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THE BIOLOGY OF LEYDIG CELL DEVELOPMENT

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SUMMARY

Greater understanding of the growth factor regulation of Leydig cells during their postnatal differentiation has been achieved. Increasingly, attention will shift toward the goal of isolating Leydig stem cells because of their therapeutic potential in the treatment of male hypogonadism and the syndrome of androgen deficiency of aging males (ADAM). Markers of Leydig stem cells such as Kit receptor can be exploited in harvesting enriched fractions from the testis for amplification *in vitro*. When subsequently implanted back into the same testis, we expect the amplified stem cells to undergo differentiation into steroidogenic Leydig cells.

Key words : Androgen deficiency; Leydig cell; Stem cell; Steroidogenesis.

INTRODUCTION

Leydig cells are the primary source of testosterone, and differentiation of Leydig cells in the testes is a signature event in the development of the male body plan. As is true for all specialized cells, Leydig cells first arise from primitive undifferentiated stem cells. They are unusual, however, in that two separate Leydig cell generations successively develop in the testis between embryogenesis and

puberty. In rats, the first generation, designated as fetal Leydig cells, differentiate from stromal cells between the nascent testis cords starting on day 12 of gestation. Once formed, fetal Leydig cells are terminally differentiated and fully competent in steroidogenesis. They reach their peak of steroidogenic activity just prior to birth on day 19 of gestation (1), and the testosterone secreted is critical for male secondary sexual differentiation (development of the penis and sex accessory glands). Fetal Leydig cells remain in the testis interstitium after birth, but rapidly involute. Although it remains unclear whether or not they ultimately die, the contribution of fetal Leydig cells to postnatal androgen secretion is negligible. Adult Leydig cells (ALCs) first become apparent by day 11 postpartum as evidenced by the onset of expression of the steroidogenic enzyme 3ß-hydroxysteroid dehydrogenase (2). It is unclear whether the fetal and adult generations of Leydig cell arise from the same stem cells. Functional differences between the two types of Leydig cell might suggest that they do not have a common origin. For example, ALCs rapidly become desensitized to bolus exposures of LH due to the presence of an inhibitory guanine nucleotide-binding protein. This same protein is not expressed in fetal Leydig cells (3, 4), which therefore have a more prolonged response to LH. This review will focus on the development of ALCs. We will review the ontogeny of both fetal

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and adult Leydig cells with respect to their origin from stem cells, and the hormonal regulation of Leydig cell development.

Fetal Leydig cell ontogeny

Fetal Leydig cells are formed during male primary sexual differentiation. Developmental commitment to the male body plan is determined at the time of fertilization by the combination of a Y chromosome from the father's sperm with an X chromosome from the mother's oocyte. During embryogenesis, gonadal differentiation in male and female embryos occurs in discrete steps, and the embryonic ovary and testis are at first morphologically indistinguishable. The testis starts as a thickening (or placode) of the coelomic epithelium on the surface of the primitive kidney (mesonephros). The placode grows into a gonadal ridge, and primordial germ cells, subsequently move into the epithelium. The primordial germ cells migrate into the genital ridge from the yolk sac (allantois), by pseudopodial motion using chemotactic signals and tracks of extracellular matrix proteins to locate their correct positions within the embryo. The gonadal ridge then, during the indifferent gonad stage, develops medullary cords of epithelial tissue. The first identifiable step separating the ovarian and testicular pathways of differentiation is the movement of primordial germ cells into the medullary cords on day 12.5 of gestation in the mouse (day 14 in the rat). The primordial germ cells, enveloped by Sertoli cell precursors are thereafter referred to as seminiferous cords, and this transition creates two compartments within the testis : seminiferous and interstitial.

The morphogenetic events of early testis differentiation are controlled by the *SRY* (Sex-determining Region on the *Y* chromosome) gene. The *SRY* gene product, a nuclear transcription factor, acts in concert with other transcription factors, including Wilm's Tumor-associated gene (WT-1), SOX-9 and DAX-1 to initiate male sexual differentiation (5, 6). Differentiation of fetal Leydig cells is one of the downstream events of *SRY* signaling. Fibroblastic cells in the testis interstitium become identifiable as fetal Leydig cells by day 12.5. Recent studies by Capel and colleagues have shown that the morphogen Desert Hedgehog and platelet derived growth factor α (PDGFα) induce the fibroblastic cells to express the androgen-synthesizing cholesterol side-chain cleavage enzyme detected by immunocytochemistry on day 12.5 (7, 8). The origin of the fibroblastic stem cells of fetal Leydig cells is a subject of ongoing debate. The most widely held view has been that mesenchymal cells of the mesonephros, originally derived from embryonic mesoderm, migrate into the testis and furnish a source of fetal Leydig stem cells (9). Yao *et al.*, however, have discovered that interference with the mesonephric migratory process does not perturb the eventual differentiation of fetal Leydig cells (8), and has proposed that their stem cells move in from the coelomic epithelium overlying the developing gonad (7). Neural crest cells provide yet another potential source of fetal Leydig stem cells (10). The neural crest is an ephemeral body that extends along the rostro-caudal axis of the developing vertebrate embryo. Formed during neurulation, the migratory neural crest cells give rise to most of the peripheral nervous system, facial skeleton and numerous other derivatives throughout the embryo, including neuroendocrine cells and possibly fetal Leydig cells. It is tempting to speculate that these cells are the source of the Leydig cell lineage, because a number of proteins co-localize in Leydig cells and the brain (11). Although fetal Leydig cells do not express the Wnt I proto-oncogene (7), a marker for the neural crest lineage, dramatic cytological transformation is typical for this lineage and markers may not persist throughout the development. More research will be needed to discriminate between alternative hypotheses for the origin of fetal Leydig stem cells.

The signals triggering the initial differentiation of Leydig stem cells into the Leydig cell lineage is unknown. Fetal Leydig cell function appears to be independent of luteinizing hormone (LH) because it is normal in hypogonadal mice that lack endogenous circulating gonadotropins, LH receptor knockout (LHRKO) males that do not have functional LH receptors, and increased steroidogenesis in fetal testis is not coincident with increase in LH (12-16). Studies suggest that steroidogenic factor 1 (SF-1), a nuclear transcription factor, directs fetal Leydig stem cells towards the steroidogenic lineage (17). SF-1, produced under the direction of *SRY*, induces expression of the cytochrome P450 steroidogenic enzymes in Leydig cells, and also promotes differentiation of Sertoli cells and pituitary gonadotropes (17). Consequences of the actions of SF-1 in embryonic Sertoli cells most likely include the secretion of Desert Hedgehog, PDGFα and other paracrine regulatory factors such as IGF-I (18) and vasoactive intestinal peptide (12) that promote the differentiation and function of fetal Leydig cells. Negative regulatory factors, such as transforming growth factor ß3 (TGF ß3) (20) and Mullerian inhibiting substance (MIS, also known as anti-Mullerian hormone)(19) partially inhibit fetal Leydig cell steroidogenesis. Fetal Leydig cells maintain high rates of steroidogenic activity, secreting primarily testosterone, during the last week of gestation leading up to birth (21).

The organization of fetal Leydig cells in the testis is clustered and less uniform in contrast to the more evenly distributed ALCs (22). The fetal Leydig cell clusters are enveloped in a basal lamina that disintegrates after the first week postpartum, but it is unclear whether the cells subsequently disperse (23). The majority of fetal Leydig cells dedifferentiate or undergo apoptosis after birth (24, 25) and therefore, they do not contribute significantly to postnatal steroidogenesis. The possibility that fetal Leydig cells persist in the testis postnatally has implications for the study of ALC ontogeny. For example, the testes of adult Desert Hedgehog knockout mice are reported to contain only fetal Leydig cells (26) but it is now apparent from the recent study by Capel and colleagues that these cells cannot be fetal in the strict sense, because Leydig cells fail to differentiate before birth in these animals (8). It is reasonable to conclude that fetal Leydig cells do not progress beyond stem cells, and that ALCs arrest at a later stage of their differentiation, in the absence of stimulation by Desert Hedgehog. This underscores the fact that fetal and adult Leydig cells are regulated distinctly.

Adult Leydig cell ontogeny

Leydig stem cells continue to exist in the testes after birth and these cells are most likely to be the undifferentiated fibroblastic cells in the interstitial spaces of the testis. Postnatal development and differentiation of adult Leydig cells can be conceptualized as three separate transitions based on morphological and functional characteristics. The first recognizable stage in the adult Leydig cell lineage is defined as the progenitor Leydig cell (PLC), which appears in the testis during days 11 to 28 postpartum. The PLCs are small, spindle shaped cells, and are similar to the undifferentiated fibroblastic stem cells from which they are derived. During PLC proliferation, the differentiated functions of mature Leydig cells begin to appear, such as weak 3ß-hydroxysteroid dehydrogenase (3ß-HSD) activity (22). Paradoxically, PLCs contain negligible amounts of smooth endoplasmic reticulum (SER), the organelle needed to support steroidogenic enzymes, yet these cells are competent to secrete steroids, mainly androsterone (AO) (27). PLCs gradually enlarge, become round and reduce their proliferative capacity. This second transition results in another intermediate, the immature Leydig cell (ILC), most commonly seen in the testis during days 28 to 56 postpartum. ILCs have more SER compared to PLC and, in addition, contain cytoplasmic lipid droplets, which support a high level of steroidogenic capacity, primarily 5α reduced androgens. ILCs

undergo a final division before adult Leydig cell (ALC) function develops by day 56 (28). ALCs are the largest of the three stages, with an abundance of SER, few lipid droplets, high levels of steroidogenic enzyme activities, and high levels of testosterone secretion. ALCs are the major population of Leydig cells in the sexually mature testis. Leydig cell renewal is thought to occur very slowly under physiological conditions (29). There are circumstances, however, where Leydig cells can actively form from pre-existing PLC even in mature animals, such as when adult Leydig cells are destroyed by the drug, ethane dimethyl sulfonate (EDS)(30) or as seen in seasonally breeding species (31).

Testosterone biosynthesis and metabolism

Testosterone is synthesized from cholesterol in a mulistep process catalyzed by four enzymes: cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc), 3ß-HSD, P450-17α and 17ß-hydroxysteroid dehydrogenase (17ß-HSD) (32). In Leydig cells, cholesterol is mainly synthesized from acetate in the SER. Movement of cholesterol to the mitochondria is then promoted by a peroxisomal protein, sterol carrier protein-2 (SCP-2), together with microtubules and intermediate filaments (33). Cholesterol is transported from the outer to inner mitochondrial membranes by two shuttle proteins. The first is comprised of the polypeptide diazepam binding inhibitor (DBI) and its receptor, the mitochondrial peripheral-type benzodiazepine receptor (PBR). DBI and PBR directly promote loading of P450scc with cholesterol (34). The second shuttle protein, steroidogenic acute regulatory protein (StAR), transports cholesterol to the inner mitochondrial membrane-bound P450scc upon activation by the LH-induced cyclic AMP second messenger pathway (35). This is the rate-limiting step in steroidogenesis. The P450scc then cleaves the 27-carbon cholesterol to the 21-carbon pregnenolone. After that, pregnenolone diffuses to the SER, where the other three enzymes, 3ß-HSD, P450-17α and 17ß-HSD, are situated and subsequent testosterone biosynthetic reactions occur. There are two testosterone biosynthetic pathways depending on the species. In the Δ^4 pathway of rodent Leydig cells, 3β-HSD first catalyzes dehydrogenation of pregnenolone and forms progesterone. Subsequently, P450-17α catalyzes conversion of progesterone into 17α-hydroxyprogesterone, and then androstenedione (ADIONE). 17ß-HSD catalyzes the last step, conversion of androstenedione to testosterone. In rabbit and human Leydig cells, testosterone biosynthesis follows the Δ^5 pathway. Initially, P450-17α catalyzes conversion of pregnenolone to 17α-hydroxypregnenolone, and then to dehydroepiandrosterone (DHEA). After that, DHEA is converted by 3ß-HSD into androstenedione, which is finally converted to testosterone by 17ß-HSD. The last reaction is reversible. Its oxidative and reductive reactions are regulated by the different isoforms of 17ß-HSD as well as by the substrate and product concentrations (36).

The changes of steroidogenic and metabolizing enzymes within the Leydig cell lead to different androgen end products, at the three stages of Leydig cell differentiation (27). Due to high levels of 5α-reductase and 3α-HSD activity and, with low levels of 17ß-HSD, androsterone is the major steroid produced by rat PLCs. In the ILC, more 5α-reduced androgens are produced as a result of increased 17ß-HSD activity, which increases testosterone levels. High 5α-reductase as well as 3α-HSD activity then convert testosterone into 5α-androstane-3α,17ß-diol (3α-DIOL), the major end product for ILC. Testosterone secretion prevails in adult Leydig cell due to increases in 17ß-HSD activity coupled to a significantly decreased 5a-reductase activity. 5α-reduction and aromatization are two major pathways of testosterone metabolism (37). 5α-Reductase converts testosterone into the more potent androgen, dihydrotestosterone (DHT) which is converted by 3α-hydroxysteroid dehydrogenase (3α-HSD) into a weak androgen, androstane-3α,17ß-diol. Cytochrome P450 aromatase (P450arom) metabolizes testosterone into estradiol. The final pathway of

testosterone metabolism involves 7α-hydroxylase and conjugating enzymes that lead to the elimination of the steroid from the body. 7α-hydroxylase is a member of the cytochrome P450 enzyme family in the liver and testis, catalyzing degradation of testosterone into water-soluble derivatives that can be excreted in the urine. Two groups of conjugating enzymes: glucuronyltransferase in the liver and sulfotransferase in the testis, liver, and adrenal conjugate testosterone with glucuronic acid and sulfates, respectively, prior to excretion by the kidney (37).

In addition to the regulators already discussed, a balance between testosterone biosynthetic and degradative enzyme activities determine the amount of testosterone secreted by Leydig cells (38). Studies focussed on the hormonal regulation of androgen metabolism are scarce, but it has been shown that PLC mRNA levels for testosterone biosynthetic enzymes are significantly lower compared to steroidogenic enzyme expression in ILC and ALC. In addition, enzymes involved in androgen metabolism exhibit transitory changes during Leydig cell development. Levels of 5α-reductase are highest in ILC, followed by PLC and ALC. The androgen metabolizing enzyme, 3α-HSD, which catalyses the interconversion of DHT and 3α-DIOL, changes its oxidative and reductive activities as the Leydig cell develops. The rate of 3α-HSD reduction is highest in PLC and lowest in ALC, whereas, the rate of 3α-HSD oxidation follows the opposite pattern (39). Using a series of sequence specific probes, we identified the gene product that confers 3α-HSD activity in adult Leydig cells as type 2 retinol dehydrogenase (RoDH-2) (40).

Factors affecting Leydig cell development in puberty

Leydig cells are the primary target for LH action in the testis, although it has been shown that testicular vascular endothelial cells also express LH receptors, and LH transport probably involves transcytosis through the endothelial cell layer into the interstitium (41). LH binding to its receptor triggers the cAMP signaling pathway and a cascade of intercellular events such as increased gene transcription, increased steroidogenic enzyme activity, testosterone production, and synthesis of the protein regulators, SCP-2 and StAR (42-44). In contrast, lack of LH stimulation results in loss of both steroidogenic organelles and steroidogenic enzyme activities as well as Leydig cell atrophy (44). LH stimulation is required for Leydig cell development, but it is unlikely to be the initial stimulus for stem cell differentiation into the Leydig cell lineage nor is it the trigger for initial expression of Leydig cell specific genes. Evidence for this conclusion comes from the fact that the LH receptor protein is truncated in stem cells and progenitor Leydig cells, providing a dull response to gonadotropin stimulation (45). LH is the master control factor for completing and maintaining fully differentiated structure and testosterone biosynthetic function of Leydig cells. However, several hormones, locally produced growth factors, nuclear transcription factor and enzymatic modulators are also thought to be involved in the regulation of proliferation, differentiation and function in Leydig cells. In this model, the action of LH occurs later, stimulating development of adult Leydig cells during prepubertal life. Thus, the critical role of LH in the development of Leydig cells is apparent from the studies of GnRHhpg mice, which are deficient in circulating LH. In these mice Leydig cell numbers are about 10% of control (46). Moreover, Leydig cells are severely hypoplastic in LH receptor knockout (LHRKO) mice (13, 16). Increase of Leydig cell proliferative activity occurs following LH/hCG administration *in vivo* (46, 47), but events subsequent to LHR binding, leading to increased cell division, have not been identified. In adult Snell dwarf mice, deficiency in plasma gonadotropin prevents full differentiation of Leydig cells without affecting their numbers (48, 49). Neither long-term suppression of LH or the return of LH to control values in adult rat has a significant effect on Leydig cell numbers (50, 51). In

addition, although LH stimulates DNA synthesis in immature rat Leydig cells *in vitro,* these increases are limited (52-58). LH and hCG stimulate IGF secretion and upregulate type I-IGF receptor gene expression in rodent Leydig cells (59-62). IGF-1 stimulates the proliferation of Leydig cell precursors and pretreatment of these cells with LH augments the mitogenic effect (51, 63, 64). The hypothesis that IGF-1 facilitates Leydig cell differentiation and maturation in conjunction with LH is based on the fact that 1) progenitor Leydig cells, fibroblastic precursors observed in early neonatal testes, possess few LHR and are relatively insensitive to LH (65, 66); 2) Leydig cells differentiate in GnRHhpg mice (45) which are deficient in circulating LH; 3) IGF-1 and its receptor mRNAs are highly expressed in progenitor and immature Leydig cells; and 4) IGF-1 enhances hCG-stimulated T formation (67). This suggests that there is a requirement for IGF-1 preceding LH-mediated events of Leydig cell differentiation, and that IGF-1 acts in conjunction with LH to further stimulate Leydig cell maturation. *In vitro* studies have also shown that IGF-1 stimulates maturation of rat immature Leydig cells by increasing expression of steroidogenic enzymes and T production (68, 69). GH-deficient Snell dwarf mice have very low circulating IGF-1 levels and treatment of these mice with IGF-1 *in vivo* induces a marked increase in the number of LHRs and in the steroidogenic response (70), providing further evidence for the importance of IGF-1 for Leydig cell maturation. Mice with an IGF-1 null mutation have marked reductions in circulating T levels (18% of wildtype control), associated with decrease in testis size and Leydig cell numbers (71). This led to the hypothesis that the dramatic declines seen in circulating T levels in adult IGF-1 null mutants result from abnormal testis development, and specifically from an imbalance in T biosynthetic and metabolizing enzyme activities in Leydig cells. Significant enhancement of the LH effect is achieved by co-administration of growth factors such as IGF-1 (51, 52). These data raised the possibility that the action of LH on Leydig cell proliferation requires the participation of other factors.

Insulin like growth factor - 1 (IGF-1)

The testis is a site of IGF-1 biosynthesis and action. IGF-1 mRNA, protein, and specific IGF-1 receptors are present in the testis and have been identified in Leydig cells, peritubular cells and spermatocytes (53-58). Testicular levels of IGF-1 are highest at four week postpartum, at the beginning of the pubertal rise in testosterone secretion (59). LH and hCG stimulate IGF-1 secretion and upregulate type I-IGF receptor gene expression in rodent Leydig cells (59-62). IGF-1 stimulates the proliferation of Leydig cell precursors and pretreatment of these cells with LH augments the mitogenic effect (52, 63, 64). The hypothesis that IGF-1 facilitates Leydig cell differentiation and maturation in conjunction with LH is based on the fact that 1) PLCs, fibroblastic precursors observed in early neonatal testes, possess few LHR and are relatively insensitive to LH (65, 66); 2) Leydig cells differentiate in GnRH-deficient mice (46) which are deficient in circulating LH; 3) IGF-1 and its receptor mRNAs are highly expressed in PLCs and ILCs and 4) IGF-1 enhances hCG-stimulated T formation (67). This suggests that there is a requirement for IGF-1 preceding LH-mediated events of Leydig cell differentiation, and that IGF-1 acts in conjunction with LH to further stimulate Leydig cell maturation. *In vitro* studies have also shown that IGF-1 stimulates maturation of rat immature Leydig cells by increasing expression of steroidogenic enzymes and T production (68, 69). GH-deficient Snell dwarf mice have very low circulating IGF-1 levels and treatment of these mice with IGF-1 *in vivo* induces a marked increase in the number of LHRs and in the steroidogenic response (70), providing further evidence for the importance of IGF-1 for Leydig cell maturation. Mice with an IGF-1 null mutation have marked reductions in circulating T levels (18% of wildtype control), associated with decrease in testis size and Leydig cell numbers (71). This led to the hypothesis that the

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Attempts to culture Leydig cells for periods longer than one week have generally been unsuccessful. Part of the problem is in replicating *in vitro* the full range of factors present *in vivo* that are required to sustain Leydig cell steroidogenic function. *In vitro* conditions have been steadily optimized by, lowering ambient oxygen tension during culture from 21% down to 5% to reduce free oxygen radical generation, and by supplementing the culture medium with lipoprotein and culturing the cells on collagen extracellular matrix protein (72, 73). However, to realize the goal of therapeutic androgen replacement during aging and treatment of hypogonadal males, it will be necessary to create populations of Leydig cells that can be expanded and maintained *in vitro* prior to implantation. To do so will entail working with Leydig stem cells. Many different growth factors and hormones have been investigated for effects on Leydig cell differentiation (74), but IGF-1 uniquely acts to stimulate both cell proliferation (63) and steroidogenic function (75). In addition, IGF-1 stimulates increased LH receptor numbers in immature pig Leydig cells. In the mouse, IGF-1 has been shown to attain peak concentrations in the interstitial fluid postnatally (76, 77). Taken together, these data form a compelling rationale to test for the role of IGF-1 on Leydig stem cells, and the role of LH in mediating early developing Leydig cells that have acquired gonadotropic sensitivity, which we designate as PLCs.

Mullerian inhibiting substance (MIS)

Involvement of MIS in the regulation of Leydig cell proliferation became apparent in 1994 when Richard Behringer and colleagues reported their findings on MIS deficient mice (78). There is a pronounced hyperplasia and focal neoplasia of Leydig cells in these males. Recombinant mice lacking MIS type-II receptor or both MIS and MIS type-II receptor also developed Leydig cell hyperplasia (78). On the other hand, adult transgenic mice overexpressing MIS have 50% fewer immature and 80% fewer mature Leydig cells than wild-type littermates (79). The loss of control over Leydig cell proliferation in the MIS knockout males indicates that MIS normally has an essential function in limiting the size of Leydig cell populations in the testis.

Leydig cells express the MIS type-II receptor and are responsive to MIS *in vitro* (80). Although the secretion of MIS by Sertoli cells gradually declines after birth, it remains detectable in a stage-specific pattern even in the adult animals (81). Moreover, the expression of the MIS type-II receptor, on Leydig cells increases from day 9 to day 14 and remains abundant in the pubertal and adult animal (80, 81). It was found MIS can inhibit Leydig cell proliferation *in vitro* (80). MIS also inhibit Leydig cell steroidogenesis primarily by inhibiting 17α-hydroxylase (80, 82, 83). When MIS on Leydig cell proliferation and steroidogenesis were further analyzed in ethylene dimethanesulphonate (EDS) treated rats, which can destroy the adult generation of Leydig cells and initialize a new round of Leydig cell development. Following EDS ablation of differentiated Leydig cells in young adult rats, recombinant MIS was delivered by intratesticular injection to evaluate its effects. MIS-treated animals had fewer mesenchymal precursors on day 15 and fewer differentiated Leydig cells on day 35 with decreased numbers of BrdU+ nuclei. Apoptotic interstitial cells were observed only in the MIS-treated testes. These data suggest that MIS inhibits regeneration of Leydig cells in EDS treated rats by enhancing apoptotic cell death as well as by decreasing proliferative capacity (84).

Stem cell factor

Just as MIS exerts its effect on Leydig cells via a tyrosine kinase coupled receptor, another Sertoli cell regulatory protein, stem cell factor (also referred to as Kit ligand), also uses this type of receptor. Kit ligand acts through a tyrosine kinase receptor (Kit), and Kit is expressed by Leydig cells. Kit signaling has an established function in melanogenesis and stimulation of hematopoiesis. In the testis, Kit regulates the dynamics of germ cell proliferation, differentiation and renewal. The presence of Kit in Leydig cells reinforces the concept that coordination in the activities of Sertoli and Leydig cells is essential for spermatogenesis. The experiments focussed on a line of mice bearing a knock-in mutation in Kit that eliminates one of its intracellular signaling pathways. The substitution of the tyrosine residue at the 719 position in Kit with phenylalanine, prevents this receptor from binding with the P85 subunit of phosphatidylinositol-3-kinase (PI3 kinase). These mutant mice (719F) have normal kit function for melanogenesis and hematopoiesis, but the males are sterile. Serum testosterone levels in 719F males did not differ from values seen in normal wildtype mice. However, the serum levels of LH were sharply elevated. The testicular histology was consistent with Leydig cell hyperplasia in mutants (71, 85). Under this scenario, the negative feedback system would reset testosterone production in the mutant back to control levels by increasing the number of Leydig cells per testis. This hypothesis was tested in a parallel study of the responses of normal adult mouse Leydig cells to stimulation by Kit ligand. Kit signaling increased Leydig cell steroidogenesis about two-fold above basal levels. Moreover, the stimulation is blocked by a chemical inhibitor of PI3 kinase, wortmannin (86, 87). These data, together with the germ cell defects in the 719F male mice, indicate that Kit activation of PI3 kinase signaling in the testis is critical for fertility. MIS and Kit ligand are hormonal effectors of testicular origin that now appear to play prominent roles in Leydig cell development.

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