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 gonadotropin 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 gonadotropin 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 GnR 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, PROKR2/PROKR2, CHD7, HS6ST1, WDR11, FEZF1, NTF2, and SEMA3A) in genes controlling GnRH secretion and action (GNRHR, GPR54 [also known as KISS1], TAC3/TACR3, LEP/LEPR, and GNRHR, 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 (13). Thus, disordered miRNA activity has been linked 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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miRNAs (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 to be a key player in normal physiology and disease. We identify miR-7a2 as a positive regulator of FSH and LH synthesis by controlling a network involving the β subunit of Dicer1 in GnRH neurons and gonadotrophs (21), resulting in hypogonadism and infertility. The 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 thread-like atrophied uteri compared with WT or mir-7a1 KO mice (Figure 2, A 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 \( \text{mir-7a1} \) or \( \text{mir-7a2} \). Interestingly, total miR-7a expression was reduced by approximately 80% in \( \text{mir-7a2} \)-deficient mice, while miR-7a levels appeared to be unchanged in mice lacking \( \text{mir-7a1} \) when compared with WT littermate animals (Supplemental Figure 2, C–F). Expression of miR-7b was unchanged in \( \text{mir-7a1} \)– and \( \text{mir-7a2} \)–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 \( \text{mir-7a2} \) 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 \( \text{mir-7a2} \) KO mice. We measured a dramatic decrease of gonadotropin expression with a reduction of \( \text{Fshb} \) mRNA by 86% (FDR = 5.94 × 10^{-52}) and \( \text{Lhb} \) mRNA by 78% (FDR = 1.26 × 10^{-82}) in \( \text{mir-7a2} \)-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 (\( \text{Cga} \)) and prolactin (\( \text{Prl} \)) mRNAs was reduced, while the expression of the GnRH receptor (\( \text{Gnrhr} \)) and other hormones of the anterior pituitary, including thyroid stimulating hormone \( \beta \) (\( \text{Tshb} \)), growth hormone (\( \text{Gh} \)), and pro-opiomelanocortin \(-\alpha \) (\( \text{Pomc} \)), remained unchanged. Since lack of \( \text{Prl} \) 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 \( \text{mir-7a2} \) 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, n = 4, mir-7a2 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-7a 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 (Tca2) were significantly increased in hypothalami of mir-7a2 KO mice, which corroborates findings that Kiss1 and Tca2 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...
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-
**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) or Glg1 (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 Glg1 (D) (n = 4). (E) Relative expression levels of gonadotropin genes, Cga, Fshb, and Lhb in cells overexpressing Ptgfrn (gray bars) or Glg1 (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).

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

CRE (36) to allow tamoxifen-inducible global ablation of mir-7a2 (referred to as UBC-Cre × mir-7a2fl/fl). We treated 6-week-old male UBC-Cre × mir-7a2fl/fl or mir-7a2fl/fl mice with tamoxifen or vehicle 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-7a2fl/fl mice as compared with control mir-7a2fl/fl mice or UBC-Cre × mir-7a2fl/fl mice that received only vehicle (Figure 4B).

**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) or Glg1 (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 Glg1 (D) (n = 4). (E) Relative expression levels of gonadotropin genes, Cga, Fshb, and Lhb in cells overexpressing Ptgfrn (gray bars) or Glg1 (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).
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**Figure 7. Model illustrating the molecular pathways by which miR-7a regulates gonadotropic hormone secretion in the pituitary.**

Tamoxifen-treated UBC-Cre × mir-7a2fl/fl mice as compared with control mir-7a2fl/fl mice or UBC-Cre × mir-7a2fl/fl mice that received only vehicle (Figure 4G). Moreover, testicular expression of the Leydig cell marker CYP17A1 was reduced by 35% (Figure 4H). Together, these data demonstrate that acute ablation of mir-7a2 in adult mice is sufficient to recapitulate hypopituitarism and hypogonadism observed in constitutive mir-7a2 KO mice.

**Dysregulated pituitary gene expression and miR-7a target depression in mir-7a2 ablated mice.** To gain insights into the molecular mechanisms of mir-7a2-dependent pituitary dysfunction, we performed RNA-Seq of mir-7a2 KO and WT littermate mice and analyzed the expression of genes known to be involved in pituitary development, gonadotrope function, and/or in LH or FSH subunit gene expression. Established key factors of early pituitary function (Gnrhr, Acvr1, Acvr1b, Acvr1c, Acvr2a, Smad1, Smad2, Smad4-7, Smad9, and Foxl2) (Supplemental Figure 4A) and development (Gli2, Gli3, Nr5a1, Pitx1, Pitx2, Isil, Tbx19, Pouf1, Prop1, Hes1) remained unchanged between constitutive mir-7a2 KO and control mice, while Grem1, coding for gremlin 1, a secreted BMP4 antagonist (37), the zinc finger protein Gli3, and the LIM homeobox protein 4 (Lhx4) were strongly reduced in mir-7a2 KO mice (Figure 5A). In tamoxifen-induced UBC-Cre × mir-7a2fl/fl mice, we confirmed that mRNA levels of Grem1, Gli3, and Lhx4 were similarly reduced, suggesting that these genes may be relevant for mir-7a2-dependent effects in the pituitary (Figure 5B). Direct target genes of miRNAs were expected to be upregulated in miRNA loss-of-function models. To identify potential direct targets of miR-7, we performed bioinformatic analysis and found that miRNAs carrying a miR-7 response element were preferentially upregulated in mir-7a2 KO mice (Figure 5C). No seed enrichment was observed for predicted targets of ubiquitously expressed mir-16, which is also abundant in the pituitary (Figure 5C). Interestingly, we did not measure a significant upregulation (except Pfn2 and Prkcb) of the strongest mir-7 targets previously described in pancreatic islets to regulate insulin secretion (22) (Supplemental Figure 4B), indicating a tissue-specific regulation of mir-7 target genes. Using a cut-off of 1.3-fold upregulation, RNA-Seq revealed 55 potential direct target transcripts of miR-7. We analyzed expression of these genes in tamoxifen-induced UBC-Cre × mir-7a2fl/fl mice and confirmed derepression of several predicted miR-7 targets (Figure 5D). Notably, Ptgfrn, the negative regulator of prostaglandin receptor F2α (38, 39), was as strongly upregulated as the established miR-7 target Cplx2 (Ptgfrn, 1.41-fold; Cplx2, 1.32-fold) (Figure 5D). We further validated by quantitative PCR (qPCR) that the mRNA transcripts of Ptgfrn and golgi glycoprotein 1 (Glg1), a member of the cysteine-rich fibroblast growth factor receptor family (40), were increased in the pituitaries of mir-7a2 KO mice (Figure 5E). To test whether predicted mir-7 targets were repressed by increased miR-7 levels, we overexpressed mir-7a2 using a recombinant adenovirus (Ad-miR-7a2) in 2 gonadotroph cell lines, aT3 and LbT2, and observed significant repression 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-7a (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 dinoprost, on its cognate receptor, PTGFR (38, 39). Activation of PTGFR by dinoprost 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 dinoprost. Indeed, administration of dinoprost 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 dinoprost for 8 hours. We found that loss of Ptgfrn further amplified the dinoprost-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...
that the BMP4 antagonist Grem1 was strongly reduced in miR-7a2 KO mice, we first tested to determine whether Grem1 overexpression would increase gonadotroph expression and detected a modest, but significant, increase in Fshb mRNA levels (Supplemental Figure 6C). In addition, we treated cells exogenously with recombinant GREM1 and analyzed its effect on hormone production. Interestingly, we detected an induction of hormone transcription that was comparable to that of dinoprost (Figure 6I).

Most strikingly, when dinoprost and GREM1 were coadministered, we measured an additional upregulation of hormone expression (Figure 6I). Mechanistically, GREM1 inhibited BMP4-induced phosphorylation of SMAD1 in LbT2 cells when these cells were pretreated with recombinant GREM1 (Figure 6I). Moreover, we observed that GREM1 was upregulated in cells depleted in GLG1, while being decreased in cells overexpressing GLG1, indicating that the direct target GLG1 was necessary for repression of GREM1 levels (Supplemental Figure 6, D and E). Together, these data identify Ptgfrn and Glgl as direct targets of miR-7 that negatively regulate pituitary hormone transcription and secretion through regulation of both prostaglandin- and BMP-dependent signaling pathways.

Discussion

The results presented in this study highlight the role of miR-7a2 in the regulation of pituitary function and the development of hypogonadotropic hypogonadism and infertility. Our data demonstrate that miR-7a2 is the predominant member of the miR-7 family in the pituitary and that constitutive genetic ablation of miR-7a2, but not miR-7a1, is sufficient to induce hypopituitarism and infertility. Deletion of miR-7a2 leads to decreased expression and profoundly reduced plasma levels of the pituitary gonadotropins FSH and LH, resulting in hypogonadism in both male and female mir-7a2 KO mice, as indicated by decreased steroidogenic gene expression and reduced sex steroid levels and gonadal sizes. We also measured decreased expression levels of prolactin expression in constitutional and inducible mir-7a2 KO mice. This finding warrants further investigation and suggests that miR-7a2 either is also essential for lactotroph cell function in the pituitary or represses the function of dopaminergic neurons in the arcuate nucleus of the hypothalamus that are known to inhibit prolactin secretion (44). Interestingly, aged male and female mir-7a2 KO mice have increased fat mass, a phenotype that may be caused by a long-lasting and physiologically relevant depletion of gonadal steroid hormones (45). However, we cannot exclude that miR-7a2 also influences body weight and energy expenditure in a partially sex steroid-independent manner, for example, through neurons in the hypothalamus, where miR-7a2 expression has also been reported (46). The observed hypogonadism stems from a dysfunction of upstream components of the HPG axis, since ovarian function and ovulation are functionally rescued when pituitary hormones are replaced in mir-7a2 KO mice in a superovulation test. This phenotype is reminiscent of the disease pathology of human hypogonadotropic hypogonadism, prompting the question of whether some cases of congenital IHH of pituitary origin with as-yet-unknown genetic causes may be attributed to abnormal mir-7a2 levels. Further investigations 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-7 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 Glgl 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 dinoprostone, 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/dinoprostone 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 development defect 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-7a1 KO and mir-7a2 KO mice was described previously (22). UBC-Cre mice (Tg[UBC-cre/ERT2]1Ejb) were purchased from Jackson Laboratories.

Generation of mir-7b KO mice. A 15.7-kb fragment encompassing mmu-mir-7b was recombined into a minimal vector using homology arms-containing primers according to the protocol provided by Gene-Bridges (http://www.genebridges.com; primers listed in Supplemental Table 2). The mir-7b gene was juxtaposed with a loxP-containing neo' cassette 97 bp upstream of the miRNA precursor sequence. Following expression of the Cre in bacteria, the neo' cassette was removed and a second Frt/loxP/neo' cassette was introduced 498 bp downstream of the mir-7b gene (Supplemental Figure 1A). Upon linearization, the targeting vector was electroporated in C57BL/6N embryonic stem cells. Upon selection with G418 (Invitrogen), clones were picked, expanded, and verified for integration by Southern blotting. Positive clones were microinjected in BALB/c blastocytes and subsequently transferred into pseudopregnant females to generate chimeric offspring. Chimeras were then bred with C57BL/6 females to produce heterozygotes, which were crossed with Flipase mice to delete the neo' cassette. Mice were bred with B6.C-Tg(CMV-cre)1Cgn/j mice (Jackson Laboratory) for deleting mir-7b. Genotyping of mir-7b KO mice was performed by PCR using primers listed in Supplemental Table 2.

DEXA. DEXA measurements of fat and lean body mass were performed as whole animal scans using an animal CT-Scanner (LaTheta) at 1-mm intervals from the head to the base of the tail. Images were analyzed using the LaTheta software.

Immunostainings. Mice were perfused with 4% paraformaldehyde, and organs were postfixed overnight at 4°C and embedded in paraffin. Microtome sections were subjected to antigen retrieval in a pressure oven in 10 mM sodium citrate, pH 6.0, for 10 minutes at 95°C. Sections were permeabilized and blocked in PBS containing 0.1% Triton X-100, 1% BSA, and 5% goat serum. Primary antibody binding was performed overnight at 4°C, while secondary antibody incubation was carried out at room temperature for 1 hour.

For morphometric analysis of pituitaries, 5-μm sections were cut and 5 sections from each pituitary were stained with anti-LH or anti-FSH antibodies and Hoechst dye to stain nuclei. Sections were scanned using a 20× objective of a Zeiss Apotome microscope. The number of FSH- or LH-positive cells compared to total cell number was determined using CellProfiler software. For morphometrical quantification of testicular Leydig cells, 4-μm paraffin sections were cut and 5 sections per testis were stained with anti-CYP17A1 antibody and Hoechst dye to stain nuclei. Sections were scanned using a 20× objective of the Panoramic 250 Slide Scanner (3D Histech). The area fractions of CYP17A1-positive cells relative to total cell area were determined using ImageJ (Fiji, NIH) software.

Western blots. Cells were lysed in RIPA buffer containing protease inhibitors (Roche) and HALT phosphatase inhibitors (Pierce). Protein concentrations were measured by the Bradford method. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blocked in 5% milk/TBS-T or 5% BSA/TBS-T for 1 hour. Membranes were incubated with appropriate antibodies overnight at 4°C. Membranes were exposed to secondary antibodies for 1 hour at room temperature and developed using ECL Western Blotting Substrate.

Illumina RNA-Seq and analysis. 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, endrepaired, 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...
utes prior to the test. Mice were transferred to an empty cage, while a
and then were allowed to acclimate in the experimental cage for 5 min-
previously described (57). Nine-week-old mice were fasted for 24 hours
instructions. For intratesticular testosterone analysis, lipid extraction
both sera and tissue homogenates were extracted twice with 2 ml of
previously (55). For measuring intratesticular and serum testosterone,
raw reads. Reads were aligned to the genome and transcriptome with
on the cBOT. Sequencing was performed on the Illumina HiSeq 2000
v3-HS (Illumina Inc.).
Data analysis. RNA-Seq reads were quality checked with FastQC
Babraham Bioinformatics), which computes various quality metrics for
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. Iso-
form expression was quantified with the RSEM algorithm (http://www.
bimedcentral.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,
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 proto-
col. 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 Biosys-
tems). 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 pmirGLO 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 pmirGLO 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 col-
lected after 15 minutes for FSH and LH measurements.
Hormone measurements. Testosterone and estradiol were quan-
tified 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 concentra-
tions 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 pre-
viously described (57). Nine-week-old mice were fasted for 24 hours
and then were allowed to acclimate in the experimental cage for 5 min-
utes 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 experi-
mental 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 pAda5 for adenovirus production (Viraqwest). The miR-7a adenovi-
ruses 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 pur-
chased 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
obtained from A. Parlow (National Hormone and Peptide Program,
Harbor-UCLA Medical Center, Torrance, California, USA). TUBB3
(catalog 4466), β-actin (catalog 4970), SMAD1 (catalog 6944), and
phospho-SMAD1/5/9 (catalog 13820) were purchased from Cell Sig-
naling Technology. CYP17A1 antibody was purchased from Santa Cruz
Biotechnology Inc. (catalog sc-46081).
Primer sequences. See Supplemental Table 3.
Statistics. For all experiments, data are shown as mean ± SD if
not stated otherwise. To determine statistical significance between 2
groups, 2-tailed Student’s t test was used. For multiple comparisons,
1-way ANOVA followed by Tukey’s post-test was used. A P value
of less than 0.05 was considered statistically significant. Statistical anal-
ysis was performed using GraphPad Prism 4 (GraphPad software).
Study approval. All animal experiments were approved by the Kan-
tonal Veterinäramt Zürich, Zurich, Switzerland.
Accession codes. All raw RNA-Seq data were deposited in the Euro-
pean 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 immu-
nohistochemistry. JK performed FSH and LH measurements. RD
performed seed enrichment analysis. EG performed hypothalamus
immunohistochemistry. YY contributed to cell culture experiments.
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