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MicroRNAs (miRNAs) are negative modulators of gene expression that fine-tune numerous biological processes. miRNA loss-of-function rarely results in highly penetrant phenotypes, but rather, influences cellular responses to physiologic and pathophysiologic stresses. Here, we have reported that a single member of the evolutionarily conserved miR-7 family, miR-7a2, is essential for normal pituitary development and hypothalamic-pituitary-gonadal (HPG) function in adulthood. Genetic deletion of mir-7a2 causes infertility, with low levels of gonadotropic and sex steroid hormones, small testes or ovaries, impaired spermatogenesis, and lack of ovulation in male and female mice, respectively. We found that miR-7a2 is highly expressed in the pituitary, where it suppresses golgi glycoprotein 1 (GLG1) expression and downstream bone morphogenetic protein 4 (BMP4) signaling and also reduces expression of the prostaglandin F2a receptor negative regulator (PTGFRN), an inhibitor of prostaglandin signaling and follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion. Our results reveal that miR-7a2 critically regulates sexual maturation and reproductive function by interconnecting miR-7 genomic circuits that regulate FSH and LH synthesis and secretion through their effects on pituitary prostaglandin and BMP4 signaling.

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Loss of microRNA-7a2 induces hypogonadotropic hypogonadism and infertility

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MicroRNAs (miRNAs) are negative modulators of gene expression that fine-tune numerous biological processes. miRNA loss-of-function rarely results in highly penetrant phenotypes, but rather, influences cellular responses to physiologic and pathophysiologic stresses. Here, we have reported that a single member of the evolutionarily conserved miR-7 family, miR-7a2, is essential for normal pituitary development and hypothalamic-pituitary-gonadal (HPG) function in adulthood. Genetic deletion of mir-7a2 causes infertility, with low levels of gonadotropic and sex steroid hormones, small testes or ovaries, impaired spermatogenesis, and lack of ovulation in male and female mice, respectively. We found that miR-7a2 is highly expressed in the pituitary, where it suppresses golgi glycoprotein 1 (GLG1) expression and downstream bone morphogenetic protein 4 (BMP4) signaling and also reduces expression of the prostaglandin F2a receptor negative regulator (PTGFRN), an inhibitor of prostaglandin signaling and follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion. Our results reveal that miR-7a2 critically regulates sexual maturation and reproductive function by interconnecting miR-7 genomic circuits that regulate FSH and LH synthesis and secretion through their effects on pituitary prostaglandin and BMP4 signaling.

Introduction

Sexual maturation and reproduction are regulated by the hypothalamus, pituitary, and gonads. These endocrine organs form an integrated system known as the hypothalamic-pituitary-gonadal (HPG) axis, which is responsible for the adequate secretion of male and female sex hormones (1). The endocrine components of the reproductive system are hierarchically organized and integrated in a classical endocrine feedback loop. The HPG axis is active during development and shortly after birth, silenced during childhood by inhibitory neurotransmitters, and reawakened at the onset of puberty by pulsatile secretion of gonadotropin-releasing hormone (GnRH), a decapeptide that is synthesized by neurons located in the mediobasal hypothalamus (2, 3). During development, GnRH neurons originate from the olfactory placode of the olfactory system and migrate to the hypothalamus, where they control reproduction by secreting GnRH into a capillary network that transmits GnRH to the anterior pituitary to stimulate secretion of follicle-stimulating hormone (FSH, encoded by Fshb) and luteinizing hormone (LH, encoded by Lhb). Secreted GnRH acts via the GnRH receptor, which is expressed on gonadotropic cells in the anterior pituitary gland. This action regulates synthesis and release of both gonadotropins, LH and FSH, that control gonadal maturation and adult reproductive physiology via stimulation of sex steroid synthesis (1). In males, FSH stimulates proliferation of immature Sertoli cells and spermatogenesis, whereas LH stimulates Leydig cells to produce testosterone (4). In females, LH triggers ovulation, promotes development of the corpus luteum, and stimulates theca cells to produce androgens, whereas FSH stimulates recruitment of secondary ovarian follicles and the secretion of estradiol from granulosa cells, thereby promoting follicular maturation, which ultimately leads to ovulation (5).

Lack of activation of the HPG axis leads to hypogonadotropic hypogonadism, which is defined as low levels of the sex steroids testosterone or estrogen (hypogonadism) in male or female patients, respectively, and inappropriately low levels of gonadotropins FSH and LH (6). An increasing number of genes have been implicated in the molecular pathogenesis of congenital isolated hypogonadotropic hypogonadism (IHH), underlining the genetic and clinical heterogeneity and complexity of this condition (7–9). Genetic causes of GnRH and gonadotropin deficiency can be grouped according to the nature of their pathogenic mechanisms, which comprise defects in neuropeptides and proteins involved in the development and migration of GnRH neurons (caused by mutations in KAL1, FGFR8/FGFR1, PROK2/PROKR2, CHD7, HS6ST1, WDR11, FZETF1, NSMF and SEMA3A) in genes controlling GnRH secretion and action (GNRHR, GPR54 [also known as KISS1], TAC3/TACR3, LEP/LEPR, and GNHR, respectively) and genes participating in synthesis of LH or FSH β subunits (7–9). Interestingly, homozygous inactivating mutations in the genes encoding the ubiquitin E3 ligase RNF216 and the deubiquitinase OTUD4 have recently been identified by whole-exome sequencing in patients exhibiting a syndrome of hypogonadotropic hypogonadism, progressive ataxia, and dementia.
MicroRNAs (miRNAs) are small noncoding RNAs that act as negative regulators of gene expression by binding to miRNA-response elements (MREs) located primarily in 3’ UTRs of mRNA targets to regulate their stability and translation (12). Mammalian genomes have more than 500 miRNA genes, with miRNAs from individual gene families often targeting hundreds of different genes, thereby linking disordered ubiquitination to neurodegeneration and reproductive dysfunction (10). Despite these recent advances, the genetic basis of many cases of congenital IHH remains unknown, as these mutations account for approximately 50% of all IHH cases (11). Therefore, discovering additional gene mutations will continue to advance our understanding of this syndrome.

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Figure 1. Ablation of mir-7a2 leads to male hypogonadism. (A–C) Representative images of mir-7a2 WT (+/+) control and KO (−/−) testes (A) and quantification of testes weights (B) of mir-7a1 KO, mir-7a2 KO, or respective WT controls (mir-7a1 control, n = 7; mir-7a2 control, n = 6; mir-7a2 KO, n = 9) at 6 to 8 weeks of age or (C) at 14 days (mir-7a2 control, n = 4; mir-7a2 KO, n = 2). Scale bar: 5 mm. (D and E) Immunohistological images (D) and quantification (E) of CYP17A1-positive Leydig cells (WT, mir-7a2 KO, n = 3). Scale bar: 50 μm. Arrows show Leydig cells. (F) Relative expression levels of steroidogenic genes in testes of mir-7a2 KO or control mice (WT, n = 9; mir-7a2 KO, n = 7). (G and H) Representative images of seminal vesicles (G) and quantification of weights (H) of control (upper) or mir-7a2 KO (lower) mice (WT, n = 6; mir-7a2 KO, n = 4). Scale bar: 5 mm. (I) Intratesticular testosterone levels normalized per total protein content in mir-7a2 KO or control mice (WT, n = 5; mir-7a2 KO, n = 3). (J) Total sperm count of the cauda epididymidis of 12-week-old mir-7a2 KO or control mice (WT, n = 5; mir-7a2 KO, n = 3). All data are represented as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001, ANOVA (B); t test (C, E, F, H, I, J).
mRNAs (13, 14). With more than half of all human mRNAs estimated to be conserved miRNA targets, miRNAs are thought to have widespread effects on gene regulation. Many miRNA knock-out models in mice show no apparent defect under normal conditions; however, they frequently exhibit severe miRNA-dependent phenotypes when specific stresses are applied (15). Moreover, the importance of individual miRNAs in normal physiology and disease has been established by the discovery of mutations in miRNAs and their targets (16–18). Recently, the collective role of miRNAs in the HPG axis was demonstrated by genetic ablation of Dicer1 in GnRH neurons (19) and gonadotrophs (20), resulting in hypogonadism and infertility. Furthermore, miR-200b and miR-429, both members of the miR-200 family, as well as their target, ZEB1, are required for female ovulation and reproduction in mice (21).

The miR-7 family is evolutionarily highly conserved and considered a prototypical neuroendocrine miRNA, being expressed at high levels in neurons and neuroendocrine organs, most notably the endocrine pancreas, pituitary, and adrenal glands (14, 22, 23). Mature mir-7 harbors a conserved seed sequence that is processed from 3 precursors − mir-7a1, mir-7a2, and mir-7b − that are transcribed from unlinked loci on murine chromosomes 13, 7, and 17, respectively. A novel mechanism of miR-7 regulation was recently described in neuronal cells through the identification of a brain-enriched circular RNA (known as antisense to the cerebellar degeneration–related protein 1 transcript [Cdr1as]) comprising several dozens of conserved miR-7–binding sites counteracting repression by this miRNA (24, 25). Functionally, miR-7 has been implicated in organ differentiation and development (26) as well as in mammalian neurodegenerative diseases and cancer (27, 28). Mouse genetic studies so far have revealed a role in insulin secretion and differentiation of pancreatic β cells (22).

In this study, we describe the unexpected finding of a hypogonadotropic hypogonadism phenotype in male and female mice as a result of the single mir-7a2 deletion. We identify miR-7a2 as a positive regulator of FSH and LH synthesis by controlling a network involving bone morphogenetic protein 4 (BMP4) and prostaglandin signaling. Our data reveal the indispensability of a single miRNA for normal pituitary gonadotroph function, raising the question of whether genetic or acquired defects leading to altered miR-7 levels may be responsible for puberty and infertility disorders of pituitary origin.

Results

Genetic ablation of mir-7a2, but not mir-7a1 or mir-7b, leads to hypogonadism and infertility in mice. To investigate the physiological role of miR-7, we generated mice constitutively lacking mir-7a1 (referred to as mir-7a1 KO), mir-7a2 (referred to as mir-7a2 KO), or mir-7b (referred to as mir-7b KO) (Supplemental Figure 1, A–D; supplemental material available online with this article; https://doi.org/10.1172/JCI90031DS1). Heterozygous breedings of all 3 mouse strains were viable, had unaltered body weight and body length, and were born at normal Mendelian ratios (Supplemental Figure 1, E–H). Interestingly, homozygous mir-7a2 KO male and female mice were infertile, while homozygous mir-7a1 KO or mir-7b KO mice remained fertile, with unaltered reproductive rates (Supplemental Table 1).

Homozygous mir-7a2 KO male mice failed to undergo sexual maturation and exhibited microphallus and hypogonadism with approximately 50% reduced testes weights at pre- and pubertal ages (2 and 6 to 8 weeks old, respectively) (Figure 1, A–C). The body weights and lengths of mir-7a2 KO mice were comparable with those of WT or mir-7a1 KO animals (Supplemental Figure 1, E and F). Histological analysis of mir-7a2 KO testes revealed smaller but developed seminiferous tubules, with diminished numbers of spermatocytes, spermatids, and spermatozoa (Supplemental Figure 1G). Immunohistochemical quantification of the Leydig cell marker CYP17A1 showed that testosterone–producing Leydig cells were reduced by more than 50% in mir-7a2 KO mice (Figure 1, D and E). We analyzed expression levels of steroidogenic enzymes in testes and measured an approximately 50% reduction of Cyp17a1 and Hsd3b6 mRNAs, which are required for dehydroepiandrosterone and testosterone synthesis, respectively (Figure 1F). Interestingly, expression levels of the FSH receptor (Fshr) and LH receptor (Lhcg) were increased by 2-fold, most likely due to a compensatory response to the decreased plasma gonadotrophin levels (29).

The sizes and weights of seminal vesicles, a classic target organ of testosterone, were reduced by 75% (Figure 1, G and H). Concomitantly, levels of intratesticular testosterone were decreased in mir-7a2 KO mice (Figure 1I) and the total sperm count of the cauda epididymidis, where mature sperm is stored, revealed a 75% decrease in mir-7a2 KO mice as compared with WT controls (Figure 1J). Finally, the inguinal fat pad weights were increased 260% in 16-week-old mice (Supplemental Figure 1H).

Analogous to the phenotype observed in males, mir-7a2 KO females had normal body weight and length (Supplemental Figure 1, I and J), but exhibited a reduced size of estrogen hormone target organs, as evidenced by reduced ovary weights and threadlike atrophied uteri compared with WT or mir-7a1 KO mice (Figure 2A and B). Histological analysis revealed that ovaries of mir-7a2 KO mice were able to form secondary and tertiary follicles that were comparable to those of WT mice; however, they lacked late-stage antral follicles or corpora lutea, consistent with failure of folliculogenesis and ovulation (Figure 2, C and D). Expression analysis of steroidogenic genes in mir-7a2 KO ovaries revealed a drastic decrease of Cyp19a1 mRNA, also known as aromatase, the key enzyme in estradiol biosynthesis (Figure 2E). Consequently, plasma levels of estradiol were reduced by 35% in mir-7a2 KO female mice (Figure 2F). Furthermore, as in male mice, gonadal fat pad weights were increased and dual energy x-ray absorptiometry (DEXA) measurements of fat and lean body mass revealed increased fat and decreased lean mass in 16-week-old mir-7a2 KO mice compared with WT littermate mice (Supplemental Figure 1, K–M), consistent with the hypogonadal-induced obesity observed in mice and humans (30, 31). Together, these data illustrate that loss of mir-7a2, but not mir-7a1 or mir-7b, is sufficient to induce hypogonadism and infertility in mice.

mir-7a2 is the predominant mir-7 family member and is enriched in the pituitary. Based on the increased testicular expression of Fshr and Lhcg, which suggests decreased plasma levels of the pituitary hormones FSH and LH, we hypothesized that the gonadal dysfunction and infertility observed in male and female mir-7a2 KO mice resulted from impaired pituitary function. First, we systematically analyzed expression levels of miR-7a and miR-7b by absolute quantification in tissues of the hypothalamic-pituitary-adrenal/gonadal axis. We found that miR-7a and miR-7b
were enriched in pituitary as compared with hypothalamus (10-fold less), adrenal gland (50-fold less), or gonads (up to 500-fold less) and that miR-7a was 2.5-fold more abundant than miR-7b in pituitary (Supplemental Figure 2, A and B). Given the low expression of miR-7a in male and female gonads, it is unlikely that miR-7a would repress target genes and exert a biological function in testis and ovary (32).

As pre-miR-7a1 and pre-miR-7a2 are processed into an identical mature miR-7a sequence, the TaqMan probe of the miRNA assay cannot distinguish between the 2 miRNA gene products and thus measures combined miR-7a1 and miR-7a2 levels. To assess the contribution of miR-7a1 and miR-7a2 to the total pool of miR-7a in the pituitary, we analyzed expression levels of miR-7a in mice lacking either mir-7a1 or mir-7a2. Interestingly, total miR-7a expression was reduced by approximately 80% in mir-7a2–deficient mice, while miR-7a levels appeared to be unchanged in mice lacking mir-7a1 when compared with WT littermate animals (Supplemental Figure 2, C–F). Expression of miR-7b was unchanged in mir-7a1– and mir-7a2–deficient mice compared with WT animals, thereby confirming the specificity of the TaqMan assay.

In conclusion, miR-7a2 is highly enriched in pituitary compared with other neuroendocrine tissues; furthermore, it accounts for the large majority of total pituitary miR-7 copies, and miR-7a1 and miR-7b cannot compensate for loss of miR-7a2 expression. miR-7a2 ablation causes hypopituitarism and hypogonadotropic hypogonadism. To test whether the hypogonadism observed in mir-7a2 KO mice was caused by altered gene expression of essential pituitary genes, we performed RNA sequencing (RNA-Seq) of pituitaries from 6-week-old WT and mir-7a2 KO mice. We measured a dramatic decrease of gonadotropin expression with a reduction of Fshb mRNA by 86% (FDR = 5.94 × 10\(^{-5}\)) and Lhb mRNA by 78% (FDR = 1.26 × 10\(^{-8}\)) in mir-7a2–deficient pituitaries that was already apparent in 2-week-old mice (Figure 3A and Supplemental Figure 3A). In addition, expression of the glycoprotein hormone \(\alpha\) subunit (Cga) and prolactin (Prl) mRNAs was reduced, while the expression of the GnRH receptor (Gnrhr) and other hormones of the anterior pituitary, including thyroid stimulating hormone \(\beta\) (Tshb), growth hormone (Gh), and pro-opiomelanocortin \(\alpha\) (Pomc), remained unchanged. Since lack of Prl does not lead to hypogonadism (33), we focused our investigation on the characterization of gonadotroph cells. Immunohistochemical analysis of pituitary sections revealed reduced numbers of FSH-expressing (–66%) and LH-expressing (–60%) cells in mir-7a2 KO mice (Figure 3, B–E). We also observed that pituitary weights were reduced.
in both male and female mir-7a2 KO mice (Figure 3, F and G). Importantly, the observed alterations of gene expression resulted in strongly decreased plasma concentrations of FSH and LH in male and female mir-7a2 KO mice (Figure 3, H–K). Furthermore, a GnRH stimulation test with buserelin revealed an increase of plasma FSH and LH levels in WT, but not mir-7a2 KO mice, further confirming that the response of gonadotropic cells to GnRH is impaired and that the defect in mir-7a2 KO mice is primarily at the level of the pituitary (Supplemental Figure 3, B and C). We then tested whether the hypopituitarism observed in mir-7a2 KO mice was responsible for the impaired gonadal function and anovulation by subjecting female mice to a standard superovulation treatment using pregnant mare serum. Ovulation was induced in hormone-treated mir-7a2 KO mice, indicating that ovaries were

Figure 3. Constitutive genetic ablation of mir-7a2 causes hypogonadotropic hypogonadism. (A) Expression of pituitary hormones in male mir-7a2 KO or control mice shown as heat map analysis from RNA-Seq (WT, mir-7a2 KO, n = 3). (B and C) Representative immunohistological images of pituitary sections (n = 3) stained for LH (B) or FSH (C) of control (upper images) or mir-7a2 KO mice (lower images). Scale bars: 200 μm. (D and E) Quantification of LH-positive (D) or FSH-positive (E) cells in pituitary of mir-7a2 KO or control mice (WT, mir-7a2 KO, n = 3). (F and G) Pituitary weights of male (F) or female (G) mir-7a1 KO, mir-7a2 KO, or respective control mice (males, mir-7a1 control, mir-7a1 KO, n = 4; mir-7a2 control, mir-7a2 KO, n = 8; mir-7a1 control, n = 8; mir-7a1 KO, n = 10; mir-7a2 control, n = 6; mir-7a2 KO, n = 7). (H and I) Plasma levels of FSH (H) or LH (I) in male mir-7a2 KO or control mice (WT, mir-7a2 KO, n = 7). (J and K) Plasma levels of FSH (J) or LH (K) in female mir-7a2 KO or control mice (WT, mir-7a2 KO, n = 4). (L) Number of oocytes collected after superovulation test in 5-week-old mir-7a2 KO or control mice (WT, n = 4; mir-7a2 KO, n = 3). All data are represented as mean ± SD except in (H, K), where data are represented as ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001, ANOVA (F, G); t test (D, E, H, I, J, K).
principally functional and that hypophyseal-pituitary dysfunction was the primary cause for the hypogonadism in mir-7a2-ablated mice (Figure 3L). We can exclude that the relatively low expression of miR-7 in the hypothalamus affects GnRH-expressing neurons, as *Gnrh* mRNA levels, morphology, and expected projections of GnRH-expressing neurons were unaltered in mir-7a2 KO mice (Supplemental Figure 3, D–F). Furthermore, transcript levels of neuropeptides and genes involved in the development and migration of GnRH neurons and expression of genes controlling GnRH secretion and action were similar in hypothalami of mir-7a2 KO and control littermates (Supplemental Figure 3, G and H). Interestingly, the neuropeptides kisspeptin 1 (*Kiss1*) and tachykinin 2 (*Tac2*) were significantly increased in hypothalami of mir-7a2 KO mice, which corroborates findings that *Kiss1* and *Tac2* expression are under inhibitory modulation from circulating gonadal hormones (34, 35). In addition, olfactory bulbs appeared to have normal size and architecture and no difference was detected in behavioral olfactory testing of male and female mice lacking

Figure 4. Acute ablation of mir-7a2 in adult mice recapitulates hypogonadotropic hypogonadism. (A) TaqMan assays showing relative expression levels of miR-7a2, miR-7b, or miR-16 in pituitary of male *UBC-Cre × mir-7a2fl/fl* or Cre-negative *mir-7a2fl/fl* mice 10 weeks after a 5-day treatment with tamoxifen (TAM) or vehicle (*UBC-Cre × mir-7a2fl/fl* + vehicle, *n* = 5, *mir-7a2fl/fl* + TAM, *UBC-Cre × mir-7a2fl/fl* + TAM, *n* = 4). (B) Relative expression levels of pituitary hormones in *UBC-Cre × mir-7a2fl/fl* or *mir-7a2fl/fl* mice after treatment with tamoxifen or vehicle (*UBC-Cre × mir-7a2fl/fl* + vehicle, *n* = 5, *mir-7a2fl/fl* + TAM, *UBC-Cre × mir-7a2fl/fl* + Tamp, *n* = 4). (C and D) Immunohistological quantification of LH-positive (C) or FSH-positive (D) cells in pituitary sections of *UBC-Cre × mir-7a2fl/fl* or *mir-7a2fl/fl* mice after treatment with tamoxifen or vehicle (*mir-7a2fl/fl* + TAM, *n* = 4; *UBC-Cre × mir-7a2fl/fl* + TAM, *n* = 3). (E and F) Plasma levels of FSH (E) or LH (F) of male *UBC-Cre × mir-7a2fl/fl* or *mir-7a2fl/fl* mice after treatment with tamoxifen or vehicle (FSH, *UBC-Cre × mir-7a2fl/fl* + vehicle, *n* = 5, *mir-7a2fl/fl* + TAM, *UBC-Cre × mir-7a2fl/fl* + TAM, *n* = 4; LH, *UBC-Cre × mir-7a2fl/fl* + vehicle, *mir-7a2fl/fl* + TAM, *UBC-Cre × mir-7a2fl/fl* + TAM, *n* = 3). (G) Testes weights of *UBC-Cre × mir-7a2fl/fl* or *mir-7a2fl/fl* mice after treatment with tamoxifen or vehicle (*UBC-Cre × mir-7a2fl/fl* + vehicle, *mir-7a2fl/fl* + TAM, *n* = 6; *mir-7a2fl/fl* + TAM, *n* = 10; *UBC-Cre × mir-7a2fl/fl* + TAM, *n* = 8). (H) Relative expression levels of the Leydig cell marker CYP17A1 in testes of *UBC-Cre × mir-7a2fl/fl* or *mir-7a2fl/fl* mice after treatment with tamoxifen or vehicle (*UBC-Cre × mir-7a2fl/fl* + vehicle, *mir-7a2fl/fl* + TAM, *UBC-Cre × mir-7a2fl/fl* + TAM, *n* = 4). All data are represented as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001, ANOVA (A, B, E, F, G, H); t test (C and D).
mir-7a2 compared with littermate controls (data not shown, Supplemental Figure 3), suggesting that the hypogonadotropic phenotype was not due to defective GnRH neuron migration from the olfactory placode into the correct hypothalamic location during development. Collectively, these data demonstrate that mir-7a2 deficiency causes pituitary dysfunction and mirrors the disease pathology of human hypogonadotropic hypogonadism.

Acute genetic ablation of mir-7a2 in adult mice recapitulates hypogonadotropic hypogonadism. To circumvent potential developmental effects caused by constitutive mir-7a2 ablation, we bred mice carrying a floxed mir-7a2 allele (referred to as mir-7a2fl/fl) with transgenic mice expressing Cre-recombinase fused to a mutant form of the estrogen receptor ERT2 under the transcriptional control of the human ubiquitin C promoter (UBC-
that received only vehicle (Figure 4B). Immunohistochemical quantification revealed that FSH cell area was reduced by 55% in tamoxifen-treated UBC-Cre × mir-7a2fl/fl mice, while LH cell area decreased to a lesser extent, with borderline significance (Figure 4, C and D). Most notably, acute ablation of mir-7a2 in adult mice led to a drastic reduction of plasma FSH and LH levels (Figure 4, E and F), and importantly, these hormones were unaltered between vehicle-treated UBC-Cre × mir-7a2fl/fl and mir-7a2fl/fl mice that received tamoxifen (Figure 4, E and F). The decreased gonadotropin expression and secretion resulted in reduced testes weights in

Figure 6. miR-7a2 regulates gonadotropin production through BMP and prostaglandin signaling. (A and B) Relative luciferase levels of plasmids carrying WT or mutated 3′ UTRs of Ptgfrn (A) orGil1 (B) cotransfected in LbT2 cells with or without forced expression of mir-7a2 (n = 3). (C and D) Relative expression levels of gonadotropin genes in cells transfected with siRNA against Ptgfrn (C) or Gil1 (D) (n = 4). (E) Relative expression levels of gonadotropin genes, Cga, Fshb, and Lhb in cells overexpressing Ptgfrn (gray bars) or Gil1 (black bars) for 72 hours (n = 4). (F and G) Relative expression levels of Fshb (F) or Lhb (G) in LbT2 cells that were transfected with siPtgfrn or siCtrl and treated with 100 nM dinoprost or PBS for 4 hours (n = 4). (H) Concentration of LH in supernatants of cells 72 hours after silencing of Ptgfrn (n = 3). (I) Expression levels of Lhb in cells treated with dinoprost (100 nM), GREM1 (0.25 μg/ml), or dinoprost and GREM1 together (n = 3). (J) Western blot analysis of phospho-SMAD1/5/9 or total SMAD1 in lysates of cells pretreated with or without GREM1 (0.25 μg/ml) for 1 hour, followed by 30 minutes of stimulation with BMP4 (50 ng/ml). Shown is 1 representative experiment of 3. All data are represented as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001, ANOVA (E, F, G, I); t test (A, B, C, D, H).

Cre) (36) to allow tamoxifen-inducible global ablation of mir-7a2 (referred to as UBC-Cre × mir-7a2Cre/Cre). We treated 6-week-old male UBC-Cre × mir-7a2Cre/Cre or mir-7a2Cre/Cre mice with tamoxifen and confirmed ablation of mir-7a2 using a TaqMan miRNA assay 8 weeks after treatment (Figure 4A). While pituitary weight and body weight remained unchanged between groups (Supplemental Figure 3, J and K), a striking 50% reduction of Fshb and Lhb mRNA expression levels was observed in the pituitaries of tamoxifen-treated UBC-Cre × mir-7a2Cre/Cre mice as compared with control mir-7a2Cre/Cre mice or UBC-Cre × mir-7a2Cre/Cre mice that received only vehicle (Figure 4B).
Smad4 development (control genes in tamoxifen-induced indicating a tissue-specific regulation of miR-7 target genes. Using of the strongest miR-7 targets previously described in pancreatic not measure a significant upregulation (except also abundant in the pituitary (Figure 5C). Interestingly, we did for predicted targets of ubiquitously expressed KO mice (Figure 5C). No seed enrichment was observed we performed bioinformatic analysis and found that mRNAs car-genes of miRNAs were expected to be upregulated in miRNA loss-ly reduced, suggesting that these genes may be relevant for miR-confirmed that mRNA levels of Grem1 and Pitx1 were strongly reduced in the pituitary (Figure 5B). Direct target confirmed derepression of several predicted miR-7 target candidates (Supplemental Figure 4, C and D). Together, these results reveal a tissue-specific regulation of direct and secondary miR-7 targets in the pituitary, with strong effects on upstream regulators of prostaglandin and BMP4 signaling. miR-7 targets Ptgfrn and Glg1 regulate expression and secretion of pituitary gonadotropins. To investigate the role of the identified miR-7 target genes in pituitary function, we undertook an unbiased approach and silenced the 55 most regulated miR-7 targets using smart-pool siRNAs in the aT3 and LbT2 pituitary cell lines and analyzed the effect on expression levels of Fshb and Cga. We found that silencing of both Ptgfrn and Glg1 induced hormone transcription, indicating that these genes may act as negative regulators of pituitary hormone transcription (Supplemental Figure 5, A and B). The 3’ UTRs of Ptgfrn and Glg1 contain 1 or 2 7mer miRNA-recognition sequences for miR-7, respectively, suggesting they are direct targets of miR-7. To confirm this, we cloned the WT or mutated 3’ UTRs of Ptgfrn and Glg1 into luciferase reporter constructs, performed luciferase assays, and demonstrated that both Ptgfrn and Glg1 were direct target genes of miR-7 (Figure 6, A and B). Next, we validated the findings from the siRNA screen in a separate experiment and detected a more than 1.5-fold upregulation of Fshb when Ptgfrn or Glg1 was silenced by RNA interference (Figure 6, C and D). In addition, knockdown of Ptgfrn increased Lhb expression by 1.4-fold in LbT2 cells, while Cga was modestly regulated (Figure 6C). In contrast, overexpression of Ptgfrn or Glg1 downregulated expression of Fshb, Lhb, and Cga (Figure 6E).

PTGFRN is known to antagonize the physiological action of prostaglandin F2α (PGF2α), pharmacologically also termed dinopro, on its cognate receptor, PTGFR (38, 39). Activation of PTGFR by dinopro is coupled to stimulatory Gq/G11-type G proteins, which leads to an increase in intracellular calcium levels (41). To investigate a potential mechanistic role of PGF2α signaling in gonadotroph cells, we tested whether activation of PTGFR would induce the expression of pituitary hormones in LbT2 cells following treatment with 10 nM dinopro. Indeed, administration of dinopro resulted in increased expression levels of both gonadotropin genes, Fshb and Lhb, after 4 and 8 hours (Supplemental Figure 6, A and B). We then tested whether reduced activity of PTGFRN, the negative regulator of PTGFR, would further enhance the observed pharmacological effects of PGF2α on pituitary hormone expression. To this end, we silenced Ptgfrn in gonadotroph cells and treated them with dinopro for 8 hours. We found that loss of Ptgfrn further amplified the dinopro-dependent induction of Fshb and Lhb (Figure 6, F and G), underscoring the specificity of a PGF2α/PTGFRN/PTGFR axis to regulate hormone expression. In addition, silencing of Ptgfrn resulted in increased secretion of LH in LbT2 cells (Figure 6H).

In addition to the negative regulation of gonadotropin production by PTGFRN, BMP signaling has also been shown to interfere with hormone production in pituitary gonadotrophs (42, 43). Given

Figure 7. Model illustrating the molecular pathways by which miR-7a regulates gonadotropic hormone secretion in the pituitary.
that the BMP4 antagonist Greml1 was strongly reduced in mir-7a2 KO mice, we first tested to determine whether Greml1 overexpression would increase gonadotroph expression and detected a modest, but significant, increase in Fshb, Lhb, and Cga mRNA levels (Supplemental Figure 6C). In addition, we treated cells exogenously with recombinant GREML1 and analyzed its effect on hormone production. Interestingly, we detected an induction of hormone transcription that was comparable to that of dinoprostone (Figure 6I). Most strikingly, when dinoprostone and GREML1 were coadministered, we measured an additional upregulation of hormone expression (Figure 6I). Mechanistically, GREML1 inhibited BMP4-induced phosphorylation of SMAD1 in Lbt2 cells when these cells were pretreated with recombinant GREML1 (Figure 6J). Moreover, we measured an additional upregulation of hormone expression that was comparable to that of dinoprostone (Figure 6I). The results presented in this study highlight the role of miR-7a2 in the regulation of pituitary function and the development of hypogonadotropic hypogonadism, are often accompanied by a loss of smell, and are warranted to explore this possibility. Cases of Kallmann’s syndrome, a form of human hypogonadotropic hypogonadism, are often accompanied by a loss of smell, termed anosmia, due to defective migration of GnRH neurons from the olfactory placode into the hypothalamus during development and the subsequent lack of GnRH release to the pituitary, leading to hypopituitarism (47). Our results show that the hypogonadotropic hypogonadism in mir-7a2 KO mice is highly unlikely to be causally linked to mir-7 function in hypothalamic neurons because (a) absolute quantification of mir-7 levels demonstrates that mir-7a expression is 10-fold lower in hypothalamus as compared with pituitary and therefore, based on mir-7b levels, in a range where no phenotype and target gene regulation is expected; (b) hypothalamic GnRH expression levels are indistinguishable between mir-7a2 KO and control littermates; (c) mir-7a2 KO mice show unaltered olfaction and normal expression of hypothalamic genes that are known to be involved in hypothalamic-dependent hypopituitarism; and (d) expression of the GnRH receptor in the pituitary, which is downregulated in animal models of GnRH deficiency, is expressed at similar levels in mir-7a2 KO and WT control mice (48). In addition, mir-7 is not among the 53 miRNAs that are enriched in GnRH neurons compared with the surrounding non-GnRH cells (19), further suggesting that mir-7 is unlikely to have a specific function in these neurons.

We have previously shown that loss of mir-7a2 in pancreatic β cells leads to increased β cell function and enhanced glucose-stimulated insulin secretion (22). Interestingly, ablation of mir-7a2 in the pituitary leads to impaired gonadotroph cell function and hormone secretion. These opposing phenotypes, elicited by a loss of the same miRNA in 2 different neuroendocrine cell types, suggest that mir-7a regulates different networks in a tissue-specific and context-dependent manner. The molecular mechanism for this observation is currently elusive, but will likely be due to different miRNA and target abundances and synergistic regulation of target genes by differentially expressed miRNAs as well as diverse occupancy of transcripts by RNA-binding proteins, which may influence binding of miRNAs to their target transcripts, thereby regulating the susceptibility of miRNA regulation.

The mir-7 family has previously been implicated in organ differentiation and development (22). Our results showing already reduced gonad sizes and dysregulated expression of FSH and LH as well as direct and indirect mir-7 targets in prepubertal mice indicate that a developmental defect contributes to the hypogonadotropic phenotype observed in mice with constitutive genetic ablation of mir-7a2. However, our data obtained from temporally induced mir-7a2 ablation in postdevelopmental adult mice provide genetic evidence that impaired gonadotroph cell functions cannot solely be ascribed to a developmental defect and demonstrate a direct role of mir-7a in the maintenance of pituitary gonadotroph function in adult animals. Mechanistically, we identify the negative regulator of Pgf2α receptor, Ptgfrn, and Ggl1 as relevant direct target genes of mir-7a that negatively regulate gonadotropin production and secretion. While only these targets could be linked to gonadotropin regulation in our pituitary cell models, we cannot rule out that other mir-7 targets also contribute to the defect in gonadotroph development and function of mir-7a2 KO mice. The physiological effects of prostaglandins on pituitary gonadotrophs have not been fully elucidated and have been in part discussed controversially. While it is accepted that prostaglandins, particularly PGE1, act on the anterior pituitary and can induce the formation of
cyclic AMP, a direct effect on LH and FSH could not be established (49). In the case of PGF2α, known as dinoprost, however, existing data suggest that PGF2α treatment may regulate LH stores and release (50–52). Our data now demonstrate that in pituitary gonadotroph cell lines, PGF2α/dinoprost increases gonadotropin production and that the direct mir-7a target PTGFRN acts as a negative modulator in a PGF2α/PTGFRN/PTGFR regulatory circuit.

In addition, we here describe the inhibitory role of the mir-7a target GLG1 in gonadotroph cell functions. While the precise physiological functions of GLG1 in pituitary hormone homeostasis are unknown, our data suggest that GLG1 negatively affects expression of the BMP antagonist GREM1. BMP4 plays a crucial role in the formation of the anterior pituitary by regulating diverse cellular responses, such as cell differentiation, migration, adhesion, and proliferation (53), but also has been described as inhibiting FSH production (42, 54). BMPs activate BMP receptors, which in turn recruit and phosphorylate SMAD proteins that translocate to the nucleus to repress Fshb subunit gene expression. Furthermore, BMPs can antagonize the activin-mediated effects known for stimulating FSH release (42). We propose that in mir-7a2 KO mice, the drastic reduction in GREM1 levels is partly mediated by dysinhibition of its negative regulator and mir-7 target GLG1, leading to derepression of BMP4 and, consequently, increased BMP activity, which affects pituitary developmental defects and impairs gonadotroph function in adult mice (Figure 7). Indeed, in this context, we demonstrate that GREM1 acts as a BMP4 antagonist to reduce phosphorylation of SMAD proteins in pituitary LbT2 cells.

In summary, our data reveal mir-7a2 as a crucial regulator of pituitary gonadotroph cell function and describe how the loss of mir-7a2 leads to hypogonadotropic hypogonadism. Mutations in the mir-7a2 gene and within the binding sites in the 3′ UTRs of its most regulated targets should be considered as potential genetic causes for hypogonadotropic hypogonadism in humans. Our findings also characterize PTGFRN and GLG1 as two novel target genes of mir-7 that negatively regulate gonadotropin production. Pharmacological stimulation of mir-7 activity and/or direct inhibition of its targets PTGFRN and GLG1 may provide a basis to develop strategies to improve pituitary function in human disease.

Methods

Animal husbandry and mouse strains. All mice were on a pure C57BL/6N background. Mice were housed in a pathogen-free animal facility at the Institute of Molecular Health Sciences at ETH Zurich. The animals were maintained in a temperature- and humidity-controlled room on a 12-hour light/12-hour dark cycle (lights on from 6 am to 6 pm). Mice were fed a standard laboratory chow diet. Generation of mir-7b KO mice was described previously (22).

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Library preparation was as follows: the quality of the isolated RNA was determined with a Qubit (1.0) Fluorometer (Life Technologies) and a Bioanalyzer 2100 (Agilent). Only those samples with a 260/280 nm ratio between 1.8 and 2.1 and a 28S/18S ratio within 1.5 and 2.0 were further processed. The TruSeq RNA Sample Prep Kit v2 (Illumina) was used in the succeeding steps. Briefly, total RNA samples (100–1000 ng) were poly-A enriched and then reverse transcribed into double-strand cDNA. The cDNA samples were fragmented, end repaired, and polyadenylated before ligation of TruSeq adapters containing the index sequence for multiplex sequencing. Multiplexing fragments containing TruSeq adapters on both ends were selectively enriched with PCR. The quality and quantity of the enriched libraries were validated using Qubit (1.0) Fluorometer and the Caliper GX LabChip GX (Caliper Life Sciences Inc.). The product is a smear with an average fragment size of approximately
Cluster generation and sequencing. The TruSeq PE Cluster Kit v3-cBot-HS or TruSeq SR Cluster Kit v3-cBot-HS (Illumina Inc.) was used for cluster generation using 10 pM of pooled normalized libraries on the cBOT. Sequencing was performed on the Illumina HiSeq 2000 paired end at 2 × 101 bp or single-end 100 bp using the TruSeq SBS Kit v3-HS (Illumina Inc.).

Data analysis. RNA-Seq reads were quality checked with FastQC (Babraham Bioinformatics), which computes various quality metrics for raw reads. Reads were aligned to the genome and transcriptome with TopHat v 1.3.3 (https://ccb.jhu.edu/software/tophat/manual.shtml). Before mapping, the low-quality ends of the reads were clipped (3 bases from the read start and 10 bases from the read end). TopHat was run with default options. The fragment length parameter was set to 100 bases, with an SD of 100 bases. Based on these alignments, the distribution of the reads across genomic features was assessed. Isoform expression was quantified with the RSEM algorithm (http://www.biomedcentral.com/1471-2105/12/323; BMC Bioinformatics) with the option for estimation of the read start position distribution turned on. For seed enrichment analysis, only genes with FPKM above 1.0 were considered. For cumulative distribution function calculations, log 2 fold-change values were corrected for 3’ UTR length biases (55).

RNA isolation and real-time pPCR. RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. RNA was subjected to DNaseI treatment with the DNA-free kit (Ambion) when necessary. RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed with a LC480 II Lightcycler (Roche) and SYBR Fast Universal Mastermix (Kapa). Results were normalized to 36B4 mRNA levels. miRNA levels were measured using the TaqMan microRNA Assay Kit (Applied Biosystems), and the results were normalized to sno-202 levels. For absolute quantification, synthesized mature miRNAs were used (Sigma-Aldrich).

 Luciferase assays. LBT2 or aT3 cells were cultured in 24-well plates and transfected with 100 ng of pmiRGO reporters. Cells were assayed 48 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega). Results were normalized to the Renilla luciferase control contained in pmiRGO and expressed relative to the average value of the controls.

GnRH stimulation test. Female nullipara mice were given an intraperitoneal injection of Buserelin (GnRH agonist, Sigma-Aldrich; 0.01 μg/μl) in 200 μl of vehicle (PBS) or vehicle alone and blood was collected after 15 minutes for FSH and LH measurements.

Hormone measurements. Testosterone and estradiol were quantified using the ELISA Kit from Alpco following the manufacturer’s instructions. For intratesticular testosterone analysis, lipid extraction was performed using 2x diethylether. Serum LH and FSH concentrations were measured using immunofluorometric assays as described previously (55). For measuring intratesticular and serum testosterone, both sera and tissue homogenates were extracted twice with 2 ml of diethyl ether, followed by conventional RIA (56).

Olfaction behavioral test. A buried food test was conducted as previously described (57). Nine-week-old mice were fasted for 24 hours and then were allowed to acclimate in the experimental cage for 5 minutes prior to the test. Mice were transferred to an empty cage, while a 1.5 g pellet of chow was buried 1 cm below the bedding in the experimental cage. Mice were reintroduced into the experimental cage, and the elapsed time was recorded once they uncovered the chow pellet.

Recombinant adenovirus. Mouse miR-7a2–expressing vectors were generated by PCR amplification of a 250-bp fragment spanning the pre-miR-7a2 genomic sequences. Fragments were cloned at Xhol sites of pAD5 for adenovirus production (Viraquset). The miR-7a adenoviruses expressed GFP from an independent promoter, and the control adenovirus (Ad-GFP) was identical except that it lacked the miRNA. Expression was confirmed by TagMan qPCR.

Chemical compounds. BMP4, dinoprost, and GnRH were purchased from Sigma-Aldrich. Grem1 was obtained from R&D Systems.

Cell lines. Pituitary cell lines aT3 and L β T2 were provided by P. Mellon (UCD, Davis, California, USA) (58, 59).

Antibodies. Antibodies recognizing mouse FSHb and LHb were purchased from Sigma-Aldrich. Grem1 was obtained from R & D Systems.

Accession codes. All raw RNA-Seq data were deposited in the European Nucleotide Archive (ENA PRJEB12612; http://www.ebi.ac.uk/ena).

Author contributions
KA designed and performed most of the experiments, analyzed and interpreted data, and wrote the manuscript. ML generated constitutive and conditional mir-7a1, mir-7a2, and mir-7b KO mice. MPL contributed to in vivo and cell culture experiments and immunohistochemistry. JK performed FSH and LH measurements. RD performed seed enrichment analysis. EG performed hypothalamus immunohistochemistry. YY contributed to cell culture experiments. JK performed FSH and LH measurements. RD performed seed enrichment analysis. EG performed hypothalamus immunohistochemistry. YY contributed to cell culture experiments. TR performed blastocyst injections. MS conceived and supervised the project, analyzed and interpreted data, and wrote the manuscript. All authors read and commented on the manuscript.

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