Sex-specific regulation of weight and puberty by the Lin28/let-7 axis

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Abstract

Growth and pubertal timing differ in boys and girls. Variants in/near LIN28B associate with age at menarche (AAM) in genome-wide association studies and some AAM-related variants associate with growth in a sex-specific manner. Sex-specific growth patterns in response to Lin28b perturbation have been detected in mice, and overexpression of Lin28a has been shown to alter pubertal timing in female mice. To investigate further how Lin28a and Lin28b affect growth and puberty in both males and females, we evaluated Lin28b loss-of-function (LOF) mice and Lin28a gain-of-function (GOF) mice. As reported previously, Lin28b LOF led to lighter body weights only in male mice while Lin28a GOF yielded heavier mice of both sexes. Let-7 GOF mice weighed less than controls, and males were more affected than females. Timing of puberty was assessed by vaginal opening (VO) and preputial separation (PS). Male Lin28b LOF and male let-7 GOF, but not female, mice displayed alteration of pubertal timing, with later PS than controls. In contrast, both male and female Lin28a GOF mice displayed late onset of puberty. Together, these data point toward a complex system of regulation by Lin28a, Lin28b, and let-7, in which Lin28b and let-7 can impact both puberty and growth in a sex-specific manner, raising the possibility that this pathway may contribute to differential regulation of male and female growth and puberty in humans.

Key Words

puberty
Lin28
let-7
sex-specific

Introduction

Women tend to be shorter than men, and girls have their growth spurt at an earlier phase in puberty than boys (Styne & Grumbach 2012). Girls also experience puberty at younger ages than boys and are more likely to exhibit precocious puberty, while boys are more likely to have delayed puberty (Carel & Leger 2008, Walvoord 2010, Palmert & Dunkel 2012, Styne & Grumbach 2012). The factors that contribute to these differences are unknown but important because variations in the timing of puberty have been associated with higher risk for adult metabolic abnormalities, cancer, and osteoporosis (Weir et al. 1998, Golub et al. 2008, Lakshman et al. 2009,..

Growth and the timing of puberty are highlyheritable, and genome wide association studies (GWAS) have identified many loci that modulate both traits. Interestingly, variants in/near LIN28B affect both adult height and the age at menarche (AAM; Lettre et al. 2008, He et al. 2009, Ong et al. 2009, Perry et al. 2009, Sulem et al. 2009, Elks et al. 2010, Widen et al. 2010, Leinonen et al. 2012, Cousminner et al. 2013, Perry et al. 2014), and two of the puberty-related single nucleotide polymorphism (SNPs) associate with growth and/or adult BMI in a sex-specific manner (Lettre et al. 2008, Widen et al. 2010, Ong et al. 2011, Leinonen et al. 2012). That this axis regulates these traits in a sex-specific manner is further supported by the finding that male but not female Lin28b loss-of-function (LOF) transgenic mice are lighter than controls (Shinoda et al. 2013b).


How this pathway regulates the timing of puberty is largely unknown. However, data from model systems indicate that the causative gene at the AAM-associated GWAS locus is indeed LIN28B. In C. elegans, lin28 LOF as well as let-7 gain-of-function (GOF) lead to precocious larval development (Ambros & Horvitz 1984, Reinhart et al. 2000). Analogously, Lin28a GOF female mice display delayed puberty (Zhu et al. 2010). This sex-specific regulation of body size by LIN28B in humans and mice and the involvement of this pathway in the regulation of pubertal timing lead us to examine whether Lin28a and Lin28b also regulate puberty in a sex-specific manner and whether let-7 is mediating such effects. We first utilized Lin28b LOF, Lin28a GOF, and let-7 GOF mice to validate the effects of perturbation of Lin28a, Lin28b, and let-7 expression on body weight in male and female mice. We then extended our analysis to include investigation of the roles of Lin28a, Lin28b, and let-7 in the timing of pubertal onset in male and female mice. Our results suggest that the regulation of these traits by Lin28a and Lin28b may differ and that some effects of Lin28b and let-7 are sex-specific.

Materials and methods

Animals

Studies were approved by the Toronto Centre for Phenogenomics (TCP) Animal Care Committee (AUP 09-08-0097) in accordance with recommendations of the Canadian Council on Animal Care, the requirements under Animals for Research Act, RSO 1980, and the TCP Committee Policies and Guidelines. Mice were maintained under controlled conditions (25°C and 10 h light:14 h darkness cycle) in sterile, individually ventilated cages, provided irradiated chow and sterile water, and allowed to feed ad libitum via an automated watering system. In Lin28b LOF (Lin28b<sup>−/−</sup>) mice, exon 2 of Lin28b has been excised by targeted mutagenesis resulting in global absence of the Lin28b protein (Shinoda et al. 2013b). In the let-7 GOF strain, a M2-rtTA element has been inserted in the Rosa26 locus and a tetracycline-responsive element, followed by a transgenic let-7g sequence, has been inserted in the Col1A1 locus (Zhu et al. 2011, Shinoda et al. 2013b), leading to global upregulation of let-7g expression after administration of doxycycline (Beard et al. 2006). Normally, Lin28b binds to the stem loop of the let-7 pre-miRNA and prevents its processing to mature let-7 miRNA (Heo et al. 2008). To prevent such downregulation, the transgenic let-7 element has been engineered so that it cannot be bound by Lin28a or Lin28b (Zhu et al. 2011). Both strains have been used previously, and alterations in
expression of Lin28b and let-7g have been validated (Zhu et al. 2011, Shinoda et al. 2013b). In the Lin28a GOF mice, a tetracycline-responsive element, followed by a transgenic Lin28a sequence, has been inserted in the Col1A1 locus. This transgene is ‘leaky’ and leads to global upregulation of Lin28a even in the absence of the M2-rtTA element and doxycycline administration (Zhu et al. 2010). The Lin28b LOF and let-7 GOF strains were created in the Daley laboratory and maintained on a mixed background of C57BL6/J, 129, and Balb/C; Lin28a GOF mice were maintained on a C57BL6/J background (Zhu et al. 2011, Shinoda et al. 2013b). For our studies, male mice were imported and mated with superovulated C57BL6/J females for strain rederivation at the TCP Transgenic Core. Embryos were transferred to an in-house C57BL6/J female and offspring were bred to each other to maintain the mixed genetic background. Prior to phenotyping the mice in our facility, LOF was confirmed by the absence of Lin28b protein in Lin28b LOF mice in the adult testes and let-7 GOF was confirmed by upregulated let-7g levels in livers of doxycycline-fed let-7 GOF mice. Successful rederivation of the Lin28a GOF mice was verified by recapitulation of the previously observed enlarged body sizes of Lin28a GOF mice.

Mice were fed a standard chow (Harlan, Teklad Global 18% Protein Rodent Diet, 2018, Madison, WI) or the equivalent diet containing 625 mg/kg doxycycline (Harlan, TD.01306). For let-7 GOF experiments, mice were fed the doxycycline chow from birth, which induced transgenic let-7g expression in mice carrying the transgene, but not in littermate controls, by feeding the doxycycline chow to the lactating mother (beginning on the date of birth of her litter) and directly to the pups after weaning. Feeding doxycycline to lactating mothers is known to cause upregulation of doxycycline-inducible transgenes in pups (Sun et al. 2012). The Lin28a transgene is known to be leaky with increased expression that is sufficient to delay puberty in females and increase body weights in both males and females without administration of doxycycline (Zhu et al. 2010); thus, these mice were fed standard chow.

In every experiment mice were mated for one week, after which the male was removed. Females were transferred to an individual cage prior to giving birth. Date of birth was monitored daily between 0009 h and noon, and weaning was performed on postnatal day 21 with males and females then housed separately with a target of 3–4 weaned pups per cage. Littermate controls were used for all experiments. Phenotyping was done without knowledge of genotype, between 0009 h and noon every day. The timing of puberty was assessed by monitoring vaginal opening (VO) (Danilovich et al. 1999, Seminara et al. 2003, Krewson et al. 2004, Fernandez-Fernandez et al. 2006, Sanchez-Garrido et al. 2013) and preputial separation (PS) (Korenbrot et al. 1977, Nathan et al. 2006, Takashima-Sasaki et al. 2006, Deboer & Li 2011, Smith & Spencer 2012, Sanchez-Garrido et al. 2013, Novaia et al. 2014) every day post-weaning. VO and PS are estradiol- and testosterone-dependent processes respectively, and altered day of VO correlates with other measures of pubertal timing (Chehab et al. 1997, Divall et al. 2010, Zhu et al. 2010, Caron et al. 2012, Dicken et al. 2012, True et al. 2015). Body weights were measured to 0.1 g. Experiments were performed a minimum of two times to ensure that results were reproducible. Mouse genotypes from tail biopsies were determined using real-time PCR with specific probes designed for each gene (Transnetyx, Cordova, TN, USA).

### Tissue dissection

Tissues were harvested following decapitation under isoflurane anesthesia. For hypothalamic tissue, whole brains were dissected and flash frozen in liquid nitrogen. The hypothalamus was then microdissected while frozen, according to established coordinates and landmarks (Baker et al. 1983, Grieco et al. 2013, Sangiao-Alvarellos et al. 2013). All other tissues were kept in RNAlater (Ambion, AM7021, Foster City, CA), at −20 °C until RNA extraction.

### Gene expression

Tissues were homogenized in QIAsol solution (Qiagen, 1023537, Hilden, Germany) with a sonicator using a microtip, and RNA was extracted using the miRNeasy kit (Qiagen, 217004). DNase treatment was performed during the RNA extraction using RNase free DNase (Qiagen, 79254). RT-reactions were performed using the Taqman microRNA reverse transcription kit (ABI, 4366596, Foster City, CA) with specific RT-primers (ABI, 442795, assay id 377, 2282, and 1231, for let-7a, let-7g, and sno142 (housekeeping gene for normalization) respectively). Twenty nanogram total RNA was used in each RT reaction. As a standard, ovarian RNA from several 20-day-old C57BL6/J females was pooled and used in every experiment. Following reverse transcription 60 μl of 10 mM Tris pH 8 was added to each 15 μl sample reaction and 6.65 μl of the diluted sample was added to each Q-PCR reaction. For all Q-PCRs, the PefecTaq FastMix II (Quanta, 95120-012,
Gaithersburg, MD, USA) was used along with the specific TM-primer for the Taqman microRNA assay.

Q-PCR was performed on a MX3 Pro Q-PCR system from Stratagene. Thermal cycling was performed with an initial denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Every reaction was run in duplicate. Quantification used the standard curve method.

Dexa

Lean mass (g) and fat mass (g) were measured following euthanasia using peripheral dual-energy X-ray absorptiometry (pDXA, PIXImusII, GE Lunar Corp., Madison, WI, USA) at the Clinical Phenotyping Core at The Toronto Centre for Phenogenomics.

Intraperitoneal glucose tolerance tests

Intraperitoneal glucose tolerance tests (IP-GTT) were performed after a 6-h fast. A baseline blood sample was obtained from the tail vein and glucose was measured with an Ascencia glucometer (Bayer). A 10% glucose solution (Sigma, G8644) was injected IP at a dose of 1 g glucose per kg bodyweight. Glucose was remeasured at 30, 60, and 120 min after the injection.

Statistical analysis

Statistical analyses, except linear regressions, were performed using Graphpad (Prism, La Jolla, CA, USA). Although experimental and control groups displayed equal variances, puberty data were not invariably normally distributed; thus PS and VO data were analyzed with Mann–Whitney non-parametric t-tests. Body weights displayed normal distribution and were analyzed with the student’s t-test. Body weights on postnatal day 80 were also analyzed with a two-way ANOVA to test for sex-specificity. Linear regression of weight and age at puberty data was done using glm package in R. The regression coefficients were estimated using the control data and the fit of the mutant data to the wild type regression was estimated with the Kolmogorov–Smirnov test. DEXA and GTT data displayed unequal variances between experimental and control groups, and therefore t-tests assuming unequal variances were employed. Gene expression data were analyzed using the student’s t-test. Results are reported as mean ± s.d., with statistical significance attributed to P<0.05.

Results

Growth

Assessing growth patterns is an important component of the assessment of pubertal timing; therefore we began our studies by re-phenotyping growth (Table 1) at our centre.

As demonstrated previously, both male and female mice globally overexpressing Lin28a weighed more than littermate controls (Fig. 1A and B) (Zhu et al. 2010), while global Lin28b LOF males, but not females, weighed significantly less than littermate controls from postnatal day 21 onward (Fig. 1C and D) (Shinoda et al. 2013b). The sex-specific effect on weight among adult Lin28b LOF mice (postnatal day 80) was statistically significant after assessment with two-way ANOVA (P=0.04 after Bonferroni correction).

To begin to examine whether Lin28a/Lin28b regulate growth phenotypes via let-7, we phenotyped the let-7g GOF mice (Zhu et al. 2011) in which global overexpression was induced by feeding doxycycline chow to the pups from birth. Doxycycline administration was accomplished initially via transfer to the pups through the mother’s milk (Sun et al. 2012) and subsequently by feeding directly to the pups after weaning. We chose to not induce let-7 overexpression during embryogenesis because this could be detrimental, as seen in Lin28a LOF embryos that overexpress let-7 and subsequently die as neonatal pups (Shinoda et al. 2013b). Because Lin28a and Lin28b can inhibit processing of let-7 precursors into functional miRNA (Heo et al. 2008), let-7 GOF mice were expected to phenocopy the Lin28b LOF mice if the effects of the Lin28b LOF model were mediated by increases in let-7 miRNAs. Indeed, the let-7 GOF mice weighed less than littermate controls (Fig. 1E and F) and males were more affected than females. Again, the sex-specificity of genotype effect on body weight on postnatal day 80 was found to be significant (P=0.0002 after Bonferroni correction). Although the Lin28b LOF and let-7 GOF mice were similar,

<table>
<thead>
<tr>
<th>Perturbation</th>
<th>Genetic background</th>
<th>Puberty</th>
<th>Body weights</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lin28a GOF</td>
<td>C57BL6/J 129, Balb/C</td>
<td>15–33</td>
<td>12–33</td>
</tr>
<tr>
<td>Lin28b LOF</td>
<td>C57BL6/J 129, Balb/C</td>
<td>12–29</td>
<td>7–15</td>
</tr>
<tr>
<td>let-7 GOF</td>
<td>C57BL6/J 129, Balb/C</td>
<td>12–24</td>
<td>7–24</td>
</tr>
</tbody>
</table>
Next we investigated pubertal timing in the Lin28b LOF mice. Female Lin28b LOF and WT littermates displayed VO at the same age (Fig. 2C). In contrast, the Lin28b LOF males displayed PS two days later than WT littermates ($P=0.004$) (Fig. 2D). The let-7 GOF mice again phenocopied the Lin28b LOF mice: in let-7 GOF females, despite the lighter weight, VO occurred at the same age as in littermate controls (Fig. 2E) while let-7 GOF males reached PS later than controls ($P=0.004$) (Fig. 2E and F). The delay in PS resulted in mice that were older and hence heavier at PS than controls (Table 2), suggesting that, at least in this strain, delayed growth could not explain the delayed puberty.

**Age/weight relationship at puberty**

Because the body weights at puberty were altered in different ways in the different strains the relationship between growth and age at puberty was examined further. A linear regression model was estimated for weight and age differences were also apparent. For example, the lighter body weights in let-7 GOF males did not become apparent until postnatal day 30, much later than the difference seen among the Lin28b LOF mice.

**Puberty**

Having confirmed sex-specific effects on growth, we next examined whether pubertal timing was regulated in a sex-specific manner in the three mouse strains. Pubertal timing in female mice was determined by daily assessments of VO, and pubertal timing in male mice was determined by daily assessments of PS. It has previously been shown that Lin28a GOF females display delayed puberty, as measured by the day of VO, age at first estrus and time of first pregnancy (Zhu et al. 2010). To determine whether Lin28a exerts this effect in a sex-specific manner, we phenotyped male and female mice in parallel. Both Lin28a GOF females and males exhibited delayed puberty ($P \leq 0.0001$) (Fig. 2A and B) and were heavier at VO and PS than controls (Table 2).

**Figure 1**

Growth patterns in Lin28a GOF (A and B), Lin28b LOF (C and D), and let-7 GOF (E and F) mice. The number of animals per group is indicated in each chart. Error bars represent s.d. Growth data were analyzed with the student’s t-test at each time point. *$P<0.05$.

**Figure 2**

The onset of puberty, as assessed by VO and PS in Lin28a GOF, Lin28b LOF, and let-7 GOF mice. In each chart black boxes represent the GOF or LOF and open circles represent the control group. The inserted bar graphs display the mean day of VO or PS. Error bars represent s.d. $n=9–33$, exact numbers per group are indicated in Table 2. Puberty data were analyzed with the Mann–Whitney non-parametric t-test. *$P<0.05$. 

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-15-0360 © 2016 Society for Endocrinology
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Table 2 Comparison of pubertal body weight at puberty in Lin28a GOF, Lin28b LOF, and let-7 GOF male and female mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sex</th>
<th>Weight at VO/PS</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>M</td>
<td>15.4±1.3</td>
<td>33</td>
</tr>
<tr>
<td>Lin28a GOF</td>
<td>M</td>
<td>20.8±2.1</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Wt</td>
<td>F</td>
<td>15.3±1.5</td>
<td>24</td>
</tr>
<tr>
<td>Lin28a GOF</td>
<td>F</td>
<td>21.1±2.0</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Wt</td>
<td>M</td>
<td>16.8±2.0</td>
<td>29</td>
</tr>
<tr>
<td>Lin28b LOF</td>
<td>M</td>
<td>16.6±2.1</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Wt</td>
<td>F</td>
<td>15.5±0.8</td>
<td>13</td>
</tr>
<tr>
<td>Lin28b LOF</td>
<td>F</td>
<td>14.8±1.3</td>
<td>9</td>
</tr>
<tr>
<td>Control</td>
<td>M</td>
<td>16.5±1.9</td>
<td>12</td>
</tr>
<tr>
<td>let-7 GOF</td>
<td>M</td>
<td>18.2±2.1</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P=0.03</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>F</td>
<td>15.9±0.8</td>
<td>24</td>
</tr>
<tr>
<td>let-7 GOF</td>
<td>F</td>
<td>15.3±1.0</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P=0.008</td>
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</table>

P values were calculated with the student’s t-test.

Body composition and glucose metabolism in young let-7 GOF mice

Because let-7 GOF males weighed less than WT littermates, and because adult let-7 GOF mice have less body-fat than controls (Brill & Moenter 2009, Frost & Olson 2011, Zhu et al. 2011), we hypothesized that the delay in puberty could be due to a lower fat content in the male GOF mice. However, DEXA scans of pre-pubertal let-7 GOF mice showed no significant difference in body fat content or percent body fat compared with control littermates (Fig. 4A and B).

Let-7 also affects glucose metabolism in adult mice and glucose metabolism could affect pubertal onset (Brill & Moenter 2009, Frost & Olson 2011, Zhu et al. 2011); at VO/PS for the littermate controls, and then we examined whether the data from the LOF/GOF mice showed the same relationship (that is, did the data from the transgenic mice fit on the line for the control mice). For both female and male mice, the Lin28a GOF weight/age relationship was significantly different from the weight/age relationship observed among controls (P=2.5×10⁻⁵ and P=0.0015 respectively, Fig. 3A and B), indicating that the alteration in Lin28a expression changed the relationship between growth and age of puberty in these mice. The relationship was changed similarly in female and male mice.

The Lin28b LOF females displayed body weights and VO similar to controls, and also exhibited a similar age/weight relationship at VO (Fig. 3C). The male Lin28b LOF mice displayed both slowed growth and delayed puberty, and the relationship between time of puberty and weight at PS was similar among the male Lin28b LOF mice and controls (Fig. 3D).

let-7 GOF females displayed a different age/weight relationship at VO compared with controls (P=2.4×10⁻⁵) (Fig. 3E), perhaps indicating that the let-7 GOF affected growth but not age at VO. As for Lin28b LOF males, the age/weight relationship at PS was not different among male let-7 GOF mice and controls (Fig. 3F). These results provide further evidence that let-7 exerts its function differently in males and females.

<table>
<thead>
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<th>Figure 3</th>
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<tr>
<td>Weight and age at puberty for each control (open circles) and transgenic (black boxes) mouse. The line represents linear regression of weight and age at VO/PS for controls. Significant differences in the relationship of age and weight at puberty between controls and transgenic mice were detected in both male and female Lin28a GOF mice and among let-7 GOF females.</td>
</tr>
</tbody>
</table>
Because previous studies have demonstrated that Lin28b and let-7 can regulate growth peripherally (Frost & Olson 2011, Shinoda et al. 2013b), we also investigated if the sex differences in puberty and body weight could derive from sexually dimorphic peripheral let-7 expression. let-7 levels were assessed in pituitary and gonads (as part of the HPG axis) and liver (as a non-HPG tissue) in Lin28b LOF mice. No differential regulation of let-7a or let-7g was detected in any tissue among Lin28b LOF female and male mice (Fig. 6A and B). We also assessed whether the lack of sufficient changes in peripheral let-7 expression in female mice might explain why these mice were less affected than the males. However, contrary to this hypothesis, overexpression was either similar in both sexes or greater in females than in males (Fig. 6C and D).

**Discussion**

Differences exist between boys and girls and men and women in many growth-related traits such as height, body mass index, waist to hip ratio, and the timing of puberty. The basis for these differences is not fully understood, but understanding the differences is important because they are linked to cardiovascular and other health outcomes (Weir et al. 1998, Golub et al. 2008, Lakshman et al. 2009, Joinson et al. 2011, Seselj et al. 2012, Widen et al. 2012, Ritte et al. 2013, Day et al. 2015). Certainly sex steroids play a role in this sex specificity and so do sex chromosomes as is evidenced by the height in women with complete androgen insensitivity and Turner syndrome. GWAS present a new opportunity to further our understanding of sex specificity among complex traits.
Peripheral let-7 expression levels were unaltered in Lin28b LOF mice, but upregulated in in let-7 GOF mice. (A and B) let-7a and let-7g levels in pituitary, gonads, and liver in Lin28b LOF mice on the mean day of VO (postnatal day 30) or PS (postnatal day 27) among control mice, n = 3–4. (C and D) let-7g levels in pituitary, gonads, and liver on the mean day of VO (postnatal day 29) or PS (postnatal day 26) in let-7 GOF mice, n = 3–4. Error bars represent S.D. Expression data were analyzed using the student’s t-test. *P<0.05.

(Sangiao-Alvarellos et al. 2013, Sangiao-Alvarellos et al. 2015). Our findings highlight the importance of understanding the sex-specific effects of this complex pathway and its contribution to differences in growth and puberty seen in boys and girls.

Following the association of LIN28B with AAM (He et al. 2009, Ong et al. 2009, Perry et al. 2009, Sulem et al. 2009, Elks et al. 2010), researchers investigated whether variants in LIN28B underlie central precocious puberty in girls (Silveira-Neto et al. 2012) or constitutional delay of growth and puberty in boys (Tommiska et al. 2010). The results indicate that such variation is not a common cause of these extreme phenotypes and suggest that the primary role of LIN28B may be in regulating the timing of puberty within the general population. In contrast to other studies using more severely affected animals (Good et al. 1997, Corradi et al. 2003, Seminara et al. 2003, Pask et al. 2005, Brüll & Moenter 2009, DeBoer et al. 2010, Deboer & Li 2011, Elias & Purohit 2013), all three strains of our mice were healthy and fertile and were thus a good model for studying genes that affect timing of puberty in the general population. Similarly, the two-day delay detected in our transgenic male mice is of comparable magnitude to studies of litter-size and diet manipulations in male mice (DeBoer et al. 2010, Smith & Spencer 2012, Sanchez-Garrido et al. 2013). In contrast, when the HPG axis is severely perturbed with impaired fertility as a result, PS and VO can be delayed 7–11 and 9 days, respectively (Novaira et al. 2014). The five-day delay in puberty seen among our Lin28a GOF mice may indicate that Lin28a plays a larger role than Lin28b in regulating the reproductive endocrine axis in mice (Zhu et al. 2010).

Although the physiology of puberty in mice and humans is not identical, decades of experiments have demonstrated that the mouse is an informative model in the study of reproductive endocrinology. Mice, like humans, display sex-differences in the timing of puberty, although in mice the direction of this sex difference can be strain-specific (Krewson et al. 2004, Nathan et al. 2006, Tyl et al. 2008a,b, Divall et al. 2010). While the effects of SNPs and knockouts may also be species-specific, our data underscore that the Lin28a/Lin28b/let-7 pathway is a key regulator of growth and pubertal timing and suggest that it may be responsible for some of the sex-specific differences seen in these traits among humans.

Lin28a and Lin28b are paralogues that appear to have overlapping as well as separate functions. Here we show that some aspects of Lin28a GOF are mirrored by Lin28b LOF mice (Lin28a GOF mice are larger and Lin28b LOF mice are smaller than controls). Other aspects differ, such as one regulator of the Lin28a, Lin28b, and let-7 pathway (Magi et al. 2010, Gilks et al. 2014). Although the vast majority of the identified loci are likely not to exert sex-specific effects, recent studies have identified sex-specific (Winkler et al. 2015) and sex-chromosome dependent loci (Lunetta et al. 2015) that affect body size and pubertal timing. Moreover, even in the absence of genetic variants with sex specific effects, the study of the genes and pathways identified by GWAS provides an opportunity to uncover new factors that contribute to male-female differences in traits such as growth and the timing of puberty since, for example, these genes and pathways may be modulated by differential sex-steroid exposures or epigenetic mechanisms.
as presence of sex-specificity and the direction of changes in pubertal timing. The age/weight relationship at puberty is also perturbed in the Lin28a GOF mice when compared with controls, whereas the relationship is unaltered in Lin28b LOF mice. Functions in humans may differ too. GWAS have not demonstrated that variants in LIN28A associate with AAM in humans, but this could stem from this gene having tolerated less sequence variation during evolution, thus lacking variants that could associate with AAM. Consistent with this idea is the observation that Lin28a LOF mice either die young or display reduced fertility as adults (Zhu et al. 2010, Shinoda et al. 2013a).

In addition, a recent study by Sangiao-Alvarellos demonstrates that expression levels of Lin28a and Lin28b are regulated in opposite directions in the testes after hypophysectomy, supporting our findings that Lin28a and Lin28b act in different ways in the HPG axis (Sangiao-Alvarellos et al. 2015). That Lin28a and Lin28b may have separate functions is further supported by cancer studies that often identify either Lin28a or Lin28b as a regulator of cell fates (Zhou et al. 2013) and by different expression patterns that have been observed in mouse (Grieco et al. 2013) and rat tissues (Sangiao-Alvarellos et al. 2013). On a cellular level, Lin28a and Lin28b operate in separate compartments of the cell (Piskounova et al. 2011).

Lin28a and Lin28b exert most of their effects through inhibition of let-7 levels but also have let-7 independent functions (Polesskaya et al. 2007, Xu & Huang 2009, Xu et al. 2009, Balzer et al. 2010, Qiu et al. 2010, Peng et al. 2011, Wilbert et al. 2012). For example, it has been shown that Lin28a can bind mRNA directly and stimulate translation (Polesskaya et al. 2007, Xu et al. 2009, Xu & Huang 2009, Qiu et al. 2010) via motifs that are found in many genes (Peng et al. 2011, Wilbert et al. 2012). Our data are consistent with the concept of both let-7 dependent and independent functions of Lin28a/Lin28b. For example, let-7 GOF mice show similar weight and puberty phenotypes as the Lin28b LOF mice, including sex specificity, and the let-7 GOF mice display body weight phenotypes in the opposite direction of Lin28a GOF mice. On the other hand, the Lin28a GOF mice display no sex specificity, and in the Lin28b LOF males, lighter body weights are apparent at younger ages than in the let-7 GOF strain. In addition, the relationship between age/weight at puberty is not perturbed in the Lin28b LOF mice, but female let-7 GOF mice display an altered relationship compared with controls, suggesting that some effects of let-7 may be independent of Lin28a and Lin28b.

Because Lin28b can downregulate let-7, we expected elevated let-7 levels in Lin28b LOF mice. However, as reported by several others using animals with modulated Lin28a or Lin28b expression, there were no apparent differences among let-7 levels in the tissues examined in Lin28b LOF mice (Zhu et al. 2010, Faas et al. 2013, Shinoda et al. 2013b, Ouchi et al. 2014). It is possible that, as shown in recent studies in Xenopus and C. elegans, Lin28a and Lin28b may modulate let-7 levels only during specific developmental stages (Vadla et al. 2012, Faas et al. 2013), and we may not have assessed the specific timepoint(s) at which this modulation occurs. This possibility is further supported by the observation that the sex-specificity in the body weight reduction is detected when the let-7 GOF is induced from birth, but not when induced from weaning (Zhu et al. 2011).

The concept of age/developmental stage-specific regulation is supported by the finding that changes in Lin28b expression in the fetus have greater effects on growth than changes later in life (Shinoda et al. 2013b). Thus, elevated let-7 levels during a short, but critical, window during development may be sufficient to induce phenotypic effects later in life. In WT animals, gene expression of Lin28a and Lin28b decrease and let-7 levels increase from birth to adulthood, but the gene expression levels are relatively stable between juvenile and pubertal stages, raising the possibility that the puberty-modulating phase may occur earlier during development (Grieco et al. 2013, Sangiao-Alvarellos et al. 2013). Further studies of the Lin28a/Lin28b/let-7 axis at different times during development are needed to inform us of critical windows during which this axis may program future phenotypes. A time-course of Lin28a/Lin28b/let-7 expression throughout postnatal development and across the pubertal transition in mice with perturbed Lin28a/Lin28b/let-7 pathways might be particularly informative.

Despite the limitations related to critical windows, we examined levels of let-7 gene expression in several tissues to explore the origin of the male-specific responses to let-7 GOF. Although males exhibited more and stronger phenotypes than females, overall males displayed a lower degree of let-7 overexpression than females, raising the possibility that regulation of growth and puberty may be more let-7 sensitive in males than in females. Interestingly, the degree of let-7g upregulation in the let-7 GOF mice varied among tissues, and was absent in the CNS as has been reported by others (Frost & Olson 2011, Zhu et al. 2011), suggesting that either we assessed expression at a time point at which let-7 is resistant to upregulation, or that weight and puberty phenotypes may not be mediated by let-7 action in the hypothalamus. Indeed, others have previously demonstrated that phenotypes detected in
CNS-restricted let-7 GOF mice are not always detected in global let-7 GOF mice, and vice versa, perhaps suggesting a combination of central and peripheral effects (Frost & Olson 2011). Further studies using tissue-specific GOF or LOF of let-7 miRNAs are needed to determine the precise site(s) of regulation of growth and puberty by let-7.

In summary, the data from our LOF and GOF strains demonstrate complex and sex-specific regulation of growth and puberty by Lin28a, Lin28b, and let-7. The data also point to possible critical windows and peripheral vs central regulation. This complexity consistent with human data showing that the dominant allele at rs314276 (the SNP that displays the strongest association with AAM) is associated with shorter height and heavier weight in women but not men (Ong et al. 2011). In addition, two other LIN28B SNPs that associate with AAM and adult height, rs314277 and rs7759938, contribute differentially to adult height in females and males (Widen et al. 2020). These findings in humans and our data in mice suggest that further study of the Lin28b LOF and let-7 GOF strains will provide an important opportunity to expand our understanding of how puberty and growth are modulated in males and females, including the basis of some sex-specific effects. In addition, our models have no apparent underlying illness, in keeping with humans with genetic variants in LIN28B; indicating that these strains also provide an opportunity to study a pathway identified through GWAS that regulates the onset of puberty within the ‘normal’ population.

Declaration of interests
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was funded by the Canadian Institutes for Health Research and by Department of Paediatrics, The Hospital for Sick Children.

Author contribution statement
C C carried out experiments, performed data analysis, and wrote the manuscript. D L C, C C, C C, and C B carried out experiments. G S, H Z, G Q D, A G, and M R P conceived the study, assisted in interpretation of results, and edited the manuscript. A G performed linear regression analysis.

Acknowledgements
The authors wish to acknowledge the contribution from the Clinical Phenotyping Core at The Toronto Centre for Phenogenomics (TCP) for their assistance with DEXA scans and the TCP Transgenic Core for their assistance with rederivation.

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Received in final form 25 November 2015
Accepted 22 December 2015
Accepted Preprint published online 23 December 2015