude and direction. For instance, the correlations of SPS with milk, fat, and protein yields were close to zero and statistically not different from zero, meaning SPS could be selected without affecting production traits. This is direct contrast with currently evaluated fertility traits, which largely present unfavorable correlations with production (Oliveira Junior et al., 2021).

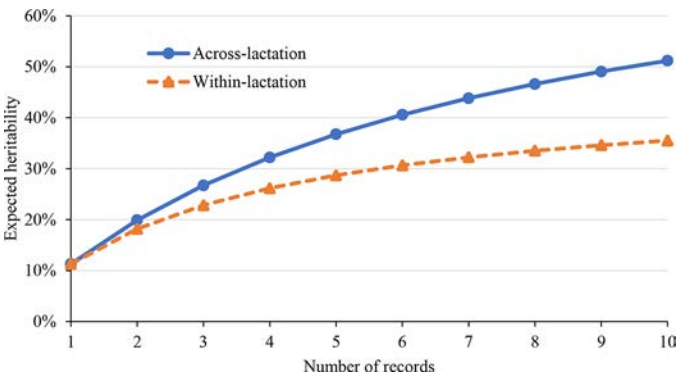

Figure 2. Change in expected heritability of reproductive tract size and position score with an increased number of repeated measurements per cow across and within lactation.

The high genetic correlation between SPS and BCS (0. 632) is noteworthy. Even though BCS is known as an indicator of fertility, the underlying mechanism is not clearly defined (Berry et al., 2016; Miglior et al., 2017; Lucy, 2019). Interestingly, Madureira et al. (2020) reported no association between SPS and BCS at the phenotypic level. We reached the same conclusion, with a nonsignificant phenotypic correlation between the 2 traits (see Table 5). Moreover, Baez et al. (2016) found that cows with a smaller uterine volume, a trait similar to SPS, had a greater pregnancy per insemination than those with a larger uterine volume, regardless of BCS. When Baez et al. (2016) compared the uterine volume within each category of the BCS scale, the relationship appeared to be stronger in thinner cows. This reflects the complex relationship between SPS and BCS, which may not be properly captured by a linear correlation, as BCS is an intermediate optimum trait; a quadratic correlation may be more relevant to study this relationship. A possible explanation for the positive genetic correlation between SPS and BCS could be the number and size of the adipocytes around and within the

Table 5. Posterior mean, posterior standard deviation (PSD), and highest posterior density (HPD) interval of the phenotypic correlations resulting from the bivariate animal model between reproductive tract size and position score (SPS) and currently selected traits, estimated on records from 490 Holstein cows over 3 yr in 1 herd

	Phenotypic correlation		
Trait	Posterior mean (± PSD)	Low HPD	High HPD
Conformation			
BCS	0.180 (± 0.023)	0.001	0.145
Thurl placement	−0.101 (± 0.025)	−0.088	−0.220
Rump angle	−0.119 (± 0.024)	−0.155	−0.079
Production			
Milk yield	−0.006 (± 0.018)	−0.034	0.024
Protein yield	−0.018 (± 0.018)	−0.046	−0.013
Fat yield	−0.000 (± 0.018)	−0.030	0.028
Fertility			
Age at first service	0.045 (± 0.024)	0.006	0.085
Nonreturn rate at 56 d	−0.049 (± 0.019)	−0.078	−0.017
First service to conception	0.058 (± 0.030)	0.012	0.107
Calving to first service	0.009 (± 0.021)	−0.027	0.041
Days open	0.005 (± 0.019)	−0.030	0.033
Number of services	0.120 (± 0.019)	0.090	0.153
Calving			
Calving ease	0.014 (± 0.021)	−0.019	0.046

reproductive tract (Crociati et al., 2018). The SPS phenotype is measured by transrectal palpation, which can be affected by fat accumulation around the tract. Accordingly, higher BCS cows could have a higher SPS measurement.

This association should be further investigated to assess the real relationship between BCS and SPS and evaluate the need to adjust for BCS when genetically evaluating cows for SPS. In our preliminary analyses, BCS was significant as an explanatory variable in the model, but it explained less than 1% of the variation in SPS. Moreover, the variance component estimates from models with and without BCS as a covariate were similar. As BCS is already a trait included in the LPI (albeit with a small contribution) and given the high genetic correlation between SPS and BCS (0.632), BCS was excluded from the univariate animal model for the final analyses.

For other conformation traits, the estimated genetic correlations were −0.263 (thurl placement) and −0.739 (rump angle). Selection for SPS should aim for lower scores to improve fertility, whereas thurl placement and rump angle optimum values are intermediate, making this negative linear association difficult to interpret.

For traits related to reproduction, the significant genetic correlations were desirable; that is, low SPS scores were related to better fertility, with the exception of calving to first service (−0.371). Age at first service, days open, first service to conception, nonreturn rate at 56 d, and number of services all showed moderate to strong estimated genetic correlations (−0.730 to 0.931), whereas calving ease had an estimated genetic correlation with SPS close to 0 (0.061), which was not statistically significant. However, it is important to note that only number of services had a relatively small PSD. The highest genetic correlation was estimated between SPS and number of services (0.931), which was expected because SPS was initially proposed as an indicator trait for number of services.

Supplemental Table S2 (https://zenodo.org/record/6925896#.YuKfsXbMJhE; Martin et al., 2022) presents the heritability estimates from the bivariate analyses for all other reproductive traits, which used only animals with SPS records available. The heritability estimates for the reproductive traits that are strongly genetically correlated with SPS were higher than those from Oliveira Junior et al. (2021). This indicates that these traits benefited from the additional information provided by SPS through their strong genetic correlation with SPS, and ended up with a significantly higher heritability estimates compared with those from Oliveira Junior et al. (2021), who used large data sets for their analyses. For comparison, heritability estimates (± PSD) from the univariate analyses for first service to conception, nonreturn rate at 56 d, and number of services were 0.090 (±0.080), 0.041 (±0.035), and 0.081 (±0.061), respectively, using only cows with an SPS record, which were within the range of the estimates from Oliveira Junior et al. (2021).

The efficiency of indirectly selecting for other reproduction traits based on selecting for SPS could be assessed using the estimated genetic correlations and heritability of the traits. Assuming the same selection intensity and using heritability estimates from the uni-

variate analyses for nonreturn rate at 56 d and number of services, selection based on a single measurement of SPS would lead to an indirect selection response on nonreturn rate at 56 d and number of services that would be 1.21 and 1.10 times greater, respectively, than the direct selection response on these traits. More interestingly, there would not be an antagonistic indirect selection response on production traits. In addition, the Pearson correlations between SPS EBV and the Canadian index values LPI and Pro$ were −0.232 and −0.226, respectively, which are favorable in both cases. Therefore, SPS showed encouraging results for selection for fertility, with favorable indirect selection response on fertility traits and no indirect selection response on production traits.

CONCLUSIONS

The SPS is a new fertility trait, based on transrectal palpation of the reproductive tract, that has been developed as an indicator of pregnancy rate, number of services per pregnancy, and pregnancy loss. This novel trait had no genetic correlation with production traits and had favorable genetic correlations with fertility traits, varying from −0.730 (nonreturn rate at 56 d) to 0.931 (number of services). Although preliminary, these results are encouraging, because SPS seems to be more heritable and highly genetically correlated with number of services to conception and strongly correlated with nonreturn rate at 56 d and first service to conception, indicating potential for effective indirect selection response on these traits. Further studies with larger data sets are warranted to validate these findings.

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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.

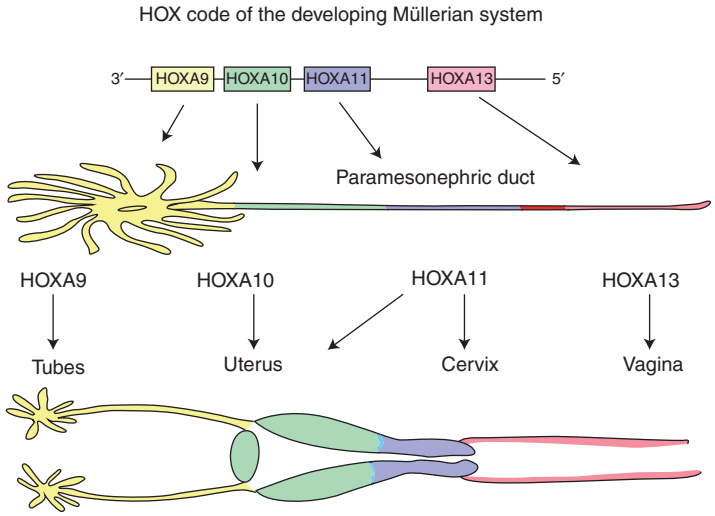

Figure 1. *HOX* code of the developing Müllerian system (adapted from Taylor 2000).

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

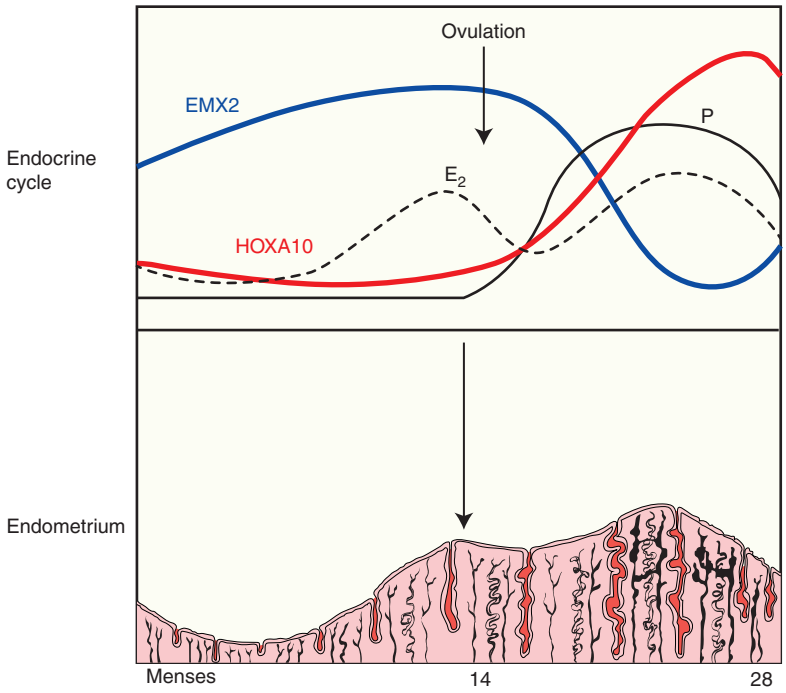

Figure 2. The pattern of *HOXA10* expression in the human endometrium through the menstrual cycle (adapted from Taylor 2000). *HOXA11* expression closely parallels that of *HOXA10*.

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 β3-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

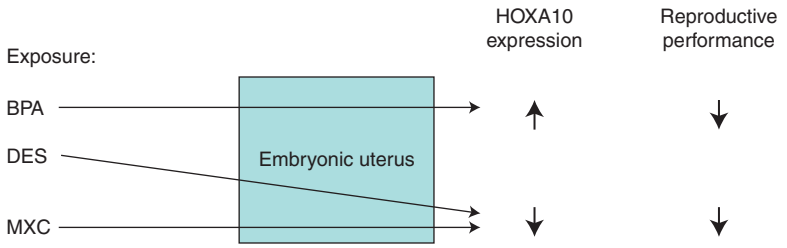

Figure 3. Exposure to various xenoestrogens alters *HOXA10* gene expression in the developing reproductive tract. BPA, bisphenol A; DES, diethylstilbestrol; and MXC, methoxychlor.

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. β3-integrin is expressed in endometrium at the time of implantation, and the disruption of in-

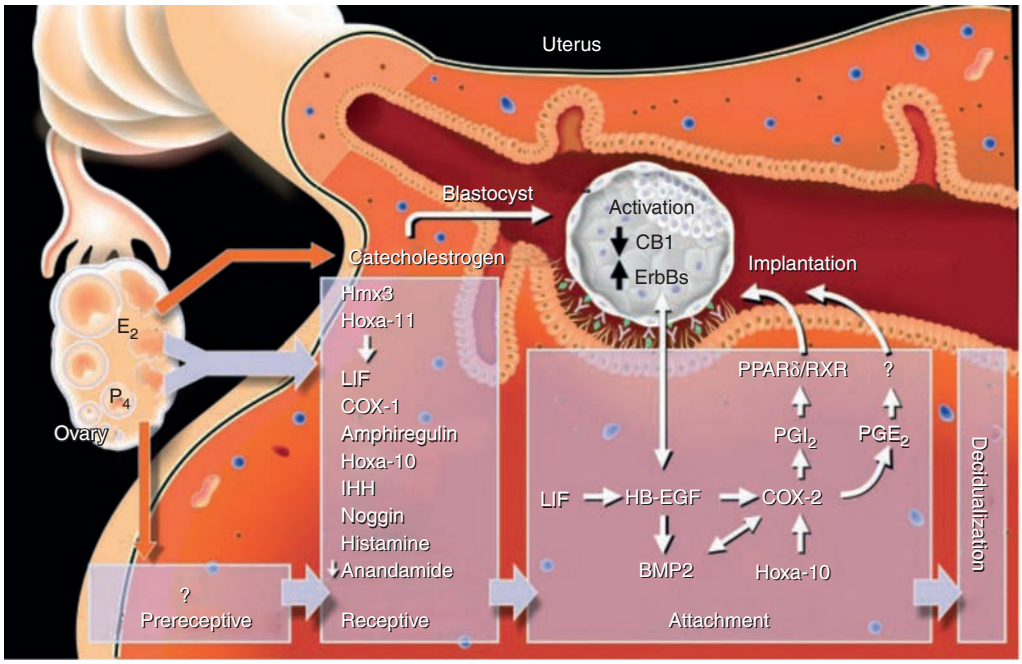

Figure 4. Molecular signaling during implantation in the mouse and human. (From Paria et al. 2002, reprinted, with permission, from The American Association for the Advancement of Science ©2002.)

tegrin expression is associated with decreased uterine receptivity and infertility (Lessey and Young 1997). Interestingly, β3-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-β, and E-cadherin or hypomethylation of genes for estrogen receptor-β 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, β3-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 TGFβ secreted by myomas leads to decreased BMP receptor expression and subsequent *HOXA10* repression (Sinclair et al. 2011). Leiomyoma alter endometrial receptivity by secreting TGFβ 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-

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, β3-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 β3-integrin is also reduced. Interestingly, two thirds of patients with hydrosalpinx who underwent salpingectomy also showed return of *HOXA10* and β3-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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Review

Review

An evo-devo perspective of the female reproductive tract

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Abstract

The vertebrate female reproductive tract has undergone considerable diversification over evolution, having become physiologically adapted to different reproductive strategies. This review considers the female reproductive tract from the perspective of evolutionary developmental biology (evo-devo). Very little is known about how the evolution of this organ system has been driven at the molecular level. In most vertebrates, the female reproductive tract develops from paired embryonic tubes, the Müllerian ducts. We propose that formation of the Müllerian duct is a conserved process that has involved co-option of genes and molecular pathways involved in tubulogenesis in the adjacent mesonephric kidney and Wolffian duct. Downstream of this conservation, genetic regulatory divergence has occurred, generating diversity in duct structure. Plasticity of the *Hox* gene code and wnt signaling, in particular, may underlie morphological variation of the uterus in mammals, and evolution of the vagina. This developmental plasticity in *Hox* and *Wnt* activity may also apply to other vertebrates, generating the morphological diversity of female reproductive tracts evident today.

Key words: Müllerian duct, sex determination, female reproductive tract, oviduct, *Hox* genes, evo-devo.

Introduction

A remarkable ability to thrive and reproduce in diverse habitats has underpinned the evolutionary success of vertebrates. This has been greatly facilitated by adaptations of the female reproductive tract. First appearing in fishes as a means of extruding gametes, the vertebrate female reproductive tract has undergone considerable modifications over evolution as lineages adopted internal fertilization, oviparity (egg-laying) and viviparity (live birth). A simple tube-like structure present at embryogenesis develops into a specialized oviduct in egg-laying species or a highly vascularised structure for supporting complete embryogenesis in therian mammals. Different reproductive strategies among vertebrates have required modifications of the female reproductive tract. Among bony fishes, a reproductive tract comparable to that seen in tetrapods (land vertebrates) is lacking in most species, while chondrichthyans (cartilaginous fishes) have well developed oviducts for oviparity or viviparity. The emergence of vertebrates onto land and freedom from water to reproduce was accompanied by differentiation of the female ducts for the development of hard-shelled eggs among reptilian and avian lineages. In therian mammals, the female reproductive tract takes on a greater role than in other lineages, being the site of fertilization, embryonic development and live birth [1].

How the diversity of female reproductive tract has been generated at the molecular genetic level is largely unknown. This review considers the female reproductive tract from a novel perspective; evolutionary developmental biology (evo-devo). We firstly summarise the comparative anatomy and physiology of the female reproductive tract among vertebrates. This sets the stage for a consideration of the molecular genetics regulating the formation and differentiation of the female reproductive tract from an evolutionary perspective.

In most vertebrates, the female reproductive tract develops during embryogenesis from paired epithelial tubes, the Müllerian ducts. We propose that morphogenesis of the Müllerian duct has involved co-option of gene regulatory pathways that play a role in tubulogenesis of the adjacent mesonephric kidney and Wolffian (pronephric) duct. Studies in mouse and chicken show that genetic regulation of early Müllerian duct formation is conserved [2]. In contrast, the subsequent duct differentiation stages have so far not been shown to be conserved between mammals and birds. This likely reflects the functional divergence of the ducts in the two clades. However, we speculate that regional differentiation of the vertebrate Müllerian duct may be under-pinned by the *Hox* gene code, as it is in mammals.

Comparative anatomy of the female reproductive tract

The female reproductive tract of tetrapods (land vertebrates) derives from a pair of embryonic epithelial tubes, the Müllerian (paramesonephric) ducts. In humans, Müllerian ducts are initially present in both sexes, together with another set of tubes, the Wolffian ducts (Fig. 1a). The ducts, gonads and associated embryonic (mesonephric) kidneys are of mesodermal origin. As both involve transfer of biological agents to the exterior, development of the reproductive tract (in both sexes) is intimately linked to the excretory system. The paired Wolffian ducts (the pronephric or mesonephric ducts) form within the body of the mesonephric kidneys. They function at embryonic stages as excretory canals, transferring nitrogenous waste from the mesonephric kidneys to allantois. At the early undifferentiated stage, (weeks 5–6 in human embryos) the Müllerian duct is a simple structure, comprising a meso-epithelial tube, the so-called Müllerian epithelium, surrounded by loose mesenchyme, and with an outer layer of surface (coelomic) epithelium [3, 4] (Fig. 1b). In therian ("placental") mammals such as human and mouse, the epithelial and mesenchymal compartments give rise to regionalised differentiation of the duct during the late embryonic and postnatal periods, generating the Fallopoian tubes, uterus and upper vagina (Fig. 1c-d). In males, Mullerian ducts typically regress during embryonic life under the influence of testis-derived Anti-Müllerian Hormone (AMH).

During vertebrate evolution, the female reproductive tract has changed anatomically to reflect a transition from the production of shelled eggs to directly supporting embryonic development (Fig. 2). In agnathans (jawless fishes such as lampreys), Müllerian duct derivatives are absent and gametes are shed directly into the coelom, then extruded. This is likely to represent the ancestral vertebrate condition. Among teleost fishes, Müllerian ducts are absent. Instead, a different structure, the gonoduct, arises from the gonad of both sexes, derived from the dorsal peritoneum. It transfers sperm in males and the ova in females. External fertilization is typical of bony fishes, but some teleosts are viviparous and the young (or eggs) develop inside simple glandular oviducts or in the ovaries themselves. These include groups such as the Poeciliidae [5, 6]. *D. rerio*, the zebrafish used widely as a developmental model, has external fertilization and the gonaduct serves purely as a vehicle to transfer gametes [7]. Interestingly, despite the lack of Müllerian ducts, teleost fishes have AMH, which has other (presumably ancestral) functions related to gonadal soma and the germ cells [8]. Most chondrichthyans (cartilaginous sharks, rays and skates) are viviparous (live-bearing) and an oviduct develops that is analogous to that of tetrapods [9–11] (Fig. 2). Embryos can develop directly in a "uterus" in such viviparous shark species [10, 12–14]. Phylogenetically, the *AMH* gene first appears among cartilaginous fishes, the most ancient gnathostome lineage, in line with the appearance of paired Müllerian ducts. Hence, these ducts degenerate in males, though rudiments are retained in the adult [8].

Most amphibians (frogs, newts, salamanders et al.) have external fertilization. The paired Müllerian ducts differentiate into oviducts, ciliated epithelial tubes that serve to transfer non-calcified eggs via the cloaca to the exterior (Fig. 2) [15]. At the cranial pole, the oviduct differentiates into the infundibulum (ostium), a ciliated slit that receives the oocyte. In frogs, the infundibulum typically lacks fimbriae (finger-like projections). Posterior to the infundibulum is the atrium, a short segment that leads into the secretory ampulla of the oviduct, a region that becomes highly convoluted when hormonally stimulated (Fig. 2) [16, 17]. Hence, over evolution, the first signs of substantial regionalised differentiation of the oviduct (Müllerian duct) are apparent in amphibians, linked to a semi-terrestrial lifestyle and a need to physically and osmotically protect eggs laid in freshwater. Evolution of Müllerian duct derivatives beyond amphibians has involved more marked regional differentiation. The complete transition from water to land necessitated internal fertilisation, facilitating evolution of the amniote egg (the amniotic membrane, in addition to the chorion, allantois and yolk sac). This transition required the production of shelled (calcified) eggs that minimise water loss and could hold large amounts of yolk (Fig. 2). Hence, reptiles and birds have specialized regions of the oviduct that facilitate these functions [18–23]. While fertilisation takes place in the infundibular region, the development of shelled egg has led to significant structural differentiation of the oviduct. Birds and reptiles have four anatomically and histologically distinct regions of the oviduct. Adjacent to the infundibulum, the magnum is specialized to secrete albumen, a liquid medium for supporting embryonic development, containing high levels of protein. The isthmus secretes the egg shell membrane, while the most posterior region, the shell gland ("uterus") lays down the calcified shell (Fig. 2) [24–27]. Most reptiles are oviparous (egg laying), but many squamates (lizards and snakes) are viviparous (live bearing) or ovo-viviparous (eggs hatch in the oviducts, then live birth). In these species, the oviduct has evolved to bear live young. It has structural and physiological adaptations that facilitate formation of a chorioallantoic placenta, allowing embryonic gas exchange and delivery of nutrients [22, 28, 29]. In most (but not all) birds, the right oviduct and ovary are vestigial. This is probably due to physical constraints precluding two gravid ducts each holding fragile hard-shelled eggs [30]. Mammalian evolution has been accompanied by a reduced reliance on yolk and advanced development of the uterus as a secretory organ that supports embryonic development (Fig. 2) [31]. The Müllerian ducts of mammalian embryos differentiate into Fallopian tubes (called "oviduct" in mice), uterus and, in therians, upper portion of the vagina. Among therian mammals, the cloaca (a common urogenital and anal canal) has been lost (Fig. 2).

Evo-devo of Müllerian duct development; conservation and divergence

The diverse female reproductive tracts described above are all derived from a common precursor structure, the Müllerian duct. This embryonic organ is structurally very similar across groups. The Müllerian ducts are bilateral meso-epithelial tubes surrounded by mesenchyme that develop on the surface of the mesonephric kidneys at embryonic or larval stages in amphibians, reptiles, birds and mammals [2, 32–36]. Genetic regulation of duct formation

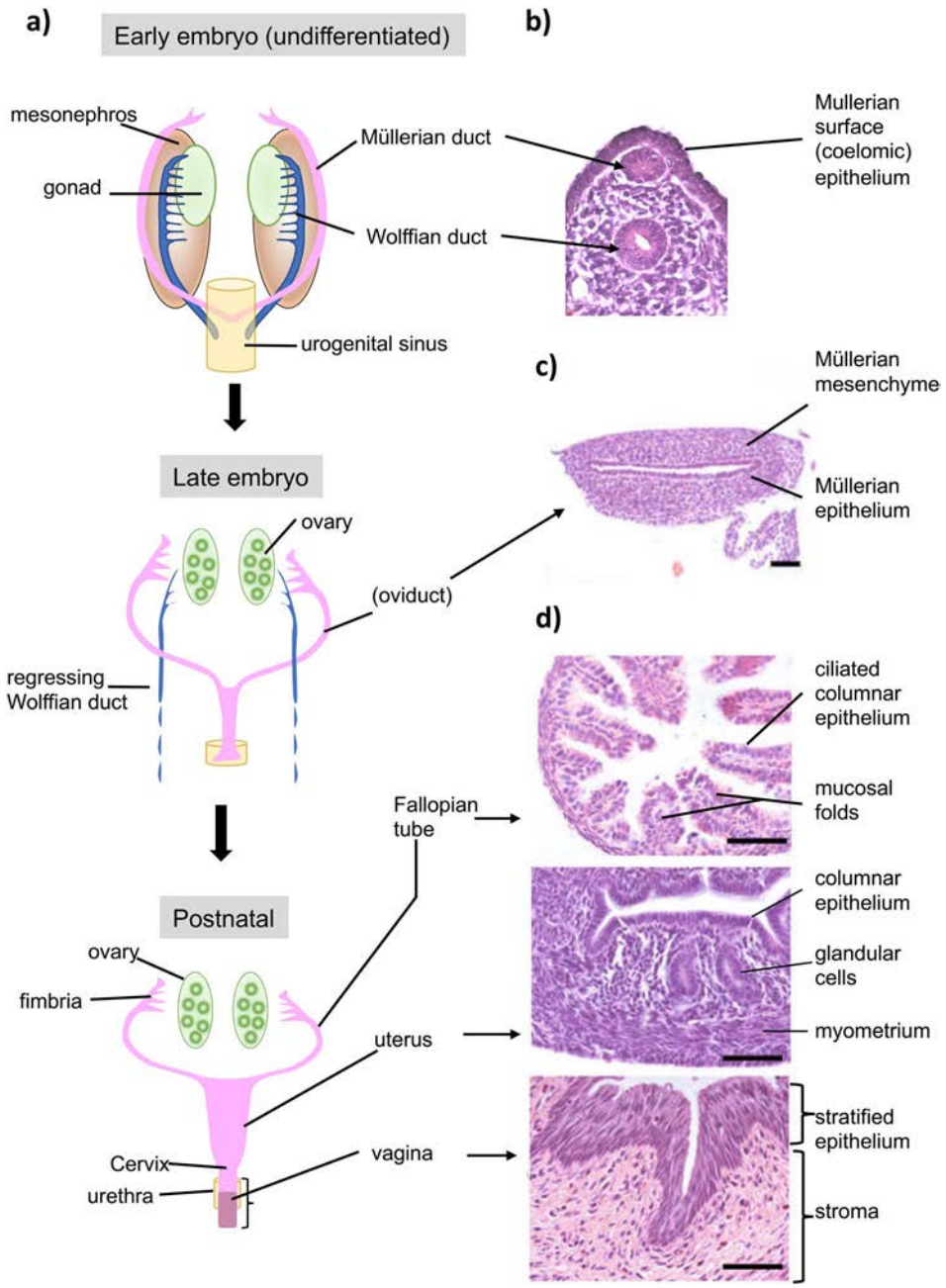

Figure 1. Development of the female reproductive tract in therian mammals. a) Schematic overview of reproductive tract formation (ventral view). In the early embryo (4–5 weeks in humans, up to 13–14.5 days postcoitum in mouse) the urogenital system is morphologically undifferentiated, characterised by paired Müllerian ducts (pink) and Wolffian ducts (blue). Later in embryogenesis, the Wolffian ducts regress in females, and the Müllerian ducts are retained. Subsequently, at postnatal stages, the Müllerian duct undergoes regionalised differentiation in Fallopian tubes, uterus, cervix / upper vagina (fused as a simplex in humans, shown here). Adapted from Roly et al. [4], with permission. b) Histology of the female reproductive tract in the mouse (transverse H&E stained sections). At the embryonic stages, the Müllerian duct is present as a tube, comprising mesenchyme and an inner Müllerian epithelium. The duct develops in close association with the Wolffian duct, adjacent to the mesonephric kidney. Image from Fujino et al [88] with permission. c) At postnatal day 3, the oviduct is not differentiated, comprising an inner epithelial layer (Müllerian epithelium), underlying mesenchyme and an outer epithelium layer, derived from the coelomic epithelium ("surface epithelium"). d) Postnatal day 28 in mouse, showing regional differentiation of the duct into Fallopian tube (ciliated columnar epithelium lining mucosal folds), uterus (glandular and columnar epithelium of the endometrium) and vagina (stratified epithelium). Images in c) and d) taken from Dunlap et al. (2011) [160], with permission. Bar = 50 μm.

is likely to be a deeply conserved process among vertebrate embryos. How, then, is the diverse comparative anatomy generated? This must involve changes to the duct differentiation process downstream of the formation stage. This may involve changes in the timing of gene regulation, or the co-option of novel genes into developmental pathways. Both of these possibilities would involve alterations to cis- and trans- regulatory regions of genes. We propose here that Müllerian duct formation has entailed co-option

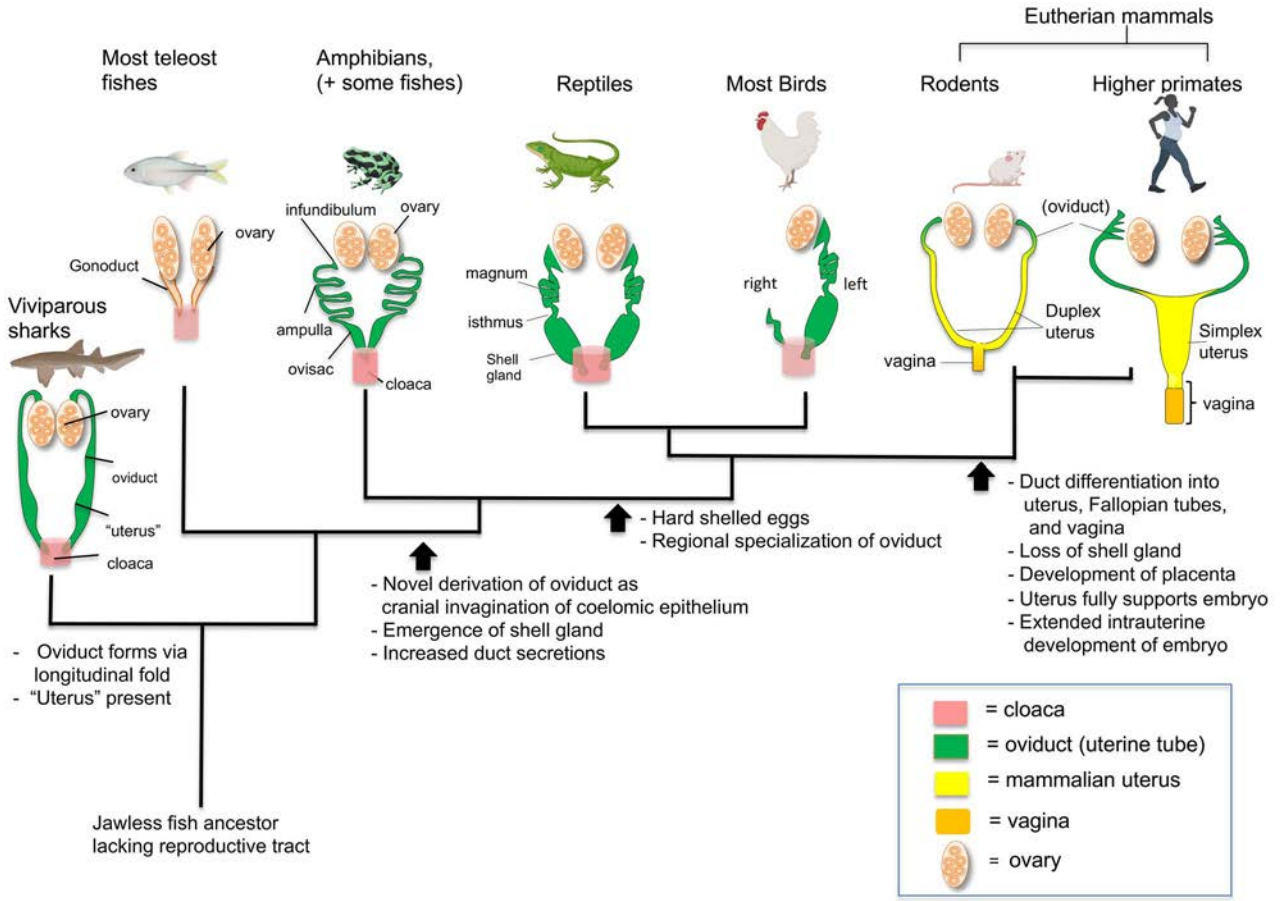

Figure 2. Comparative anatomy of the adult female reproductive tract in vertebrates, showing major evolutionary steps. Figure prepared using BioRender.com.

for genetic programs that underlie mesonephric kidney and nephric duct formation. We further propose that the early stage of duct specification is developmentally conserved, at the cellular and genetic level, whereas duct elongation and differentiation involve divergent gene regulatory networks that reflect the different reproductive strategies across vertebrate groups. We speculate that a major point of divergence between egg-laying vertebrates and mammals may centre around the changing role of oestrogen in duct development. Lastly, we consider patterning of the duct in the context of the *Hox* gene code, which has only been explored in mammals, but may apply to other vertebrates. We speculate on the developmental origin of mammalian-specific Müllerian derivative such as the vagina, which may have involved plasticity of the *Hox* code.

Co-option of nephric regulatory genes to the Müllerian duct

The cell biology and molecular genetics of Müllerian duct formation in the mouse model has been extensively reviewed [2–4, 36, 37]. Duct regression in males under the influence of Anti-Müllerian Hormone has also been well described [38–42]. Here, we focus on genetic regulation of duct morphogenesis from a novel evolutionary development biology perspective. We find that morphogenesis of the Müllerian duct has involved co-option of genetic programs that regulate nephric duct and (mesonephric) tubule formation. All three tissues feature tubulogenesis - the formation of epithelial tubes - via inductive signalling, EMT events, invagination, cell migration and mesenchyme maturation [43, 44].

Developmental studies in mouse and chicken embryos have shown that the Müllerian duct develops from a group of precursor cells, specified in the coelomic epithelium at the cranial pole of the mesonephros [44, 45]. These cells proliferate and invaginate, forming a meso-epithelial tube that migrates caudally, similar to migration of the Wolffian duct [36]. In mouse and/or chicken models, duct progenitor cells express the transcription factors, *Pax2*, *Lim1* and *Emx2* and signalling factors such as FGF and BMP family members (BMP 2,3, 4, and 7) [44, 46–49]. The meso-epithelial tube, called the Müllerian epithelium, is surrounded by mesenchyme (Müllerian mesenchyme), which also derives from the surface coelomic epithelium, via an EMT (Epithelial to Mesenchyme transition) [50, 51]. Müllerian duct formation is morphologically conserved among tetrapods, involving the same types of cellular events.

Müllerian duct morphogenesis is very reminiscent of tubulogenesis in the adjacent mesonephros and nephric duct. The nephric duct (i.e. the pronephric or mesonephric duct, which becomes the Wolffian duct) pre-dates evolution of the Müllerian duct. Nephric ducts are present in the most ancient vertebrate lineage, the jawless fishes, which lack Müllerian ducts [52]. Similarly, the pronephric kidney, an excretory organ derived from the nephric duct and featuring a few nephric tubules, pre-dates the Müllerian duct. Evolution of the Müllerian duct might therefore be viewed as a process that has involved recruitment of pre-existing developmental pathways that regulate the formation of tubular nephric structures (pronephros/mesonephric

tubules and Wolffian duct). Important genes expressed in these tissues are also expressed during stages of Müllerian duct development. Table 1 summarises key common genes implicated in both nephric formation (duct or tubule) and Müllerian duct morphogenesis, based on data primarily derived from mammal (rodent) and bird (chicken) studies. Several transcription factors expressed at the early stages of duct morphogenesis (specification and /or invagination), are also required for Wolffian duct/mesonephric morphogenesis These regulators include the transcription factor gene, *Wt1*, which is required for posterior mesonephric tubule formation [53], and for activating *Amhr2* in Müllerian duct [54], and *Osr1*, an essential nephrogenic regulator [55–57]. The paired box transcription factor gene, *Pax2*, is also crucial for multiple steps in urogenital development, including both kidney and Müllerian duct formation [44, 48, 58]. Another critical transcription factor gene for mesonephric kidney, Wolffian and Müllerian duct formation is *Lim1* (also known an *Lhx1*) [46, 59]. Mice lacking *Lim1* fail to develop Wolffian and Müllerian ducts [1, 46]. In fact *Lim1* is specifically linked to tubular morphogenesis in the reproductive tract [59]. The requirement for *Lim1* and *Pax2* in vertebrate urogenital tubulogenesis would appear deeply conserved, as both are also expressed in the zebrafish mesonephros [60]. Another important homeobox gene for both nephric and Müllerian duct development is *Emx2*. This gene is expressed in the epithelium of mesonephric tubules, Wolffian and Müllerian ducts, and both pairs of ducts do not form in its absence [49]. All of these transcription factors regulate specification of duct/tubule precursors, EMT or mesenchyme development during tubulogenesis of both nephric structures and the Müllerian duct (Table 1).

Conserved signalling pathways for tubulogenesis are also shared between the nephric duct/tubules and the Müllerian duct. Specifically, Wnt and Fgf signalling feature prominently in both Müllerian duct formation and Wolffian and mesonephric duct development. In the Wolffian duct, FGF7 and FGF10 are expressed in duct mesenchyme, signalling through FGFR2, a process required for proper Wolffian duct development [61]. Similarly, FGF is required for the early specification and invagination phases of Müllerian duct development, as reveal by mouse and chicken studies (Table 1) [44]. Key Wnt growth factors include WNT4, required in mesenchyme for both mesonephric tubule differentiation and Müllerian duct development, via the canonical β-catenin pathway (mouse or chicken models) [43, 62, 63]. In the mouse Müllerian duct, mesenchyme-secreted WNT4 is required for invagination of overlying epithelial Müllerian precursor cells, and coordinates cell migration and extension of the Müllerian duct [62, 64]. *Wnt4* null mice lack Müllerian ducts [64]. Similarly, waves of inductive signalling that involve WNT4 are required for differentiation in the mesonephros in chicken embryos [43] and for pronephric tubulogenesis in the amphibian, *X. laevis* [65].

Conserved Hox gene expression in the Mullerian duct and nephric structures

Consistent with their graded expression along the rostral-caudal body axis generally, *Hox* genes play a pervasive role in segmental patterning structures of the urogenital system. Patterning of the Müllerian and nephric (Wolffian) ducts involves *Hox*-mediated positional cues [66]. In mammals, anterior regions of both the Wolffian and Müllerian ducts express *Hoxa9*, while more posterior regions express *Hoxa11* and *Hoxa13* [67, 68]. In both tissues, loss of function mutations can cause partial homeotic transformations [69, 70]. Regional differentiation of the Müllerian duct under the influence of *Hox* genes again appears to reflect co-option from pre-exiting nephric *Hox* regulatory pathways (Table 1). In mouse and/or human, *Hoxa9 – Hoxa11* control regional differentiation of the Wolffian duct mesenchyme into epididymis, vas deferens and seminal vesicle [71, 72]. This *Hox* axis is conserved in the Müllerian duct of mouse and human, where *Hoxa9-Hoxa13* regulate regional patterning into oviduct, uterus, cervix and upper vagina [73]. *Hoxd13* is also implicated in both Wolffian and Müllerian duct formation, with expression being restricted to the caudal region (vas deferens and seminal vesicle in the male, and upper vagina in female). $Hoxa13^{+/-}/Hoxd13^{-/-}$ compound mutant mice show a transformation of cervix at the uterus/vagina boundary to uterus in females, while the same compound mutants show disrupted seminal vesicle formation in males (Table 1) [68, 74, 75]. Nephrogenesis also involves *Hox* gene regulation, most notably, the requirement of *Hox11* trans-paralogs form branching of the ureteric buds and interaction with metanephric mesenchyme [76]. The ontogenetic timeline of urogenital development (pronephros followed by mesonephros, Wolffian and then Müllerian duct formation) is collinear with the evolutionary appearance of these structures. It is likely that a subset of *Hox* genes was first co-opted to nephric duct formation from the cranio-cadual body axis, and then recruited to regional segmentation of the Wolffian and Müllerian ducts.

At the cell biology level, morphogenetic mechanisms are conserved between Müllerian and Wolffian ducts. Mesenchyme-epithelium interactions are fundamental to morphogenesis of both the Müllerian and nephric ducts. Regionally specified mesenchyme regulates cell fate specification of the epithelium during duct differentiation in both the Müllerian duct and in Wolffian duct and in nephric structures [66]. This is demonstrated by tissue recombination experiments. When mouse presumptive uterine epithelium is grown with presumptive vaginal mesenchyme, for example, the epithelium adopts a squamous vaginal cell fate [77].

Müllerian duct specification is developmentally conserved, while later stages are divergent

The early stages of Müllerian duct formation are conserved among tetrapods, involving the same cellular processes [2, 32–35]. This implies that the underlying molecular control is likely to be conserved. Indeed, the early stages of Müllerian duct formation involve a genetic program among that appear to be conserved among vertebrates. This program drives tubulogenesis. However, later stages of duct development are developmentally divergent.

Conservation of master regulators

Duct formation during embryogenesis can be divided into three stages; specification/invagination, elongation and patterning. This is shown schematically in Figure 3, which also shows genes expressed at these stages across vertebrate lineages. The duct forms through specification of Müllerian precursors cells in the cranial coelomic epithelium overlying the mesonephros, followed by delamination and invagination of these cells. The first cells to invaginate form a mesoepithelial tube (characteristics of both mesenchyme and epithelium) and has recently been called pEMT (partial Epithelium to Mesenchyme Transition) [51]. This process of mesoepithelial induction produces the duct luminal epithelium and it's formation is deeply conserved, from fishes with ducts (sturgeon) through to

Table 1. Known developmental genes shared between the embryonic nephric duct/or tubules and the Müllerian duct, based on mammal (rodent/human) or avian (chicken) models

Gene	Protein product	Nephric duct/tubule	Müllerian duct	References
Wt1	Wilms' Tumor 1, a zinc finger transcription factor	Formation of caudal mesonephric tubules	Regulates *AmhrII* during duct regression	[53, 54]
Osr1	Odd-skipped related transcription factor (often a repressor)	Formation of urogenital system – kidneys and gonads	Expressed in ducts (conditional gene knockout not reported)	[55–57]
Pax2	Paired homeodomain Transcription Factor	Nephric lineage specification	Müllerian duct lineage progenitor specification	[48, 58] [44]
Lim1	Homeodomain Transcription factor	Formation of nephric progenitors	Formation of Müllerian progenitors	[44, 46, 59]
Emx2	Homeodomain Transcription factor	Required in epithelial cells of mesonephric (Wolffian) duct and mesonephric tubules	Required in epithelial cells of Müllerian duct	[49]
Gata3	Transcription factor	Required in mesonephric kidney development	Required for mouse Müllerian duct elongation (expressed in Wolffian epithelial cells)	[91, 160, 161]
Fgf	Secreted Fibroblast growth factors	Fgf8 required for kidney tubulogenesis	Fgf/ERK signalling required for duct specification	[162] [44]
Wnt4	Wnt secreted growth factor	Mesonephric tubule differentiation	Required for duct formation and later patterning	[43] [64]
Hoxa9	Homeodomain Transcription factor	Patterning anterior Wolffian duct into epididymis	Patterning anterior Müllerian duct into oviduct	[71]
Hoxa10	Homeodomain Transcription factor	Patterning posterior Wolffian duct into vas deferens and seminal vesicle	Patterning posterior Müllerian duct into uterus	[72]
Hoxa11	Homeodomain Transcription factor	Patterning posterior Wolffian duct into vas deferens	Patterning anterior Müllerian duct into uterus	[163] [75]
Hoxa13 *Hoxd13*	Homeodomain Transcription factors	Patterning posterior Wolffian duct into seminal vesicle	Patterning posterior Müllerian duct into upper vagina	[67, 68]
Hnf1b	Hepatic nuclear factor 1, a Homeodomain transcription factor	Required in epithelium of Wolffian duct, and for kidney tubule development	Expressed in Müllerian duct epithelia; human mutations cause abnormalities of Müllerian derivatives	[164, 165]

tetrapods [2, 78–81]. At the genetic level, this stage has only been examined in any detail in mouse and chicken embryos, revealing conserved expression of master duct initiators, LIM1, PAX2 and EMX2 transcription factors, together with FGF and BMP signalling [46, 47], and WNT9B, derived from the mesonephros (summarised in [4, 51] (Fig. 3). In chicken, BMPs have been shown to induce PAX2 expresison, while FGFs induce LIM1 expression [44].

In mouse, the POU homeodomian transcription factor, HNF1B, is also required for Müllerian duct specification [82], and we have also noted its expression in the chicken model [57], pointing to a conserved role (Fig. 3). Similarly, retinoic acid (RA) signaling is required for proper duct elongation in mouse [83], and probably also in chicken, due to expression of RA-synthesising enzymes and receptors in rthe forming duct [57]. Interestingly, the transcriptional co-activators, *Dach1* and *Dach2*, are redundantly required for Müllerian duct development in mouse, and they could have a deep evolutionarily conserved role, as *Drosophila* dachshund mutant also have a female reproductive tract phenotype [84]. These genes are also expressed in the chicken Müllerian duct [57]. However, overall, as highlighted on Fig. 3, very little is known about the conservation of duct formation at the genetic level beyond the mammalian and avian models.

Conservation of Fgf signaling

As noted above, there is a key role for Fgf signaling in Mullerian duct formation, as revealed in rodent and chicken models. In the chicken embryo, PAX2 expression in the coelomic epithelial duct progenitor cells induces FGF expression, which then activates LIM1 and triggers the pEMT process [44] (reviewed in [4], [51]). FGF action in the avian model is mediated by FGFR2 activation of the ERK/MAPK pathway [44]. The likely ligands are FGF2, FGF8 and/or FGF9, which are expressed in chicken mesonephric tissue [85], or in the nacsent duct itself [57]. In the mouse model, an early role for FGF singlaing has not been established, although later Müllerian duct epithelial fate commitment (vaginal vs uterine development) depends upon FGF/MAPK signaling, along with other mesenchymal paracrine factors, such as BMP/SMAD [86, 87]. The migration stage of Müllerian duct development in rodents involves the The phosphatidylinositol 3-kinase (PI3K)/AKT pathway, active at the tip of the migrating duct [88]. This intracellular pathweay is activated by FGF, though the exact ligand is unclear, and whether the process is conserved is also presently unknown.

Deep conservation of WNT4 signaling

During the invagination and elongation phases, there is a deeply conserved role for canonical WNT signalling, inferred from studies across fishes, amphibians, birds and mammals. This applies most notably to WNT4. WNT4 is required for Müllerian duct formation in mouse, where it is expressed in the mesenchyme and signals the duct progenitor cells in the coelomic epithelium to form a meso-epithelial tube and extend caudally [62, 64]. Consequently, *Wnt4*

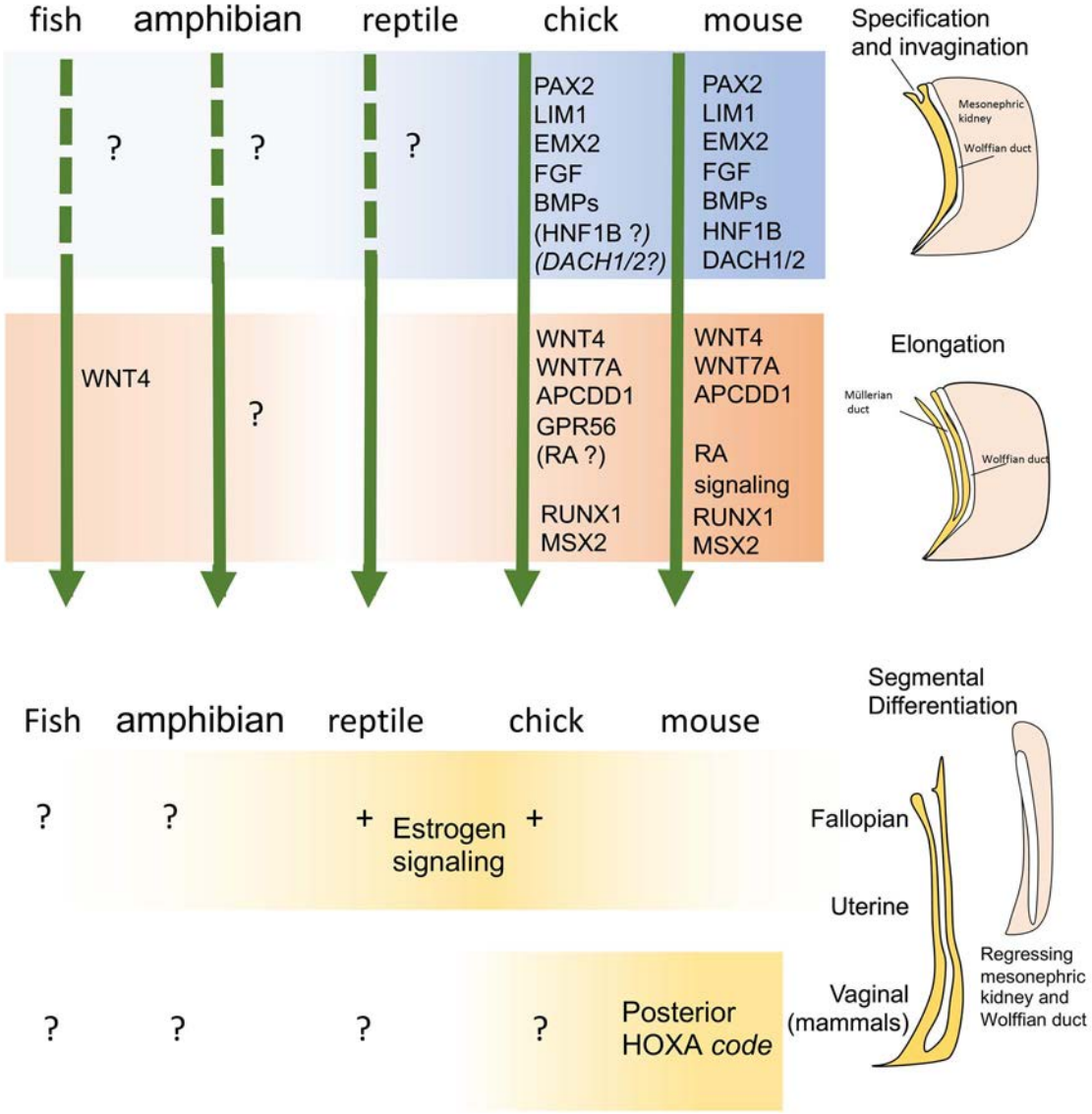

Figure 3. Conserved and divergent gene expression implicated in Müllerian duct development across vertebrates. Some genes show deep conservation of expression, while others appear novel. Conservation applies to the earlier stages of duct formation.

null mutant mice lack Müllerian ducts [64]. *Wnt4* has a conserved expression profile in duct mesenchyme of the chicken embryo, where it is inferred to play the same role [34]. A conserved role has recently been shown in the zebrafish, which has Müllerian ducts, and expresses the osteichthyan homologue, *Wnt4a*. In zebrafish, as in mouse, targeted deletion of *Wnt4a* results in failure of duct development [89]. WNT4 would therefore appear to be a deeply conserved regulator of Müllerian duct formation, though it has not been examined during duct development in amphibians or reptiles. WNT7A is also expressed in the developing duct in both chicken and mouse (in the mesoepithelium, and later during duct differentiation) [57, 90] but, again, expression of this gene not been examined in other species. In mouse, WNT9b derived from the Wolffian duct, acts as a diffusible signal during the Müllerian duct elongation phase [91]. This factor has not been examined in other vertbrates, although Wnt9b mesonephric (wolffian) duct expression is conserved in chicken [92].

Divergent gene expression

From the duct elongation phase, molecular signals show some divergence among vertebrate groups, presumably due to divergence of function. In avians, for example, a muscular oviduct develops, adapted to deposition of a calcified shell. In eutherian mammals, the uterus develops to facilitate placentation. Interestingly, a comparison of recent RNA-seq data for chicken versus mouse shows that there is little overlap in gene expression during Müllerian duct differentiation [40, 57]. For example, the G-protein coupled receptor, GPR56, is required for duct elongation in chicken [50], though this is not the case in mouse [93]. We identified a number of transcription factors or signalling molecules that show strong expression during chicken duct development and are inferred to have an important role, such as *FOXE1, SMARCA2*, *APCDD1*, and *TSHZ3* [57]. However, in mouse, targeted deletion of these genes is not accompanied by any reported Müllerian duct abnormalities [94–96]. In mouse, a role during duct cell fate commitment (vagina) has been shown for the

transcription factors *Six1*, *Runx1* and SMAD signal transduction [86, 97]. We recently found conserved expression of *Runx1* in the chicken model, earlier than in mouse, though functional data are lacking. The homeobox gene, *Msx2*, is required for vaginal epithelia differentiation in mouse [98]. Expression of this gene is also conserved in chicken [99], but again functional data are lacking. Overall, however, gene expression and functional analysis during Müllerian duct elongation and regional segmentation have not been characterised among other vertebrates (fishes, amphibians, reptiles) to allow any firm vertebrate-wide conclusions to be drawn.

The role of estrogen action

Estrogens have a central role in ovarian development among egg-laying vertebrates. The estrogen-synthesising enzyme, aromatase, is expressed in female gonads and estrogen is required for proper ovary formation during embryogenesis among fishes, amphibians, reptiles and birds [100, 101]. However, in therian mammals, oestrogen is required after birth to maintain the ovarian phenotype [102, 103]. Estrogen also plays a role in Müllerian duct differentiation among oviparous vertebrates. Estrogen receptors are expressed in the duct mesenchyme of avian (chicken) and reptilian (turtle) embryos [104–106] (Fig. 3). The developing duct is dynamically sensitive to estrogen in reptiles and birds; exposure to estrogen during duct elongation blocks the elongation process, but causes duct hypertrophy when administered after elongation is complete [107, 108]. Furthermore, local estrogen blocks induction of duct regression by AMH, at least in the chicken [107, 109, 110]. In the chicken embryo, the right duct regresses in line with regression of the right gonad, mediated by AMH, but the left duct is thought to be protected by estrogen, although this remains to be definitively proven. After hatching, oestrogen stimulates oviduct growth in chickens [111, 112]. It regulates the formation of tubular glands and the differentiation of ductal epithelium into goblet and ciliated cells. In chicken, oestrogen also stimulates the synthesis and secretion of materials from the duct, such as albumen and ovomucoid [110, 113, 114]. Altogether, the data indicate that estrogens may have a role in normal differentiation of the Müllerian duct in oviparous species. However, the effects on the ducts of complete abolition of oestrogen action via genetic deletion of either aromatase or oestrogen receptor (ESR1) in non-mammals have not been reported. Advances in genome editing for reptiles and birds now make these experiments feasible [115, 116].

While exposure to exogenous estrogen and diethylstilbestrol (DES) can cause Müllerian duct adenocarcinoma and genital tract abnormalities, duct hypertrophy has not been reported in mammals [117]. Müllerian ducts develops normally in the Aromatase knock out mouse, although uteri are under-developed, as oestrogen has a postnatal role [118]. Postnatally, estrogen regulates epithelial cell differentiation of the mammalian female reproductive tract, by modulating the *Six1-Runx1* axis noted above [97]. Postnatally, estrogens regulate epithelial cell height and degree of ciliation in mammals, concomitant with elevated levels during the menstrual/estrus cycle (reviewed in [119] Estrogen also plays a role in postnatal oviduct function in mammals by regulating epithelial cell proliferation. In rodent and human, the uterus is a primary target of estogen, where the steroid stimulates proliferation of both stroma and eptithelial cells of the endometrium [120–122]. In the mammalian Fallopian tube, estrogen also has a mitogenic effect [123]. Hence, while there is a clear physiological role of estrogen in the mature female reproductive tract, formation and early differentiation of the Müllerian duct is less influenced by endogenous estogen in mammals compared to oviparous species. Altogether, the data suggest a shift away from steroid regulation of embryonic duct differentiation in the (eutherian) mammalian lineage. This might be related to chorioallantoic placentation and requirement for the foetal duct to be refractory to maternal estrogens during its formative stages.

Developmental plasticity of the mammalian female reproductive tract

Among mammals, there is great variability with regard to the anatomy of the female reproductive tract [124]. Developmentally, this variability is due to different degrees of fusion of the paired Müllerian ducts late in embryogenesis. Four main types of uteri are recognised, based on degree of caudal duct fusion; duplex (no fusion), bipartite (some fusion), bicornate (more extensive fusion) and simplex (complete fusion). This is shown in a phylogenetic context in Figure 4. There is an overall evolutionary trend in mammals as a whole from separate uteri, through duplex to bipartite/bicornate and simplex uteri (Fig. 4). Monotremes (egg-laying mammals) have two separate uteri that open into the urogenital sinus, and they lack a vagina. The uteri secret the egg shell around the egg, and is essentially homologous to the bird/reptile oviduct. This condition can be considered ancestral among mammals. Beyond the monotremes (in therian mammals), a vagina evolved. Marsupials also have two uteri, but associated with two lateral vaginae and a medial birth canal that can be permanent (kangaroos) or transient (phalangers) (Fig. 4) [125]. (The paired vaginae in marsupials are related to the fact that the ureters pass medially between the two vaginae, anatomically preventing their fusion). Among the three major clades of eutherian mammals ("placentals") a variety of uteri is observed, without any clear phylogenetic restrictions (Fig. 4) [126–129]. This means that the various of uterine types across eutherians have arisen via a degree of convergence. Bipartite and bicornate uteri show partial duct fusion to generate uterine horns and a uterine body (more extensive in the bicornate form (Fig. 4). Most members of the archaic Xenarthra (armadillo, anteater) have a simplex uterus, although some species have less caudal duct fusion, resulting in a bicornate form [130, 131]. A bicornate uterus is typical of the Afrotherian clade (elephants, hyrax, and aquatic manatee and dugong) [132–134]. However, among the largest eutherian clade, the boreoeutherians, all four types are apparent, with no clear phylogenetic groupings of uterine types (Fig. 4) [135–139]. Among higher primates, including humans, the paired uterine progenitors have fused along their entire length into a single (simplex) uterus (Fig. 4) [1, 30, 124].

The functional morphology of these diverse uterine types is often considered to be linked to reproductive strategy. Eutherian mammals that have litters of multiple offspring, such as carnivores, have bicornate uteri with uterine horns to facilitate the development of many embryos. Higher primates, by contrast, have a simplex uterus that is adapted to the development of one or two embryos of relatively large size (due in part to advanced encephalization *in utero*). However, this is far from a universal trend. In fact, a reappraisal of the literature does not strongly support the notion that litter size correlates with the type of uterus. Cetaceans, elephants and aardvarks (Tubulidentata) have single offspring but bicornate uteri, while armadillos have up to 12 offspring and a simplex uterus (though armadillos have polyembryonic clones in which a single blastocyst divides into multiple embryos). Meanwhile, the panda

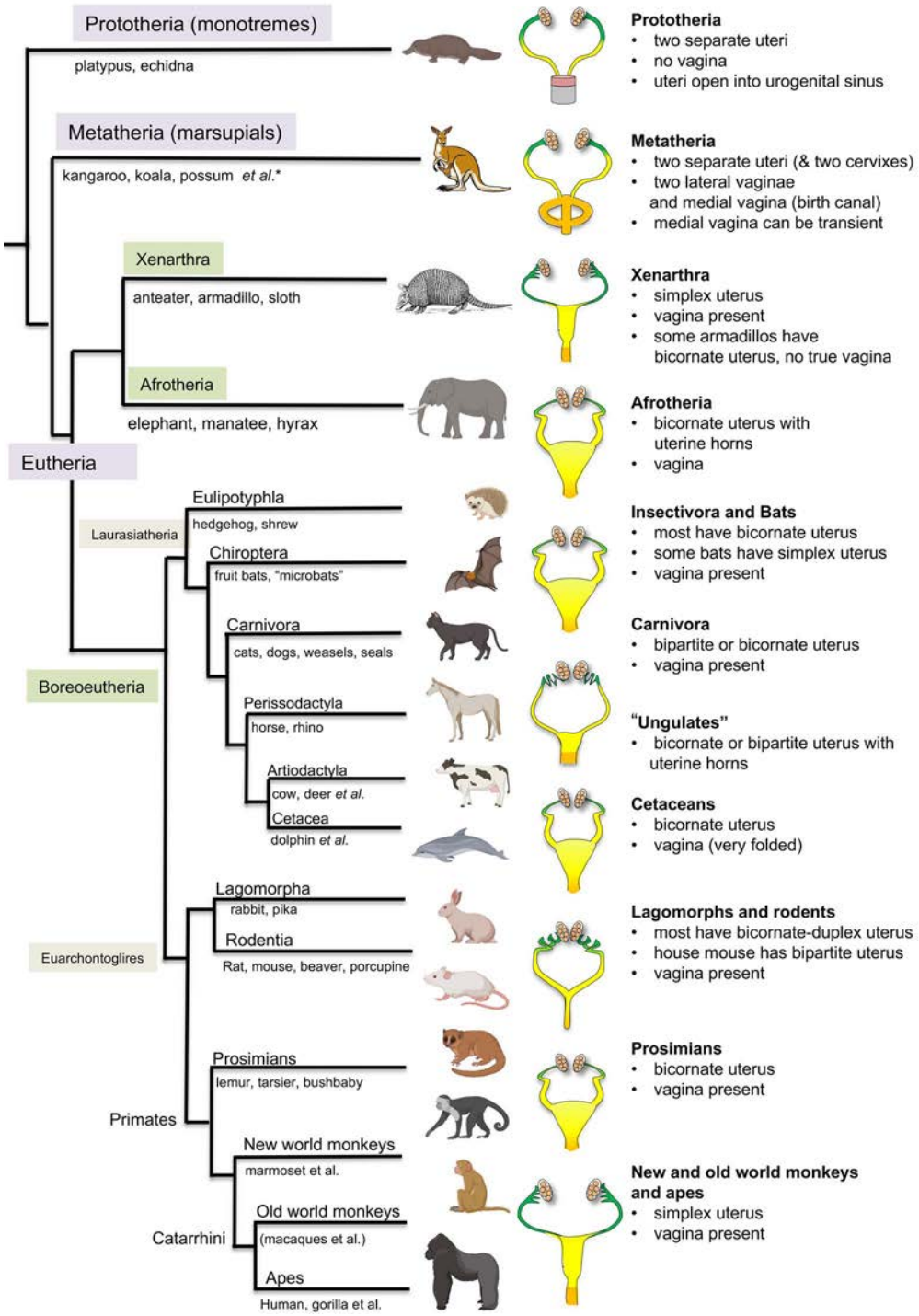

Figure 4. Diversity of adult female reproductive tracts across the mammalian phylogeny. Prototherians (monotremes) have separate uteri and lack a vagina, the likely ancestral state. Metatherians (marsupials) have two separate uteri and cervixes together with two lateral vaginae and a medial vagina (which can be transient, as in phalangers), and a vagina is present. Among eutherians ("placentals") diverse uterine structures are evident, reflecting different degrees of caudal embryonic duct fusion, from duplex to bipartite, bicornate and simplex uteri. Phylogeny based upon integrated current molecular genetic data available on TimeTree (http://www.timetree.org/). Figure partly prepared using BioRender.com. = oviduct (uterine tube, or Fallopian tube in humans). = uterus. = vagina. = urogenital sinus and cloaca. = ovary.

bear, sea otter and pinnipeds (seals, sea lions) have tubular bicornate or bipartite uterus but only one or two embryos [126]. Chiropterans (bats) are an interesting case. Among bats, most species have a bicornate uterus, but some have a simplex structure [140, 141]. Bat neonates are relatively large relative to maternal body weight. Overall, duplex uteri in eutherians are associated with multiple

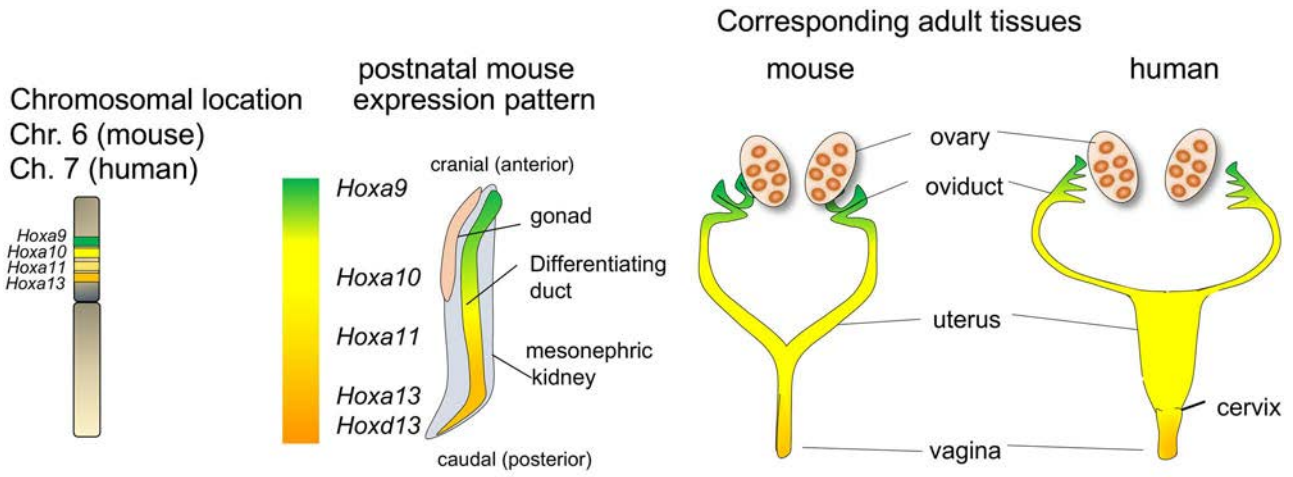

Figure 5. Expression patterns of 5′ *Hox*a and *Hoxd* genes in the mouse Müllerian duct and the relationship to adult female reproductive tracts in mouse and human.

smaller embryos while the trend towards the simplex uterus is associated with one or two larger embryos (either altricial or precocial). However, the adaptive significance of the different mammalian uterine types remains to be fully understood.

In humans and mouse models, patterning and differentiation of the female reproductive tract at the molecular level has been correlated with nested expression domains of posterior *Hox* genes (Fig. 5). In both species, *Hoxa* genes are differentially expressed along the length of the Müllerian duct, induced, at least in part, by graded retinoic acid signalling [69, 142]. As in other tissues along the A-P axis, these genes show spatial expression domains along the Müllerian duct in postnatal mouse that are collinear with their chromosomal ogranisation [73, 143]. In mouse, *Hoxa9, Hoxa10, Hoxa11* and *Hoxa13/Hoxd13* exhibit embryonic expression domains that demarcate the future oviduct, uterus, cervix-vagina and vagina, respectively (Fig. 5) [70, 73]. Loss of function mutations in these genes can cause homeotic transformations of the female mouse reproductive tract [68, 69, 73, 143]. Segmental differentiation of the uterus requires *Hoxa10* and *Hoxa11*. In mouse, mutations in *Hoxa10* can cause partial transformation of the uterus into oviduct [74]. Disruption of *Hoxa11* in mouse embryos also causes partial homeotic transformation and affects radial uterine patterning [144]. (*Hoxa12* is lost in mammals). Interestingly, while these *Hox* genes show the spatial collinearity along the duct, they do not exhibit temporal collinearity, at least in the postnatal mouse. They are all expressed simultaneously [73]. It has been suggested that this may afford developmental plasticity as the Müllerian duct differentiates unusually late (after embryogenesis) compared to other organs [73].

Postnatally, WNT signalling also plays a role in patterning the uterus, at least in mice. *Wnt7a* and *Wnt5a* are expressed in the uterine horns of postnatal mice, in epithelium and mesenchyme, respectively [145]. Wnt expression in the female reproductive tract is conserved in the neonatal sheep [146]. In mouse, expression of these genes is highly regionalised. *Wnt7a* mutant mice show posteriorized female reproductive tracts. Postnatally, the posterior part of the oviduct appears uterine and the posterior part of uterus (normally simple columnar epithelium) appears vaginal (stratified squamous epithelium) [147]. *Wnt5a* mutant mice have shortened and coiled uterine horns [148]. The different uterine morphologies seen in different mammalian clades could be regulated by altered expression domains of *Hox* and/or *Wnt* genes, either at embryonic or postnatal stages, given that homeotic transformations or fusion defects can prevail when these factors are ablated in mice. *Hoxa10*, for example, which is implicated in uterine differentiation, may have temporally or spatially altered expression domains in the Müllerian ducts of diverse mammalian species that have duplex vs bicornate vs simplex uteri.

Among mammals, the vagina is a reproductive innovation not present in other vertebrates and that may have evolved through modified *Hox* or *Wnt* gene expression domains. In the mouse model, *Hoxa13* and *Hoxd13* are expressed in the posterior pole of the Müllerian duct and are required for differentiation of the vagina. Compound *Hoxa13* and *Hoxd13* mutant mice fail to complete caudal duct fusion to form the vagina [68]. Humans with *HOXA13* coding region mutations exhibit hand-foot-genital syndrome, featuring a similar duct fusion defect to the mouse *Hoxa13/Hoxd13* model [149, 150]. As monotreme mammals lack a vagina, while marsupials have three (Fig. 4), it would be of interest to examine Müllerian ducts (or postnatal ducts) of these animals in the context of embryonic *Hoxa13* and *Hoxd13* expression. In terms of WNT expression, *Wnt5a* mouse mutants lack clear cervical and vaginal structures [148]. Plasticity in WNT signalling may be another mechanism that has driven evolution of the vagina among the therian clade of mammals. Evolution of cis-regulatory elements controlling *Hox* and *Wnt* gene expression may be drivers of reproductive tract diversity. However, it has been shown that structural changes to the coding sequences of *Hox* genes themselves may have been important for female reproductive tract evolution. There has been strong directional selection of *Hoxa11* and *Hoxa13* in the therian lineages, which are linked to cervix and vagina development [31, 151]. It is posited that novel or expanded functions of these transcription factors has facilitated new protein–protein interactions and cellular functions that have been important for the evolution of the vagina, uterus, implantation and *in utero* embryonic development. More broadly, it would be informative to examine *Hox* and *Wnt* expression (and indeed global gene expression) at the time of caudal Müllerian duct fusion in accessible model species that have different uterine structures. This would be is feasible in bats, which have diverse uterine structures and where embryonic development has been described in some species [141, 152].

Most recently, unbiased global gene expression studies have been applied to the question of female reproductive tract evolution, focussing on the transition from egg laying to live birth in mammals. Lynch and colleagues used high throughput RNA-seq to characterise the transcriptome of the (adult) uterine endometrium coincident with the emergence of pregnancy. They detected thousands of genes recruited to, or lost from, the uterus during the evolution of eutherian pregnancy. Using ChIP-seq and related methods, they found that changes to cis-regulatory regions mediated via transposable elements have played a major role during uterine evolution [153]. It would be worthwhile to apply such a detailed comparative analysis to late stage vertebrate embryos during the segmental differentiation of the female reproductive tract across mammalian embryos from the major clades.

Regional differentiation of the Müllerian duct among vertebrates beyond mammals may also involve plasticity of the *HOX* code and /or WNT signalling outlined above. There is currently no information on the role of *Hox* genes or indeed other master developmental regulators in regionalised differentiation of the female reproductive tract among non-mammals. The 5' *Hoxa* genes are expressed in embryonic chicken Müllerian ducts [57], but their role in avian duct differentiation is unknown. Regional differentiation of the reptilian and bird female reproductive tracts (magnum, isthmus, shell gland) may be regulated by graded *Hox* signals as in mammals. Some previous studies have described the transcriptional landscape of the mature chicken oviduct, comparing magnum, and "uterus" or shell gland, with emphasis on genes expressed for egg production [154, 155]. However, how these compartments are genetically specified during embryogenesis remains unknown. In an evo-devo context, it would be of interest to examine the evolutionary conservation of the *Hox* code in the Müllerian duct including among cartilaginous fishes (sharks), where the ducts form in a fundamentally different way to those of tetrapods. While the diversity of female reproductive tract development may be underpinned by alterations in the Müllerian *Hox* code, this is at present an unexplored area of comparative reproductive anatomy.

Conclusion and outlook

Morphogenesis of the female reproductive tract has been fundamental to vertebrate development and evolution. The evolution of jawed vertebrates from agnathan ancestors has involved the development of a dedicated pair of ducts for transporting the oocyte, fertilisation, egg development and, in many cases, directly supporting embryogenesis. Developmentally, the female reproductive tract derives from paired Müllerian ducts, which appear to have arisen via co-option of gene regulatory pathways pre-existing in the pronephric (Wolffian) duct and mesonephric kidney. Over evolution, the female reproductive tract has exhibited remarkable diversification, becoming adapted to different reproductive modes. How this plasticity is genetically regulated is still largely unknown, but is ripe for detailed molecular studies. Some insights into duct evolution could actually come from genetic analysis of human females with atypical reproductive tract development. Approximately 4% of women have irregular uterine anatomy, such as didelphys (paired) or bicornate uterus, in which the caudal parts of the duct fail to fuse [156]. Genes found to be mutated in these cases would be candidate regulators of uterus evolution. In animal diverse animal models, forward and reverse genetics screens will also enhance our understanding of Müllerian duct differentiation during embryogenesis. Methods such as histone Chromatin immunoprecipitation and ATAC-seq can be combined with RNA-seq studies of whole transcriptome profiling to provide a more complete view of the gene regulatory pathways governing differentiation of the duct across different vertebrate clades [157].

Of particular value will be the application of RNA-seq and single cell RNA-seq to species beyond mammals. RNA-seq will reveal a great deal about the conserved and divergent mRNA expression patterns in the Müllerian duct during its development across animal groups. Single cell RNA-seq will also inform our understanding of cell lineage specification during duct formation across groups and whether the cell biology is conserved of divergent. Currently, single cell technology has not been applied to the Müllerian duct (not even in mouse). Most recently, fluorescent mouse reporter lines have been used to define regional segmentation of the mouse oviduct at the molecular level [158], while single cell sequencing has been conducted on the neonatal mouse uterus [159]. However, detailed single cell RNA-seq analysis during Müllerian duct morphogenesis is completely lacking. All of these approaches will yield new information regarding the evo-devo of female duct development. The female reproductive tract provides a fascinating biological model for exploring intersecting questions relating to reproduction, development and evo-devo. Understanding how the female reproductive tract develops in different vertebrate groups, and the developmental mechanisms involved, will shed light on the genetics that underpin a major step in evolution.

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