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Kisspeptin/KISS1R 系統在小鼠睪丸發育過程中所扮演的角色

The Role of Kisspeptin/KISS1R System in Mice Testicular Development

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漆黑潮濕的空間讓人感到不自在。我正等待將再次現身的底片。時間慢慢消磨，內心的不安也漸漸膨脹。始終沒等到底片，以為出了大事。慌亂之中找到了學長，巧手一修，洗片機最終是安然無恙了。壓力釋放的瞬間，我在內心翻躍了好幾圈。像那次在暗房被內心恐懼吞噬後，因旁人拉了一把而使我可從黑暗中逃出一樣，在這四、五年的研究路上，每當徬徨無助時，總是能接受到及時又影響深遠的幫助，讓我重新振作去突破困境。如今，終於順利地走到完成論文的這一刻。

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中文摘要

近年研究指出，Kisspeptin 與其受器 KISS1R 在動物生殖生理的調控機制中扮演相當重要的角色。Kisspeptin 是由 *Kiss1* 基因轉譯而來的蛋白質產物，而 KISS1R 則是典型的穿膜蛋白。當 kisspeptin 與促性腺激素釋放素神經元 (GnRH neuron) 上的 KISS1R 結合時，便會刺激 GnRH neuron 興奮，進而調控下視丘-腦垂腺-性腺軸 (HPG axis)，促使性腺發育成熟並開始表現生殖能力。除了下視丘之外，先前研究也顯示人類和嚙齒類的睪丸組織皆有 *Kiss1* 和 *Kiss1r* 基因表現其中。然而，對於 kisseptin 和 KISS1R 在睪丸中的位置分佈和所執行的功能目前仍不是很清楚。

首先，為了獲得高專一性的抗體來偵測 KISS1R 在睪丸中的位置分佈，我們選用母雞作為抗體生產的宿主。成功獲得具專一性之抗體後，我們更進一步利用免疫組織染色法證實了，kisspeptin 主要表現在萊迪氏細胞中，而 KISS1R 則主要分布在精細胞細胞膜上。此外，透過即時定量聚合酶鏈鎖反應，我們也發現睪丸中的 *Kiss1* 和 *Kiss1r* 基因表現量隨著時間進展而有不同的變化。自小鼠出生後，睪丸中的 *Kiss1r* 基因表現量便維持恆定，然而，*Kiss1* 基因卻是從小鼠出生後第四周才開始大量表現。有鑑於 *Kiss1* 基因表現量有所轉變且其時間點與小鼠性成熟初始時間重疊，因此我們進一步比較 *Kiss1* 基因與其他睪丸發育相關基因之表現情形。有趣的是，*Insl3* (參與生精作用與睪丸沉降) 以及 *Cyp19a1* (負責雌二醇生成) 兩個基因表現型態皆與 *Kiss1* 基因相似，皆是從小鼠出生後第四周才開始被大量表現。另一基因，*Lhcgr*，雖同樣有漸趨上升的基因表現形態，卻比前述三個基因早約一周於睪丸中表現。此結果顯示，睪丸中的 kisspeptin/KISS1R 系統可能受到 LH 和其受器下游訊號所調控。為了證實這項假設，我們分別將初代萊迪氏細胞處理綿羊排卵素 (ovine luteinizing hormone, oLH) 以及將 MA-10 細胞株處理 LH 下游第二訊息傳遞因子，Br-cAMP。結果顯示，不論是 oLH 或 Br-cAMP 皆會增加萊迪氏細胞內的 *Kiss1* 基因表現量 ($p < 0.05$)。此外，在 Br-cAMP 及 RP-cAMPS (PKA 抑制劑)共處理之下，萊迪氏細胞內的 *Kiss1* 基因表現量明顯受到抑制 ($p < 0.05$)。

綜觀上述，小鼠萊迪式細胞中可觀察到 kisspeptin 的表現，且 *Kiss1* 基因表現量可透過排卵素的刺激及下游訊息傳遞而有所增加；反之，主要位於細精管內部的 *Kiss1r* 其基因表現量則不受排卵素調控。此外，由於發現可接受 kisspeptin 訊號的 KISS1R 表現位置以及 *kiss1* 基因表現時間點與生精作用相關之基因表現時間相似，故我們猜測 kisspeptin 可能會協同其他負責生精作用的相關因子一起調控生精作用的運作。然而，關於 kisspeptin 於睪丸中所扮有的實際角色仍須進行更深入的實驗才能證實。

關鍵詞：kisspeptin、KISS1R、排卵素、生精作用、睪丸發育

Abstract

Kisspeptin and KISS1R had been considered as essential regulators of animal reproductive functions in recent year. Kisspeptin, peptide encoded by the *Kiss1* gene, is synthesized in the discrete neurons of hypothalamus. When kisspeptin binds with its receptor, KISS1R, the following signals induce GnRH secretion to regulate the HPG axis. Interestingly, not only in the hypothalamus, previous data has already revealed the presence of *Kiss1* and *Kiss1r* mRNAs in the testis of human beings and rodents. However, the precise location and possible physiological role of kisspeptin/KISS1R system in testis remain unclear.

To further address the exact site of KISS1R in testis, we successfully produced an anti-KISS1R IgY antibody. Later, by conducting IHC assays, we detected that kisspeptin was expressed in Leydig cells, while KISS1R localized in seminiferous tubules, especially on spermatid membrane. Besides, real-time PCR results showed that mouse testis constantly expressed *Kiss1r* mRNA from birth but it didn't express *Kiss1* mRNA until postnatal fourth week. With comparing testicular gene expression patterns in the first 12 weeks after birth, we found the mRNA expression pattern of *Kiss1* was synchronized with *Insl3* and *Cyp19a1* which are genes important for testis descent and spermatogenesis, and estrogen synthesis. Moreover, *Lhcgr* (LH receptor encoding gene) expression was increased one week earlier than *Kiss1* expression, which indicated that the

Kisspeptin/KISS1R system in testis might be under control of LH and its receptor-mediated signaling such as cAMP pathway. To confirm this hypothesis, we treated primary Leydig cell with LH and MA-10 (Leydig cell line) with Br-cAMP, respectively. Indeed, these treatments obviously increased *Kiss1* mRNA expression in both two types of cells ($p < 0.05$). In addition, co-treatment of Br-cAMP and RP-cAMPS, a PKA inhibitor, significantly suppressed 50% Br-cAMP-induced *Kiss1* expression ($p < 0.05$).

From our present study, it is concluded that LH act as an upstream initiator to induce *Kiss1* expression in mouse Leydig cells through cAMP/PKA pathway. Since receptors for Kisspeptin are present on spermatids and the similar timing and location of gene expression between *Kiss1* and several genes regulating spermatogenesis, it is tempting to assume the synergistic action of Kisspeptin and development-related factors on spermatogenesis. However, the definitive role for Kisspeptin/KISS1R system in testicular development needs more studies to elucidate.

Keywords: kisspeptin, KISS1R, luteinizing hormone, spermatogenesis, testicular development

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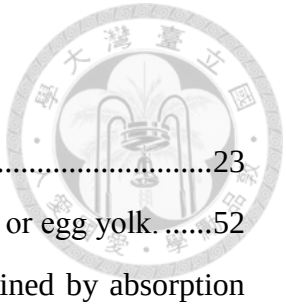

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1. Introduction

Reproduction is an indispensable function for perpetuating the species. Because reproduction counts for lasting a population and is under the control of a complex network of regulatory signals, studying the physiology and pathology of reproduction has been a primary work in the biomedical field all the time. As we have known that one of the important events controlling reproduction is the pubertal onset. Without the successful transition of puberty, the individual would remain under the immature condition and hardly produce mature gametes for fertilization. After longtime searching on the regulatory factors of pubertal onset, it is clear that the hypothalamic-pituitary-gonadal (HPG) axis is the central control of initiating the puberty by integrating signals in all direction (Ebling, 2005).

Recently, kisspeptin/KISS1R system had been discovered as the upstream regulator of HPG axis (Gottsch *et al.*, 2004) and its mechanisms of regulating gonadotropin releasing hormone (GnRH) release were constantly uncovered and reviewed in the past years (Colledge, 2009; Han *et al.*, 2005). Being inspired by the observation that *KISS1* and *KISS1R* genes are expressed not only in brain but also in a number of other tissues (Ohtaki *et al.*, 2001), scientists continuously found the local functions of kisspeptin/KISS1R system in the ovary, testis and other tissues. However, those direct functions, like ovulation or spermatogenesis in gonads, are not confirmed certainly

because there are insufficient and partly discrepant data presented (Pinilla *et al.*, 2012).

In our study, we are the first trying to produce an antibody against KISS1R in hens which have farther genetic distance to mice providing the antigen and we expect an improved antibody response in this system. Then, we used our own anti-KISSS1R antibody and commercial anti-kisspeptin antibody to detect the sites where KISS1R and kisspeptin are expressed in the testis. In addition, we also analyzed the pattern of *Kiss1* and *Kiss1r* expression during the testicular development and the connection between *Kiss1* gene and other development-related genes to find the possible role of kisspeptin/KISS1R system in the testis.

2. Literature Review

2.1 Male reproduction

Reproduction is the process to maintain one's species by the production of offspring. With the reproductive system, the individual can produce, nourish and transport the gamete (female oocyte or male sperm), then create a new individual following the union of two kinds of gametes. Though both two sexual reproductive systems are involved in the same physiological purpose, they are different in shape, structure and, needless to say, in function. Becoming a male, it requires the presence of testicular determining factor (TDF) to develop the male reproductive system (Berta *et al.*, 1990). TDF is controlled by the Y chromosome. When TDF is synthesized within the bipotential gonad, the development and differentiation of the testes and other organs within the male reproductive system is stimulated (Svingen and Koopman, 2013).

2.1.1 Testicular constitution

The testes are considered the primary reproductive organs in the male because they produce testosterone and spermatozoa. In addition, they produce other substances such as inhibin, estrogen and a variety of proteins believed to be important to spermatozoal function. There are other organs belong to the reproductive system, such as spermatic cord, epididymis, ductus deferens, prostate, seminal vesicles, penis and so on. All of them contribute to a successful fertile ability.

The testes occur as a pair of oval-shaped organs originally in abdomen. They descended into the scrotum late in gestation or when a male individual is going through the puberty, depending on the species. The testis is generally divided into two compartments: one is interstitium in which Leydig cells located; the other is seminiferous tubules consisting of Sertoli cells and germ cells. Although being anatomically separated, both compartments are closely connected with each other.

The interstitium consists of all cells and materials outside the seminiferous tubules such as blood vessels, connective tissue, lymphatics, nerves and Leydig cells. This compartment comprises about 2.6% of the total testicular volume in experimental animals. Nevertheless, the interstitium in the human testis resides about 12–15% of the total testicular volume, and 10–20% of which is occupied by Leydig cells (Nieschlag *et al.*, 2010).

Leydig cells clustering between the seminiferous tubules were first described in 1850 by German anatomist Franz von Leydig (1821–1908). However, scientists spent almost a century to identify an endocrine role for Leydig cells. A 3β-HSD (a critical dehydrogenase in the biosynthesis of all steroid hormones) histochemical localization in cells of the interstitial compartment was illustrated in the end of 1950s (Wattenberg, 1958). In 1965, Christensen and Mason further indicated that androgen biosynthesis is much more active in isolated interstitium than in isolated seminiferous tubules of the rat testis (Christensen

and Mason, 1965). With these two striking evidences, the fact that androgen production by Leydig cells had been fairly confirmed and accepted. Not only testosterone but also insulin-like factor 3 (INSL3) is the major secretory product of Leydig cells in the testis. Although INSL3 was discovered only recently, it have been known so far that INSL3 is essential for the process testicular descent (Nef and Parada, 1999) and is also involved in germ cell survival (Kawamura *et al.*, 2004). Due to the great relevance to testicular function, testosterone and INSL3 are considered the indispensable hormones for male reproduction.

According to discrete phases of testosterone secretion during the life cycle, separate populations of Leydig cells could be distinguished. In the mammalian, there are two different populations of Leydig cells existing in the testis at different periods, fetal Leydig cells (FLCs) and adult Leydig cells (ALCs). FLCs are terminally differentiated cells in the fetal testis. After birth, FLCs start to regress and ALCs are formed during pubertal development, processing through a series of Leydig cells lineages (precursor stem cells, progenitors, immature and mature adult Leydig cells) (Benton *et al.*, 1995). Both FLCs and ALCs express abundant 3β-HSD in their membranes of SER and secrete testosterone for male sexual differentiation in the fetus and secondary sexual maturation and fertility after birth.

The seminiferous tubules in which spermatogenesis takes place represents about 60–

80% of total testicular volume. It contains the germ cells and two types of somatic cells, the peritubular cells and the Sertoli cells. The Sertoli cell occupies about 37% of the epithelium in human and about 15-20% in the rodent species (Nieschlag *et al.*, 2010).

The peritubular cells form the contractile, basal membrane around the seminiferous tubules. Inside the tubules, the various germ cells arrange from marginal (basal) to central (adluminal) tubules as the spermatogenic process. The process of spermatogenesis can be subdivided into three phases. It begins with the proliferation of spermatogonia. Then, spermatogonia go through the meiosis to generate spermatocytes and spermatids. Finally, spermatids undergo a remarkable transformation that creates fully differentiated spermatozoa. Among the spermatogonia of the seminiferous tubules are the Sertoli cells, they support the structure of the germinal epithelium, convert testosterone to estradiol and secrete numerous factors to coordinate the spermatogenesis (Walker and Cheng, 2005).

2.1.2 Regulation of HPG axis

Puberty is the process of acquiring reproductive competence, namely the ability to accomplish reproduction successfully in a male or female. The onset of puberty occurs when the activity of HPG axis increases, and results in the production of gonadal steroids and other growth-associated hormones.

HPG axis is one of the central neuroendocrine system relying on the dynamic interaction of three endocrine glands, hypothalamus, pituitary and gonad (male testis or

female ovary). In male, HPG axis operate as a three-tiered functional hierarchy. The first level is that the gonadotropin-releasing hormone GnRH is secreted from the neurons which reside almost exclusively in the preoptic area and hypothalamus and terminate in the median eminence (Colledge, 2009; King and Anthony, 1984). Unlike a preovulatory surge of GnRH every few weeks in the female, the release of GnRH in the male occurs in frequent, intermittent bursts that appear throughout the day and night. Generally, these bursts of GnRH last for a few minutes. At the second level, released GnRH is transported to the anterior pituitary, where it can bind and activate GnRH receptors on gonadotropes, thereafter stimulating synthesis and secretion of the gonadotropins, lutenizing hormone (LH) and follicle-stimulating hormone (FSH). The episodes of LH occur between four to more than eight times every 24 hours and each time last from 10 to 20 minutes. In contrast to the pattern of LH secretion, the pulses of FSH are longer duration, but lower concentrations than that of LH. Then, the activation of LH and FSH on Leydig cells and Sertoli cells respectively is considered as the last level. Through the circulation system, LH induces steroidogenesis and testosterone secretion from Leydig cells for spermatogenesis and maturation of peripheral reproductive tissues, and FSH supports the development and spermatogenic activities of Sertoli cells. Leydig cells synthesize and secrete testosterone less than 30 minutes after an appearance of LH episode, and this response usually lasts for 20 to 60 minutes. Testosterone from Leydig cells and estradiol

and inhibin from Sertoli cells then negatively feedback to the hypothalamus and pituitary, resulting in a decrease in GnRH secretion (Knobil *et al.*, 2006).

2.1.3 GnRH pulse generators for the pubertal onset

In early studies, the pituitary, testes and ovaries of immature animals are all potentially functional as they become active when transplanted into adult animals or when stimulated by GnRH (Mason *et al.*, 1986; Wildt *et al.*, 1980). Moreover, other observations also revealed that a sustained increase in pulsatile GnRH released from GnRH neurons is the dominant signal required for heralding the puberty (Ebling, 2005). Therefore, neuroendocrinologists believed that the hypothalamus, rather than the pituitary or gonads, is the major site that launches the preliminary signal for the onset of puberty.

In past few decades, many strong cases have been built for afferent synaptic neurotransmitters, glial cell products, growth factors, and prostaglandins as contributing the cascade of signals that trigger the GnRH pulse in pubertal animals (Ojeda *et al.*, 2010).

Multiple components of these regulatory systems have been identified. Neurotransmitters, like glutamate and the peptide kisspeptin, are secreted from the specific neurons which compose a complex network and integrate neuronal excitatory or inhibitory afferents to GnRH neurons. For example, kisspeptin from Kiss1 neurons fires the GnRH neurons by intracellular calcium pathways (Oakley *et al.*, 2009). Kiss1 neurons also produce neurokinin B, a peptide recently implicated in the control of human puberty

(Topaloglu *et al.*, 2009), and dynorphin, an opioid peptide which inhibits GnRH secretion (Navarro *et al.*, 2009). Other inhibitory counterpart of this neuronal circuit for the GnRH pulse is provided by both GABAergic neurons and opiatergic neurons (Terasawa and Fernandez, 2001). In addition, cell-cell signalling molecules produced from glial cells stimulate GnRH release, and have been shown to be critical for the correct timing of the pubertal process (Ojeda *et al.*, 2008).

Not only the neuroendocrine substrates but certainly other factors such as body mass, leptin and environmental cues are important to regulate the beginning of puberty (Falconer, 1984; Fernandez-Fernandez *et al.*, 2006). Recently, novel regulatory elements, such as epigenetics and miRNA pathways are also considered in the control of the pubertal awakening (Lomniczi *et al.*, 2013).

Although there are multiple factors influencing the GnRH secretion, it is not much different that the timing of puberty within the same species. It is about 12.5 to 13 years for the woman and 14 years for the man. With regard to the rodents, the puberty in both female and male usually starts between postnatal fourth and sixth weeks (Falconer, 1984; Ojeda *et al.*, 1980).

2.1.4 Steroidogenesis

All steroid hormones are synthesized from cholesterol through a series of enzymatic pathways. This process of conversions is so called steroidogenesis and usually takes place

in adrenal glands and gonads. The end-product of steroidogenesis would be controlled by the signals given from the upstream tropic hormones.

The production of testosterone in testicular Leydig cells is primarily under the control of LH which is the tropic hormone released from the pituitary. In detail, LH binds with its corresponding receptor (luteinizing hormone/choriogonadotropin receptor, LHCGR) on the membrane surface. Then, the activated G-protein acts on a membrane bound adenylyl cyclase to increase the concentration of intracellular second messenger cAMP. cAMP thereafter activates a family of protein kinase, mostly protein kinase A (PKA), to induce the activity of enzymes and the transcription of genes which are necessary for steroidogenesis. No matter what the end-product is, steroidogenesis always begins with two key pathways: cholesterol in the cytoplasm is transported into the mitochondria via the steroidogenic acute regulatory protein (StAR) and is cleavage to pregnenolone by cholesterol side chain enzyme system (CYP11A1). After these two processes, pregnenolone is metabolized into various intermediates and active steroid hormones by other specific enzymes (Ghayee and Auchus, 2007).

When testosterone is finally secreted from Leydig cells, it would respond to spermatogenesis or be transferred into Sertoli cells for aromatase (CYP19A1)–induced estradiol production. Combining testosterone, estradiol and other proteins, the testis could complete the circuit of reproductive regulation.

2.1.5 Spermatogenesis

While steroid hormones are secreted from the interstitium, the mature and haploid spermatozoa essential for the fertility are produced within the seminiferous tubules with the stimulation of diffused testosterone from the interstitium. The developmental process during which spermatogonia enter the differentiation pathway and ultimately create spermatozoa usually taking about 75 days in man and 35 days in mice is defined as spermatogenesis (Hess and de Franca, 2008).

Spermatogenesis is generally divided into three phases. The first phase, namely the proliferation or mitotic phase, is composed of all mitotic divisions of spermatogonia. The division of spermatogonia gives rise to either the production of two new stem cells for keeping the stem cell pool or undifferentiated spermatogonia that are destined to develop into sperm. The second phase is termed the meiotic phase which involves primary and secondary spermatocytes. During this phase, spermatocytes continue to mature as they go through the sequential phases of meiosis. At the end of meiotic phase, haploid spermatids are formed by the second (final) meiotic division. Subsequently, the spermatids head to the differentiation phase. During the final phase, spermatids undergo spermiogenesis that they are compacted and elongated into the mature spermatozoa containing a head (nuclear materials) and a flagellum involving mitochondria (Jan *et al.*, 2012). Under the viewing of cross-section of a seminiferous tubule, these germ cells move from the basement

membrane toward the lumen of a seminiferous tubule as they proliferate and mature.

Testosterone is the major androgen in testis that regulates spermatogenesis. With androgen receptors in the nucleus and cytoplasm, testosterone can initiate the functional responses required to support spermatogenesis in both Sertoli cells and peritubular myoid cells. It is clear that Sertoli cells create a micro-environment that enables the sustained generation of spermatozoa and peritubular myoid cells provide growth factors and assist the movement of fluid and sperm through the tubule lumen (Smith and Walker, 2014).

2.2 Kisspeptin/KISS1R system

KISS1 gene was discovered in 1996 when scientists try to find the gene(s) responsible for metastasis suppression. By conducting subtractive hybridization in melanoma cell lines with different metastatic capacity, Lee *et al.* (1996) cloned *KISS1* mRNA and identified the gene as a selectively overexpressed transcript in tumor cells with low invasiveness.

Later, in 1999, another Lee's group (Lee *et al.*, 1999) found the gene coding for an orphan receptor, GPR54, which shares a significant sequence identity with the gene coding for galanin receptor. Although these two receptors have up to 40% similar amino acids in the transmembrane regions, there is no specific binding observed between GPR54 and galanin which is a ligand of galanin receptor.

It was not until 2001 that the high binding affinity between KISS1 and GPR54 (later

designated KISS1R) was described by three independent groups and then KISS1R was identified as a receptor for KISS1-derived peptides (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001). With the studies providing complete characterization of the peptide products of the *KISS1* gene, the term kisspeptins displaced the initial terminology of metastin. Recently, following the discovery of being metastasis suppressor, kisspeptin and KISS1R were also attributed to regulate the reproduction. Table 1 shows the major functions of kisspeptin/KISS1R system.

2.2.1 Elements

Kisspeptins represents a family of related products which are encoded by the *KISS1* gene. Since the prepro-kisspeptin was generated from the gene, this 145-amino acid precursor with a putative 19-amino acid signal sequence usually undergoes the differential proteolytic processing to form the biologically active peptides with different lengths of fragment (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001). These proteolytic peptides were collectively named kisspeptins.

Among kisspeptins, kisspeptin-54 (kp-54) is the major product of the *KISS1* gene and is originally named metastin for its ability to inhibit tumor metastasis. Kp-14, kp-13 and kp-10 are the other peptides fragments from proteolysis of kisspeptin precursor (Ohtaki *et al.*, 2001). These shorter proteolytic kisspeptins share the same COOH-terminal 10-amino acid stretch of kp-54. This stretch is designated as kp-10 and contains

Table 1. The major functions of kisspeptin/KISS1R system.

Category	Function	Model	Brief description	Reference
Reproduction	Puberty onset	Human	First study discovered the connection between kisspeptin/KISS1R system to iHH (idiopathic hypogonadotropic hypogonadism)	de Roux *et al.* (2003)
		Rat	Infusion of kisspeptin antagonist delays the puberty onset.	Pineda *et al.* (2010)
		Rhesus monkeys	Infusion of Kp-10 into the hypothalamus increases the GnRH secretion	Guerriero *et al.* (2012)
	Spermatogenesis	Mouse	Impaired spermatogenesis in KISS1R and kisspeptin knockout mice	Mei *et al.* (2011)
		Human	Expressions and functions of kisspeptin and KISS1R in human spermatozoa.	Pinto *et al.* (2012)
		Mouse	kisspeptin and KISS1R might be involved in the fertilization process (capacitation).	Hsu *et al.* (2014)
	Ovulation	Rat	Kisspeptin's location and time of expression in ovary.	Castellano *et al.* (2006)
		Siberian hamster	Kisspeptin and seasonal breeding	Shahed and Young (2009)
		Human	Kisspeptin's location and time of expression in ovary.	Gaytan *et al.* (2009)
		Marmoset Rat	Kisspeptin expression is blocked by inhibition of prostaglandin synthesis.	
		Musk Shrew (Suncus murinus)	Effect on reflex ovulation	Inoue *et al.* (2011)
		Mouse	*Kiss1r* haplo-insufficiency causes premature ovarian failure	Gaytan *et al.* (2014)
Cell migration	Metastasis suppressor	Melanoma	First study identified *Kiss1* gene which is responsible for metastasis suppression	Lee *et al.* (1996)
		Human tumor tissues and cell lines	Metastin can suppress tumor metastasis	Ohtaki *et al.* (2001)
		HT-1080 cell line	Kisspeptin suppresses cell invasiveness with modulation of metalloproteinase.	Yan *et al.* (2001)
		Renal cell carcinoma cell	Kisspeptin suppresses cell invasiveness with modulation of metalloproteinase.	Yoshioka *et al.* (2008)
	Trophoblast invasion	Trophoblast cells	Kp-10 inhibits trophoblast migration and invasion.	Bilban *et al.* (2004)

an Arg-Phe-NH_2 motif characteristic of the RF-amide peptide family composed of a variety of neuroactive peptides.

It has been known that kp-10 with C-terminal amidation is the minimal sequence required for full receptor binding and activation of KISS1R signaling pathways (Kotani *et al.*, 2001; Roseweir *et al.*, 2009). So far, it has not defined which the primary forms of kisspeptins are produced in different tissues, though scientists has been detected abundant kp-54 in placenta extracts (Ohtaki *et al.*, 2001). With regard to the rodents, the largest product of kisspeptins is composed of only 52 amino acids and the terminal RF-amide sequence is replaced by Arg-Tyr-NH_2 motif (Terao *et al.*, 2004).

Through the binding and functional assays, kisspeptins were identified as the specific ligand of KISS1R which belongs to the rhodopsin family of G protein-coupled receptor (Muir *et al.*, 2001; Ohtaki *et al.*, 2001). KISS1R has a typical seven transmembrane domain and pairs with $G_{q/11}$ protein, whose activation increases intracellular calcium concentration instead of cAMP levels associated with G_s and/or $G_{i/o}$ proteins (Kotani *et al.*, 2001; Neves *et al.*, 2002). Thereafter, with several broad searches on its distribution, KISS1R was found to be expressed in a variety of tissues, including in the central nervous system, gut, pancreas, testes and placenta (Harms *et al.*, 2003; Kotani *et al.*, 2001). In terms of the identity of amino acid level, mouse and rat KISS1R are nearly 95% identical to each other, and both are approximately 85% identical to human KISS1R

(Clements *et al.*, 2001).

2.2.2 Kisspeptin/KISS1R system in reproduction

Although kisspeptin/KISS1R system faced us with its function on suppressing cell invasion in the beginning, it didn't take a long time that scientists indicated an important role of the system on the control of puberty after they discovered the combination between kisspeptins and KISS1R in 2001.

In 2003, de Roux *et al.* (2003) and Seminara *et al.* (2003) firstly observed that genetic inactivation of KISS1R led to impaired puberty progression and fail sexual maturation in humans and mice. Later, a similar phenotype of defective reproductive capacity was also described in mice engineered to lack functional *kiss1* gene (d'Anglemont de Tassigny *et al.*, 2007; Lapatto *et al.*, 2007). Also, the phenomenon of precocious puberty was observed in humans with activating mutations of *KISS1* (Silveira *et al.*, 2010) or *KISS1R* (Teles *et al.*, 2008) and it is probably because a greater stability of Kp-54 or decreased desensitization of KISS1R caused by the missense mutations leading to amino-acid substitutions. In addition to the above investigations on gene deficiency, studies in female rats (Pineda *et al.*, 2010) and monkeys (Guerriero *et al.*, 2012) also demonstrated that central infusion of an effective kisspeptin antagonist, peptide 234 (p234), results in delayed onset of puberty. Based on those multifaceted evidences, it is significantly confirmed that kisspeptin/KISS1R system administrate the

key role on the pubertal onset and reproductive capacity (Pinilla *et al.*, 2012).

Because these above data demonstrated the decrease concentration of gonadotropins (FSH and LH) by abnormality of kisspeptins or/and its receptor in patients and experimental animal models, scientists suggested that there might be a physiological relevance of kisspeptin/KISS1R system in the regulation of HPG axis and a variety of studies continuously documented the ability of kisspeptins to potently elicit gonadotropin secretion. Being inspired by the hypothesis that kisspeptin/KISS1R system governing the HPG axis, a number of groups evaluated the ability of kisspeptin to elicit LH release in various species, such as rat (Gutierrez-Pascual *et al.*, 2007), bovine (Ezzat *et al.*, 2010) and porcine (Suzuki *et al.*, 2008), and then suggested a direct stimulation of pituitary LH secretion by kisspeptin.

However, this affirmation of direct kisspeptin action at the pituitary level was later denied by the study on *hpg* mice which is severely deficient in GnRH secretion and unable to respond to kisspeptin stimulation for LH secretion (Gill *et al.*, 2010). In other words, kisspeptin-induced LH release must be arranged by hypothalamic GnRH. Besides, several expression data from immunofluorescence indicated that there is a direct connection between Kisspeptin neurons and GnRH neurons in mouse hypothalamus, majorly in the medial preoptic area (POA) region (Clarkson and Herbison, 2006; Kinoshita *et al.*, 2005). Although kisspeptin neurons are mostly expressed in the anteroventral periventricular

nucleus (AVPV) region and the arcuate (ARC) nucleus of the hypothalamus, it seems like that kisspeptin fibres could extend into POA and median eminence (ME) region to give the signal to GnRH neurons (Clarkson *et al.*, 2008; Herbison, 2008). Not only expression studies but electrophysiological experiments within GnRH neuron also supported the physiologic relevance of kisspeptin signaling in the control of gonadotropin secretion (Han *et al.*, 2005; Zhang *et al.*, 2007). Therefore, those further proofs have substantiated that the dominant site of action of kisspeptins in the regulation of the gonadotropic axis is located at hypothalamic GnRH neurons (Roa *et al.*, 2009).

All in all, the above set of data have solidly confirmed that initiating the pubertal onset by increasing GnRH secretion is one of the physiological role of kisspeptin/KISS1R system in reproduction.

In spite of abundant data focusing on functions of cerebral kisspeptins, compelling evidences obtained in different species implies additional regulatory effects of kisspeptins at other levels of the reproductive system. Since 2001, it has been known that *Kiss1* and *Kiss1r* gene are expressed in variant tissues (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001). Because the presence of *Kiss1* and *Kiss1r* gene is also discovered in the testis and the ovary, peripheral or local actions of kisspeptin and KISS1R in the gonad might be considered as responsible for cooperating individual's reproduction with the central control.

In terms of the ovary, Castellano *et al.* (2006) demonstrated that ovarian KiSS-1 levels increase during the pubertal transition and fluctuated in a cyclic-dependent manner with a peak at the proestrus, i.e. preceding ovulation while Gpr54 mRNA levels remained rather low and stable across the ovarian cycle. Besides, by immunohistochemistry, both kisspeptin-IR and KISS1R-IR were observed in theca layers of growing follicles, corpora lutea, and interstitial gland. They also found that gonadotropin priming enhanced levels of *Kiss1* mRNA which is previously negligible in immature ovaries. Gaytan *et al.* (2009) further confirmed the observations of kisspeptin and KISS1R expression in the ovary of human and monkey and also suggested that gonadotropin-induced ovarian *Kiss1* mRNA levels was inhibited by the inhibition of prostaglandin synthesis. Nevertheless, human with impaired KISS1R signaling can still ovulate if properly primed with gonadotropins (Pallais *et al.*, 2006) while ovulation linked to the absence of KISS1R cannot be fully rescued by gonadotropin replacement in mouse (Gaytan *et al.*, 2014). Because of those inconsistent observations, the indispensability of locally produced Kisspeptins in the control of ovulation is still doubtful so far.

It contrast to the ovary, there is much less data on the potential local expression or actions of kisspeptins in the testis. Based on the observations of established *Kiss1*$^{-/-}$ and *Kiss1r*$^{-/-}$ mutant mouse model (d'Anglemont de Tassigny *et al.*, 2007; Kauffman *et al.*, 2007b; Mei *et al.*, 2011; Seminara *et al.*, 2003), scientists noted that mutant male mice of

either genotype have failed reproductive capability with lower testicular weights and reduced serum testosterone levels compared to wild-type. In addition, spermatogenesis is severely impaired with spermatogenic arrest at the meiotic-division stage (primary spermatocytes were expressed but few haploid spermatids were shown), resulting in the absence of spermatozoa in the seminiferous tubules and epididymides. However, there are some mutant mice presenting complete spermatogenesis and producing few spermatozoa though they also have those abnormal phenotypes of male reproductive system (Lapatto *et al.*, 2007).

Recently, two studies in human and mouse spermatozoa sequentially demonstrated the specific sites where kisspeptins and KISS1R exist in and the regulation of kisspeptin/KISS1R system on the fertilization ability of sperm. Pinto *et al.* (2012) first indicated both Kisspeptin IR and KISS1R IR were presented in post-acrosomal region of the mature sperm head and also localized around the sperm neck while Kisspeptin IR is particularly intense in the equatorial segment. Moreover, this group also investigated calcium concentration in sperms and calcium-regulated sperm function, like motility, hyperactivation and the acrosome reaction under the addition of kisspeptin. According to the data, they suggested that kisspeptin, binding with KISS1R, could induce intracellular calcium-signal transduction to modulate sperm moltility and hyperactivation but not acrosome reaction.

Later, our previous data further showed extensive data related to expression profiles and functions of kisspeptin and KISS1R in the mouse reproductive tissues (Hsu *et al.*, 2014). In contrast to the immunodetective results from described data (Pinto *et al.*, 2012), we found that only KISS1R is expressed in mouse mature spermatozoa, specifically on the acrosome, and kisspeptin is localized in the interstitial testicular compartment and epididymis instead. While focusing on female reproductive tissues, Kisspeptin but no KISS1R immunoreactivity was detected specifically in follicles, corpora lutea, and interstitial glands, the results which are consistent with previous demonstration (Castellano *et al.*, 2006). In the test of fertilization capacity, we also confirmed the regulation of kisspeptin on sperm capacitation, though acrosome reaction is not include, by treating noncapacitated and capacitated sperm with p234. Combining those line of data, we suggested that kisspeptin coming from the epididymis and oviduct regulates the fertilization capacities of spermatozoa during capacitation in reproductive tract.

Overall, it seems like that kisspeptin/KISS1R system acts not only on central neuronendocrine system but also on gonad locally to integrate the reproduction and fertility.

2.2.3 Kisspeptin signaling via KISS1R

Since the fact that kisspeptins constitute natural agonists of KISS1R was found in 2001, the functions and signaling pathways regulated by kisspeptin/KISS1R system has

been investigated widely in several cell models, such as CHO-K1, HEK203, and B16-BL6. At first scientists found an increasing calcium concentration in KISS1R-transfected cells after cells were treated with kisspeptin, but these increase didn't disappear under the treatment of pertussis toxin which blocks the activity of G_i protein. More, intracellular cAMP were not modulated by Kisspeptin in cells transiently expressing KISS1R. Those data suggested that KISS1R is a G_q-coupled receptor without the connection to G_s and/or G_i proteins. Also, PLC–PKC and MAPK pathways which often activated by G protein-coupled receptors were also disclosed by using specific inhibitors in kisspeptin-treated cells (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001).

Because the function on GnRH secretion was discovered later, many researches used GnRH neurons as the model and revealed that intracellular calcium increased by kisspeptin via KISS1R also regulated multiple ion channels [such as potassium channel, transient receptor potential cation (TRPC) channel and so on] to depolarized the GnRH neuron and induce GnRH release (Liu *et al.*, 2008; Zhang *et al.*, 2008).

In summary, the binding of kisspeptin and KISS1R activates phospholipase C (PLC), then subsequently converts phosphatidylinositol bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3), which induces the mobilization of Ca^{2+} from intracellular reservoirs. The increase of intracellular Ca^{2+} may results in phosphorylation of MAPK by diacylglycerol (DAG) and protein kinase C (PKC) activation to regulate many cell

functions (like anti-metastasis and proliferation) or changes in ion channel permeability thus causing depolarization responses (Fig. 1).

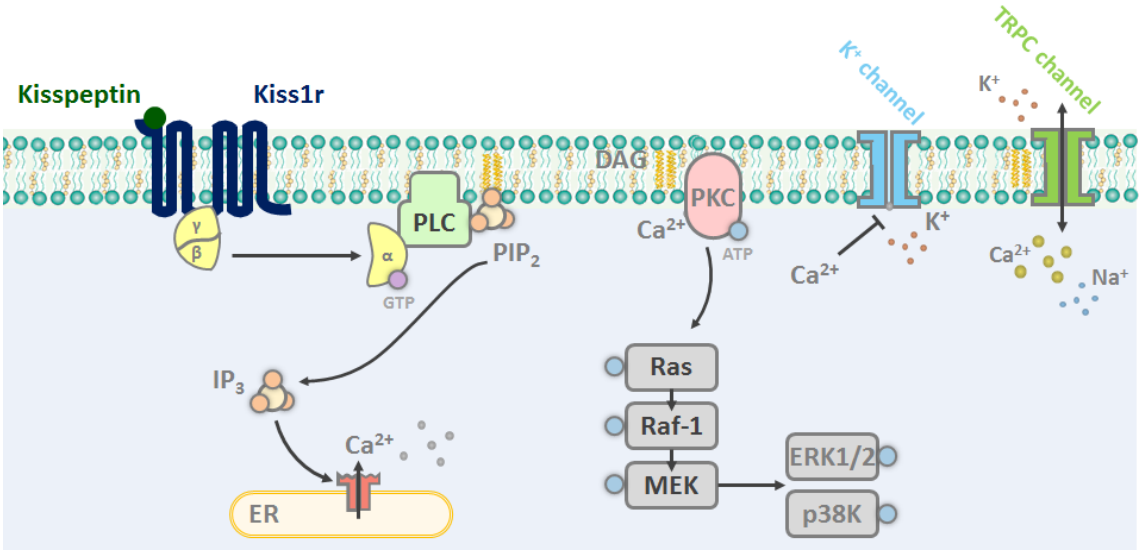

Fig. 1. Kisspeptin via GPR54 signaling at a glance. Schematic presentation of the major signaling pathways recruited upon GPR54 activation by kisspeptins.

3. Materials and Methods

3.1 Synthetic peptides

KISS1R peptides (H-NASDDPGSAPRPLD-C) were synthesized from Kelowna International Scientific Inc. (Taipei, Taiwan). For immunogens, KISS1R peptides were conjugated with Keyhole limpet hemocyanin (KLH).

3.2 Preparation of antibodies

Modified immunization protocols were demonstrated previously (Narat, 2003), two ISA Brown hens (40-week-old) were immunized by intramuscular injection at multiple sites on breast. Primary immunization was performed with 400 μg of KISS1R peptides-KLH in 0.5 mL of saline and equal volume of Freund's complete adjuvant (Sigma-Aldrich, USA) for each hen. Total three boosters with 300 μg of KISS1R peptides-KLH in 0.5 mL of saline and equal volume of Freund's incomplete adjuvant were performed. The first two boosters were performed at 1-week-intervals and the third booster was performed 4 weeks after the second one. The health status of hens was monitored daily and their blood were taken weekly or laid eggs were collected daily. All of the samples were stored at -20 or 4℃ until further processing.

3.3 Purification of egg yolk antibody

IgY was purified by the polyethylene glycol (PEG) precipitation method as previously described (Atha and Ingham, 1981). In order to analyze the average quality

of antibody along the weeks after immunization, eggs laid weekly (about 5-7 eggs) from each hen were pooled prior to IgY extraction. Because IgY in the serum is selectively transferred to the yolk, we only retained egg yolk. After recording total volume of weekly yolk, the yolk was mixed with double yolk volume of PBS. Then, 3.5 % PEG 6000 (Sigma-Aldrich, USA) of the total volume (yolk + PBS) was added, followed by 10 min mixing on a rolling mixer. The tubes were centrifuged at 13,000*g* and 4℃ for 20 min. After centrifugation, the supernatant was passed through a folded filter and transferred to a new tube. 8.5 % PEG 6000 in gram (calculation based on the new volume) were added into the tube. The tube was rolled on a rolling mixer and centrifuged as above mentioned. The pellet was dissolved in 1 mL PBS by means of a glass stick and the vortex. PBS was added to a final volume of 10 mL. The solution was mixed with 12 % PEG 6000 (w/v, 1.2 gram), followed by rolling and centrifugation again. The pellet was carefully dissolved in 2 mL PBS buffer and the solution was dialyzed for 24 hr in PBS. Thereafter, the IgY-extract was taken out from the dialysis bag (Membrane Filtration Products, INC., USA) and stored at -20℃ until further processing. The protein content (mg/mL) of the samples was measured by Pierce™ BCA protein assay kit (Thermo Fisher Scientific Inc., USA) and the quality of the antibody was analyzed by simple SDS-PAGE.

3.4 Enzyme-linked immunosorbent assay (ELSIA)

The titers of chicken IgY anti-KISS1R production and its avidity were evaluated by an indirect ELISA. Briefly, high affinity microtiter plates (Costar Corning Inc., USA) were coated with KISS1R peptide (10 mg/mL) in coating buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9.6) and incubated overnight at 4℃. Plates were washed twice with washing buffer (6.1 mM $Na_2HPO_4 \cdot 2H_2O$, 3.9 mM $NaH_2PO_4 \cdot H_2O$, 0.1% Tween-20, pH 7.0) and blocked with blocking buffer (0.25% gelatin, 0.15 M NaCl, 0.05 M Tris-base, 6 mM EDTA, 0.05% Tween-20, pH 8.0) overnight at 4℃. Antibodies (100 mg/mL) in weekly serum or yolk extract were diluted 1:10,000 in assay buffer, added to the wells in duplicate and incubated for 1 hr at room temperature. After washing, plates were incubated with peroxidase-conjugated goat anti-chicken IgY antibody (Abcam plc, Cambridge, UK), diluted 1:20,000 in assay buffer, for 1 hr at room temperature. The color was revealed by adding 3.7 mM o-phneylenediamine in 0.03% H_2O_2 within 5 min, and the color presenting reaction was stopped by addition of 8 N H_2SO_4. The optical density (OD) was determined at 490 nm with the ELISA reader (Dynatech, Dnkendort, Germany).

3.5 Animals and tissues collection

ICR mice were purchased from National Taiwan University, maintained under a 12-h light cycle and given chow diet and water ad libitum. All procedures conformed to the National Institutes of Health Guide for the care and use of laboratory animals. Testes used

for RNA extraction and Immunohistochemistry were obtained from male mice aged 0, 1, 2, 3, 4, 6, 8 and 12 weeks postpartum (wpp). Brain slices of the hypothalamus, testes, epididymis, kidney, liver, and heart used for protein extraction were obtained from male mice aged 8 wpp. For analyzing the specific site, the brain was positioned on the brain blocker with the plane of section of the mouse brain and cut sagitally into 2-mm thick slices containing the hypothalamus area. Also, brain slices and ovaries collected from 8-week-old female mice were used as the positive control in immunohistochemical staining. Female mice were pretreated with 10 IU pregnant mares' serum gonadotropin (PMSG; Sigma–Aldrich) for 48 hr to maintain their estrous cycle during a preovulatory stage before sacrifice and tissue collection.

3.6 Primary Mouse Leydig cell culture

Mice up to the age of 12 weeks were sacrificed by decapitation. Testes were immediately collected and put into isolation buffer (10 mg collagenase and 10 mg BSA in HBSS buffer). Buffer was renewed once for removing red blood cells and tissue debris. Then, testes in isolation buffer were incubated at room temperature for 5 min. After separating the seminiferous tubules by filtration through sterile stainless steel net with nylon mesh, the filtrate was centrifuged at 300*g* for 5 min at room temperature. The dissociated cells were resuspended in 15 mL Medium 199 (M-199) and incubated in at 37℃ with 5% CO_2.

In order to identify Leydig cells, 3β-HSD staining was carried out by a modification of the method described previously (Payne *et al.*, 1980). 2 x 10^5 cells were seeded on 6-well plate 24 hr before staining. Cells were allowed to dry on well for 15 min at 37℃. After drying was complete, cells were covered with staining solution (1% BSA, 1.5 mM β-NAD, 0.25 mM NBT, 0.2 mM DHEA, 80% PBS) for 8 hr. Then, cells were rinsed in PBS, and fixed in 4% paraformaldehyde in PBS. Cells were observed at 400X magnification for the presence of blue-purple formazan granules.

Before treatment, cells were counted and seeded (2 x 10^5) with M-199 FBS on 6-well plate for 24 hr. Then, Cells were treated with ovine luteinizing hormone (oLH) and RP-cAMPS (Enzo Life Science, Inc., NY, USA), a PKA inhibitor, in serum-free medium for additional 24 hr. Later, we extracted RNA from cells of each treatment for cDNA synthesis and real-time PCR analysis.

3.7 Cell line culture

We used MA-10 mouse Leydig tumor cells as cell model to confirm the expression of KISS1R on Leydig cell's membrane by Immunohistochemistry and investigate the mechanism of LH-dependent *Kiss1* gene expression by real-time PCR. Cells were maintained in DMEM/F-12 medium supplemented with 10% FBS, 2.2 mg/mL $NaHCO_3$, 100U/mL penicillin and 0.1 mg/mL streptomycin under the condition of 37℃ and 5% CO_2. For conducting the experiment, MA-10 cells were plated at 2 x 10^5 cells/well and

allowed to adhere for 24 hr. Then, we treated the cells with or without oLH and RP-cAMPS in the following 24 hr. After the treatment, total RNA was extracted from cells for gene analysis.

3.8 Immunohistochemistry

Formalin-fixed mouse tissues were embedded in paraffin, sectioned into 5-mm thick slies, and adhered to poly-L-lysine-coated slides. Tissue sections were deparaffined in xylene and rehydrated through descending concentrations of ethanol, washed with H_2O, then immersed in 10 mM citrate buffer (pH 6.0) with 0.05% Tween-20, and finally heated twice in a microwave for 10 min at 750 W with the 5 min interval. The sections was removed and allowed to cool by a brief wash in tap water and then in PBS. After quenching endogenous peroxidase activity with 1% (v/v) H_2O_2 in methanol for 30 min, the sections were rinsed three times with PBS for 5 min each. Nonspecific binding sites were blocked with goat serum in PBS [3% (v/v) normal goat serum and 0.2% (v/v) Triton X-100 in PBS] for 1 hr. A commercial rabbit polyclonal antibody raised against mouse kisspeptin 145 (1:100 dilution; Abcam) was used to visualize Kisspeptin. For KISS1R, we used our own chicken-anti mouse KISS1R antibody described above at a dilution of 1 : 5,000. This antibody specificity had been validated by showing gradually obscure bands when antibodies were pre-incubated with grading concentrations of antigen in absorption test. Negative controls for antibodies were established using blocking buffer

alone. After 2 hr (KISS1R) or 20 hr (kisspeptin) of incubation at 4℃, antibodies were revealed with a biotinylated secondary antibody directed against rabbit immunoglobulin G (for kisspeptin) or chicken immunoglobulin G and Y (for KISS1R) for 1 hr. Slides were washed three times with PBS for 5 min each at room temperature and incubated with an avidin–biotin–HRP complex in the Vectastain Universal ELITE ABC kit (Vector Laboratories, Burlington, ON, Canada) for 30 min according to the manufacturer's instructions. After rinsing again, slides were incubated for 10–20 min at room temperature with diaminobenzidine to visualize immunostaining. Finally, slides were rinsed with distilled water twice for 10 min each, counterstained with hematoxylin for 30 s and hydrated with ethanol and xylene before adding mounting medium (Hecht-Assistent; Sondheim, Germany). Sections were observed using optical microscope (Axioskop 40, Carl Zeiss, Göttingen, Germany), and images were collected using an AxioCam ERc 5s (Carl Zeiss) digital camera.

3.9 Western Blot

The tissues or cells were rinsed once with cold PBS and collected. They were ground with a mechanical homogenizer in cold lysis buffer [150 nM NaCl, 0.1% Triton-X 100, 50 mM Tris-HCl (pH 8.0), protease inhibitor, phosphatase inhibitor]. Protein concentrations were determined by using the Pierce™ BCA protein assay kit according to the manufactures instructions. Samples containing 30–60 μg protein were separated by

15% SDS–PAGE. The separated proteins were transferred onto a PVDF membrane. The membrane was blocked by immersing in PBS containing 0.01% Tween 20 (PBST) and 2.5% BSA for 8 hr at room temperature, followed by incubation with our own chicken-anti mouse KISS1R antibody (1:200,000 dilution or a serial diluted concentrations) in PBST with 0.5% BSA for 18 hr at room temperature. Then, the membrane was washed three times with PBST and incubated for 2 hr with peroxidase-conjugated goat anti-rabbit IgG (1:25,000 dilution; Jackson ImmunoResearch Laboratory, PA, USA) or goat anti-chicken IgY. The membrane was washed with PBST and bound antibodies were visualized by the ECL system (Merck Millipore). The images were presented on Kodak X-OMAT film (Eastman Kodak Co.).

3.10 RNA extraction and cDNA synthesis

Total RNA was extracted from tissues or cells with TRIsure reagent (Bioline Inc., USA) according to the manufacturer's instructions. We used PrimeScript™ RT reagent Kit (Takara Bio Inc., Japan) to synthesize cDNA. Total RNA (500 ng) was mixed with 25 pM oligo(dT) primer, 50 pM random hexamers, enzyme mix and reaction buffer, then incubated at 37℃ for 15 min. The reverse transcriptase was inactivated by heating to 85℃ for 5 s, and cDNA products were stored at 4℃ for further analysis.

3.11 Quantitative real-time PCR

Relative levels of target mRNA was examined with the StepOne Real-Time PCR

System (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Transcripts were quantified using the Fast SYBR Green Master Mix (Applied Biosystems) in a total volume of 10 μL. Samples were heated for 10 min at 95℃, followed by 40 cycles of 15 s at 95℃, 30 s at 60℃ and 30 s at 72℃. Then, melting curve analysis was conducted to observe the appearance of non-target products which may affect the final data. The primer sequences are shown in table 2.

3.12 Statistical analysis

Each experiment was replicated at least three times. Data were expressed as mean ± SD. Data were analyzed by Student's t-test or one-way ANOVA followed by Duncan's method with Sigma Plot Software (Systat Software, CA, USA); $p < 0.05$ indicated significance.

Table 2. Primers used for quantitative real-time PCR in this study

Transcript	Forward primer	Reverse primer	Length (bp)	Accession number
Kiss1	CTGCTGCTTCTCCTCTGTGTC	TCCCAGGCATTAACGAGTTCC	107	NM_178260.3
Kiss1r	GTGCAAATTCGTCAACTACATCC	AGCGGGAACACAGTCACATAC	103	NM_053244.5
Insl3	AAGCTCTGCGGCCACCA	GTCTCTGCTCTAGCCACTGC	127	NM_013564.7
Lhcgr	GCCCGACTATCTCTCACCTATC	CCTTTCCAGGGAATCACTCTGA	111	NM_013582.2
Fshr	GGAACGCCATTGAACTGAGATT	TGGAGAACACATCTGCCTCTAT	141	NM_013523.3
Hsd3b-I	TTTGCTCTCTCAGTTGTGACCA	GCCTGCTTCGTGACCATATTTATT	134	NM_008293.3
Cyp19a1	ACGTGGATGTGTTGACCCTC	GATGAGGAGAGCTTGCCAGG	137	NM_007810.3
Ar	GTATCCTGGTGGAGTTGTGAAC	GGTACTGTCCAAACGCATGTC	121	NM_013476.3
Erα	TGGACAGGAATCAAGGTAAATG	TTGAGGCACACAAACTCTTCTC	118	NM_007956.4

4. Results

4.1 Titer of chicken-anti KISS1R antibody

In order to confirm when the hens had expressed the optimal quality of the antibody, we took ELISA assay to test the titer of antibody in serum and yolk. Two hens were immunized for total 7 weeks with 4 injections and both of them successfully responded to the injected antigen. The titer analysis was conducted every week. As shown in Fig. 2, antibodies in serum extracted on the second week after the third injection has the highest titer among all of the collected sera. However, being compared to serum antibodies, there is a delay of the highest titer performance for yolk antibody which expressed the optimal titer on the fourth week after the third injection. The different appearance of the peak in Fig. 2 indicated antibodies in egg yolk got the optimal titer later than antibodies in serum did even though antibodies from two different sources developed synchronically after the second injection (first boost).

4.2 Specificity of chicken-anti KISS1R antibody

Since KISS1R were expressed on GnRH neurons and GnRH neurons located majorly in the preoptic area (POA) of hypothalamus (Clarkson and Herbison, 2006), we used mouse hypothalamus as the positive control to confirm that our anti-KISS1R antibody could particularly recognize its antigen, KISS1R. Each screening lane included about 7.5 μg total proteins extracted from hypothalamus when total 150 μg proteins were

transferred to the membrane. A serial diluted concentrations (1:200,000; 1:400,000; 1:800,000; 1:1,600,000) of yolk-extracted proteins from hens with or without antigen injection were respectively loaded in duplicate into the lanes and they hybridized with samples about 16 hr.

The molecular weight of KISS1R is about 43 kDa, so the immunoreactive bands are near the 48 kDa band of marker ladder. The density of band gradually decreased when the dilution of antibodies is increasing (Fig. 3A). However, yolk-extracted proteins from hen without antigen injection didn't detect any bands which means the antibodies against mouse KISS1R were produced only after KISS1R peptide immunization. Clearly, in 1:800,000 dilution, there were only a single band presented but the specific band was almost absent under 1:1,600,000 dilution. Furthermore, to avoid the query of non-specific binding, we conducted the adsorption test by pre-incubating our antibody with antigen. The experimental procedures are as same as the specificity analysis while those yolk-extracted proteins were pre-mixed with grading concentrations of KISS1R peptides without KLH (immunogenic peptide) overnight. When pre-binding with 100 μM immunogenic peptide, the antibodies had no ability to bind with KISS1R in loading samples and its immunoreactive bands were vaguer than other bands (Fig. 3B). According to the gradually obscure band, we ensured that the antibodies we produced from hens are specifically against the extracellular domain (H-NASDDPGSAPRPLD-C) of K1SSR

because pre-incubation of the given antigens prohibited the antibodies from later binding with KISS1R in loading samples.

4.3 Specific detection of KISS1R in various tissues of mouse

After confirmed the specificity of the antibody, we applied it on detecting the protein expression of KISS1R in several mouse tissues (Fig. 4). Each screening lane was loaded 30 μg total proteins extracted from tissues. The KISS1R immunoreactive band with an apparent molecular weight of 43 kDa was revealed in the hypothalamus, testis and slightly in epididymis, whereas the specific immunoreactive band was absent in epididymal fat, kidney, liver and heart even though other non-specific bands were still visualized in kidney, liver and heart.

4.4 Immunohistochemical staining of kisspeptin and KISS1R in mouse testis

To obtain a greater understanding of the specific location of protein expression of kisspeptin and KISS1R in testis, we collected the testis sections from mice aged from 0 to 12 wpp and immunostained those sections with commercial rabbit-anti kisspeptin antibody and our own chicken-anti KISS1R antibody (Fig. 5 and 6). After staining, brown color was revealed to represent the immunoreactive targets. Kisspeptin is particularly expressed in the cytoplasm of the Leydig cells which locate beside seminiferous tubules from postnatal third week to 12th week (Fig. 5A-H). It seemed that the expression of kisspeptin increased as the population of adult Leydig cells was growing. Immunoreactive

cells on the arcuate nucleus of hypothalamus were presented as the positive control (Fig. 5I). Section of 12-week-old testis incubated without primary antibody was shown as the negative control without any brown-color staining observed (Fig. 5J).

However, the location of KISS1R in the testis is completely contrast to where kisspeptin is expressed. With 2-hour-hybridization of our own chicken-anti KISS1R antibody, we observed that KISS1R locates in seminiferous tubules from postnatal third week to 12th week (Fig. 6A-H). Under higher magnification (1,000X), it is obvious to see that KISS1R is specifically expressed on the membrane of round spermatids (Fig. 6H). Here, we used the oviduct as the positive control (Cejudo Roman *et al.*, 2012). KISS1R was detected on ciliated epithelium of the oviduct (Fig. 6I). As the negative control for the immunohistochemical procedure, section of 12-week-old testis was incubated without primary antibodies (Fig. 6J).

4.5 Gene expression of *Kiss1* and *Kiss1r* during testicular development

Preliminary results from Western blotting and IHC indicated that there is a local expression of kisspeptin/KISS1R system in the testis. In detail, kisspeptin is expressed in Leydig cells while KISS1R locates on the membrane of spermatids. To further address when the testis expresses kisspeptin and KISS1R, we analyzed the gene expression pattern of *Kiss1* and *Kiss1r* in mouse testis during developmental process by quantitative real-time PCR. *Kiss1r* mRNA is constantly expressed by mouse testis from infancy to

adulthood (Fig. 7B); however, its transcript levels during development are significantly lower in the testis than in the adult female hypothalamus. In contrast, the gene expression of Kiss1 has an obvious change at puberty.

As shown in Fig. 7A, testis doesn't express *Kiss1* mRNA until postnatal fourth week. Along with the growth, *Kiss1* transcript level gradually increases to the highest level which is expressed at 8 and 12 wpp.

4.6 *Kiss1* gene expression is related to testicular development

According to the records of body and testes weights, both growth curves are similar and they have steady rises from birth to adulthood (Fig. 8A). However, it is interesting that the ratio of testes and body weight hardly gets change after postnatal third week (Fig. 8B). In other words, the proportion of testes in the whole body keep stably after the initiation of puberty (4 wpp). This result implied that the stage of testis is changing from mass growing to reproductive-function performing during postnatal fourth week.

Combining the data of testicular growth (Fig. 8B) and *Kiss1* gene expression (Fig. 7A), both two patterns have a dramatic transition during 12 weeks after birth. It seemed that testes stop changing its proportion to the whole body but begin to express *Kiss1* gene at postnatal fourth week. Therefore, we suggested that the expression of *kiss1* gene beginning from postnatal fourth week might relate to developmental events, such as hormone secretion, testis descent, spermatogenesis and so on in the testis.

We selected several genes related to testicular development and compared the fluctuations of those transcript level with *Kiss1* gene to figure out the relevance of *Kiss1* gene to testis maturation. There are three genes are similar to *Kiss1* gene as the expression pattern is concerned (Fig. 9). *Insl3, Cyp19a1* as well as *Kiss1* mRNA levels increase significantly at the same time and maintain at the highest level throughout pubertal stage in the testis (Fig. 9B and 9C). Although *Lhcgr* also has a rising pattern of gene expression, its expression appears one week earlier than *Kiss1* and the other 2 genes do (Fig. 9A).

Still, we also found that the genes relate to Sertoli cell maturation and hormone signaling have different expression pattern to *Kiss1* gene. The gene expression of *Fshr* and *Hsd3b* varies inversely with *Kiss1*. In general, they decrease their mRNA levels after 1 or 2 wpp (Fig. 10A and 10B). The other two steroid hormone receptor genes, *Ar* and *Er* have tiny fluctuations on transcript level during whole developmental process (Fig. 10C and 10D).

4.7 *Kiss1* gene expression depends on LH signaling

Due to both *Lhcgr* gene expression (Fig. 9A) and LH concentration (O'Shaughnessy *et al.*, 2009) are increased from postnatal second to third week, earlier than *Kiss1* gene expression, we proposed that the expression of *Kiss1* might be dependent on LH and its receptor-mediated signaling. To investigate the relevance of LH to *Kiss1* gene expression, we used primary Leydig cell and MA-10 cell line (MA-10) as cell models to confirm our

hypothesis.

In primary Leydig cell experiments, cells were treated with different dose of oLH in serum-free medium for 4 hr. Adding 50 ng/ml oLH increased *Kiss1* gene expression by one and half folds (Fig. 11A) but it didn't affect the *Kiss1r* (Fig. 11B) and *Lhcgr* (Fig. 11C) gene expression in primary Leydig cells. Being consistent with the results in primary Ledig cell experiment, *Kiss1* gene expression in MA-10 was also significantly increased by the addition of 50 and 100 μM Br-cAMP, the second messenger in LH signaling (Fig. 12A). In addition, co-treatment of Br-cAMP and RP-cAMPS, a PKA inhibitor, suppressed about 50% Br-cAMP-induced *Kiss1* gene expression while RP-cAMPS didn't alter the expression along (Fig. 12B).

5. Discussion

5.1 The establishment of anti-KISS1R IgY antibody

Evidences for the expression of *KISS1* and *KISS1R* genes in human testis have been presented (Kotani *et al.*, 2001; Ohtaki *et al.*, 2001) to suggest direct actions of kisspeptin exerting in the male gonad. To evaluate the expression of KISS1R proteins in the testis, we produced a KISS1R-specific antibodies suitable for immunohistochemistry and western blotting.

Immunoglobulin Y (IgY), Immunoglobulin A (IgA), and Immunoglobulin M (IgM) are three classes of antibody found in the chicken and IgY is the major group of antibody which composes about 75% of the total immunoglobulin pool (Leslie and Martin, 1973). Avian IgY is the functional equivalent of mammalian immunoglobulin G (IgG) but they are actually different with regard to the structure. The major difference is the number of constant regions (C_H) in heavy chains: IgY has four regions without the hinge between C_H1 and C_H2, while IgG only has three regions connected with the hinge at the first two C_H (Shimizu *et al.*, 1992). Due to the difference in Fc region, IgY lacks the typical IgG-Fc dependent functions and decreases many unnecessary bindings when it is applied in mammalian researches, for example diagnostics, medical application and biotechnology (Narat, 2003). Combining the benefit brought from the distinct structure and other advantages of IgY, such as large and accessible amounts (Narat, 2003), non-invasive

procedure as collecting laid eggs and a better response of chicken to mammalian antigens due to farther evolutionary distance (Larsson and Sjoquist, 1990), we chose the hen as the host for antibody-production.

After analyzing the titer of chicken-anti KISS1R antibody extracted from serum and egg yolk, we found that antibodies from both two different sources developed quickly after the first boost but their presenting titer rose to the highest level at different time (Fig. 2). Interestingly, the peak appeared later in the yolk group than in that of serum group. This lag of the appearance of the optimal titer might be related to a period ranging from three to six days which is required for the transovarial passage and condensation of newly-produced IgY in egg yolk (Patterson *et al.*, 1962; Woolley and Landon, 1995). However, the span of the lag in our data is two weeks which is longer than usual. We supposed that it was influenced by the different methods of extracting antibody from two sources. Based on the results of ELISA, we knew that hens had a quick but not long response to the given immunogen and we would use the optimal antibodies extracted from egg yolk laid within the sixth week of immunization for further analyses.

It is necessary to detect the specificity of our own anti-KISS1R antibody before the further application. We used western blotting technology to check if our antibody has the non-specific binding and the appropriate dilution for our antibody. Because a single band was only presented by antibody of 1:800,000 dilution (Fig. 3A), we suggested that

1:800,000 dilution is the optimal dilution for our own antibody. Moreover, it is greatly higher than the working dilution of commercial anti-KISS1R antibodies (Cartwright and Williams, 2012; Shoji *et al.*, 2010). Thereafter, we also conducted the adsorption test to eliminate the possibility of non-specific binding. Indeed, the immunoreactive bands were vague when antibodies had pre-bond with immunogenic peptide (Fig. 3B). It supported that our own antibodies are specifically against the extracellular domain (H-NASDDPGSAPRPLD-C) of K1SSR and could be a reliable alternative to be applied in other researches.

5.2 Expression of KISS1R in mouse tissues

Previous studies have shown that *KISS1R* mRNA were expressed in numerous human tissues by quantitative RT-PCR. According to their data, the highest level of *KISS1R* mRNA expression was observed in the placenta, next are pancreas and brain (Kotani *et al.*, 2001; Ohtaki *et al.*, 2001). Besides, other studies also indicated that *KISS1R* mRNA is majorly expressed in the POA region of hypothalamus (Kinoshita *et al.*, 2005).

In our study, we focused on the expression of KISS1R protein in several mouse tissues. By using the hypothalamus (where the POA region is) as the positive control of KISS1R immunoreactive detection, we found that the KISS1R immunoreactive bands with apparent molecular weight of 43 kDa are only in the testis and epididymis rather than in the epididymal fat, kidney, liver and heart (Fig. 4). This result is consistent to the

previous data which demonstrating relatively low or non-existent *KISS1R* expression in the kidney, liver and heart whereas the testis expresses relatively high *KISS1R* mRNA (Kotani *et al.*, 2001; Ohtaki *et al.*, 2001). However, there is no denying that the expression of KISS1R proteins changed with the species, stages of life and pathological status and then might be expressed or degraded under the specific conditions (Pinilla *et al.*, 2012).

5.3 Expression of kisspeptin and KISS1R in mouse testis

The testis is comprised of interstitium and seminiferous tubules. After confirming the expression of kisspeptin and KISS1R in the testis, we next investigated the cellular distribution of this two proteins in the testis. We collected the testis sections from mice aged from 0 to 12 wpp and immunostained those sections with commercial rabbit-anti kisspeptin antibody and chicken-anti KISS1R antibody generated in this study (Fig. 5 and 6). The peptides of Kisspeptin/KISS1R system displayed completely different patterns of cellular distribution in mouse testis (Fig. 5 and 6). The staining results revealed that kisspeptin is particularly expressed in the cytoplasm of the Leydig cells (Fig. 5), whereas KISS1R locates in seminiferous tubules, exactly on the membrane of round spermatids (Fig. 6). The reason why KISS1R particularly locates on the membrane is probably because the G-coupled receptor must be expressed on the membrane to contact with its ligand. Moreover, the different location of kisspeptin and KISS1R might imply that there is a paracrine function of kisspeptin and KISS1R system in the testis.

So far, the fact that kisspeptin is expressed in Leydig cells has been demonstrated by our group (Hsu *et al.*, 2014) and Tena-Sempere's group (Pinilla *et al.*, 2012). However, the location of KISS1R is still controversial. The pattern of KISS1R expression revealed here is inconsistent to our previous data (Hsu *et al.*, 2014) which indicated that KISS1R locates on acrosome rather than membrane of spermatids. Although it needs more evaluations to clarify the described disparity, we confirm the specificity of our chicken anti-KISS1R antibody with the evidence given by the pre-absorption test.

5.4 *Kiss1* gene expression is related to testicular development

In figure 5 and 6, we also observed that the relative level of immunoreactive color representing kisspeptin and KISS1R expression varied with mice development. In order to check the patterns of *Kiss1* and *Kiss1r* expression have the relevance with the testicular development, we analyzed the gene expression of *Kiss1* and *Kiss1r* in the testis during postnatal development by using quantitative real-time PCR (Fig. 7). It was shown that *Kiss1r* mRNA is constantly expressed by mouse testis from infancy to adulthood (Fig. 7B), while the level of *Kiss1* expression has an obvious transition between 3 and 4 wpp (Fig. 7A), the period that mouse undergoes the pubertal onset (Falconer, 1984). In addition, we also observed another interesting phenomenon that the ratio of testes and body weight begins to maintain unchanged level at the same time, which means growing of testis is arrested and the testis is going to possess mature functions for fertility at that

time (Fig. 8B). Based on these data, we suggested that the induction of *Kiss1* expression is positively correlated with reproductive performance during testicular development.

Several functions and landmark events in mouse testicular development have been well-reviewed in a variety of studies (Nieschlag *et al.*, 2010; O'Shaughnessy *et al.*, 2009), which include hormone secretion, testis descent, cellular differentiation, spermatogenesis and formation of blood-testis barrier. In our study, we chose *Lhcgr*, *Fshr*, *Insl3*, *Hsd3b*, *Cyp19a1*, *Ar* and *Er* gene which are related to above mentioned testicular development and compared the patterns of those transcript expression with *Kiss1* gene to investigate the correlation between *Kiss1* gene expression and testis maturation.

The similar patterns observed in *Kiss1*, *Insl3* and *Cyp19a1* gene expression during testicular development (Fig. 7A, 9B and 9C), imply that the increasing synthesis of kisspeptin might be in charge of spermatogenesis which is under the control of INSL3 produced from adult Leydig cells (Ivell and Anand-Ivell, 2009) and estrogen synthesized by aromatase (CYP19A1) in Sertoli cells (Robertson *et al.*, 1999). Moreover, analysis of RXFP2 (the receptor of INSL3) expression showed that RFXP2 locates on Leydig cells themselves, and on both pre- and post-meiotic stages of germ cells, with most being on post-meiotic spermatids (Feng *et al.*, 2007; Filonzi *et al.*, 2007). These data suggest that INSL3 secreted from Leydig cells could drive its actions on spermatogenesis in spermatids expressing RFXP2. Based on the equivalent of gene-induction period,

secretory cell and receptor-presenting site between *Kiss1* and *Insl3* gene, we suppose a synergistic effect of kisspeptin and INSL3 on spermatogenesis and look forward to confirming this hypothesis with further experiments.

In addition to two genes mentioned above, *Lhcgr* gene has the similar pattern of gene expression to *Kiss1* gene. However, *Lhcgr* gene expression appears one week earlier than *Kiss1* and the other 2 genes do (Fig. 7A and 9). The earlier expression pattern of *Lhcgr* gene imply that the expression of *Lhcgr* gene probably has an upstream regulation on *Kiss1*, *Insl3* and *Cyp19a1* gene. This implication could be supported by previous studies on LH-receptor knockout mice which fail to express INSL3 protein (Anand-Ivell *et al.*, 2006) and *Cyp19a1* gene (Ma *et al.*, 2004).

5.5 *Kiss1* gene expression depends on LH signaling

To understand whether LH regulates *Kiss1* gene expression, we used primary Leydig cell and MA-10 cell line (MA-10) as cell models to evaluate our hypothesis and possible mechanisms involved in. As shown in Fig. 11A, *Kiss1* gene expression significantly increased by the addition of oLH in primary Leydig cells. This data was further supported by the success of Br-cAMP treatment to induce *Kiss1* gene expression in MA-10 (Fig. 12A). Furthermore, the results of adding PKA inhibitor in MA-10 revealed that the rising of *Kiss1* gene expression is dependent on the stimulation of cAMP/PKA pathway (Fig. 12B), which is a well-known pathway induced by LH signaling and equivalent to the way

LH acts on testosterone secretion at the time of puberty (Nieschlag *et al.*, 2010). Based on the fact that receiving the signal from the same initiator at the similar time, it supports the idea that not only testosterone release but also *Kiss1* gene expression could be induced by LH during puberty.

In 2005, it have been found that the mouse *Kiss1* promoter includes two putative functional cAMP response element (CRE) half-sites (TGACT) located at - 127 and - 758 bp upstream of the *Kiss1* transcription start site (Zhang *et al.*, 2005). Recently, Song *et al.* (2014) also confirmed that intracellular concentration of cAMP induced by adenylyl cyclase activator forskolin (fsk) plus phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) treatment significantly increases *Kiss1* expression in mouse hepatocytes. In luciferase reporter assay, they also successfully showed *Kiss1*'s transcriptional responses to fsk/IBMX is decreased by the mutation of either of the CRE half-sites on *Kiss1* promoter, and further decreased by the mutation of both CRE half-sites on *Kiss1* promoter.

Therefore, it is inferred from those previous data and our present results that LH secretion and LH receptor-mediated signaling, cAMP/PKA pathway, is the primary regulator of *Kiss1* gene expression in several tissues, like testis and liver.

Interestingly, the phenomenon of increasing *Kiss1* mRNA levels and steady *Kiss1r* mRNA levels during puberty was also observed in the hypothalamus. Previous studies

have already indicated a dramatic upsurge in *Kiss1* mRNA levels and/or kisspeptin-IR at the AVPV and ARC of hypothalamus along puberty in mice (Clarkson and Herbison, 2006; Gill *et al.*, 2010), rats (Iijima *et al.*, 2011; Takumi *et al.*, 2011) and monkeys (Shahab *et al.*, 2005). And, one of these data demonstrated that hypothalamic *Kiss1r* mRNA expression is similar before and after puberty (Shahab *et al.*, 2005). These observations as well as our present data indicate that there must be some factors controlling the expression of *Kiss1* gene in the hypothalamus during puberty.

Unlike LH signaling we discovered in Leydig cells, it is the organizing effect of sex steroids that gives rise to such an increase in the synthesis of kisspeptin and/or the number of Kiss1 neurons in the hypothalamus around the time of puberty. It has been known that the expression of kisspeptin is decreased in the exposure to high dose of androgen (Kauffman *et al.*, 2007a) or in conditions of low estrogenic input (Clarkson *et al.*, 2009; Gill *et al.*, 2010) before puberty. However, we didn't find any effect of estrogen (estradiol) and androgen (testosterone) on *Kiss1* gene expression in MA-10 cell line so far.

Overall, our data verify the local expression of kisspeptin and KISS1R in the testis again. Although *Kiss1* mRNA level increases in both central (hypothalamus) and local (testis) system during puberty, its upstream regulation is certainly different in those two tissues. *Kiss1* gene expression is controlled by steroid hormones in hypothalamus but by LH signaling in the testis, which means *Kiss1* mRNA expression is governed under

different conditions and signals in hypothalamus and testis during puberty. Just because of the distinct regulation in the tissues, it is possible to speculate that varied functions of kisspeptin might occur in different tissues. In this study, from the time and place of expression we suppose that kisspeptin/KISS1R system might play a role in spermatogenesis of the testis.

6. Conclusion

The kisspeptin/KISS1R system was also observed in the testis by immunohistochemistry; while kisspeptin locates in Leydig cells, KISS1R is expressed on membrane of spermatids.

During puberty, LH signaling increases testosterone concentration to improve spermatogenesis and maturation of peripheral reproductive tissues. According to our data, it seems like that LH signaling increases not only testosterone concentration but also *kiss1* gene expression by cAMP/PKA pathway. In addition, the level of *Insl3*, *Cyp19a1* and *Kiss1* gene expression increases at the same time after the occurrence of rising *Lhcgr* gene expression. That is, there are three possible reasons for supporting the local function of kisspeptin/KISS1R system in the testis: (1) both testosterone and *Kiss1* gene are produced by Leydig cell, (2) KISS1R, receptor for Kisspeptin is presented on spermatids, and (3) the similar timing and location of gene expression between *Kiss1* and two genes (*Insl3* and *Cyp19a1*) regulating spermatogenesis. Due to above mentioned reasons, it is tempting to assume the synergistic action of Kisspeptin and development-related factors on spermatogenesis (Fig. 13). However, the definitive role for Kisspeptin/KISS1R system in testicular development needs more studies to elucidate.

7. Figures

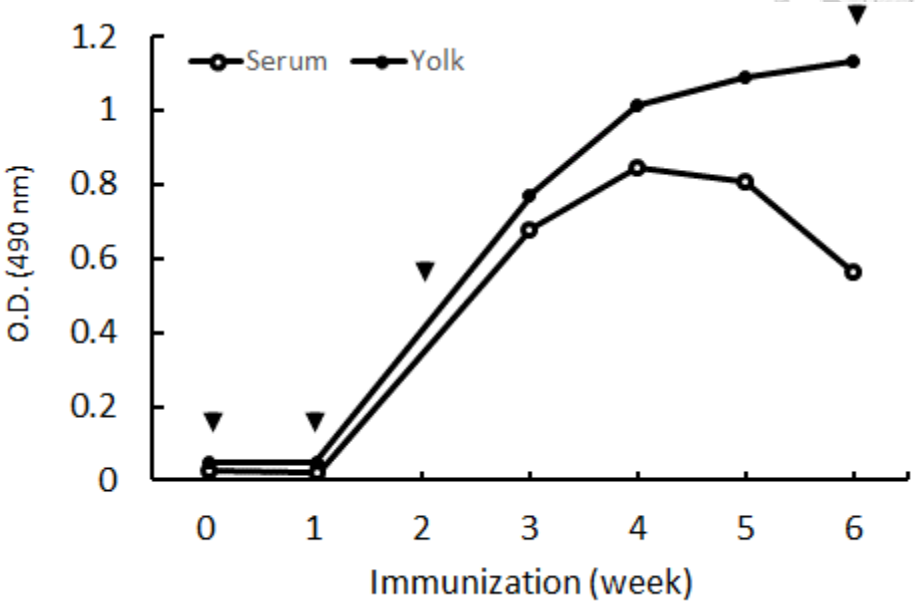

Fig. 2. The titer of anti-KISS1R polyclonal antibodies in hen's serum or egg yolk. The titer was determined by the reactive binding to the peptide of KISS1R's extracellular domain (antigen). 10 μg/mL antigen, 1:100 dilution of weekly anti-serum or egg yolk and 1:20,000 dilution of anti-IgY secondary antibody were used in the test. The unit of titration was expressed as the optical density in 490 nm. Arrowheads denote immunogen injections.

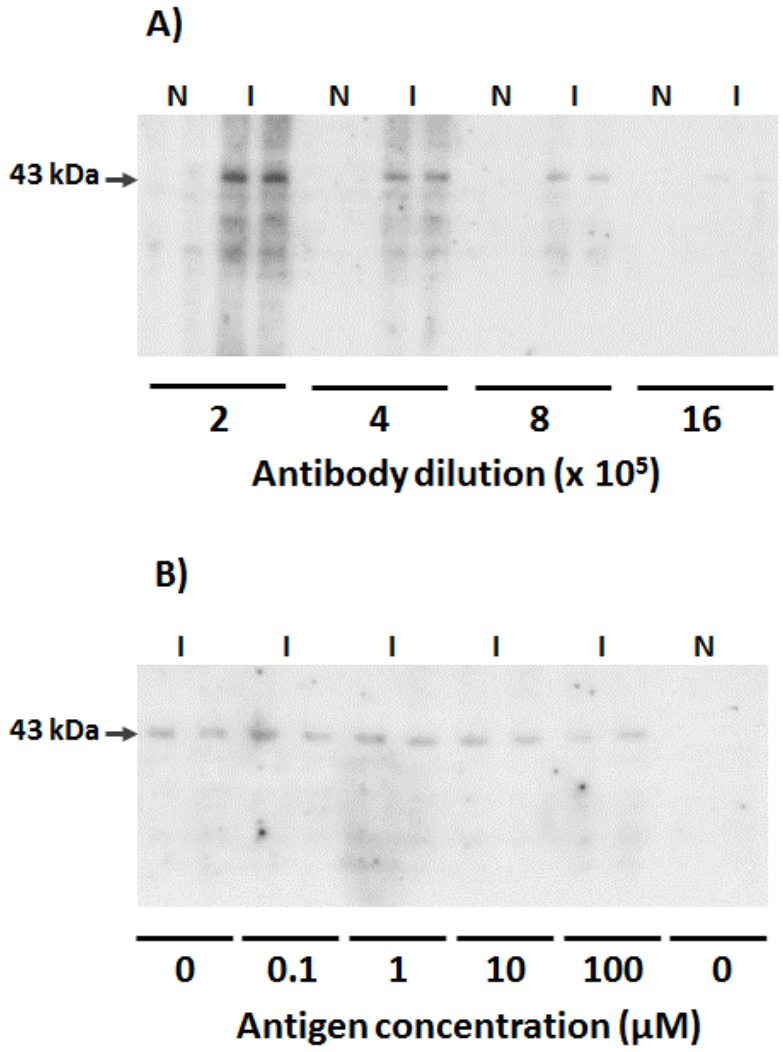

Fig. 3. The specificity of anti-KISS1R polyclone antibodies determined by absorption control. Protein samples were extracted from hypothalamus of 8-week-old mouse. **A)** Protein samples were hybridized with sequentially diluted antibody (1 mg/ml) from egg yolk of non-immunized (N) or immunized hen (I). **B)** 800,000 dilution of antibody (1 mg/ml) from immunized hen was pre-absorbed by different concentration (0-100 μM) of antigen for overnight at 4˚C before hybridizing with protein samples. 1:40,000 dilution of anti-IgY secondary antibody were used in the test. Arrow indicated the presenting bands of KISS1R.

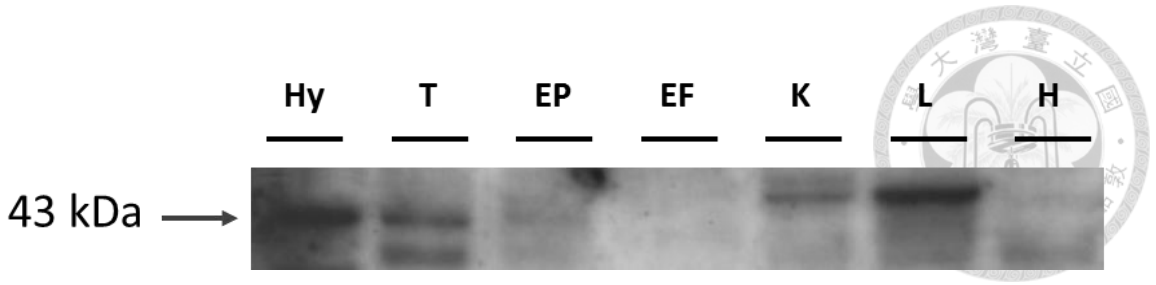

Fig. 4. KISS1R is expressed in the hypothalamus and testis of 8-week-old mouse. Protein samples were extracted from the hypothalamus (Hy), testis (T), epididymis (EP), epididymal fat (EF), kidney (K), liver (L) and heart (H) of 8-week-old mouse. Arrow indicated the presenting band of KISS1R.

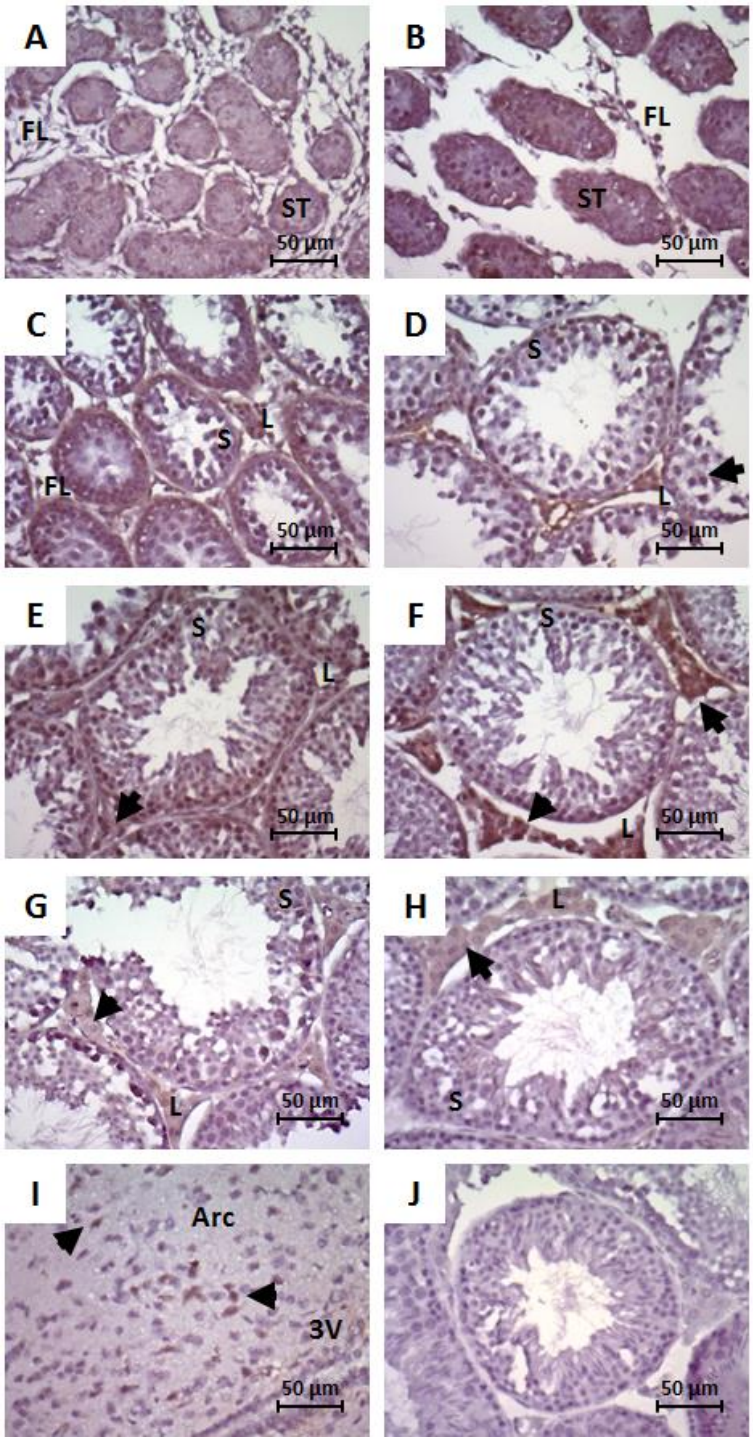

Fig. 5. The Kisspeptin is expressed on Leydig cells of 3 to 12-week-old mouse testis. **A)** 0 (birth), **B)** 1, **C)** 2, **D)** 3, **E)** 4, **F)** 6, **G)** 8 and **H)** 12 week-old male ICR mice testis sections were stained for Kisseptin with specific antibody. Kisspeptin expressed on **I)** arcuate nucleus in 8-week-old female mouse is used as positive control. Section incubated without primary antibody is shown as the negative control **(J)**. (Arc = arcuate nucleus; FL = fetal Leydig cell; L = Leydig cell; S = spermatid; ST = seminiferous tubule)

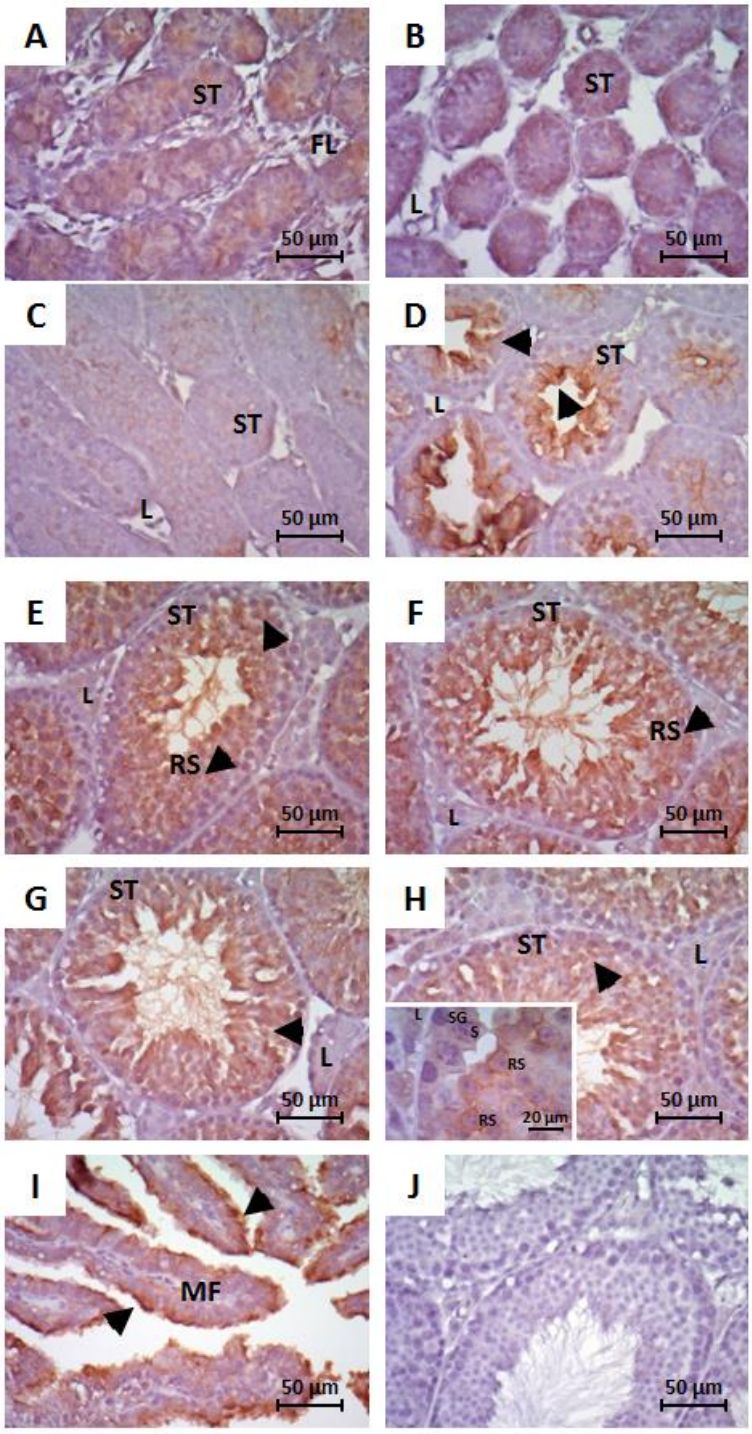

Fig. 6. KISS1R is located on germ cells, especially spermatids, in seminiferous tubules of 2 to 12-week-old mouse testis. A) 0 (birth), **B)** 1, **C)** 2, **D)** 3, **E)** 4, **F)** 6, **G)** 8 and **H)** 12 week-old male ICR mice testis sections were stained for KISS1R with our own IgY antibody. The insert panel shows the original image at 5X magnification.KISS1R expressed on **I)** oviduct epithelial cells in 12-week-old female mouse is used as the positive control. Section incubated without primary antibody is shown as the negative control **(J)**. (Arc = arcuate nucleus; FL = fetal Leydig cell; L = Leydig cell; RS = round spermatid; ST = seminiferous tubule; MF = mucosal fold)

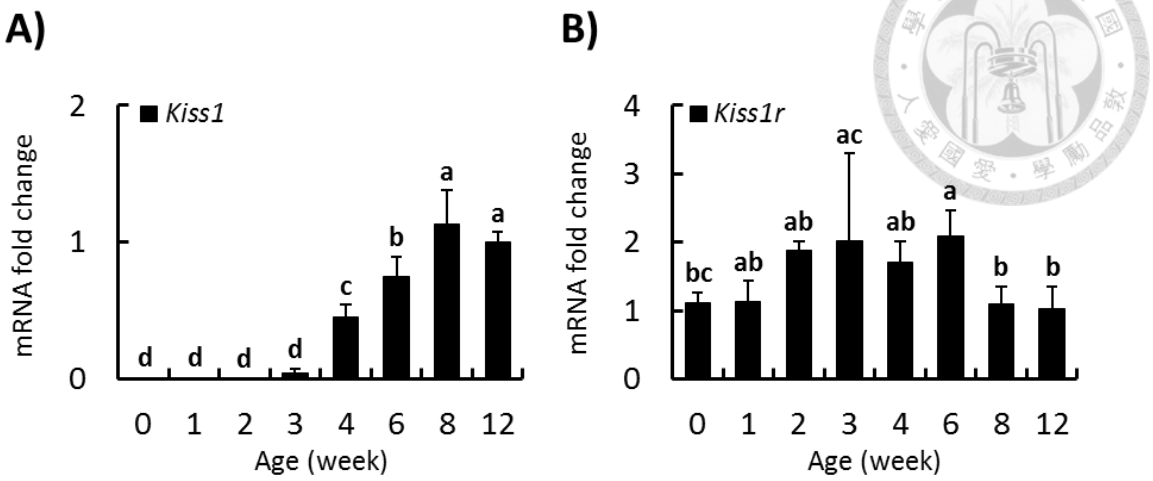

Fig. 7. qPCR results of *Kiss1* and *Kiss1r* mRNA expression levels in mouse testis at different ages. Total mRNA were extracted from whole testis which range from 0 to 12-week-old age. **A)** *Kiss1* and **B)** *Kiss1r* data were shown as fold changes compared to expression levels of *Kiss1* and *Kiss1r* in 12-week-old mice. All mRNA expression levels were normalized with *Rpl19* as the internal control. Bar values are means ± S.D. Bar with different letters are significantly different, $p < 0.05$. (n=3)

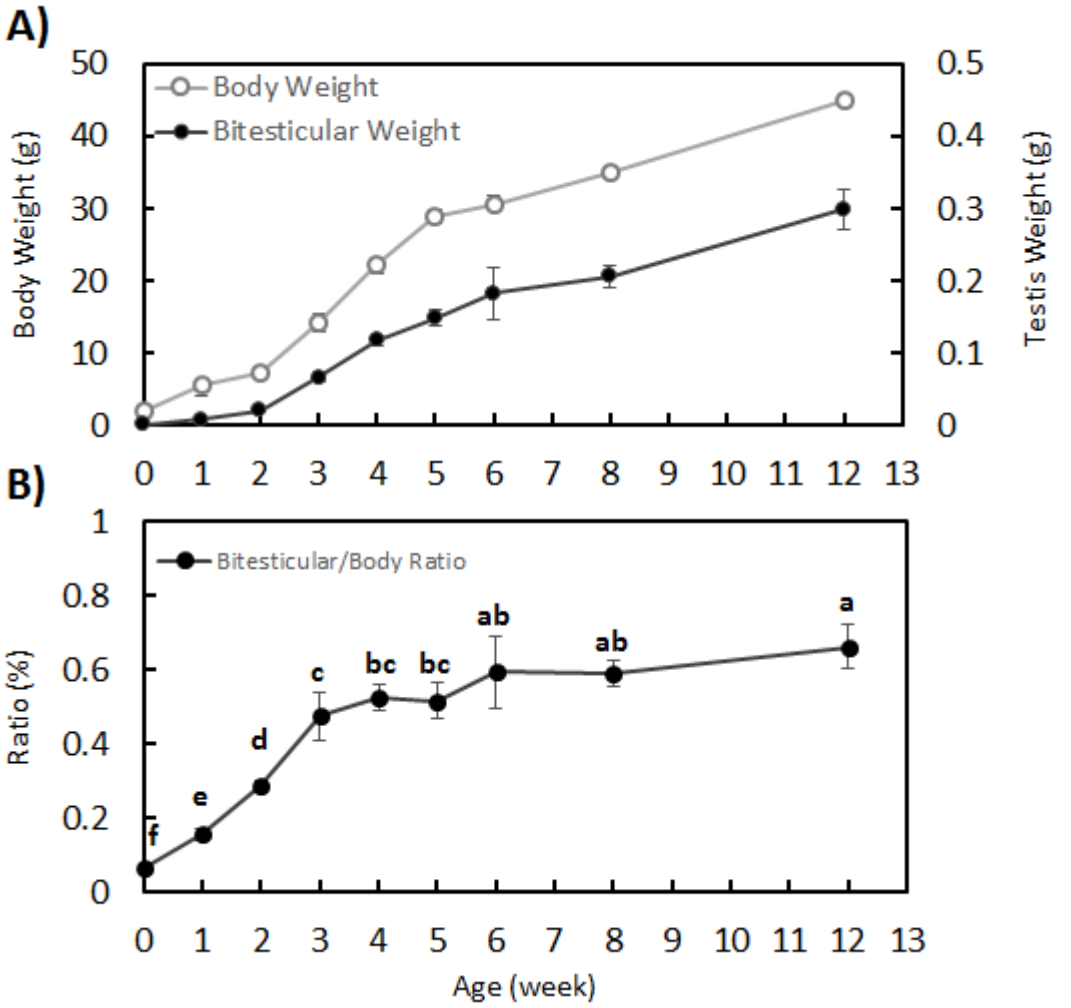

Fig. 8. Body and testes weights and their related ratios of male mice at different ages. **A)** Body and testes weights of mouse and **B)** the related ratio of testes and body weights were recorded from birth to 12-week-old age. Point values are means ± S.D. Different letters are significantly different, $p < 0.05$. (n=3)

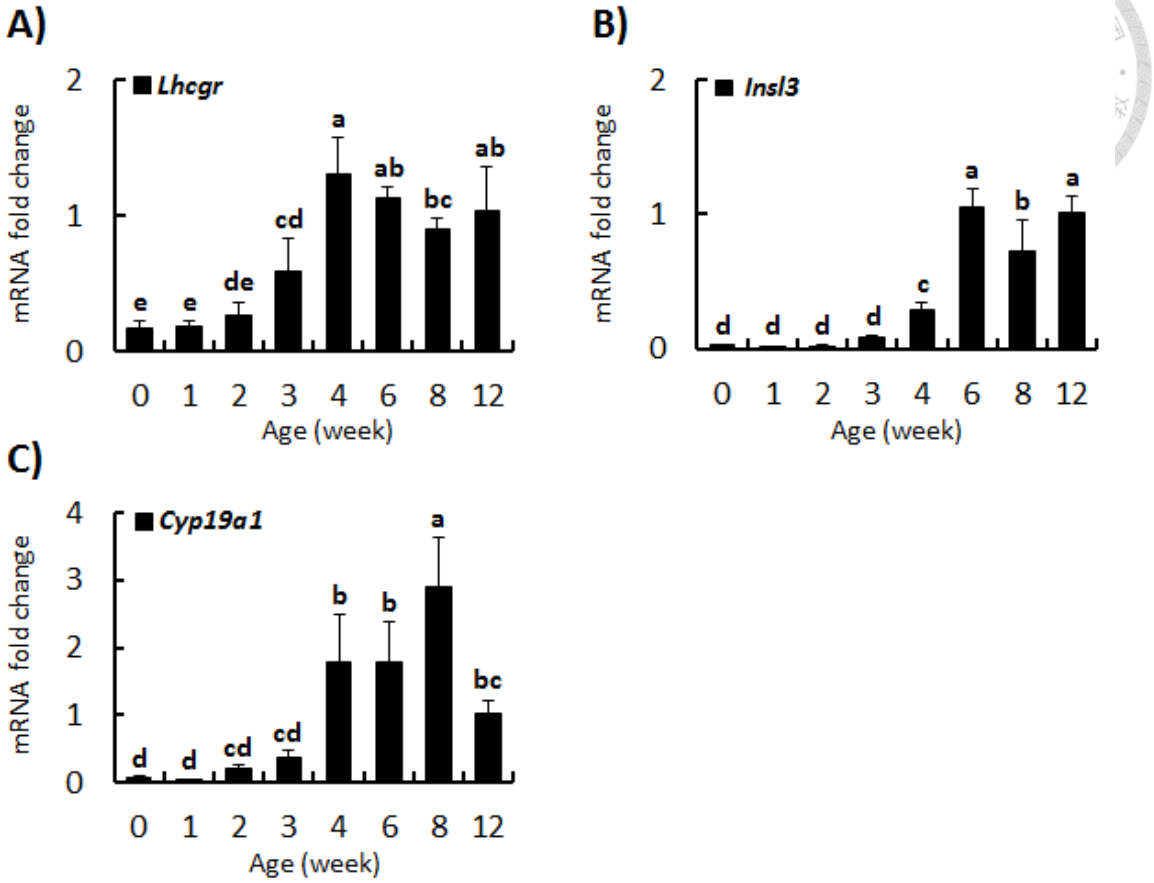

Fig. 9. qPCR results of *Lhcgr*, *Insl3* and *Cyp19a1* mRNA expression levels in mouse testis at different ages. Total mRNA were extracted from whole testis which range from 0 to 12-week-old age. **A)** *Lhcgr*, **B)** *Insl3* and **C)** *Cyp19a1* data were shown as fold change compared to expression levels in 12-week-old mice. All mRNA expression levels were normalized with *Rpl19* as the internal control. Bar values are means ± S.D. Bar with different letters are significantly different, $p < 0.05$. (n=3)

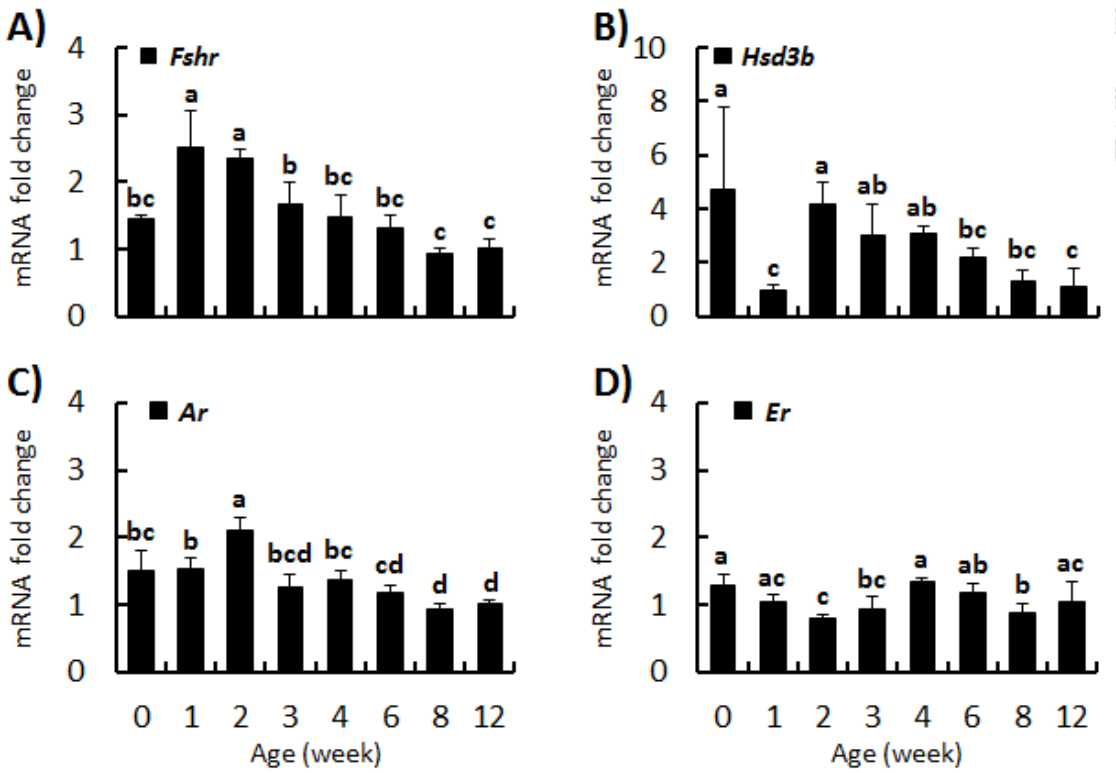

Fig. 10. qPCR results of *Fshr*, *Hsd3b*, *Ar* and *Er* mRNA expression levels in mouse testis at different ages. Total mRNA were extracted from whole testis which range from 0 to 12-week-old age. **A)** *Fshr*, **B)** *Hsd3b*, **C)** *Ar* and **D)** *Er* data were shown as fold change compared to expression levels in 12-week-old mice. All mRNA expression levels were normalized with *Rpl19* as the internal control. Bar values are means ± S.D. Bar with different letters are significantly different, $p < 0.05$. (n=3)

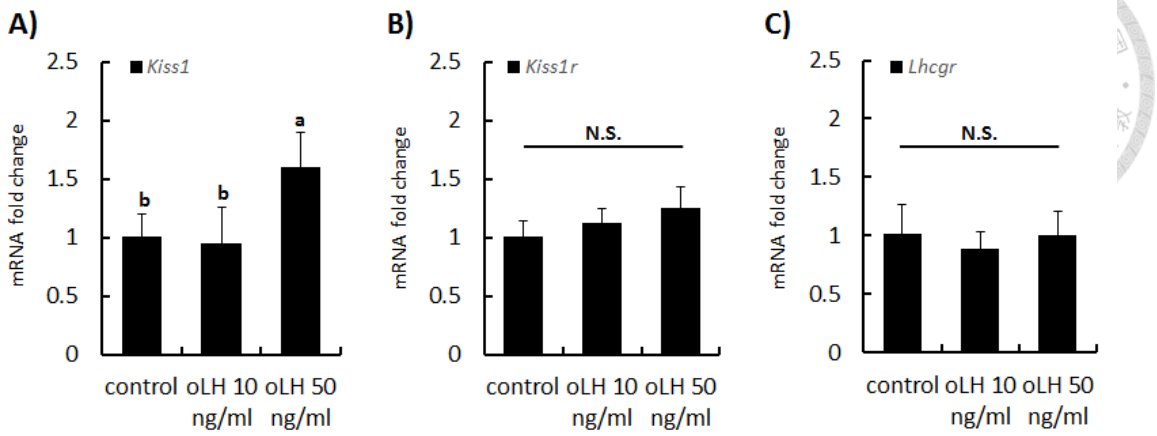

Fig. 11. qPCR results of *Kiss1*, *Kiss1r* and *Lhcgr* mRNA expression levels in oLH-treated primary Leydig cells. Total mRNA were extracted from 15-week-old primary Leydig cells treated without or with 10 and 50 ng/ml oLH for 4 hours. Data were shown as fold changes compared to expression levels of **A)** *Kiss1*, **B)** *Kiss1r* and **C)** *Lhcgr* in the cells of control group. All mRNA expression levels were normalized with *Rpl19* as the internal control. Bar values are means ± S.D. Bar with different letters are significantly different, $p < 0.05$. (n=3)

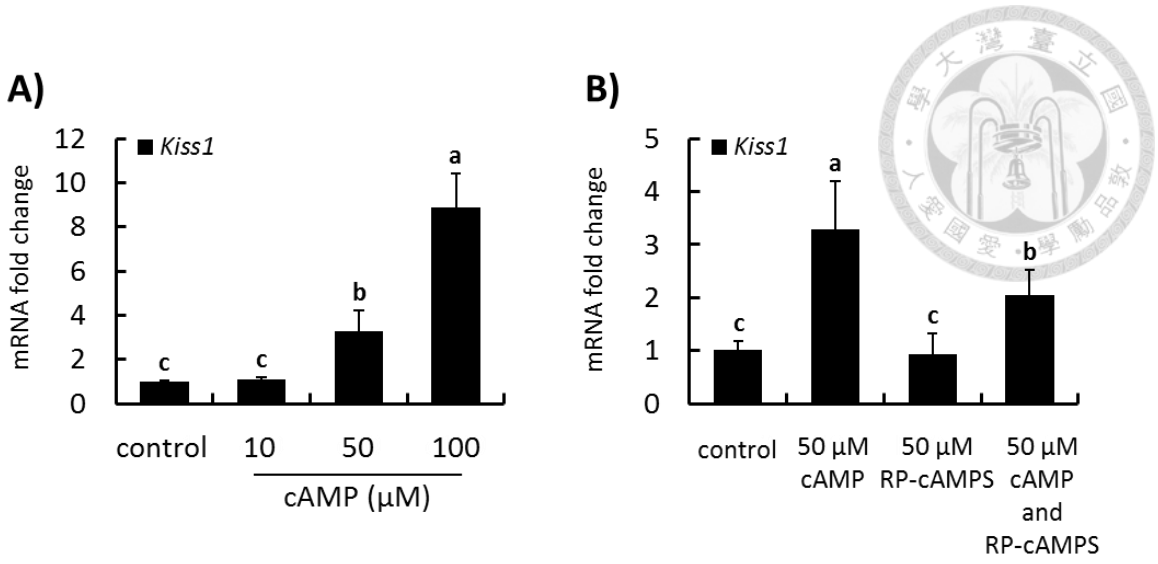

Fig. 12. *Kiss1* mRNA expression levels were induced by cAMP through PKA pathway. MA-10 Leydig cells were treated with cAMP, RP-cAMPS (PKA inhibitor) or both for 24 hours. Data were shown as fold changes compared to expression levels of *Kiss1* in the cells of control group. All mRNA expression levels were normalized with *RPL19* as the internal control. Bar with different letters are significantly different, $p < 0.05$. Bar values are means ± S.D. (n=3)

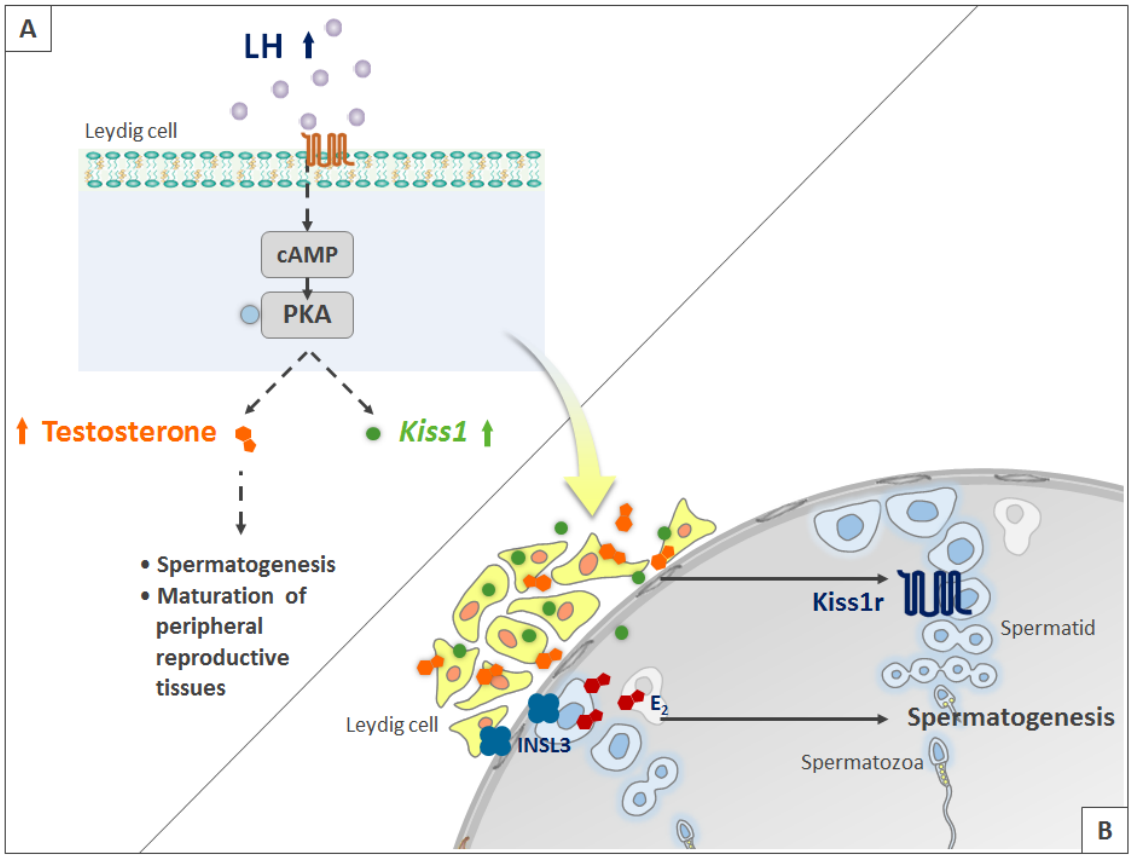

Fig. 13. The kisspeptin/KISS1R system has local function in the testis. A) LH signaling increases not only testosterone concentration but also *kiss1* gene expression by cAMP/PKA pathway in Leydig cells. **B)** The synergistic action of Kisspeptin and development-related factors (INSL3, E_2 and so on) on spermatogenesis might happen in the testis.

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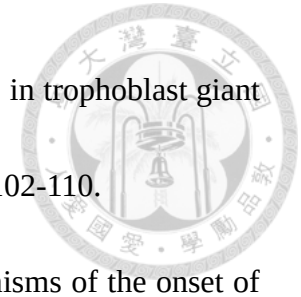

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Appendix List of Abbreviations

Abbreviation	Meaning
ALCs	Adult Leydig cells
AR	Androgen receptor
ARC	Arcuate nucleus
AVPV	Anteroventral periventricular nucleus
Br-cAMP	8-Bromoadenosine-3',5'-cyclic monophosphate
cAMP response element	CRE
C_H	Constant regions
CYP11A1	Cholesterol side chain enzyme system
CYP19A1	Aromatase
DAG	Diacylglycerol
ER	Estrogen receptor
FLCs	Fetal Leydig cells
FSH	Follicle-stimulating hormone
fsk	Forskolin
GnRH	Gonadotropin releasing hormone
HPG axis	Hypothalamic–pituitary–gonadal axis
hpg mice	Hypogonadal mice
IBMX	3-isobutyl-1-methylxanthine
IgY	Immunoglobulin Y
IP3	Inositol 1,4,5-trisphosphate
LH	Lutenizing hormone
LHCGR	Luteinizinghormone/choriogonadotropin receptor
MAPK	Mitogen-activated protein kinase
ME	Median eminence
MF	Mucosal fold
p234	Peptide 234
PIP2	Phosphatidylinositol bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
POA	Preoptic area
RP-cAMPS	Rp-Adenosine 3′,5′-cyclic monophosphorothioate
RS	Round spermatid
RXFP2	Relaxin/insulin-like family peptide receptor 2
SER	Smooth endoplasmic reticulum

Abbreviation	Meaning
SER	Smooth endoplasmic reticulum
ST	Seminiferous tubule
StAR	Steroidogenic acute regulatory protein
TDF	Testicular determining factor
TRPC	Transient receptor potential cation
wpp	Weeks postpartum