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Changes in hypothalamic expression of the Lin28/let-7 system and related MicroRNAs during postnatal maturation and after experimental manipulations of puberty

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Abstract

Lin28 and Lin28b are related RNA-binding proteins that inhibit the maturation of miRNAs of the let-7 family and participate in the control of cellular stemness and early embryonic development. Considerable interest has arisen recently concerning other physiological roles of the Lin28/let-7 axis, including its potential involvement in the control of puberty, as suggested by genome-wide association studies and functional genomics. We report herein the expression profiles of Lin28 and let-7 members in the rat hypothalamus during postnatal maturation and in selected models of altered puberty. The expression patterns of c-Myc (upstream positive regulator of Lin28), mir-145 (negative regulator of c-Myc), and mir-132 and mir-9 (putative miRNA repressors of Lin28, predicted by bioinformatic algorithms) were also explored. In male and female rats, Lin28, Lin28b, and c-Myc mRNAs displayed very high hypothalamic expression during the neonatal period, markedly decreased during the infantile-to-juvenile transition and reached minimal levels before/around puberty. A similar puberty-related decline was observed for Lin28b in monkey hypothalamus but not in the rat cortex, suggesting species conservation and tissue specificity. Conversely, let-7a, let-7b, mir-132, and mir-145, but not mir-9, showed opposite expression profiles. Perturbation of brain sex differentiation and puberty, by neonatal treatment with estrogen or androgen, altered the expression ratios of Lin28/let-7 at the time of puberty. Changes in the c-Myc/Lin28b/let-7 pathway were also detected in models of delayed puberty linked to early photoperiod manipulation and, to a lesser extent, postnatal underfeeding or chronic subnutrition. Altogether, our data are the first to document dramatic changes in the expression of the Lin28/let-7 axis in the rat hypothalamus during the postnatal maturation and after different manipulations that disturb puberty, thus suggesting the potential involvement of developmental changes in hypothalamic Lin28/let-7 expression in the mechanisms permitting/leading to puberty onset.

The heterochronic gene, *Lin28*, was first identified in the nematode *Caenorhabditis elegans*, where it displays tissue- and stage-specific patterns of expression. Lin28 mutations results in either advanced or delayed maturational events (1). In mammals, two *Lin28*-related genes, named *Lin28* (also termed *Lin28a*) and *Lin28b*, have been described (2–4). *Lin28* paralogs are highly conserved across evolution (5), therefore suggesting similar regulatory functions. In addition to various coding mRNAs, *Lin28* proteins have been shown to bind to the terminal loops of precursors of the *let-7* family of microRNAs (miRNAs), blocking their processing into mature miRNAs (6). MiRNAs are small, noncoding RNAs that operate as ubiquitous post-transcriptional regulators of gene expression (7), *let-7* miRNAs being highly conserved across phyla, and widely and abundantly expressed in numerous species (8). Compelling evidence has documented the involvement of *let-7* and *Lin28* members in the regulation of essential events in the development of different species, including mammals. Thus, as in *C. elegans*, drosophila *let-7* mutants display maturational alterations with clear juvenile features in their neuromuscular system, which lead to defects in adult behaviors such as flight, motility, and fertility (9). Several studies have shown an increase in the expression of *let-7* miRNAs in invertebrate and vertebrate species during development in different tissues (10–13). In turn, *Lin28* has been implicated in the maintenance of cellular stemness and several groups have reported the reprogramming of adult human fibroblasts to induced pluripotent stem cells by targeted expression of various factors, including *Lin28* (14). Indeed, in mammals, *Lin28* is widely expressed during embryonic development, but its expression becomes restricted to specific tissues in adulthood (15).

Recent evidence has unveiled the central position of *Lin28*/*Lin28b* within a key regulatory network involving also *c-Myc* and *let-7* (16–19), which targets various cellular pathways. The complexity of this regulatory system is illustrated by the fact that *Lin28/Lin28b* redundantly represses the synthesis of mature *let-7* miRNAs, which in turn are able to suppress *Lin28* levels, therefore creating a double-negative feedback loop. A second (positive) feedback loop exists between *Lin28* and c-Myc: *Lin28*/*Lin28b* derepresses *c-Myc* by suppressing mature *let-7* synthesis, whereas *c-Myc* transcriptionally activates both *Lin28* and *Lin28b* expression (18, 19). While the upstream regulators of the c-Myc/*Lin28*/*let-7* triad are likely numerous, some of them have been identified recently. For instance, it has been shown that *mir-145* causes inhibition of *c-Myc* expression at the posttranscriptional level (20, 21). In addition, *mir-132* and *mir-9* have been predicted as putative miRNA repressors of *Lin28* by the use of bioinformatic algorithms (see *Materials and Methods* and *Results*). A schematic summary of the regulatory interactions between the aforementioned factors is provided in Supplemental Figure 1 (please see Supplemental Figure 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

In the last few years, genome-wide association studies (GWAS) have disclosed the putative link between variations in/around the *LIN28b* locus and changes in key developmental phenomena in humans. Thus, in 2008, Lettre and coworkers reported 12 loci strongly associated with variations in height, accounting for ~2% changes in human height, which included *LIN28b* and several *let-7* targets (22). In addition, in 2009, four independent studies related the age of menarche in humans with changes in or around the *LIN28b* locus (23–26), while two additional studies supported the involvement of *LIN28b* in the regulation of human growth (27, 28). The strong association of changes in the *LIN28b* locus and the age of menarche in humans were later confirmed by a large meta-analysis (29). In good agreement, *Lin28* overexpression in mice resulted in delayed puberty and increased body size, confirming the putative role of *Lin28* members in the regulation of postnatal growth and puberty (30). Admittedly, however, the few genetic studies on this topic conducted so far in humans with either isolated central precocious puberty or constitutive delay of puberty have failed to identify mutations in *LIN28b* gene as possible cause for these conditions (28, 31, 32).

Puberty is a crucial developmental event in sexual and somatic maturation, and hence in the life-cycle of any individual (33–35). Not surprisingly, puberty is under the control of a sophisticated network of regulatory signals of central and peripheral origin that ensures its appropriate timing and appropriate response to endogenous variables and environmental cues (34). Puberty critically relies on precedent developmental events, such as brain sexual differentiation, and can be considered as the end-point of a maturational continuum that leads to reproductive competence. Recently, system biology approaches have resulted in the identification of sets of

genes/proteins that may contribute to the system-wide control of the time of puberty. This has led to the proposal that, rather than the consequence of the action of a single trigger, puberty is the end-point of the concerted and hierarchical activation/inactivation of excitatory and inhibitory networks (35, 36) whose timed regulation would require precise and multifaceted control mechanisms that are yet to be fully identified.

In this context, miRNAs, whose expression is finely regulated (37), and are capable to regulate the activity of numerous target genes, appear to be specially well suited for a role in the regulation of complex biological processes such as puberty. Yet, despite the suggestive evidence from GWAS and functional genomic studies summarized above, the potential involvement of miRNA regulatory pathways in general, and of *Lin28/let-7* system in particular, in the orchestrated control of the central developmental events that enable/lead to pubertal activation, has not been directly addressed. Here we report the expression profiles of key components of the above regulatory pathway, and several associated factors, in the hypothalamus during male and female postnatal/pubertal maturation, and document changes in their expression patterns in various preclinical models of altered puberty.

Materials and Methods

Animals and drugs

All experiments and animal protocols included in this study were reviewed and approved by the Ethics Committee of the University of Córdoba, and were conducted in accordance with European Union Normative for the use and care of experimental animals. Male and female Wistar rats of different ages were used in this study; the animals were housed in a temperature-controlled room with a standard 14-hour, 10-hour, light–dark cycle, unless otherwise stated. The day the litters were born was considered day 1 of age. All rats were provided with ad libitum access to water. Estradiol benzoate (EB) and testosterone propionate (TP) were purchased from Sigma Chemical Co. (St. Louis, Missouri).

Tissue dissection

Rats were euthanized by cervical dislocation, and trunk blood was extracted. The hypothalami were dissected as previously reported, taking as limits the posterior margin of the optic chiasm (rostrally) and the anterior margin of the mammillary bodies (caudally), with a dissection depth of approximately 2 mm (38), and stored at −80 ºC until further processing and assays. In addition, selected expression analyses were conducted using either the suprachiasmatic region (hereinafter referred to as the preoptic area, POA) or the medial basal hypothalamus (MBH). Dissection of these fragments was conducted as previously recommended (39). The nonhuman primate brain tissue used derived from female rhesus monkeys (*Macaca mulatta*) obtained through the Oregon National Primate Research Center Tissue Distribution Program. The MBH of female monkeys was dissected as previously described (40), from animals classified into different stages of development according to the criteria reported by Watanabe and Terasawa (41).

Bioinformatic analysis

To search for potential regulatory miRNAs of *Lin28b* and potential targets of miRNA of interest, we used four different algorithms: TargetScan (42)(http://www.targetscan.org/); miRanda (43) (http://www.microrna.org/microrna/home.do); PicTar (44) (http://pictar.mdc-berlin.de/); and Diana Lab (45) (http://diana.cslab.ece.ntua.gr/microT/).

Experimental design

Tissue distribution and differential expression of Lin28 and Lin28b genes in rats

In experiment 1, the expression of profiles of *Lin28 and Lin28b* mRNAs was explored in a broad panel of tissues from adult (>75-days-old, n = 3) male and female rats. The tissues were removed, snap frozen and stored at −80ºC until used for RT-PCR expression analyses.

Expression of c-Myc/Lin28/let-7 system and related miRNAs in the hypothalamus during postnatal maturation

In experiment 2, the expression profiles of *c-Myc*, *Lin28*, and *Lin28b* mRNAs, as well as *let-7a*, *let-7b*, *mir-145*, *mir-132*, and *mir-9* miRNAs were determined in the hypothalamus of male and female rats at different age-points during postnatal maturation: neonatal (postnatal day [PND]-1), late neonatal (PND-7), infantile (PND-15), juvenile (PND-24 in females; PND-30 in males), early pubertal (PND-30 in females; PND-38 in males), pubertal (PND-35 in females; PND-45 in males), and adult (>PND-75) ages; size = 7-8 per group. *Lin28b* expression was also comparatively analyzed in the cortex and hypothalamus of neonatal (PND-1) and pubertal male and female rats (experiment 3), as well as in the MBH of infantile, late juvenile, early pubertal, and late pubertal female rhesus monkeys (experiment 4). In addition, in experiment 5 *Lin28*, *Lin28b*, *let-7a*, and *let-7b* expression was studied in the POA region and MBH, obtained from male and female rats at the neonatal, infantile, juvenile, and pubertal stages of postnatal maturation.

Changes in hypothalamic expression of the c-Myc/Lin28/let-7 system in models of perturbed puberty

To provide further evidence for the putative roles of this system in the maturational program leading to puberty, we conducted a comprehensive series of expression analyses in various preclinical models of disturbed puberty. In experiment 6, neonatal male and female rats were exposed to high doses of TP or EB as a model of disrupted brain sexual differentiation and altered puberty. Alterations of the sex steroid milieu during the critical neonatal period of sexual differentiation are known to disrupt pubertal maturation and gonadotropic function later in life (46–48). Male and female rats (n = 10 per group) were injected subcutaneously (sc) on PND-1 with olive oil alone (100 μl) (vehicle: control group) or EB (neonatal estrogenization: 100 μg/rat in females; 500 μg/rat in males) dissolved in olive oil. An additional group of females (n = 10) was sc injected on PND-1 with TP (1.25 mg/rat), as recommended (46).

In experiment 7, photoperiodic manipulation was used as another model of perturbed puberty. On the basis of previous evidence showing that either changes in melatonin levels or photoperiod/day length modify the timing of puberty (49–51), a model of constant darkness (CD) during lactation, between PND-5 and -10, was used. Subsets of animals (n = 8–12) were euthanized and hypothalamic tissues collected at PND-15 (ie, immediately after completion of CD) and at puberty (PND-35 in females; PND-45 in males). Pubertal maturation was monitored by detecting vaginal opening (VO) in females and balano-preputial separation (BPS) in males.

In experiment 8, the impact of undernourishing during lactation on the hypothalamic expression of the *c-Myc/Lin28/let-7* system was explored using female rats bred in large litters (20 pups/dam), as model of delayed puberty (52). This model has been proposed to mimic nutritional challenges during the last trimester of human gestation (53). After weaning, the rats were reared with ad libitum access to water and food. Subsets of rats were sacrificed at PND-5, -15, and -35 (females) or -45 (males). In experiment 9, the effects of persistent caloric restriction after weaning on the expression profiles of the *c-Myc/Lin28/let-7* system were studied. Immature rats were submitted to a 30% reduction in their daily food intake. Hypothalamic tissue was obtained at about the time of puberty (PND-35, females, and PND-45, males).

Qualitative final time RT-PCR

Total RNA was isolated from a panel of adult rat tissue samples using the Trizol reagent (Invitrogen, CA), following the instructions of the manufacturer. Expression of *Lin28*, *Lin28b*, and S11 ribosomal protein was assayed by final-time RT-PCR (Bio-Rad Laboratories, Hercules, CA). The PCR cycling conditions were as follows: 95ºC for 5 minutes, followed by 34 cycles at 95ºC for 30 seconds, 60ºC (*Lin28* and *Lin28b*) or 58ºC (S11) for 30 seconds, and 72ºC for 10 seconds. The primer pairs used were: *Lin28*-forward: 5′-cccggtggacgtctttgtg-3′; *Lin28*-reverse: 5′-cactgcctcaccctccttga-3′; *Lin28b*-forward: 5′-ggatcagatgtggactgt gagaga-3′; and *Lin28b*-reverse: 5′-ggaggtagaccgcattctttagc-3′; S11-forward: 5′-cattcagacggagcgtgcttac-3′ S11-reverse: 5′-tgcatcttcatcttcgtcac-3′. PCR products were visualized by 2% agarose gel electrophoresis and their identity confirmed by direct sequencing.

Quantitative real-time PCR

Total RNA was extracted using the Trizol reagent (Invitrogen), and employed for measurement of *c-Myc*, *Lin28*, and *Lin28b* mRNAs. For assays of miRNA levels, total RNA was extracted with the Ambion® *mir*Vana™ miRNA Isolation Kit (Ambion). For real-time PCR, we used SYBR Green qPCR Master Mix (Promega) with primer pairs for *Lin28* and *Lin28b* as described above. The primers used for *c-Myc* were as follows: *c-Myc*-forward: 5′-gagctcctcgcgttatttgaag-3′; *c-Myc*-reverse: 5′-gagtcgtagtcgaggtcatagttcct-3′. For data analysis, relative standard curves were constructed from serial dilutions of one reference sample cDNA and the input value of the target gene was standardized to HPRT levels in each sample. *HPRT*-forward: 5′-agccgaccggttctgtcat-3′; *HPRT*-reverse: 3′-ggtcataacctggttcatcatcac-5′. PCR was initiated by one cycle of 95ºC for 10 minutes, followed by 40 cycles of 15 seconds at 95ºC, 35 seconds at 60ºC, and 10 seconds at 72ºC, followed by one hold of 72ºC for 10 minutes.

For miRNA quantification, cDNA was synthesized by using 10 ng total RNA with TaqMan-specific RT primers and the TaqMan microRNA reverse transcription kit (Applied Biosystems, Foster City, CA). Thereafter, quantitative RT-PCR was performed using predesigned assays for *Let-7a*, *Let-7b*, as we studied two representative miRNAs of the *let-7* family belonging to different clusters (8, 54), *mir-132*, *mir-145*, *mir-9*, and *RNU6* (Applied Biosystems). PCR reactions were carried out as follows: 50ºC for 2 minutes, 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minutes. For quantitative miRNA determination, *RNU6* served as the internal reference.

Real-time PCR analysis of monkey LIN28b

Four hundred nanograms of total RNA were reverse transcribed (RT) using the Omni RT Kit (Qiagen, Valencia, California) in the presence of random hexamer primers (Invitrogen, Carlsbad, California), as suggested by the manufacturer. The PCR reactions were performed using an ABI Prism 7900HT Real-Time PCR system. The primers used to detect *18S* rRNA were purchased as a kit (Applied Biosystems). Primers for *LIN28b* mRNA were as follows: sense 5′-gcaaaggtggtggagaagag –3′ and antisense 5′-ggcttccctctcggtttatc-3′. To determine the relative abundance of *LIN28b*, we used the SYBR GreenER™ qPCR SuperMix system for the ABI PRISM® instrument (Invitrogen). The real-time PCR program used consisted of 2 minutes at 50ºC, followed by 10 minutes at 95ºC, and 40 cycles of 15 seconds at 95ºC and 1 minutes at 60ºC. For data analysis, relative standard curves were constructed from serial dilutions of one reference sample cDNA, and the input value of the target gene was standardized to 18S levels in each sample.

Statistical analysis

Expression data were analyzed using SigmaStat 3.1 (Systat Software, Inc.), expressed as percentage of the control group in each experiment, and presented as mean ± SEM. Statistical significance was determined by *t* test (experiments with two groups), one-way ANOVA with post hoc Tukey test (experiments with more than two groups and one variable), or two-way ANOVA with post hoc Tukey test (experiments with more than two groups and two variables). $P < .05$ was considered significant. Different letters above bars or asterisks indicate statistical significance.

Results

Tissue distribution and differential expression of Lin28 and Lin28b genes in rats

As shown in Figure 1A, *Lin28* mRNA is prominently expressed in placenta, testis, ovary, and pituitary, with modest expression in the hypothalamus, and weak expression in adipose tissue, heart, lung, liver, and stomach. No expression was observed in kidney, spleen, and skeletal muscle. Similarly, *Lin28b* transcripts were abundantly expressed in adult rat testis and placenta, with RNA expression being also observed in the hypothalamus. In addition, modest but detectable levels of *Lin28b* mRNA were found in skeletal and cardiac muscle; other tissues such as the pituitary gland pancreas and stomach had very low or undetectable *Lin28b* mRNA levels (Figure 1B).

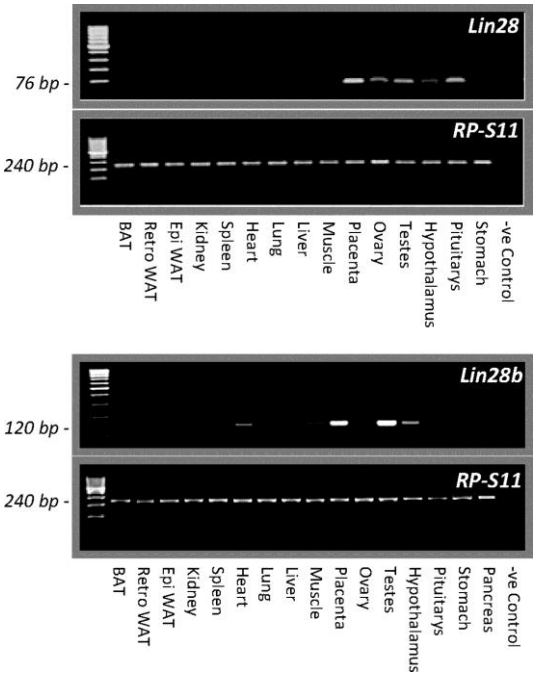

Figure 1. Expression profiles of Lin28 and Lin28b genes in different tissues in the adult rat. A panel of tissues from adult male and female (ovary) rats were screened for expression of Lin28 and Lin28b mRNAs, using conventional RT-PCR, as described in Materials and Methods.

Bioinformatic analyses

It is well established that the *Lin28/let-7* tandem is subjected to a dual negative feedback regulatory loop. *Lin28* and *Lin28b* repress the synthesis of mature *let-7* miRNAs, which in turn posttranscriptionally suppress *Lin28*/*Lin28b* expression. Besides the known regulatory roles of *c-Myc* (transcriptional activator of *Lin28* (18)) and *mir-145* (suppressor of *c-Myc* (21)) in this network, we searched for additional miRNAs and genes that may be involved in *Lin28/let-7* modulation, using the four bioinformatic algorithms listed in *Materials and Methods*. A summary of the most salient results is shown in Supplemental Table 1. Considering the biological profiles of the potential candidates to regulate *Lin28b*, and their relatively large number (which precluded systematic analyses of them all), we included in our assays *mir-132* and *mir-9*, as both are highly conserved across most vertebrates species and they are highly expressed in the rat hypothalamus, as compared with other miRNAs (for instance, *mir-208* and *mir-144* are not expressed in rat hypothalamus while *mir-499* expression is very low; data not shown). A schematic representation of the miRNA regulatory network examined in the present study, including miRNAs predicted by TargetScan is provided in Supplemental Figure 1. We also used bioinformatic tools to identify potential target genes of the *let-7* family. Besides *Lin28/Lin28b*, other targets include genes involved in the control of diverse cellular functions, such as membrane fusion, proteolysis, peroxisome biogenesis, endosome sorting (*Fignl2, Fidgetin*), chromosomal architecture (*Hmga2, Arid3b*), embryonic patterning, cell lineage gene regulation, cell cycle control, cellular growth and division (A*rid3b, Znf512b, Ddx19b*), embryonic survival and neuronal/brain maturation or central nervous system formation (*Trim71, Acvr1c, Igf2bp2, Prtg*), neurodegenerative disease (*Pde12*), signal transduction cascades or receptors (*Map3k1, Pgrmc1, Adrb3, Tgfbr1, Znf512b*), and tumorogenesis (*N-Myc, Znf512b, Yod1, Tgfbr1, Hic2, Prtg, Igf1r*). Additional putative targets of *let-7* miRNAs are genes encoding TGF-beta family members (*Tgfbr1, Gdf6*) and proteins related to insulin-like growth factors, insulin signaling, or diabetes (*Igfr1, Igf2bp2, Igf2bp1*). Interestingly, many of these genes (*Hmga1, Hmga2, Igf1r, Igf2bp1, Myc, Lin28a, Lin28b* and *Map3k3*) have been identified by GWAS studies in humans as related to final height (for further details, see Supplemental Table 2).

Expression of c-Myc/Lin28/let-7 and related miRNAs in the hypothalamus during postnatal maturation

In male rats, very high levels of *Lin28* and *Lin28b* mRNA were detected in the hypothalamus during the neonatal period (Figure 2). *Lin28* mRNA levels declined precipitously thereafter, remaining at low values throughout adulthood (Figure 2). *Lin28b* mRNA abundance declined less pronouncedly, by about 50% during the infantile period, remaining at these values until early puberty. At puberty, the values declined further remaining low in adulthood (Figure 2). A similar pattern of expression of *Lin28b* was detected in the hypothalamus of female rats during postnatal maturation (Figure 2). Opposite patterns of *Lin28b* expression were found in the frontal cortex of male and female rats, with mRNA levels increasing, instead of decreasing, between the neonatal and pubertal periods (Supplemental Figure 2), thereby suggesting that the changes observed in the hypothalamus are tissue-specific.

Expression levels of c-Myc mRNA in rat hypothalamus mirrored those of Lin28b in both male and female rats. In clear contrast, let-7a or let-7b, mir-132, and mir-145 miRNA levels were minimal neonatally and progressively increased during postnatal maturation in both male and female rats. These trends, however, were not detected for mir-9, whose hypothalamic levels showed the highest expression during the neonatal period and declined gradually during postnatal maturation (Figure 2).

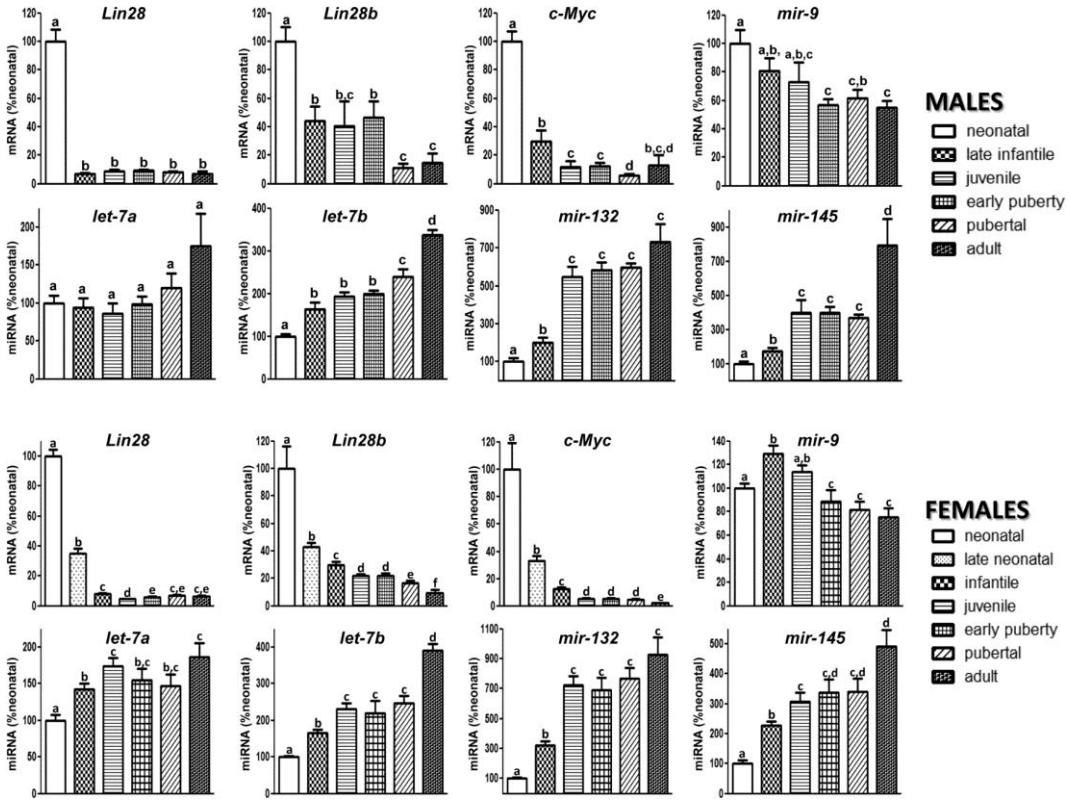

Figure 3. Expression profiles of the components of the Lin28/let-7 axis in preoptic area (POA) and medial basal hypothalamus (MBH) regions from male and female rats and female rhesus monkeys at different stages of postnatal/pubertal maturation (A, B). *P ≤ .05 vs neonatal in each region. (a) P ≤ .05 vs MBH for each age. (b) P ≤ .05 vs infantile in each region. (c) P ≤ .05 vs juvenile in each region (two-way ANOVA with post hoc Tukey test). In addition, expression levels of LIN28b measured by qPCR in the MBH of female rhesus monkeys during postnatal development are shown in panel C. *P ≤ .05 vs juvenile group (ANOVA with post hoc Tukey test). Abbreviations: NEO, neonatal; INF, infantile; JUV or J, juvenile; PUB, pubertal; EP, early pubertal; LP, late pubertal.

Expression analyses of Lin28, Lin28b, let-7a, and let-7b during postnatal maturation were conducted in the POA or the MBH, as a means to regionalize the observed changes in our previous studies using whole hypothalamic explants (Figure 3). Overall, the expression profiles of the above targets in these two hypothalamic areas were broadly similar to those shown in Figure 2 for the hypothalamus as a whole. High expression of Lin28 and Lin28b was detected in the POA and MBH in neonatal male and female rats, and markedly declined thereafter, with virtually negligible levels of Lin28 and moderate but detectable levels of Lin28b at later stages (infantile, juvenile, pubertal) of postnatal/pubertal development (Figure 3). In clear contrast, let-7a and let-7b expression levels were low in neonatal samples from the POA and MBH, in both male and female rats, and progressively increased thereafter, with subtle differences being detected between let-7a and let-7b profiles depending on the sex and hypothalamic region. Thus, in male rats, the expression levels of let-7a and let-7b progressively rose between during postnatal maturation in both the POA and MBH, with a trend for higher expression levels of both targets (at least at some age-points) in the MBH. In females, let-7a and let-7b expression was notably higher in the MBH along postnatal maturation, with clear-cut increases during the study period. In contrast, in the POA, the rise of expression of let-7 miRNAs was more modest and only statistically significant for let-7a (Figure 3, A and B).

Finally, measurement of Lin28b mRNA levels was also conducted in the MBH of female rhesus monkeys during postnatal development. These analyses revealed that, as in rats, Lin28b mRNA abundance declines from high levels during the infantile period to low values at the time of puberty (Figure 3C).

Changes in the profiles of hypothalamic expression of c-Myc/Lin28/let-7 in models of perturbed puberty

Neonatal administration of EB to male rats perturbed pubertal maturation, as evidenced by decreased serum levels of both gonadotropins, LH and FSH, absent BPS and diminished testicular weights (data not shown), in agreement with previous publications (46–48). This protocol of neonatal estrogenization resulted also in detectable changes in the c-Myc/*Lin28*/*let-7* axis at the expected time of puberty. Thus, on PND-45, neonatally estrogenized male rats displayed increased *Lin28b* and *c-Myc* mRNA levels at the hypothalamus; *Lin28* levels tended also to be higher but this change did not reach statistical significance. In contrast, hypothalamic *let-7a* and *mir-132* miRNA levels were significantly lower than in controls. Curiously, *mir-9* miRNA levels were also lower in neonatally estrogenized male rats, whereas *let-7b* and *mir-145* expression remained unchanged (Figure 4).

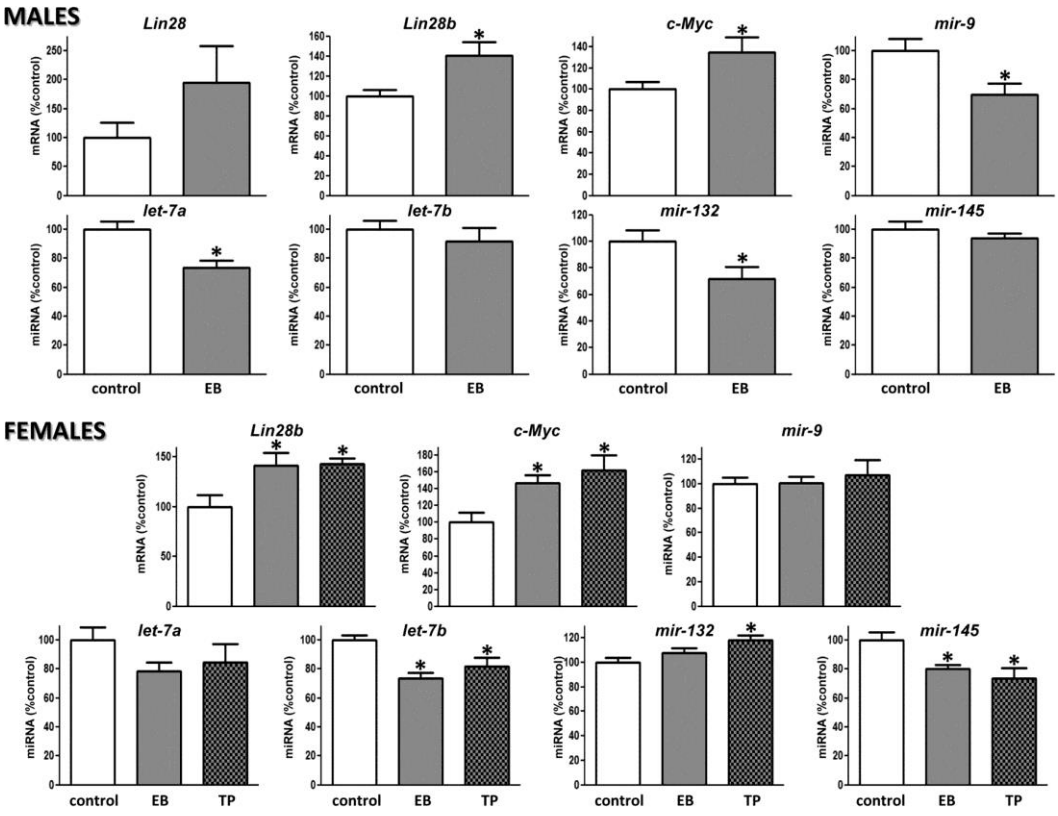

Figure 4. Expression profiles of the components of the Lin28/let-7 axis and related factors in the hypothalamus of pubertal male and female rats following neonatal estrogenization or androgenization. In males (upper panels), expression analyses were conducted in whole hypothalamic fragments from rats subjected to a standard protocol of neonatal estrogenization (estradiol benzoate, EB); samples were obtained from peripubertal (postnatal day, PND-45) animals. In females (lower panels), expression analyses were conducted in whole hypothalamic fragments from rats subjected to a standard protocol of neonatal estrogenization (EB) or androgenization (testosterone propionate, TP); samples were obtained from peripubertal (PND-35) animals. Expression analyses included Lin28 (males only), Lin28b, and c-Myc mRNAs, as well as let-7a, let-7b, mir-9, mir-132, and mir-145 miRNAs. *$P \leq .05$; vs control (vehicle) (t Student test).

Likewise, sex steroid milieu manipulation during the neonatal period in female rats (namely, injection on PND-1 of EB or TP) resulted in hormonal (ie, perturbed gonadotropin levels) and phenotypic (ie, altered timing of VO) indices of disrupted puberty. These were associated with detectable changes in the expression patters of the c-Myc*/Lin28/let-7* system in the hypothalamus at the expected time of puberty. Thus, as in EB-treated males, expression levels of *Lin28b* and *c-Myc* mRNA were significantly increased in neonatally EB- and TP-treated female rats (Figure 4). In turn, neonatal treatments with EB or TP decreased the hypothalamic expression levels of *let-7b* and *mir-145*, while there was a trend for a drop of *let-7a* miRNA levels, especially in EB-treated females, which did not reach statistical significance. In addition, hypothalamic expression of *mir-9* and *mir-132* did not change with neonatal EB or TP treatments, except for a modest increase in *mir-132* levels that was observed in androgenized female rats (Figure 4).

Other rat models of perturbed puberty, including timed manipulation of photoperiod during postnatal maturation in both male and female rats, were also studied. Males that were submitted to CD between PND 10 and 15 displayed a significant delay in the age of BPS. Thus, at PND-45, 91.7% of control animals (reared under conventional photoperiod; 14-hour light) showed complete BPS, as compared with only 27.3% of CD males. Likewise, at PND-35 only 12.5% of CD females had vaginal opening as compared with 90% of control females. No differences were observed in terms of pubertal body weight between control and CD animals (data not shown).

Analysis of CD males at PND-15 revealed a significant increase in hypothalamic *Lin28b* and *c-Myc* mRNA levels. In contrast, these animals exhibited a consistent decline in *let-7a*, *let-7b*, *mir-132*, and *mir-145* miRNA abundance, while *mir-9* remained unchanged (Figure 5). Surprisingly, these changes were transient, as no consistent modifications in the hypothalamic expression of the above targets were detected in CD males when studied at PND-45, the expected time of puberty (data not shown). In agreement with data for males, data for females submitted to the above CD regimen displayed increased expression levels of *Lin28b* and *c-Myc* mRNA levels in the hypothalamus at PND-15; this rise persisted on PND-35 in the case of *Lin28*. CD females had also significantly lower hypothalamic *let-7a*, *let-7b*, *mir-132*, and *mir-145* miRNA levels, and no changes in *mir-9*. However, these changes were detected only on PND-35, instead ofPND-15, with the exception of *mir-145* whose expression levels were also decreased on PND-15 (Figure 5).

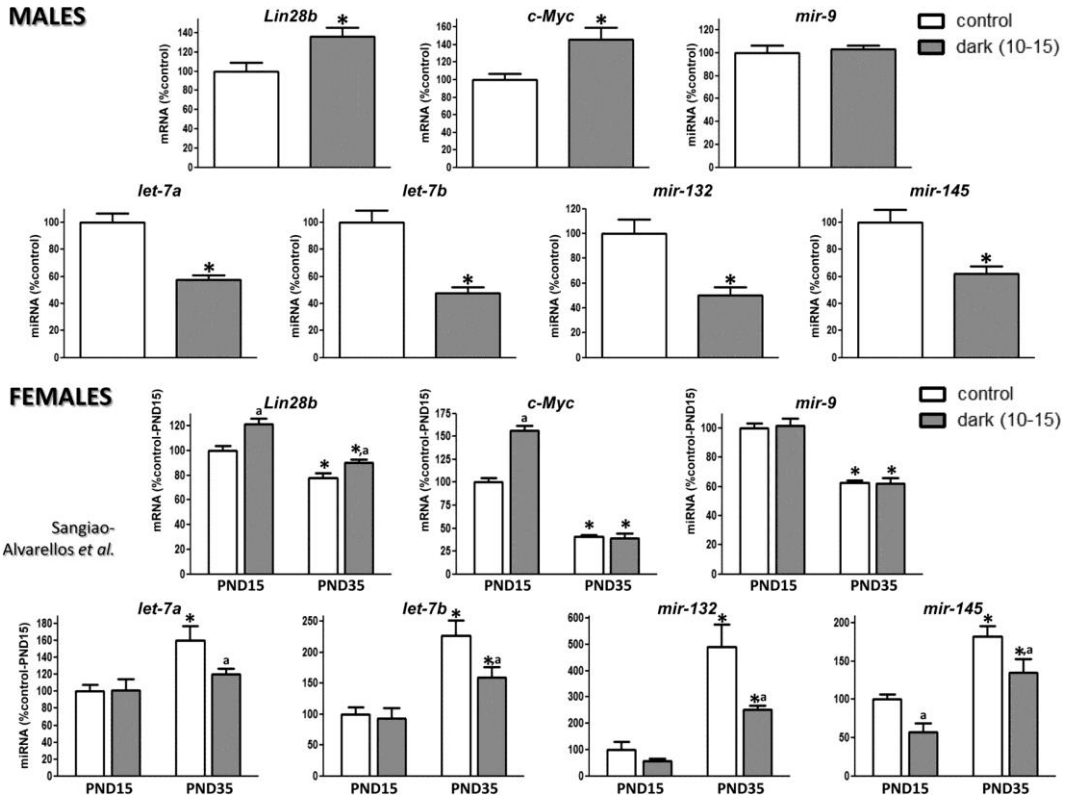

Figure 5. Expression profiles of the components of the Lin28/let-7 axis and related factors in the hypothalamus of male and female rats following photoperiod manipulation (CD, constant darkness from postnatal day [PND]10–15). In males (upper panels), studies in hypothalamic samples obtained at PND-15 are shown. In females (lower panels), studies were conducted at PND-15 and PND-35. For male studies, *P ≤ .05 vs control (normal photoperiod) group (t Student test). For female studies, *P ≤ .05 vs. PND-15 group for each photoperiodic regimen. (a) P ≤ .05 vs control (normal photoperiod) group for each age. (Two-way ANOVA with post hoc Tukey test.)

Models of perturbed puberty due to metabolic distress associated to different forms of undernutrition were also evaluated. First, a protocol of subnutrition during lactation, generated by breeding in large litters, was applied as a means to induce partially delayed puberty, as reported recently elsewhere Breeding in large litters caused an overt delay in the timing of puberty (at PND-45, only 22% of males reared in large litters had BPS, while 92.6% of control males did, and at PND-35, 37.5% of females from large litters had complete VO, in comparison with 90% of control females). Postnatal underfeeding of males and females resulted in increased Lin28b and c-Myc mRNA levels during the neonatal and infantile periods (Figure 6). Changes in hypothalamic miRNA expression induced by postnatal subnutrition were, however, less consistent and displayed some degree of sexual dimorphism. Thus, while in males and females let-7b miRNA levels remained unchanged, let-7a modestly decreased in underfed males during the neonatal period, but increased in infantile females. Hypothalamic levels of mir-132 tended to diminish in pubertal female rats subjected to postnatal undernutrition, but did not significantly change in underfed males. Finally, hypothalamic mir-145 miRNA content showed opposite profiles, with lower levels during neonatal and infantile periods in males subjected to postnatal underfeeding and significantly increased expression in similarly treated infantile females (Figure 6).

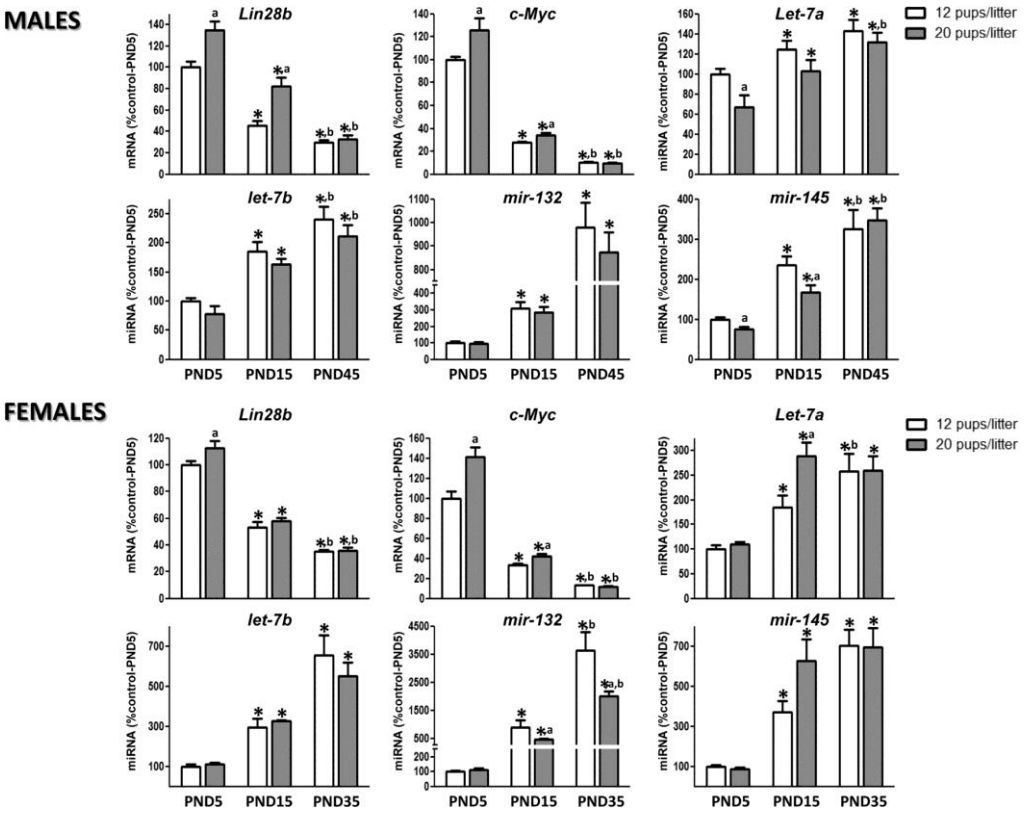

Figure 6. Expression profiles of the components of the Lin28/let-7 axis and related factors in the hypothalamus of male and female rats following postnatal undernutrition. In males (upper panels), analyses were conducted at postnatal day (PND)–5, -15, and -45. *P ≤ .05 vs PND-5 group for each pups litter group. (a) P ≤ .05 vs. 12 pups/litter for each age. (b) P ≤ .05 vs corresponding PND-15 in each pups litter (two-way ANOVA with post hoc Tukey test). In females (lower panels), analyses were conducted at PND-5, -15, and -35. *P ≤ .05 vs PND-5 in each pups litter group. (a) P ≤ .05 vs. 12 pups/litter in each age. (b) P ≤ .05 vs PND-15 in each pups litter group (two-way ANOVA with post hoc Tukey test). P ≤ .05 was considered significant. - See more at: http://press.endocrine.org/doi/full/10.1210/en.2012-2006#sthash.lsJWWRAZ.dpuf

Discussion

From a neurobiological perspective, the initiation of puberty requires an increased secretion of GnRH by a discrete neuronal population of the hypothalamus. Although it appears clear that this change is driven by the coordinated modification of transsynaptic inputs to GnRH neurons of excitatory and inhibitory nature, in conjunction with glial stimulatory signals, the mechanisms setting in motion these coordinated events remain poorly understood. Recent system biology approaches have led to the hypothesis that, rather than being the result of a single trigger, puberty is brought about by the concerted activation of genetic networks hierarchically arranged and endowed with major regulatory hubs connected not only to a plethora of subordinate genes, but also to upper-echelon genes of other regulatory networks (56). According to this concept, such coordinated changes in network activity would ensure proper timing of the pubertal process (40, 57, 58). The existence of regulatory systems able to integrate at different levels the functional output of such networks appears likely, but these systems remain to be identified. MiRNAs might be especially well suited to serve some of these functions, because of their ability to modulate gene expression at multiple hierarchical levels (7, 59). The present study begins to address this issue by providing an initial characterization of the potential roles that miRNA regulatory pathways may have in the central control of puberty. Considering recent GWAS/functional genomic data (23–26, 29), we focused our attention on the *Lin28/Let-7* system. Because this system is linked to other regulators (including transcriptional factors and other miRNAs),

additional components of this putative regulatory pathway were also included in our analyses (as depicted in Supplemental Figure 1).

Consistent with a role of the *Lin28/let-7* system as one of the factors involved in the control of reproductive function, its various components were found to be expressed in key reproductive tissues, including the hypothalamus. *Lin28* mRNA is prominently expressed only in placenta, testis, ovary, and pituitary of adult rats, with modest expression in the adult hypothalamus. *Lin28b* transcripts were also expressed in adult rat testis and placenta; however, contrasting with *Lin28*, substantial *Lin28b* mRNA levels were observed in the hypothalamus of adult rats, whereas no expression was detected in the ovary. Taking into account the putative relationship (as predicted by different GWAS) between *Lin28b* and the age at menarche (23–26) and the low/negligible expression of *Lin28b* in the ovary, we focused our expression analyses on the hypothalamus.

Our results demonstrate that *Lin28* and *Lin28b* (as well as *c-Myc*) mRNAs are abundant in the rat hypothalamus of both sexes during the neonatal period, declining strikingly during the infantile-to-juvenile transition. The fact that a similar trend was not observed in other brain areas, such as the cortex, where *Lin28b* mRNA levels actually increased between the neonatal and the pubertal phases of development, suggests that postnatal loss of *Lin28b* expression is specific to the hypothalamus. Furthermore, a similar decline in hypothalamic *Lin28b* mRNA levels was detected in the hypothalamus of female rhesus monkeys during postnatal development, suggesting that this maturational change is evolutionary conserved.

In contrast to the decline in the hypothalamic expression of *Lin28/Lin28b*, *let-7* (mainly, *let-7b*), *mir-132*, and *mir-145* miRNAs abundance was minimal in the hypothalamus of neonatal rats, increasing progressively thereafter. These results are coherent with previous studies showing that *mir-145* suppresses *c-Myc* expression, resulting in reduced *Lin28/Lin28b* transcription and derepression of *let-7* family maturation. Moreover, a recent study showed that seven of the eight miRNAs of the *let-7* family are highly expressed in hypothalamic arcuate and paraventricular nuclei of adult rats (60). The above developmental profile is reminiscent of that found in *C. elegans*, in which high levels of *Lin28* at the beginning of first larval stage (L1) are followed by an abrupt drop at the end of the L1 phase to allow progression to late larval stage, while *let-7* levels increase progressively to reach maximum values at the adult stage (61). Moreover, overexpression of *let-7* at early stages of worm development causes premature adoption of adult fates, while *let-7* underexpression results in failure to terminally differentiate at the larval to adult transition. Altogether, these data suggest that the level and timing of mature *let-7* miRNA expression, in a reciprocal equilibrium with *Lin28*, plays a vital role in defining key aspects of development in *C. elegans* (61). An inverse relationship between the expression levels of *Lin28* and *let-7* had been also reported in mammals, suggesting that *Lin28* is an important regulator of *let-7* biogenesis across species (8, 62–67). We also studied the changes in hypothalamic levels of *mir-132* and *mir-9* miRNAs, two putative negative regulators of *Lin28b*. We observed that *mir-132* levels increased gradually during postnatal/prepubertal maturation, in keeping with a potential role of this miRNA in the negative regulation of *Lin28b*. In contrast, *mir-9* miRNA levels were the highest during the neonatal period, declining gradually thereafter. This expression pattern suggests that *mir-9* does not play a major role in repressing hypothalamic *Lin28/Lin28b* expression during postnatal maturation. Instead, *mir-9* may be primarily involved in regulating neurogenesis and maturational events at earlier, fetal stages of brain development (68–70).

In order to provide further evidence for a role of the *Lin28/let-7* system in the development of the hypothalamic systems leading to the initiation of puberty, we conducted a series of expression analyses using various models of perturbed puberty. One of these models uses rats in which sexual differentiation of the brain is altered by neonatal exposure to high doses of estrogen or androgens. Consistent with previous studies (46), these treatments induced persistently decreased LH and FSH levels and prevented normal puberty onset. In these animals, hypothalamic levels of *c-Myc* and *Lin28b* mRNAs were enhanced, while *let-7* and *mir-145* miRNA levels were decreased at the expected time of puberty, resembling, but not quite mimicking, the high *Lin28*/low *let-7* levels detected in the hypothalamus of immature animals. The association between enhanced *Lin28* expression and delayed puberty observed in rats exposed to high sex steroid levels during neonatal life is in agreement with the delay of puberty onset reported in *Lin28a* transgenic mice (30), and

further supports the concept that decreasing hypothalamic *Lin28* expression is permissive for the correct timing of puberty. A discordant finding is the sex difference in *mir-9* and *mir-132* miRNA levels after neonatal estrogenization. Although in males, both *mir-9* and *mir-132* miRNA levels diminished with estrogenization, no such changes occurred in females rats treated with EB. In fact, *mir-132* levels actually increased in females treated with TP. The physiological importance of these differences remains to be established. A trend for an increased ratio of *Lin28*/*let-7* expression was also observed in male and female rats with delayed puberty caused by early manipulation of the photoperiodic conditions. Animals subjected to CD during a discrete postnatal window not only displayed delayed puberty onset, but also disturbed expression levels of *Lin28* transcripts and several miRNAs in the hypothalamus at different stages of postnatal development. As was the case of immature rats and neonatally estrogenized/androgenized animals, *Lin28b* and *c-Myc* mRNA levels were significantly increased, whereas *let-7a* and *let-7b* miRNA levels were decreased in the hypothalamus of CD male and female rats. However, a sex dimorphism was detected for the time course of these changes, since altered *Lin28*/*let-7* ratios were detected in infantile (but not peripubertal) males, whereas in females such alteration was only detectable around the time of puberty. As observed in rats exposed to sex steroids during neonatal life, *mir-132* and *mir-145* miRNA levels, but not those of *mir-9*, were decreased in male and female rats exposed to CD, suggesting that delayed puberty caused by either altering neonatal sex differentiation of the brain or disrupting photoperiod cues during infantile development is associated with alterations of the *Lin28*/*let-7*regulatory system, a finding that indirectly supports a tentative inhibitory role of this system on the timing of puberty. The functional relevance of these changes, and of their temporal differences, for the alteration of puberty observed after photoperiodic manipulation awaits further investigation.

In contrast, altered puberty due to manipulation of the metabolic/feeding status did not result in comparable changes. Neither early subnutrition, by means of rearing large litters, nor a condition of negative energy balance around puberty, which was effective in delaying puberty onset, affected the hypothalamic *Lin28*/*let-7* system as did early androgenization, estrogenization, and CD conditions. For instance, *let-7b* miRNA levels remained unchanged in the hypothalamus of male and female rats subjected to early subnutrition, whereas *Let-7a* levels decreased in males during the neonatal period, but increased in infantile females reared in large litters. Likewise, while *mir-132* levels were lower in pubertal female rats subjected to postweaning undernutrition, *mir-145* miRNA levels in the hypothalamus displayed opposite profiles between males (lower levels) and females (higher levels) after postweaning underfeeding. All in all, these observations strongly suggest that, even though conditions of early metabolic stress causing pubertal alterations can perturb the expression of some of the elements of the *Lin28*/*let-7* axis in the hypothalamus, the underlying mechanisms and cellular substrate for such alterations are likely different from those caused by early manipulations of brain sex differentiation or photoperiod.

System biology approaches have allowed identification of a putative gene network operating within the female hypothalamus to control the initiation of puberty. Among the elements of this network, a set of tumor suppressor genes (TSG) was shown to be activated and to play a major role in the timing of puberty. These include enhanced at puberty protein 1 (*EAP1*) (40, 71), the POU gene, octamer transcription factor 2 (*OCT2*) (58), and thyroid transcription factor 1 (*TTF1/NKX2.1*) (57), which are transcription factors that are deregulated in several types of cancer (72–74). Hypothalamic TSG networks are hierarchically controlled by specific regulatory hubs; one of the predicted major hubs is p53, whose expression has been shown to increase in the hypothalamus during the pubertal transition in female rhesus monkeys (56). Importantly, p53 is a tumor suppressor that negatively regulates *c-Myc*, while it induces the transcription of *mir-145*, which in turn represses a number of oncogenes, including *c-Myc* itself (20, 21, 75). Given the positive regulatory loop between *c-Myc* and *Lin28*/*Lin28b* expression (18, 19), it is possible that the decline in *c-Myc/Lin28* expression along postnatal maturation reported herein might be caused by the increase in p53 abundance (and hence, *mir-145*) that takes place during postnatal development. In turn, decreased levels of *Lin28* binding proteins might cause the observed increase in *let-7* miRNA levels. Because bioinformatic analysis predicts that *EAP1*, *OCT-2*, and *TTF1* are targets of *let*-7 or related miRNAs (according to the computational analysis, *EAP1* is only regulated by *let-7e* and not by several members of the *let-7* family), these genes might serve as portals conveying the regulatory actions of the *Lin28*/*let-7* axis to the neuroendocrine complex

that controls the timing of puberty. Since *let-7* and other miRNAs are considered to be tumor suppressors, it is tempting to assume that they represent an additional component of the regulatory TSG network postulated to be involved in the physiological regulation of puberty.

In conclusion, our study documents for the first time the changes in *mir-145/c-Myc*/*Lin-28*/*let-7* axis and *mir-132* miRNA expression that occur in the rat hypothalamus during postnatal maturation in male and female rats. Our results show a marked decline in *c-Myc*/*Lin28*a/*Lin28b* mRNA levels coupled to significant increases in *let-7*, *mir-145*, and *mir-132* miRNA abundance during prepubertal maturation. We also report that delayed puberty induced by either neonatal exposure to sex steroids or infantile exposure to constant darkness hampers these changes. Because the alterations in hypothalamic expression of the *Lin28/let-7* system occur gradually during prepubertal maturation, our results suggest that *Lin28b* and its associated miRNA complex play a permissive role in the initiation of puberty. Such a role is consistent with the earlier finding of delayed puberty in *Lin28*-overexpressing mice (30), which exhibit delayed sexual maturation despite of enhanced body growth. Our results pave the way for functional analyses involving manipulation of these elements in vivo, including the generation of models of transient or constitutive overexpression of *Lin28b*, which will be required to conclusively define the precise role that the *Lin28b*–miRNA complex plays in the hypothalamic regulation of the pubertal process.

Abreviations

BPS Balano-preputial separation; CD constant darkness; EB estradiol benzoate; GWAS genome-wide association studies; MBH medial basal hypothalamus; PND postnatal day; POA preoptic area; TP testosterone propionate; TSG tumor suppressor genes; VO vaginal opening.

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