REVIEW

Thyroid hormone action in the developing testis: intergenerational epigenetics

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Abstract

Male fertility involves the successful transmission of the genetic code to the next generation. It requires appropriately timed cellular processes during testis development, adequate support of spermatogenesis by hormonal cues from the reproductive axis and cellular cross-talk between germ and somatic cells. In addition to being the vessel of the father's genome, increasing evidence shows that the mature sperm carries valuable epigenetic information – the epigenome – that, after fecundation, influences the development of the next generation, affecting biological traits and disease susceptibility. The epigenome of the germ line is susceptible to environmental factors, including exogenous chemicals and diet, but it is also affected by endogenous molecules and pathophysiological conditions. Factors affecting testis development and the epigenetic information of the germ line are critical for fertility and of relevance to the non-genetic but heritable component in the etiology of complex conditions. Thyroid hormones are one of those factors and their action, when untimely, produces profound effects on the developing testis, affecting spermatogenesis, steroidogenesis, testis size, reproductive hormones and fertility. Altered thyroid hormone states can also change the epigenetic information of the male germ line, with phenotypic consequences for future generations. In the context of past literature concerning the consequences of altered thyroid hormone action for testis development, here we review recent findings about the pathophysiological roles of the principal determinants of testicular thyroid hormone action. We also discuss limited work on the effects of thyroid hormone on the male germ line epigenome and the implications for the intergenerational transmission of phenotypes via epigenetic mechanisms.

Key Words

- thyroid hormone
- thyroid hormone receptors
- deiodinases
- Thra
- Thrb
- Dio2
- Dio3
- developing testis
- transgenerational epigenetics
- Sertoli cells
- spermatogonia
- Leydig cells
- spermatogenesis
- gonadal axis
- steroidogenesis
- male fertility
- retinoic acid

Journal of Endocrinology (2020) **244**, R33–R46

Introduction

Testicular development and function are critical for normal spermatogenesis and male reproduction. The growth and maturation of the testes require the timely occurrence of multiple processes of cell proliferation, differentiation and cross-talk to ensure adequate cell function and hormone action in testicular tissue to support the maturation of germ cells into functional sperm (Makela *et al.* 2019). The disruption of these processes may impair spermatogenesis and compromise male reproductive function. Understanding these processes and the factors that disrupt them is more relevant than ever, considering the continuous decrease in sperm quantity and quality observed in humans during the last decades (Levine *et al.* 2017).

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https://doi.org/10.1530/JOE-19-0550

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Reproduction not only implies the accurate transmission of the genetic code to the next generation. Sperm can carry a wealth of non-genetic (epigenetic) information that reflects the variable environmental and pathophysiological circumstances of an individual (Dunn *et al.* 2005, Hochberg *et al.* 2011, Guerrero-Bosagna & Skinner 2012). Importantly, this epigenetic information may also influence the development, physiology and disease susceptibility of descendants. The intergenerational transmission of epigenetic information may partly explain the etiology of complex human conditions that exhibit a high degree of heritability but for which genetic factors alone fall short of explaining a majority of clinical cases (Koch 2014*a*).

In this context of testis development and intergenerational transmission of epigenetic information, thyroid hormones (TH) have pleiotropic effects in mammalian systems, and the developing testis is also a critical target organ (Hernandez 2018*b*). Alterations in TH status and signaling have profound effects on multiple cellular processes and functions of the testis, especially during development, ultimately impacting male fertility. In addition, limited but consistent evidence reveals an influence of TH on the epigenetic information of the germ line and the biological traits of descendants. Here, we review the most critical observations about the effects of TH on the developing testis and the implications for male reproduction. We further highlight key findings concerning the intergenerational epigenetic effects of TH on the pathophysiology of descendants.

Thyroid hormone action in the developing testis

Basic mechanisms regulating thyroid hormone action

The canonical mechanism of TH action involves the binding of the most active TH, 3,5,3′-triiodothyronine (T3), to its nuclear receptors. These receptors are DNA-binding transcription factors and are encoded by two different genes, *Thra* and *Thrb* (Pascual & Aranda 2013). Upon receptor binding and subsequent cofactor recruitment and chromatin modifications, T3 regulates the transcription of target genes (Pascual & Aranda 2013).

Prior to nuclear receptor binding, T3 secreted by the thyroid gland into the circulation can enter the target cell via cell membrane specific transporters, among which mono-carboxylate transporter 8 (*Mct8* or *Slc16a2*) shows the highest affinity for T3 transport (Heuer & Visser 2013). In addition, T3 can be generated in peripheral tissues from thyroxine (T4), which is produced by the thyroid gland in more abundance than T3 and is largely considered a pro-hormone for canonical signaling, as its affinity for thyroid hormone receptors is approximately 10% that of T3. The conversion of T4 to T3 is accomplished by the type 1 and type 2 deiodinases (DIO1 and DIO2, respectively), and their action may modify local T3 availability as well as contribute to circulating levels of TH (St Germain *et al.* 2009).

Finally, the type 3 deiodinase (DIO3) converts both T3 and T4 into metabolites with very low affinity for the TH receptors, effectively clearing TH and reducing T3 signaling (Charalambous & Hernandez 2013). Thus, it is increasingly appreciated that T3 action does depend not only on circulating levels of TH, but also on the complement of transporters (Heuer & Visser 2009), deiodinases (Bianco *et al.* 2019), receptors (Forrest & Visser 2013) and receptor cofactors (Astapova & Hollenberg 2013) present in a particular cell or tissue. This complement of factors can markedly enhance or reduce local T3 action in a manner that is largely independent of circulating levels of TH (Ng *et al.* 2004).

Effects of altered TH status on the developing testis

This subject has been recently reviewed in more detail (Wagner *et al.* 2008, Hernandez 2018*a*,*b*). Here we highlight the most important findings in the context of the present article.

Developmental thyrotoxicosis

Probably the first observation of testicular abnormalities as a result of TH excess (thyrotoxicosis) during development was reported in the rat model of neonatal T4 administration ('neo T4'). In this model, newborn rats were administered large doses of T4 daily for 4–10 days after birth (Bakke *et al.* 1975, Martin & Moberg 1981). This protocol resulted in very high neonatal levels of serum T3 and T4 (Martin & Moberg 1981). Neo-T4 rats exhibited growth retardation and developmental delays and, as adults, moderate central hypothyroidism and an abnormal set-point and physiological response of the hypothalamic-pituitary-thyroid (HPT) axis. They also exhibited a significant reduction in testis size (Bakke *et al.* 1975).

Similar mouse models of TH administration during neonatal life have also shown a decrease in testis size (Cooke *et al.* 1994). This is largely due to T3 causing an

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arrest of Sertoli cell (SC) proliferation (van Haaster *et al.* 1993, Palmero *et al.* 1995), a critical process in the testis that is affected by different molecular pathways (Holsberger & Cooke 2005, Meroni *et al.* 2019), including those dependent on TH. The small adult testis size and increased levels of follicle-stimulating hormone (FSH) caused by neonatal T3 administration can be fully normalized by a genetic loss of functional THRA (Holsberger *et al.* 2005*b*), demonstrating that this receptor is the main mediator of the testicular effects caused by neonatal thyrotoxicosis.

T3 excess also increases gonocyte differentiation, reduces the proliferation of spermatogonia and may promote the apoptosis of germ cells (Boulogne *et al.* 2003, Rijntjes *et al.* 2008, Faraone-Mennella *et al.* 2009) in the neonatal testis.

Hypothyroidism

Developmental hypothyroidism also leads to consistent abnormalities in testis development and the hormonal pathophysiology of the gonadal axis (Hernandez 2018*b*). The occurrence and severity of these abnormalities may vary depending on the degree and length of the hypothyroidism and the stage of development affected (from early neonatal to pubertal age). However, the observations consistently show a delay in SC differentiation, leading to increased SC number and testis size in adulthood (Bunick *et al.* 1994, Cooke *et al.* 1994, Sakai *et al.* 2004, Oluwole *et al.* 2013), along with alterations in the serum levels of testosterone, progesterone and prolactin (Kimura & Furudate 1996, Maran *et al.* 2000).

Developmental hypothyroidism also affects spermatogenesis, by reducing the differentiation of spermatogonia (Kirby *et al.* 1996, Tousson *et al.* 2011), although this may result in increased sperm production in adulthood due to a larger reserve of undifferentiated germ cells (Auharek & de Franca 2010).

Insufficient neonatal levels of TH cause a reduction in the number of mesenchymal cells that differentiate into Leydig cell (LC) progenitors and ultimately into adult LCs (Rijntjes *et al.* 2009, Kobayashi *et al.* 2014). This effect is associated with decreased expression of steroidogenic genes including *Sf1* (*Nr5a1*), *Star*, *Cyp11a1* and *Hsd17b3* (Sarkar & Singh 2017*a*).

In summary, with some caveats, T3 signaling during testis development generally promotes the differentiation of germ and somatic cells, ultimately affecting steroidogenesis, spermatogenesis, testis size, testicular cell homeostasis and the hormonal physiology of the adult gonadal axis.

Determinants of TH action in the developing testis

A first determinant of circulating TH and T3 action in the developing testis is the HPT axis. Mammalian development is characterized by a steady increase in circulating levels of TH until the full maturation of the HPT axis. In rodents (and humans), TH levels in early fetal development are generally much lower than those in adults (Morreale de Escobar *et al.* 1990). This is due to the absence of thyroid gland function at that age and the effective clearance of maternal TH by DIO3, which is highly expressed in uterine decidual tissue, placenta and fetal tissues (Galton *et al.* 1999, Huang *et al.* 2003). Murine TH levels peak around 2–3 weeks after birth, with several factors accounting for the systemic increase in TH during mammalian development. These include: the reduction of DIO3 activity in most tissues as development progresses (Hernandez 2013), the start of thyroid function in late gestation (Ruiz de Oña *et al.* 1991), the neonatal maturation of the hypothalamic-pituitary thyroid axis (Dussault & Labrie 1975) and the developmental increase in DIO1 and DIO2 expression, mostly in liver and brain (Bates *et al.* 1999), which aid in the generation of T3 from circulating T4. During fetal and neonatal life, tissue-specific changes in the expression of TH transporters, receptors and mostly deiodinases are often responsible for timely and abrupt changes in local levels of TH action. These changes may markedly depart from TH levels in the circulation as they meet the specific T3 needs of particular developmental processes (Ng *et al.* 2004).

In the face of rising levels of TH in the serum, the developing testis expresses its own local determinants of TH action, most notably the transporter MCT8, the deiodinase DIO3 (Martinez *et al.* 2016*b*) and the receptor THRA (Jannini *et al.* 1990, 1994, Canale *et al.* 2001). Recently published comprehensive transcriptomic data across tissues, species and developmental ages (Fig. 1) (Cardoso-Moreira *et al.* 2019) indicate that the testicular expression of *Mct8*, *Dio3* and *Thra* mRNAs in mice and humans (and other species) is higher during development than in post-pubertal life (Cardoso-Moreira *et al.* 2019). Despite this general parallelism, the translation to humans of findings from rodent studies should be taken with caution given the marked difference between these species in the velocity of maturation of the testis and reproductive function, making models of human testis development difficult to establish (Hutka *et al.* 2017, Tharmalingam *et al.* 2018). In addition, these human datasets lack information on the second half of gestation and on the childhood years during which the human gonadal axis

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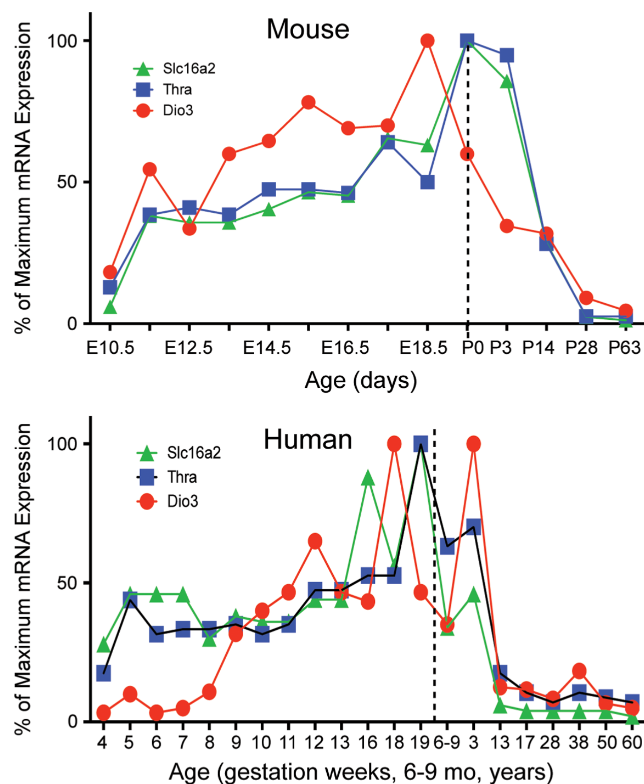

Figure 1
Testicular mRNA expression profile of key determinants of thyroid hormone action. Figures are built based on transcriptomic datasets in developing tissues (Cardoso-Moreira *et al*. 2019) (https://apps.kaessmannlab.org/evodevoapp/) and expressed as a percentage of the maximum expression found. Dotted line indicates birth. (Note that important aspects of testicular development may differ between mice and humans and that human data points available in this work skip the second half of gestation and pre-pubertal childhood years.)

remains relatively dormant. Overall, mouse data on the developmental testis transcriptomes are consistent with specific published data on the testicular expression of these genes at both the mRNA and protein levels (Tagami *et al*. 1990, Jannini *et al*. 1994, Martinez *et al*. 2016*b*). The marked decrease in the testicular expression of *Thra* and *Mct8* in adult life suggests that testicular sensitivity to TH at this age is much more limited than during development.

Although the THRB receptor can be detected in the testis and spermatocytes (Vanacker *et al*. 1998, Rao *et al*. 2003), its mRNA abundance is less than 5% that of THRA and its functional significance is yet to be determined. Similarly, absolute levels of testicular *Dio2* mRNA are very low, even though a sharp increase is noted in late neonatal life and adulthood (Fig. 1) (Bates *et al*. 1999, Cardoso-Moreira *et al*. 2019). The expression of the DIO1 in the testis is negligible.

In summary, the abundance of transporter MCT8 and receptor THRA suggests that the developing testis is highly sensitive to TH action (Fig. 1). On the other hand, the relatively high expression of *Dio3* indicates that TH action in the testis needs to be appropriately limited during perinatal life. Initial observations in transgenic mouse models provide some insight into the influence of these genes on testis development and function.

THRA and DIO3 deficiencies

One would predict that the consequences of a deficiency in THRA, the main TH receptor in the testis and responsible for mediating T3 action in this tissue, would be consistent with those in neonatal hypothyroidism. Indeed, global and SC-specific dominant negative THRA mutations in mice produce small testes and a extended period of SC proliferation (Fumel *et al*. 2012). Comparable results have been described in mouse models lacking a p43 isoform of THRA in the mitochondria or expressing a dominant negative THRA from the aromatase promoter (Fumel *et al*. 2013, 2015).

On the other hand, DIO3, which exhibits a marked developmental pattern of expression in most tissues (Hernandez 2005) including the testis, (Martinez *et al*. 2016*b*) (Fig. 1), would protect the immature testis from premature T3 action. Mice with global DIO3 deficiency (*Dio3-/-* mice) exhibit impaired TH clearance and subsequent serum thyrotoxicosis during fetal and neonatal life (Hernandez *et al*. 2006), resulting in a 75% reduction in testis size by adult age (Martinez *et al*. 2016*b*). This is consistent with observations in pharmacological models of neonatal thyrotoxicosis (Bakke *et al*. 1975, Holsberger *et al*. 2005*b*). However, in the latter models, the observed reduction in testis size is more modest despite achieving much higher levels of neonatal T3 in the circulation (Martin & Moberg 1981, Hernandez *et al*. 2006). This suggests that the active DIO3 present in those models is still able to provide substantial protection from T3 excess.

Also consistent with previously published observations in models of neonatal thyrotoxicosis, *Dio3-/-* mice exhibit proliferation arrest of SCs and premature lumen formation in seminiferous tubules (Martinez *et al*. 2016*b*). They further show impaired spermatogenesis, delayed puberty, poor fertility and hormonal abnormalities in the reproductive axis. The latter include reduced serum testosterone, elevated serum levels of FSH and LH and elevated mRNA expression of hypothalamic *Gnrh* and pituitary *Fshb* and *Lhb* (Martinez *et al*. 2016*b*). It remains to be determined to what extent the central abnormalities in the reproductive axis of *Dio3-/-* mice result from direct effects of T3 in the hypothalamus and pituitary or from abnormal testicular hormones feedback during development.

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Genes regulated by T3 in the neonatal testis The sensitivity of the developing testis to TH and the severe testicular and reproductive phenotype of *Dio3-/-* mice imply broad and profound underlying changes in gene expression patterns. Gene expression profiling of the postnatal day 5 *Dio3-/-* testis identified 5K+ genes with altered expression when compared to wild type littermates (Martinez *et al.* 2019). Genes showing increased expression in the neonatal *Dio3-/-* testis were enriched in biological and ontology terms associated with cell membrane function, while genes repressed by TH included cell cycle genes and most histone genes and were greatly enriched in terms associated with nuclear function, mitosis and chromatin structure (Martinez *et al.* 2019). These results are consistent with previous studies suggesting a general role of T3 in promoting testis cell differentiation and halting cell proliferation.

Although T3 generally induces the differentiation of most types of testicular cells, it is SCs that are most affected, as extensively showed in pharmacological models of neonatal thyrotoxicosis mentioned above. In the *Dio3-/-* testis, down-regulation of anti-mullerian hormone (*Amh*) and many mitosis-related genes and up-regulation of genes associated with the establishment of the blood-testis barrier such as Connexin 43, *Cldn11* and *Esyt3* (Sarkar & Singh 2017*b*, Martinez *et al.* 2019, Meroni *et al.* 2019) are consistent with proliferation arrest and subsequent differentiation of SCs.

Pathway analysis of differentially expressed genes in the neonatal *Dio3-/-* testis provides interesting mechanistic insight (Table 1). Pathways predicted as activated include those regulated by *Trp53* and *Cdkn1a* (also p21), androgen receptor, dexamethasone and progesterone. Of these, p21 and androgen signaling have been previously shown to induce the differentiation of SCs (Holsberger & Cooke 2005, Holsberger *et al.* 2005*a*, Meroni *et al.* 2019). Additional pathways predicted as activated include those regulated by lipopolysaccharide, interleukin 6, tumor necrosis factor and interferon gamma (Table 1), which are suggestive of inflammation. In contrast, pathways predicted to be inhibited in the neonatal *Dio3-/-* testis are mostly involved in cell proliferation (Table 1) including 17β-estradiol, which has been shown to enhance SC proliferation (Meroni *et al.* 2019).

One pathway predicted to be strongly upregulated is that of tretinoin, an agonist of the retinoic acid (RA) receptors. This is particularly interesting, as RA signaling not only promotes SC differentiation (Meroni *et al.* 2019), but also is known to be a main driver of spermatogenesis (Evans *et al.* 2014). Furthermore, the regulatory effects

Table 1 Regulated pathways in postnatal day 5 *Dio3-/-* testis.

Upstream regulator	Name/type of compound	Pathway prediction	Z-score	*P* value
AR	Androgen receptor	Activation	2.119	2.53E-36
IL6	Interleukin 6	Activation	3.747	5.03E-30
TNF	Tumor necrosis factor	Activation	4.32	7.74E-29
LPS	Lipopolysacharide	Activation	3.31	9.02E-29
DEX	Dexamethasone	Activation	3.378	2.88E-26
Progesterone	Progesterone	Activation	2.647	2.75E-24
TP53	Transformation related protein 53	Activation	3.664	1.21E-23
TGFB1	Transforming growth factor beta	Activation	2.706	2.16E-23
tretinoin	Retionic acid receptor agonist	Activation	6.059	7.25E-22
TPA	Phorbol ester	Activation	3.916	5.16E-19
CDKN1A	Cyclin-dependent Kinase inhibitor 1a	Activation	3.16	6.1E-19
CEBPB	CAAT-enhancer binding protein beta	Activation	2.329	1.64E-18
CTNNB1	beta-Catenin	Activation	2.263	2.04E-17
IFNG	Interferon gamma	Activation	3.8	4.25E-17
ERBB2	erb-b2 receptor tyrosine kinase 2	Inhibition	−4.661	1.34E-26
CSF2	Colony stimulating factor 2	Inhibition	−2.264	2.17E-24
PTGER2	Prostaglandin E receptor 2	Inhibition	−5.364	1.14E-23
RABL6	RAB, RAS oncogene family like 6	Inhibition	−5.303	1.47E-22
beta-Estradiol	17β-Estradiol	Inhibition	−2.11	2.88E-20
FOXM1	forkhead box M1	Inhibition	−3.949	4.38E-19
AREG	Amphiregulin	Inhibition	−3.524	1.08E-17
TBX2	T-box transcription factor 2	Inhibition	−5.282	8E-17
EP400	E1A binding protein P400	Inhibition	−4.021	1.12E-16
T3	L-triiodothyronine	Activation	3.132	1.53E-09

Top upstream regulators identified by Ingenuity Pathway Analysis with lowest *P* values and Z-scores >2 or <−2. Parameters obtained for the T3 pathway are shown below for reference.

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of DIO3 deficiency on a subset of genes specific to spermatogonia strongly correlate with those produced by RA administration (Evans *et al.* 2014, Martinez *et al.* 2019). This suggests that T3 may enhance RA regulation of spermatogenesis. This can be facilitated by the down-regulation of DIO3 by RA (Evans *et al.* 2014).

Although TH positively regulates steroidogenesis in the prepubertal testis (Sarkar & Singh 2017*a*), results from early neonatal *Dio3-/-* testis indicate a marked repression of *Hsd17b3* and *Hsd17b1* (Martinez *et al.* 2019), which are steroidogenic genes responsible for the last step in testosterone synthesis. However, other steroidogenic genes such as *Star* and *Cyp11a1* were up-regulated in *Dio3-/-* testis. It is possible that certain steroidogenic genes are not regulated in the same manner in the neonatal and pre-pubertal testis, possibly because fetal and adult LCs respond differently to T3. In this regard, markers of fetal LC progenitors (McClelland *et al.* 2015) were largely down-regulated in *Dio3-/-* neonatal testis (Martinez *et al.* 2019), suggesting a role for T3 in the differentiation of fetal LCs. The expression changes in steroidogenic genes may also reflect impairment in the maturation of adult LCs caused by T3. The ultimate impact on perinatal serum testosterone remains to be determined.

The overlap of T3 regulated genes in *Dio3-/-* testis and in the testis of newborn mice carrying a dominant negative *Thra* mutation in SCs (Chatonnet *et al.* 2014) is consistent, but also revealed a few genes apparently regulated in opposite directions by TH signaling (Martinez *et al.* 2019). This may reflect differences between models in the developmental windows of response or in the populations of cells that express those genes rather than a direct regulation.

Genetic rescue of the testis phenotype of DIO3 deficiency Diminishing TH action by genetic inactivation of other determinants of TH signaling ameliorates the severe testicular phenotype caused by global DIO3 deficiency. *Dio3-/-* mice lacking THRA exhibit normal size testis and normalized T3-dependent expression of testicular genes (Martinez *et al.* 2016*b*), further demonstrating that this receptor subtype mediates most of the detrimental testicular effects of T3 excess, as shown before in a pharmacological mouse model of neonatal thyrotoxicosis (Holsberger *et al.* 2005*b*).

The testis phenotype of the *Dio3-/-* mice was improved, but not fully normalized, by a global deficiency in the MCT8 transporter (Martinez *et al.* 2016*b*). The partial rescue of the phenotype included testis size and T3-dependent expression genes in the neonatal testis. Although *Mct8* mRNA is present in several cell types of the neonatal testis according to single cell RNA seq data (Evans *et al.* 2014, Green *et al.* 2018), the function of MCT8 in SCs probably accounts for most of the phenotypic rescue. Some adult parameters in testis, pituitary and hypothalamus of *Dio3-/-* mice were not improved by global MCT8 deficiency, suggesting additional, more complex effects in tissues of the reproductive axis.

Similarly, a global loss in DIO2 also improved to a partial extent the testis size phenotype of *Dio3-/-* mice, as well as their testicular neonatal expression of T3 regulated genes (Martinez *et al.* 2016*b*). Since transcriptome datasets and enzymatic activity data indicates that DIO2 is expressed at very low levels in the developing testis, it is likely that the observed phenotypic rescue derives from a reduction in systemic T3 availability due to lack of DIO2 function in other neonatal tissues.

DIO3 deficiency in germ cells The observations in *Dio3-/-* mice raise an important question about the cellular expression of *Dio3* in the testis. *Dio3* expression in SCs seems to be functionally irrelevant, as genetic inactivation of DIO3 in SCs does not cause any change in DIO3 activity in the neonatal testis (Martinez *et al.* 2019). However, *Dio3* inactivation in neonatal spermatogonia (using a mouse model with a floxed *Dio3* and a cre transgene regulated by the *Stra8* promoter) reduces testicular DIO3 activity by 90% (Martinez *et al.* 2019). This observation indicates that spermatogonia are the cell type predominantly expressing *Dio3* during testicular development and possibly in the adult testis. As the marked reduction in testicular *Dio3* expression in adulthood (Martinez *et al.* 2016*b*) is consistent with the much lower percentage that undifferentiated spermatogonia represent in the cell population of the adult testis. It is unknown whether this finding is translatable to humans, as no information is available about what testicular cell types in humans express DIO3 or other determinants of TH action. A recent published work describes single cell RNA-sequencing data on the human testis during puberty and other ages and could provide important insights in this regard, but the data could not be timely analyzed for the present review (Guo *et al.* 2020).

Consistent with the presence of DIO3 specifically in early stage spermatogonia, genes down-regulated in the testis of *Dio3-/-* neonates exhibit an enrichment in markers specific to stage III spermatogonia (Martinez *et al.* 2019). Treatment with retinoic acid in a synchronized model of neonatal spermatogenesis results in a marked decrease in *Dio3* expression (Evans *et al.* 2014), suggesting

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a decrease in *Dio3* expression at the initial stages of spermatogenesis. Furthermore, although expressed at lower levels in the adult testes, *Dio3* expression peaks at stages 2–7 of the seminiferous epithelium, when undifferentiated spermatogonia accumulate (Green *et al.* 2018). Finally, single cell RNA sequencing data also show germ cell *Dio3* expression peaking perinatally when the percentage of undifferentiated spermatogonia is highest in the testis (Law *et al.* 2019).

Neonatal spermatogonia-specific inactivation of *Dio3* is sufficient to cause local thyrotoxicosis in the testis, as indicated by reduced testis size at weaning age and expression changes in TH-dependent genes (Martinez *et al.* 2019). Importantly, this local excess of T3 affects the expression of genes specific to SCs and LCs, indicating that TH-dependent gene expression patterns in testicular somatic cells are influenced by spermatogonial *Dio3* (Fig. 2), with consequences for steroidogenic enzymes and SC differentiation and testis growth (Martinez *et al.* 2019). These observations reveal a cross-talk between testicular cells that involves the regulation of T3 action.

Intergenerational epigenetic effects of thyroid hormone

Unidentified etiology of complex conditions

A large component in the etiology of many complex human conditions remains undefined. Schizophrenia, autistic spectrum disorders, infertility, cardiovascular disease, metabolic syndrome and obesity are but a few examples of disorders that exhibit a relatively high heritability according to familial studies (Lee *et al.* 2011), but for which genetic factors identified in genome-wide association studies (GWAS) can only explain a minority of actual clinical cases.

This marked discrepancy between disease heritability and genetic variation initiated the quest to identify the origins for the 'missing heritability' of such conditions (Lee *et al.* 2011, Koch 2014*b*), which can be partly explained by the inheritance of altered epigenetic information. In this regard, increasing evidence is showing that paternal and maternal germ lines carry a complement of epigenetic information (the epigenome, in the broadest sense) that influences gene expression programs and development in the offspring, generating phenotypes of relevance to disease (Anway *et al.* 2005, Skinner 2007, Guerrero-Bosagna & Skinner 2012). Critically, the germ cell epigenome, including DNA methylation patterns (Waterland *et al.* 2007), microRNA population, chromatin signatures, concentration of metabolites and other small molecules (Rodgers *et al.* 2013, 2015), is susceptible to change as a result of environmental influences such as paternal diet, stress and glucocorticoid exposure, infection and inflammatory states and exposure to drugs or chemicals in the environment.

In this context, the extensive roles of TH in development and metabolism, its role in influencing the chromatin landscape and its targeting of the developing testis and spermatogonia all make a strong case for these hormones as an important influence on the germ line epigenome. These effects of TH are of clinical relevance, as abnormal TH states frequently occur in humans due to thyroid disease (McLachlan & Rapoport 2014) or to exposure to chemicals that disrupt TH physiology and signaling (Brent *et al.* 2007).

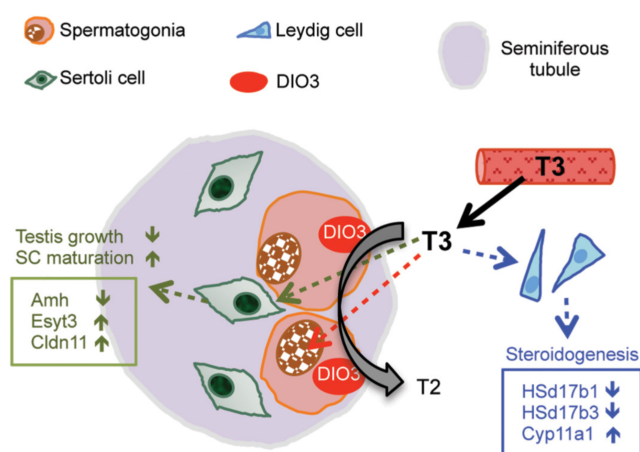

Figure 2
DIO3 role in the developing testis. By converting T3 into T2 (thick grey arrow), spermatogonial DIO3 modulates thyroid hormone action in different testicular cells (dotted arrows), influencing the expression of genes in neighboring cells involved in LC steroidogenesis (blue color) and SC proliferation and differentiation (green color) (Martinez *et al.* 2019).

Intergenerational epigenetic effects of thyroid hormones: seminal observations

As mentioned previously, the work of Bakke *et al.* using the rat 'neo-T4' model was one of the first to report a reduction in testis size as a result of developmental overexposure to TH (Bakke *et al.* 1975). Interestingly, using this animal model in two seminal articles published in the early seventies, Bakke and colleagues were also the first to our knowledge to report intergenerational epigenetic effects of altered TH states (Bakke *et al.* 1976). These investigators observed that the offspring of neo-T4 male rats (Fig. 3A) manifested delayed eye opening, enlarged thyroid gland

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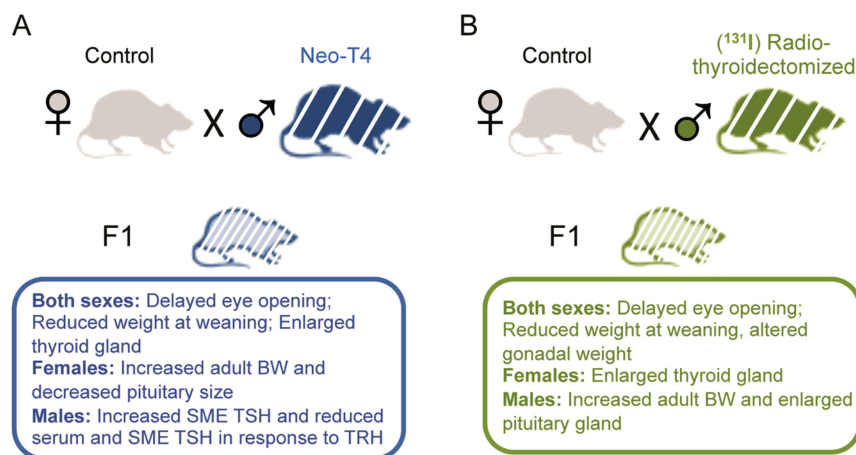

Figure 3
Phenotypes in the offspring of male rats with altered thyroid status. (A and B) Phenotypes in the offspring of male rats overexposed to thyroid hormone during development ('neo-T4' model, A) or made hypothyroid as adults (B). BW, body weight; TSH, thyroid stimulating hormone; TRH, thyrotropin releasing hormone; SME, stalk-median eminence (Bakke *et al*. 1976).

(goiter) and reduced weight at weaning. Other noted abnormalities were sexually dimorphic and included increased adult body weight and reduced pituitary size (females), increased stalk-median eminence thyroid stimulating hormone (SME-TSH) and reduced serum and SME TSH in response to stimulation by thyropin releasing hormone (TRH) (males) (Bakke *et al*. 1976).

Since neo-T4 animals experience thyrotoxicosis during neonatal life leading to moderate central hypothyroidism in adulthood, Bakke and colleagues also studied the offspring of male with severe, adult-onset hypothyroidism accomplished by radio-ablation of the thyroid gland (Fig. 3B). Both sexes of this offspring also exhibited delayed eye opening and reduced weight at weaning, but also alterations in gonadal weight (Bakke *et al*. 1976). Again, these mice also showed sexually dimorphic features, including goiter in females and increased adult body weight and pituitary size in males.

The authors could barely speculate about a potential mechanism to explain these observations at a time when the word 'epigenetic' was a rarity. However, their work is a critical precedent supporting the notion that altered TH status modifies the epigenetic information of the male germ line, affecting phenotypic traits in the offspring.

Intergenerational epigenetic effects of DIO3 deficiency

Intergenerational epigenetic effects have also been observed using a mouse model of DIO3 deficiency (Martinez *et al*. 2018) (Fig. 4). This is not surprising given the large phenotypic similarities and testis outcomes in neo-T4 rats and *Dio3-/-* mice.

Studies on wild type, second-generation descendants of *Dio3-/-* mice through the paternal line reveal alterations in neonatal patterns of gene expression in several brain regions, including the hypothalamus, striatum and hipocampus (Martinez *et al*. 2018) (Fig. 4). A disproportionate number of the down-regulated genes were specific to neurons, while a large proportion of up-regulated genes were specific to oligodendrocytes and myelination (Martinez *et al*. 2018). Since myelination and myelination-related genes are highly dependent on TH action and correlate with the neonatal increase in systemic and brain TH, intergenerational epigenetic marks controlling the maturation of the HPT axis or local T3 availability in the CNS may have been affected by overexposure to TH in *Dio3-/-* ancestors. It is also possible that these T3-regulated genes are epigenetically altered as a result of the ancestral exposure, but the modest overrepresentation of known T3-regulated genes among differentially expressed genes in the brain of descendants (Martinez *et al*. 2018) suggests that the effects observed cannot be only explained by epigenetic alterations in the brain T3 targetome.

The descendants' changes in neonatal brain gene expression were associated with abnormal behavior, including decreased anxiety-like behavior, reduced levels of physical activity and increased marble burying behavior, with no changes in depression-like behavior (Martinez *et al*. 2018). The first behavioral trait was also described in *Dio3-/-* mice, while the other three are opposite or inconsistent with the behavioral traits in *Dio3-/-* ancestors (Stohn *et al*. 2016). These observations indicate that the intergenerational epigenetic effects of TH overexposure may lead to a maintenance, correction or reversion of the original ancestors' neurobehavioral phenotypes.

These observations, as well as the previous ones by Bakke's group, suggest that the male germ line of *Dio3-/-* mice and neo-T4 rats is transmitting altered epigenetic information to their offspring and descendants. Consistent with this idea, neonatal germ cells of *Dio3-/-* male mice

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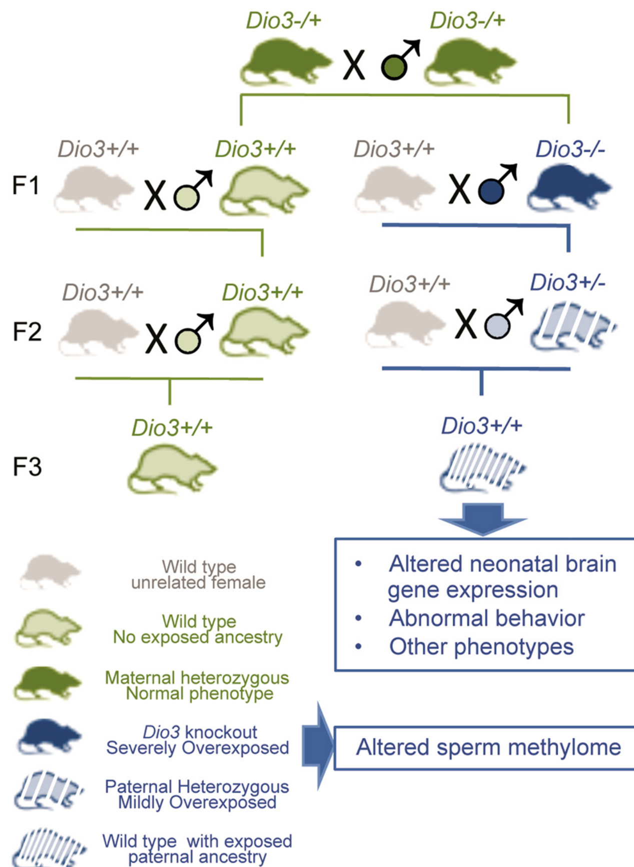

Figure 4
Intergenerational epigenetic effects in descendants of *Dio3-/-* mice. Male *Dio3-/-* mice showed alterations in the sperm methylome, and their F2 generation descendants in paternal lineage exhibit abnormal brain gene expression and behavior compared to F2 generation descendants of male *Dio3+/+* littermates (Martinez *et al.* 2018).

exhibited hypomethylation, and the methylation profile of the mature sperm of these mice was altered (Martinez *et al.* 2018). Interestingly, the *Dio3-/-* sperm methylome features a similar number of hypermethylated and hypomethylated CpG residues across the genome, but exhibits a marked bias toward hypomethylation in CpG islands associated with gene promoters (Martinez *et al.* 2018). The majority of genes associated with the most profound promoter hypomethylation are specific to brain tissue and implicated in brain development (Martinez *et al.* 2018) and may contribute to the behavioral abnormalities in descendants. However, for the most part, the hypomethylated genes in ancestral sperm are not the same as the abnormally expressed genes in the brain of descendant neonates. The absence of a major gene overlap in this regard suggests that the abnormal behavioral traits and brain gene expression patterns in *Dio3-/-* descendants could be secondary to abnormalities in earlier brain developmental processes and/or partially result from alterations in other molecular types of epigenetic information caused by TH overexposure in ancestors.

Although less studied, available evidence suggests that the female germ line is likewise susceptible to epigenetic changes as a result of TH overexposure. Wild type mice with a *Dio3-/-* paternal grandmother also exhibit changes in neonatal brain gene expression patterns, and these are strikingly similar to those observed in mice with a *Dio3-/-* paternal grandfather (Martinez *et al.* 2018). Furthermore, second generation descendants of neo-T4 female rats through the paternal line exhibit similar phenotypes as those in descendants of neo-T4 males (Bakke *et al.* 1977). These overlapping phenotypes in the descendants of both types of male and female T3-overexposed animals implicate comparable TH-driven epigenetic changes in both the male and female germ lines.

Transgenerational epigenetic inheritance in humans caused by thyroid hormone

In addition to rodents, evidence for a comparable role for TH status in transgenerational epigenetic effects has been described in humans. In this regard, Anselmo and colleagues have worked with individuals from different generations of a relatively isolated Azorean population carrying mutations in the *TRHB* gene (Anselmo *et al.* 2019). THRB is the receptor that specifically mediates the central negative feedback of TH on the HPT axis (Forrest *et al.* 1996).

Carrying one copy of this mutation is not sufficient to cause an abnormal TH status. However, due to the especial thyroid function needs during pregnancy, pregnant women carriers develop hyperthyroidism, which may result in excessive TH action in the fetus. Patients of either sex not carrying the mutation but exposed to an excess of TH *in utero* because of maternal THRB mutation exhibit as adults a HPT axis with decreased central sensitivity to TH (Fig. 5), as demonstrated by impaired serum TSH suppression by T3 treatment (Srichomkwun *et al.* 2017). This phenotype is also observed in a mouse model carrying the mutation (Srichomkwun *et al.* 2017). Interestingly, this physiological impairment is transmitted for two generations along the paternal lineage, but the transmission is halted at any female descendant in the ancestry tree (Anselmo *et al.* 2019) (Fig. 5).

This study suggests that the epigenetic information of the human male germ line is also affected by a developmental excess of TH, affecting the HPT axis

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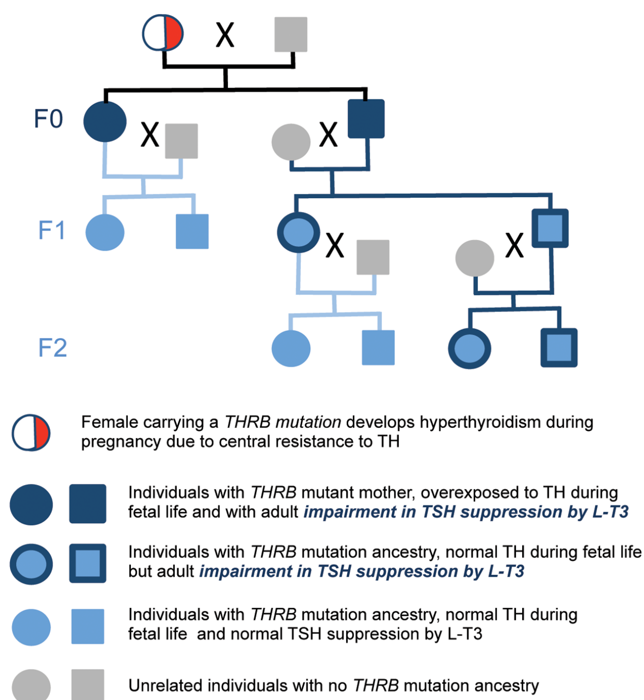

Figure 5
Epigenetic inheritance of TSH suppression in humans overexposed to thyroid hormones *in utero*. Genetically normal children of mothers that develop hyperthyroidism during pregnancy due to a *THRB* mutation exhibit impaired TSH suppression by T3 (F0 generation), and this phenotype is transmitted for two generations (F1 and F2) through the paternal line (darker lines), but not through the maternal line (light lines) (Anselmo *et al.* 2019).

of non-exposed descendants for two generations and demonstrating stable epigenetic inheritance (Anselmo *et al.* 2019). It is intriguing why the female germ line is not affected. Perhaps sex differences exist in the timing of germ cell maturation and susceptibility to TH. Alternatively, the maternal germ line may be altered in a different fashion, influencing descendants' phenotypes not yet identified.

The HPT axis phenotype observed in this human model of transgenerational inheritance (Anselmo *et al.* 2019) is comparable to the phenotype reported in the offspring of neo-T4 male rats (Bakke *et al.* 1975). In addition, unpublished results in our laboratory indicate an HPT phenotype in descendants of *Dio3-/-* mice. The consistency of HPT set point phenotypes in three different animal and human models significantly strengthen a case for the epigenetic inheritance of HPT phenotypes as a result of ancestral TH status. This may partly explain the inter-individual variation within the normal clinical range of serum parameters of HPT function (Browning *et al.* 1986).

Differences between mouse and humans in the physiological regulation and timing of biological processes affecting testicular and reproductive development may lead to inter-species differences in the susceptibility of the germ line to epigenetic alterations caused by abnormal levels of TH action. The current work of Anselmo and colleagues indicates that fetal life is one such period of susceptibility.

Mechanisms of intergenerational epigenetic effects driven by TH

Little is known about the molecular underpinnings of the intergenerational epigenetic effects of TH or other factors. Considering what is known about the canonical signaling pathway of T3 and its effects, and the presence of THRA in male germ cells (Evans *et al.* 2014), changes in T3 availability in undifferentiated spermatogonia may cause specific changes in the broad epigenetic signature of these cells. Presumably, these changes may be maintained or lead to additional changes in the mature sperm, with consequences for the pathophysiology of the next generation.

The sexual dimorphisms (Bakke *et al.* 1976) and parental lineage effects (Anselmo *et al.* 2019) observed in TH-driven intergenerational epigenetic suggest the implication of genes subject to genomic imprinting. This phenomenon involves the establishment of sex-specific germ line epigenetic marks, preferential or exclusive allelic expression and is considered highly susceptible to environmental factors (Jirtle & Skinner 2007). It is thus intriguing to consider that, as an imprinted gene in mouse and humans (Hernandez *et al.* 2002, Tsai *et al.* 2002, Martinez *et al.* 2016*a*), *Dio3* epigenetic alterations may be contributing to the intergenerational epigenetic effects of T3. Several findings support this hypothesis: *Dio3* is strongly up-regulated by T3 in multiple tissues (Barca-Mayo *et al.* 2011, Hernandez *et al.* 2012); *Dio3* is a critical determinant of the HPT axis set point (Hernandez *et al.* 2006, 2007), a phenotype involved in T3-driven intergenerational effects; and *Dio3* is specifically and highly present in the male germ line (Martinez *et al.* 2019). Interestingly, contrary to other tissues showing preferential expression from the paternal allele (Charalambous & Hernandez 2013), *Dio3* exhibits biallelic expression in the neonatal testis (Martinez *et al.* 2019) and, presumably, in spermatogonia.

The evidence currently available thus supports a direct effect of T3 on spermatogonia as likely responsible for its intergenerational epigenetic effects. However, these effects may be secondary to changes in other cellular and biochemical factors that are regulated by T3 systemically or in the testis. Thus, TH has broad effects on metabolism and altered metabolic states are known to cause intergenerational

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epigenetic effects (Zambrano *et al.* 2005, Godfrey *et al.* 2010, Nilsson & Skinner 2015, Skinner 2016). In addition, as reviewed here, T3 may also impact the physiology of somatic cells in the testis, notably LCs and SCs, which exert critical functions in supporting spermatogonia differentiation and could also influence their epigenetic information. Much work is needed to understand the molecular and cellular mechanisms underlying T3-driven germ line epigenetics, and to delineate the developmental gene expression programs and disease-relevant phenotypes that are affected as a result.

Conclusions

Appropriately timed, cell-specific TH action is essential for normal testis development and to regulate steroidogenesis, spermatogenesis and male fertility. Spermatogonial DIO3 is critically positioned to adequately modulate MCT8- and THRA-mediated T3 availability and action in somatic and germ cells of the developing testis, impacting their proliferation and differentiation and the physiology of the male reproductive axis. Abnormal TH levels during development, and possibly during adulthood, can alter the epigenetic information of the male germ line, with consequences for brain gene expression programs, behavior, development and the HPT axis set point of future generations. This may be relevant to the missing heritability of complex conditions in humans, as abnormalities in TH physiology may occur due to thyroid disease or exposure to endocrine disruptors.

Declaration of interest

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

Funding

This work was partly supported by grants DK095908, MH096050 and DE028732 from the National Institutes of Health.

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Received in final form 15 January 2020
Accepted 24 January 2020
Accepted Manuscript published online 24 January 2020

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