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Functions and mechanism of noncoding RNA in the somatic cells of the testis

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Review

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Summary

ncRNAs are involved in numerous biological processes by regulating gene expression and cell stability. Studies have shown that ncRNAs also contribute to spermatogenesis. Leydig cells (LCs) and Sertoli cells (SCs) are somatic cells of the testis that support spermatogenesis and are vital to male fertility. In this review, we summarized the findings from studies on ncRNAs in SCs and LCs. In SCs, ncRNAs play key roles in phagocytosis, immunoprotection and development of SCs. In LCs, ncRNAs are involved in steroidogenesis, in particular production of testosterone as well as development of LCs. Here, we discuss the possible target genes and functions of ncRNAs in both types of cells. These ncRNAs regulate the expression of target genes or mRNA coding sequence regions, resulting in a chain reaction that influences cell function. In addition, microRNAs, lncRNAs, piRNA-like RNAs (pilRNAs) and natural antisense transcripts (NATs) are discussed in this review. In summary, we suggest that these ncRNAs might act in coordination to control spermatogenesis and maintain the environmental homeostasis of the testis.

Introduction

Because of the work of the Encyclopedia of DNA Elements (ENCODE) Project Consortium, we have a comprehensive understanding of DNA sequences in the human genome (The ENCODE Project Consortium, 2004, 2012; Birney *et al.*, 2007). Eighty per cent of the genome is transcribed into RNA, while only a small proportion encodes proteins (The ENCODE Project Consortium, 2012). Noncoding RNAs, formerly called 'junk', have been proven to conduct 'pervasive transcription' and contain a great deal of functional regulatory elements (Ohno, 1972; Kapranov *et al.*, 2007; Neph *et al.*, 2012). According to the length of noncoding RNA, they are divided into long noncoding RNAs (lncRNAs; >200 nt) and small noncoding RNAs. Small noncoding RNAs include microRNAs (miRNAs), endogenous small interfering RNAs (endosiRNAs) and PIWI-interacting RNAs (piRNAs) (Lucas and Raikhel, 2013). Noncoding RNAs (ncRNAs) play important roles in gene expression and stability in processes from embryonic development to adult homeostasis (Geisler and Coller, 2013; Patil *et al.*, 2014). According to their functional features, ncRNAs are divided into housekeeping ncRNAs and regulatory ncRNAs. The regulatory ncRNAs comprise miRNAs, siRNAs, lncRNAs, piRNAs and intermediate ncRNAs such as small nucleolar RNAs. They are expressed in specific cells or a specific stage during cell development and differentiation or in response to environmental stimuli (Brosnan and Voinnet, 2009; Guan *et al.*, 2013). Increasing evidence shows that ncRNAs are involved in spermatogenesis and maintenance of male fertility in germ cells (de Mateo and Sassone-Corsi, 2014; Salviano-Silva *et al.*, 2018). Here we summarize the findings of studies on the noncoding RNAs involved in male fertility in somatic cells.

Sertoli cells

Niche cells, which are devoted to subtle coordination of the testicular microenvironment, are mostly composed of SCs. SCs, which are proximal to spermatogonial stem cells (SSCs), play auxiliary roles in spermatogenesis as 'mother' or 'nurse' cells for SSCs by supplying structural, immunological and nutritional support (Oatley and Brinster, 2012; Hai *et al.*, 2014). As supportive cells, SCs also produce a great number of growth factors and define the fate of SSCs, as stem cell factors (SCFs), bone morphogenetic proteins (BMPs) or glial cell line-derived neurotrophic factors (GDNFs) (Jan *et al.*, 2012; Hai *et al.*, 2014). Moreover, SCs are important constituents of the blood–testis barrier, which is an essential ultrastructure for male fertility (Setchell, 2008; McCabe *et al.*, 2016). The biological functions of SCs have been summarized as follows: they expand SSCs as feeder cells and activate SSC differentiation, phagocytosis and immunoprotection (Zhang *et al.*, 2007; Hai *et al.*, 2014). In 2013, a study using computer-assisted annotation of the small RNA transcriptome in murine SCs was completed (Ortogero *et al.*, 2013). Large numbers of studies have also shown that the disruption in the

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expression of noncoding RNAs in SCs might effect male fertility and these studies also made efforts to determine the functions of SCs in spermatogenesis and male fertility.

microRNAs in Sertoli cells

A microRNA is a small RNA of approximately 22 nucleotides in length (Kim, 2005). Growing evidence has shown that microRNAs regulate many biological processes by influencing post-transcriptional gene expression, such as mRNA degradation, translational repression, DNA methylation and chromatin modification (Krol *et al.*, 2010). MicroRNAs target specific mRNAs and stimulate the degeneration or inhibition of translated mRNAs (Tay *et al.*, 2008). Recently, some studies have shown that miRNAs are also involved in modulating many functions in SCs.

Apoptosis and phagocytosis

Phagocytosis is indispensable for the maintenance of tissue homeostasis. Sufficient evidence has shown that phagocytosis by SCs plays an essential role in the development and differentiation of germ cells. More than one-half of spermatogenic stem cells was cleared and degraded by SCs (Wang *et al.*, 2006). Several miRNAs have been reported to be involved in conventional phagocytosis or LC3-associated phagocytosis (LAP) or cell apoptosis (Jovanovic and Hengartner, 2006; Niu *et al.*, 2011). miR-471-5p regulates the level of Dock180, which interacts with autophagy-related proteins and make up LC3-dependent phagocytic complexes. It has been proven that SCs recruit autophagy-related proteins via LAP and that these proteins play crucial roles in the clearance of apoptotic germ cells. Overexpression of miR-471-5p in SCs from transgenic mice increased the number of apoptotic germ cells and damaged male fertility as miR-471-5p targets Dock180, LC3, Atg12, Rab5, Rubicon and Becn1 and represses their expression. This evidence suggested that SC phagocytosis and the clearance of apoptotic germ cells are regulated by miR-471-5p and its target proteins (Panneerdoss *et al.*, 2017).

miR-758 and miR-98-5p were predicted to be participants in germ cell apoptosis by binding to the 3′UTR of *mitogen-activated protein kinase 11* (*MAPK11*, *p38 β isoform*) gene. The expression of MAPK11 in SCs could induce the expression of tumour necrosis factor α (TNF-α), which interacts with TNF receptor 1 (TNFR1) and leads to germ cell apoptosis (Chen *et al.*, 2016). In the SCs of underfed sheep, the lack of let-7/miR-98 increased the expression of Fas mRNA and Fas protein and the presence of let-7/miR-98 reduced cell sensitivity to Fas-induced apoptosis (Wang *et al.*, 2011). Overexpressed miR-202-3p increased the number of apoptotic SCs and inhibited the proliferation and synthesis function of SCs by targeting LRP6 and cyclin D1 (Yang C *et al.*, 2019). In addition, miR-125a-3p, miR-872 and miR-24 possibly induced cell apoptosis by targeting SOD-1, a Cu/Zn superoxide dismutase in SCs (Papaioannou *et al.*, 2011).

Proliferation and development:

Dicer, an RNaseIII endonuclease, has been deemed crucial for miRNA production (Bernstein *et al.*, 2001). Studies have shown that it is highly correlated with maturation and survival of SCs, which suggests that the development, survival and function of SCs might be regulated by miRNAs related to Dicer (Papaioannou *et al.*, 2009, 2011). Dicer is critical to SCs as well as to spermatogenesis. The absence of Dicer leads to infertility. Several miRNAs (miR-299, miR-381, miR-409-5p, miR-376a and miR-674) in SCs were suppressed in Dicer ablated mice, indicating that these RNAs may take part in the development and function of SCs (Papaioannou *et al.*, 2009).

miR-34c has been proved to be involved in the development of male germ cells (Lian *et al.*, 2012). In fact, the development of seminiferous tubules also requires the participation of miR-34c. *Platelet-derived growth factor receptor alpha* (*PDGFRA*), which is mainly expressed in support cells such as SCs, has been identified as one target gene of miR-34c in swine. In addition, the expression level of *PDGFRA* in 2 days was much higher than it was in 5 months, indicating that *PDGFRA* may be involved in the early stages of SC development (Zhang X et al., 2015). miR-762 also played an important role in pig testis. miR-762 promoted immature SC proliferation and controlled apoptosis by targeting the 3′UTR of *ring finger protein 4* (*RNF4*), thereby decreasing AR protein expression and the transcriptional regulatory AR activity in SCs (Ma C *et al.*, 2016). In addition, miR-762 could also promote DNA damage repair in SCs (Ma C *et al.*, 2016). As mentioned above, miR-202-3p also inhibited the proliferation and synthesis functions of SCs (Yang C *et al.*, 2019).

Compared with those of patients with obstructive azoospermia (OA), the SCs of patients with Sertoli-cell-only syndrome (SCOS) expressed higher levels of miRNA-133b. Studies have shown that miR-133b promotes SC proliferation in humans by targeting GLI3 and activating cyclin B1 and cyclin D1 (Yao *et al.*, 2016). In addition, the level of miR-375 expression is negatively correlated with the mRNA levels of rearranged L-*myc* fusion (RLF) and hypoxia-induced gene domain protein 1A (HIGD1A) in pig SCs. MTS analysis showed that miR-375 might inhibit SC proliferation (Guo *et al.*, 2018).

Junction of the blood–testis barrier

The BTB is one of the most impermeable blood–tissue barriers in the living body, It is which was composed of tight junctions (TJs), gap junctions (GJs) and desmosome-like junctions and adherens junctions (AJs). The AJs include the basal tubulobulbar complex (basal TBC) and the basal ectoplasmic specialization (basal ES) (Wong and Cheng, 2005; Cheng and Mruk, 2012). The BTB separates the seminiferous epithelium into basal and apical parts. Spermatogenesis involves five orderly processes and the BTB provides a physical barrier that compartmentalizes three of these processes: the cell cycle that enables the spermatocyte transition from the zygotene to the diplotene stage, the procession of round spermatids to spermatozoa and spermiation. In addition, it enables these processes to progress in an immune-privileged environment, indicating that the BTB plays an important role in spermatogenesis and male fertility (Cheng and Mruk, 2012). In mice, considerable evidence has proven that the miR-17-92 cluster plays an important role in spermatogenesis (Bjork *et al.*, 2010; Tong *et al.*, 2012). miR-20a, a member of the miR-17–92 cluster, might be related to genistein (GEN)-induced abnormal spermatogenesis (Gu *et al.*, 2017). As the target gene of miR-20a, *Limk1* (Gu *et al.*, 2017), is involved in the RhoB/ROCK/LIMK1 pathway and regulates the adherens junction dynamics of Sertoli germ cells (Lui *et al.*, 2003).

Other findings about microRNAs in Sertoli cells

Circular RNAs (circRNAs) have loop structures and perform important functions in many biological processes (Rybak-Wolf *et al.*, 2015; Ebbesen *et al.*, 2016), including those of inflammatory reactions (Ng *et al.*, 2016). Evidence has shown that circRNA-9119 acts as a miRNA sponge and inhibits miR-136 and miR-26a expression. miR-136 and miR-26a inhibited the expression of retinoic acid inducible gene-I (RIG-I) and Toll-like receptor 3 (TLR3)

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by binding to the 3′UTR of RIG-I and TLR3 respectively in Sertoli cells and Leydig cells (LCs). By targeting TLR3 and RIG-I during orchitis in SCs and LCs, miR-136 and miR-26a modulate circRNA-9119-mediated inflammatory reactions, indicating that miRNAs and circRNAs are crucial to the immune microenvironment (Qin *et al.*, 2019).

miR-202-5p, regulated by the testis-determining factor SOX9, is a let-7 family member (Wainwright *et al.*, 2013). miR-202-5p is selectively expressed in SCs. The expression of miR-202-5p is related to testicular development and maturation (Dabaja *et al.*, 2015). However, no miR-202-5p expression was detected in SCs from SCOS patients. The distinct expression of levels of miR-202-5p in fertile and infertile men suggests that it might play crucial roles in normal male fertility (Dabaja *et al.*, 2015).

A study on the expression of miRNAs and their target genes in SCs after exposure to nonylphenol (NP) has been completed. The expression of 186 miRNAs is significantly distinct from that of the control group. In addition, it has been proven that miR-135a* can mediate the generation of reactive oxygen species (ROS) by regulating the Wnt/beta-catenin signalling pathway (Choi *et al.*, 2011). In 2018, a study showing the regulation of microRNA signalling by doxorubicin in LCs and SCs was also reported (Akinjo *et al.*, 2018).

Long noncoding RNAs

LncRNAs are longer than 200 nucleotides, with tissue- or cell-type specificity and without protein-coding capacity (Derrien *et al.*, 2012). Large numbers of lncRNAs have been identified, however few of these have been deeply explored (Ma L *et al.*, 2015; Reon *et al.*, 2016; Liu *et al.*, 2019). The main functions of lncRNAs were summarized as mediators of nuclear trafficking, altering splicing, changing mRNA stability and translation at both the transcriptional and post-transcriptional levels (Ayupe *et al.*, 2015). For post-transcriptional regulation, lncRNAs also organized protein complexes to influence cell signalling and regulate allosteric proteins (Geisler and Coller, 2013). The *Catsper1* gene, expressed in male germ cells, is essential for sperm motility and fertilization. Evidence suggests that the promoter of the *Catsper1 gene* can regulate a new gene, named *Catsper1au* (Catsper1 antisense upstream transcript). Analysis of the whole genome sequence revealed that Catsper1au has 1402 bp and is a polyadenylated lncRNA with no intron. It is found in the nucleus of SCs and germ cells of adult male mouse testis as well as in LCs, suggesting that it might have an effect on spermatogenesis and male fertility (Jimenez-Badillo *et al.*, 2017). Nevertheless, the detailed mechanism is still unclear and remains to be explored. Notably, many lncRNAs have been found in SCs, but their specific targets are also unknown (Yang *et al.*, 2018).

PiRNA-like RNAs

PIWI-interacting RNAs (piRNAs) are small noncoding RNAs that are exclusively expressed in the germ cells of mammalian gonads. They have been regarded for a long time as germ-cell-specific small RNAs. The piRNAs are categorized into repetitive sequence-derived piRNAs and non-repetitive sequence-derived piRNAs (Unhavaithaya *et al.*, 2009). The former silences transposons by DNA methylation and the latter is intergenic or intragenic and some have other non-repetitive regions with relatively unclear functions (Aravin *et al.*, 2007; Carmell *et al.*, 2007). However, in 2014, a novel class of somatic small RNAs, which were similar to piRNAs, were detected in somatic cells and named piRNA-like RNAs (pilRNAs). The pilRNAs have a distinct ping–pong signature and might target mRNAs 3′UTRs in a unique and complementary way (Ortogero *et al.*, 2014). These data provided one possibility that pilRNAs, whose functions are similar to those of germ cell piRNAs in SCs, might play a role in male fertility.

Leydig cells

Androgen greatly influences male health. Lack of testosterone can influence general health in males, such as by downregulating bone density, impairing muscle mass, injuring cognitive function and damaging immunity (Huhtaniemi, 2014). As males age, serum testosterone levels decrease gradually following an increased in serum follicle stimulating hormone (FSH) levels that either enhance or do not affect LH levels, indicating that the loss of testosterone results from changes in the ability of LCs to respond to LH. The loss is at the gonadal level rather than the hypothalamic–pituitary level (Wang *et al.*, 2017). Many chronic and age-related clinical symptoms are associated with a low level of androgen, including cardiovascular diseases, obesity and metabolic syndrome (Kupelian *et al.*, 2006; Saad and Gooren, 2009; Kloner *et al.*, 2016). Testosterone is mainly produced by LCs. It plays a critical role in maintaining secondary sexual characteristics and spermatogenesis regulation in adults (Matzkin *et al.*, 2013).

The LCs are divided into fetal LCs and postnatal LCs. The LH and hypothalamic–pituitary–gonadal axis regulates testosterone synthesis in adult interstitial LCs (Huhtaniemi I, 2015). However, fetal LCs produce androstenedione instead of testosterone directly without the presence of 17β-hydroxysteroid dehydrogenase-type 3 and androstenedione is converted into testosterone by fetal SCs (Shima *et al.*, 2013). Recent studies have shown that large numbers of ncRNAs are also expressed in LCs, Here, we summarized the findings of ncRNA function in LCs.

MicroRNAs

MicroRNAs related to LC steroidogenesis

Basic fibroblast growth factors (bFGF), including acidic and basic fibroblast growth factors, play diverse and specific roles in specific stages of LC steroidogenesis (Laslett *et al.*, 1997). It was reported that five miRNAs (miR-29a, miR-29c, miR-142-3p, miR-451 and miR-335) are regulated by both bFGF and LH and are involved in the regulation of androgen production in immature LCs (Liu *et al.*, 2014). miR-142-3p plays a vital role in cAMP production and PKA biological function to influence the cAMP/PKA signalling cascade, a secondary messenger pathway for steroid synthesis (Huang *et al.*, 2009; Manna *et al.*, 2014). Scavenger receptor class B type I (SR-BI), a HDL (high-density lipoprotein) receptor, is essential for the selective uptake of HDL CEs (cholesteryl esters) in steroidogenic cells (Shen *et al.*, 2018). The expression of SR-BI and the selective uptake of HDL CEs were inhibited after the transfection of pre-miRNA-125a and pre-miRNA-455 in LCs, implying that miRNA-125a and miRNA-455 also play roles in steroidogenesis. Evidence has shown that miRNA-125a and miRNA-455 can bind to the 3′UTR of the *SR-BI* gene and negatively regulate SR-BI functions in rat steroidogenic cells. The two miRNAs were sensitive to changes in trophic hormones (ACTH or gonadotropin) and cAMP (Hu *et al.*, 2012). In addition, treatment with Bt2cAMP increased the levels of miRNA-96, miRNA-132, miRNA-182 miRNA-183 and miRNA-212 and decreased the expression levels of miRNA-19a and miRNA-138 in MLTC-1 cells. All of these miRNAs can be found in the adrenal glands and are sensitive hormones similar to ACTH. In addition, miRNA-132 and miRNA-214 could inhibit

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the expression of SREBP-1c and LDLR by combining with the 3′UTR of SREBP-1c and LDLR respectively (Hu *et al.*, 2013).

MicroRNAs associated with Leydig cell development

miR-140-3p was the most highly expressed miRNA expressed in a sexually dimorphic pattern, while the expression level of miR-140-5p in the testis was low. Evidence showed that the absence of miR-140-5p/miR-140-3p increased the number of mouse LCs, indicating that miR-140-3p and miR-140-5p might be related to the development of gonad as well as testis differentiation in mice (Rakoczy *et al.*, 2013). As explained above, miR-136 and miR-26a played roles in the immune microenvironment, which is crucial to LC development (Qin *et al.*, 2019).

Other findings of microRNAs in Leydig cells

Currently, in the zearalenone (ZEN)-exposed TM3 LC line, the analysis of miRNAs expression has been completed. Approximately 197 miRNAs were found to be significantly distinct from those of the control group. The predicted target genes participate in many signalling pathways, suggesting that ZEN, an important environmental pollutant, is regulated by miRNAs in LCs.(Wang M *et al.*, 2019)

Long noncoding RNAs

In 2018, in total, 33,883 lncRNAs were identified from sheep testes. The sheep LCs with knocked out lncRNA TCONS_00863147 expressed lower levels of *PRKCD* (*protein kinase C,delta*), indicating that the *PRKCD* could interact with lncRNA TCONS_00863147 in a trans-activation mechanism and then played a role in spermatogenesis (Yang *et al.*, 2018). This study also revealed a large number of lncRNAs in the LCs, however their specific roles in male fertility remain unknown.

Tesra, a novel testis-specific lncRNA in mice, has been proved to be present in germ cells and the cytoplasm of LCs, as shown by *in situ* hybridization. *Tesra* activated *Prss42/Tessp-2* gene expression by binding to the *Prss42/Tessp-2* promoter and then enhancing promoter activity. *Prss42/Tessp-2* played important roles in the progression of meiosis as well as in germ cell survival. It was found that *Tesra*, similar to other lncRNAs, might recruit histone modification enzymes or transcription factors such as *GClnc1* to the *Prss42/Tessp-2* promoter region. However the specific mechanism of *Tesra* in LCs is still unknown (Satoh *et al.*, 2019).

Natural antisense transcripts

Natural antisense transcripts (NAT) are RNA sequences that complement a sense transcript and either encode a protein or do not encode a protein (Balbin *et al.*, 2015; Latge *et al.*, 2018). In fact, many NATs were mistakenly regarded as lncRNAs (Latge *et al.*, 2018). Similar to lncRNAs, NAT expression was regulated by promoters and enhancers. Notably, their sense genes or the neighbouring genes are closely connected to their expression levels (Lin *et al.*, 2015). Growing evidence implicates NATs as participants with a unique mechanism of action in gene expression (Pelechano and Steinmetz, 2013; Nishizawa *et al.*, 2015; Latge *et al.*, 2018).

Translocator protein (Tspo), with rate-limiting step activity in steroidogenesis in LC steroidogenesis, can transport cholesterol into mitochondria (Chung *et al.*, 2013). Evidence showed that the expression of the *Tspo gene* and its function in steroidogenesis were regulated by a NAT that was specific for *Tspo* (*Tspo*-NAT) in LCs (Fan and Papadopoulos, 2012). The extension of the SINE (short interspersed repetitive element) B2 element-mediated transcript formed *Tspo*-NAT in mouse tumour LCs. It has been proven that endogenous *Tspo*-NAT was more likely to suppress endogenous Tspo levels. In addition, the evidence also revealed that the expression of *Tspo*-NAT was regulated by cAMP and in this way maintained Tspo at a proper level for optimal LCs steroid production (Fan and Papadopoulos, 2012).

The steroidogenic acute regulatory (StAR) protein is a key protein that transports cholesterol located in mitochondria from outer membrane to the inner membrane (Manna *et al.*, 2013). *StAR* NAT was complementary to the spliced StAR sense 3.5-kb transcript and was highly expressed in LC and steroidogenic tissues. Evidence has shown that the *StAR* RNAs sense strands and the *StAR* RNAs antisense strands might be regulated in coordination as they were both expressed in the same cells. It has been proven that *StAR* NAT could downregulate the expression of StAR protein, as well as progesterone, by regulating cAMP (Castillo *et al.*, 2011). In this way, *StAR* RNAs play a role in regulating steroid biosynthesis.

Conclusion

With the development of large-scale genomic technologies and bioinformatics analyses, an increasing number of ncRNAs have been identified in SCs and LCs. Noncoding RNAs especially miRNAs including Dicer-dependent and Dicer-independent miRNAs in SCs play key roles in phagocytosis, immunoprotection and SCs development. These miRNAs are essential for the junction of BTB which maintains the testicular microenvironment for spermatogenesis LC ncRNAs are involved in steroidogenesis and the production of testosterone as well as development of LCs. Many miRNAs directly target genes involved in steroidogenesis and many of these are regulated by cAMP. Recently several novel lncRNAs such as *Tesra* have been identified. In addition, the discovery of NATs has provided another prospect for the regulation of gene expression. Compared with the vast number of ncRNAs in somatic cells, the numbers of current functional studies are exploring only the tip of the iceberg. In addition, the discovery of pilRNAs, which are similar to piRNAs and are present in somatic cells, open new horizons for researchers.

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