EVALUATION OF THE EXPRESSION OF LIN28A AND LIN28B WITHIN THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS

by

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EVALUATION OF THE EXPRESSION OF LIN28A AND LIN28B WITHIN THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS

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ABSTRACT

The genes that regulate pubertal timing in the general population are not well understood. Recently, genome-wide association studies have demonstrated that genetic variants near LIN28B associate with variation in pubertal timing in humans. To investigate where within the hypothalamic-pituitary-ovarian (HPO) axis Lin28b, and its homologue Lin28a, regulate pubertal timing, expression of these genes was assessed across the pubertal transition. The finding that Lin28a/b expression decreases only in the ovary suggests that the Lin28 pathway may exert its regulatory effects with respect to puberty in the ovary. Another aim of this thesis was to examine the effect of estrogen on Lin28b expression in immortalized GnRH neuronal cells, but the data remains equivocal and detailed future studies are needed to make definitive conclusions. The ovarian expression data lay the foundation for further studies using conditional knockout mice to verify the importance of the tissue and age specific developmental pattern that was identified.
I’d like to thank, first and foremost, my supervisor Dr. Mark Palmert, whose guidance during my Master’s thesis has been invaluable. He has taught me not only how to be a better scientist, but also how to succeed outside the realm of research. Second, thanks must go out to Elizabeth Rodgers – without her none of my committee meetings would have come to fruition. I’d also like to thank my committee members, Dr. Denise Belsham and Dr. Annie Huang, for their support and assistance.

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<table>
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<th>Definition</th>
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<tbody>
<tr>
<td>AAM</td>
<td>age at menarche</td>
</tr>
<tr>
<td>ACC</td>
<td>Animal Care Committee</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>C. elegans</td>
<td><em>Caenorhabditis elegans</em></td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>$C_T$</td>
<td>cycle threshold</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
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<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DPN</td>
<td>diarylpropionitrile</td>
</tr>
<tr>
<td>E$_2$</td>
<td>17$\beta$-estradiol</td>
</tr>
<tr>
<td>EGR-1</td>
<td>early growth response factor-1</td>
</tr>
<tr>
<td>ERs</td>
<td>estrogen receptors</td>
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<td>estrogen receptor alpha</td>
</tr>
<tr>
<td>ER$\beta$</td>
<td>estrogen receptor beta</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin releasing hormone</td>
</tr>
<tr>
<td>GPR54</td>
<td>G-protein-coupled receptor 54 (kisspeptin receptor)</td>
</tr>
<tr>
<td>GWA</td>
<td>genome wide association</td>
</tr>
<tr>
<td>hnRNPA1</td>
<td>heteronuclear ribonucleoprotein A1</td>
</tr>
<tr>
<td>HPG</td>
<td>hypothalamic-pituitary-gonadal</td>
</tr>
<tr>
<td>HPO</td>
<td>hypothalamic-pituitary-ovarian</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>LD</td>
<td>linkage disequilibrium</td>
</tr>
<tr>
<td>LIN28A</td>
<td><em>lin28</em> homologue A</td>
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</table>
**LIN28B**  *lin28* homologue B
miRNA  micro RNA
OCT-4  octamer-binding transcription factor-4
P₄  progesterone
PBS  phosphate buffered saline
PPT  propyl-pyrazole-triol
PTX-1  pan-pituitary activator of transcription-1
qRT-PCR  quantitative reverse-transcriptase PCR
QTL  quantitative trait loci
RISC  RNA-induced silencing complex
RNA  ribonucleic acid
RNase  ribonuclease
RT  reverse transcription
RT-PCR  reverse-transcriptase PCR
rtTA  reverse tetracycline transactivator
SEM  standard error of the mean
SF-1  steroidogenic factor-1
siRNA  short interfering RNA
snoRNA142  small nucleolar RNA142
SNPs  single nucleotide polymorphisms
SOX-2  (sex determining region Y)-box-2
SV40  simian virus 40
TCP  Toronto Centre for Phenogenomics
TEMED  tetramethylethylenediamine
TUTase  terminal uridylyltransferase
VNN  vomeronasal nerve
ZP3  zona pellucida 3
CHAPTER 1: INTRODUCTION
1. INTRODUCTION

Why does initiation of puberty for some females occur at 9 years of age, while others at 12 years of age? Variation in the onset of puberty has been shown to follow a nearly normal distribution (Palmert and Boepple, 2001), but the scientific community has yet to fully understand what regulates the timing. This thesis, in two parts, begins to identify the biology of the newly discovered regulators of pubertal timing, namely lin28 homologue A (LIN28A) and lin28 homologue B (LIN28B), through the use of both mouse tissues and immortalized cell lines. This introductory chapter provides a review of pubertal development, the hypothalamic-pituitary-ovarian (HPO) axis, and the genes LIN28A and LIN28B.

1.1 DEVELOPMENT AND MATURATION OF THE HPO AXIS

Although pubertal onset occurs during the juvenile period, development of the HPO axis within females begins much earlier. During embryogenesis, several important developmental processes, namely migration of gonadotropin releasing hormone (GnRH) neurons, pituitary organ development, and development of follicles within the ovaries, are required in order to produce a functional HPO axis. Once the components of the HPO axis have developed, initial activation of GnRH neurons and maturation of the ovarian tissue are required for the initiation of puberty and eventual attainment of reproductive capacity, respectively.

1.1.1 Migration and activation of GnRH neurons
GnRH neurons originate from the olfactory placode during embryonic development and then migrate to the preoptic nucleus in the rostral region of the hypothalamus (Krsmanovic et al., 1992; Merchenthaler et al., 1984). From the olfactory placode, GnRH neurons migrate caudally towards the brain by associating with the vomeronasal nerve (VNN). After successfully reaching their destination, GnRH neurons dissociate from the VNN and form into a scattered network known as the GnRH pulse generator (Yoshida et al., 1995). Evidence of this developmental phenomenon was first established by Schwanzel-Fukuda and Pfaff (1989), when they used both immunocytochemistry and autoradiography in fetal mice to map the migratory path of GnRH neurons. Recent studies have confirmed this migration; one of which used green fluorescent protein (GFP)-GnRH transgenic mice to map the migratory path of GnRH neurons in real-time (Bless et al., 2005).

Pubertal onset is highlighted by activation of GnRH neurons within the hypothalamus, which leads to episodic secretion of GnRH, and eventual release of gonadotrophic hormones from the pituitary and gonadal maturation (Knobil et al., 1981; reviewed in Krsmanovic et al., 2009; Krsmanovic et al., 1992; Smith and Vale, 1981). This phenomenon was initially identified by Boyar et al. (1972), where pubertal children showed an increase in the pulsatile release of GnRH, which was not apparent in pre-pubertal children. Further to this, Boyar et al. (1974) identified that the increase in GnRH release in pubertal boys stimulate release of testosterone, demonstrating the importance of GnRH neurons and the central nervous system.
with respect to gonadal maturation and development of secondary sexual characteristics.

What controls activation of GnRH neurons, however, is largely unknown. Some studies have suggested that the control of pubertal timing, and thus activation of GnRH neurons, is mediated through a complex and hierarchal gene network. These networks are composed of upstream genes that regulate and control the expression of genes further down in the hierarchal network, and thus these “master regulators” control the onset of pubertal timing (Ojeda et al., 2010). Central to this hierarchal gene network is the gene encoding for the master hormone GnRH.

Evidence of a possible regulator of pubertal timing was discovered in 2003, when it was reported that humans with mutations in the kisspeptin receptor (GPR54) and transgenic mice lacking expression of GPR54 displayed defective sexual development (Seminara et al., 2003). Moreover, a publication by Irwig et al. (2004) discovered that intracerebroventricular administration of kisspeptin-52 activated GnRH neurons, suggesting that these neurons are a direct target of the kisspeptin-GPR54 pathway. One group further established the kisspeptin-GPR54 pathway as a key regulator of pubertal timing by establishing that kisspeptin-producing neurons in the hypothalamus project to and activate GnRH neurons (Han et al., 2005). However, recent evidence has suggested that the kisspeptin-GPR54 pathway does not regulate the onset of pubertal timing, but rather the pathway itself is activated
by developmental changes in estradiol (Clarkson et al., 2009), and is therefore likely not a higher order gene. Although other higher order genes are currently unknown, given that the lin28 family functions as an important developmental regulator in the nematode Caenorhabditis elegans and in mammals (described in section 1.3), they may play an important role in modulating pubertal timing.

1.1.2 Development of the pituitary tissue during embryogenesis

The fully developed pituitary gland is derived from two separate ectodermal tissues, namely the oral and neural ectoderm, which give rise to the anterior and posterior pituitary, respectively (Eagleson et al., 1990; Osumi-Yamashita et al., 1994). Initial invagination of these ectodermal layers leads to the formation of the primordial pituitary gland, or Rathke’s pouch (Jacobson et al., 1979). Through signaling gradients working both in concert and in opposition, distinct expression patterns of various transcription factors are established within Rathke’s pouch, initiating the formation of the various pituitary cell types (Ericson et al., 1998; Takuma et al., 1998; Treier et. al, 1998; Treier and Rosenfeld, 1996). For proper development of gonadotropin producing cells of the anterior pituitary, expression of steroidogenic factor-1 (SF-1), early growth response factor-1 (EGR-1), and the pan-pituitary activator of transcription-1 (PTX-1) are required (Ingraham et al., 1994; Lee et al., 1996; Szeto et al., 1999; Tremblay et al., 1998, Tremblay and Drouin, 1999). During the final stages of development, these cell types express differentiation markers in
distinct temporal and spatial patterns, ultimately leading to maturation of the pituitary tissue (Treier et al., 2001).

1.1.3 Ovarian tissue development and maturation

The ovarian tissue undergoes a process known as follicular assembly during embryogenesis, where the ooctyes become engulfed by a single layer of squamous granulosa cells, and form the primordial follicles (Lintern-Moore and Moore, 1979). These primordial follicles remain static until transitioning into primary follicles, where the granulosa cells adopt a cuboidal (cube-like) morphology (Hirshfield, 1991; Lintern-Moore and Moore, 1979). These primary follicles continue to grow and develop into secondary follicles, which become surrounded by multiple layers of cuboidal granulosa cells (Hirshfield, 1991; Kezele and Skinner, 2003; Skinner, 2005). Up until this point, this entire process is gonadotropin independent (Richards et al., 1976), and prior to pubertal onset, these follicles undergo a controlled degenerative process known as atresia (Hirshfield, 1991). After pubertal onset and activation of GnRH neurons and secretion of the gonadotropins from the anterior pituitary, the tertiary follicles are able to fully mature, leading to ovarian maturation and attainment of reproductive capacity (Chun et al., 1996; McGee et al., 1997).

Ovarian maturation is evidenced by the ability of the ovary to secrete estrogen (Ojeda and Skinner, 2006). Estrogen secretion from the ovary has both a positive
and negative effect on GnRH release from the hypothalamus and gonadotropin release from the pituitary, and this can be illustrated during the female menstrual cycle. During the late follicular phase of the menstrual cycle, the dominant tertiary follicle secretes increasing amounts of estrogen, leading to a sustained rise in circulating estrogen levels (Van Dessel et al., 1996). This rise stimulates a preovulatory surge in both GnRH from the hypothalamus and gonadotropin release from the pituitary tissue (Moenter et al., 1990; Sarkar et al., 1976). This surge, in turn, promotes ovulation of the oocyte within the follicle. The remaining follicles degenerate via atresia (Chun et al., 1996; McGee et al., 1997). During the rest of the menstrual cycle, low levels of estrogen negatively feedback on the hypothalamus and pituitary (Caraty, et al., 1989; Evans et al., 1994). Given that estrogen plays an essential role in the regulation of the female HPO axis centrally, an important goal of this thesis was to investigate whether estrogen modulates expression of the newly identified HPO axis regulators, *Lin28a* and *Lin28b* (see section 1.2.1), in hypothalamic GnRH neurons.

### 1.1.4 Secondary sexual characteristics that are evidence of pubertal onset

Although pubertal onset is a result of GnRH neuronal activation, several secondary sexual characteristics can be used to document pubertal onset in females indirectly. The earliest characteristic of pubertal development in females is the initiation of breast development (Tanner State B2), or thelarche (Marshall and Tanner, 1969), and is usually characterized by a physician (Hergenroeder et al., 1999). Pubic hair
development, or adrenarche, is attributed to a rise in circulating levels of androgens (Marshall and Tanner, 1969) from either the adrenal glands or the ovaries (Parent et al., 2003). The onset of first menstruation, or age at menarche (AAM), usually occurs 2 – 3 years after the onset of pubertal development (Marshall and Tanner, 1969), and is another characteristic of pubertal onset in females. Measuring AAM usually relies on retrospective assessment using the recall method (Henneberg and Louw, 1995; Parent et al., 2003). AAM in the general population is variable and normally distributed, and average AAM varies depending on the population of females; this varying onset is exemplified in several studies that documented AAM, where the average ranged from 12.4 – 13.6 years (Elks et al., 2010 He et al., 2009; Ong, et al., 2009; Perry et al., 2009; Sulem et al., 2009; Towne et al., 2005).

1.2 PUBERTAL TIMING IS A GENETICALLY INHERITED TRAIT

Within the general population, timing of puberty is quite variable and is believed to be affected by both environmental and physiological factors (Kaprio et al., 1995; Zacharias and Wurtman, 1969). It is also evident, however, that similarity in pubertal timing exists within ethnic groups, families and monozygotic twins, which provides evidence that that onset of puberty is a genetically inherited trait (Herman-Giddens et al., 1997; Zacharias and Wurtman, 1969). Indeed, various reports have suggested that 50 – 80 % of the variation in pubertal timing can be explained by genetic factors (Fischbein et al., 1977; Kaprio et al., 1995; Loesch et al., 1995; Meyer et al., 1991; Sklad, 1977). However, despite this high level of
heritability, little is known about the genetic regulation of the variation in pubertal timing.

Discovering which genes regulate normal variation in pubertal timing within the general population has proven difficult. Two methods commonly used to discover genes that may regulate normal pubertal timing are candidate gene-based association studies and linkage analyses. The former approach uses common variants, or single nucleotide polymorphisms (SNPs), and determines if these SNPs correlate with variation in pubertal onset. One key requirement is that one must select certain SNPs to perform analyses on, and therefore requiring previous knowledge regarding genes and pathways. The latter approach scans the whole genome to identify quantitative trait loci (QTL) that may contain genes of interest (Gajdos et al., 2010). Although successful in identifying genes and pathways that regulate single gene disorders such as Kallman syndrome (Franco et al., 1991; Hardelin et al., 1993; Hardelin et al., 1992) these methods of gene identification have not proven fruitful in discovering genes that regulate normal variation in pubertal timing in the general population.

1.2.1 LIN28B is the first gene to associate with timing of puberty in humans

1.2.1.1 Discovery of LIN28B through genome-wide association (GWA) studies

Recently, four independent groups identified genetic variations near LIN28B to associate with AAM in females in the human population through the use of GWA
studies (He et al., 2009; Ong et al., 2009; Perry et al., 2009; Sulem et al., 2009). The groups scanned hundreds of thousands of SNPs throughout the entire genome in tens of thousands of women, and assessed whether the SNPs associate with AAM. Several SNPs reached genome wide significance, after whole genome correction. A group of SNPs were localized to the 9q31.2 region of the genome, which is an intergenic region with no known genes. A second clustering of SNPs was observed in the 6q21 region of the human genome, where LIN28B is located. A schematic of the SNPs within region 6q21, from all four studies, can be seen in Figure 1.1. Six of the SNPs localized to intron 2 of the LIN28B gene, while eight SNPs localized upstream of the LIN28B gene. All of these SNPs were in linkage disequilibrium (LD) and had an $r^2 > 0.94$, which can be seen in Figure 1.2. The SNPs were within the same LD block of about 100 kb, suggesting that they represented the same signal. A recent publication, where all four of these studies were combined into a meta-analysis totaling 87,802 women, confirmed the 6q21 region of the genome as associating with AAM in women, where $p < 5.4 \times 10^{-60}$ (Elks et al., 2010).
The single nucleotide polymorphisms (SNPs) discovered from recent genome-wide association studies localized to region 6q21 of the genome, which contains the LIN28B gene. Six SNPs were located within intron 2 of LIN28B, while eight SNPs were located upstream of LIN28B. Adapted from the International HapMap Project.
Figure 1.2: A linkage disequilibrium plot of the SNPs located within the 6q21 region of the human genome.

The SNPs that associated with age at menarche in the four recently published genome-wide association studies in *Nature Genetics* are plotted in this diagram, using Haploview. The SNPs are in linkage disequilibrium, ($r^2>94\%$) and are part of the same LD block of about 100 kb, suggesting they represent the same signal.
1.3 LIN28B AS A DEVELOPMENTAL REGULATOR

Although previously having no known link to the regulation of pubertal timing, LIN28B, and its homologues, had been implicated previously in regulation of development. Within the nematode *C. elegans* for example, *lin28* acts at a herteochronic gene and controls developmental timing within its four larval stages (Moss et al., 1997; Yang and Moss, 2003). More recently in mammals, both *LIN28A* and *LIN28B* have been shown to regulate pluripotency and differentiation (Ji and Wang, 2010; Viswanathan and Daley, 2010).

1.3.1 Initial discovery of lin28 in *C. elegans*

Within the nematode *C. elegans*, the heterochronic genes control developmental timing by regulating cell fates within each of its four larval stages. *lin28* is a heterochronic gene that regulates cell fates and developmental events specific to the second larval stage of development (Moss et al., 1997; Reinhart et al., 2000; Yang and Moss, 2003). When *lin28* expression is altered, the nematode undergoes altered developmental timing (Ambros and Horvitz, 1984; Moss et al., 1997; Yang and Moss, 2003). Specifically, when there is a gain-of-function mutation, leading to an increase in *lin28* expression, the *C. elegans* has delayed development. This is shown in Figure 1.3, where events specific to the second stage of development are reiterated and transition into the third stage of development is delayed. Conversely, when the nematode undergoes a loss-of-function mutation, where expression of *lin28* is reduced, events specific to the second larval stage are skipped, and events of the
third larval stage occur earlier than normal, resulting in precocious development (Moss et al., 1997; Yang and Moss, 2003). For example, in *C. elegans*, the pattern of division of the lateral hypodermal cells usually occurs in the third larval stage; however, with a loss-of-function mutation, this event occurs in the second larval stage, thereby resulting in earlier than normal larval stages (Moss et al., 1997).
*lin28* gain-of-function Mutation

*lin28* loss-of-function Mutation

**DELAYED DEVELOPMENT**

**PRECOCIOUS DEVELOPMENT**

Figure 1.3: *lin28* acts as a key developmental regulator in the nematode *C. elegans*.

Within the nematode *C. elegans*, larval development proceeds through four stages. If there is a gain-of-function mutation in *lin28*, events specific to the 2nd larval stage are reiterated, leading to delayed transition into the 3rd larval stage and delayed development. With a loss-of-function mutation in *lin28*, events specific to the 2nd larval stage are skipped and the nematode proceeds directly to the third larval stage, resulting in precocious development. Adapted from Moss et al., 1997.
1.3.2 *LIN28B* as a developmental regulator in mammals

Despite being located on different chromosomes, namely chromosomes 1 and 6, *LIN28A* and *LIN28B* are functionally redundant cytoplasmic RNA binding proteins (Balzer and Moss, 2007; Moss et al., 1997), as evidenced by the similarity of their RNA binding domains, namely the N-terminal cold-shock domain and a pair of C-terminal zinc finger domains (Guo et al., 2006). Both *LIN28A* and *LIN28B* have been implicated in a number of developmental processes in mammals. Firstly, *LIN28A* was used in combination with a variety of factors, namely *OCT4, SOX2, NANOG*, to reprogram adult human somatic cells into induced pluripotent stem (iPS) cells (Yu et al., 2007). Related to this, the expression of *Lin28a* and *Lin28b* are high in embryonic stem cells, and persist at high levels in the embryonic mouse until day 10.5 (Yang and Moss, 2003). Moreover, one study demonstrated that repressing *Lin28a* expression in mouse embryonic stem cells leads to decreased cell proliferation, while over expression of *Lin28a* leads to accelerated cell proliferation (Xu et al., 2009). Together, these studies highlight *LIN28A* and *LIN28B* as important regulators of pluripotency, differentiation and proliferation.

In addition to controlling normal differentiation, *LIN28A* and *LIN28B* have been implicated in a variety of cancers. One study found that both *LIN28B* mRNA and protein levels are elevated in human hepatocellular carcinoma (HCC) tissues and in a variety of HCC cell line models, when compared to normal tissue (Guo et al., 2006). Moreover, both *LIN28A* and *LIN28B* were detected in epithelial ovarian cancers (Lu
et al., 2009). Finally, Viswanathan et al. (2009) discovered that both \textit{LIN28A} and \textit{LIN28B} were over expressed in approximately 15 \% of primary human tumours and in cancer cell lines, particularly those with poor differentiation and prognosis. In addition, these tumours had relatively low levels of mature \textit{Let-7} miRNA, presumably due to elevated levels of \textit{LIN28A/LIN28B} (see section 1.3.4). Therefore, there is some evidence to suggest that pluripotency, developmental timing, and oncogenesis are regulated by a similar \textit{LIN28/Let-7} signalling pathway (Ji and Wang, 2010; Viswanathan and Daley, 2010).

\textbf{1.3.3 Expression profile of \textit{LIN28A} and \textit{LIN28B} in adulthood}

Although established as developmental regulators, the expression profile of \textit{LIN28A} and \textit{LIN28B} in normal adult tissue is largely unknown. In the developing mouse, \textit{Lin28a} and \textit{Lin28b} have high levels of expression up until embryonic day 10.5. Into adulthood, their expression patterns diverge, where \textit{Lin28a} is largely restricted to the skeletal muscle, heart, intestinal crypts, ovary (Yang and Moss, 2003), and testis (Zheng et al., 2009), and \textit{Lin28b} is expressed in the blood, adult brain, testis, fetal liver and placenta (Guo et al., 2006). To better understand the roles of \textit{Lin28a} and \textit{Lin28b} in pubertal development, examining their expression more fully within the HPO axis of female mice was one focus of this thesis.

\textbf{1.3.4 LIN28 prevents maturation of \textit{Let-7} microRNA (miRNA)}
Regulation of development by the LIN28 family of proteins is mediated largely through repression of Let-7 miRNA maturation (Viswanathan and Daley, 2010). In mice and humans, Let-7 miRNAs are a fourteen and thirteen-member family of noncoding RNAs, respectively (Lagos-Quintana et al., 2001; Roush and Slack, 2008), of about 20 – 24 nucleotides in length, that work to repress the expression of target genes and play an important role in regulating developmental pathways (Reinhart et al., 2000). Let-7 miRNAs are initially transcribed within the nucleus, by RNA polymerase II, as an immature primary Let-7 transcript. This transcript is then recognized by the RNA-binding Microprocessor complex (Denli et al., 2004; Gregory et al., 2004), which contains the RNase III enzyme DROSHA that subsequently processes the transcript into a 60-80 nucleotide hairpin structure (Lee et al., 2003) known as pre-Let-7 miRNA, as demonstrated in Figure 1.4. This pre-Let-7 miRNA then gets exported from the nucleus into the cytosol by exportin 5 (Bohsack et al., 2004; Lund et al., 2004; Yi et al., 2003), at which point it gets recognized and cleaved by the DICER ribonuclease III enzyme, to yield mature double-stranded 20 – 24 nucleotide miRNAs duplexes (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001). At this point, the Let-7 miRNA duplex binds to the RNA-induced silencing complex (RISC). One strand of the duplex remains stably bound to the RISC complex (Schwarz et al., 2003), and this miRNA-RISC complex drives the other strand to target and repress gene expression at both the mRNA (Zeng et al., 2002) and protein level (Olsen et al., 1999).
LIN28A and LIN28B proteins work to reduce the expression of *Let-7* miRNA by preventing its maturation, and this was shown by both Viswanathan et al. (2008) and Piskounova et al. (2008), where mature *Let-7* miRNA transcripts were down-regulated when LIN28A was over-expressed. Further studies by Hagan et al. (2009) and Heo et al. (2008) demonstrated that LIN28A and LIN28B target *Let-7* miRNA within the cytoplasm, prior to maturation from pre-*Let-7* to the *Let-7* miRNA duplex. Instead of normally getting processed by DICER, the pre-*Let-7* miRNA transcript is terminally uridylated by the terminal uridylyltransferase (TUTase) Zcchc11, which is recruited by LIN28A and LIN28B. A schematic of this process is displayed in Figure 1.4. This UTP-dependant process prevents processing of pre-*Let-7* miRNA by DICER, and is instead targeted for degradation (Hagan et al., 2009; Heo et al., 2008). Therefore, LIN28A and LIN28B proteins work to prevent the maturation of *Let-7* miRNA, which in turn, prevents *Let-7* miRNA mediated repression of gene expression.
Let-7 miRNA is initially produced in the nucleus as a primary Let-7 transcript, and gets processed to pre-Let-7 miRNA by DROSHA and its associated proteins. Pre-Let-7 miRNA is then exported out of the nucleus and into the cytoplasm. Normally, pre-Let-7 miRNA is recognized by DICER, and is processed into 20-24 nucleotide fragments. These fragments associate with RNA-induced silencing complexes (RISC), which act to decrease expression of target genes. Lin28 acts to prevent pre-Let-7 maturation by promoting terminal uridylation, by recruiting a 3' terminal uridyl transferase (TUTase). Adapted from Hagan et al., 2009.
1.4 ALTERING LIN28A EXPRESSION ALTERS PUBERTAL TIMING IN MICE

To investigate whether \textit{Lin28b} was a causal gene with respect to pubertal timing, a collaboration was initiated with Dr. George Daley’s laboratory, where a transgenic mouse model designed to over express \textit{Lin28a}, a homologue of \textit{Lin28b}, was utilized. These mice were designed with a doxycycline inducible reverse tetracycline transactivator (rtTA) under the expression of the \textit{Rosa26} locus, and the \textit{Lin28a} gene under the direction of the \textit{Collagen 1a1} locus. However, even in the absence of doxycycline-induced rtTA activation, these transgenic mice over-expressed \textit{Lin28a}, and represented a global model of \textit{Lin28a} over-expression. For the collaborative studies, these transgenic mice were characterized for a variety of pubertal markers, which are described below (Zhu et al., 2010).

The female transgenic mice were assessed for timing of vaginal opening, a commonly used marker of pubertal timing in female mice. In transgenic mice, an approximate 2-day delay in the timing of vaginal opening was observed, when compared to wild type mice, suggesting an overall delay in the timing of pubertal onset. Prior to vaginal opening, transgenic and wild type mice were also assessed for ovarian and uterine weights to determine if they had altered sexual development. Despite the overall increased size into adulthood, transgenic mice had lower ovarian and uterine weights at 26 days of age, also indicating a delay in sexual development and maturation. The transgenic mice also displayed delayed age at first estrus, when compared to wild-type mice. Finally, the transgenic mice
displayed delayed first litter and larger litter sizes when compared to wild-type mice (Zhu et al., 2010).

Larger facial structure, coarser hair, and increased crown-rump length were also observed in transgenic mice when compared to wild type mice. Interestingly, these size differences were observed after weaning, suggesting transgenic mice were able to grow for longer periods of time. It is also important to note that the body fat percentage of the transgenic and wild type mice was similar, indicating there was no change in body composition (Zhu et al., 2010).

Results from this study verified that $LIN28B$ was the causal gene underlying the associations that were found in the GWA studies, and demonstrated that the $LIN28A/LIN28B$ pathway is important for regulation of puberty. Interestingly, the phenotypes observed in the transgenic mice were similar to the phenotypes observed in the gain-of-function mutants in *C. elegans* (see section 1.3.1), where these mutants displayed delayed development (Moss et al., 1997; Yang and Moss, 2003). Moreover, when analyzing the expression pattern of *Lin28a* in the transgenic versus wild type mice, an increase in expression was localized to various tissues, including the hypothalamus and ovary. Specifically, expression of *Lin28a* within hypothalamic and ovarian tissue increased by 4-fold and 5-fold, respectively, when compared to wild-type mice (Zhu et al., 2010). This suggests that the locus (loci) of
control for Lin28a/Lin28b regulation of puberty may be either the hypothalamus or ovary or both within the HPO axis.

1.5 OVERVIEW OF THESIS

1.5.1 Overall aims and hypotheses

In this thesis, these initial data were followed up in two separate chapters. In chapter 2, the patterns of Lin28a and Lin28b expression was analyzed across the pubertal transition in the HPO axis in female mice to determine the site(s) where Lin28a and Lin28b exert their effects on pubertal timing. It was hypothesized that both Lin28a and Lin28b expression would decrease across the pubertal transition within the HPO axis, and discovering where the decrease was localized to would offer insight into a possible locus of control for pubertal timing. In chapter 3, the effect of 17β-estradiol (E2) on Lin28b expression was investigated in immortalized GnRH neurons, in order to determine if estrogen from the ovaries regulate Lin28b in those neurons. It was hypothesized that E2 would enhance expression of Lin28b within these immortalized GnRH neurons.

1.5.2 Models used in this thesis

In chapter two, tissues derived from inbred C57Bl/6 female mice were used to analyze the expression of Lin28a and Lin28b across the pubertal transition. Mouse tissues were used in this chapter because, like humans, puberty has been shown to be regulated by the Lin28 pathway (Zhu et al., 2010). In addition, since the tissues
were derived from genetically identical mice, the confounding effect of genetic variation on pubertal timing was reduced. Moreover, even though mice and humans exhibit some differences (Ebling, 2005), they share common reproductive pathways (Beier and Dluhy, 2003). Therefore, results obtained from chapter 2 can likely be applied to pubertal timing in humans.

In chapter three, two clonal immortalized cells lines were used in order to investigate the effect of E$_2$ on Lin28b expression in GnRH neuronal cells. GT1-7 cells were derived via targeted oncogenesis using the simian virus 40 T-antigen (SV40 T-antigen) under the direction of the rat GnRH promoter. Various hypothalamic tumours formed, and GT1-7 neuronal cells were established via serial dilution (Mellon et al., 1990). GN11 cells were also derived from targeted oncogenesis using the SV40 T-antigen, although under the direction of the human GnRH promoter. Expression of SV40 T-antigen resulted in migratory arrest of GnRH neurons and formation of olfactory bulb tumours along the migratory pathway. Similar to GT1-7s, GN11 cells were established via serial dilution (Radovick et al., 1991). Although these cell lines represent single, isolated, clonal populations of GnRH neurons, they are useful for investigating the direct effect of E$_2$ on Lin28b expression.
CHAPTER 2: EXPRESSION OF LIN28A AND LIN28B DECREASE ACROSS THE PUBERTAL TRANSITION
2.1 OBJECTIVE AND HYPOTHESIS

Given that over-expression of lin28 in the nematode C. elegans resulted in delayed development (Moss et al., 1997; Yang and Moss 2003), and delayed puberty was observed in transgenic mice designed to over express Lin28a (Zhu et al., 2010), it was hypothesized that Lin28a and Lin28b expression would decrease across the pubertal transition. Moreover, it was reasoned that identifying where within the hypothalamic-pituitary-ovarian (HPO) axis Lin28a/b decreased may provide an indication of the site(s) where Lin28a/b exerts its effects that regulate the timing of puberty. Therefore the objective in this chapter was to determine the expression of Lin28a and Lin28b across the pubertal transition in the hypothalamus, pituitary, and ovary in female mice.

2.2 METHODS

2.2.1 Animals

C57Bl/6 mice used in this study were housed at the Toronto Centre for Phenogenomics (TCP) in a controlled lighting (12 h light/dark cycle) and temperature environment and were given ad libitum access to mouse chow (Tekland Standard Chow, Harland Laboratories, Mississauga, ON, CAN) and water, as described previously (Mastronardi et al., 2006). The Animal Care Committee (ACC) at the TCP approved all experimental protocols.
2.2.2 Collection of tissues for quantitative reverse-transcriptase PCR (qRT-PCR) and immunohistochemistry (IHC)

On the day of collection, C57Bl/6 female mice were euthanized with carbon dioxide (CO₂) for 1 – 2 minutes followed by cervical dislocation and rapid decapitation.

For qRT-PCR, hypothalamic, pituitary, and ovarian tissue were collected at 20, 25, 30, 35, and 45 days of age from female C57Bl/6 mice. For hypothalamic tissue, the whole brain was removed and placed ventral side up, where an en bloc dissection approach was taken with the following boundaries: rostral; posterior to the optic chiasm, lateral; the choroidal fissures, and caudal; the rostral pole of the mammillary bodies (Baker et al., 1983). Hypothalamic tissue was excised via a 2 mm cut from the ventral side of the brain as described by Baker et al. (1983). After collection, tissues were stored in RNAlater® (Ambion, Foster City, CA, USA) at 4°C for 24 hours, followed by long-term storage at -20°C. This reagent was used to denature proteins, including RNases, and preserve RNA integrity. For immunohistochemistry, ovarian tissue at 20 and 30 days of age were collected and stored in neutral-buffered formalin at room temperature for 24 hours, followed by long-term storage in 70% ethanol at 4°C.

2.2.3 RNA extraction

Tissues were removed from RNAlater® and placed in either 600 µL of Buffer RLT (Qiagen, Mississauga, ON, CAN) or 700 µL of QIAzol® (for Let-7 miRNA targets) and
were homogenized using sonication. Total RNA extraction was performed using a modified guanidine isothiocyanate and silica membrane isolation procedure with Qiagen’s RNeasy® Mini Kit or Qiagen’s miRNeasy Mini Kit (for Let-7 miRNA targets), following the manufacturer’s recommended protocol, with an on-column DNase digestion using Qiagen’s RNase free DNase Set. RNA concentrations were quantified using ultraviolet-visible spectroscopy, and the integrity of total RNA was assessed using a denaturing formaldehyde agarose gel (1.2 % (w/v) agarose, 10 % (v/v) MOPS buffer, 3 % (v/v) formaldehyde, and 0.05 mg/mL ethidium bromide).

2.2.4 Two-step qRT-PCR

qRT-PCR for Lin28a/b Targets.

Either 1250 ng (for hypothalamic and ovarian tissue) or 385 ng (for pituitary tissue) of total RNA was reverse transcribed into cDNA using the SuperScript® III Reverse Transcriptase First Strand System (Invitrogen, Carlsbad, CA, USA). For standard curves, either 5000 ng or 1925 ng of pooled RNA from hypothalamic or ovarian tissues were used, and were serially diluted (see below). Less total RNA was used for standard curves to quantify expression within pituitary tissue since the RNA yield was lower than ovarian and hypothalamic tissue.

After reverse transcription, samples were diluted in RNase free H₂O (1:5), and either 50 ng (hypothalamic and ovarian tissue) or 25 ng (pituitary tissue) was used. Samples and standards were run in triplicate on 96-well qRT-PCR plates. Standard
curves were generated by diluting hypothalamic and ovarian standard tissues from 1:10 to 1:10000 (200 - 0.02 ng for hypothalamic and ovarian tissue, 125 – 0.0125 ng for pituitary tissue). Quantification of initial template quantity was performed using the standard curve method. Gene specific primers were designed to span exon-exon boundaries. The primers used for qRT-PCR (Lin28a, Lin28b, and β-actin) are listed in Table 2.1. A total volume of 20 µL was used, where each reaction contained 4 – 6.5 µL of reverse-transcribed sample or standard, 10 µL of SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 600 nM of forward and reverse Lin28a/b primers, or 50 nM of forward and reverse β-actin primers. The real-time polymerase chain reaction was performed using the following conditions:

1. Initial 10 minute denaturation step at 95°C
2. 40 cycles (denaturation, annealing/extension): 95°C for 15 seconds and 60°C for 1 minute
3. Dissociation curve segment: 95°C for 1 minute, 60°C for 30 seconds, and 95°C for 30 seconds
Table 2.1: Primers used for RT-PCR and qRT-PCR.

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>Amplicon (bp)</th>
<th>Cross-intron?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lin28a</td>
<td>F 5’-AGGCGGTGGAGTTCACCTTTAAGA-3’</td>
<td>190</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>R 5’-AGCTTTGCATTCTTCCTTGCGATGATGG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lin28b</td>
<td>F 5’-CAACATGGGCGAAAGCGGGG-3’</td>
<td>123</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>R 5’-CCCATGCGACGTTGACCA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>F 5’-CCACACCGGGCCACAGTTTCG-3’</td>
<td>112</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>R 5’-TACAGCGCGGGGAGCATGT-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
An example of the raw data from qRT-PCR is depicted in Figure 2.1, 2.2 and 2.3. In Figure 2.1, 2.2, and 2.3 A, the amplification plots of Lin28a, Lin28b, and β-actin are shown in one set of samples, where 20-day-old ovarian tissue is denoted by the blue line, and 25-, 30-, 35- and 45-day-old ovarian tissue are denoted by the red, green, grey, and yellow lines, respectively. The amplification plots for Lin28a and Lin28b show that 20-day-old tissue had more initial template quantity, where the fluorescence level significantly increased above background and reached the threshold earlier, translating into a smaller cycle threshold (Ct), which corroborates what was observed in Figure 2.6 C. Initial template quantity for β-actin mRNA within the variously aged ovarian tissue was similar, as evidenced by the similar (Ct) values (19.76, 19.80, 19.23, 20.00, and 20.16 for 20-, 25-, 30-, 35- and 45-day-old tissue, respectively). In addition, dissociation curve for Lin28a, Lin28b, and β-actin had a single peak, centred around 84–86°C, 84–88°C, and 86–90°C respectively, as depicted in Figures 2.1, 2.2, and 2.3 B, which indicated single products specific to Lin28a, Lin28b and β-actin were amplified.
Figure 2.1: Amplification plot and dissociation curve of Lin28a mRNA in variously aged ovarian tissue.

In (A), the amplification plot shows the various ages in one set of samples (20, 25, 30, 35, and 45 days corresponds to the blue, red, green, grey and yellow line, respectively). The fluorescence in the 20-day-old tissue increases above the threshold level earlier than 25-, 30-, 35-, and 45-day-old tissue, therefore translating into a lower C_T value and higher initial template quantity (24.45 versus 28.10, 26.94, 27.49, and 26.74, respectively), which corroborates with what was observed in Figure 2.6. In (B), one peak is observed in the dissociation curve (at 84-86°C), suggesting a single product specific to Lin28a was amplified.
In (A), the amplification plot shows the various ages in one set of samples (20, 25, 30, 35, and 45 days corresponds to the blue, red, green, grey and yellow line, respectively). The fluorescence in the 20-day-old tissue increases above the threshold level earlier than 25-, 30-, 35-, and 45-day-old tissue, therefore translating into a lower $C_T$ value and higher initial template quantity (29.35 versus 31.73, 31.38, 32.43 and 31.75, respectively), which corroborates with what was observed in Figure 2.6. In (B), one peak is observed in the dissociation curve (at 84-88°C), suggesting a single product specific to Lin28b was amplified.
Figure 2.3: Amplification plot and dissociation curve of β-actin mRNA in variously aged ovarian tissue.

In (A), the amplification plot shows the various ages in one set of samples (20, 25, 30, 35, and 45 days corresponds to the blue, red, green, grey and yellow line, respectively). The fluorescence in all of the tissues increases above the threshold at a similar cycle, therefore resulting in similar C_{T} value and similar initial template quantity (19.76, 19.80, 19.23, 20.00, and 20.16, respectively). In (B), one peak is observed in the dissociation curve (at 86-90°C), suggesting a single product specific to β-actin was amplified.
**qRT-PCR for Let-7 targets**

Five (5) nanograms of total RNA were reverse transcribed from variously aged ovarian tissue using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems), with specific reverse transcription (RT) primers designed to reverse transcribe *Let-7a* (Assay ID: 000377, Applied Biosystems), *Let-7g* (Assay ID: 002282, Applied Biosystems), and the housekeeping gene snoRNA142 (Assay ID: 001231, Applied Biosystems). For standard curves, 10 ng of RNA from 30-day-old ovarian tissue was used, with *Let-7a*, *Let-7g*, and snoRNA142 RT primers.

After reverse transcription, samples and standards were run on 96-well qRT-PCR plates in triplicate. Standard curves were generated by diluting the standards from 1:10 to 1:40 (10 - 0.25 ng input RNA). Quantification of mature *Let-7a* or *Let-7g* was performed using the standard curve method. A total volume of 20 µL was used, where each reaction contained 1.33 µL of reverse-transcribed sample or standard, 10 µL of TaqMan Universal PCR Master Mix no AmpErase® UNG (Applied Biosystems), and 1 µL of either 20x *Let-7a* (Assay ID: 000377, Applied Biosystems), *Let-7g* (Assay ID: 002282, Applied Biosystems), or housekeeping gene snoRNA142 (Assay ID: 001231, Applied Biosystems) TaqMan® MicroRNA Assay (Applied Biosystems). The real-time polymerase chain reaction was performed using the following conditions:

1. Initial 10 minute denaturation step at 95°C
2. 40 cycles (denaturation, annealing/extension): 95°C for 15 seconds and
An example of the raw data from qRT-PCR is depicted in Figure 2.4. In this figure, the amplification plot of snoRNA142 is shown in one set of samples, where 20-day-old ovarian tissue is denoted by the blue line, and 25 and 30-day-old ovarian tissue are denoted by the red and green lines, respectively. Initial template quantity for snoRNA142 within the variously aged ovarian tissue was similar, as evidenced by the similar (C<sub>T</sub>) values (27.80, 28.39, and 28.53 for 20-, 25-, 30-day-old tissue, respectively).
The amplification plot shows the various ages in one set of samples (20, 25, and 30 days corresponds to the blue, red, green lines, respectively). The fluorescence in all of the tissues increases above the threshold at a similar cycle, therefore resulting in similar $C_T$ value and similar initial template quantity (27.80, 28.39, and 28.53, respectively).
2.2.5 RT-PCR

Total RNA was extracted from hypothalamic, pituitary, and ovarian tissue using Qiagen’s RNeasy® Mini Kit, and reverse transcribed using the SuperScript® III Reverse Transcriptase First Strand System (Invitrogen), as described above. The no template control (NTC) in Figure 2.5 corresponds to the cDNA reagents in the absence of total RNA.

A total of 50-80 ng of cDNA were loaded with 0.625 units of Taq polymerase (Fermentas, Burlington, ON, CAN), 0.4 µM of forward and reverse primers for Lin28a/b or 0.1 µM of forward and reverse primers for β-actin, 0.4 mM of dNTP mix, 2 mM of MgCl₂, and 10x Taq buffer (with (NH₄)₂SO₄) (Fermentas). The polymerase chain reaction was performed using the following conditions:

1. 95°C for 2 minutes
2. 40 cycles (denaturation, annealing, extension): 95°C for 1 minute, 55°C for 1 minute, and 72°C for 90 seconds
3. 72°C for 5 minutes
4. HOLD at 4°C

The primers used to amplify targets are described in Table 2.1. Primer pairs were designed to span exon-exon boundaries (i.e. cross-intron). Amplified products were separated via polyacrylamide gel electrophoresis (100 mL 6 % acrylamide gel mixture, 100 µL TEMED, and 1000 µL 10 % APS) and visualized by ethidium bromide staining (1 mg/mL) followed by ultraviolet illumination.

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2.2.6 Immunohistochemistry (IHC)

Immunohistochemical staining was performed by the Centre for Modeling Human Disease (CMHD) at the Toronto Centre for Phenogenomics. Tissues were incubated with polyclonal antibodies specifically designed to recognize murine Lin28a protein (1:100, Cell Signaling Technology, Beverly, MA, USA; Catalogue Number 3978), for 24 hours at 4°C, followed by incubation with secondary goat anti-rabbit biotinylated antibody (1:200, Vector labs, Burlingame, CA, USA, catalogue number: BA-1000) for 30 minutes at room temperature. Tissues were then incubated with ABC reagent (1:50, Vector Labs, catalogue number PK-6100) and visualized with diaminobenzidine reagent. Samples were counterstained with haematoxylin. Two biological replicates at each time point were used.

2.2.7 Statistical analysis

For qRT-PCR, all time points (20, 25, 30, 35, and 45 days for Lin28a/b targets; 20, 25 and 30 days for Let-7a/g targets) were compared against each other within each tissue, and differences were analyzed by one-way Analysis of Variance (ANOVA), followed by the Bonferroni Multiple Comparison Test. Data are expressed as mean ± standard error of the mean (SEM), with n ≥ 3.

2.3 RESULTS

2.3.1 Expression of Lin28a and Lin28b in the female mouse
2.3.1.1 *Lin28a* and *Lin28b* are expressed in hypothalamic, pituitary, and ovarian tissue

Both *Lin28a* and *Lin28b* are expressed in 25-day-old hypothalamic, pituitary, and ovarian tissue (Figure 2.5 A). Using qRT-PCR, (Figure 2.5 B) it was determined that expression of *Lin28b* within hypothalamic tissue was 34 times higher than *Lin28a*, while expression of *Lin28b* within pituitary tissue was 2 times higher than *Lin28a*. Conversely, ovarian tissue had higher levels of *Lin28a* than *Lin28b*, where 62 times as much mRNA was observed. The relative ratio of *Lin28b* to *Lin28a* mRNA was stable over multiple ages (20, 25, 30, 35 and 45 days), as no statistical differences were detected.
Figure 2.5: Hypothalamic, pituitary, and ovarian tissue express Lin28a and Lin28b.

Expression of Lin28a (190 bp) and Lin28b (123bp) mRNA in 25-day-old hypothalamic (hypo), pituitary, and ovarian tissue from C57Bl/6 female mice as measured by (A) RT-PCR and (B) qRT-PCR, where Lin28a and Lin28b mRNA levels were normalized to β-actin, and the relative levels of Lin28b/Lin28a underwent a Log2 data transformation. Lin28b is more highly expressed than Lin28a in hypothalamic tissue and pituitary tissue, while Lin28a is more highly expressed than Lin28b in ovarian tissue. RNA was extracted from the tissues and was reverse-transcribed into cDNA and 50-80ng of cDNA template was used for RT-PCR. NTC = no template control.
2.3.1.2 Expression of *Lin28a* and *Lin28b* across the pubertal transition in hypothalamic, pituitary, and ovarian tissue

A recent publication demonstrated that over expression of *Lin28a* led to delayed pubertal onset in female mice, as measured by vaginal opening, time to first estrus, ovarian and uterine weights, and time to first pregnancy (Zhu et al., 2010). Moreover, expression of *Lin28a* was increased by 4-fold and 5-fold in hypothalamic and ovarian tissue, respectively, suggesting that the *Lin28* pathway may regulate the HPG axis by exerting effects in one or both of these tissues. Therefore, to determine any changes in expression, both *Lin28a* and *Lin28b* were measured across the pubertal transition in female C57Bl/6 female mice in these tissues. To assess the HPG axis fully, expression was also examined in the pituitary.

2.3.2 Hypothalamic tissue

*Lin28a* and *Lin28b* mRNA levels do not change across the time course

Hypothalamic tissue (20, 25, 30, 35, and 45 days) was collected from female C57Bl/6 mice, and mRNA levels of *Lin28a* and *Lin28b* were measured via qRT-PCR, which is displayed in Figure 2.6 A. Neither *Lin28a* nor *Lin28b* mRNA levels changed significantly across the time course, with differences being limited to statistically non-significant fluctuations in expression.
Figure 2.6: Lin28a and Lin28b mRNA expression levels in hypothalamic, pituitary, and ovarian tissue across the pubertal transition.

Expression of Lin28a and Lin28b mRNA in tissue (20, 25, 30, 35, and 45 days) from C57Bl/6 female mice, as measured by qRT-PCR, normalized to β-actin housekeeping gene. Average age of vaginal opening in the mouse colony is denoted by the red arrow, which is 31.5 ± 2.4 days. (A), neither Lin28a nor Lin28b mRNA levels change in expression across the pubertal transition in hypothalamic tissue. (B), Lin28a but not Lin28b mRNA levels significantly change in expression in pituitary tissue after the pubertal transition. (C), Lin28a and Lin28b significantly decrease in expression across the pubertal transition in ovarian tissue. Values are displayed as means ± SEM (n=6-8 for each time point). For Lin28a in pituitary tissue; p<0.001 (***), and p<0.01(**) when comparing 20 and 25 day old tissue to 45 day old tissue, respectively. For ovarian tissue, * = p<0.05, **=p<0.01, ***=P<0.001, when compared to 20 day old tissue.
2.3.3 Pituitary tissue

An increase in Lin28a but not Lin28b mRNA levels is observed after the pubertal transition

To determine if Lin28a and Lin28b levels change across the pubertal transition in the pituitary, tissue (20, 25, 30, 35, and 45 days) was collected, and mRNA levels were measured by qRT-PCR. As observed in Figure 2.6 B, Lin28b mRNA levels did not change significantly across the time course. However, Lin28a mRNA levels show a trend towards increasing expression across the time course. In particular, expression in 45-day-old tissue is significantly higher than both 20-day and 25-day-old tissue, at a p<0.001 and p<0.01, respectively. The observed increase in expression is after the timing of puberty in female C57Bl/6 female mice, which occurs at 31.5 ± 2.4 days in the colony.

2.3.4 Ovarian tissue

2.3.4.1 A decrease in mRNA levels was detected by qRT-PCR across the pubertal transition

The expression of Lin28a and Lin28b mRNA was measured in 20-, 25-, 30-, 35- and 45-day-old ovarian tissue, via qRT-PCR, as seen in Figure 2.6 C. With respect to Lin28b, a significant decrease is observed. Specifically, 25, 30, 35 and 45-day-old tissues had 42 %, 52 %, 61 %, and 54 % lower levels of Lin28b, when compared to 20-day-old tissue (p<0.05). A significant decrease was also observed with respect to
Lin28a, where 20, 25, 30, 35 and 45-day-old tissue had 56 %, 66%, 69%, and 70% lower expression levels than 20-day-old tissue, respectively (p<0.05).

### 2.3.4.2 Protein levels within the ovary decrease across the time course

To examine whether the decrease in mRNA levels within the ovarian tissue translates into a decrease in protein levels, IHC was used to examine Lin28a protein levels in 20-day-old and 30-day-old tissue. In Figure 2.7, Lin28a protein is identified by diaminobenzidine (brown) staining. From 20 days to 30 days (Figure 2.7 A and D), an overall decrease in Lin28a protein levels is observed, agreeing with what was found in Figure 2.6 C regarding expression level. Antibodies that are useful in IHC have not yet been successfully generated for Lin28b protein, so it was not possible to examine this protein within ovarian tissue.

In 20-day-old tissue (Figure 2.7 B and C), Lin28a protein is apparent within oocytes of early developing follicles (arrow # 1). Further, Lin28a protein persists in the oocytes within secondary follicles (arrows # 2, 3, and 4), which are surrounded by multiple layers of cuboidal granulosa cells. As the secondary follicles develop, the levels of Lin28a protein appear to decrease (arrow # 5), where the diaminobenzidine staining becomes less intense. The multi-layered cuboidal granulosa cells surrounding secondary follicles are also positive for Lin28a protein.
Therefore, these data suggest that Lin28a protein is evident within granulosa cells and within developing follicles, and the levels may decrease as the follicles develop.

As seen in Figure 2.7 E and F, there are atretic follicles that have lower levels of Lin28a protein in 30-day-old tissue (arrows # 7 and 8). This phenomenon was consistent in the two biological replicates for 30-day-old tissue, where an increased level of atretic follicles was observed when compared to 20-day-old tissue (see Figure 2.8 for other biological replicate). Lin28a protein is still evident within secondary follicles (arrow # 6), and early developing follicles (arrow # 9). Interestingly, Lin28a protein persists within the estrogen secreting granulosa cells surrounding secondary follicles (arrow # 6).
Figure 2.7: Lin28a protein decreases across the pubertal transition in ovarian tissue.

Immunohistochemical stain of Lin28a protein in 20 day (A-C) and 30 day (D-F) ovarian tissue from the first biological replicate. Lin28a protein expression is hallmarked by diaminobenzidine staining. In 20-day-old tissue, Lin28a is detected in early developing follicles (arrow #1), early secondary follicles (2, 3, and 4), late secondary follicles and granulosa cells surrounding secondary follicles (5). In 30-day-old tissue, Lin28a protein is less evident, due to the abundance of atretic follicles (7 and 8). Lin28a still persists in the granulosa cells and oocytes of secondary follicles (6), and in early follicles (9). This pattern of Lin28a protein distribution was consistent in the two biological replicates.
Figure 2.8: Lin28a protein decreases across the pubertal transition in ovarian tissue.

Immunohistochemical stain of Lin28a protein in 20 day (A-C) and 30 day (D-F) ovarian tissue for the second biological replicate. Lin28a protein expression is hallmarked by diaminobenzidine staining.
2.3.4.3 Examination of Let-7a and Let-7g miRNA levels in ovarian tissue across the pubertal transition

To investigate if the expression of Let-7 miRNA changes across the time course, the miRNA expression levels of two members of the Let-7 family, namely Let-7a and Let-7g, were quantified using qRT-PCR. With both Let-7a and Let-7g, a trend toward decreased expression is observed at 25 days and 30 days, when compared to 20-day-old ovarian tissue. However, the only statistically significant decrease was observed with Let-7a expression when comparing 30-day-old tissue to 20-day-old tissue, at a p<0.05 (*), as displayed in Figure 2.9. Therefore, this result suggests that along with a decrease in Lin28a and Lin28b mRNA and Lin28a protein levels across the pubertal transition within the ovary, a significant decrease in Let-7a miRNA expression at 30 days is observed as well.
Figure 2.9 Expression of Let-7a and Let-7g miRNA across the pubertal transition in ovarian tissue.

Expression of Let-7a and Let-7g miRNA decrease from 20-day-old ovarian tissue to 25- and 30-day-old ovarian tissue, as measured by qRT-PCR, normalized to snoRNA142 housekeeping gene. Let-7a miRNA expression decreases significantly in 30-day-old tissue, when compared to 20-day-old ovarian tissue (p<0.05, *). Values are displayed as means ± SEM (n=3 for each time point).
2.4 DISCUSSION

The importance of the Lin28 pathway with respect to puberty has been investigated recently, where Zhu et al. (2010) demonstrated that transgenic female mice over-expressing Lin28a had delayed puberty. Interestingly, Moss et al. (1997) initially established that within C. elegans, lin28, a homologue of Lin28a, plays an important role with respect to developmental timing. Specifically, over-expression of lin28, through gain-of-function mutations, resulted in delayed development. Since over-expression of Lin28a in mice and lin28 in C. elegans both resulted in delayed developmental phenotypes, it can be hypothesized that the Lin28 pathway acts as a brake that inhibits the pubertal process within mice. Moreover, given the fact that lack of lin28 expression in C. elegans, through loss-of-function mutations, resulted in precocious development (Moss et al., 1997), one can envision that transition into puberty within mice would be highlighted by decreases in expression in Lin28a and Lin28b.

Since Lin28 is a known repressor of Let-7 miRNA (Hagan, et al. 2009; Heo et al., 2008; Piskounova et al., 2008; Viswanathan et al., 2008), one can hypothesize that the Lin28 pathway modulates pubertal onset by regulating maturation of Let-7 miRNA. Hypothetically, over-expression of Lin28a/b would lead to an increase in protein levels and consequently result in decreased Let-7 miRNA levels. Since Let-7 miRNA function to reduce the expression of target genes, this would result in increased expression of its targets, which may function to repress pubertal onset.
The opposite would occur for transition into puberty, where Lin28a/b expression and protein levels decrease, leading to an increase in Let-7 miRNA, and subsequently leading to a decrease in the expression of target genes. This de-repression of Let-7 miRNA and repression of target gene expression leads to pubertal onset (Ojeda et al., 2010). It is important to note, however, that this hypothesis has never been validated, and whether Lin28a and Lin28b negatively regulate pubertal timing and development remains unknown.

Since pubertal onset results from the activation of GnRH neurons within the hypothalamus, control of the timing of pubertal onset is believed to be a centrally regulated process (Ojeda et al., 2010; Plant, 2001). Therefore, it was initially postulated that transition into puberty would be associated with decreases in Lin28a and Lin28b gene expression within hypothalamic tissue. In contrast to this hypothesis, Lin28a and Lin28b expression within hypothalamic tissue did not change across the pubertal transition in female mice. It is important to note, however, that since expression of these two genes was analyzed using whole hypothalamic tissue, it is possible that distinct nuclei within the hypothalamus, or regions of the brain not included in the dissection, such as the preoptic area, may show changes in expression across the time course. Although this could be addressed further, it is more important to focus on the dramatic and consistent changes in both Lin28a and Lin28b within the ovary, which is described below.
The observed increase in *Lin28a* mRNA expression within pituitary tissue was somewhat unexpected, as it is in contrast to the hypothesis that pubertal onset results from a decrease in *Lin28a* and *Lin28b* expression. Also, given that this expression pattern was not replicated with *Lin28b*, where no change was seen across the pubertal transition, the biological significance is less clear. Regardless, *Lin28a* increased in expression significantly at 45 days, when compared to 20 and 25 days, which is past the timing of puberty in the colony of female mice where the tissues were derived from (31.5 ± 2.4 days). In fact, at around 45 days of age, a female mouse is an adult, is sexually developed, and is fully competent to become pregnant and produce offspring (Zhu et al., 2010). Therefore, the observed increase in *Lin28a* at 45 days of age may have some importance with respect to fertility, and not pubertal onset. However, to fully understand this observed increase, future experimentation is necessary, which will be discussed in chapter 4.

*Lin28a* and *Lin28b* mRNA expression did not change consistently in pituitary tissue, but expression did decrease convincingly within ovarian tissue across the pubertal transition. Moreover, given that the ovary was the only tissue that showed a clear and consistent decrease in both *Lin28a* and *Lin28b* expression levels, this demonstrates that the ovary may be an important locus of control for pubertal timing. In addition, since the expression within the ovary decreased after 20 days of age, this suggests that *Lin28a* and *Lin28b* could regulate other aspects of pubertal
development, such as fertility and ovulation. To understand this fully, however, further investigation is necessary, which will be discussed in chapter 4.

A decrease in Lin28a protein levels within the ovary correlated with the decrease observed in Lin28a and Lin28b, as measured by immunohistochemistry. Consistent with the observed decrease in Lin28a expression, a general decrease in protein levels was seen in 30-day-old tissue when compared to 20-day-old tissue. Within 20-day-old tissue, Lin28a protein levels appear to be higher within the oocytes of early developing follicles, and expression levels decrease in oocytes as the follicles become more developed. Given that the oocytes within developing follicles are arrested in prophase I and subsequently undergo meiosis and maturation upon ovulation (Skinner, 2005), this is consistent with what is previously known about Lin28a, where expression is persistent in stem cell populations, and decreases as the cells differentiate (Yang and Moss, 2003; Xu et al., 2009). In addition, a recent publication demonstrated that Lin28a protein is evident within undifferentiated spermatogonia, which suggests that there may be some similarities with respect to gametogenesis between the two sexes (Zheng et al., 2009).

Within 30-day-old tissue, there appears to be an increase in atretic follicles. This phenomenon normally occurs during the late follicular phase of the menstrual cycle, where one developing follicle transforms into a dominant follicle, which begins to produce high amounts of estrogen (Van Dessel et al., 1996). This rise in estrogen
levels leads to the preovulatory surge of gonadotropins from the anterior pituitary (Moenter et al., 1990; Sarkar et al., 1976), and this, in-turn, promotes the ovulation of the oocyte within the dominant follicle, while the rest of the follicles undergo areasia (Chun et al., 1996; McGee et al., 1997). In these atretic follicles, Lin28a protein is lower.

In summary, these observations suggest that pubertal timing may be regulated at the level of the ovary. Combining Lin28a protein and Lin28a and Lin28b mRNA expression analysis, one can conclude that levels of Lin28a and Lin28b decrease across the pubertal transition in ovarian tissue. Moreover, given that over-expression of Lin28a resulted in delayed puberty (Zhu et al., 2010), it stands to reason that Lin28a may be part of the brake that inhibits pubertal onset. Further, since Lin28a and Lin28b showed similar trends in expression levels within the ovary, it stands to reason that Lin28b may also repress pubertal timing. Although one cannot definitively conclude that the site of Lin28 pathway action regarding pubertal timing is within the ovary, these findings provide important data that requires further investigation (see chapter 4).

Within C. elegans, both lin28 and the Let-7 miRNA family play important roles with respect to developmental timing, and this is best observed in mutants who aberrantly express either lin28 or Let-7 (summary in Table 2.2). Those nematodes that over-express Let-7 have precocious development, while those who lack Let-7
miRNA expression have delayed development (Reinhart et al., 2000). Interestingly, this is in direct contrast to mutants who aberrantly express *lin28*, where over-expression leads to delayed development, and lack of expression leads to precocious development.
Table 2.2: Summary of altered development phenotypes in transgenic mice and mutant *C. elegans*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Observation</th>
<th>Cause</th>
<th>Publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>1) Later Pubertal Onset 2) Delayed Sexual Maturation</td>
<td><em>Lin28a</em> over-expression</td>
<td>Zhu et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2) Precocious Development</td>
<td>1) <em>lin28</em> loss-of-function 2) <em>let-7</em> gain of function</td>
<td>1) Moss et al., 1997 2) Reinhart et al., 2000</td>
</tr>
</tbody>
</table>
Evidence of LIN28 mediated repression of Let-7 miRNA is evident in vitro, where cell lines modeling primary human tumours were found to have both high levels of LIN28A and LIN28B and lowered levels of Let-7 miRNA (Viswanathan et al., 2009). Moreover, evidence of Lin28 mediated repression of Let-7a and Let-7g was demonstrated by Viswanathan et al. (2008), where knockdown of Lin28a within P19 cells resulted in an elevation of mature Let-7a and Let-7g miRNA levels. Moreover, Piskounova et al. (2008) demonstrated that transfecting 293T cells with a Lin28a plasmid resulted in reduced levels of mature Let-7g miRNA. Similar to these studies, Newman et al. (2008) found that over expressing Lin28a and Lin28b protein within NIH-3T3 cells resulted in reduced levels of mature Let-7a and Let-7g. There was, however, one piece of data that indicated this might not be true, at least with respect to the hypothalamic-pituitary-gonadal axis. Specifically, transgenic mice that had elevated levels of Lin28a within the hypothalamus and ovary had no change in Let-7a and Let-7g miRNA levels within these tissues, when compared to wild-type mice. Similarly, Let-7a and Let-7g miRNA levels within the pituitary remained unchanged (Zhu et al., 2010).

Overall, though, it seemed reasonable to conclude that along with the decrease in Lin28a and Lin28b expression levels within the ovary, an increase in Let-7a and Let-7g would be expected. As a result, the apparent decrease in Let-7a and Let-7g miRNA levels across the pubertal transition in ovarian tissue was unexpected. It is important to note, however, that the levels of mature miRNA for all the Let-7
members were not measured. It is also important to note, though, that when analyzing the relationship between Lin28a/b and Let-7, Let-7a and Let-7g are the most commonly measured (Dangi-Garimella et al., 2009; Hagan et al., 2008; Heo et al., 2008; Jeong et al., 2009; Lu et al., 2009; Newman et al., 2008; Viswanathan et al., 2008; Viswanathan et al., 2009; Zhu et al., 2010), which is why these two members were quantified across the pubertal transition in the ovary. Moreover, several publications have demonstrated that other members of the Let-7 family respond in a similar manner as Let-7a and Let-7g, one of which demonstrated that over-expression of Lin28a and Lin28b resulted in reduced levels of Let-7b, Let-7d, Let-7e, Let-7f, and Let-7i, in addition to Let-7a and Let-7g (Newman et al., 2008). Thus, although not formally tested, the previous data and the high degree sequence homology observed within the Let-7 family (Table 2.3) make it likely that the other members of the Let-7 family changed in a similar manner across the pubertal transition in the ovary.
Table 2.3: Summary of the members of the Let-7 miRNA family in *H. sapiens* and *M. musculus*.

Adapted from Roush and Slack, 2008. Sequences downloaded from miRBase (http://www.mirbase.org/).

<table>
<thead>
<tr>
<th><em>H. sapiens</em> (human)</th>
<th><em>M. musculus</em> (mouse)</th>
<th>Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Let-7a (-1, -2, and -3)</td>
<td>Let-7a (-1 and -2)</td>
<td>UGAGGUAGUAGGUUGUAUAGUU</td>
</tr>
<tr>
<td>Let-7b</td>
<td>Let-7b</td>
<td>UGAGGUAGUAGGUUGUGGUU</td>
</tr>
<tr>
<td>Let-7c</td>
<td>Let-7c (-1 and -2)</td>
<td>UGAGGUAGUAGGUUGUGGUU</td>
</tr>
<tr>
<td>Let-7d</td>
<td>Let-7d</td>
<td>AGAGGUAGUAGGUUGCAUAGUU</td>
</tr>
<tr>
<td>Let-7e</td>
<td>Let-7e</td>
<td>UGAGGUAGGAGGUUGUAUAGUU</td>
</tr>
<tr>
<td>Let-7f (-1 and -2)</td>
<td>Let-7f (-1 and -2)</td>
<td>UGAGGUAGUAGAUUGUAUAGUU</td>
</tr>
<tr>
<td>Let-7g</td>
<td>Let-7g</td>
<td>AGAGGUAGUAGUUUGUAGGUU</td>
</tr>
<tr>
<td>Let-7i</td>
<td>Let-i</td>
<td>UGAGGUAGUAGUUUGCGUGUU</td>
</tr>
<tr>
<td>mir-98</td>
<td>mir-98</td>
<td>UGAGGUAGUAGUUGUAUUGU</td>
</tr>
<tr>
<td>mir-202</td>
<td>mir-202 (-1 and -2)</td>
<td>Human: AGAGGUAGGGCAUGGGAA Mouse: UUCCUAUGCAUAUACUUCUUU</td>
</tr>
</tbody>
</table>
The data raise the possibility that within the ovary, \textit{Lin28a/b} mediates its effects in a \textit{Let-7} independent manner. Indeed, it was recently demonstrated that although transgenic mice over-expressing \textit{Lin28a} have increased levels of this gene within the ovary, levels of \textit{Let-7a} and \textit{Let-7g} miRNA were unchanged (Zhu et al., 2010), indicating that \textit{Lin28a/b} signaling within the ovary may be independent of these two \textit{Let-7} members with respect to pubertal timing, and regulation of \textit{Let-7} is not fully dependant on \textit{Lin28a/b} levels. Related to this, a recent publication has demonstrated that with respect to neurogliogenesis, \textit{Lin28} signaling acts independently of \textit{Let-7} (Balzer and Moss, 2010). Finally, given that ovarian tissue is more complex than \textit{in vitro} models, it is possible that there are regulators of \textit{Let-7} miRNA expression other than \textit{Lin28a/b} and that \textit{Let-7} levels may vary independently of \textit{Lin28a/b}. For example, a recent publication has demonstrated that heteronuclear ribonucleoprotein A1 (hnRNP A1) blocks \textit{Let-7} miRNA maturation at the DROSHA step in somatic cells (Michlewski and Caceres, 2010). Further experimentation may improve understanding of the interaction between \textit{Lin28a/b} and \textit{Let-7} miRNA within the ovary, further experimentation is necessary (see chapter 4).

Together, the data presented in this chapter build on previous knowledge relating to the role of \textit{Lin28a} and \textit{Lin28b} in the regulation of pubertal timing. More specifically, it was determined that mRNA levels of \textit{Lin28a/b} and Lin28a protein decrease across the pubertal transition in ovarian tissue, and therefore agree with the restraint
hypothesis, where Lin28a/b may be part of the brake that inhibits pubertal onset. Moreover, Lin28a protein decreased within the oocyte of developing follicles, therefore provide evidence that the oocyte may be the primary site of Lin28a/b modulation of pubertal timing. To understand these findings further, in addition to the paradoxical decrease in Let-7 miRNA observed in the ovary, future studies using transgenic mouse models could be used, which will be discussed in chapter 4.
CHAPTER 3: EFFECT OF 17β-ESTRADIOL ON LIN28B GENE EXPRESSION
3.1 INTRODUCTION

Over the last decade, several publications have provided evidence for direct effects of estrogen on GnRH neurons. These direct effects were initially dismissed when several groups were unable to co-localize neuronal cell populations immunoreactive for estrogen receptor alpha (ERα) and GnRH, as measured by double-staining immunocytochemistry in the male and female rat (Shivers et al., 1983; Herbison and Theodosis, 1992) and in the ewe (Herbison et al., 1993; Lehman and Karsch, 1993). As a result, the scientific community believed that estrogen actions onto GnRH neurons were solely indirect, exerted through other neuronal cell populations. However, several studies have subsequently demonstrated the presence of both ERs, namely ERα and ERβ, both in vivo (Butler et al., 1999; Kallo et al., 2001; Legan and Tsai, 2003) and in vitro in the well established immortalized GnRH neurons, namely GT1-7 (Navarro et al., 2003; Roy et al., 1999) and GN11 (Ng et al., 2009), therefore demonstrating that GnRH neurons indeed express the ERs.

Evidence of direct actions of estrogen on GnRH neurons were initially reported by Roy, et al. (1999), where immortalized GT1-7 cells were treated with 1 nM estradiol concentrations over a 48 hour time course and a repression of GnRH mRNA levels were observed at 12 hours onward. Conversely, when GT1-7 cells were treated with estradiol in a manner that replicated the rat estrus cycle, significant increases in GnRH secretion were observed when compared to control treated cells, similar to the GnRH preovulatory surge in vivo (Navarro et al., 2003). This study provided
further evidence for the direct effect of estrogen on GnRH neuronal cells. A recent publication validated what was originally discovered by Roy et al. (1999), where nM levels of estrogen significantly repressed GnRH mRNA levels in both GT1-7 and GN11 GnRH neuronal cells (Ng et al., 2009).

3.2 OBJECTIVE AND HYPOTHESIS
Given the wealth of evidence demonstrating signaling between ovarian estrogen and GnRH neurons of the hypothalamus, and the fact that the Lin28a/Lin28b pathway has been established as an important regulator of pubertal timing, this chapter investigated whether 17β-estradiol (E$_2$), the predominant estrogen produced by the ovary, regulates Lin28b in GnRH neurons.

To fulfill this objective, two established immortalized cell lines were used. Specifically, a preliminary screen was performed using both GN11 and GT1-7 cells, and further experimentation was performed solely with GT1-7 cells. Both these cell lines were derived from targeted oncogenesis in transgenic mice designed to express the simian virus 40 T antigen under the direction of either the human (GN11) or rat (GT1-7) GnRH promoter. The resulting GnRH neuron specific tumours were then cloned by serial dilution and the immortalized lines were established (Mellon et al., 1990; Radovick et al., 1991). Importantly, GT1-7 cells were derived from a tumour within the anterior hypothalamus, and probably represent a more differentiated GnRH neuron (Mellon et al., 1990), while the GN11 cells were derived
from tumours formed along the migratory path of GnRH neurons (Radovick et al., 1991) and therefore represent a migrating GnRH neuron.

It is not known how Lin28b regulates the HPG axis, nor whether this regulation is subject to feedback from the gonad; the latter is the topic of this chapter. Since over-expression of Lin28a lead to delayed puberty in transgenic mice (Zhu et al., 2010) and hence established the Lin28a pathway as a negative regulator of puberty, and E2 has been shown to negatively regulate GnRH expression in GT1-7 (Ng et al., 2009; Roy et al., 1999) and GN11 neurons (Ng et al., 2009), it was hypothesized that estrogen positively regulates Lin28b mRNA expression.

3.3 METHODS

3.3.1 Cell culture

GT1-7 and GN11 cells were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum, 4.5 mg/mL glucose and 1 % penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), and maintained at 37°C and in an atmosphere of 5 % CO₂, as described previously (Roy et al., 1999).

3.3.2 Estradiol treatment

At least 4 hours prior to treatment, GT1-7 cells were washed with PBS and grown in Dulbecco’s modified Eagle medium without phenol red (HyClone, Logan, UT, USA)
supplemented with 1 % charcoal stripped FBS (Wisent, St-Bruno, QC, CAN) and 1 % penicillin/streptomycin (Invitrogen) (Dhillon and Belsham, 2011). Cells were treated with 17β-estradiol (Sigma-Aldrich, St. Louis, MO, USA) for 1, 2, 4, 8, 12, 16, 24, and 36 hours at 37°C and an atmosphere of 5 % CO₂ before RNA extraction. The estradiol was dissolved in 100 % ethanol, and a final concentration of 10 nM was obtained by serial dilution in DMEM without phenol red, whereas control cells were treated with comparable amount of ethanol, as described previously (Ng et al., 2009).

### 3.3.3 RNA extraction

RNA was extracted when the cells were at 70-90% confluency. The media was aspirated and the cells were washed in PBS, and lysed in 600 µL of Buffer RLT (Qiagen, Mississauga, ON, CAN). The cell lysate was homogenized by passing it through an RNase-free 22-gauge needle 6 times. Total RNA was extracted via a modified guanidine isothiocyanate and silica membrane isolation procedure using Qiagen's RNeasy® Mini Kit, following the manufacturer’s recommended protocol, with an on-column DNase digestion step using Qiagen’s RNase-free DNase Set (Qiagen). RNA concentrations were quantified using ultraviolet-visible spectroscopy, and the integrity of total RNA was assessed using a denaturing formaldehyde agarose gel (1.2 % (w/v) agarose, 10 % (v/v) MOPS buffer, 3 % (v/v) formaldehyde, and 0.05 mg/mL ethidium bromide).
3.3.4 Two-step qRT-PCR

A total of 1250 ng of RNA from GT1-7 cells was reverse transcribed into cDNA using Invitrogen’s SuperScript® III Reverse Transcriptase First Strand System. For standard curves, 5 mg of either hypothalamic tissue or GT1-7 cells was used.

After reverse transcription, samples were diluted (1:5), and 50 ng was used. Samples and standards were run in triplicate. Standard curves were generated by diluting hypothalamic tissue or GT1-7 cells from 1:10 to 1:10000 (200 - 0.02 ng). Quantification of initial template quantity was performed using the standard curve method. Gene specific primers were designed to span exon-exon boundaries, and the primers used for qRT-PCR (Lin28b, H3, and GnRH) are listed in Table 3.1. A total volume of 20 µL was used, where each reaction contained 4 µL of reverse-transcribed sample or standard, 10 µL of SYBR® Green PCR Master Mix, 600 nM of forward and reverse Lin28a/b primers or GnRH primers, or 50 nM of forward and reverse H3 primers. The real-time polymerase chain reaction was performed using the following conditions:

1. Initial 10 minute denaturation step at 95°C
2. 40 cycles (denaturation, annealing/extension): 95°C for 15 seconds and 60 C for 1 minute
3. Dissociation curve segment: 95°C for 1 minute, 60°C for 30 seconds, and 95°C for 30 seconds
Table 3.1: Primers used for qRT-PCR.

All primers were designed to span exon-exon junctions, and had an annealing temperature of 60 °C.

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>Amplicon (bp)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GnRH</strong></td>
<td>F 5’-CCCTTTGACTTTCCACATCC-3’</td>
<td>200</td>
<td>Ng, et al. (2009)</td>
</tr>
<tr>
<td></td>
<td>R 5’-GGGTTCCTGCCCCATGGATCCAC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>H3</strong></td>
<td>F 5’-CGCTTCCAGAGTGCGTATT-3’</td>
<td>72</td>
<td>Kim, et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>R 5’-ATCTTTAAAAAGGCGGCAACCAG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lin28b</strong></td>
<td>F 5’-TTTGGCTGAGGAGGAGTACTCAT-3’</td>
<td>113</td>
<td>Unpublished</td>
</tr>
<tr>
<td></td>
<td>R 5’-ATGGATCAGATGGACTGTGCGA-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
One example of the raw data from qRT-PCR is depicted in Figure 3.1, 3.2, and 3.3. In Figure 3.1, 3.2, and 3.3 A, the amplification plots of *Lin28b*, GnRH, and H3 mRNA in GT1-7 neuronal cells are shown. Initial template quantity for H3 mRNA in GT1-7 cells treated with either 17β-estradiol or vehicle was similar, as evidenced by the similar \( C_T \) values (19.54 – 19.92). In addition, dissociation curves for *Lin28a*, GnRH, and H3 had a single peak, centred around 78-82°C, 82-86°C, and 78-82°C respectively, as depicted in Figures 3.1, 3.2, and 3.3 B, which indicated single products specific to *Lin28b*, GnRH, and H3 were amplified.
Figure 3.1: Amplification plot and dissociation curve of Lin28b mRNA in GT1-7 cells treated with either 17β-estradiol or vehicle.

In (A), the amplification plot shows Lin28b mRNA in GT1-7 cells treated with either 17β-estradiol or vehicle at various time points. In (B), one peak is observed in the dissociation curve (at 78-82°C), suggesting a single product specific to Lin28b was amplified.
Figure 3.2: Amplification plot and dissociation curve of GnRH mRNA in GT1-7 cells treated with either 17β-estradiol or vehicle.

In (A), the amplification plot shows GnRH mRNA in GT1-7 cells treated with either 17β-estradiol or vehicle at various time points. In (B), one peak is observed in the dissociation curve (at 82-86 °C), suggesting a single product specific to GnRH was amplified.
Figure 3.3: Amplification plot and dissociation curve of H3 mRNA in GT1-7 cells treated with either 17β-estradiol or vehicle.

In (A), the amplification plot shows H3 mRNA in GT1-7 cells treated with either 17β-estradiol or vehicle at various time points. The fluorescence in all the samples increase above the threshold at a similar cycle, therefore resulting in similar C_T values (19.54 – 19.92) and similar initial template quantity. In (B), one peak is observed in the dissociation curve (at 78-82°C), suggesting a single product specific to H3 was amplified.
3.3.5 RT-PCR

Total RNA was extracted from hypothalamic tissue, GT1-7 and GN11 cells using Qiagen’s RNeasy® Mini Kit, as described above, and reverse transcribed using the SuperScript® III Reverse Transcriptase First Strand System (Invitrogen), as described above. The no template controls (NTC) were run with the cDNA reagents in the absence of total RNA.

A total of 50-80 ng of cDNA was loaded with 0.625 units of Taq polymerase, 0.4 μM of forward and reverse primers for Lin28a/b and ERα/β or 0.1 μM of forward and reverse primers for β-actin, 0.4 mM of dNTP mix, 2 mM of MgCl₂, and 10x Taq buffer (with (NH₄)₂SO₄) (Fermentas, Burlington, ON, CAN). The polymerase chain reaction was performed using the following conditions:

1. 95°C for 2 minutes
2. 40 cycles (denaturation, annealing, extension): 95°C for 1 minute, 55°C for 1 minute, and 72°C for 90 seconds
3. 72°C for 5 minutes
4. HOLD at 4°C

The primers used to amplify targets are listed in Table 3.2. The amplified products were separated via polyacrylamide gel electrophoresis (100 mL 6% acrylamide gel mixture, 100 μL TEMED, and 1000 μL 10% APS) and visualized by ethidium bromide staining (1 mg/mL) followed by ultraviolet illumination.
Table 3.2: Primers used for RT-PCR.

All primers were designed to span exon-exon junctions, and had an annealing temperature of 55 °C.

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>Amplicon (bp)</th>
<th>Source</th>
</tr>
</thead>
</table>
| B-actin | F 5’-CCACACCCGCCACCAGTTCG-3’  
R 5’-TACAGCCGGGGAGCATCGT-3’ | 112 | Unpublished |
| ERα    | F: 5’-AGAGTGCCAGGCTTTGGGGACT-3’  
R: 5’-TGCAGAACCAGCTTTGACGTAGC-3’ | 223 | Unpublished |
| ERβ    | F 5’-GCAAACCTCCTGATGCTTTTT-3’  
R 5’-TTGTACCCTCGAAGCGTGTGA-3’ | 146 | Ng, et al. (2009) |
| Lin28α | F 5’-AGGCGGTGGAGTTCACCTTTAAGA-3’  
R 5’-AGCTTGCATTCTTTGGCATGATG-3’ | 190 | Unpublished |
| Lin28β | F 5’-CAACATGGCCAGGCGGGG-3’  
R 5’-CCCATGCGACGTGAAACCA-3’ | 123 | Unpublished |
3.3.6 Statistical analysis

Data analysis was performed by using one-way Analysis of Variance (ANOVA), followed by the Bonferroni Multiple Comparison Test. Data are expressed as mean ± SEM, and 3 independent biological replicates were performed per experiment.

3.4 RESULTS

3.4.1 Preliminary screen of GN11 and GT1-7 cells

Using RT-PCR, GN11 and GT1-7 cells were screened for the presence of Lin28a, Lin28b, β-actin, ERα and ERβ, and quantification of GnRH mRNA levels was achieved by qRT-PCR.

3.4.1.1 GN11 and GT1-7 cells express Lin28b, but not Lin28a

GN11 and GT1-7 GnRH neuronal cells express Lin28b. However, levels of Lin28a mRNA were below the detection limit of the RT-PCR assay (Figure 3.4 A). This was verified using qRT-PCR, where no Lin28a mRNA transcript was detected after 40 cycles (data not shown). Both GN11 and GT1-7 cells express ERα and ERβ (Figure 3.4 A).
Both GN11 and GT1-7 cells express ERα (223 bp), ERβ (146 bp), Lin28b (123 bp), β-actin (112 bp), but not Lin28a (190 bp), as measured by RT-PCR (A). (B), GT1-7 cells have higher levels of GnRH mRNA when compared to GN11 cells, as measured by qRT-PCR using the standard curve method. For both (A) and (B), RNA was extracted and reverse-transcribed, and 50ng of cDNA template was used for RT-PCR. For (A) 25-day-old C56Bl/6 female hypothalamic(hypo) tissue was used as a positive control. NTC = no template control.
3.4.1.2 The expression profile of GnRH mRNA differ in GN11 and GT1-7 cells

It is known that GT1-7 cells express much higher levels of GnRH mRNA than GN11 cells (Mellon et al., 1990; Ng et al., 2009; Radovick et al., 1991). This was again evident in Figure 3.4 B, where the levels of GnRH mRNA were approximately 14 times higher in GT1-7 cells than GN11 cells. Therefore, for the purposes of this thesis, only GT1-7 cells were used, since they represent a more differentiated GnRH neuron within the hypothalamus, and express GnRH mRNA at higher levels than GN11 cells. Also, given that the GT1-7 cells express both ER subtypes, this suggests that they may be sensitive to estrogen treatment and are therefore suitable for investigation of the regulation of Lin28b expression by estrogen.

3.4.2 Treatment of GT1-7 cells with 17β-estradiol (E2)

To determine the effect of E2 on Lin28b and GnRH gene expression, GT1-7 cells were treated with 10 nM E2 dissolved in ethanol or vehicle (comparable amount of ethanol) over a complete time course: 1, 2, 4, 8, 12, 16, 24, and 36 hours. This concentration of E2 was used because it was previously shown to be effective in GT1-7 cells (Ng et al., 2009; Novaira et al., 2009; Titolo et al., 2006). RNA from GT1-7 cells was collected and levels of mRNA were quantified using qRT-PCR, normalized to histone H3 housekeeping gene.

3.4.2.1 Effect on Lin28b mRNA expression
From 1 – 12 hours, *Lin28b* mRNA expression did not change significantly when comparing E2-treated to vehicle-treated GT1-7 cells (Figure 3.5 A). A statistically significant increase of 1.6 fold (p<0.01) in *Lin28b* mRNA was observed at 16 hours. This statistically significant increase was no longer evident at 24 and 36 hours.
Expression of (A) *Lin28b* and (B) GnRH in GT1-7 cells treated with E₂, as measured by qRT-PCR, normalized to H3 housekeeping gene. Cells were treated with 10 nM of E₂ (dissolved in ethanol), or vehicle (comparable amounts of ethanol) for 1, 2, 4, 8, 12, 16, 24 and 36 hours. Values are displayed as means ± standard error of the mean (n=3). All E₂ treatments were normalized to vehicle at their respective time-point. For statistical analysis, all vehicle and E₂ treatments were subject to one-way ANOVA, followed by the Bonferroni multiple comparisons test, comparing each E₂ treatment with vehicle at the respective time point. **= p<0.01.
3.4.2.2 Effect on GnRH mRNA expression

Previous publications have demonstrated that treatment of GT1-7 cells with E₂ significantly represses GnRH mRNA levels (Ng et al., 2009; Novaira et al., 2009; Roy et al., 1999). Therefore, as a positive control for effects of E₂ treatments on GT1-7 cells, GnRH mRNA levels were assessed as a positive control. Although GnRH mRNA levels were expected to decrease with E₂ treatment, particularly at 12 hours onward, this was not apparent in the results (Figure 3.5 B). All that was observed were non-significant fluctuations in GnRH expression, where E₂ treated cells were within 10 – 20 % of the vehicle treated cells.

3.4.3 Troubleshooting the E₂ experiments

Given that GnRH mRNA expression did not change with E₂ treatments in GT1-7 cells, three lines of troubleshooting were performed. First, the expression of the estrogen receptor subtypes was assessed for any changes within the GT1-7 cells after 6 passages. Second, to investigate further whether the E₂ was functional, and if the E₂ treatments were effective on GT1-7 cells, ERβ mRNA levels were quantified via qRT-PCR. Third, a new aliquot of E₂ was acquired and several time-points were repeated.

3.4.3.1 Did the phenotype of GT1-7 cells change?

The expression of ERα and ERβ remains the same after 6 cell culture passages

The preliminary screen of ERα and ERβ was performed on GT1-7 cells after 13 passages, and experiments with E₂ were performed after cells were grown to
passages 15-17. Therefore, to investigate if the phenotypic profile of the GT1-7 cells changed, another RT-PCR reaction was performed, and GT1-7 cells were assessed for expression of ERα and ERβ after 19 passages. As seen in Figure 3.6, GT1-7 cells, as expected, still express the estrogen receptor subtypes. Thus, it seemed reasonable to conclude that the GT1-7 cells being used should still be able to respond to E₂ treatment.
GT1-7 neuronal cells after 6 passages still express ERα (223 bp) and ERβ (146 bp) at similar levels to GT1-7 cells at an earlier passage (see Figure 3.4). RNA was extracted and was reverse-transcribed. 50ng of cDNA template was used for RT-PCR. 25-day-old C56Bl/6 female hypothalamic (hypo) tissue was used as a positive control. NTC = no template control.

Figure 3.6: Expression of ERα and ERβ in GT1-7 cells after six passages.
3.4.3.2 Is the $E_2$ functional?

*The expression of ERβ changes with $E_2$ treatment*

According to a paper published by Titolo et al. (2006), ERβ mRNA levels were enhanced by approximately 60% when GT1-7 cells were treated with 10 nM $E_2$ for 12 hours ($p<0.01$). When this experiment was replicated with the GT1-7 cells and $E_2$ used for this thesis, a significant increase in ERβ expression was also observed ($p<0.001$) (Figure 3.7). However, although the direction was identical to what was found by Titolo et al. (2006), the expression increased by only 25%. Thus, it seemed reasonable to conclude that the $E_2$ treatments were effective, but at a lower magnitude. This led to the possibility that the $E_2$ being used might have diminished biological activity.
The expression of ER\(\beta\) mRNA increases in GT1-7 cells when treated with 10 nM of E\(_2\) when compared to vehicle (comparable amount of ethanol) for 12 hours. Values are displayed as means ± standard error of the mean (n=3). For statistical analysis, vehicle and E\(_2\) treatments were subject to an unpaired t-test. ***=p<0.001.

Figure 3.7: Effect of E\(_2\) on ER\(\beta\) mRNA expression in GT1-7 cells.
### 3.4.3.3 New stock of E₂

Although calculations were verified several times and E₂ solutions were remade, no differences in the results were observed. To explore the question of the biological effect of the E₂ further, a new stock of E₂ that has been shown to be effective in Dr. Belsham’s lab was used and GnRH mRNA levels were quantified at 4, 12, and 16 hours (Figure 3.8 A), in triplicate. Although not statistically significant, these initial data suggested that the new estradiol had a more robust biological effect, with a decrease in GnRH expression observed at 12 hours.

To explore whether the new E₂ had an effect on Lin28b expression within GT1-7 cells, Lin28b mRNA was also quantified at 4, 12, and 16 hours. When compared to vehicle-treated cells, Lin28b expression within estradiol-treated cells was not significantly altered (Figure 3.8 B). Moreover, there was no evidence that the previously observed increase in Lin28b expression at 16 hours (Figure 3.5 A) was present.
Figure 3.8: Effect of new E2 on GnRH and Lin28b mRNA levels.

Expression of (A) GnRH and (B) Lin28b in GT1-7 cells treated with E2, as measured by qRT-PCR, normalized to H3 housekeeping gene. Cells were treated with 10 nM E2 (dissolved in ethanol), or vehicle (comparable amounts of ethanol) for 4, 12 and 16 hours. Values are displayed as means ± standard error of the mean (n=3). All E2 treatments were normalized to vehicle at their respective time-point.
3.5 DISCUSSION

Despite various publications demonstrating evidence for direct effects of estrogen on GnRH neurons (Navarro et al., 2003; Ng et al., 2009; Roy et al., 1999; Titolo et al., 2006), the effect of E₂ on Lin28b mRNA expression has not yet been explored. Therefore, in this chapter, the effect of 10 nM E₂ on Lin28b mRNA expression over a complete time-course was investigated. This concentration was chosen because it has previously been shown to reduce GnRH mRNA expression (Ng et al., 2009), and also affect expression of the ERs (Titolo et al., 2006) within GT1-7 cells. Interestingly, in a study by Navarro, et al. (2003) treating GT1-7 cells with cyclic doses of E₂ and progesterone (P₄) that resembled the rat estrous cycle (E₂: 2 pM – 17 pM; P₄: 2nM – 20 nM) stimulated GnRH secretion in a manner similar to the preovulatory surge. However, since the goal was to study routine physiologic actions of E₂ and not the preovulatory surge, a concentration of 10 nM was chosen.

Although found only at 16 hours, the increase observed in Lin28b expression within GT1-7 cells treated with E₂ compared to those treated with vehicle was consistent with the hypothesis. However, the positive control (GnRH expression) did not show the expected decrease at 12, 16, 24, or 36 hours. (Figure 3.5 B). Previous publications have demonstrated that the effect of E₂ on GnRH mRNA expression in GT1-7 cells occurred at these later time-points (Ng et al., 2009; Novaira et al., 2009; Roy et al., 1999). Specifically, E₂ repressed expression of GnRH; one-such
publication demonstrated that GT1-7 cells treated with 10 nM of E_2 for 16 hours decreased expression by 61 % compared to vehicle (Ng et al., 2009). Therefore, this raised the possibility that the 16 hour *Lin28b* result was a false positive.

On the other hand, given that the GT1-7 cells still expressed both ERα and ERβ after 6 passages, it appeared that the phenotypic profile of these cells did not change and they were still able to respond to E_2. The demonstration that ERβ expression increased at 12 hours after E_2 treatment is in agreement with Titolo, et al. (2006), and this suggested that the E_2 used was indeed functional. However, the decreased magnitude of the change (25 % versus 60 %, as observed by Titolo et al., 2006) suggested that the *Lin28b* result could be valid and that the lack of change in GnRH could have stemmed from the E_2 having diminished biological activity.

Data from treatment with the new E_2 were consistent with improved biological activity and indicative of a decrease in GnRH expression at 12 hours. With a larger number of biological replicates, it is possible that GnRH expression at 12 hours will show a statistically significant decrease with E_2-treated versus vehicle-treated cells. Therefore, the results from this thesis suggest that *Lin28b* does not appear to be affected by estradiol in GT1-7 cells. However, a definitive conclusion regarding whether E_2 regulates *Lin28b* within GT1-7 neuronal cells cannot be drawn with the current data. Given that Ng et al. (2009) and Novaira et al. (2009) have demonstrated that GnRH expression is repressed by E_2 in GT1-7 cells at 16 hours,
and that this finding was not replicated in this thesis, further investigation of whether $E_2$ regulates $Lin28b$ expression in GnRH neurons is necessary, and would require a larger number of replicates, along with additional experiments to determine cause and effect more clearly, such as dose-response curves and utilization of ER agonists and/or antagonists. These additional analyses are beyond the scope of this thesis, but are among the future directions described in chapter 4.
CHAPTER 4: SUMMARY OF FINDINGS AND FUTURE DIRECTIONS
4.1 SUMMARY OF FINDINGS

Complementary to the delayed developmental phenotype observed in *C. elegans* mutants over-expressing *lin28* (Moss et al., 1997; Yang and Moss, 2003) and the delayed puberty phenotype observed in transgenic mice over-expressing *Lin28a* (Zhu et al., 2010), the findings in chapter 2 demonstrate that *Lin28a* and *Lin28b* expression decrease across the pubertal transition. Moreover, since this decrease was localized to oocytes within the ovary, this suggests that *Lin28a/b* may function to repress pubertal timing at the level of the ovary within the HPO axis. This is a hypothesis that can be tested in future studies using transgenic mouse models designed to have altered expression of *Lin28a/Lin28b*.

In chapter 3, other studies were designed to test another hypothesis about how *Lin28a/b* might regulate the HPG axis. Studies were completed to investigate whether estrogen affected *Lin28b* expression within GnRH neurons. The goal of this work was to determine if estrogen regulated *Lin28b* expression in a GnRH neuronal model system.

To better understand how these genes function to control pubertal timing, two different approaches could be used, which are described below. First, in addition to characterizing transgenic mice that over-express *Lin28a* (Zhu et al., 2010), transgenic mice without expression of *Lin28a* and *Lin28b* could be utilized and assessed for alterations in pubertal timing. Moreover, to investigate if the *Let-7*
miRNA family is an important mediator of \textit{Lin28a/b} with respect to pubertal timing, mice designed to over-express \textit{Let-7} could be characterized. Second, to complement the whole body \textit{in vivo} experiments, an \textit{in vitro} approach could be taken to investigate how \textit{Lin28a/b} is involved in the bi-directional feedback within the HPG axis.

\section*{4.2 FUTURE DIRECTIONS}

\subsection*{4.2.1 Use of transgenic mouse models}

To better understand the \textit{Lin28} pathway with respect to control of pubertal timing, in addition to characterizing transgenic mice that over-express \textit{Lin28a} (Zhu et al., 2010), transgenic mice without expression of \textit{Lin28a} and \textit{Lin28b} could be utilized and assessed for alterations in pubertal timing. These mice, where genes are globally knocked out, are currently being characterized for vaginal opening, a marker of pubertal timing in female mice. Given that over-expression of \textit{Lin28a} mice lead to delayed puberty (Zhu et al., 2010), these mice are expected to have precocious puberty. If this pubertal phenotype is indeed observed, then the next step is to investigate the effect on pubertal timing of knocking out \textit{Lin28b} within specific tissues and at specific times to explore further the mechanism of \textit{Lin28b} in modulation of the HPG axis.

To accomplish this next goal, transgenic mice, where a portion of the \textit{Lin28b} gene is surrounded by loxP sequences, could be used. Through the use of tissue-specific Cre
recombinases, one will be able to design knockouts of *Lin28b* expression. From chapter 2 of this thesis, it was established that the oocyte within the ovary may be the locus of control for *Lin28a/b* mediated repression of pubertal timing. Therefore to test the hypothesis that the oocyte is the site of *Lin28a/b* mediated regulation of pubertal timing, one could envision using a Cre recombinase with a promoter that drives expression solely within the oocyte of a female mouse. One such transgenic mouse exists from Jackson Laboratories, where the Cre recombinase is under the direction of gene regulatory sequences from the mouse zona pellucida 3 (*Zp3*) (Jackson Laboratories Stock Number 003651), which drives expression exclusively in the oocyte prior to the first meiotic division. Finding altered pubertal timing in this mouse compared, for example, to neuronal knockouts, would suggest the importance of ovarian *Lin28a/b* in the regulation of pubertal timing. Moreover, since *Lin28a* and *Lin28b* expression decreased permanently within the ovary, these genes may regulate other aspects of pubertal development. Therefore, apart from pubertal timing, ovarian and uterine weights could be measured to assess sexual maturation, and time to first pregnancy can be measured to assess fertility.

To investigate how the temporal pattern of *Lin28a* and *Lin28b* expression within the HPO axis regulates puberty, one could envision using a temporally activated Cre recombinase (Jackson Laboratories Stock Number 004847) at a certain age postnatally, which would allow the expression of these genes to be knocked-out at a specific point in development.
Given that *Lin28a* expression within pituitary tissue increased significantly at 45 days of age, further investigation could be initiated. Since the increase was beyond the timing of puberty for female mice, it may be important with respect to fertility and not pubertal timing. To test this hypothesis, one could utilize a tamoxifen-inducible Cre recombinase specific to pituitary tissue and knockout *Lin28a* at specific ages of development and assess for alterations in pubertal timing and fertility.

Finally, to complement *Lin28b* knockout studies, transgenic mice designed to over-express *Let-7* miRNA could also be studied. Moreover, considering that *Let-7* miRNA expression decreased across the pubertal transition within ovarian tissue, investigating if altering *Let-7* miRNA expression alters pubertal development will help identify if *Lin28a/b* modulate pubertal timing through changes in *Let-7* miRNA. Fortuitously, there is currently an inducible *Let-7* transgenic mouse available. These transgenic mice are designed to over-express a mutant *Let-7g* species, known as 7S21L, which contains a modification that prevents it from being bound and degraded by the Lin28 proteins (Zhu et al., unpublished data). Traditionally, it would be hypothesized that these transgenic mice would have early pubertal timing, similar to the phenotype observed with the global *Lin28b* knockout mice. However, the data from chapter two of this thesis suggests that these transgenic mice will either have no pubertal phenotype, indicating *Let-7* independent actions of *Lin28*, or
delayed puberty, similar to mice that had over-expression of Lin28a.

4.2.2 Further experimentation with GT1-7 cells

Determining if gonadal products feedback onto GnRH GT1-7 cells and regulate Lin28b is still an important issue to address. In future work, this could be pursued further by increasing the number of biological replicates and modifying the time-course experiment to include a tighter selection of treatment times, such as 10, 12, 14, 16 and 18 hours. Further, a dose-response experiment with E2 could be performed (1 nM, 10 nM, and 100 nM), at incubation times that resulted in the largest change in Lin28b expression, during the timeline experiment. The results from this experiment will help determine if E2 dose-dependently regulates Lin28b mRNA expression. Moreover, to investigate whether E2 signaling is mediated through either of the ERs, GT1-7 cells could be treated with agonists of either ERα (propyl-pyrazole-triol, PPT) or ERβ (diarylpropionitrile, DPN), and Lin28b expression could be quantified. Finally, if E2 treatment does change Lin28b expression, changes of Let-7 miRNA expression could also be measured, to determine if E2-dependant changes in Lin28b expression results in changes in Let-7 miRNA levels as well.

To investigate if Lin28b regulates GnRH secretion and expression directly within GnRH neurons, Lin28b could be knocked-down in GT1-7 cells using a short interfering RNA (siRNA) approach. After successful knockdown, both GnRH
expression and secretion could be analyzed. Together with investigating the effect of estrogen on \textit{Lin28b} expression, these findings will help determine if \textit{Lin28b} is involved in bi-directional feedback mechanisms within the HPG axis.

\textbf{4.3 OVERALL CONCLUSIONS}

From the landmark discovery, where variations in and near \textit{LIN28B} were found to associate with AAM in the general population via GWA studies (Elks et al., 2010 He et al., 2009; Ong, et al., 2009; Perry et al., 2009; Sulem et al., 2009), to verifying that \textit{Lin28a} was a casual gene that regulates pubertal timing (Zhu et al., 2010), important progress has been made with respect to understanding the genetic regulation of pubertal onset. The results presented in this thesis build on this progress, and provide further insight into how \textit{Lin28a} and \textit{Lin28b} regulate pubertal timing by identifying the ovary as the likely site of the modulatory effects of \textit{Lin28b}. 


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