The molecular pathways underlying early gonadal development

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Abstract
The body of knowledge surrounding reproductive development spans the fields of genetics, anatomy, physiology and biomedicine, to build a comprehensive understanding of the later stages of reproductive development in humans and animal models. Despite this, there remains much to learn about the bi-potential progenitor structure that the ovary and testis arise from, known as the genital ridge (GR). This tissue forms relatively late in embryonic development and has the potential to form either the ovary or testis, which in turn produce hormones required for the development of the rest of the reproductive tract. It is imperative that we understand the genetic networks underpinning GR development if we are to begin to understand abnormalities in the adult. This is particularly relevant in the contexts of disorders of sex development (DSDs) and infertility, two conditions that many individuals struggle with worldwide, with often no answers as to their aetiology. Here, we review what is known about the genetics of GR development. Investigating the genetic networks required for GR formation will not only contribute to our understanding of the genetic regulation of reproductive development, it may in turn open new avenues of investigation into reproductive abnormalities and later fertility issues in the adult.

Genital ridge formation
The mammalian GR is derived from intermediate mesoderm as paired structures that lie on either side of the dorsal mesentery in the coelomic cavity (Fig. 2). Formation of testicular/ovarian structures, accessory sex structures and secondary sexual characteristics, whereby genital ridge (GR) formation was included only as a step in gonadal sex determination or excluded from the broader picture all together. The focus of this review is formation of the GR in mice and humans, and consequences of failure of its development, rather than sex determination, which has been reviewed extensively elsewhere (Greenfield 2015, Capel 2017).

Key Words
- gonad
- urogenital ridge
- disorders of sex determination
- infertility
- molecular genetics
the mammalian GR begins with increased proliferation of coelomic epithelial cells on the ventromedial surface of the mesonephroi. Each mesonephros also contains the mesonephric duct (also known as the Wolffian duct), a primordial urogenital tissue that will give rise to the male epididymis, vas deferens and seminal vesicles following male sex determination (Hannema & Hughes 2007, Shaw & Renfree 2014). In addition, the paramesonephric duct (also known as the Müllerian duct) is also present in the mesonephros, running in parallel to the mesonephric duct (Fig. 2A). This is the female equivalent to the mesonephric duct, which will form the fallopian tubes, uterus and part of the vagina following female sex determination (Acien 1992). Together, the mesonephros and GR are known as the urogenital ridge (UGR).

Proliferation of the coelomic epithelial cells on the ventromedial surface of the mesonephros creates a dense, pseudostratified epithelial cell layer (Gropp & Ohno 1966, Pelliniemi 1975, Wartenberg et al. 1991). Alongside the proliferation of the coelomic epithelium, the underlying basement membrane becomes fragmented, allowing many epithelial cells to ingress dorsally, towards the mesonephros (Fig. 2B) (Karl & Capel 1998, Kusaka et al. 2010). As these cells delaminate from the coelomic epithelium, they undergo an epithelial-to-mesenchymal transition (EMT) (Kusaka et al. 2010) and as mesenchymal cells, begin to populate the space between the coelomic epithelium and the mesonephros (Karl & Capel 1998, Schmahl et al. 2000). These mesenchymal cells of the GR are precursor cells that can differentiate into somatic support cells and interstitial/stromal cell lineages of the early sexually differentiated gonad (Karl & Capel 1998, Ito et al. 2006, Mork et al. 2012).

In the testis, additional cells are also recruited into the GR region from the mesonephros to augment the population of mesenchymal cells. Cell-tracing and organ culture studies in mice revealed that these largely contribute to the endothelial cell population for the establishment of the male vascular system (Karl & Capel 1995, Martineau et al. 1997, Coveney et al. 2008). Migrating into the GR region from the mesonephros to augment the population of mesenchymal cells. Cell-tracing and organ culture studies in mice revealed that these largely contribute to the endothelial cell population for the establishment of the male vascular system (Karl & Capel 1995, Martineau et al. 1997, Coveney et al. 2008).
just prior to gonadal sex determination are primordial germ cells (PGC), the precursors to the germ cells that become sperm and oocytes later in life. PGCs arise near the yolk sac and travel, via the hindgut, to the GR as a result of chemotaxis (Doitsidou et al. 2002).

Genital ridge formation in mice

Most of the work investigating mammalian gonad development has been performed on mice. It is assumed that events in the human embryo, as well as other Eutherian mammals, follow the same basic pattern, albeit with some differences in the timing and anatomy.

The process of GR formation, starting with the proliferation of the coelomic epithelium, begins at about E9.5 in mice (Hu et al. 2013), which equates to about 5 weeks of gestation in humans (Jost 1972). The GR itself is not morphologically evident until about E10.0–10.5 where a clear distinction between the GR and the mesonephros can be seen under light microscopy. From E10.5 to 11.5, further proliferation of the GR coelomic epithelium and mesonephros expands the overall size on the ventral side of the mesonephros (Fig. 2A). The initiation, proliferation and expansion of the GR is indistinguishable between XX and XY embryos, and the size of the GR is determined by the length of the embryonic trunk (Wainwright et al. 2014). At around E11.5–12.0, the molecular events that determine gonad fate occur, prompting the gonad to follow either a testis- or ovary-specific developmental trajectory from this time-point. The first sign of sexual dimorphism of the early differentiated gonad becomes evident in XY gonads at about E12.5, when testis cords can be seen (Hacker et al. 1995, Schmahl et al. 2000).

The GR is not only a progenitor tissue for the gonad, but also the adrenal cortex, a critical endocrine tissue that synthesizes and secretes a variety of steroid hormones to maintain body homeostasis and regulate the stress response (Yates et al. 2013, Walczak & Hammer 2015). Consequently, several genes required for gonad development are also important for adrenal gland development (Luo et al. 1994, Gut et al. 2005, Katoh-Fukui et al. 2005, Bandiera et al. 2013, Tevosian et al. 2015). The medulla and the cortex of the adult adrenal gland have separate origins; the medulla is derived from neural crest cells, whereas the cortex is derived from cells located at the anterior-most region of the GR, indicating it has an intermediate mesoderm origin (Hatano et al. 1996). Beginning at E10.5, a small cluster of cells at the anterior region of mesenchyme separates from the GR primordium and moves dorso-medially to form the adrenal anlage (Hatano et al. 1996, Val & Swain 2010). From E11.5 to 12.5, neural crest cells invade the adrenal anlage and aggregate in the centre to form the medulla. Mesenchyme cells, hypothesized to be of a coelomic epithelial origin, form a fibrous capsule around the composite adrenal primordium by E14.5 (Xing et al. 2015).

Development of the PGC in the Genital ridge

As previously mentioned, PGCs originate from a separate location near the yolk sac, away from the mesonephros and GR (Saga 2008). Mammalian PGCs are specified via an inductive system of signalling molecules, particularly BMP4. In mice, PGCs are induced around E6.5 (equating to approximately 2 weeks gestation in humans) from the proximal epiblast by BMP4 signalling (Lawson et al. 1999). These cells subsequently move to and cluster at the base of the allantois/yolk sac wall, near the forming hindgut, which can be seen in mouse embryos at about E7.0 (week 3–4 human gestation) as a small population of ~45 cells (Lawson et al. 1999). As development proceeds, the hindgut folds and the PGCs migrate into the embryo proper (Hara et al. 2009, Harikae et al. 2013). By E9.5 the PGCs begin to migrate away from the hindgut towards the UGR and colonize the gonad between E10.0 and 11.0 (~5-week gestation in humans) (Fig. 2A; Witschi 1948, Molyneaux et al. 2001). During this mass migration of PGCs, the hindgut descends into the coelomic cavity and the last PGCs to migrate must travel through the dorsal mesentery before entering the gonads (Molyneaux et al. 2001).

The PGCs undergo several rounds of cell division to achieve a population of about ~3000 cells by ~E11.5 (Tam & Snow 1981). Around this time, PGCs begin to undergo a process known as licensing, undergoing a global change in gene expression, turning on genes required for gametogenesis, concurrently switching off their pluripotency genes over the course of the following days (Stebler et al. 2004, Gill et al. 2011, Rolland et al. 2011, Seisenberger et al. 2012). Following this transition, the PGCs are referred to as gametogenesis-competent cells and are poised to initiate either male or female differentiation, including meiosis, upon receiving cues from the somatic cells of either the forming testis or ovary and the nearby mesonephric tissue (McLaren & Southey 1997, Adams & McLaren 2002, Gill et al. 2011).
Genes essential for initial gonad formation

Mutational analysis using the mouse model, with some additional evidence from human clinical cases, have brought to light a number of genes that are required to initiate the formation and proliferation of the GR, as well as testis/ovary differentiation (Table 1). These include Wilms’ tumour suppressor 1 (WT1) (Kreidberg et al. 1993, Hammes et al. 2001), LIM homeobox gene 9 (LHX9) (Birk et al. 2000), Nuclear Receptor Subfamily 5 Group A member 1 (NR5A1; also called steroidogenic factor 1 (SF1)) (Luo et al. 1994), empty spiracles homeobox gene 2 (EMX2) (Miyamoto et al. 1997) and GATA-binding protein 4 (GATA4) (Hu et al. 2013). In mouse embryos, a homozygous null mutation in any one of these genes causes gonadal agenesis. While the function of these five genes have mainly been characterized in mice, mutations in three of these genes (NR5A1, WTI and GATA4) have also been found in patients with DSD, indicating a conserved role in reproductive development (Bashamboo et al. 2010b, Kohler et al. 2011, Lourenco et al. 2011, Swartz et al. 2017). However, these genes are expressed and function in many developing organ systems, meaning the loss of function in both mice and humans produce a range of phenotypes beyond the reproductive tract (Ingraham et al. 1994, Klamt et al. 1998, Hammes et al. 2001, Tevosian et al. 2015). Genes known to be required for GR development often show altered expression in gene knockout lines, indicating a co-regulatory relationship exists between these critical factors (discussed further below).

It is evident that many of these critical early factors discussed below have multiple roles in reproductive

<table>
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<tr>
<th>Gene</th>
<th>Mouse UGR expression</th>
<th>Mouse gonadal phenotype</th>
<th>Human phenotype</th>
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<tbody>
<tr>
<td>Nr5a1</td>
<td>Initially expressed in the anterior region of the coelomic epithelium. Expression domain expands in an anterior–posterior direction (Hu et al. 2013)</td>
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<tr>
<td>Emx2</td>
<td>Coelomic epithelium and cells that move into the underlying mesenchyme Mesonephric duct epithelia (Hu et al. 2013)</td>
<td>Absent gonads Reduced proliferation of the coelomic epithelium (Miyamoto et al. 1997, Pellegrini et al. 1997)</td>
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<td>Lhx9</td>
<td>Coelomic epithelium and cells that move into the underlying mesenchyme (Birk et al. 2000, Hu et al. 2013)</td>
<td>Absent gonads Reduced proliferation of the coelomic epithelium (Birk et al. 2000)</td>
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<tr>
<td>Gata4</td>
<td>Coelomic epithelium, expression domain expands in an anterior–posterior direction, precedes expression of Sf1 (Hu et al. 2013)</td>
<td>Absent gonads No expansion of the coelomic epithelium (Hu et al. 2013) Following sex determination, roles in ovarian and testicular development (Efimenko et al. 2013) Minor testis abnormalities (Warr et al. 2011)</td>
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<tr>
<td>MAP3K1</td>
<td>Coelomic epithelium and gonad mesenchyme (Warr et al. 2011)</td>
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development, from formation of the bi-potential gonad of both sexes, to sex-specific roles following gonadal sex determination. Currently, we are lacking a detailed gene network integrating both the roles of these transcription factors, along with the signalling pathways that regulate them, to gain a broader picture of how these components together regulate cellular processes like differentiation and proliferation to control formation of the bi-potential gonad. Future genome-wide approaches to study gene interactions will help to better define the regulatory interactions between these proteins, and others, required for GR formation.

Wilms tumour suppressor (Wt1)

*Wt1* mRNA is expressed during gonad development in the coelomic epithelium and mesonephros prior to gonadal sex determination and in the progenitor support cells of both the developing testis and ovary (*Armstrong et al. 1993*). *Wt1−/−* mice have a recognizable gonad primordium at E11.0, but this then degenerates due to apoptosis of somatic cells (*Kreidberg et al. 1993, Hammes et al. 2001*). Knockout mouse embryos, on a C57BL/6 genetic background, do not survive to parturition due to embryonic lethality from heart defects, while knockout mice generated on other genetic backgrounds, such as Balb/c, survive until birth (*Kreidberg et al. 1993, Herzer et al. 1999*).

The *Wt1* gene encodes for a zinc finger transcription factor that functions as either an activator or repressor of transcription; however, the structure and function of the WT1 protein varies depending on the cell type and promoter used to transcribe the gene, RNA editing, alternative usage of translational start sites and alternative splicing (*Bruening & Pelletier 1996, Scharnhorst et al. 1999, Dallosso et al. 2007*). Of particular interest to gonad development are two alternate splice forms of the *Wt1* protein named WT1−KTS and WT1+KTS, due to either the inclusion or exclusion of three amino acids located between the third and fourth zinc finger domains (*Hammes et al. 2001*). Mice lacking expression of the *Wt1−KTS* isoform have gonads markedly reduced in size and poorly differentiated (*Hammes et al. 2001*), suggesting that WT1−KTS is the isoform required for the proliferation and differentiation of GR cells. Knockout of the WT1+(KTS) splice transcript leads to complete male-to-female sex reversal and reduced *Sry* gene expression, yet, ovarian development proceeds normally (*Hammes et al. 2001; Fig. 3*). For both knockout lines, the overall expression of *Wt1* mRNA was similar to WT animals, due to increased expression of the alternative splice form compensating for the loss of the targeted splice form (*Hammes et al. 2001*). Thus, this resulting shift in isoform ratio (the ratio of −KTS/+KTS), leading to overexpression of the alternative isoform, may also contribute to the observed knockout phenotype. However, this has not been investigated further in mice with transgenic mouse lines. In humans, an altered splice form ratio (increased WT1−KTS, reduced WT1+KTS isoform) does severely affect gonadal development resulting in Frasier syndrome with streak gonads (*Barbaux et al. 1997, Klamt et al. 1998*).

More recently, *Chen et al. (2017)* showed that the conditional inactivation of *Wt1* just prior to sex determination at E10.5 allows gonadogenesis to proceed with reduced differentiation of Sertoli and granulosa cells from somatic cell precursors. Thus, when *Wt1* expression is lost from gonads at E13.5, most somatic cells develop into steroidogenic cell types (*Chen et al. 2017; Fig. 3*). In contrast, in traditional knockout animals where *Wt1* is inactive throughout development, development of the GR is blocked (*Kreidberg et al. 1993; Fig. 3*). Thus, the role of WT1 alters as gonad development progresses, from being required for initial cell proliferation and growth of the GR, predominately through the WT1−KTS splice form, to controlling cell fate of somatic cells, and this is likely to occur through direct regulation of Nr5a1 gene expression (*Fig. 4*, described further in the following section).
not develop beyond the early GR stage, and as a result, XY mice show complete male-to-female sex reversal (Luo et al. 1994). Gonadal regression in Nr5a1−/− embryos is due to increased apoptosis of the somatic cells (Luo et al. 1994), also observed with WT1 loss-of-function mice (Hammes et al. 2001). Recent single-cell RNA sequencing (scRNA-seq) of XY somatic cells, expressing Nr5a1 (Nr5a1-GFP labelled cells) prior to (E10.5) and following sex determination (E11.5–E16.5), confirmed the presence of a multipotent Nr5a1+ cell population in the GR that progressively forms both the supporting and steroidogenic cell lineages from E11.5 (Stevant et al. 2018).

Regulation of Nr5a1 gene expression plays a critical role in gonad development, with all key transcription factors described to date in GR development function in regulating correct Nr5a1 expression (Fig. 4; Wilhelm & Englert 2002, Katoh-Fukui et al. 2005, Hu et al. 2013, Chen et al. 2017). LHX9 and WT1−/− proteins both bind to the promoter region of Nr5a1 in vitro, and together they increase reporter gene expression in a Sertoli-like (TM4) cell line (Wilhelm & Englert 2002), a finding replicated in Leydig-like (TM3) and C2C12 (myoblast derived) cell lines (Val et al. 2007, Takasawa et al. 2014). However, in primary Leydig cells WT1 overexpression repressed Nr5a1 gene expression. Further experiments confirmed that WT1 directly binds in vivo to the Nr5a1 promoter in Sertoli cells obtained from 2-week-old mice (Chen et al. 2017). It was suggested that these conflicting results were most likely due to the use of cell lines vs primary cells in these studies (Chen et al. 2017). The TM4 and TM3 cell lines were derived from juvenile BALB/c mouse testis (Mather 1980) and both cell lines express a similar combination of cell type gene markers (Beverdam et al. 2003). It is also worth noting that Chen et al. (2017) used primary Leydig cells obtained from adult and juvenile mice on a mixed background (C57Bl/6:129/SvEv), given that strain background can strongly influence the resulting phenotypes (Herzter et al. 1999, Meeks et al. 2003, Brennan & Capel 2004, Munger et al. 2009). WT1 also may have differing roles in the regulation of Nr5a1 gene expression, as the gonad develops into a testis, perhaps being required for an initial activation of Nr5a1, with protein partner LHX9 in the early gonad and, following gonadal sex determination, WT1 reduces Nr5a1 expression in those NR5A1+ cells that are fated to become Sertoli cells. In this case, the presence or absence of certain protein partners, for instance LHX9, would impact on how WT1 regulates Nr5a1 gene expression. Previous studies have shown that WT1 is converted from an activator to a repressor protein by the protein partners such as BASP1 (McKay et al. 1999, 2002).
Carpenter et al. 2004). Given that NR5A1+ cells contribute to both cell populations, it may that Nr5a1 expression levels determine which fate, with Sertoli cells expressing significantly lower levels of Nr5a1 compared to Leydig cells (Fig. 4B; Chen et al. 2017).

LIM homeobox 9 (Lhx9)

The Lhx9 gene encodes a transcription factor expressed in a variety of regions within the developing mouse embryo, including the brain, heart, kidney, limb buds and the coelomic epithelium (Retaux et al. 1999, Birk et al. 2000, Failli et al. 2000, Molle et al. 2004, Oshima et al. 2007, Smagulova et al. 2008, Tzchori et al. 2009, Yang & Wilson 2015). Lhx9 gene expression in the UGR is first seen in the coelomic epithelium at E9.5 and later in the gonad primordium, until sexual differentiation where its expression becomes restricted to the interstitial/mesothelial regions of testes and the cortical regions of ovaries (Birk et al. 2000). Lhx9+/− mutant mice exhibit a similar gonadal phenotype to that of Wt1(−KTS)−/− mice, whereby normal GR development and PGC migration is observed but discrete gonads fail to form and genetically male mice show complete male-to-female sex reversal of their secondary sex characteristics (Birk et al. 2000). The observed underdevelopment of the GR results from disrupted proliferation of the gonad primordium (Birk et al. 2000), as opposed to increased apoptosis observed in the Wt1−/− and Nr5a1+/− mice (Kreidberg et al. 1993, Luo et al. 1994). In addition, male- and female-knockout offspring are infertile, with atrophic uteri, vaginas and oviducts, no male accessory sex organs, increased follicle-stimulating hormone levels, and undetectable levels of testosterone and oestrogen (Birk et al. 2000). Interestingly, Lhx9+/− mice show no other phenotypic abnormalities outside of gonad agenesis and male-to-female sex reversal (Birk et al. 2000, Tzchori et al. 2009), despite expression in other tissues, such as the limbs, nervous system and pancreas, during development. This may be a result of the functional redundancy of Lhx9 with its closely related parologue, LIM homeobox gene, Lhx2 (Jurata & Gill 1998, Birk et al. 2000, Tzchori et al. 2009).

Empty spiracles homeobox 2 (Emx2)

Emx2 encodes a transcription factor that is the mouse homolog of the Drosophila head gap gene empty spiracles (ems). The Emx2 transcript is expressed in the dorsal telencephalon, mesencephrons and coelomic epithelium (Miyamoto et al. 1997, Yoshida et al. 1997). Emx2−/− knockout mice embryos exhibit normal PGC migration into the UGR region, but the initial thickening of the coelomic epithelium is not prominent and the GR soon degenerates (Miyamoto et al. 1997). These mutants completely lack gonads, genital tracts and kidneys (Miyamoto et al. 1997). The gonadal dysgenesis phenotype in Emx2−/− mice is a result of impaired cell migration from the coelomic epithelium through the basement membrane, as well as increased apoptosis (Kusaka et al. 2010). Interestingly, Nr5a1 gene expression is also significantly affected in Emx2−/− embryos (Kusaka et al. 2010), suggesting that Emx2 acts upstream of Nr5a1 in the developmental cascade, but how Emx2 regulates Nr5a1 transcription, whether directly or indirectly remains unclear.

GATA-binding protein 4 (Gata4)

GATA4 is a transcription factor that is essential for the development of multiple organs such as the heart, foregut, liver, ventral pancreas and the UGR (Kuo et al. 1997, Molkentin et al. 1997, Viger et al. 1998, Hu et al. 2013). Gata4 gene expression in the GR was originally linked to a role in testis differentiation through activating transcription of Sry together with WT1 (Tevosian et al. 2002). Later studies revealed that Gata4 is expressed prior to any other gonadal factor, initially in the anterior half of the coelomic epithelium at E9.5 and expanding to the posterior region by E10.2 (Hu et al. 2013). Gata4-knockout mice show no signs of gonadal initiation, with the coelomic epithelium remaining as an undifferentiated monolayer, indicating that Gata4 is required for the initial thickening of the coelomic epithelial layer (Hu et al. 2013). In these knockout mice, Lhx9 and Nr5a1 gene expression is lost, but Wt1 and Emx2 gene expression is unaffected (Hu et al. 2013). This suggests that GATA4 acts not only upstream of the Lhx9 gene, but also Nr5a1, possibly even directly regulating both genes, as binding sites for GATA4 have been identified in the proximal promoter regions of these two genes (Tremblay & Viger 2001, Smagulova et al. 2008, Hu et al. 2013).

Genes with minor but essential roles in Genital ridge development

Other genes have been implicated in early GR development, although mouse-knockout strains for these genes exhibit a less severe phenotype than that of the null phenotype of the key genes described earlier. While the loss of function of the following genes does not cause termination of gonad development, it does result in the underdevelopment of the gonads, often in combination with sex reversal of the secondary sex characteristics.
Transcription factor 21 (Tcf21 or Podf1)

Tcf21, also known as epicardin, encapsulating or transcription factor 21, belongs to the basic helix-loop-helix family of transcription factors. It is expressed by mesodermal cell types including heart proepicardial cells, kidney and visceral smooth muscle as well as the endodermal gastrointestinal tract (Quaggin et al. 1998, 1999, Lu et al. 2000, Miyagishi et al. 2000). Tcf21-knockout mice die in the perinatal period due to lung, kidney and cardiac defects, but also display gastric and splenic defects (Cui et al. 2003, Funato et al. 2003, Plotkin & Mudunuri 2008). In addition, Tcf21 XX and XY KO mice have irregular shaped gonads, the urogenital tracts of both XX and XY mice are indistinguishable and XY pugs had feminized genitalia (Cui et al. 2004). Consistent with this phenotype, Tcf21 gene expression is initially detected throughout the GR at the bi-potential stage and continues to be expressed in the gonads of both sexes, with expression slightly higher in the testes following sex determination (Tamura et al. 2001). While the gonad does form in the Tcf21−/− embryo, morphological defects such as a shortened length were observed by E11, and vascular abnormalities were observed by E12.5 (Cui et al. 2004), suggesting a defect early in gonadal development. Further studies focused on expression analyses, these suggested a negative regulatory relationship exists between Tcf21 and Nr5a1, as Tcf21 KO results in increased Nr5a1 expression and an expanded Leydig cell population (Miyagishi et al. 2000). Despite an increase in Leydig cell numbers, the XY KO genitalia was feminized and the gonads fail to descend from an abdominal position, suggesting that testosterone levels were low, either due to later apoptosis of the gonadal tissue or a not all steroidogenic enzymes were expressed correctly (Cui et al. 2004). TCF21 also appears to repress Nr5a1 gene expression in the adrenal gland, with in vitro studies revealing that TCF21 directly represses Nr5a1 gene expression, through binding at E-box sequences located in the Nr5a1 promoter (Franca et al. 2013, 2015).

Interestingly, the Sertoli cells do differentiate in the Tcf21 KO line, but there was some evidence that this cell population was reduced in number (although it was not quantified) (Cui et al. 2004). TCF21 can cause a sex reversal-like phenotype in vitro using a rat primary ovarian cell culture system. Overexpression of TCF21 causes these cells to express Sertoli-like gene markers, in a similar pattern to that observed with Sry overexpression (Bhandari et al. 2011). Additionally, the Tcf21 gene has proposed to be a direct target of SRY (Bhandari et al. 2011). Together, these results suggest that TCF1 acts downstream of Sry to promote Sertoli cell expansion and repress steroidogenic cell lineages.

Sine oculis-related homeobox 1/4 (Six1/Six4)

Six1 and Six4 genes belong to the mammalian homolog of the Drosophila sine oculis homeobox family of transcription factors, containing a distinctive SIX domain (required for protein-protein interactions) and homeodomain (Kawakami et al. 2000). Six1 and Six4 genes are located in the same genomic regions within about 100kb of one another, and have highly overlapping tissue expression during mouse development (Boucher et al. 1996, Esteve & Bovolenta 1999, Ozaki et al. 2001, Fougerousse et al. 2002, Laclef et al. 2003). Six1/4 double KO mice show different phenotypes compared to either Six1 or Six4 single KO mice, highlighting regions of redundant function in the development of limbs, skeletal muscle, sensory neurons and kidney (Grifone et al. 2005, Konishi et al. 2006, Giordani et al. 2007, Kobayashi et al. 2007). The suggested functional redundancy between the two genes is further exhibited as both genes share a common DNA-binding site, MEF3 (Kawakami et al. 2000, Kumar 2009). In particular, only Six1/4 double KO mice embryos display smaller gonads and adrenal glands (Kobayashi et al. 2007, Fujimoto et al. 2013). Out of the essential GR genes listed previously, only Nr5a1 gene expression is significantly reduced, a finding corroborated through reporter assays showing that SIX1/4 is able to transactivate Nr5a1 transcription (Fujimoto et al. 2013). Furthermore, it was also shown that SIX1/4 is able to activate Fog2 gene expression and that interaction, together with GATA4, regulates Sry gene expression (Fujimoto et al. 2013). Overall, this indicates that SIX1/4 is required necessary for sufficient Nr5a1 gene expression in the early gonad and, a loss of Six1/4 gene expression, reduces Nr5a1 gene expression, and this may be responsible for the Six1/4 KO undersized gonad phenotype. Nonetheless, there is still sufficient Nr5a1 gene expression to form the gonad primordium in Six1/4 KO embryos.

Chromobox homolog 2 (Cbx2) (mouse polycomb group member, M33)

CBX2 is a component of the polycomb group complex of regulatory proteins involved in the repression/silencing of genes. In mice, Cbx2−/−knockout mice show XY gonadal male-to-female (testis-to-ovary) sex reversal, and XX animals have smaller or absent ovaries (Katoh-Fukui et al. 2005). Additionally, mutants show defects in adrenal and
splenic development, which was shown to be a result of a reduction in the expression of \textit{Nr5a1} (Katoh-Fukui \textit{et al.} 2005). This has led to the suggestion that CBX2 acts as an upstream regulator of \textit{Nr5a1} gene expression. \textit{Cbx2} has also been identified as a factor contributing to the differentiation of the testis through indirect regulation of \textit{Sry} (Katoh-Fukui \textit{et al.} 2012). Genome-wide identification of CBX2 target genes in a human Sertoli-like cell line suggests that CBX2 acts to stimulate male-specific genes, while suppressing female-pathway genes (Eid \textit{et al.} 2015).

In humans, a reported case study of a 46, XY child with female internal and external genitalia and histologically normal ovaries found a compound loss-of-function mutation in the coding region of \textit{CBX2} (Biason-Lauber \textit{et al.} 2009). This study further lends support to the role of CBX2 in the trans-activation of \textit{Nr5a1} and its role in both the GR and testis developmental pathways (Fig. 4A).

**Insulin receptor, \textit{Insr} and insulin-like growth factor type 1 receptor (\textit{Igf1r})**

Insulin and its related growth factors IGF1 and IGF2 regulate a variety of physiological processes including metabolism, stimulation of cell proliferation, differentiation and survival (Efstratiadis 1998). Their function is mediated by two membrane-associated tyrosine kinase receptors, the insulin receptor (INSR) and the IGF type 1 receptor (IGF1R). The genes for \textit{Insr}, \textit{Igf1r} and insulin receptor-related receptor (\textit{Irr}) have previously been shown to be necessary for the testis determination pathway, as mutant mice lacking all three genes show male-to-female sex reversal and decreased \textit{Sry} and \textit{Sox9} gene expression (Nef \textit{et al.} 2003). Recently, it has been shown that \textit{Insr} and \textit{Igf1r}, but not \textit{Irr} have roles in GR development, whereby mice lacking both \textit{Insr} and \textit{Igf1r} have reduced proliferation of the GR prior to sex determination but also extensive downregulation of hundreds of genes associated with adrenal, testicular and ovarian development (Pitetti \textit{et al.} 2013). As a result, these mice embryos exhibit agenesis of the adrenal cortex, along with male-female sex reversal due to a delay in \textit{Sry} gene upregulation. Interestingly, ovarian differentiation is also delayed in these mice, leaving the GR in an undifferentiated state until about E16.5 when the ovarian programme is eventually initiated (Pitetti \textit{et al.} 2013). Among the genes downregulated are \textit{Wt1}, \textit{Lhx9} and \textit{Nr5a1}, indicating that genes essential for GR development are under the influence of insulin/IGF signalling (Pitetti \textit{et al.} 2013), but only partially dependent on this signalling pathway, as GR development is only hindered by decreased progenitor cell numbers.

**Pre-B-cell leukaemia homeobox 1 (\textit{Pbx1})**

\textit{Pbx1} encodes a TALE (three amino acid loop extension) class homeodomain transcription factor that has been shown to be involved in a number of processes during mammalian embryogenesis including, skeletal development and patterning (Selleri \textit{et al.} 2001), maintenance of haematopoiesis (DiMartino \textit{et al.} 2001), pancreatic development (Kim \textit{et al.} 2002) and kidney and adrenal development (Schnabel \textit{et al.} 2003). The GR of \textit{Pbx1}-null mice are also smaller due to decreased proliferation of the progenitor cells in the gonad primordium (Schnabel \textit{et al.} 2003). Further experiments found that this is due to the downregulation of \textit{Nr5a1} gene expression; \textit{Pbx1} also upregulates \textit{Nr5a1} during adrenal development (Schnabel \textit{et al.} 2003). The adrenal gland primordium is initially part of the GR but then later buds off from the very rostral end around E10.5 (Schnabel \textit{et al.} 2003, Pitetti \textit{et al.} 2013). Therefore, perturbed \textit{Pbx1} gene expression prior to budding likely affects \textit{Nr5a1} expression in the rostral GR, if not the whole GR, causing reduced \textit{Nr5a1} expression and decreased proliferation of somatic progenitor cells.

**Odd-skipped related 1 (\textit{Odd1})**

\textit{Odd1} gene encodes a zinc finger transcription factor homologous to the \textit{Drosophila} odd-skipped class of transcription factors that are involved in embryonic patterning and tissue morphogenesis (Wang \textit{et al.} 2005). Targeted gene knockout of \textit{Odd1} revealed that this gene functions in both heart and intermediate mesoderm development (Wang \textit{et al.} 2005). \textit{Odd1}−/− embryos have severe heart malformations, and completely lack adrenal glands, kidneys and gonads, all of which derive from intermediate mesoderm (Wang \textit{et al.} 2005). Although gonad development was not the focus of this study, they did show that in early development the GR was hypoplastic (Wang \textit{et al.} 2005), likely due to increased apoptosis observed with the developing kidney. These hypoplastic GRs appeared to degenerate, as no visible gonad structures were observed by E15.5 (Wang \textit{et al.} 2005). Although no further investigation of \textit{Odd1} with regards to urogenital development has been done since Wang \textit{et al.} (2005), it is unclear if the GR phenotype was an effect of \textit{Odd1} acting either directly in the GR or indirectly, potentially acting via downregulation of \textit{Wt1} gene expression, which also has an apoptotic phenotype when deleted (Hammes \textit{et al.} 2001).
Genital ridge formation in humans

Much of what is known of human gonadal development is based on early embryology work and case studies of individuals with abnormal characteristics stemming from improper sexual development as a result of disruption of genes involved in this process. Recently, as sequencing technologies have become more affordable and require less material, more studies are using human-derived samples to study human sex development at the molecular level, mostly through studies of cases of disorders of sexual development (DSD) (O’Shaughnessy et al. 2007, Ostrer et al. 2007, Houmard et al. 2009, Del Valle et al. 2017, Li et al. 2017). These are congenital disorders that arise as a consequence of atypical chromosomal, gonadal or anatomical sexual development (Hughes et al. 2006), including some of the aforementioned genes involved in GR development. The term DSD replaced old and ambiguous terms such as intersex, hermaphroditism and pseudo-hermaphroditism previously used to describe such disorders in humans (Dreger et al. 2005, Lees & Tuch 2006). DSD phenotypes include a broad range of conditions such as failure of gonad formation, mixed gonadal tissue (male- and female-specific cell types within the tissue), ambiguous genitalia and failure of secondary sexual characteristics to develop normally (Cools et al. 2006). There is limited data on the incidence of DSDs, but it is estimated that the overall worldwide incidence of DSDs is 1 in 5,500 (Damiani 2007, Houk & Lee 2008).

In humans, the gonad develops from the coelomic epithelium (Fig. 5), and the first signs of sex differentiation are observed between 6 and 7 weeks with the formation of testicular cords (Gruenwald 1942, Wyndham 1943). Germ cells are first detected in the hindgut dorsal mesentery at 4 weeks (Carnegie Stage 12 (CS12)) and migrate into the GR by 5 weeks (CS16) (Mckay et al. 1953). Gondal sex determination occurs around 41–42 days in human embryos, signified by the upregulation of SRY gene expression in XY embryos ((Hanley et al. 2000, Del Valle et al. 2017, Mamsen et al. 2017). Genes required for steroidogenesis and secreted factors such as AMH are essential for the sex-specific development of genitalia and the reproductive tract, with their expression commencing between 54 and 57 dpc (CS23). Genes essential for GR development in mice are also expressed at similar levels in both XX and XY gonads (Lhx9, Emx2, Wt1 and Gata4), further supporting a role for these factors in early human gonad development (Del Valle et al. 2017, Mamsen et al. 2017).

Despite their apparent early essential roles in gonad development, the loss of function of genes required for sex development typically results in a range of phenotypes from complete gonad dysgenesis to adult infertility. Mutations in some of these early gonad development genes are also responsible for some cases of human idiopathic infertility in cases with apparently normal gonad development.

Like DSDs, infertility is a condition associated with severe emotional and mental stress, particularly in societies where there is a social emphasis placed upon the ideal of having biological children (Ashraf et al. 2014). The mechanisms of infertility in males and females are varied in both origin and functional impact and often these conditions are thought to have an underlying genetic component (Zorilla & Yatsenko 2013). Only 6–18% of infertility cases have identifiable genetic causes (mainly sex-chromosome abnormalities) indicating that for many cases of infertility (and subfertility), like DSD, the underlying genetic factors are yet to be elucidated.

Genes with mutations commonly identified in DSD studies include androgen receptor and synthesis genes (androgen receptor (AR), CYP17A1, SRD5A2), NR5A1(SF1), WT1, GATA4, SRY, DAX1 and CHD7 (Kremen et al. 2017). Those patients with DSD and a complete gonad dysgenesis phenotype carry mutations in SRY, MAP3K1, DHH and NR5A1 genes (Ono & Harley 2013). While these genes are also critical for mouse gonadal development, largely supporting the use of the mouse as a model for human gonad development, there are several exceptions. For example, sequencing screens have found gain-of-function mutations in the mitogen-activated protein kinase kinase kinase 1 (MAP3K1) gene, including patients with streak gonads and female genitalia (Pearlman et al. 2010, Loke et al. 2014, Baxter et al. 2015, Eggers et al. 2016, Granados et al. 2017), suggesting a requirement for MAP3K1 function in gonad development for both sexes. However, although the mouse orthologue, Map3k1, is expressed in

Figure 5
Histology sections through human embryos 36–44 days (Carnegie stages 14–18). At ~36 days the coelomic epithelium begins to thicken, and by 39 days a ridge of tissue is forming, facing into the gut cavity. Just prior to sex determination (~42 days), the mesenchyme has proliferated to form a gonad region that is now easily distinguished from the neighbouring mesonephros. Section images were obtained from the Virtual Human Embryo resource (https://www.prenatalorigins.org/virtual-human-embryo/). CE, coelomic epithelium; DM, dorsal mesentery; M, mesonephros; MT, mesonephric tubule; MV, mesonephric vesicle; PMD, paramesonephric duct. Scale bar = 100 μm.
the E11.5 gonad mouse, knockout mice have only minor testicular abnormalities, indicating a minimal role in XY gonadal development (Warr et al. 2011). This stresses the importance of additionally developing mouse models that replicate human gene mutations to study phenotype, as often human gene mutations do not result in a complete loss of gene/protein function but rather gain of function.

Mutations in NR5A1 were identified in 4% of patients examined with unexplained male infertility (Bashamboo et al. 2010a, Ropke et al. 2013) and in female patients within premature ovarian failure (Lourencos et al. 2009). Novel NR5A1 mutation I (R92W) leads to 46, XX ovotestis (SRY negative) (Igarashi et al. 2017, Baetens et al. 2017b). The types of NR5A1 gene mutations and phenotypes associated with these disorders are reviewed in (Ferraz-de-Souza et al. 2011). WT1 mutations have also been found in male and female cases of infertility, with ‘normal’ gonadal development (Seabra et al. 2015, Nathan et al. 2017). Humans are not the only species to present with variable phenotypes; in mice, the genetic background or strain strongly influences the adult phenotype of many genes linked to gonadal development. In one such example, gene knockout of Gadd45g (growth arrest and DNA damage-inducible protein 45g) on a mixed genetic background (129/C57BL/6), 20% of the XY homozygous mice developed as infertile males, whereas on a C57BL/6 (B6) background, 100% of XY mice were sex reversed (Johnen et al. 2013). Genetic background also influences the phenotype of Nr5a1-, Fgf9-, and Wt1-null mice (Meeks et al. 2003, Brennan & Capel 2004). The B6 mouse strain is more likely to result in a male-to-female sex reversal phenotype than the 129S1/SvImJ (129S1) strain, and differences in gonadal gene expression between these strains are observed even prior to sex determination at E11.5 (Colvin et al. 2001, Munger et al. 2009). Therefore, even though studies have identified genes critical to the early steps of gonad development, a loss-of-function mutation does not necessarily lead to complete gonadal dysgenesis. In at least some cases, genetic background and possibly environmental factors determine the phenotypic consequences of loss-of-function mutations in these genes.

**Future directions: unravelling the molecular pathways of early gonad development**

The advent and affordability of high-throughput sequencing technologies and new methods of replicating organ development in vitro, together will be valuable research tools in propelling forward both genetic and cellular biology into early GR development.

The use of single-cell RNA-seq (scRNA-seq) has rapidly furthered our understanding of developmental events, particularly within heterogeneous cell populations (Shapiro et al. 2013). Given that the GR consists of a broad variety of cellular precursors for endothelial, steroidogenic and supporting cell lineages, along with maturing germ cells, it is especially suitable for scRNA-seq analysis. Li et al. undertook scRNA-seq analysis of isolated single foetal germ cells and their surrounding ‘niche’ somatic cells from 4- to 26-week-old human embryos (Li et al. 2017). Based on gene expression profiles from this study, somatic cells in the early gonad can be divided into four groups within each sex. XX gonadal cells are comprised of endothelial cells, and three types of maturing granulosa cells (early-(10 weeks), mid-(10–20 weeks) and late-granulosa (20–26 weeks)) (Li et al. 2017). In XY gonads, somatic cells group into Sertoli cells, Leydig cell precursors, differentiated Leydig cells and endothelial cells (Li et al. 2017). Systematic examination of the expression profiles of each gonadal cell population during development will not only improve our knowledge of the in vivo mechanisms of cell differentiation but also the conditions required to induce correct cell differentiation in vitro.

Organoid systems are becoming popular in vitro models of organ development (Fatehullah et al. 2016). Isolated preparations of human somatic and germ cells can self-organize into a testicular-like organoid using an artificial scaffold to aid 3D organization (Baert et al. 2017). These studies make use of cells that have already undergone sex-specific differentiation, as the cells are isolated from post-natal tissues (Baert et al. 2017). Recently, Sepponen et al., reported using human embryonic stem cells (hESCs) culture conditions to sequentially induce the primitive streak, followed by intermediate mesoderm and finally bi-potential-like gonadal cells expressing genes such as LHX9, EMX2, WT1 and GATA4 (Sepponen et al. 2017). This study found that timing and levels of BMPs (bone morphogenetic protein), WNT/β-catenin and Activin-A signalling ligands are essential to promote differentiation of gonadal cell precursors, over other mesodermal cell types (Sepponen et al. 2017). These studies, along with new sequencing resources, lay the groundwork for future research for not only modelling the early events of human gonad development, but to also examine the functional consequences of gene mutations identified in DSD patients using cultured cells engineered with the same genetic mutation.

Several groups have taken a comprehensive targeted screening approach in order to identify genetic factors in DSD patients, and this in turn may lead to the identification
of novel genes required for GR development. Exome sequencing and targeted gene sequencing identified the genetic cause (classed as a functional gene mutation) in 28–38% of cases examined (Baxter et al. 2015, Dong et al. 2016, Fan et al. 2017, Kim et al. 2017). Deep sequencing of 64 known and 967 candidate genes improved the genetic diagnosis rate to 43% for one patient cohort (Eggers et al. 2016). Thus, despite advances in sequencing technologies, over 50% of DSD cases remain without a genetic diagnosis.

While targeted sequencing and exome sequencing studies have focused on the protein coding regions of the genome, epigenetic processes, gene regulatory elements and non-coding RNAs (ncRNAs) are just as important for correct embryonic development. Errors in any of these gene regulatory mechanisms may underlie many cases of idiopathic DSD that lack mutations in protein-coding genes. Studies in vertebrates have largely focused on identifying sex-dimorphic expression of small ncRNAs called miRNAs following sex determination (Bannister et al. 2009, Real et al. 2013, Wainwright et al. 2013, Presslauer et al. 2017). There is limited information regarding how these miRNAs function in sex determination, if their function is essential, if they act to finely adjust gene expression levels during gonad development and whether these, and others, have earlier roles in the formation of the bi-potential gonad. Currently no miRNAs have been linked to human gonad development. Long ncRNAs are another important class of ncRNAs, which function to regulate gene expression (reviewed in Moran et al. 2012). With respect to sex development, these have been most thoroughly investigated for their role in X-chromosome dosage compensation (Cerase et al. 2015). It remains to be determined if autosomally encoded long ncRNAs contribute to mammalian sex determination and gonadal development but given their important roles in the development of other organ systems, it is likely they have a role in some aspects of gonadal development.

Genome regulatory elements have been difficult to identify as most lie within the non-coding regions of the genome and can act over long distances to influence gene expression via chromatin folding. Mutations in regulatory elements range from single nucleotide sequence variants that prevent or reduce binding of a transcription factor to their DNA target sequence, to larger deletions, duplications and translocations resulting in structural changes that alter chromatin confirmation and regulatory interactions between enhancers and their target genes. Loss-of-function mutations in regulatory elements located near the SOX9 gene, a gene important for male sex determination, are the best-characterized regulatory changes associated with DSD (reviewed in Baetens et al. 2017a). As putative regulatory elements are difficult to predict, few have been mapped for human genes associated with early gonadal development. Recent genome-wide studies, including those mapping the chromatin state in specific cell lineages (Mikkelsen et al. 2007), will improve our ability to predict if DNA changes associated with DSD lie within gene regulatory regions.

Epigenetic mechanisms such as DNA methylation and histone modifications have been implicated in the regulation of Sry gene in mice and dogs (Nishino et al. 2011, Jeong et al. 2016, Kuroki et al. 2017). Loss of function of the JMJD1A gene, which encodes a H3K9 demethylase enzyme, results in XY sex reversal (Kuroki et al. 2017). Some cases of XY DSD with complete sex reversal in dogs are thought to be due to persistent DNA hypermethylation of the Sry gene (Jeong et al. 2016). Therefore, it is likely that some cases of human DSD may be the result of mutations to epigenetic regulatory factors. Regulation of gene expression through epigenetic mechanisms is also especially sensitive to environmental influences and this impacts on many developmental programmes including sex determination (Feil & Fraga 2012). DNA methylation levels, determined by environmental factors, are vital to many naturally occurring forms of sex reversal and environmental sex determination in animals (Capel 2017). While difficult to study with respect to human DSD, it is possible that DNA methylation may play a role in balancing one sex developmental trajectory over another, and thus, errors in this may lead to gonad dysgenesis or sex reversal in humans.

Summary
The complexity of reproductive development is reflected in the difficulty in assigning a genetic diagnosis in most cases of human DSD. While we know the genetic aetiology of a small number of DSDs, up to as many as 75% of individuals with a DSD will remain without a genetic diagnosis (Arboleda et al. 2014). Even with whole genome sequencing, it is often difficult to identify functional variants and causal mutations, rendering many sequencing approaches somewhat ineffective (Fan et al. 2017). Gene expression levels, epigenetic modifiers and genetic background, along with the type of mutation and its functional consequence can all influence the resulting phenotype for both humans and mice. The future development of new technologies and improvement of existing ones will provide us with a much better understanding of the processes underlying normal
gonadal development in both human and mouse models, which in turn will lead to improved diagnosis in cases of DSD and infertility.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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