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THYROID HORMONE REGULATION OF SERTOLI CELL PROLIFERATION MAY BE MEDIATED THROUGH THE CELL CYCLE INHIBITORS $p27^{Kip1}$ AND $p21^{Cip1}$

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INTRODUCTION

The Sertoli cell is the somatic cell of the seminiferous epithelium that supports all stages of male germ cell development from spermatogonia to fully mature spermatozoa. Sertoli cells support a relatively fixed number of germ cells, though the specific number of germ cells supported by each Sertoli cell varies with species. Therefore, the number of Sertoli cells in the adult testis establishes the magnitude of sperm production, and factors that control Sertoli cell proliferation and establishment of the adult population are of critical interest. Sertoli cell proliferation begins fetally and persists for variable periods after birth, depending on the species. In rats and mice Sertoli cells rapidly proliferate neonatally, then ceases by about the time of weaning, thus establishing the adult Sertoli cell population (1, 2).

Follicle-stimulating hormone (FSH) from the pituitary is the major stimulator of early Sertoli cell proliferation. However, neonatal Sertoli cells *in vivo* stop proliferating during neonatal life despite continual FSH exposure (3). Similarly, cultured neonatal Sertoli cells proliferate in response to FSH or other mitogens, while juvenile Sertoli cells do not proliferate in culture (4). A critical question is therefore what controls the developmental shift of Sertoli cells from a proliferative to non-proliferative state during early postnatal life.

Thyroid hormone regulates early Sertoli cell development

Thyroid hormone appears to play a key role in Sertoli cell development, and is a key factor in halting proliferation of developing Sertoli cells and thus establishing the adult Sertoli cell population. Work dating back half a century has indicated that thyroid hormone is not a major factor in regulation of the adult testis (5, 6). However, thyroid hormone receptors are present in high quantities in neonatal Sertoli cells, indicating that the developing Sertoli cell may be an important thyroid hormone target even though the adult testis shows less dependence on thyroid hormone (7, 8).

For example, hypothyroidism in the neonatal rat inhibits testicular growth, germ cell maturation, seminiferous tubule lumen formation and other developmental events (9-12). This results not only from decreases in thyroid hormones, but also from secondary changes in a variety of sex steroids (13). However, when rat pups are made hypothyroid by the administration of the goitrogen propylthiouracil (PTU) from birth to day 25 postnatal, then allowed to recover, they become euthyroid within a few weeks. The recovered euthyroid rats showed an increase in testis size and daily sperm production (DSP) of 80% and 140%, respectively, when they reach adulthood compared to normal

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control animals (14, 15). These surprising findings raised important questions related to the role of thyroid hormone in developing testis, and how neonatal hypothyroidism could increase adult testis size and DSP despite the known inhibitory effects on the testis during the period of hypothyroidism.

Shortly after the finding that neonatal hypothyroidism could produce an increase in adult testis size, work by van Haaster *et al.* (16) and other groups (2) established that hypothyroidism prolonged the period of early Sertoli cell proliferation, and led to increased adult populations of Sertoli cells that were responsible for the subsequent increased adult testis size and DSP following neonatal hypothyroidism. This suggested that thyroid hormone normally induced Sertoli cells to stop proliferating, and subsequent work showing that the biologically active thyroid hormone, 3,5,3'-triiodo-L-thyronine (T_3) could decrease Sertoli cell proliferation and stimulate a variety of other maturational markers indicated that this was a direct T_3 effect on Sertoli cells (17-21). Thus, T3 normally stimulates cessation of Sertoli cell proliferation and a concomitant functional maturation of these cells. When animals are made hypothyroid, Sertoli cell proliferation is extended and an increased pool of adult Sertoli cells results. When the animals recover from hypothyroidism, the increased Sertoli cell population is capable of supporting increased number of germ cells (22). The net effect is an increase in adult testis size, Sertoli cell numbers and DSP in animals that have recovered from neonatal hypothyroidism, despite the well documented deleterious effects on the testis during the period when the animal is hypothyroid (9, 1, 12).

Thyroid hormone effects on Sertoli cell proliferation may involve the cell cycle regulators $p27^{Kip1}$ and $p21^{Cip1}$

Following the identification of T_3 as a critical factor in inducing Sertoli cells to stop proliferating during development, a clear priority in this area was to determine the molecular mechanism of this effect. This goal has become more attainable during recent years due to the rapid progress in our understanding of the cell cycle and factors that control progression of cells through the cell cycle or their exit from the cell cycle into a differentiation pathway. In addition, the development of an extensive array of knockout mice lacking cell cycle proteins has provided unique tools to directly understand how T_3 regulates the Sertoli cell cycle and ultimately proliferation.

Progression of mammalian cells through the cell cycle is regulated by cyclins, which work by associating with and activating cyclin-dependent kinases (Cdks). Activation of the cyclin-dependent kinase complexes (cyclin + Cdk) results in progression of cells through the various phases of the cell cycle. The activity of the cyclin-dependent kinase complexes is further regulated by a family of proteins known as the cyclin-dependent kinase inhibitors (CDKIs). CDKIs bind and inactivate cyclin-dependent kinases and can therefore exert negative control over progression through the critical G1 checkpoint of the cell cycle.

Recent data has shown that two CDKIs, $p27^{Kip1}$ and $p21^{Cip1}$, are involved in Sertoli cell proliferation and may also be crucial links in the process by which T_3 inhibits Sertoli cell proliferation. Although many questions remain, these results are beginning to provide new mechanistic insights into the critical question of how T_3 induces cessation of Sertoli cell proliferation during development. Testis expresses high levels of $p27^{Kip1}$, primarily in Sertoli cells, and $p27^{Kip1}$ expression is inversely correlated with Sertoli cell proliferation (23). Levels of $p27^{Kip1}$ are minimal in rapidly proliferating neonatal Sertoli cells, but $p27^{Kip1}$ expression is normally

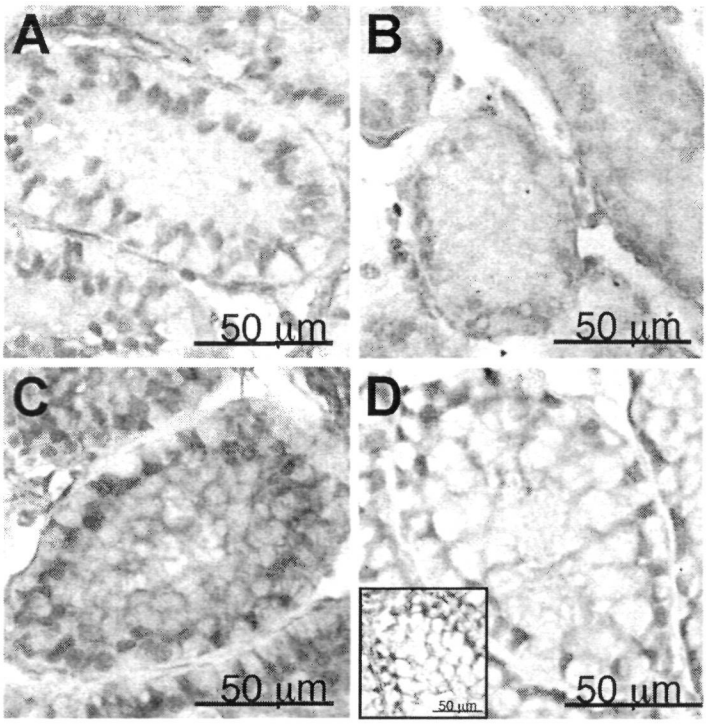

Fig.1 Immunohistochemical detection of $p27^{Klpl}$ in Sertoli cells from euthyroid (A), hypothyroid (B), and hyperthyroid (C) 10-day-old mice. Hypothyroidism decreases $p27^{Kipl}$ expression whereas hyperthyroidism increases $p27^{Kipl}$ expression in 10-day-old Sertoli cells when compared to euthyroid controls. By 25 days of age, $p27^{Kipl}$ expression in Sertoli cells from hypothyroid mice (D) is similar to age-matched euthyroid control mice (insert).

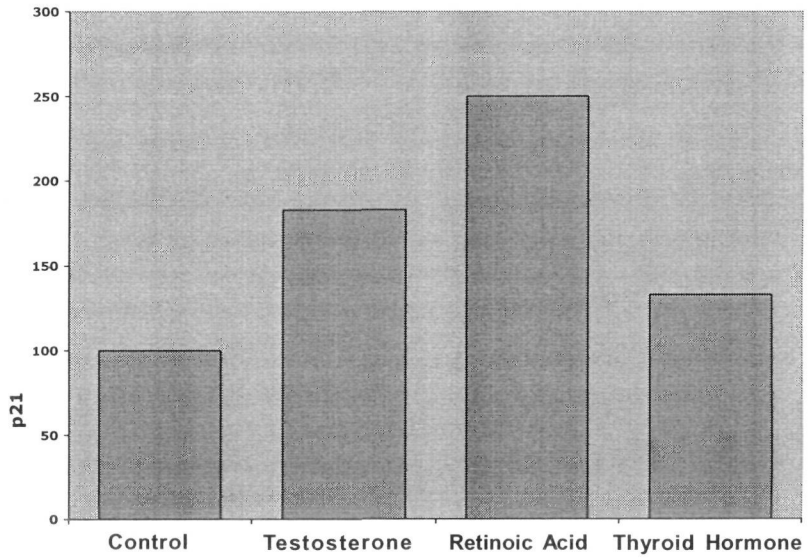

Fig.2 $p21^{Cipl}$ expression increases in six-day-old rat Sertoli cells cultured for 48 hours with testosterone, retinoic acid, thyroid hormone or vehicle control. (Data adapted from Buzzard *et al.*, 2003.)

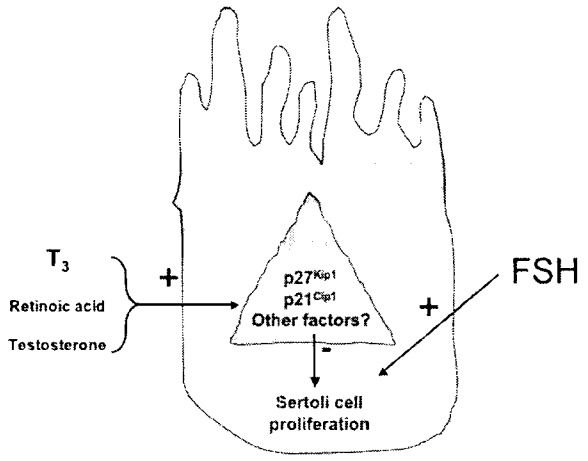

Fig.3. Schematic diagram illustrating hormonal regulation of neonatal Sertoli cell proliferation. FSH is a positive regulator of neonatal Sertoli cell proliferation, whereas testosterone, thyroid hormone, and retinoic acid are inhibitors. Testosterone, thyroid hormone, and retinoic acid may act through increasing the cell cycle inhibitors $p27^{Kipl}$ and $p21^{Cipl}$ in neonatal Sertoli cells, but also may have effects on other cell cycle targets.

high in post-mitotic adult Sertoli cells (23). Consistent with a role for $p27^{Kip1}$ in the inhibition of Sertoli cell proliferation, $p27^{Kip1}$ expression is sharply decreased in rapidly proliferating Sertoli cell tumors (24). These results indicating that $p27^{Kip1}$ could regulate Sertoli cell proliferation and the establishment of adult Sertoli cell numbers are of added significance in the light of recent work from two laboratories showing that T_3 regulates $p27^{Kip1}$. Its expression was higher in Sertoli cells from hyperthyroid neonatal mice when compared to euthyroid controls, whereas decreases in $p27^{Kip1}$ expression were observed in Sertoli cells from hypothyroid mice (Figure 1) (25). A companion paper by Buzzard *et al.* (26) showed that T_3 treatment of neonatal rat Sertoli cells increased expression of $p27^{Kip1}$ protein. In addition, T_3 treatment of neonatal rat Sertoli cells also stimulated expression of $p21^{Cip1}$, suggesting that this CDKI could also be involved in both normal Sertoli cell proliferation and the process by which T_3 turns off Sertoli cell proliferation during development (26). Similar to T_3, testosterone and retinoic acid also stimulated $p27^{Kip1}$ protein expression, and retinoic acid also stimulated $p21^{Cip1}$ as well (Figure 2) (26). Thus, $p27^{Kip1}$ and $p21^{Cip1}$ may function as regulatory factors that integrate a variety of hormonal inputs on Sertoli cell proliferation, further emphasizing the importance of these CDKIs in Sertoli cell development (Fig. 3).

The regulation of $p27^{Kip1}$ and $p21^{Cip1}$ in Sertoli cells by T_3 clearly suggests that early hypothyroidism may interfere with the normal increase in these CDKIs, which would then lead to extended Sertoli cell proliferation and the observed testicular organomegaly. However, to directly evaluate this hypothesis, it is essential to establish whether or not $p27^{Kip1}$ and $p21^{Cip1}$ are important regulators of Sertoli cell proliferation and are involved in the establishment of ultimate Sertoli cell number. The increase in testis size reported in $p27^{Kip1}$ knockout (p27KO) mice (27-29) are consistent with this hypothesis, but Sertoli cell number had not been measured in these mice. Similarly, there had been no determination of Sertoli cell number, or even testis size, in $p21^{Cip1}$ knockout (p21KO) mice. To directly test the hypothesis that $p27^{Kip1}$ and $p21^{Cip1}$ are both important regulators of Sertoli cell numbers (and that T_3 effects on these CDKIs could therefore alter Sertoli cell number), we have examined Sertoli cell number, testis weight and DSP in 4-month-old wild-type (WT), p27KO, p21KO and $p27^{Kip1}$/ $p21^{Cip1}$ double knockout (DBKO) mice.

Sertoli cell number was increased by over 100% in both p21KO and p27KO mice (Holsberger *et al.* unpublished). Sertoli cell number was greater in DBKO mice than in either the p21KO or p27KO, indicating that the increase in Sertoli cell numbers caused by loss of $p27^{Kip1}$ and $p21^{Cip1}$ are

partially additive. Testis weights were significantly increased by 25%-35% in p21KO and p27KO mice, respectively, and the increases in the DBKO mice were over 70%, indicating that the increase in testis weight induced by loss of $p27^{Kip1}$ or $p21^{Cip1}$ are additive (Holsberger *et al.* unpublished). DSP increases in the various groups were similar to those for testis weight. These data indicate that both CDKIs play an inhibitory role in regulating adult Sertoli cell number; loss of either increases Sertoli cell number, testis weight and DSP, and loss of both CDKIs causes greater increases in these parameters larger than those seen following the loss of only one of the CDKIs.

The ability of T_3 to regulate both $p27^{Kip1}$ and $p21^{Cip1}$, combined with the recent data indicating that both of these CDKIs are involved in Sertoli cell proliferation, strongly indicates that both of these proteins are involved in increasing Sertoli cell number and testis weight following neonatal hypothyroidism. However, a number of critical questions still must be answered to establish the relative roles of $p27^{Kip1}$ and $p21^{Cip1}$ in mediating the effects of T_3 on developing Sertoli cells. Even though recent data established that T_3 can increase expression of both $p27^{Kip1}$ and $p21^{Cip1}$, and both $p27^{Kip1}$ and $p21^{Cip1}$ are involved in Sertoli cell proliferation, the relative importance of each of these CDKIs in the T_3 effect on Sertoli cell proliferation still remains to be established. In addition, it is not clear that these CDKIs are the sole mediators of the T_3 effect on Sertoli cell proliferation. In other words, it is certainly possible that neonatal hypothyroidism could have additional effects over and above inhibition of $p27^{Kip1}$ and $p21^{Cip1}$, and that an additional effect(s) of T_3 not involving the CDKIs could also be an important contributor to the increase in Sertoli cell number induced by neonatal hypothyroidism.

To determine the relative importance of $p27^{Kip1}$ and $p21^{Cip1}$ in the T_3 effect on Sertoli cell proliferation, and also to establish whether these CDKIs are the sole mediators of T_3 on Sertoli cell proliferation, we have begun a set of experiments involving PTU treatment of neonatal p21KO, p27KO and DBKO mice. These experiments will allow us to determine the individual roles of $p27^{Kip1}$ and $p21^{Cip1}$ in mediating the effects on Sertoli cell proliferation induced by early hypothyroidism. In addition, they will establish that the effects of T_3 on Sertoli cell proliferation are either mediated entirely by $p27^{Kip1}$ and $p21^{Cip1}$, or that the T_3 effect involves another factor(s). For example, if early PTU exposure still produces an increase in Sertoli cell number and adult testis size in the p27KO mouse, this will indicate that the effect of neonatal hypothyroidism on Sertoli cells involves effects other than a suppression of $p27^{Kip1}$. Likewise, if PTU treatment of p21KO mice results in an increase in Sertoli cell number relative to the untreated p21KO control, this will indicate that a factor(s) other than $p21^{Cip1}$ is involved in the T_3 effect on Sertoli cell number. If PTU treatment of p27KO mice increases in Sertoli cell number, it would be tempting to postulate that this is solely due to PTU suppression of $p21^{Cip1}$ in these mice. Likewise, an increase in Sertoli cell number in p21KO mice may be solely due to the effects of hypothyroidism on $p27^{Kip1}$. However, in addition to effects of T_3 on $p27^{Kip1}$ and $p21^{Cip1}$, literature from other cell types also raises the possibility of T_3 affecting additional cell cycle proteins or other targets that have effects on the cell cycle.

Thyroid hormone effects on Sertoli cell proliferation may also involve other cell cycle regulators

There are reports that in oligodendrocytes and other cell types, T_3 has effects on the Rb protein family of cell cycle proteins, a potential mechanism by which T_3 could alter proliferation (30, 31). There are also data in the oligodendrocyte precursor cell line P19 that T_3 directly inhibits the E2F transcription factor responsible for driving cells through the G1 restriction point, so T_3-induced inhibition

of Sertoli cell proliferation could involve direct T_3 effects on E2F (30). Other work has suggested that T_3 increases other CDKIs, such as $p16^{Ink4a}$, $p18^{Ink4c}$ and $p19^{Ink4d}$ in oligodendrocytes, suggesting yet another mechanism by which T_3 could regulate Sertoli cell proliferation (32). Furthermore, previous reports suggest that T_3 can affect proliferation in other cell types as a result of changes in expression of cell cycle proteins such as cyclin D1, D2 or E (30, 33, 34), or their partners such as Cdk2 (31, 35) or Cdk4 (31).

Assuming that both $p27^{Kip1}$ and $p21^{Cip1}$ are involved in the mediation of T_3 inhibition of Sertoli cell proliferation, it will be possible to determine whether the entire effect of T_3 on Sertoli cell proliferation is mediated through these two CDKIs, or also involves T_3 effects on another target(s). By treating DBKO mice with PTU then examining their Sertoli cell number in adulthood, this question can be definitively answered. If Sertoli cell number in PTU-treated DBKO mice is not increased over that seen in untreated DBKO mice, this will indicate that the absence of $p27^{Kip1}$ and $p21^{Cip1}$ totally precludes the increase in Sertoli cell number that normally results from early hypothyroidism, and will indicate that the PTU effect is entirely mediated through effects on $p27^{Kip1}$ and $p21^{Cip1}$. Conversely, if some increase in Sertoli cell number is still seen in DBKO mice treated neonatally with PTU, this will indicate that the PTU effect is not entirely mediated through $p27^{Kip1}$ and $p21^{Cip1}$, but also may involve other effects on the cell cycle independent of these two CDKIs.

SUMMARY AND CONCLUSION

In summary, the effects of T_3 on Sertoli cell development and the establishment of adult Sertoli cell number appear to involve the CDKIs $p27^{Kip1}$ and $p21^{Cip1}$ as critical mediators of T_3 effects on Sertoli cell proliferation. Though these findings suggest a mechanistic paradigm for how T_3 acts on Sertoli cells, a number of important questions related to the precise role of $p27^{Kip1}$ and $p21^{Cip1}$, separately and in concert, still must be addressed to develop a comprehensive understanding of this important developmental process in Sertoli cells.

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