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Investigation of Peripubertal Expression of *Lin28a* and *Lin28b* in C57BL/6 Female Mice

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Abstract

Genome-wide association studies recently identified 32 loci that associate with the age at menarche (AAM) in humans. Because the locus most robustly associated with AAM is in/near *LIN28B*, the goal of this study was to investigate how the *Lin28* pathway might modulate pubertal timing by examining expression of *Lin28b*, and its homologue, *Lin28a*, across the pubertal transition in female mice. Quantitative reverse-transcriptase PCR data indicate that, prior to the onset of puberty, expression of both *Lin28b* and *Lin28a* decreases in the ovary, while expression of only *Lin28a* decreases in the hypothalamus; the expression of *Lin28a* increases after the onset of puberty in the pituitary. Immunohistochemistry in ovarian tissue verified that *Lin28a* protein levels decreased in parallel with gene expression. Although these data do not demonstrate cause and effect, they do suggest that decreased expression of *Lin28a/Lin28b* may facilitate the transition into puberty, consistent with previous data showing that overexpression of *Lin28a* in transgenic mice leads to delayed puberty. In addition, although *Lin28b* and/or *Lin28a* expression significantly decreased prior to puberty, neither *Let-7a* nor *Let-7g* miRNA levels changed significantly, raising the possibility that some effects of *Lin28b* and *Lin28a* within the hypothalamic-pituitary-gonadal (HPG) axis may be *Let-7* miRNA independent. Subsequent studies, such as tissue and age specific modulation of *Lin28b* and *Lin28a* expression, could determine whether the expression patterns observed are responsible for modulating the onset of puberty and delineate further the role of this pathway in the HPG axis.

Keywords and topic category

puberty; *Lin28a*; *Lin28b*; hypothalamic-pituitary-gonadal axis

1. INTRODUCTION

Although the timing of puberty is highly heritable, the genes that control the onset of puberty in the general population have, until recently, been largely unknown [reviewed in - Gadjos et al., 2010; Towne et al., 2005]. Thirty-two loci that associate with variation in the

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age at menarche (AAM) have now been identified through genome wide association (GWA) studies. The most robustly associated locus is in/near the gene that encodes the RNA binding protein LIN28B [Elks et al., 2010; He et al., 2009; Ong et al., 2009; Perry et al., 2009; Sulem et al., 2009].

Two lines of evidence indicate that *LIN28B* may be the responsible gene at the GWA locus. First, prior to the GWA studies, *lin28* had been shown to regulate developmental timing in *C. elegans* [Moss et al., 1997; Yang et al., 2003]. When *lin28* is mutated and expression is decreased, progression through the larval stages is accelerated; conversely, when *lin28* expression is increased, the nematode undergoes delayed development [Ambros and Horvitz, 1984; Moss et al., 1997; Yang and Moss, 2003]. Second, a relationship was found between the *Lin28* pathway components and pubertal timing when it was examined in a fortuitously available transgenic mouse model that over-expressed *Lin28a*, a functional homologue of *Lin28b* [Zhu et al., 2010]. Vaginal opening (VO), time to first estrus, and age at first pregnancy were all delayed in the female mice carrying the transgene compared to littermate controls. These data provide evidence that the *Lin28a/Lin28b* pathway regulates the hypothalamic-pituitary-gonadal (HPG) axis and suggest that *LIN28B* is likely the gene at the GWA locus responsible for regulating AAM in humans.

How the *Lin28a/Lin28b* pathway regulates the HPG axis and modulates the timing of puberty is not known. However, the data from *C. elegans* and from the transgenic mice suggest that decreased expression would be associated with progression into puberty because over-expression of *lin28* in *C. elegans* and of *Lin28a* in female mice both lead to delayed development.

To begin to assess where within the HPG axis the *Lin28a/Lin28b* pathway exerts effects and to determine whether levels of expression change across the pubertal transition, we assessed *Lin28a* and *Lin28b* expression in the hypothalamus, pituitary, and ovary of mice between prepubertal to postpubertal ages, ages 15 to 45 days, respectively. We also assessed expression of key members of the *Let-7* miRNA family, a putative downstream mediator of *Lin28a/Lin28b*. To our knowledge, this is the first study to analyze the peripubertal expression of members of the *LIN28B* pathway, which has recently been linked to variation in AAM in humans, within the HPG axis.

2. MATERIAL AND METHODS

2.1 Animals

C57BL/6 (B6) 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 [Nathan et al., 2006]. The Animal Care Committee (ACC) at the TCP approved all experimental protocols.

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 rapid decapitation.

For qRT-PCR, hypothalamic, pituitary, and ovarian tissue were collected at 15, 20, 25, 30, 35, 39, and 45 days of age from female C57BL/6 mice. Tissues collected at 35, 39, and 45 days were grouped and collectively analyzed as a ‘postpuberty’ time-point. 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, and hypothalamic tissue was then excised via a 2 mm cut from the ventral side of the brain [Baker et al., 1983]. After collection, all tissues for qRT-PCR were either stored in RNAlater®, containing 10 % w/v sodium citrate tribasic dihydrate and 60 % w/v ammonium sulphate (Ambion, Foster City, CA, USA), or flash frozen in liquid nitrogen. For immunohistochemistry, hypothalamic, and 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. Pituitary tissue was collected at 15 and 20 days. Samples from the different ages were processed together.

2.3 RNA extraction

Total RNA extraction was performed using a modified guanidine isothiocyanate and silica membrane isolation procedure with Qiagen's RNeasy® Mini Kit, AllPrep DNA/RNA Mini Kit, or miRNeasy Mini Kit (for *Let-7a* and *Let-7g* miRNA collection), following the manufacturer's recommended protocols. 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).

2.4 qRT-PCR for *Lin28a* and *Lin28b*

Total RNA from hypothalamic, pituitary, or ovarian tissue at each age was reverse transcribed into cDNA using the SuperScript® III Reverse Transcriptase First Strand System (Invitrogen, Carlsbad, CA, USA). After reverse transcription, quantification of *Lin28a* or *Lin28b* mRNA levels from 50 ng of hypothalamic and ovarian tissue, or 25 ng of pituitary tissue, was performed using threshold cycle (Ct) values compared to a standard curve of serially diluted hypothalamic or ovarian tissue. Ct is defined as the cycle where fluorescence in the reaction increased above a baseline threshold. Primers were designed to span exon-exon boundaries to ensure specificity, which was further ensured by dissociation curve analysis and sequencing (*Lin28a* F: 5'-AGGCGGTGGAGTTCACCTTTAAGA-3', R: 5'-AGCTTGCAATTCCTTGGCATGATGG-3'; *Lin28b* F: 5'-CAACATGGCCGAAGGCGGGG-3', R: 5'-CCCATGCGCACGTTGAACCA-3'; β -actin F: 5'-CCACACCCGCCACCAGTTCG-3', R: 5'-TACAGCCCCGGGAGCATCGT-3'). A total volume of 20 μ L was used, where each reaction contained 4 – 6.5 μ L of reverse-transcribed sample or standard, 10 μ L of SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 600 nM of forward and reverse *Lin28a* or *Lin28b* primers, or 50 nM of forward and reverse β -actin primers. The real-time polymerase chain reaction consisted of a 10 minute initial denaturation step at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. A final dissociation curve analysis was also performed (95°C for 1 minute, 60°C for 30 seconds, and 95°C for 30 seconds) to ensure a single product was amplified.

2.5 qRT-PCR for *Let-7a* and *Let-7g*

Five (5) nanograms of total RNA were reverse transcribed from 15-, 20-, 25- and 30-day-old hypothalamic and pituitary tissues and 20-, 25-, and 30-day-old 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 reverse transcribed using *Let-7a*, *Let-7g*, and snoRNA142 RT primers, and was serially diluted from 1:10 to 1:40.

After reverse transcription, quantification of mature *Let-7a* or *Let-7g* was performed using the standard curve method [Chen et al., 2005]. 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 consisted of an initial 10 minute denaturation step at 95°C, followed by 40 cycles of alternating denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute.

2.6 RT-PCR

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* and *Lin28b* or 0.1 μM of forward and reverse primers for β -*actin*, 0.4 mM of dNTP mix, 2 mM of MgCl_2 , and 10x Taq buffer (with $(\text{NH}_4)_2\text{SO}_4$) (Fermentas). The polymerase chain reaction consisted of an initial denaturation step at 95°C for 2 minutes, 40 cycles of denaturation (95°C for 1 minute), annealing (55°C for 1 minute) and extension (72°C for 90 seconds), followed by a final step at 72°C for 5 minutes.

The primers used to amplify targets are identical to those used for qRT-PCR analysis (see above). 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.

2.7 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. Western blotting was used to verify the specificity of the polyclonal antibodies, where only one protein was recognized at approximately 26 kDa, corresponding to Lin28a. 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 from each tissue were assessed.

2.8 Statistical analysis

For qRT-PCR, all time points were compared with 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 \pm standard error of the mean (SEM).

3. RESULTS

3.1 Expression of *Lin28a* and *Lin28b* in hypothalamic, pituitary, and ovarian tissue

Both *Lin28a* and *Lin28b* are expressed in hypothalamic, pituitary, and ovarian tissue (Figure 1A). However, the relative expression of these two genes differed among the tissues. Expression of *Lin28b* within hypothalamic tissue was approximately 30 times higher than *Lin28a* (Figure 1B), while expression of *Lin28b* within pituitary tissue was approximately 2 times higher than *Lin28a*. Conversely, in ovarian tissue, levels of *Lin28a* expression were

approximately 60 times higher than *Lin28b*, which is in agreement with data from a recent study of expression within human ovarian tissue (El-Khairi et al., 2012).

3.2 Expression of *Lin28a* and *Lin28b* across the pubertal transition in hypothalamic, pituitary, and ovarian tissue

Because increased expression of *Lin28a* has been shown to cause delayed puberty in mice [Zhu et al., 2010], it was hypothesized that a decrease in expression of *Lin28b* and/or *Lin28a* might facilitate the transition into puberty. Demonstrating a diminution in expression prior to the pubertal transition would support this hypothesis. Thus, levels of *Lin28a* and *Lin28b* expression in components of the HPG axis in female C57BL/6 mice were examined using qRT-PCR prior to the onset of puberty (at post natal days 15, 20, 25, 30) and postpubertally (between ages 35–45 days); the average age of pubertal onset (as assessed by vaginal opening) in our colony is 31.5 ± 2.4 days.

3.2.1 A decrease in *Lin28a* mRNA was observed in hypothalamic tissue prior to the pubertal transition—In hypothalamic tissue, a statistically significant decrease in *Lin28a* expression of 49%, 51%, and 46% was observed at 25 days, 30 days, and postpubertally, respectively, when compared to 20-day-old prepubertal tissue (Figure 2A). No change in *Lin28b* expression was noted.

3.2.2 Both *Lin28a* and *Lin28b* mRNA levels decrease across the pubertal transition within ovarian tissue—Statistically significant decreases in both *Lin28a* and *Lin28b* expression were observed in ovarian tissue prior to the onset of puberty (Figure 2C). Specifically, 25-day-old, 30-day-old, and postpubertal mice had 64%, 72%, and 74% lower levels of *Lin28a* expression when compared to 15-day-old mice, and 56%, 66%, 69%, lower levels of *Lin28a* expression when compared to 20-day-old mice. Similarly, 25-day-old, 30-day-old, and postpubertal mice had 48%, 57%, and 57% lower levels of *Lin28b*, when compared to 20-day-old mice ($p < 0.05$ for all).

3.2.3 Neither *Lin28a* nor *Lin28b* expression decreases across the pubertal transition within pituitary tissue—No statistically significant changes in mRNA levels were noted in the pituitary prior to the onset of puberty (Figure 2B), but there is a trend towards decreased levels of *Lin28a* and *Lin28b* mRNA between 15 and 20 days. After 20 days, *Lin28a* mRNA levels tended to increase, with a statistically significant increase being seen between 20 days and the postpuberty timepoint ($p < 0.05$). This increase postdates the onset of onset of puberty in our colony, and was not observed for *Lin28b*.

3.3 Protein levels within the ovary also decrease prior to the onset of puberty

Using immunohistochemistry (IHC), *Lin28a* protein levels were examined in hypothalamic, pituitary, and ovarian tissue (an antibody suitable for IHC is currently not available for *Lin28b*); time points were chosen according to the ages where decreases in expression were noted.

In the ovary, an overall decrease in *Lin28a* protein levels was observed from 20-day-old to 30-day-old tissue (see Figure 3), which parallels the observed decreased mRNA levels measured by qRT-PCR. Unlike the ovary, only diffuse low level IHC-staining, with no specific pattern nor apparent change over time, was observed in the pituitary and hypothalamic tissues (data not shown), indicating that only low levels of *Lin28a* protein are present in these tissues at these ages.

In 20-day-old tissue (Figure 3A, B and C), *Lin28a* protein was visible within oocytes of early developing follicles, and levels of *Lin28a* protein appear to decrease as follicles

develop. In 30-day-old tissue, an overall reduction *Lin28a* protein level was observed when compared to 20-day-old tissue, and this was associated with an increase in more developed and atretic follicles.

3.4 *Let-7a* and *Let-7g* miRNA levels do not change in hypothalamic, pituitary, and ovarian tissues prior to the onset of puberty

To investigate whether the expression of *Let-7* miRNA changed across the time course, the miRNA expression levels of *Let-7a* and *Let-7g* were quantified using qRT-PCR. As with IHC studies, ages for investigation were chosen based on the timing of observed decreases in *Lin28a* and/or *Lin28b* expression. Hypothalamus was assessed at days 15, 20, 25, and 30, and ovary at days 20, 25, and 30 (Figure 4); to be complete, pituitary was also assessed at days 15, 20, 25, and 30 even though changes in *Lin28a* and *Lin28b* expression were not observed across the pubertal transition within the pituitary. Despite the observed decreases in *Lin28b* expression within the hypothalamus, and *Lin28a* and *Lin28b* mRNA within the ovary, there were no statistically significant changes observed in *Let-7a* and *Let-7g* miRNA levels between the younger versus older mice. No changes in *Let-7a* and *Let-7g* expression levels were noted in the pituitary either.

4. DISCUSSION

Transgenic female mice that over-express *Lin28a* have delayed puberty [Zhu et al., 2010]. This finding is consistent with the earlier data from Moss et al. (1997) showing that in *C. elegans*, over-expression of *lin28*, an orthologue of *Lin28a*, through gain-of-function mutations, results in delayed development while lack of *lin28* expression, through loss-of-function mutations, results in precocious development. The decreased *Lin28a* and *Lin28b* mRNA levels we observed prior to the onset of puberty in the ovary are consistent with these previous studies. We acknowledge that our data do not demonstrate cause and effect, and it remains possible that the decreases in expression that we noted are not related to the transition into puberty but simply represent part of a developmentally programmed diminution in *Lin28a* and *Lin28b* expression with age. However, in total, data from the transgenic mice, *C. elegans*, and the current study are all consistent and all support the working hypothesis that lowered levels of *Lin28a* and *Lin28b* expression may facilitate developmental transitions, while increased levels are associated with delayed development.

Within hypothalamic tissue, a significant decrease in *Lin28a* expression was observed at 25 days, 30 days, and postpubertally, when compared to 20-day-old female mice, while there was no significant decrease in *Lin28b* expression. Although these findings are consistent with the findings of Zhu et al. (2010) and suggest that lower levels of *Lin28a* expression may facilitate the transition into puberty, they also raise several questions that will warrant further investigation: what is the biological significance of steady as opposed to decreasing levels of *Lin28b* expression at these ages? Although IHC may not be quantitative enough to detect subtle changes in protein levels, does the lack of change in IHC staining for *Lin28a* in the hypothalamus negate the importance of the changes in gene expression? Moreover, it remains possible that decreases in *Lin28a* and/or *Lin28b* expression and protein levels will be observed in different strains of mice, at different time points (including *in utero*), or within particular nuclei or cell types within the hypothalamus and/or pituitary.

Our data do provide important new information and strongly suggest that changes of *Lin28a*/*Lin28b* expression in the ovary may play an important role in modulating maturation of the HPG axis. The most convincing and consistent changes in expression, for both *Lin28a* and *Lin28b*, prior to the onset of puberty were observed in the ovary. These data indicate that the ovary may be an important site where the *Lin28* pathway exerts effects that modulate the timing of puberty. Although traditionally much attention has been focused on hypothalamic

factors that modulate pubertal timing [Ojeda et al., 2006] and it is well accepted that the hypothalamus is the central site of regulation of the onset of puberty, it is also clear that gonadal products are also critical components of the network that modulates the onset of puberty [Mayer et al., 2010]. The hypothalamus receives modulating signals from the periphery (such as nutritional status and other inputs) and responds to them via a complex gene network/hierarchy that factors these inputs in when controlling the activation of GnRH neurons and onset of puberty [Ojeda et al., 2010]. Thus, it is possible that the *Lin28a/Lin28b* pathway influences the timing of puberty, at least in part, through indirect effects at the level of the ovary or perhaps even at other tissues in the periphery. For example, two groups have recently demonstrated that altering *Lin28b* expression in peripheral tissues, such as muscle, alters glucose metabolism and affects body size [Frost and Olson, 2012; Zhu et al., 2011, Zhu et al., 2010].

Importantly, the changes we observed in *Lin28a* gene expression were correlated with a decrease in protein levels in 30-day-old compared to 20-day-old ovaries. Moreover, *Lin28a* protein levels appear to be higher within early developing follicles (which are more prominent at 20 days) and lower as the follicles become more developed and/or atretic (which is more prominent in the 30-day-old samples), suggesting that the level of *Lin28a* protein is dependent on the stage of follicular development. Demonstrating that the alterations in expression that we observed modulate pubertal onset will require future experimentation. For example, one could envision using transgenic methodologies to modulate *Lin28a* and/or *Lin28b* expression in a tissue or even age-dependent manner and investigating whether changes of expression in the ovary (or other components of the HPG axis) alter pubertal timing. Such studies would also allow for investigation of whether the *Lin28a/Lin28b* pathway modulates aspects of reproductive endocrine function other than pubertal timing, such as follicle development, ovulation, and fertility.

Lin28 is known to repress generation of mature *Let-7* miRNAs [Hagan et al., 2009; Heo et al., 2008; Piskounova et al., 2008; Viswanathan et al., 2008]. Thus, it is currently thought that the *Lin28* pathway modulates pubertal onset by affecting levels of *Let-7* miRNAs and, subsequently, affecting levels of expression of *Let-7* targets. Based on this reasoning, it would follow that increased expression of *Lin28a/Lin28b* would lead to decreased *Let-7* miRNA levels, which would then lead to increased expression of the *Let-7* target genes. If these target genes usually restrain pubertal onset [Ojeda et al., 2010; Viswanathan and Daley, 2010; Zhu et al., 2010], this cascade of events would result in later pubertal onset. Indeed, this reasoning fits with the observation that increased *Lin28a* expression leads to later puberty [Zhu et al., 2010] and our finding of decreased *Lin28a* and *Lin28b* expression prior to the onset of puberty.

However, other data from the transgenic mice that overexpress *Lin28a* and the *Let-7* data from the present studies raise the possibility that at least some of the *Lin28a/Lin28b* pathway effects in the HPG axis may be *Let-7* independent. The transgenic mice displayed increased levels of *Lin28a* in the hypothalamus and ovary, but the expected decrease in *Let-7a* and *Let-7g* miRNA levels was not observed [Zhu et al., 2010]. Our current data show that, although *Lin28a* expression decreased in the hypothalamus and *Lin28a* and *Lin28b* expression decreased in the ovary, neither *Let-7a* nor *Let-7g* miRNA levels increased significantly across the time course. It is possible that subtle changes occurred that we did not detect or that changes in other *Let-7* family members occurred that were not assessed by our assays. This last possibility seems unlikely, however, because *Let-7a* and *Let-7g* are prominent members of the *Let-7* family, and it has been demonstrated that changes in these two miRNAs are reflective of changes within the whole family [Newman et al., 2008; Zhu et al., 2011].

That modulation of the timing of puberty in female mice may be *Let-7* independent has important implications. Our data suggest that *Lin28a* and *Lin28b*'s effects may be mediated, at least in part, through a different miRNA pathway within the HPG axis [Balzer et al., 2010] or that *Lin28a* and *Lin28b* effects within the HPG axis may be miRNA independent. Indeed, a recent publication about regulation of metabolic pathways by the *Lin28* system has demonstrated that the *Lin28* pathway can affect phenotypes through both *Let-7* dependent and independent actions [Zhu et al., 2011]. It is clear that investigation of the downstream mediators of *Lin28a* and *Lin28b*'s effects within the HPG axis will be an important area for further study. Determining whether modulation of pubertal timing is *Let-7* dependent will require model systems, such as transgenic animals, where *Let-7* levels can be increased or decreased and the effect on pubertal timing assessed.

An unexpected but interesting finding is that *Lin28a*, but not *Lin28b*, expression increased after puberty in the pituitary. The observed increase in *Lin28a* expression may indicate a role for *Lin28a* in adult neuroendocrine function and suggests that the role of the *Lin28* pathway within the pituitary is an important area for further investigation.

In summary, we report what we believe are the first published data examining peripubertal expression of *Lin28a*, *Lin28b*, and *Let-7* family members in the HPG axis. Consistent with the delayed developmental phenotype observed in *C. elegans* mutants over-expressing *lin28* [Moss et al., 1997; Yang and Moss, 2003] and the delayed puberty observed in transgenic mice that over-express *Lin28a* [Zhu et al., 2010], our current findings demonstrate that *Lin28a* and *Lin28b* expression decrease across the pubertal transition. Moreover, our data raise the possibility that *Lin28a* and *Lin28b* expression in the ovary may be an important regulator of the HPG axis, affecting the timing of puberty and/or follicular development. Although this pathway clearly warrants further investigation in the hypothalamus and pituitary, our data point to the importance of gaining further understanding of how the *Lin28* pathway modulates ovarian function and how that modulation may then regulate maturation of the HPG axis.

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Highlights

- Expression of *Lin28a* and *Lin28b* decreases in the ovary prior to pubertal onset
- Expression of *Lin28a* decreases in hypothalamus prior to the onset puberty
- A decrease *Lin28a* protein levels mirrored the decline in *Lin28a* in ovarian tissue
- *Lin28* regulation of pubertal onset may occur at the ovarian and hypothalamic level
- The effects of the *Lin28* pathway may be mediated, in part, independently of *Let-7*

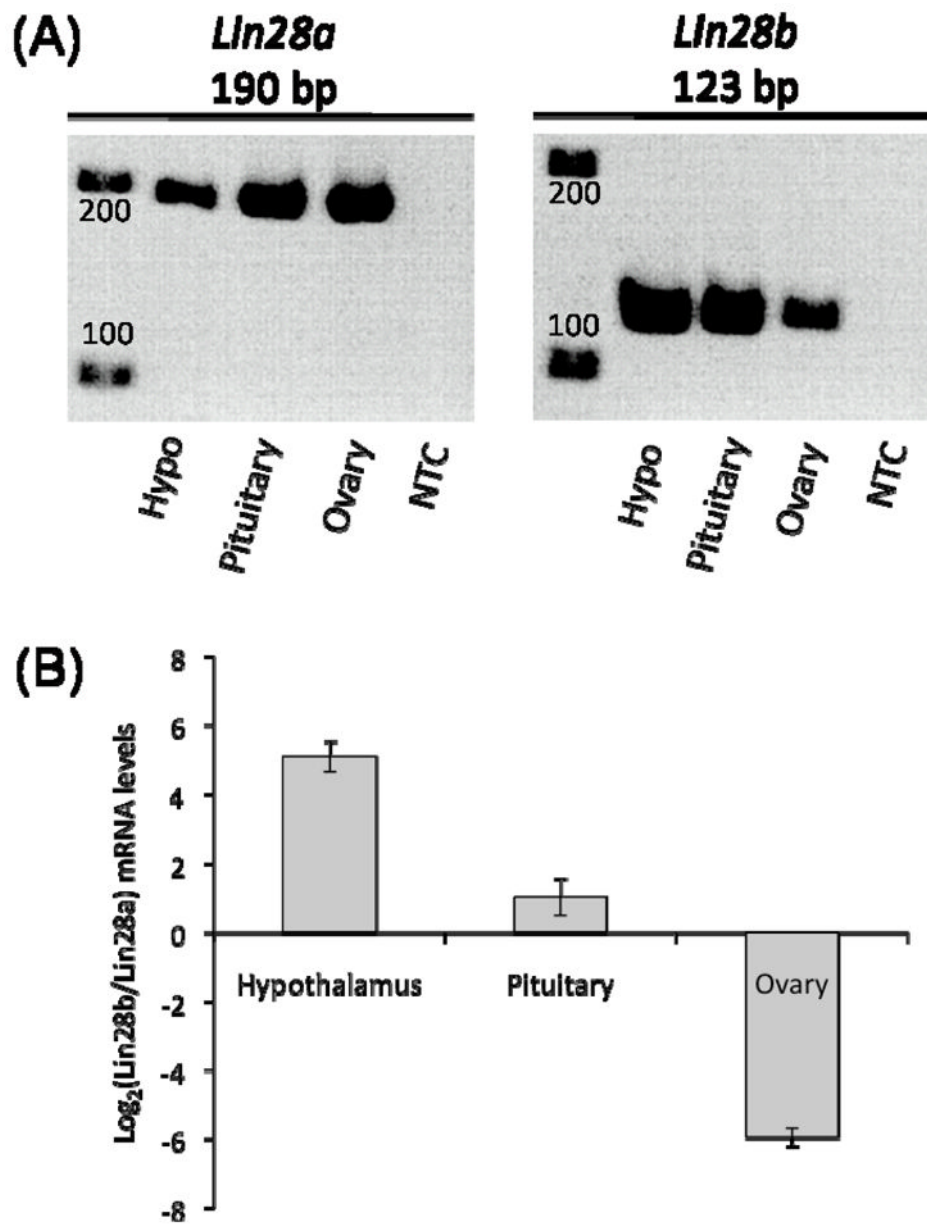


Figure 1. Expression of *Lin28a* and *Lin28b* in hypothalamic, pituitary, and ovarian tissue
(A) RT-PCR of *Lin28a* (190 bp) and *Lin28b* (123 bp) mRNA in 25-day-old hypothalamic (hypo), pituitary, and ovarian tissue from C57BL/6 female mice. NTC = no template control.
(B) Relative expression levels of *Lin28a* and *Lin28b* in different tissues at 25 days as determined by qRT-PCR, normalized to β -actin mRNA.

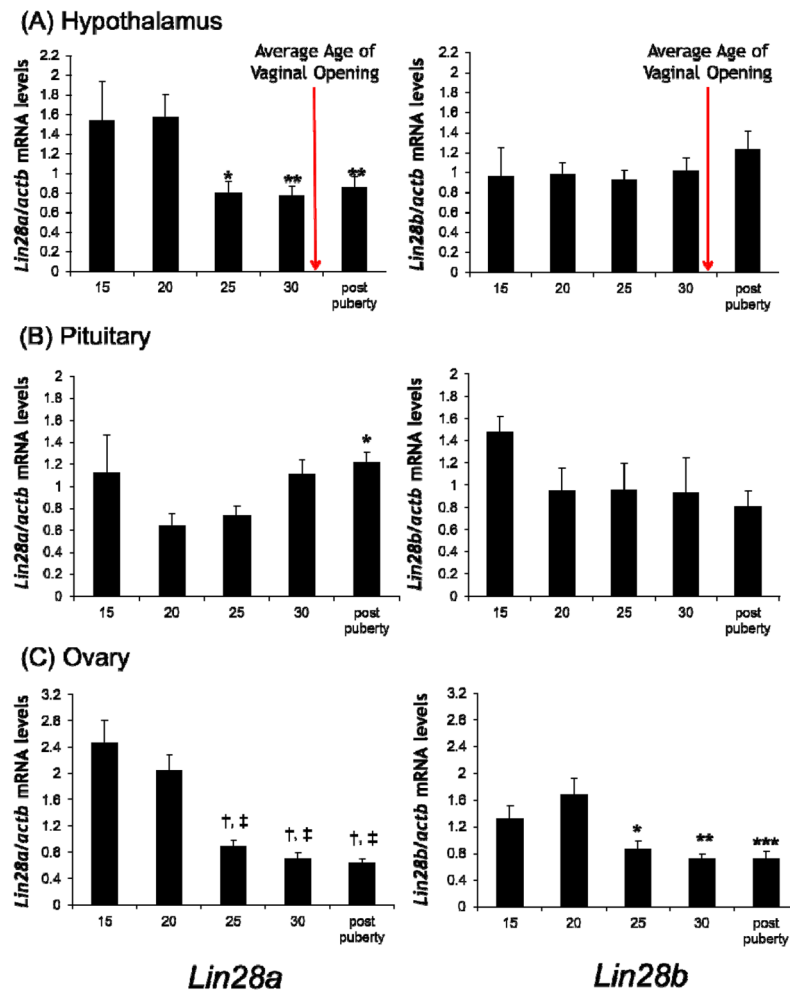


Figure 2. *Lin28a* and *Lin28b* mRNA expression levels in hypothalamic, pituitary, and ovarian tissue across the pubertal transition
qRT-PCR for *Lin28a* and *Lin28b* mRNA in tissues from prepubertal 15, 20, 25, 30 day old and postpubertal (35–45 days old) C57BL/6 female mice, normalized to β -actin in hypothalamus (A), pituitary (B), and ovary (C). Average age of vaginal opening in the mouse colony is indicated by the arrow (31.5 ± 2.4 days). Values are displayed as means \pm SEM ($n=5-21$ mice for each time point). For *Lin28a* in hypothalamic tissue, $*=p<0.05$, and $**=p<0.01$, when compared to 20-day-old tissue. For *Lin28a* in pituitary tissue, $*=p<0.05$, when compared to 20-day-old tissue. For *Lin28a* in ovarian tissue, $\dagger=p<0.001$, and $\ddagger=p<0.001$, when compared to 15 and 20-day-old tissue, respectively. For *Lin28b* in ovarian tissue, $*=p<0.05$, $**=p<0.01$, and $***=p<0.001$, when compared to 20 day old tissue.

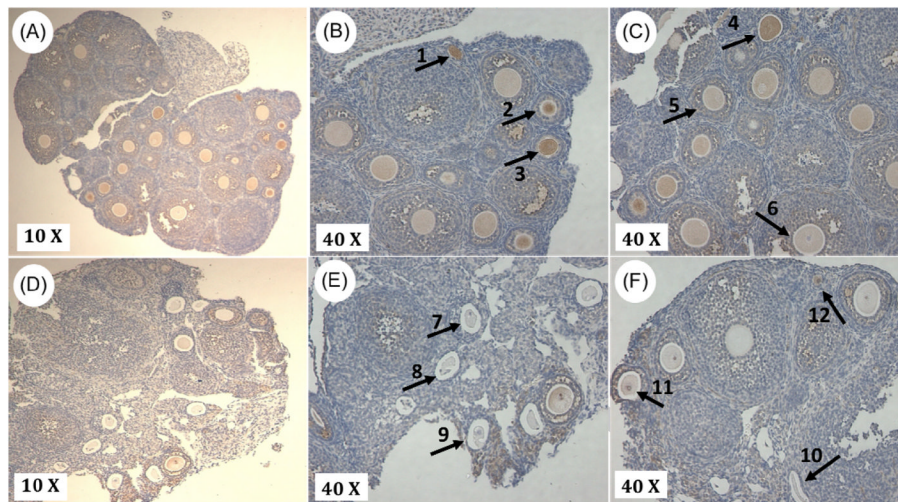


Figure 3. Lin28a protein levels decrease prior to the onset of puberty in ovarian tissue
 Immunohistochemical staining of Lin28A protein in 20 day (A–C) and 30 day (D–F) ovarian tissue. Lin28a protein expression is identified by diaminobenzidine staining. In 20-day-old tissue, Lin28a is detected in early developing follicles (arrow 1), early secondary follicles (arrows 2, 3, and 4), late secondary follicles and granulosa cells surrounding secondary follicles (arrow 5). In 30-day-old tissue, Lin28a protein is less evident, due to the abundance of atretic follicles (arrows 7, 8, 9 and 10). Lin28a still persists in the granulosa cells and oocytes of secondary follicles (arrow 11), and in early follicles (arrow 12). This pattern of Lin28a protein distribution was consistent in the two biological replicates that were examined

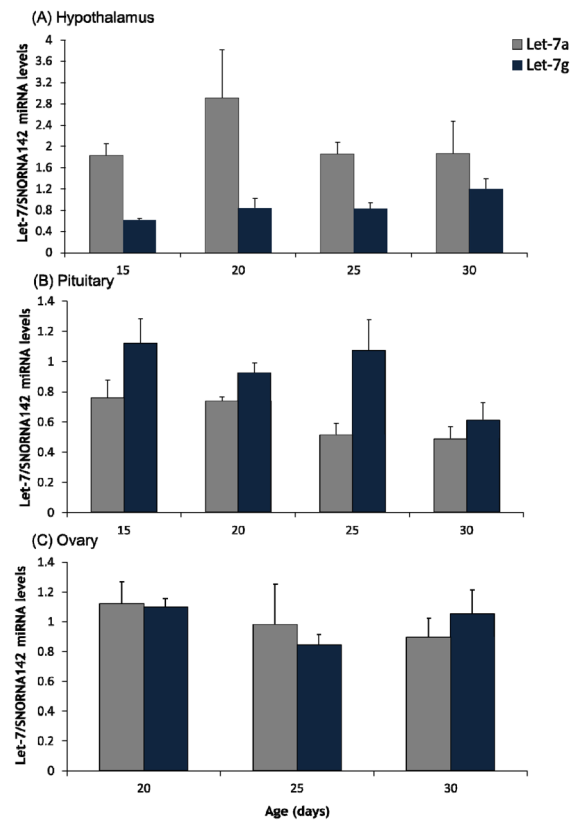


Figure 4. Expression of *Let-7a* and *Let-7g* miRNA in hypothalamic, pituitary, and ovarian tissue qRT-PCR for *Let-7a* and *Let-7g* miRNA in 15, 20, 25, and 30-day-old hypothalamic (A) and pituitary (B) tissue, and 20, 25, and 30-day-old ovarian (C) tissue, normalized to snoRNA142. Values are displayed as means \pm SEM (n=3–7 for each time point).