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Fetal Leydig Cell Origin and Development

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Abstract

Male sexual differentiation is a complex process requiring the hormone-producing function of somatic cells in the gonad, including Sertoli cells and fetal Leydig cells (FLCs). FLCs are essential for virilization of the male embryo, but despite their crucial function, relatively little is known about their origins or development. Adult Leydig cells (ALCs), which arise at puberty, have been studied extensively and much of what has been learned about this cell population has been extrapolated to FLCs. This approach is problematic in that prevailing dogma in the field asserts that these 2 populations are distinct in origin. As such, it is imprudent to assume that FLCs arise and develop in a similar manner to ALCs. This review provides a critical assessment of studies performed on FLC populations, rather than those extrapolated from ALC studies to assemble a model for FLC origins and development. Furthermore, we underscore the need for conclusive identification of the source population of fetal Leydig cells.

Keywords

Adrenogonadal primordium; Adult Leydig cells; Fetal Leydig cells; Leydig stem cells; Multipotent progenitor; SF-1; Testis development

Testicular Leydig cells are responsible for the production of androgens and other hormones that are critical to male reproductive system development and male reproductive health. These cells reside in the interstitial spaces of the testis and consist of several populations over the course of an organism's development (reviewed by Habert et al., 2001; Haider, 2004). In rodents, there are 2 populations – fetal Leydig cells (FLCs) and adult Leydig cells (ALCs) (Roosen-Runge and Anderson, 1959). FLCs are present in the embryonic testis from shortly after testis determination to birth, at which point their numbers rapidly decline. A few FLCs persist in the postnatal interstitium, but these are eventually replaced by the ALC population that expands at puberty. Humans and other primates also have an infantile Leydig cell (ILC) population that contributes to a 'minipuberty' event a few months after birth. These ILCs are also eventually replaced by ALCs. The ALC population arises at puberty and maintains androgen production throughout adult life, functionally replacing

earlier populations (fig. 1). While it appears that ALCs arise from a lineage specific peritubular stem cell, it is unclear whether the earlier populations are also derived from the same stem cell (Ge et al., 2006). There has been some speculation that FLCs and ALCs are distinct populations with distinct origins, but this hypothesis has not been supported by experimental evidence.

FLCs are the primary source of androgens and other virilizing hormones that are responsible for driving male sexual differentiation in developing mammals. FLCs are the front line of male development and maintenance, eventually giving way to later populations of Leydig cells that drive sexual maturation at puberty. While the ALC population has been studied extensively in mammals, very little is known of FLC development. This is due to several reasons; most obviously that FLC development occurs almost entirely in utero making direct and continuous observation of FLCs over the course of their development difficult. Furthermore, rodents are the model organism of choice for most mammalian studies because of their relatively short generation time and high fecundity as compared to other laboratory-adapted mammals. In the case of sexual development, however, this has both benefits and drawbacks. While a short gestation period yields many animals very quickly, it also means that the dramatic changes that occur during an embryo's sexual development happen quickly as well, making a holistic and thorough observation of FLC development quite challenging. As such, most of what is 'known' about FLC development has been extrapolated from what is known of ALC development, which lends itself better to extended time-point analysis. This extrapolation is somewhat problematic as there exists a long-standing dogma in the field that FLCs and ALCs are in fact distinct populations, arising from separate progenitors (Roosen-Runge and Anderson, 1959; Lording and De Kretser, 1972; Baker et al., 1999; Nef and Parada, 1999; O'Shaughnessy et al., 2003). This hypothesis is largely based on subtle differences in expression profiles between FLC and ALC populations, which might indicate distinct lineages, but more likely reflects the different environments (prenatal and post-pubertal) in which these populations develop. Though this separate origin hypothesis has not been directly or satisfactorily demonstrated, it remains a popular idea and is complicated by the fact that the predominant model for FLC development is derived from a condensed ALC developmental scheme despite the purported non-relationship between these two populations. This review synthesizes empirical data regarding FLC development so as to gain a more comprehensive and accurate model of FLC development based on studies of this population of cells, rather than extrapolation from a potentially unrelated population.

FLC Function

Sexual differentiation is driven by the somatic cells of the gonad. FLCs play a critical role in male sexual differentiation and development in that they are the primary source of androgens in the developing embryo and are thus responsible for virilizing the male reproductive tract. Differentiation towards a sexually dimorphic phenotype begins with sex determination. Prior to sex determination the gonads are bipotential in that they can give rise to either testes or ovaries given the appropriate genetic signals. Sex determination is a unique process in which the bipotential gonad is directed toward either a testicular or ovarian fate by the presence or absence of *Sry* expression from the Y chromosome (Koopman et al., 1990, 1991; Fechner et al., 1993; Birnbacher and Frisch, 1995; Greenfield

and Koopman, 1996). In males, the onset of *Sry* expression is followed closely by expression of *Sox9* in the early testis, which marks pre-Sertoli cells (Kent et al., 1996; Morais da Silva et al., 1996). Either of these two factors is capable of driving the bipotential gonad to a testicular fate (Koopman et al., 1991; Vidal et al., 2001; Qin and Bishop, 2005). In the absence of *Sry* expression as in XX embryos, *Sox9* is not upregulated and the gonad adopts an ovarian fate. In XY embryos, *Sry* and *Sox9* are expressed in pre-Sertoli cells, and it is the Sertoli cells along with FLCs that will drive further differentiation of the rest of the reproductive tract. Sertoli cells, along with FLCs, direct sexual differentiation and development by patterning the reproductive tract toward a male fate via the production of various hormones. Differentiations of structures outside the gonad itself are benchmarks of sexual development. Embryos contain two complete sets of ductal structures, the Wolffian and Müllerian ducts which are competent to give rise to the male and female reproductive tracts, respectively. Normal sexual differentiation results in an individual possessing only a single complement of structures, either male or female. In the case of XY individuals the somatic cells of the testis must direct the proper development and regression of the appropriate primordial ducts. Specifically, the androgens produced by FLCs are critical for maintaining the Wolffian duct structures and promoting their differentiation into the epididymis, vas deferens, and seminal vesicles. Meanwhile, Sertoli cells produce anti-Müllerian hormone (AMH), which is responsible for the regression of the Müllerian ducts. In the absence of androgens from FLCs (i.e., in the female embryo), the Wolffian ducts regress, and no male structures are formed. Likewise, without AMH from the Sertoli cells the Müllerian ducts are maintained and then differentiate into the oviducts, uterus, and upper portion of the vagina. Thus, due the hormonal products of the male somatic cells in the testis, Müllerian ducts regress and Wolffian ducts differentiate, giving rise to a male reproductive system. If the bipotential gonad becomes an ovary, the absence of hormonal products from male somatic cells leads to regression of the Wolffian ducts and differentiation of the Müllerian ducts into the female reproductive tract (fig. 2). Beyond the differentiation of the ductal structures toward the male fate, FLCs are also involved in other virilizing processes. Androgens produced by FLCs are critical for virilization of the external genitalia. Disruption of androgen signaling in male embryos causes feminization of external genitalia, and inappropriate exposure of female embryos to excessive levels of testosterone during early gestation can result in pseudohermaphroditism (reviewed by Klonisch et al., 2004). It is widely believed that fetal androgens have significant influence on brain development, driving the subtle differences in brain patterning and subsequent sex-specific behaviors later in life (Barker, 1990; Vilain and McCabe, 1998; Robinson, 2006). Furthermore, FLCs also produce a hormone, insulin-like 3 (INSL3), which induces differentiation of the gubernaculum ligament that controls the transabdominal phase of testicular descent toward the scrotum (Adham et al., 2000, 2002; Emmen et al., 2000a; Bogatcheva et al., 2003; Tomiyama et al., 2003). In the absence of this factor, the testes fail to descend and are retained in the body cavity, a condition known as cryptorchidism (Nef and Parada, 1999; Zimmermann et al., 1999; Kumagai et al., 2002; Tomiyama et al., 2003; Hutson and Hasthorpe, 2005). Cryptorchidism can lead to infertility later in life as the higher temperature of the body cavity is detrimental to spermatogenesis. Cryptorchidism can also lead to germ cell tumors such as dysgerminomas and is also associated with Leydig cell tumors (Kirsch et al., 1993; Nef and Parada, 1999; Hutson and Hasthorpe, 2005). FLCs play

a crucial role in the differentiation and development of the male reproductive system and possibly male-specific behaviors, thus they have a profound impact on sexual function in the adult.

Androgen Synthesis

The primary function of FLCs is to produce androgens which virilize the reproductive tract. Testosterone is often considered the end product of androgen biosynthesis, but this idea is somewhat misleading. Testosterone may be synthesized *de novo* from the primary substrate cholesterol or from intermediate products of the estrogen or androgen synthesis pathway. Androgen synthesis is further complicated by the fact that some enzymes catalyze multiple steps in the pathway, and some mediate both the forward and reverse reactions (fig. 3). Therefore, while it is helpful to imagine testosterone biosynthesis as a linear reaction proceeding directly from the cholesterol substrate, it is more accurate to describe this as a dynamic process involving multiple substrates and dynamic equilibria established between intermediate products of the reaction.

Because androgen synthesis is the primary function of FLCs, the expression of steroidogenic enzymes proves a useful measure of functional differentiation. In expression studies in ALCs, the differential expression of these enzymes can be used to indicate the stage of cellular development within the lineage. These marker-defined stages have been extrapolated to the FLC population as a model for defining various stages of FLC development. However, given the shorter time period over which this population develops and the concurrent acquisition of most of these markers as reported in the literature, it is unlikely that differential expression of steroidogenic enzymes accurately define stages of FLC development.

FLC Development

Relatively little empirical data is available for FLC development in large part due to the accelerated time frame over which the process occurs and also since the population develops almost entirely in utero. As such, extensive analysis of this population has been very challenging and therefore the prevailing model for FLC development is mostly derived from an extrapolation of the better studied ALC population. Thus it is important to focus on studies of the FLC population with respect to the timing of morphological changes and molecular markers within this population in hopes of better defining FLC development in its own right. Some studies of ALC development will also be included for the purpose of comparison.

Putative Sources of FLCs

While the progenitor pool of FLCs is currently unknown, there is much speculation. FLCs have been purported to arise from migrating neural crest cells, mesonephric or epithelial populations of the adjacent mesonephros, and the coelomic epithelium, leaving the less popular formal possibility that FLC progenitors are present in the bipotential gonad at the onset of gonadal development and are specified toward their fate by an unknown signal upon male sex determination. Furthermore, there has been strong suggestion that FLCs share

a common origin with adrenocortical cells that split off from the early adrenogonadal primordium (AGP). While none of these proposed progenitor pools has been demonstrated to give rise to FLCs directly, none have, as of yet, been ruled out either. Thus, it is useful to outline the circumstantial evidence for each of these putative progenitor pools in order to gain a more complete idea of possible sources of FLCs.

Neural Crest Cells

It is well known that the migratory trunk neural crest cells (NCCs) can contribute to neuroendocrine populations in the developing embryo, such as the steroidogenic population of the adrenal medulla (reviewed by Huber, 2006). It was previously believed that several neuroendocrine populations in the pancreas and foregut were also derived from NCCs, and while it seems that there is sufficient evidence to contradict this hypothesis with regards to the pancreatic and gut populations, it remains a persistent and popular idea (Pictet et al., 1976; Andrew and Kramer, 1979; Kirchgessner et al., 1992; Andrew et al., 1998). Similarly, early hypotheses of FLC origins also suggested NCCs as putative precursors. Despite a lack of definitive evidence in favor of, and potentially some evidence against this hypothesis, it has persisted for some time. Taking into account the fact that the adrenal glands and gonads share a common primordium early in development as well as the idea that NCCs might contribute to several other neuroendocrine populations, it was perhaps not so far-fetched to hypothesize that steroidogenic cells of the testis might also be derived from NCCs. This hypothesis has been furthered by studies of molecular marker expression in Leydig cells in which it is demonstrated that Leydig cells do indeed express a number of neural markers including nestin, NCAM, S-100, and neurofilament protein 200 to name a few (Middendorff et al., 1993; Mayerhofer et al., 1996; Davidoff et al., 2002; Lobo et al., 2004). However, this reasoning fails to account for the fact that many cells of non-neural or NCC origin express some 'neural' markers. Furthermore, Leydig cells are known to participate in the neuroendocrine system and so expression of common genes necessary to this interaction is expected, but does not necessarily imply a common cellular origin (Davidoff et al., 1993). At least one group has pursued this hypothesis briefly and has reported (but not shown data) that NCCs do not contribute to the FLC population (Brennan et al., 2003). However, as the hypothesis has yet to be definitively disproven, NCCs remain a putative cellular origin that must be considered. Further research on the possible contribution of NCCs to the FLC population is warranted.

Coelomic Epithelium

The gonadal ridge that gives rise to the bipotential gonad is itself derived from an epithelial to mesenchymal transition of cells from the overlying coelomic epithelium, but it is unclear which of the somatic cells of the gonad are directly derived from these cells and which are recruited from the underlying mesenchyme once the gonadal ridge has been formed. In an elegant study, coelomic epithelial (CE) cells were labeled by single cell lipophilic vital dye injection and followed in ex vivo organ explant cultures for 12–24 h (Karl and Capel, 1998). This group found that depending on the stage of the embryo at the time of injection, the testicular compartment to which the labeled cells migrated is restricted. Earlier in development the labeled CE cells could migrate into the developing testis and eventually be found either within the seminiferous tubules, implying a Sertoli cell fate, or in the

interstitium. Slightly later in development labeled CE cells are excluded from the seminiferous tubules and restricted to the interstitium implying a possible FLC fate. However, this was not confirmed by expression analysis for FLC lineage markers and so it is unclear whether these cells give rise to FLCs or some other interstitial cell type. It is unclear whether these labeled cells from the CE do in fact give rise to FLCs. As such, the CE remains a putative source of FLCs, and further studies are required to determine the possible contribution of the CE to the FLC population.

Mesonephros – Mesenchyme and Epithelia

The mesonephros has long been suspected of contributing to the FLC lineage due in no small part to the dramatic morphological events that take place in the male gonad shortly after sex determination. It has been demonstrated by a number of researchers that the male gonad induces a migratory event from the adjacent mesonephros at embryonic day 12.5 (E12.5) in the mouse (Buehr et al., 1993; Merchant-Larios et al., 1993; Merchant-Larios and Moreno-Mendoza, 1998). This process occurs exclusively in male gonads and while the donor tissue must be mesonephric, the sex of the mesonephros is irrelevant (Martineau et al., 1997). Recombinant organ culture experiments (in which a male gonad is cultured alongside a genetically marked mesonephros prior to this migratory event) demonstrate that male gonads cultured with female mesonephroi can still recruit the migratory population; however, male gonads cultured with limb bud mesenchyme do not. Female gonads do not recruit a migratory population regardless of the origin or sex of the adjacent tissue. Furthermore, this migratory process is required for sexual development to proceed normally, as testes that are deprived of this migratory population fail to form the cords that will give rise to the seminiferous tubules. At least one study in rats has used marker analysis to implicate the mesonephric epithelia as a putative source for the FLC population. This study found that the mesonephric epithelium expresses NCAM at E14.5, and that an interstitial population concentrated near the gonad-mesonephros border also expresses this same marker at E17.5 (Mayerhofer et al., 1996). This does not exclude the possibility that these interstitial cells could be derived from some unrelated population but happen to express this common marker independent of origin. It is currently unclear whether the mesonephric epithelium, or mesenchyme, or both contribute to the population of cells that migrate into the testis. In any case, an eventual FLC fate has not been definitively ruled out for these cells, and further research is required to determine which, if any, of the mesonephric cells can contribute to the FLC population.

Resident Unspecified Somatic Cells of the Gonad

Early studies on mesonephric migration suggest a progenitor pool of unspecified cells residing within the gonad as opposed to a migratory population from some other tissue. In these studies, male gonads separated from the mesonephros at E11.5, prior to migration of mesonephric cells into the gonad, were cultured in the absence of mesonephroi for several days. These testes failed to develop testis cords but did develop a subpopulation of cells with FLC characteristics (Merchant-Larios et al., 1993). This suggests that FLC precursors are present in the gonad prior to a migration of cells from the adjacent mesonephros. It is known that both Sertoli and Leydig cells express *Nr5a1/steroidogenic factor 1(SF-1)/Ad4BP* and so

it has been assumed that they arise from progenitor cells that express this factor (Luo et al., 1994, 1995). *SF-1* is expressed quite early in adrenogonadal development and it appears that some of these SF-1 positive cells go on to become steroidogenic cells of the adrenal cortex suggesting that this transcription factor specifies several hormone-producing cell lineages (Hatano et al., 1994). However, it has not been demonstrated whether FLC progenitors are among these early adrenogonadal primordial cells or if they arise from a separate, possibly migratory population and initiate *SF-1* expression independently. Further tests of this hypothesis are required.

The Adrenogonadal Primordium: A Common Source for all Steroidogenic Cells of the Urogenital System?

Genetic disruption of the mouse *Nr5a1* (*Ftz-F1*) gene, which encodes SF-1, revealed an essential role for SF-1 function in early adrenal gland development and slightly later in the development of the gonads (Luo et al., 1994, 1995; Ikeda et al., 1995). While development of the genital ridge is initiated in *SF-1* null animals, the tissue fails to differentiate at E12.0, and cells in that region undergo programmed cell death. As a result, *SF-1* null animals lack both adrenals and gonads after birth, suggesting a critical role for SF-1 in the development of both organs. To follow up on this finding, it has been demonstrated that a single population of SF-1 positive cells is present prior to the development of the gonadal region in this same area between the coelomic epithelium and the dorsal aorta (Hatano et al., 1996; Morohashi, 1997). Interestingly, this study observed a number of morphological events that point to a common origin for the gonads and adrenal cortex, both of which contain steroidogenic cells that are derived from this shared SF-1 immunoreactive population. The SF-1 population is first visible as a line of cells roughly 3–4 cell layers thick beneath the coelomic epithelium, extending along the length of what will later be identifiable as the urogenital ridge. As primordial germ cells (PGCs) migrate into this region, they are evenly distributed among the SF-1 positive cells. Over time, the SF-1 positive cells expand to a more cylindrical area along the presumptive gonadal ridge and then begin to separate into two different groups in the rostral most region: those closest to the dorsal aorta which exclude PGCs and those remaining adjacent to the coelomic epithelium which include PGCs and begin to express SF-1 more strongly and more abundantly. In the more caudal regions the 1st group (that closest to the dorsal aorta) is absent, indicating that the rostral group excluding PGCs splits off from the common population to give rise to the adrenal cortex, while the rest of the SF-1 positive cells intermingling with PGCs will become the gonad (fig. 4). It should be noted that while the presence or absence of PGCs is generally a good indicator of gonadal and adrenal compartments it may not be perfect as PGCs are sometimes ectopically observed in the adrenal gland (Upadhyay and Zamboni, 1982). These morphological events occur before sex determination and no differences in morphology or expression were observed between XX and XY embryos. It has since been demonstrated that all hormone-producing cells of the adrenal cortices and gonads express SF-1 at early stages of differentiation, and it is also required later for expression of steroidogenic enzymes (Jeyasuria et al., 2004). Of note to the origins of FLCs is the observation that the SF-1 positive cells in the gonadal primordium ‘seem to proliferate’ after the split from the adrenal primordium (Hatano et al., 1996). Without the use of proliferation markers or quantitative

analysis, this suggestion is based on the observation that there appear to be more SF-1 positive cells in the gonadal primordium as well as a stronger signal. This could result from several possibilities: proliferation of SF-1 positive cells, upregulation of SF-1 expression, or local recruitment of neighboring cells to take on SF-1 expression, or some combination of these events.

Several other genes play an early role in both adrenal and gonadal development. *Dax1* (*Nr0b1*) encodes an X-linked nuclear receptor that acts as a transcriptional antagonist to SF-1 in vitro (Zazopoulos et al., 1997; Lalli et al., 1998; Nachtigal et al., 1998; Tabarin et al., 2000; Wang et al., 2001; Salvi et al., 2002; Suzuki et al., 2003). *Dax1* hemizygous males exhibit testicular dysgenesis and delayed FLC marker expression as well as delays in adrenal gland development (Yu et al., 1998). Surprisingly, *Dax1/SF-1* compound mutants (*Dax1*^{-/-}; *SF-1*^{+/-}) demonstrate a cooperative rather than antagonistic role for these two factors in the context of testicular development. The compound mutants exhibit further delay of FLC marker expression and the Sertoli cell product *desert hedgehog* (*Dhh*), which is also essential for FLC development, as compared to *SF-1* heterozygotes (Yao et al., 2002; Park et al., 2007). This cooperative interaction between factors important for both gonadal and adrenal development further underscores the developmental relationship between these two organs.

Given the shared primordium of the gonads and adrenal glands as well as expression of common transcription factors, including SF-1, in all steroidogenic lineages, it is not unlikely that steroidogenic lineages in both the adrenal glands and the gonads are derived from a common SF-1 positive progenitor cell in the adrenogonadal primordium. In support of this common origin, several groups have observed expression of established adrenal markers in the developing testis (Hatano et al., 1994, 1996; Heikkila et al., 2002; Wang et al., 2002; O'Shaughnessy et al., 2003). More recently, a subpopulation of steroidogenic 'adrenal-like cells' has been characterized in the fetal testis (Val et al., 2006; Hu et al., 2007). The adrenal-like cells respond to ACTH and likely contribute to testicular production of corticosterone, an established adrenal hormone. These cells express transcripts of adrenal steroidogenic enzymes *Cyp11b1* and *Cyp21* as well as *Cyp17a1* and *Cyp11a1* (encoding the P450_{c17} and P450_{scc} steroidogenic enzymes, respectively, both of which are expressed by Leydig cells) but not the Leydig cell peptide hormone *Insl3*, indicating that these adrenal-like cells share some properties with Leydig cells but are nonetheless distinct (Val et al., 2006). It appears that the adrenal-like cells of the testis originate at the gonad-mesonephros border and subsequently migrate into the testis differentiating concurrently with FLCs, which is not inconsistent with a shared adrenogonadal origin, but this has not been conclusively demonstrated.

Currently, none of the putative migratory populations, nor a resident gonad population have been conclusively demonstrated to be an exclusive source of FLCs. It is important that the source population of FLCs be defined so as to better understand the properties of this lineage and the nature of its development. Identification of the FLC progenitor pool will allow for further characterization of the earliest cell in the FLC lineage and perhaps whether this cell can contribute to other Leydig populations throughout the lifetime of the organism. This has important therapeutic implications in that most somatic testicular tumors are of Leydig cell

origin and occur quite early, suggesting an early Leydig cell population as the origin of the tumor. Furthermore, Leydig cell hypoplasia in adults is frequently correlated to infertility and endocrine disorders. As such, characterizing the possible contribution of FLCs or their progenitors to the ALC population could provide opportunities for early intervention in these cases.

Morphological Changes in FLCs

The earliest studies defining various stages of FLC development were primarily ultrastructural studies conducted in rat testis (Roosen-Runge and Anderson, 1959; Haider, 2004). Briefly, FLCs are first morphologically identifiable by their interstitial location and large, numerous lipid droplets present in the cytoplasm. At the ultrastructural level, FLCs have mitochondria with tubulovesicular cristae and abundant smooth endoplasmic reticula (SER). FLCs form clusters in the interstitium and form cell contacts with other FLCs within their clusters (Haider et al., 1995; Tran et al., 2006). Early cells in the FLC lineage appear morphologically similar to fibroblasts which are also present in the testis interstitium, so it is useful to apply molecular markers when attempting to identify these cells. The same is true of the ALC lineage, and positive identification of these cells is further complicated by the persistence of some FLCs in the interstitium postnatally (Mendis-Handagama et al., 1987, 1998; Kerr and Knell, 1988). While these persisting FLCs do appear grossly similar to the developing ALCs, some subtle differences do occur. FLCs have many large cytoplasmic lipid droplets while ALCs have relatively smaller droplets in fewer numbers (Haider et al., 1995). Furthermore, some steroidogenic enzyme isoforms are expressed exclusively by ALCs including 17 β -HSD type III and 11 β -HSD type I, allowing for a more accurate method for distinguishing ALCs from persisting FLCs in the postnatal testis (Baker et al., 1997; Haider et al., 1990, 1997).

FLC marker Expression and Regulation

The primary function of FLCs is the regulation of male sexual differentiation via the production of androgens. Thus, it is no surprise that the molecular markers characterizing FLC development consist primarily of steroidogenic enzymes and their regulators. As with many models of FLC development, much of the information available is gained through experiments in adult tissues or in cell culture which may not be representative of FLC development in vivo (reviewed by Habert et al., 2001; Haider, 2004; O'Shaughnessy et al., 2006). However, some studies have been conducted which place the expression of relevant markers within their proper context.

The most widely used markers of FLC lineage and development are the steroidogenic enzymes that catalyze the conversion of cholesterol to steroid hormones, with the end result in FLCs being testosterone. This is a multistep process, with some enzymes functioning at several steps along the synthesis pathway and some that catalyze both the forward and reverse reactions between one substrate and the next (fig. 5). Furthermore, many steroidogenic enzymes are also expressed by other steroidogenic tissues within the urogenital system – most notably the adrenal glands and prostate, and the ovaries in the case of the females – such that no single molecule stands out as an exclusive marker of FLCs.

Therefore, a combination of markers will prove most useful in defining both the lineage and the stages of development in this population.

Steroidogenic enzymes of FLC populations include cytochrome P450 cholesterol side chain cleavage enzyme (P450_{scc}), cytochrome P450 17 α -hydroxylase/C₁₇₋₂₀ lyase (P450_{c17}), and 3 β -hydroxysteroid dehydrogenase/ 5-4-isomerase (3 β -HSD). Also of note is 17 β -hydroxysteroid dehydrogenase/ketosteroid reductase (17 β -HSD), which catalyzes the final step in testosterone biosynthesis, from androstenedione to testosterone, although the paucity of antibodies against this molecule limits its usefulness for defining stages of FLC development *in vivo*. Furthermore, the peptide hormone INSL3 is known to be expressed by terminally differentiated FLCs, but not by earlier cells in the lineage (Mendis-Handagama et al., 2007).

Many of the steroidogenic enzymes belong to a class of cytochrome P450s. In the case of androgen synthesis, these consist of P450_{scc} and P450_{c17}, which mediate the 1st, 4th and 5th step of testosterone biosynthesis, respectively (P450_{c17} catalyzes two steps: from progesterone to 17 α -hydroxyprogesterone to androstenedione). P450_{scc} is active in the inner mitochondrial membrane, requiring the substrate cholesterol to be actively transported to this subcellular compartment by steroidogenic acute regulatory protein (StAR). P450_{c17} is instead active in the smooth endoplasmic reticulum (SER), along with other enzymes in the pathway. FLCs also express a third cytochrome enzyme P450 aromatase (P450_{arom}) which can convert testosterone to estradiol (E₂). Since this enzyme is not critical to *de novo* testosterone synthesis it will not be discussed further in this review.

Currently, it is known that P450_{scc} is expressed as early as E12.5 in the mouse testis as detected by *in situ* hybridization, and that all three early steroidogenic enzymes (P450_{scc}, P450_{c17}, and 3 β -HSD) are detectable by RT-PCR by E13.5 (Greco and Payne, 1994; Baker et al., 1999). Some controversy surrounds the expression of 17 β -HSD. It has long been assumed that this final enzyme in testosterone biosynthesis is expressed by FLCs, but some isoforms have been shown to be more strongly expressed by Sertoli cells in the fetal testis, whereas they are expressed exclusively in Leydig cells in the adult population (Baker et al., 1997; O'Shaughnessy et al., 2000). Given the strong evidence for a common SF-1 positive progenitor for both Sertoli cells and FLCs, expression of steroidogenic enzymes in both cell types is perhaps not unexpected. In contrast to ALCs, FLCs acquire expression of steroidogenic enzymes concurrently or in very rapid succession (Mack et al., 2000). As such, acquisition of steroidogenic enzymes in FLCs is a useful marker in identifying members of the FLC lineage, but expression of individual enzymes cannot define different stages of FLC development.

SF-1 is an orphan nuclear receptor that is expressed in the earliest stages of hormone producing cell lineages. It is known to be expressed by Sertoli and Leydig cells as well as by steroidogenic cells of the adrenal glands. SF-1 is expressed very early in the development of these cells when they are all located in the AGP and is required for expression of steroidogenic enzymes (Hatano et al., 1994, 1996; Chau et al., 1997; Leers-Sucheta et al., 1997). *SF-1* null mice exhibit gonadal and adrenal agenesis, indicating an essential role of SF-1 in the development of these hormone producing glands (Luo et al., 1994, 1995; Ikeda

et al., 1995; Sadovsky et al., 1995). A Leydig cell-specific *SF-1* knockout, using the *Amhr2-Cre* allele abrogates SF-1 expression in the testis from E11.5 (Jeyasuria et al., 2004). These embryos exhibit grossly delayed testicular differentiation. Cords do not form until E16.5, whereas they are present at E12.5 in wild-type testes. Furthermore, adult *SF-1* conditional knock-out (*SF-1* CKO) male mice exhibit external genitalia that are indistinguishable from wild-type females, suggesting FLC dysfunction. Internal reproductive structures are recognizably male but very hypoplastic, and the testes are significantly smaller and are retained at the level of the bladder. Furthermore, FLCs in *SF-1* CKO testis do not express P450_{scc} enzyme at E14.5, nor do they express the early steroidogenic cell marker StAR at E16.5, indicating an inability to produce testosterone. These results suggest a drastic delay in FLC development, although it seems that they do finally gain some function, as indicated by some transabdominal testicular descent directed by INSL3, a product of mature FLCs. The failure of the testes to descend to the scrotum is attributable to insufficient androgens, which control this second phase of testicular descent. Histology of adult *SF-1* CKO testes reveals the presence of interstitial cells which may be surviving ALCs, indicating that SF-1 is not required for Leydig cell survival, but rather for functional differentiation, although it is possible to attribute this phenotype to mosaic Cre activity in FLCs, allowing some to escape recombination and maintain SF-1 function. SF-1 also appears to promote cell division in the male gonad, as indicated by the marked decrease in proliferation in *SF-1* CKO testis at E12.5.

DHH is produced by Sertoli cells and acts as a paracrine factor on the Patched-1 (PTCH1) receptor expressed by FLCs and other interstitial cells (Clark et al., 2000; Pierucci-Alves et al., 2001; Yao et al., 2002). While DHH is not a marker of FLCs, its action is required for proper differentiation. FLCs fail to differentiate in *Dhh*^{-/-} XY gonads, but Sertoli cell differentiation and mesonephric cell migration into the testis is unaffected, suggesting that DHH produced in Sertoli cells signals to nearby cells in the interstitium to initiate a differentiation program toward the FLC fate, but does not affect the origins of FLCs (Yao et al., 2002).

StAR is an important regulator of steroidogenesis (Clark et al., 1994). This protein is responsible for the transport of cholesterol to the inner mitochondrial membrane, where it undergoes side chain cleavage, the first step toward androgen synthesis (Clark et al., 1994; Stocco and Clark, 1996). As such, StAR may be considered both an early marker of the FLC lineage as well as a regulator of FLC functional development.

The platelet-derived growth factor receptor alpha (PDGFRA) is 1 of 2 receptors for PDGF signaling. The receptors can form homo- or heterodimers with differing affinities for the secreted ligands in their various dimeric forms, and these various combinations appear to differentially regulate many developmental processes including the formation of the male gonad (Gnessi et al., 1995; Loveland et al., 1995; Basciani et al., 2002; Mariani et al., 2002). From studies done in ALCs, it is clear that PDGFRA is expressed in the ALC stem cells, which can self-renew and give rise to ALCs both in vivo and in vitro, and it is the only known marker for this particular stage in the ALC lineage (Ge et al., 2006). This is interesting from the perspective of FLCs primarily because it has not yet been demonstrated whether the ALC stem cells are derived from the FLC lineage. Furthermore, *Pdgfra* knock-

out (KO) testes fail to initiate migration of cells from the mesonephros, fail to develop testis cords, and never develop FLCs in ex vivo organ cultures (Brennan et al., 2003). This has not yet been shown in vivo as the *Pdgfra*^{-/-} mice are embryonic lethal and an appropriate testis-specific Cre for creating conditional knock-out embryos has not been applied to this system (Soriano, 1997). Interestingly, PDGFRA is required in the testis, but not the mesonephros, to induce migration and cord formation. The formation of cords is dependent upon this migratory event, and the development of FLCs appears to require cord formation. This study suggests that PDGF signaling through the α -receptor is required to initiate a secondary signal or program that induces the cells of the neighboring mesonephros to migrate into the testis. However, it is unclear whether the precursors of FLCs are already present in the gonad and are induced to differentiate by this event, or if FLC precursors are among the migratory cells of the mesonephros, and upon failure to migrate into the appropriate environment, they fail to differentiate.

INSL3 is a peptide hormone produced by mature terminally differentiated Leydig cells in both the FLC and ALC populations (McKinnell et al., 2005; Anand-Ivell et al., 2006; AgoulNIK, 2007; Mendis-Handagama et al., 2007). INSL3 from FLCs has a critical role on male sexual development, as this hormone acts as a ligand for the relaxin/insulin-like family peptide receptor 2 (RXFP2/LGR8) in the gubernaculum ligament (Kumagai et al., 2002; Bogatcheva et al., 2003). Activation of the receptor by INSL3 induces differentiation of the gubernaculum, which pulls the testis caudally, thus driving the transabdominal phase of testicular descent (Adham et al., 2000; Emmen et al., 2000a, b; Tomboc et al., 2000). Loss of *Ins3* causes cryptorchidism, or retention of the testes in the body cavity, which is associated with low serum testosterone and infertility as well as a higher risk for developing testicular tumors (Zimmermann et al., 1999).

Other Factors Required for FLC Formation and Development

While the molecular markers discussed above are useful for identifying cells of the FLC lineage and defining stages of functional development, most are not required for FLC differentiation and survival. Table 1 provides a brief overview of several factors affecting FLC development and function.

Summary

Based on available data from studies conducted in FLCs, and not from extrapolation of studies on ALC populations, we suggest a new working model for the stages of FLC development (fig. 6). While the cellular origins of FLCs are still unclear, it is known that SF-1 is required very early in the specification of steroidogenic cells. Therefore we propose a multipotent 'steroidogenic progenitor' (STpro) that expresses SF-1 early in adrenogonadal development. However, it should be cautioned that the SF-1 positive population is not specified to the FLC fate but can also give rise to Sertoli and adrenocortical cells, so the expression of this marker alone is insufficient to identify committed FLC progenitors among other cells of the gonad. More analysis will be needed to further characterize the properties of this cell. Since *Pdgfra* is expressed by FLCs and not Sertoli cells, and appears to be required for the differentiation of any FLCs, we propose an SF-1+; PDGFRA+ population in

the testis interstitium as the ‘precursor FLC’ (preFLC) stage of differentiation. The earliest visually distinguishable cells of the FLC lineage appear in the testis interstitium at E12.5 and are characterized by their spindle shaped, fibroblast-like morphology and their expression of steroidogenic enzymes including P450_{scc}, P450_{c17}, and 3 β -HSD, as well as StAR and luteinizing hormone receptor (LHR). We suggest the name ‘immature FLCs’ (iFLCs) for this class of cells. Later, these cells take on a more differentiated morphology and ultrastructure including polyhedral shape, vesicular mitochondria, and cytoplasmic lipid droplets as well as acquiring *Insl3* expression. We propose that this stage be termed ‘mature FLCs’ (mFLCs). Further study is required to completely characterize the earlier phases of FLC development, particularly with respect to the acquisition of markers relative to specification toward the FLC fate. It will also be important to determine at which stages in FLC development the cells retain or lose proliferative ability as well as their capacity for self-renewal. This will help to elucidate the nature of the earliest FLCs in the lineage – are they derived from a lineage restricted stem cell and if so, does this stem cell also give rise to the ALC population? Finally, a definitive identification of the source population of FLCs is necessary to fully understand the origin and nature of these cells. Currently most of the evidence for putative FLC source populations relies heavily on expression patterns of distinctive factors that might define the FLC lineage. These types of studies can suggest origins for specific cell types based on earlier expression of the same factor in possible progenitor pools, but these lines of evidence are not conclusive. While it would appear that FLCs might arise from SF-1 positive cells of the AGP, it is unclear whether they are derived from the original SF-1 positive population, or from other cells in the gonadal primordium that are recruited to express SF-1, or from cells that migrate into the gonad from neighboring tissue and then become committed to the FLC fate. As such, it is imperative to conduct thorough fate mapping experiments for each of the putative FLC source populations in order to definitively determine which of these gives rise to FLCs (Lawson, 1999; Joyner and Zervas, 2006; Bhattacharyya et al., 2008). Understanding these aspects of FLC development will yield a more comprehensive model of their contribution to sexual differentiation and the disorders arising from FLC dysfunction.

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Fig. 1.

Leydig cell populations. Three distinct populations of Leydig cells have been described during testis development. The fetal Leydig cell (FLC) population appears shortly after sex determination, peaks shortly before birth, and declines postnatally. The infantile Leydig cell (ILC) population is present in humans and other primates. It appears a few months after birth and declines shortly thereafter. The adult Leydig cell (ALC) population arises at puberty and maintains androgen production throughout adult life, functionally replacing earlier populations. In some species, this population declines with aging. Red: FLC population; green: ILC population; blue: ALC population.

Fig. 2.

Sexual differentiation. **A** At E11.5 both XX and XY embryos possess a pair of bipotential gonads, fully formed Wolffian ducts, and developing Müllerian ducts. **B** *Sry* from the Y chromosome initiates male sex determination, *Sry* upregulates *Sox9*, Sertoli and fetal Leydig cells differentiate. **C** Müllerian ducts regress in response to AMH. Androgens support differentiation of the Wolffian ducts. The gubernaculum ligament shortens, initiating testicular descent. **D** Complete male reproductive tract including descended testes and Wolffian duct derivatives epididymis, vas deferens, and seminal vesicles. **E** *Sry* is not expressed in XX gonads, the gonad adopts an ovarian fate. **F** Müllerian ducts differentiate and Wolffian ducts regress. **G** Complete female reproductive tract including oviducts, uterus, cervix, and anterior portion of vagina derived from the Müllerian ducts. AMH: anti-Müllerian hormone; C: cervix; E: epididymis; FLC: fetal Leydig cells; Gu: gubernaculum ligament; Ins13: insulin-like 3; K: kidney; Mes: mesonephros; MD: Müllerian duct; Od: oviduct; Ov: ovary; SC: Sertoli cells; SV: seminal vesicle; T: testis; UB: ureteric bud; Ur: ureter; Ut: uterus; V: vagina; VD: vas deferens; W: Wolffian duct.

Fig. 3.

Androgen synthesis. Androgens are synthesized de novo from cholesterol. This process involves multiple enzymes and intermediate products. P450_{scc} mediates conversion of cholesterol to pregnenolone (P5). Other cytochrome P450 enzymes and hydroxysteroid dehydrogenases mediate production of intermediate products and final androgens and estrogens. 17αOH P5: 17α-hydroxy pregnenolone; 17αOH P4: 17α-hydroxy progesterone; 17β-HSD: 17β-hydroxysteroid dehydrogenase/ketosteroid reductase; 3β-HSD: 3β-hydroxysteroid dehydrogenase/ 5- 4-isomerase; 5α-red: 5α-reductase; DHEA: dehydroepiandrosterone; DHT: dihydrotestosterone; P450_{arom}: cytochrome P450 aromatase; P450_{c17}: cytochrome P450 17α-hydroxylase/C 17–20 lyase; P450_{scc}: cytochrome P450 cholesterol side chain cleavage enzyme.

Fig. 4.

SF-1 subpopulation in the adrenogonadal primordium. **A** Adrenogonadal primordium containing a subpopulation of SF-1 positive cells (purple) concentrated in the coelomic epithelium covering the medial region of the urogenital ridge. **B** Primordial germ cells (PGCs, green) are evenly distributed throughout the adrenogonadal primordium. SF-1 expression is upregulated in the rostral region of the adrenogonadal primordium near the coelomic epithelium. **C** Adrenal primordium begins to separate from gonadal primordium. PGCs are found only in the presumptive gonadal primordium, while strongly SF-1 expressing cells (dark purple) are preferentially found in the adrenal primordium. **D** Gonad contains both PGCs and SF-1 positive cells. Adrenal contains only SF-1 positive cells. SF-1: cells expressing *Ad4BP/SF-1*; High SF-1: cells strongly expressing *Ad4BP/SF-1* (presumptive adrenal primordium cells); PGCs: primordial germ cells.

Fig. 5.

Sub-cellular localization of known FLC markers. Markers of the FLC lineage consist of several classes of molecules including transmembrane signaling molecules, transcription factors, and steroidogenic enzymes. 3 β -HSD: 3 β -hydroxysteroid dehydrogenase/ 5 α -4 α -isomerase; DHH: Desert hedgehog; GATA4: GATA-binding protein 4; INSL3: insulin-like 3; P450_{c17}: cytochrome P450 17 α -hydroxylase/C₁₇₋₂₀ lyase; P450_{scc}: cytochrome P450 cholesterol side chain cleavage enzyme; PDGFA, platelet-derived growth factor A; PDGFRA, platelet-derived growth factor receptor α ; PTCH1: Patched 1; SF-1: Ad4BP/SF-1/steroidogenic factor 1; StAR: steroidogenic acute regulatory protein.

Fig. 6.

Model of FLC development. FLCs are derived from a steroidogenic lineage progenitor population which may contribute to multiple steroidogenic lineages in the gonads and adrenal glands. Precursor FLCs are restricted to the FLC lineage and are identified by PDGFRA expression. It is unknown whether the precursor FLC can also give rise to the ALC population. Immature FLCs are characterized by the acquisition of functional markers including steroidogenic enzyme expression. Mature FLCs are characterized by expression of *Ins13* and a distinctive polyhedral shape. 3β-HSD: 3β-hydroxysteroid dehydrogenase/ 5- 4- isomerase; GATA4: GATA-binding protein 4; iFLC: immature fetal Leydig cell; INSL3: insulin-like 3; mFLC: mature fetal Leydig cell; P450_{c17}: cytochrome P450 17α-hydroxylase/C₁₇₋₂₀ lyase; P450_{scc}: cytochrome P450 cholesterol side chain cleavage enzyme; PDGFRA: platelet-derived growth factor receptorα; preALC: adult Leydig cell precursor; preFLC: fetal Leydig cell precursor; SF-1: AD4BP/SF-1, steroidogenic factor 1; StAR: steroidogenic acute regulatory protein; STpro: steroidogenic progenitor.

Table 1

Mouse mutants affecting FLC development

Gene	Endogenous expression	Loss-of-function phenotype	Effect on FLCs	References
<i>Arx</i>	Forebrain, floorplate, testis interstitium	Small brain, aberrant neuronal migration, loss of FLCs	Arx required for FLC development	Kitamura et al., 2002
<i>AR (ARKO)</i>	Sertoli cells, peritubular myoid cells and FLCs	XY sex reversal (variable penetrance), androgen insensitivity syndrome in adult males	Unclear: variably penetrant sex reversal indicates impaired Sertoli cell and FLC function, other studies report no FLC phenotype	O'Shaughnessy et al., 2002; De Gendt et al., 2004, 2005; Merlet et al., 2007
<i>AR</i> , Sertoli cell specific (SCARKO)	As above, Cre used in this study expressed in Sertoli cells	FLCs normal, but smaller testes with reduced ALC number postnatally	Androgens function via Sertoli cells to influence ALC population; not required for FLC development	Chang et al., 2004, De Gendt et al., 2004; Holdcraft and Braun, 2004; De Gendt et al., 2005
<i>Dhh</i>	Sertoli cells	Malformed testis cords, extracordal germ cells, loss of Patched (PTCH1) receptor expression, failure to develop FLCs	DHH required to specify FLCs via PTCH1 receptor	Bitgood et al., 1996; Pierucci-Alves et al., 2001; Yao and Capel, 2002; Yao et al., 2002; Park et al., 2007
<i>Igf1</i>	Sertoli cells, FLCs	Severe dwarfism in homozygous null mice; small testes relative to body size; small, fewer LCs; low testosterone	IGF1 stimulates FLC proliferation, differentiation, and function	Liu et al., 1993; Rouiller-Fabre et al., 1998; Wang et al., 2003; Wang and Hardy, 2004
<i>Lhb</i> (luteinizing hormone β subunit/LH β)	Secreted molecule expressed by the pituitary gland	Normal FLC development but hypoplastic ALCs	FLCs are LH independent; ALCs require LH stimulation	Ma et al., 2004
<i>Lhcgr</i> (LHR)	FLCs	Normal FLC development but failure to develop ALC population; (Humans: LC hypoplasia, aplasia, hypogonadism)	FLCs are LH independent; ALCs require LH stimulation	Lei et al., 2001; Zhang et al., 2001; Latronico and Arnhold, 2006
<i>Pdgfa</i>	Sertoli cells; ALCs	Normal virilization, small testes postnatally, loss of ALCs	PDGFA required for ALC development	Gnessi et al., 2000
<i>Pdgfra</i>	FLCs	Embryonic lethal but gonads can be cultured – no testis cords and no FLC formation	PDGFRA required for FLC lineage differentiation	Soriano, 1997; Brennan et al., 2003
<i>Nr5a1</i> (SF-1)	Somatic cells of AGP; Sertoli, FLC, and adrenocortical cells	Gonadal/adrenal agenesis	SF-1 required for formation of steroidogenic organs	Luo et al., 1994; Ikeda et al., 1995; Luo et al., 1995
<i>Nr5a1</i> (SF-1); Leydig cell-specific	As above; Cre used in this conditional knock-out expressed in FLCs	Severely delayed/underdeveloped testes, decrease in steroidogenic enzyme, cryptorchidism	SF-1 is required for functional differentiation of FLCs	Jeyasuria et al., 2004
<i>Tgfb</i>	Various members of this pathway expressed in somatic cells of the gonad	Overexpression of some family members yields incomplete virilization	TGF-beta signalling inhibits steroidogenesis in FLCs	Teerds and Dorrington, 1993; Levacher et al., 1996; Mishina et al., 1996; Gautier et al., 1997; Olaso et al., 1997; Racine et al., 1998