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Recent progress in understanding the mechanisms of Leydig cell differentiation

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ABSTRACT

Leydig cells in fetal and adult testes play pivotal roles in eliciting male characteristics by producing androgen. Although numerous studies of Leydig cells have been performed, the mechanisms for differentiation of the two cell types (fetal Leydig and adult Leydig cells), their developmental and functional relationship, and their differential characteristics remain largely unclear. Based on recent technical progress in genome-wide analysis and *in vitro* investigation, novel and fascinating observations concerning the issues above have been obtained. Focusing on fetal and adult Leydig cells, this review summarizes the recent progress that has advanced our understanding of the cells.

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1. Introduction

Mammalian gonadal sex is determined by the presence or absence of the *SRY* gene: *the sex determining region of the Y chromosome.* Once SRY is expressed in sexually indifferent gonadal somatic cells, they fix their cell fate to develop into Sertoli cells (Gubbay et al., 1990; Hawkins et al., 1992; Koopman et al., 1991; Lovell-Badge and Robertson, 1990). SRY triggers the expression of *SRY-box-containing gene 9* (*SOX9*), and SOX9-positive Sertoli cells then immediately surround the primordial germ cells to form a tubular structure called a testicular cord (Sekido et al., 2004; Wilhelm et al., 2007). As a consequence, the basic structure, consisting of two distinct compartments: inside the testicular cords and outside interstitial space, is formed in the fetal testis. While Sertoli and germ cells are localized inside testicular cords, a variety of cell types, such as Leydig cells, peritubular myoid cells, endothelial cells, macrophages, and uncharacterized interstitial cells, are localized in the interstitial space (reviewed in Martin, 2016; Potter and DeFalco, 2017; Svingen and Koopman, 2013).

In 1850, a German scientist, Franz Leydig, first described the cells residing in the interstitial space (Leydig, 1850). Thereafter, evidence supporting the notion that these cells were responsible for the synthesis of androgen accumulated gradually, resulting in recognition of their physiological role. For this historical reason, the androgen producing cells localized in the interstitial space are called 'Leydig cells'. Steroid hormones are divided into five classes in terms of their physiological functions: glucocorticoid, mineralocorticoid, androgen, estrogen, and progestin. Since these steroid hormones are mainly synthesized in functionally specialized steroidogenic cells in the adrenal gland, testis, and ovary, the genes implicated in steroidogenesis have been utilized as markers for steroidogenic cells.

Two types of Leydig cells, fetal and adult, develop in mammalian testes at the prenatal and postnatal stage, respectively (Roosen-Runge and Anderson, 1959). In mice, fetal Leydig cells appear in the interstitial space shortly after sex determination on embryonic day 12.5 (E12.5), and thereafter increase in number during fetal days (reviewed in Griswold and Behringer, 2009; Habert et al., 2001; O'Shaughnessy et al., 2006). Since fetal Leydig cells rarely proliferate (Miyabayashi et al., 2013; Orth, 1982), their increase in number is primarily due to their differentiation from progenitor cells (reviewed in Barsoum and Yao, 2010). Interestingly, it has been shown that non-steroidogenic (negative for fetal Leydig marker genes) interstitial cells contain the progenitor for fetal Leydig cells (Inoue et al., 2016).

Adult Leydig cells that emerge after birth play pivotal roles to establish and maintain male-specific secondary sexual characteristics through testosterone production. For studies of adult Leydig cell differentiation, a unique model using ethylene dimethane sulfonate (EDS) has been established. Interestingly, an EDS treatment completely depletes adult Leydig cells from rat testes (Kerr et al., 1985; Molenaar et al., 1985), and soon after the cells regenerate again (Jackson et al., 1986; Teerds et al., 1988). Studies exploiting this experimental model revealed the presence of stem/progenitor cells of adult Leydig cells among peritubular and perivascular cells contain (Davidoff et al., 2009; Ge et al., 2006) and implication of multiple factors in this differentiation process (reviewed in Teerds and Huhtaniemi, 2015; Ye et al., 2017).

A type VI intermediate filament protein, Nestin, has been a marker of stem cells localized in various tissues (reviewed in Wiese et al., 2004). As for Leydig cells in adults, Nestin-positive stem cells are present in the interstitial space of adult testis, and have the potential to differentiate into many cell types, including steroidogenic Leydig cells (Jiang et al., 2014).

Several studies have suggested that fetal Leydig cells are completely replaced by adult Leydig cells in postnatal testes (Hardy et al., 1989; Lording and De Kretser, 1972; Roosen-Runge and Anderson, 1959; Zirkin and Ewing, 1987; reviewed in Griswold and Behringer, 2009). Fetal Leydig cells contribute to the synthesis of androstenedione, which is thereafter converted into testosterone by Sertoli cells and thus masculinizes male fetuses, while adult Leydig cells are essential for secondary male specific sexual maturation through the production of testosterone. Recent cell-lineage tracing studies showed that fetal Leydig cells persist in postnatal mouse testes, together with adult Leydig cells (Kaftanovskaya et al., 2015; Shima et al., 2015).

Over the past two decades, the molecular mechanisms of fetal gonad development and sex differentiation have been primarily studied using genetically modified animals. These studies have identified one-by-one the genes necessary for these processes. As a consequence, many transcription factors and growth factors have been discovered, and our understanding of the involved molecular mechanisms has been advanced considerably (reviewed in Shima and Morohashi, 2017). However, we do not thoroughly understand all of the mechanisms yet. This is likely because we have not yet grasped the whole genes whose expressions are regulated by particular transcription factors and growth factors. A recently developed instrument, the next generation sequencer, has enabled genome-wide analyses and thus is a powerful tool contributing to the study of genes responsible for Leydig differentiation and functions (Inoue et al., 2016; McClelland et al., 2015; Miyabayashi et al., 2017).

In addition to newly established sequencing techniques, *in vitro* culture methods that enable the reproduction of Leydig cell differentiation are informative techniques. The *in vitro* culture to reproduce the differentiation of male germ cells has been employed extensively to respond to queries from medical and reproductive research fields, and as a consequence this technique has been successfully used to reproduce germ cell differentiation (Sato et al., 2011, 2013; Yokonishi et al., 2013). Unfortunately, *in vitro* research focusing on gonadal somatic cells has been lagging far behind compared with germ cell studies. However, interesting methods have been reported recently for the investigation of the somatic cells of fetal and adult testes (Inoue et al., 2016; Stanley et al., 2012). In this review, we will summarize the studies of Leydig cells that have utilized the recently developed novel techniques, and discuss the remaining issues to be resolved.

2. Genome-wide analyses of Leydig cells

In general, to comprehend the molecular mechanisms underlying the differentiation of certain cell types via the functions of the transcription factors of interest, it is essential to know all of the factor's target genes. New sequencing technologies enable us to obtain such genome-wide data sets. Although the methods have been fine-tuned, there are a few issues that should be identified. One of these issues is purity of the cells to be analyzed. In studies of testicular somatic cells, the somatic cell fraction is easily contaminated by the various stages of germ cells. Such contamination may result in detection of expression of germ cell specific genes in the transcriptomes of the somatic cells. As a consequence, further bioinformatic analyses using such data sets could be affected.

2.1. Preparation of Leydig cells

2.1.1. Transgenic lines whose somatic cells in the fetal testes are labeled with EGFP

Due to the reason outlined above, it is critical for genome-wide studies of certain tissues or organs that are comprised of multiple cell types to obtain pure fractions of the cells of interest. Many

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