RESEARCH

miR-29 family regulates the puberty onset mediated by a novel *Gnrh1* transcription factor TBX21

Xiaoning Li¹, Junhua Xiao¹, Yating Fan¹, Kan Yang¹, Kai Li¹, Xin Wang¹, Yanhua Lu² and Yuxun Zhou^{1,3}¹College of Chemistry, Chemical Engineering & Biotechnology, Donghua University, Shanghai, China²State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, China³Human Phenome Institute, Fudan University, Shanghai, ChinaCorrespondence should be addressed to Y Zhou: zhouyuxun@dhu.edu.cn

Abstract

Gonadotropin-releasing hormone (GnRH) is the ultimate signal by which the neuroendocrine system controls the puberty onset and fertility in mammals. The pulsatile release of GnRH is regulated by numerous extracellular and intracellular factors, including miRNAs. Here, we report a novel regulation mechanism mediated by miR-29 family. We found that the absence of miR-29s resulted in elevated expression of *Gnrh1* in GT1-7 cells. Through *in silico* and *wet* analysis, we identified *Tbx21*, a target gene of miR-29, as the main effector. As a transcription activator, TBX21 stimulates the expression of *Gnrh1* directly by binding to its promoter region, and indirectly by activating the expression of *Dlx1*, another transcription activator of *Gnrh1*. Stereotactic brain infusion of miR-29 inhibitor into the hypothalamus caused earlier puberty onset in prepubertal female mice than that of intact controls. The female mice with ectopic expression of *Tbx21* in the hypothalamus were affected in both puberty onset and fertility, as they had higher level of serum LH and FSH, larger litter size but steeper decline of fertility compared with those of controls. Our results revealed that miR-29-3p and its target *Tbx21* played a role in regulating the mammalian puberty onset and reproduction by modulating the *Gnrh1* expression.

Key Words

- ▶ puberty onset
- ▶ GnRH
- ▶ miR-29 family
- ▶ *Tbx21*
- ▶ GT1-7 cells
- ▶ CRISPR/Cas9
- ▶ stereotactic brain infusions
- ▶ female mouse

Journal of Endocrinology
(2019) **242**, 185–197

Introduction

Puberty is the attainment of fertility, a process encompassing morphological, physiological and behavioral development. In neuroendocrine terms, puberty onset is the result of the reactivation of hypothalamus–pituitary–gonad (HPG) axis, symbolized by the pulsatile release of gonadotropin-releasing hormone (GnRH) (Ebling 2005). GnRH is a peptide hormone composed of ten amino acids (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂) (Lee *et al.* 2008) and represents the final output of neuroendocrine system that regulates sexual

development and reproductive function (Herbison 2016). A great number of extracellular and intracellular factors have been reported to regulate the *Gnrh1* expression (Lee *et al.* 2008, Herbison 2016, Avendano *et al.* 2017), among which, miRNAs have received increasing attention (Messina *et al.* 2016). miRNAs are genome encoded, noncoding, ~21 nucleotide-long RNA molecules which are involved in post-transcriptional regulation of gene expression and control many developmental and cellular processes in eukaryotic organisms (Krol *et al.* 2010).

miRNAs are particularly abundant in the brain where they act as ‘master regulators’ or ‘fine-tuners’ of gene expression to play regulatory roles ranging from neuronal development to fulfilling function (Saba & Schratt 2010, Rajman & Schratt 2017). Although more than 210 miRNAs were detected in the arcuate (ARC) and paraventricular (PVN) nuclei of rat hypothalamus where the puberty onset modulating machinery is located (Amar *et al.* 2012), only a small portion of them have been proved with direct evidences in regulating the puberty onset of mammals. Deletion of miR-9 and miR-200 resulted in altered GnRH neuron development (Garaffo *et al.* 2015), miR-200 and miR-155 have been demonstrated to promote the expression of *Gnrh1* by inhibiting the repressors of *Gnrh1* promoter *Zeb1* and *Cebpb*, respectively (Messina *et al.* 2016) in mouse GnRH neurons. miR-505-3p was proved to play a role in regulating the puberty onset of female mice (Zhou *et al.* 2018).

miR-29 family (miR-29s) consists of four members: miR-29a, miR-29b1, miR-29b2 and miR-29c, which have been postulated to potentially regulate more than 4000 gene products. In the central nervous system, miR-29 family is involved in the development of nervous system, the maintenance of mature neuron and the plasticity of nerve cell regulation (Lippi *et al.* 2011, Annis *et al.* 2016, Ripa *et al.* 2017).

In our previous work, we found that the expression levels of miR-29s in mouse hypothalamus gradually increased during the sexual development (Omariba *et al.* 2018). Similarly, Zhang *et al.* showed that miR-29a-3p was expressed differentially in the developing hypothalamus of porcine (Zhang *et al.* 2013a). Messina *et al.* also found that the expression level of miR-29 family members in mouse GnRH neurons were higher at postnatal day 12 than that at postnatal day 7, and their abundance was higher in GnRH neuron than that in non-GnRH neurons (Messina *et al.* 2016). Moreover, mice with brain-specific miR-29 knockdown showed decreased reproductive capacity in males, in contrast with female mice (Takeda & Tanabe 2016). Turquoise killifish with neuron-specific miR-29 knockdown also showed the phenotype of decreased fertility (Ripa *et al.* 2017). These collective evidences imply that miR-29s can be an important regulator of puberty onset and reproduction.

GT1-7 cells are immortalized GnRH neurons derived from mouse hypothalamic neurons and provide an ideal model system for studying the regulation of *Gnrh1* expression (Keynes *et al.* 1990). In this study, we generated a miR-29s-knockout GT1-7 cell strain using the CRISPR/Cas9 technology. In miR-29s-knockout GT1-7 cells,

the level of *Gnrh1* expression increased by two-fold. *Tbx21*, a target gene of miR-29 family, acts as a transcriptional activator of *Gnrh1* in this process, TBX21 also activates the expression of *Dlx1*, another activator of *Gnrh1* promoter. Both inhibition of miR-29s and promotion of *Tbx21* in the hypothalamus of prepubertal female mice affects the timing of puberty onset, and some reproductive phenotype could be affected by the ectopic expression of *Tbx21* significantly.

Methods

Construction of vectors

The plasmid 42230-multi-sgRNAs-miR-29, an all-in-one vector targeting four miR-29 family members, was constructed by firstly inserting the 20bp proto-spacer sequence of miR-29a into the *Bbs* I site of pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmid (Addgene; 42230), and then inserting the sgRNA cassettes targeting miR-29b1, miR-29b2 and miR-29c respectively into the *EcoR* I site step by step. These sgRNAs were designed for each miRNA according to CRISPR DESIGN (<http://crispr.mit.edu/>). The sequences were listed in Supplementary Table 1 (see section on supplementary data given at the end of this article). To facilitate the screening for the cells with miR-29 family knockout, we also introduced a cassette of puromycin N-acetyltransferase (PuroR) which was transcribed by its own mouse phosphoglycerate kinase 1 (PGK) promoter at the end of sgRNA cassettes. A 42230-sgRNA-Dlx1 used to generate a *Dlx1*-knockout cell model was also constructed by using the same method.

Cell culture and transfection

GT1-7 and N6 cells were cultured in high-glucose DMEM (KeyGENBioTECH, Nanjing, China), containing v/v 10% FBS (Thermo Fisher Scientific), and 100 U/mL penicillin-100 µg/mL streptomycin in a humidified incubator with 5% CO₂ at 37°C.

Plasmids, miRNA inhibitors (a single-stranded RNA complementary to miR-29a-3p chemically synthesized by Gene Pharma, Shanghai, China) or miRNA mimics (a single-stranded RNA identical to miR-29a-3p chemically synthesized by Gene Pharma) was used in transient transfection following a protocol recommended by the manufacturer of Lipofectamine™ 3000 reagent (ThermoFisher). Cells were seeded into 24-well plates 1 day prior to transfection at a density of 150,000 cells/well.

For miR-29 family knockout, 1 µg of 42230-multi-sgRNAs-miR-29 plasmid was used. For miR-29 family knockdown, 20 pmol of miR-29a inhibitor was used. For overexpression of *Tbx21*, 1 µg of pCMV6-Tbx21 (OriGene Technologies, Inc., Rockville, MD, USA) was used.

Generation of stable cell lines

The miR-29 family-deleted GT1-7 cell lines, *Dlx1*-knockout cell line and *Tbx21*-overexpressed cell line were generated following the same protocol as following (except for the antibiotics employed for screening according to the antibiotic resistant genes carried by the plasmid): 300 ng/mL puromycin were added into the culture medium 48 h after transfection and incubated for additional 4 days before changing the medium into DMEM plus 10% FBS. After cultivated for another 7 days, colonies were picked and cultivated expansively. To confirm the deletion of miR-29 family in GT1-7 cells, the targeted regions were amplified by PCR using the genomic DNA extracted from the puromycin-resistant clones as template. The primer sequences were listed in Supplementary Table 1. PCR products with correct length were confirmed by Sanger sequencing. Potential off-target sites of all miR-29 sgRNAs were predicted by CRISPR DESIGN, and their off-target effects were examined in top 10 loci of each sgRNA accordingly. These predicted off-target loci were PCR amplified from miR-29-knockout cells and detected by three-round multiplex PCR for genotyping with next-generation sequencing (Chen *et al.* 2016). The primer sequences were listed in Supplementary Table 2.

RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Total RNA from miR-29-knockout cell line was extracted using RNAiso Plus (TaKaRaBio Inc.) according to the manufacturer's recommendations. To extract total RNA from hypothalamus, the hypothalamus of mice was taken out and shredded in RNAiso Plus and subsequent steps were the same as that for cells. Genomic DNA was eliminated by DNase I digestion (ThermoFisher), and then the cDNA was synthesized by using Thermo Scientific RevertAid First-Strand cDNA Synthesis Kit (ThermoFisher). Specific stem-loop reverse transcription (RT) primers for mature miRNAs were designed respectively, and miR-16-5p was used as the internal control (Tong *et al.* 2015). The mRNA was reverse transcribed with oligo-dT as primer. Quantitative PCR were carried out on Applied Biosystems 7500 Real-Time PCR system (ThermoFisher) using SYBR

Green SuperRealPreMixPlus (TIANGEN, Beijing, China); all reactions were run in triplicate and no template controls were included for each gene. $2^{-\Delta\Delta C_t}$ was used to calculate the fold change of the expression level of a gene between the test and control samples. The primers for each gene were listed in Supplementary Table 1.

Western blot

The cells were seeded and cultured in a six-well plate. After removal of the media, the cells were washed twice with ice-cold PBS, and then lysed using Nucleoprotein Extraction Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's recommendations. The samples were boiled for 5 min before they were loaded onto SDS-PAGE; electrophoresis was run at 80 V for 30 min in the 4% stacking gel and at 120 V for 60 min in 10% separation gel. And then the proteins were transferred onto 0.45 µm PVDF membrane (Merck Millipore) at 300 mA for 100 min. The membrane was blocked at room temperature for 1 h with blocking solution (w/v 5% BSA in TBST) and incubated overnight at 4°C with the appropriate primary antibody diluted in blocking buffer (mouse monoclonal anti-PCNA, D190751, 1:1,000 (Sangon Biotech, Shanghai, China); mouse monoclonal anti-Tbx21, sc-21749, 1:5000 (Santa Cruz Biotechnology)). The membranes were washed three times with TBST before being incubated with HRP-conjugated secondary antibody (anti-mouse IgG; Beyotime, Shanghai, China) 1:1000 for anti-PCNA, 1:5000 for anti-Tbx21 diluted in blocking buffer for 1 h at room temperature. The ECL detection kit was used for visualization of PCNA (Thermo Fisher) and SuperSignal West Femto Trial Kit was used for that of TBX21 (Thermo Fisher). Immunoblots were scanned and analyzed using Image Lab software (Bio-Rad).

Dual-luciferase gene reporter assay

The full-length of WT sequence of the 3'UTR of *Tbx21* containing miR-29-binding sites was amplified by PCR and cloned into the psiCHECK-2 vector (Promega Corp.). The plasmid was named Tbx21-3'UTR-WT. The mutant Tbx21-3'UTR-MU was constructed by QuickMutation™ Site-Directed Mutagenesis Kit (Beyotime) according to the manufacturer's instructions. For the detection of dual-luciferase activity, GT1-7 cells were co-transfected with miR-29 mimic (20 mM) or scramble (20 mM) and Tbx21-3'UTR-WT (500 ng) or Tbx21-3'UTR-MU (500 ng). Forty-eight hours later, cells were lysed using Reporter Lysis Buffer, and the activities of Renilla and Firefly

luciferase were analyzed respectively, using a Dual-Luciferase Reporter Gene Assay Kit (Beyotime) according to the manufacturer's recommendations.

Chromatin immunoprecipitation assays

The chromatin isolation and enzymatic digestion of GT1-7 cells was performed using the Pierce Chromatin Prep Module Kit (ThermoFisher) following the manufacturer's instructions. Then, chromatin immunoprecipitation (ChIP) was carried out by using the Pierce Agarose ChIP Kit (ThermoFisher). After shearing by MNase, protein-bound DNA was immunoprecipitated at 4°C overnight using 5 µg of a mouse monoclonal anti-Tbx21 antibody or goat IgG control. ChIP-DNA fragments were analyzed using quantitative PCR. The primers used for ChIP was designed according to the prediction of JASPAR, a database of transcription factor-binding profiles (<http://jaspar.genereg.net>). The productions of each pair of primers covered two to four binding sites of TBX21 with score higher than 5.5. The primers for each gene were listed in Supplementary Table 1.

Animals

All animal procedures were approved by the Animal Ethics Committee of Donghua University, and all experiments were conducted in strict compliance with the National Institutes of Health Guide for the Use of Laboratory Animals. C57BL/6 mice were group-housed under specific pathogen-free conditions in a temperature-controlled room (21–22°C) with a 12-h light–darkness cycle and *ad libitum* access to food and water. Female mice were weighed at postnatal day (PND) 1, the day of birth and weaned at PND 20. Vaginal opening (VO) was monitored daily from the time of weaning. The vaginal smears of females were taken daily and analyzed under an inverted microscope to identify the specific day of the estrous cycle (Byers *et al.* 2012). The fertility indices of 3-month-old females were calculated from the size of litters and the frequency of fertility during a 60-day mating with WT male.

Stereotactic brain infusions

Female mice were placed in a stereotaxic frame (RWD Life Science, Shenzhen, China) under anesthesia (amobarbital sodium), and a burr hole was drilled at the bregma. A 10 µL Hamilton syringe was slowly inserted into the bottom of the left lateral ventricle (5.75 mm deep relative to the dura) and 1 µL of the different treatment solutions

(antagomiR-NC, Gene Pharma, 50 µmol/L; antagomiR-29a, Gene Pharma, 50 µmol/L; lenti-null-P2A-eGFP, a lentivirus for control, 10⁸ TU/mL; lenti-Tbx21-P2A-eGFP, a lentivirus for *Tbx21* overexpression, 10⁸ TU/mL) was injected using an infusion pump (KD Scientific, MA, USA) over 20 min. Mice were randomly assigned for injection. Animals were subjected to intracranial surgery at PND15.

Tissue preparation and digital image acquisition

Mice were anesthetized with amobarbital sodium and perfused transcardially with 30 mL of saline, followed by 20 mL of w/v 4% PFA, pH 7.4. Brains were collected, post fixed in the same fixative for 24 h at 4°C, dehydrated in w/v 20% sucrose for 24 h at 4°C, followed by soaking in w/v 30% sucrose for 24 h at 4°C. The brains were embedded in OCT embedding medium (Tissue-Tek, Sakura Finetek USA, Inc., CA, USA), frozen at –20°C and cryosectioned (ThermoFisher) at 10 µm. Sections were analyzed using fluorescence microscope.

Immunofluorescence assay

The brain of mouse was taken as mentioned above. Brains were cut and divided in three sets of coronal sections (30 µm) in a freezing microtome. The slides were washed for 15 min with 0.01 M PBS, blocked for 1 h with 5% BSA in PBS and incubated with appropriate antibodies (diluted 1:100 in 0.01 M PBS with 5% BSA; rabbit polyclonal anti-GnRH, NB300-506, Novus Biologicals; mouse monoclonal anti-Tbx21, sc-21749, Santa Cruz Biotechnology) for 48 h. After washing, the sections were incubated with m-IgGκ BP-CFL 555 (sc-516177, Santa Cruz Biotechnology) and Alexa Fluor 488-labeled Goat Anti-Rabbit IgG (Beyotime) for 2 h (1:100 in 1% PBS with 5% BSA). After incubation, the slides were washed with PBS and treated with 4,6'-diamidino-2-phenylindole (DAPI) for 10 min, rinsed and covered with fluorescent mounting medium.

Hormone assays

Serum hormone levels were measured by ELISA. Blood of mice in the estrous was drawn from the orbital sinus, placed for 30 min at room temperature and centrifuged at 10,000 *g* for 15 min at 4°C. The serum was collected and stored at –80°C until assayed. LH and FSH were measured using a colorimetric ELISA kit (Elabscience Biotechnology Co., Ltd, Wuhan, China) following the manufacturer's instructions.

Figure 2

The relationship between miR-29s and *Tbx21*, *Tbx21* and the *Gnrh1* promoter, *Tbx21* and the *Gnrh1* activator *Dlx1*. (A) A screen of puberty-related genes influenced by miR-29s knockout. Unpaired two-tailed Student's *t*-test, $n = 5-6$ per group, $P_{(Gnrh1)} = 0.0021$, $P_{(Kiss1)} = 0.8147$, $P_{(Tac2)} = 0.7275$, $P_{(Gpr54)} = 0.2502$, $P_{(Igf1r)} = 0.9384$, $P_{(Irs1)} = 0.8523$, $P_{(Lepr)} = 0.1739$, $P_{(Npy4r)} = 0.7556$. (B) A screen of *Gnrh1* transcription factors influenced by miR-29s knockout. Unpaired two-tailed Student's *t*-test, $n = 3$ per group, $P_{(Pou2f1)} = 0.5587$, $P_{(Aes)} = 0.5960$, $P_{(Dlx1)} = 0.0018$, $P_{(Pbx1)} = 0.9506$, $P_{(Pknx1)} = 0.2877$, $P_{(Cebpb)} = 0.3554$, $P_{(Mx1)} = 0.3801$, $P_{(Tle4)} = 0.1123$, $P_{(Zeb1)} = 0.4109$. (C) The interacting genes of *Dlx1* analyzed by BioGRID. (D and E) The mRNA and protein level of *Tbx21* expression in GT1-7-miR-29s-KO. Unpaired two-tailed Student's *t*-test, for qPCR, $n = 5$ per group, $P_{(mRNA)} < 0.0001$; for Western blot, $n = 3$ per group, $P_{(protein)} = 0.0011$. (F) The interaction between miR-29s and *Tbx21* mRNA 3'UTR in GT1-7 detected by dual-luciferase reporter assay. Unpaired two-tailed Student's *t*-test, $n = 6$ per group for *Tbx21*-3'UTR-WT, $P_{(miR-29a)} = 0.0010$, $P_{(miR-29b)} = 0.0117$, $P_{(miR-29c)} = 0.0023$; for *Tbx21*-3'UTR-MU, $P_{(miR-29a)} = 0.1152$, $P_{(miR-29b)} = 0.0945$, $P_{(miR-29c)} = 0.0776$. (G) The relative luciferase activity of dual-luciferase reporter in GT1-7 with or without miR-29s. Unpaired two-tailed Student's *t*-test, $n = 6$ per group, for GT1-7-miR-29s-WT (WT), $P = 0.0281$; for GT1-7-miR-29s-KO (KO), $P = 0.9468$. (H) The verification of miR-29s effect on *Tbx21*, *Dlx1* and *Gnrh1* by miR-29s knockdown in GT1-7 cell. Unpaired two-tailed Student's *t*-test, $n = 6$ per group, $P_{(miR-29a)} < 0.0001$, $P_{(miR-29b)} = 0.0007$, $P_{(miR-29c)} = 0.0011$, $P_{(Tbx21)} = 0.0018$, $P_{(Dlx1)} = 0.0271$, $P_{(Gnrh1)} = 0.0403$. (I) The verification of miR-29s effect on *Tbx21*, *Dlx1* and *Gnrh1* by miR-29s knockdown in N6 cell. Unpaired two-tailed Student's *t*-test, $n = 6$ per group, $P_{(miR-29a)} = 0.0051$, $P_{(miR-29b)} = 0.0687$, $P_{(miR-29c)} = 0.0042$, $P_{(Tbx21)} = 0.0930$, $P_{(Dlx1)} = 0.0895$, $P_{(Gnrh1)} = 0.0099$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0082>.

a single miR-29 had played the predominant role in this process or not, each of the four members of miR-29s were knocked out individually in GT1-7 cells using similar method, but the significant change in *Gnrh1* expression was not observed in any of these cells (Supplementary Fig. 2), which implied that miR-29s functioned in a redundant way here.

Since the 3' untranslated region (3'UTR) of *Gnrh1* mRNA had no binding sites for miR-29s, we speculated that some transcription factor might have played a role in this modulation. The expression pattern of nine transcription

factors of *Gnrh1* (Messina *et al.* 2016) were checked; and among the five activators and four repressors, one transcription activator, Distal-less homeobox-1 (*Dlx1*), manifested significant increase in the expression level in the absence of miR-29s (Fig. 2B), while *Dlx1* was not the target of miR-29s either.

The interactive partners of *Dlx1* were searched on BioGRID (<https://thebiogrid.org>), counting on some targets of miR-29s playing the role (Fig. 2C). TBX21 was screened out as the working partner of DLX1 by the mammalian two-hybrid system (Ravasi *et al.* 2010),

its coding gene *Tbx21* was targeted by all of the three members of miR-29 family (Steiner *et al.* 2011), and it has binding sites in the promoter regions of *Dlx1* and *Gnrh1* predicted by JASPAR. The expression of *Tbx21* in the miR-29s-null GT1-7 cell was checked, and the accumulation of both the mRNA and protein of *Tbx21* increased as expected (Fig. 2D and E). Dual-luciferase gene reporter assay was carried out to verify the interaction between miR-29-3p and *Tbx21* in GT1-7 cells. The data showed miR-29-3p could bind to 3' UTR of *Tbx21* mRNA and reduce the amount of luciferase (Fig. 2F). On the other hand, the relative luciferase activity was recovered due to the mutations of the miR-29-binding site on *Tbx21* 3'UTR in WT cells. While in the miR-29s-null cells, the luciferase activity was no longer affected by the form of miR-29-binding site (Fig. 2G).

The off-target loci of the four sgRNAs were predicted using CRISPR DESIGN algorithm (<http://crispr.mit.edu/>), and the top 10 off-target suspects for each locus were PCR amplified and sequenced by next-generation sequencing accordingly. At least in these 40 loci, no off-target effect was detected (Supplementary Table 3).

To further ensure the consequences caused by miR-29s reduction, both GT1-7 and N6 cells, another GnRH-expressing hypothalamic neuronal cell model (Belsham *et al.* 2004), were transfected with miR-29a inhibitor. After 48h of transfection, the accumulation of all miR-29 members dropped and the *Tbx21* expression increased (Fig. 2H and I). We then treated GT1-7 cells with miR-29 mimics to test whether the miR-29 overexpression interfered with the *Gnrh1* expression. The results showed that the supply of any combination of miR-29 family members could not cause significant differences in the amount of *Gnrh1* mRNA (Supplementary Fig. 3).

TBX21 is a novel transcription factor of *Gnrh1*

Tbx21 is a member of *Tbr1* subfamily of *T-box* transcription factor genes that mainly regulate brain development. The expression of *Tbx21* was detected in the olfactory bulbs (OBs) where GnRH neurons originated (Mihalas & Hevner 2017). The Allen Human Brain Atlas (<http://portal.brain-map.org/>) also showed that the region where the TBX21 expressed largely overlapped with that of GNRH1 and DLX1 in the hypothalamus. Meanwhile, the RNA-seq data of GT1-7 and GN11, a cell strain derived from immature, migratory GnRH neurons, measured by Huang *et al.*, displayed the expression level of *Tbx21* was higher in the GT1-7 than that in the GN11 cells (Huang *et al.* 2016), implying *Tbx21* had similar expression trend with

Gnrh1 (Supplementary Table 4). Taken together, these data support the hypothesis that the increased *Gnrh1* and *Dlx1* in the absence of miR-29s could be mediated by *Tbx21*. Immunofluorescence colocalization results showed that *Tbx21* was co-expressed with *Gnrh1* in the medial eminence (ME) where GnRH neuron distributed (Fig. 3A). Consistent with *Gnrh1*, the expression level of *Tbx21* rose continuously during sexual development. Unexpectedly, the expression of *Dlx1* decreased from infancy to puberty but upregulated from puberty to adulthood (Fig. 3B). Then, the effect of *Tbx21* on *Gnrh1* and *Dlx1* was studied *in vitro*. The expression of both *Gnrh1* and *Dlx1* were promoted by *Tbx21* overexpression in GT1-7 and N6 cells (Fig. 3C, D and E). In a *Tbx21* stably overexpressed GT1-7 cell line, the *Gnrh1* expression was obviously enhanced (Fig. 3C and F). As TBX21 was predicted to bind to the promoters of both *Gnrh1* and *Dlx1* and function as a transcription activator, a ChIP assay was carried out and DNA fragments with predicted binding sites upstream of *Gnrh1* and *Dlx1* were significantly enriched (Fig. 3G and H).

Since DLX1 is a transcription activator of *Gnrh1*, and predicted to interact with TBX21, it is rational to propose that TBX21 may probably form a complex with DLX1 when binding to the *Gnrh1* promoter region, besides binding to it directly. Therefore, we constructed a *Dlx1*-knockout GT1-7 cell model, in which frame shift mutation existed in the *Dlx1*-coding region (Fig. 3I), resulting in a nonfunctional product of *Dlx1* with the deletion of DNA-binding domain. However, in this *Dlx1*-null cell line, the activation of *Gnrh1* expression caused by *Tbx21* overexpression was not impaired (Fig. 3J), which suggests that DLX1 may not be essential for TBX21 binding to the *Gnrh1* promoter.

Repression of miR-29a promotes the puberty onset

To assess the functional involvement of miR-29 family members in the puberty-onset regulation, we performed stereotactic brain infusions of antagomiR-29a in hypothalamus of prepubertal female mice. AntagomiR-29a is a cholesterol-conjugated and cy3-labeled single-stranded RNA analogue complementary to miR-29a-3p, which prevents miR-29-3p from attaching to the 3'UTR of its target genes, scrambled-sequence antagomiR-NC was used as control. Similar to the miR-29a inhibitor, the expression of miR-29s were repressed by antagomiR-29a significantly in GT1-7 cells (Fig. 4A). However, due to the limited area of the injection, miR-29s of the whole hypothalamus were unaffected (Fig. 4B). Given that the silencing duration of antagomiRs was as long as 23 days

Figure 3

The regulatory role of *Tbx21* in the expression of *Gnrh1* both *in vivo* and *in vitro*. (A) Fluorescence micrographs of the expression of GnRH and TBX21 in the ME of female mouse. Upper panel: low magnification images of GnRH (Left), TBX21 (Central Left) and DAPI (nuclear staining; Central Right), final merge of the three signals (right), scale bar: 100 μ m; lower panel: the higher magnification of the upper panel, scale bar: 50 μ m. (B) The expression of *Gnrh1*/*Dlx1*/*Tbx21* from infants to adults in mouse hypothalamus detected by qRT-PCR. One-way ANOVA *post hoc* Dunnett's test, $n = 6$ per group, compared with PND10, for *Gnrh1* $P_{(PND25)} = 0.1910$, $P_{(PND30)} = 0.0389$, $P_{(PND120)} < 0.0001$; for *Tbx21* $P_{(PND25)} < 0.0001$, $P_{(PND30)} = 0.0002$, $P_{(PND120)} < 0.0001$; for *Dlx1* $P_{(PND25)} = 0.1910$, $P_{(PND30)} = 0.0389$, $P_{(PND120)} < 0.0001$, compared with PND25, $P_{(PND120)} = 0.0190$. (C) The level of *Tbx21* expression in pCMV-Tbx21 transiently transfected GT1-7, N6 and stably *Tbx21*-overexpressed GT1-7-Tbx21-OE. Unpaired two-tailed Student's *t*-test, $n = 6$ per group, $P_{(GT1-7)} < 0.0001$, $P_{(N6)} = 0.0024$, $P_{(GT1-7-Tbx21-OE)} < 0.0001$. (D and E) The effect of transient *Tbx21* overexpression on *Gnrh1* and *Dlx1* in GT1-7 and N6. Unpaired two-tailed Student's *t*-test, $n = 6$ per group, for GT1-7, $P_{(Gnrh1)} = 0.0034$, $P_{(Dlx1)} = 0.0026$; For N6, $P_{(Gnrh1)} = 0.0057$, $P_{(Dlx1)} = 0.0202$. (F) The expression of *Gnrh1* and *Dlx1* in GT1-7-Tbx21-OE. Unpaired two-tailed Student's *t*-test, $n = 6$ per group, $P_{(Gnrh1)} = 0.0042$, $P_{(Dlx1)} = 0.0003$. (G and H) The distribution of putative TBX21-binding sites on the mouse *Gnrh1* and *Dlx1* gene (upper panel) and their validation using a TBX21 chromatin immunoprecipitation assay in GT1-7. The gray squares represent enhancers and promoter of *Gnrh1*. The red vertical line represent putative TBX21-binding sites. The arrow indicates the location of the qPCR primer. Unpaired two-tailed Student's *t*-test, $n = 3$ per group, $P_{(Gnrh1-p1)} = 0.0017$, $P_{(Gnrh1-p2)} = 0.0075$, $P_{(Gnrh1-p3)} = 0.1071$, $P_{(Dlx1-p1)} = 0.0160$, $P_{(Dlx1-p2)} = 0.0136$, $P_{(Dlx1-p3)} = 0.031$, $P_{(Dlx1-p4)} = 0.0340$. (I) The genotype of *Dlx1*-knockout GT1-7 cell. The underlined bases are the sgRNAs recognition sites. (J) The effect of transient *Tbx21*-overexpression on *Gnrh1* and *Dlx1* in *Dlx1*-knockout GT1-7. Unpaired two-tailed Student's *t*-test, $n = 5$ per group, $P_{(Tbx21)} = 0.0001$, $P_{(Gnrh1)} = 0.0018$, $P_{(Dlx1)} = 0.0007$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. 3V, 3rd ventricle; ME, median eminence.

after intravenous injection (Krutzfeldt *et al.* 2005), we carried out stereotactic brain infusions at PND15. The mice were killed at 20 days after injection, and the brain sections were made to inspect the site of action.

The signal of antagomiR-29a was concentrated near the third ventricle (Fig. 4C and Supplementary Fig. 4). Though the growth rate was similar between the antagomiR-29a-3p-injected and control mice (Fig. 4D and G),

Figure 4

Effect of repression of miR-29a on the puberty onset. (A) The relative expression levels of miR-29s in GT1-7 treated with antagomiRs. Unpaired two-tailed Student's *t*-test, $n = 5$ per group, $P_{(\text{miR-29a})} = 0.0009$, $P_{(\text{miR-29b})} = 0.0015$, $P_{(\text{miR-29c})} = 0.0013$. (B) The relative expression levels of miR-29s in hypothalamus treated with antagomiRs. Unpaired two-tailed Student's *t*-test, $n = 6$ per group, $P_{(\text{miR-29a})} = 0.0831$, $P_{(\text{miR-29b})} = 0.0924$, $P_{(\text{miR-29c})} = 0.1985$. (C) Fluorescent photograph of brain tissue sections of antagomiRs-injected mice. 3V, third ventricle. Scale bar: 100 μm . (D) Growth curve of antagomiRs-injected mice. Two-way repeated-measures ANOVA, $n = 8$ and 15 mice per group, $P = 0.6096$. (E) Cumulative percentage of antagomiRs-injected mice over time (days) with VO. Log-rank (Mantel-Cox) test, $n = 8$ and 15 mice per group, $P = 0.0394$. (F) Age at vaginal opening of antagomiRs-injected mice. Mann-Whitney test, $n = 8$ and 15 per group, $P = 0.0610$. (G) Weight of antagomiRs-injected mice at vaginal opening. Unpaired two-tailed Student's *t*-test, $n = 8$ and 15 mice per group, $P = 0.1241$. $^{**}P < 0.01$, $^{***}P < 0.001$. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0082>.

antagomiR-29a-injected mice exhibited a precocious onset of puberty when compared with the control (Fig. 4E and F).

Overexpression of *Tbx21* promotes the puberty onset and reproduction in mice

Tbx21 hypothalamic ectopic expression mouse model was created by injecting viral particle carrying *Tbx21* and eGFP cassette into the mouse, mouse injected with the eGFP-expressing virus was used as control. The fluorescence of eGFP was detectable in brain 7 days after injection (Fig. 5A). Compared with the control mice, the *Tbx21* overexpressed mice had similar growth rate but attained VO significantly earlier (Fig. 5B, C, D and E). Most of the mice with *Tbx21* ectopic expression showed a slight prolongation in estrus cycle (Fig. 5F), and higher serum FSH and LH levels during estrus cycles (Fig. 5G and H). The assay for LH and FSH has a sensitivity of 0.28 ng/mL and 0.94 ng/mL, respectively, and the intraassay coefficient of variation of the quality controls was 0.6742 (control) and 0.5665 (*Tbx21*) for FSH, 0.7654 (control) and 0.5093 (*Tbx21*) for LH. Moreover, the mice with *Tbx21* overexpression had a larger first litter size (Fig. 5I), while the pregnant frequency decreased within 2 months (Fig. 5J).

Discussion

The regulation of *Gnrh1* expression is of great significance for the puberty onset and reproduction in mammals. Our study discovered a novel regulation mechanism mediated by miRNA-29 family. In GT1-7 cells, both the knockout and knockdown of miR-29s resulted in the increased *Gnrh1* expression which paralleled the consequence of their target *Tbx21* overexpression. We found that TBX21 was co-expressed with GnRH in the same increasing trend in the hypothalamus during mouse puberty onset. *In vitro*, TBX21 activated *Gnrh1* expression as a transcription factor in both direct and indirect ways. The effect of both miR-29s and TBX21 on the puberty-onset regulation was demonstrated by experiments in mouse models. Although TBX21 was predicted as an interacting protein of DLX1, our data from the *Dlx1*-knockout cells provided that TBX21 activated the *Gnrh1* expression independent of working together with DLX1 in the form of mutual cofactors.

miR-29 family consists of four members, and they have identical 'seed' sequence and differ with each other in only a few nucleotides, so they share many identical targets. When we destroyed any one of miR-29s individually in GT1-7 cells, we could hardly find variation in

Figure 5

Effect of overexpression of *Tbx21* on puberty onset and reproduction. (A) Fluorescent image of brain tissue sections of virus-injected mice. 3V, third ventricle; ME, median eminence. Scale bar: 500 μ m (left) and 100 μ m (right). (B) Growth curve of virus-injected mice. Two-way repeated-measures ANOVA, $n = 13$ and 12 mice per group, $P = 0.427$. (C) Cumulative percentage of virus-injected mice with vaginal opening over time (days). Log-rank (Mantel-Cox) test, $n = 13$ and 12 mice per group, $P = 0.0220$. (D) Age at vaginal opening of virus-injected mice. Mann-Whitney test, $n = 13$ and 12 mice per group, $P = 0.0423$. (E) Weight of virus-injected mice at vaginal opening. (F) Estrous cyclicity of virus-injected mice at the age of four months. Di, diestrus; E, estrus; P, proestrus. (G and H) Serum levels of FSH/LH during estrus of virus-injected mice. Unpaired two-tailed Student's t -test, $n = 5$ mice per group, $P_{(FSH)} = 0.0201$, $P_{(LH)} = 0.0349$. (I) First litter size of virus-injected mice. Unpaired two-tailed Student's t -test, $n = 12$ mice per group, $P = 0.0304$. (J) Pregnancy frequency within two months of virus injection. Unpaired two-tailed Student's t -test, $n = 12$ mice per group, $P = 0.0721$. * $P < 0.05$. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0082>.

Gnrh1 expression, which was because family members usually function redundantly. The expression of any member of a miRNA family can usually rescue the family mutant phenotypes (Alvarez-Saavedra & Horvitz 2010), while the others regulate their targets in a primarily additive fashion (Subasic *et al.* 2015). Benefiting from the compact distribution of the miR-29 genes in the mouse genome and the CRISPR/cas9 technology, we managed to destroy the four miR-29 genes simultaneously and observed robust increase of *Gnrh1* expression in miR-29 family knockout cells. In GT1-7 cells, all miRNA-29a, miRNA-29b and miR-29c can be expressed and have binding site on the 3'UTR of *Tbx21* gene. When any one of them is absent, its inhibitory effect on *Tbx21*

gene maybe compensated by other family members. On the other hand, the overexpression of miR29s made undetectable difference on the mRNA amount of *Gnrh1* gene, which could be due to the relatively low abundance of *Tbx21* in the GT1-7 cells, and the decrease of *Tbx21* gene expression caused by miR-29s made limited influence on *Gnrh1* expression. In contrast, Ahmed *et al.* found a single member of the miR-7 family, miR-7a2, was essential for normal pituitary development that mice lacking miR-7a2, not miR-7a1 or miR-7b, develop hypogonadotropic hypogonadism and infertility. They ascribed the unique character of miR-7a2 to that it is the predominant member of miR-7 family in the pituitary and that constitutive genetic ablation of miR-7a2 was

sufficient to cause the phenotypic variation (Ahmed *et al.* 2017).

The rise in the level of *Gnrh1* expression in *Tbx21*-overexpressed cells was lower than that in miR-29s-knockout cells, which could be interpreted by the multiple targets of miRNAs. The product of miR-29 family distributed ubiquitously in tissues and organs, participating in a variety of biological processes, including the signal transduction, proliferation and apoptosis of neurons. The predicted target genes of miR-29 family are as many as 1051 in TargetScanMouse, a lot of them have been verified by experiments (<http://mirtarbase.mbc.nctu.edu.tw/php/index.php>). They are likely to differ in the expression patterns between tissues and response to the immediate cellular milieu (Slusarz & Pulakat 2015). Studies also showed that miR-29 family synergistically regulated the same biological process, which could be supported by the fact that more than 20 target genes of miR-29s had been confirmed to be involved in the extracellular matrix synthesis (Villarreal *et al.* 2011). In addition to *Tbx21*, miR-29s may also regulate the epigenetic status of *Gnrh1* gene by targeting the 3'UTR of *Tet1/2/3*, *Dnmt3a/b*, *Tdg* (Zhang *et al.* 2013b, Hysolli *et al.* 2016), which are the regulators of DNA methylation, including *Gnrh1* (Kurian *et al.* 2010, 2016). Moreover, some other mechanism can be responsible for the increased expression of *Gnrh1* caused by miR-29s absent as well.

We applied stereotactic brain infusion of antagomiR-29a-3p to generate the hypothalamic miR-29-knockdown mouse model other than destroyed the coding genes by gene editing technology, because it could be highly laborious and time consuming to obtain the homozygous mice with four genomic loci of miR-29 family members damaged simultaneously. The cholesterol-conjugated antagomiRs can diffuse in the cells readily and have an effective window of 23 days *in vivo* (Krutzfeldt *et al.* 2005), which provides long enough period to investigate the puberty-onset phenotypes of female mice with hypothalamus locating injection. We observed the advanced VO in miR-29-knockdown female mice actually, which was consistent with the previous reports that miR-29s were promoters of female fertility. Because of the different phenotype caused by miR-29s knockdown between the female and male (Takeda & Tanabe 2016), their effect on the fertility of male mice needs further exploring.

With the same method, we made *Tbx21* overexpressed mouse model by hypothalamus infusion of lentivirus carrying a *Tbx21* expression cassette. The female mice of this model were affected more intensively in the puberty

onset and fertility than miR-29-knockdown mice, which might result from that lentivirus integrated its genome into the host chromosome when it entered the host cell, and the foreign genes it carried could be expressed with the host genes all along.

The pulsatile release of GnRH is the result of the synergy between its stimulation and suppression signals. Messina *et al.* reported a miRNA switch including miR-155 and miR-200 that controlled the rise of hypothalamus GnRH production before puberty in mice (Messina *et al.* 2016). These results described the permitting roles of miRNAs in sexual maturation process and suggested the existence of a multilayered array of miRNAs and their target genes in the HPG axis that modulated the puberty timing and reproduction (Messina *et al.* 2016). Our elucidation about the repressive function of miR-29 family in *Gnrh1* expression suggested multiplex roles of miRNAs in the puberty-onset regulation. Unexpectedly, during the initiation of puberty, the expression level of miR-29 rose continuously. Nevertheless, it was not the unique phenomenon that the inhibitors changed synchronously with their effector genes. For example, Li *et al.* found the expression level of miR-378 and its target gene *PGC1 β* increased simultaneously under certain conditions (Li *et al.* 2018). Besides, the level of *Tet1/2/3*, the vital DNA demethylation genes, elevated while the overall genomic methylation level decreased during the embryonic development of mouse (Jessop *et al.* 2018). These imply that during the period of rapid change, some mechanisms like brakes are also needed to participate in the mediation.

To conclude, our data indicated one of the mechanisms which miR-29 family takes part in the regulation of *Gnrh1* expression. Transcription factor *Tbx21*, as the target of miR-29 family, promotes *Gnrh1* expression directly and indirectly, and then influences the puberty onset and reproduction of mice.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0082>.

Declaration of interest

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

Funding

This study was supported by the National Nature Science Foundation of China (Grant no 31772550), the Open Project Funding of Human Phenome

Institute (No. HUPIKF2018204), Fudan University. Open Funding Project of the State Key Laboratory of Bioreactor Engineering. The Fundamental Research Funds for the Central Universities (CUSF-DH-D-2017054).

Acknowledgments

The authors thank Prof. Zilong Qiu in Institute of Neuroscience, CAS Center for Excellence in Brain Science and Intelligence Technology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences for his help of stereotactic brain infusions, and Prof. Xiaoying Li in Shanghai Clinical Center for Endocrine and Metabolic Diseases, Shanghai Jiaotong University for providing GT1-7 cells.

References

- Ahmed K, Lapierre MP, Gasser E, Denzler R, Yang Y, Rulicke T, Kero J, Latreille M & Stoffel M 2017 Loss of microRNA-7a2 induces hypogonadotropic hypogonadism and infertility. *Journal of Clinical Investigation* **127** 1061–1074. (<https://doi.org/10.1172/JCI90031>)
- Alvarez-Saavedra E & Horvitz HR 2010 Many families of *C. elegans* microRNAs are not essential for development or viability. *Current Biology* **20** 367–373. (<https://doi.org/10.1016/j.cub.2009.12.051>)
- Amar L, Benoit C, Beaumont G, Vacher CM, Crepin D, Taouis M & Baroin-Tourancheau A 2012 MicroRNA expression profiling of hypothalamic arcuate and paraventricular nuclei from single rats using Illumina sequencing technology. *Journal of Neuroscience Methods* **209** 134–143. (<https://doi.org/10.1016/j.jneumeth.2012.05.033>)
- Annis RP, Swahari V, Nakamura A, Xie AX, Hammond SM & Deshmukh M 2016 Mature neurons dynamically restrict apoptosis via redundant premitochondrial brakes. *FEBS Journal* **283** 4569–4582. (<https://doi.org/10.1111/febs.13944>)
- Avendano MS, Vazquez MJ & Tena-sempere M 2017 Disentangling puberty: novel neuroendocrine pathways and mechanisms for the control of mammalian puberty. *Human Reproduction Update* **23** 737–763. (<https://doi.org/10.1093/humupd/dmx025>)
- Belsham DD, Cai F, Cui H, Smukler SR, Salapatek AM & Shkreta L 2004 Generation of a phenotypic array of hypothalamic neuronal cell models to study complex neuroendocrine disorders. *Endocrinology* **145** 393–400. (<https://doi.org/10.1210/en.2003-0946>)
- Byers SL, Wiles MV, Dunn SL & Taft RA 2012 Mouse estrous cycle identification tool and images. *PLoS ONE* **7** e35538. (<https://doi.org/10.1371/journal.pone.0035538>)
- Chen K, Zhou YX, Li K, Qi LX, Zhang QF, Wang MC & Xiao JH 2016 A novel three-round multiplex PCR for SNP genotyping with next generation sequencing. *Analytical and Bioanalytical Chemistry* **408** 4371–4377. (<https://doi.org/10.1007/s00216-016-9536-6>)
- Ebling FJ 2005 The neuroendocrine timing of puberty. *Reproduction* **129** 675–683. (<https://doi.org/10.1530/rep.1.00367>)
- Garaffo G, Conte D, Provero P, Tomaiuolo D, Luo Z, Pinciroli P, Peano C, D'atri I, Gitton Y, Etzion T, *et al.* 2015 The *Dlx5* and *Foxg1* transcription factors, linked via miRNA-9 and -200, are required for the development of the olfactory and GnRH system. *Molecular and Cellular Neurosciences* **68** 103–119. (<https://doi.org/10.1016/j.mcn.2015.04.007>)
- Herbison AE 2016 Control of puberty onset and fertility by gonadotropin-releasing hormone neurons. *Nature Reviews: Endocrinology* **12** 452–466. (<https://doi.org/10.1038/nrendo.2016.70>)
- Huang PP, Brusman LE, Iyer AK, Webster NJ & Mellon PL 2016 A novel gonadotropin-releasing hormone 1 (*Gnrh1*) enhancer-derived noncoding RNA regulates *Gnrh1* gene expression in GnRH neuronal cell models. *PLoS ONE* **11** e0158597. (<https://doi.org/10.1371/journal.pone.0158597>)
- Hysolli E, Tanaka Y, Su J, Kim KY, Zhong T, Janknecht R, Zhou XL, Geng L, Qiu C, Pan X, *et al.* 2016 Regulation of the DNA methylation landscape in human somatic cell reprogramming by the miR-29 family. *Stem Cell Reports* **7** 43–54. (<https://doi.org/10.1016/j.stemcr.2016.05.014>)
- Jessop P, Ruzov A & Gering M 2018 Developmental functions of the dynamic DNA methylome and hydroxymethylome in the mouse and zebrafish: similarities and differences. *Frontiers in Cell and Developmental Biology* **6** 27. (<https://doi.org/10.3389/fcell.2018.00027>)
- Keynes R & Lumsden A 1990 Segmentation and the origin of regional diversity in the vertebrate central nervous system. *Neuron* **4** 1–9. ([https://doi.org/10.1016/0896-6273\(90\)90438-L](https://doi.org/10.1016/0896-6273(90)90438-L))
- Krol J, Loedige I & Filipowicz W 2010 The widespread regulation of microRNA biogenesis, function and decay. *Nature Reviews: Genetics* **11** 597–610. (<https://doi.org/10.1038/nrg2843>)
- Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M & Stoffel M 2005 Silencing of microRNAs in vivo with 'antagomirs'. *Nature* **438** 685–689. (<https://doi.org/10.1038/nature04303>)
- Kurian JR, Keen KL & Terasawa E 2010 Epigenetic changes coincide with in vitro primate GnRH neuronal maturation. *Endocrinology* **151** 5359–5368. (<https://doi.org/10.1210/en.2010-0555>)
- Kurian JR, Louis S, Keen KL, Wolfe A, Terasawa E & Levine JE 2016 The methylcytosine dioxygenase ten-eleven translocase-2 (*tet2*) enables elevated GnRH gene expression and maintenance of male reproductive function. *Endocrinology* **157** 3588–3603. (<https://doi.org/10.1210/en.2016-1087>)
- Lee VH, Lee LT & Chow BK 2008 Gonadotropin-releasing hormone: regulation of the GnRH gene. *FEBS Journal* **275** 5458–5478. (<https://doi.org/10.1111/j.1742-4658.2008.06676.x>)
- Li Y, Jiang J, Liu W, Wang H, Zhao L, Liu S, Li P, Zhang S, Sun C, Wu Y, *et al.* 2018 microRNA-378 promotes autophagy and inhibits apoptosis in skeletal muscle. *PNAS* **115** E10849–E10858. (<https://doi.org/10.1073/pnas.1803377115>)
- Lippi G, Steinert JR, Marczylo EL, D'oro S, Fiore R, Forsythe ID, Schrat G, Zoli M, Nicotera P & Young KW 2011 Targeting of the Arp3 actin nucleation factor by miR-29a/b regulates dendritic spine morphology. *Journal of Cell Biology* **194** 889–904. (<https://doi.org/10.1083/jcb.201103006>)
- Luque RM, Kineman RD & Tena-Sempere M 2007 Regulation of hypothalamic expression of *Kiss-1* and *GPR54* genes by metabolic factors: analyses using mouse models and a cell line. *Endocrinology* **148** 4601–4611. (<https://doi.org/10.1210/en.2007-0500>)
- Messina A, Langlet F, Chachlaki K, Roa J, Rasika S, Jouy N, Gallet S, Gaytan F, Parkash J, Tena-Sempere M, *et al.* 2016 A microRNA switch regulates the rise in hypothalamic GnRH production before puberty. *Nature Neuroscience* **19** 835–844. (<https://doi.org/10.1038/nn.4298>)
- Mihalas AB & Hevner RF 2017 Control of neuronal development by T-box genes in the brain. *Current Topics in Developmental Biology* **122** 279–312. (<https://doi.org/10.1016/bs.ctdb.2016.08.001>)
- Omariba G, Tong L, Wang M, Li K, Zhou Y & Xiao J 2018 Characterization of the dynamic change of microRNA expression in mice hypothalamus during the time of female puberty. *Genes and Genomics* **40** 295–304. (<https://doi.org/10.1007/s13258-017-0633-5>)
- Rajman M & Schrat G 2017 MicroRNAs in neural development: from master regulators to fine-tuners. *Development* **144** 2310–2322. (<https://doi.org/10.1242/dev.144337>)
- Ravasi T, Suzuki H, Cannistraci CV, Katayama S, Bajic VB, Tan K, Akalin A, Schmeier S, Kanamori-Katayama M, Bertin N, *et al.* 2010 An atlas of combinatorial transcriptional regulation in mouse and man. *Cell* **140** 744–752. (<https://doi.org/10.1016/j.cell.2010.01.044>)
- Ripa R, Dolfi L, Terrigno M, Pandolfini L, Savino A, Arcucci V, Groth M, Terzibasi Tozzini E, Baumgart M & Cellerino A 2017 MicroRNA miR-29 controls a compensatory response to limit neuronal iron accumulation during adult life and aging. *BMC Biology* **15** 9. (<https://doi.org/10.1186/s12915-017-0354-x>)

- Saba R & Schratt GM 2010 MicroRNAs in neuronal development, function and dysfunction. *Brain Research* **1338** 3–13. (<https://doi.org/10.1016/j.brainres.2010.03.107>)
- Slusarz A & Pulakat L 2015 The two faces of miR-29. *Journal of Cardiovascular Medicine* **16** 480–490. (<https://doi.org/10.2459/JCM.0000000000000246>)
- Steiner DF, Thomas MF, Hu JK, Yang Z, Babiarz JE, Allen CD, Matloubian M, Belloch R & Ansel KM 2011 MicroRNA-29 regulates T-box transcription factors and interferon-gamma production in helper T cells. *Immunity* **35** 169–181. (<https://doi.org/10.1016/j.immuni.2011.07.009>)
- Subasic D, Brummer A, Wu Y, Pinto SM, Imig J, Keller M, Jovanovic M, Lightfoot HL, Nasso S, Goetze S, *et al.* 2015 Cooperative target mRNA destabilization and translation inhibition by miR-58 microRNA family in *C. elegans*. *Genome Research* **25** 1680–1691. (<https://doi.org/10.1101/gr.183160.114>)
- Takeda T & Tanabe H 2016 Lifespan and reproduction in brain-specific miR-29-knockdown mouse. *Biochemical and Biophysical Research Communications* **471** 454–458. (<https://doi.org/10.1016/j.bbrc.2016.02.055>)
- Tong L, Xue H, Xiong L, Xiao J & Zhou Y 2015 Improved RT-PCR assay to quantitate the pri-, pre-, and mature microRNAs with higher efficiency and accuracy. *Molecular Biotechnology* **57** 939–946. (<https://doi.org/10.1007/s12033-015-9885-y>)
- Villarreal G, Jr, Oh DJ, Kang MH & Rhee DJ 2011 Coordinated regulation of extracellular matrix synthesis by the microRNA-29 family in the trabecular meshwork. *Investigative Ophthalmology and Visual Science* **52** 3391–3397. (<https://doi.org/10.1167/iops.10-6165>)
- Zhang L, Cai Z, Wei S, Zhou H, Zhou H, Jiang X & Xu N 2013a MicroRNA expression profiling of the porcine developing hypothalamus and pituitary tissue. *International Journal of Molecular Sciences* **14** 20326–20339. (<https://doi.org/10.3390/ijms141020326>)
- Zhang P, Huang B, Xu X & Sessa WC 2013b Ten-eleven translocation (Tet) and thymine DNA glycosylase (TDG), components of the demethylation pathway, are direct targets of miRNA-29a. *Biochemical and Biophysical Research Communications* **437** 368–373. (<https://doi.org/10.1016/j.bbrc.2013.06.082>)
- Zhou Y, Tong L, Wang M, Chang X, Wang S, Li K & Xiao J 2018 MiR-505-3p is a repressor of the puberty onset in female mice. *Journal of Endocrinology* **240** 379–392. (<https://doi.org/10.1530/JOE-18-0533>)

Received in final form 17 June 2019

Accepted 28 June 2019

Accepted Preprint published online 28 June 2019