1   **MicroRNA-202 *(miR-202)* controls female fecundity by**

2   **regulating medaka oogenesis**

3

4   **Short title**

5   Fish oogenesis and microARN-202

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16   Key words

17   Fish fertility, ovary, gonads, microRNAs, CRSIPR/Cas9, quantitative image analyses,

18   microarray

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ABSTRACT

Female gamete production relies on coordinated molecular and cellular processes that occur in the ovary throughout oogenesis. In fish, as in other vertebrates, these processes have been extensively studied both in terms of endocrine/paracrine regulation and protein expression and activity. The role of small non-coding RNAs in the regulation of animal reproduction remains however largely unknown and poorly investigated, despite a growing interest for the importance of miRNAs in a wide variety of biological processes. Here, we analyzed the role of *miR-202*, a miRNA predominantly expressed in male and female gonads in several vertebrate species. We studied its expression in the medaka ovary and generated a mutant line (using CRISPR/Cas9 genome engineering) to determine its importance for reproductive success with special interest for egg production. Our results show that miR-202-5p is the biologically active form of the miRNA and that it is expressed in granulosa cells and in the unfertilized egg. The knock out (KO) of *miR-202* resulted in a strong phenotype both in terms of number and quality of eggs produced. Mutant females exhibited either no egg production or produced a drastically reduced number of eggs that could not be fertilized, ultimately leading to no reproductive success. We quantified the size distribution of the oocytes in the ovary of KO females and performed a genome-wide transcriptomic analysis approach to identified dysregulated molecular pathways. Together, cellular and molecular analyses indicate that lack of *miR-202* impairs the early steps of oogenesis/folliculogenesis and decreases the number of large (*i.e.* vitellogenic) follicles, ultimately leading to dramatically reduced female fecundity. This study sheds new light on the regulatory mechanisms that control the early steps of follicular development and provides the first

1   *in vivo* functional evidence that an ovarian-predominant microRNA may have a major

2   role in female reproduction.

Author summary

4   The role of small non-coding RNAs in the regulation of animal reproduction remains

5   poorly investigated, despite a growing interest for the importance of miRNAs in a wide

6   variety of biological processes. Here, we analyzed the role of *miR-202*, a miRNA

7   predominantly expressed in gonads in vertebrate. We studied its expression in the

8   medaka ovary and knocked out the *miR-202* genes to study its importance for

9   reproductive success. We showed that the lack of *miR-202* results in the sterility of both

10   females and males. In particular, it lead to a drastic reduction of both the number and

11   the quality of eggs produced by females. Mutant females exhibited either no egg

12   production or produced a drastically reduced number of eggs that could not be

13   fertilized, ultimately leading to no reproductive success. Quantitative histological and

14   molecular analyses indicated that *miR-202* KO impairs oocyte development and is also

15   associated with the dysregulation of many genes that are critical for reproduction. This

16   study sheds new light on the regulatory mechanisms that control oogenesis and

17   provides the first *in vivo* functional evidence that an ovarian-predominant microRNA

18   may have a major role in female reproduction.

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1 INTRODUCTION

2 In fish, female fecundity is tightly linked to the proper completion of oogenesis in the ovary,

3 whereby undifferentiated germinal stem cells undergo meiosis, a dramatic increase in size

4 and ultimately form the eggs (1). Such an important differentiation processes requires

5 coordinated interactions between the oocyte and the surrounding somatic cells (granulosa

6 and theca cells), which together form the ovarian follicles (2). While the role of endocrine

7 and intra-ovarian factors in these processes has been extensively studied, the regulation by

8 the small non-coding RNAs (microRNAs) has received far less attention (3)(4).

9 MicroRNAs (approximately 22 nucleotides in length) play many different biological

10 functions through the post-transcriptional regulation of protein-coding genes. In

11 mammals, most researches that investigate the role of miRNAs in the ovary have mainly

12 relied on *in vitro* analyses. Numerous studies that have highlighted the role played by

13 miRNAs in ovarian development and oogenesis, as shown for miR-224 (5)(6)(7). In

14 contrast, data documenting the role played by miRNAs in fish reproduction remain

15 scarce and mainly rely on expression studies. In zebrafish, the expression and regulation

16 of specific miRNAs has been associated with vitellogenesis and follicular development

17 (8). More specifically, expression and regulation of miR-17 and miR-430b in the ovary

18 suggest a role in follicular development and oocyte maturation. Large scale differential

19 transcriptomic analyses were also performed during sex differentiation, gonadal

20 development or vitellogenesis in various fish species, including rainbow trout (9),

21 zebrafish (10)(11), Atlantic halibut (12), yellow cat fish (13), Tilapia (14)(15) and

22 medaka (16)(17). The identification of differentially expressed miRNAs through the

23 different stages of the reproductive cycle is consistent with a participation of miRNAs in

4

1   oogenesis and spermatogenesis. Among the most abundantly expressed miRNA was

2   *miR-202*, a miRNA known to be predominantly expressed in gonads in several vertebrate

3   species, including frog (18), Atlantic halibut (19), human, mouse, rat (20)(21), chicken

4   (22)(23), rainbow trout (24) and medaka (25). For protein-coding genes, such a

5   predominant expression in gonads is usually associated with a major role in

6   reproduction, as illustrated by many maternal-effect genes (26). There is however no

7   functional evidence of the major role in fish reproduction of either *miR-202* or any other

8   gonad-predominant miRNAs, although some recent *in vitro* studies reported a role for

9   *miR-202* in mouse during spermatogenesis (27). Furthermore, it is currently unknown if

10   such a critical feature, observed for protein-coding genes, would also be observed for

11   miRNAs that are usually seen only as fine modulators of gene regulatory networks

12   rather than major regulators of biological processes (28).

13   Here, we investigated the role of *miR-202* in fish reproduction with special attention for

14   its role in the ovary. We thoroughly analyzed its cellular expression within the ovary and

15   characterized the reproductive phenotype of *miR-202* knock-out (KO) fish, generated by

16   CRISPR/Cas9 genome engineering. We showed that miR-202-5p is the predominant

17   mature form expressed in granulosa cells. The lack of miR-202 resulted in a dramatic

18   decrease of fertility in both male and females. In females, both egg number and quality

19   were reduced, ultimately leading to the absence of any viable offspring. Quantitative

20   image analysis of mutant ovaries showed that *miR-202* impairs the early steps of

21   oogenesis/folliculogenesis. Along with this drastic phenotype, we observed the

22   dysregulation of several key genes known for their role throughout oogenesis, as well as

23   the dysregulation of genes, the role of which is not yet known in the ovary. Overall, this

24   study sheds new light on the regulatory mechanisms that control the early steps of

1 follicular development and provides the first *in vivo* functional evidence that an ovarian-

2 predominant microRNA has a major role in female reproduction.

3 **RESULTS**

4 **MiR-202-5p is highly expressed in granulosa cells**

5 The medaka miR-202 gene harbors two mature miRNAs sequences, miR-202-5p and

6 miR-202-3p (Fig 1A), whose expression levels were surveyed by quantitative reverse

7 transcription TaqMan PCR (TaqManPCR) in eleven different tissues of adult fish and

8 during embryonic development. The miR-202-5p mature form was found specifically

9 expressed at high levels in both gonads (*i.e.* ovary and testis), in comparison to all other

10 tissues (Fig 1B). During embryonic development, the highest expression levels of miR-

11 202-5p were detected in non-fertilized eggs at stage 0 (st.0), thus corresponding to a

12 maternal accumulation (Fig 1C). Lower levels of miR-202-5p were detected from the

13 one-cell stage (st.2) onward. A slight increased expression was detected just before

14 hatching (st.39), likely corresponding to a zygotic transcription. The miR-202-3p mature

15 form presented similar expression profiles in adult tissues but expression levels were

16 approximately 1500 times lower in ovary and testis, in comparison to miR-202-5p an

17 undetectable in other tissues. During development, miR-202-3p also exhibited a profile

18 similar to the -5p form and could be detected in unfertilized eggs at levels 500 times

19 lower, while it could not be detected above background levels at any other

20 developmental stages (data not shown).

21 The cellular expression pattern of *miR-202* was analyzed in the ovary by fluorescent *in*

22 *situ* hybridization (FISH) on ovarian sections (Fig 2). Ovaries were dissected either from

23 juvenile females (*i.e.* before the first spawning) or from adult females (*i.e.* reproductively

6

1   active fishes). In both juvenile and adult ovaries, miR-202-5p was detected in follicles at

2   all vitellogenic and post-vitellogenic stages, in granulosa cells surrounding the oocyte

3   (Figs 2A' and 2B', arrowhead), but not in theca cells (Figs 2A' and 2B'', arrow). To

4   visualize *sox9*-expressing cells in the germinal cradle, we performed FISH on ovaries

5   from adult transgenic medaka *Tg*(*sox9b*::EGFP). MiR-202-5p was detected in a subset of

6   GFP-positive cells surrounding early pre-vitellogenic follicles (primordial follicles, Fig

7   2B'''). FISH performed with a specific MiR-202-3p probe (Fig 2C, D) or with a scramble

8   control probe (S1 Fig) revealed no detectable signal above background.

9   ### *MiR-202* knock-out drastically reduces female fertility

10   To determine the role of miR-202 on female reproduction, we inactivated the *miR-202*

11   gene using the CRISPR/Cas9 technology. Small insertion/deletion (INDEL) mutations

12   were inserted in the genome, in the miR-202-3p mature sequence (Fig 3A). Fishes

13   displaying the same INDEL (-7+3) were selected to establish a mutant fish line. In

14   homozygotes mutants (*miR-202-/-*), the processing of the pri-miR-202 was impaired and

15   the miR-202-5p mature form was absent (Fig 3B). The sex ratio for both heterozygous

16   and homozygous was the same as in wild-type siblings. This mutant line was used in all

17   further experiments.

18   We thoroughly analyzed the reproductive phenotype of *miR-202-/-* adult females. The

19   frequency of spawning was analyzed during ten consecutive cycles (Fig 3C). Two

20   categories of mutant females were distinguishable by different reproductive

21   phenotypes. The mildly affected females (subfertile females), which represented 85% of

22   all mutant females analyzed, displayed an irregular frequency of spawning compared to

23   wild-type females that spawned every day within one hour of the onset of the light. The

1   most strongly affected females (sterile females), which represented 15% of all mutant

2   females analyzed, spawned only once in ten days. Further analysis of the quantity of

3   spawned eggs (female fecundity) revealed that subfertile females spawned a

4   significantly reduced number of eggs (5.8 eggs per clutch on average) compared to wild-

5   type females (18.8 eggs per clutch on average, Fig 3D). In addition, the viability of

6   embryos (*i.e.* capability of eggs to be fertilized and to develop correctly) was

7   dramatically reduced when subfertile females were outcrossed with wild-type males

8   (Fig 3E). Similar results were obtained when subfertile females were mated with mutant

9   males. Conversely, when mutant males were outcrossed with wild-type females, the

10   viability of embryos was also drastically affected compared to wild-type siblings.

11   Further analysis of siblings revealed that, eggs originating from subfertile females

12   fertilized by either a wild-type or a mutant male could not be fertilized, while eggs

13   originating from wild-type females fertilized by a mutant male were fertilized but

14   arrested during the first cleavage stages of embryonic development (st.4-5, Fig 3F). The

15   overall reproductive success of mutant females was therefore reduced to 0.83 viable

16   eggs per clutch for subfertile females (*i.e.* mutant females that exhibited irregular

17   spawning, reduced fecundity and low embryonic survival) and to zero viable egg for

18   sterile females that exhibited the most severe phenotype, while wild-type female

19   exhibited an average of 15.3 viable eggs per clutch. To maintain the mutant fish line

20   (INDEL -7+3), heterozygous fishes were thus systematically outcrossed with wild-type fishes

21   at each generation (see Material and Methods). Another mutant line harboring another

22   INDEL mutation (-8) was used to confirm this reproductive phenotype but this line was

23   not used for further histological and molecular phenotyping analyses (data not shown).

1 Altogether, our results indicate that *miR-202* is required for the formation of functional

2 gametes in both female and male gonads.

3 ***MiR-202* knock-out affects the early steps of follicular growth in juvenile**

4 **females**

5 We further analyzed the role of *miR-202* during oogenesis in juvenile females (*i.e.* before

6 the first spawning). To this aim, the follicular content of wild-type and mutant ovaries

7 were analyzed and compared. For each ovary, the number and size of follicles were

8 determined on the median ovarian section. Nuclei of somatic cells, including ganulosa

9 cells surrounding each oocyte, were stained with DAPI, which allowed delineating all

10 follicles and automatically individualizing them, using a computational automatic

11 segmentation procedure (Fig 4A). The section area, the mean follicle diameter and the

12 number of follicles were determined (Fig 4B). In *miR-202-/-* ovarian sections, the mean

13 follicular diameter was significantly reduced (70 μm), as compared to wild-type ovarian

14 sections (80 μm), and the number of follicles appeared higher (500 μm) as compared to

15 wild-type (300 μm), although not significantly. These data suggest an impairment of the

16 early follicular growth in juvenile mutant ovaries.

17 The size distribution of follicles on sections was thoroughly analyzed in mutant and

18 wild-type ovaries. Follicles were classified according to their diameter into five different

19 classes (<50 μm, 50-100 μm, 100-150 μm, 150-200 μm and 200-350 μm) (Fig 4C).

20 Comparison of the resulting profiles revealed different size distributions profiles in

21 mutant and wild-type ovaries. In mutant ovarian sections, the number of small follicles

22 appeared higher compared to wild-type, with a significant increased in the 50-100 μm

23 size class, whereas the number of follicles in the larger diameter class (200-350 μm)

9

1 tended to decrease, although not significantly. This clearly suggests an important defect

2 of the early follicular growth in juvenile *miR-202-/-* females, leading to an accumulation

3 of small and medium oocytes (*i.e.* pre-vitellogenic and early-vitellogenic oocytes) to the

4 detriment of larger late-vitellogenic and maturing follicles.

5 **_MiR-202_ knock-out decreases the number of growing follicles in subfertile**

6 **adult females**

7 The role of *miR-202* during oogenesis was analyzed at the adult stage (reproductively

8 active fish), in both subfertile and sterile females (Fig 5A). Section areas, mean follicular

9 diameters and numbers of follicles were measured on median sections. The follicular

10 size distribution was also analyzed. Follicles were classified according to their diameter

11 into three different size classes based on their diameter: small (<100 μm), medium

12 (100-400 μm) and large (400-1200 μm). In sterile females, ovaries displayed a

13 significant decrease of the mean follicular diameter (170 μm) in comparison to wild-

14 type ovaries (220 μm), but similar numbers of follicles were found in both cases.

15 Consistently, ovarian sections were significantly reduced in mutant compared to wild-

16 type (Fig 5B). The analysis of the size distributions also revealed distinct profiles in

17 wild-types and mutants. The number of large follicles (>400 μm) was significantly

18 reduced in mutant, while small follicles (<100 μm) tended to accumulate, although not

19 significantly (Fig 5C). These observations suggest a strong impairment of the early steps

20 of the follicular growth in sterile females, similarly to juvenile mutant females analyzed

21 during the first reproductive cycle (see above).

22 In subfertile females, which exhibited a milder reproductive phenotype (*i.e.* occurrence

23 of spawning but a reduced number of eggs and reduced egg survival), ovarian sections

10

1   displayed a significantly reduced number of follicles. This was associated with a

2   significant reduction of ovarian section areas (Fig 5B). No modification of the mean

3   follicular diameter was however observed, suggesting that although fewer follicles were

4   engaged into growth, they were all able to reach their correct final size. This was

5   supported by the follicular size distribution profiles, which showed a significant and

6   important reduction of the number of medium-size follicles (100-400 μm in diameter)

7   in mutant compared to wilt-type, and no accumulation of small-size follicles (<100 μm

8   in diameter, Fig 5C). These data likely reflect a milder effect of the *miR-202* deficiency on

9   follicular growth in subfertile adult females compared to sterile fishes, indicating a

10 partial recovery of the phenotype at this stage.

11 **Genome-wide analysis reveals major dysregulation of key ovarian genes**

12 To get further insight into the molecular pathways that are affected in absence of miR-

13 202, we performed a genome-wide transcriptomic analysis on ovaries of wild-type and

14 *miR-202-/-* females. This analysis was conducted on ovaries from juvenile females,

15 before the first spawning, when ovaries display a more homogenous follicle population

16 (from 30 to 350 μm in diameter) compared to ovaries from reproductively active adult

17 fish (from 30 to 1100 μm in diameter, see Figs 4 and 5). We identified 52 differentially

18 expressed genes, including 11 genes that were up-regulated in mutant and 41 genes

19 down-regulated in mutant (S1 Table). The most differentially expressed genes were

20 validated by QPCR (*wnt2bb*, *wnt4a*, *klhl23*, *setd4*, *npr1b and* srgap3) along with other

21 major genes involved in fish oogenesis selected from the literature (*cyp19a1a, cyp17,*

22 *gsdf, inh, foxl2b, sycp3, foxl3, Sox9b, olvas*). Results are shown on figure 6 and are

23 described bellow.

1 **Down-regulation of known genes expressed in the somatic germ-cell supporting cells (Fig**

2 **6A)**

3 Expression analysis revealed a marked expression decrease of two genes encoding key

4 steroidogenic enzymes (*cyp19a1a* and *cyp17*). *Cyp19a1a* (also known as aromatase) is

5 an ovarian-specific steroidogenic enzyme that mediates estradiol (E2) production and

6 therefore plays a major role in both sex differentiation and vitellogenesis (Lubzens et al.

7 2010, for review). An even more pronounced under-expression was observed for *cyp17*

8 that acts upstream of *cyp19a1a*. Consistently, data obtained from microarray analysis

9 also revealed the down-regulation of the *steroidogenic acute regulatory* gene (*star*, S1

10 Table). *Star* is involved in the cholesterol shuttling across the inner mitochondrial

11 membrane, which is a rate-limiting step of the steroidogenesis (29)(30). A decrease in

12 *star* expression indicates an overall decrease in ovarian steroidogenic production.

13 We also observed the down-regulation of *gsdf* and *inhibin*, two members of the TGFβ

14 family, in the ovary of mutant females. *Gsdf* is known to be expressed in granulosa cells

15 and to be involved in the maintenance and maturation of these cells (31)(32). *Inhibin*

16 consists of a unique α subunit and an activin β subunit. In the zebrafish ovary, members

17 of the activin-inhibin-follistatin system exhibit dynamic changes in expression during

18 folliculogenesis and the α subunit (*inha*) is expressed during vitellogenesis, especially

19 during final oocyte growth (*i.e.* prior to final oocyte maturation)(33).

20 Finally, QPCR analysis revealed a significant down-regulation of the transcription factor

21 *foxl2b*, the only *foxl2* copy retained in medaka after teleost-specific genome duplication

22 (34). In medaka, *foxl2b* is a female-specific gene expressed in somatic cells surrounding

23 germ-cells in the germinal cradle. At later stages, *foxl2b* continue to be expressed in

24 granulosa cells and in a minority of theca cells in pre-vitellogenic and vitellogenic

12

1   follicles, but expression ceased in post-vitellogenic follicles (35)(36). In mouse, *foxl2* is

2   known as a key regulator of oogenesis and plays a critical role in ovarian differentiation

3   (37)(38)(39).

4   **Down-regulation of known genes expressed in the primary oocyte within the germinal**

5   **cradle (Fig 6B)**

6   We analyzed the expression of *sycp3, foxl3* (a *foxl2*-relative)*, sox9b* and the medaka *vasa-*

7   like gene (*olvas*)*. Sycp3 and foxl3* are both expressed in germ cells in the ovary and are

8   both involved in the first meiotic division (4)(40). *Olvas* is known as a specific marker of

9   early germ cells, which are nested in the cords of *sox9b*-expressing cells, referred to as

10 the germinal cradle (41)(4). QPCR analysis revealed a down-regulation of *sycp3* and

11 *foxl3* in the ovary of mutant females, although the diminution of *sycp3* expression is not

12 significant. In contrast, the expression of *olvas* and *sox9b* was similar in wild-type and

13 mutant ovaries. Together these observations suggest that germinal cradles of mutant

14 ovaries are subjected to a reduced meiotic activity of primordial oogonia, while the

15 mitotic activity of germline stem cells is likely to be maintained.

16 **Down-regulation of other genes that belong to the WNT and KELCH families (Fig 6C)**

17 Among the most differentially expressed genes were several other genes coding for

18 proteins of the WNT signaling pathway, including *wnt2bb* and *wnt4a*. In ovary of mutant

19 females, QPCR revealed a clear and significant down-regulation of *wnt2bb* and *wnt4a*.

20 *Wnt4a* is expressed in fish ovary, while the expression of *wnt2bb* remains

21 uncharacterized to date (42). The microarray analysis also revealed a dramatic down-

22 regulation of *klhl23*, a member of the KELCH Like Family. KELCH proteins were initially

23 characterized due to their important role in oocyte-somatic cells communication during

13

1   drosophila oogenesis. Several members of this large family (over 30 members) are

2   known to be expressed in the ovary of several vertebrates even though they remain

3   poorly studied (43).

4   **Identification of novel molecular players of oogenesis (Fig 6D)**

5   Finally, transcriptomic analysis revealed three other differentially expressed genes

6   (*setd4*, *npr1b* and *srgap3*) that have never been previously described as molecular

7   players of oogenesis. The *set domain-containing protein 4* (*setd4*) gene displayed a

8   significantly reduced expression in ovary of mutant females. *Setd4* encodes for a

9   recently characterized methyltransferase involved in breast cancer cell proliferation

10 (44). While the role of *setd4* in the ovary remains unknown, a recent report

11 demonstrated its role in the regulation of cell quiescence during the diapause of artemia

12 embryos (45). The *natriuretic peptide receptor 1b* gene (*npr1b*) exhibited a marked over-

13 expression. Although the role for *npr1b* in oogenesis has never been reported, the mouse

14 *nrp2* gene has already been detected in granulosa cells and shown to maintain meiotic

15 arrest of oocytes in mouse ovary (46). The *slit-robo Rho GTPase activating protein 3*

16 (*srgap3*) gene displayed a milder, yet significant, over-expression. Along with a well-

17 established function in axon guidance, several studies suggested that the SLIT/ROBO

18 pathway could also have important functions in the reproductive system (47). These

19 results suggest that *setd4*, *npr1b* and *srgap3* are indeed novel molecular players of

20 oogenesis. Given the greater number of down-regulated genes, in comparison to up-

21 regulated genes, a non-annotated gene was selected for QPCR as a control and the

22 profile observed on microarrays could be validated.

14

1 DISCUSSION

2 Most of our current understanding of the regulatory mechanisms of oogenesis in fish is

3 due to several decades of research on hormones, secreted factors or intrinsic signaling

4 pathways. However, miRNAs are well known for their role in many physiological

5 processes through post-transcriptional gene regulations. Here, we showed that this

6 fundamental class of molecules, endowed with pleiotropic functions, is also necessary

7 for oogenesis in medaka ovary. In particular, we showed that *miR-202* plays a key role in

8 follicular recruitment and growth, and ultimately in the female reproductive success, as

9 shown by the severe phenotype of *miR-202* KO fishes.

10 Mir-202-5p is the biologically active mature form in the ovary

11 Our present results show that miR-202-5p is the predominant mature form processed

12 from *miR-202* gene, while the miR-202-3p form is detected at much lower levels. This is

13 consistent with existing data in which one miRNA form is biologically active, while the

14 other is degraded. More specifically, our ISH experiments reveal that miR-202-5p is

15 highly expressed in the somatic granulosa cells surrounding the oocyte. Consistently,

16 recent transcriptomic studies in zebrafish reported either an increased expression of

17 miR-202-5p during folliculogenesis or an increased expression in developing oocytes

18 and unfertilized eggs (48)(49). Yet, it has also been claimed that *miR-202* could by

19 expressed only in early medaka oocytes and absent at later stages, based on ISH

20 experiments (16). Since our ISH did no reveal any expression of miR-202-5p above

21 background levels in early oocytes, we presume that it is more likely accumulated

22 during vitellogenesis in vitellogenic and/or post-vitellogenic oocytes, as suggested by

23 the detectable expression in unfertilized eggs,

15

1 *Mir-202* **is required for both male and female reproductive success, but not**

2 **for sex determination**

3 Here, we observed that the inactivation of *miR-202* in medaka does not lead to any

4 modification of the sex ratio, since mutant siblings give rise to 50% of adult males and

5 50% of adult females. This rules out the possibility of a key role of *miR-202* in sex-

6 determination. It should however be stressed that the *miR-202* KO results in the sterility

7 of both females and males. More particularly, when *miR-202* KO males were crossed

8 with wild-type females, embryonic viability was dramatically reduced since most

9 embryos were arrested during the first cleavage stages (st.4-5). To our knowledge, this

10 phenotypic defect of male reproduction as never been reported before in fish, and it

11 clearly indicates that *miR-202* is also required for spermatogenesis. This is in agreement

12 with results obtained after *miR-202* KO in cultured spermatogonial stem cell in mouse,

13 indicating a role in the control of the cellular proliferation/differentiation balance (27).

14 Further studies are now required to unravel the precise role of *miR-202* in fish testis

15 during spermatogenesis.

16 **Females lacking *miR-202* produce eggs that cannot be fertilized**

17 In this study, we paid special attention to the severe phenotype displayed by *miR-202*

18 KO females. The overall reproductive success of mutant females was reduced to 0.83 or,

19 even more, to 0 viable eggs per clutch, compared to 15.3 viable eggs per clutch for wild-

20 type females. This phenotype is the consequence of both a reduced fecundity (*i.e.*

21 reduced number of spawn eggs) and reduced egg viability. Mutant females indeed

22 produce a large proportion of eggs that cannot be fertilized, subsequently resulting in no

23 embryonic development. During folliculogenesis, the somatic cells that surround the

16

1   growing oocyte (granulosa and theca cells) contribute to the formation of the chorion,

2   and the micropyle, which is primordial for the egg fertilization (50). It is thus likely that

3   *miR-202*, which is expressed in granulosa cells throughout folliculogenesis, contributes

4   to the formation of a functional chorion and micropyle. However, we cannot totally

5   exclude an additional role of *miR-202* in oocytes during the growth and maturation

6   steps, given the possible accumulation of *miR-202* inside the oocyte.

7   **MiR-202 deficiency severely affects early folliculogenesis and female**

8   **fecundity**

9   The very low fecundity of *miR-202* KO females strongly indicates that *miR-202* plays an

10   important role in the oogenesis processes in the ovary, which was further investigated.

11   One of the most remarkable features observed in mutant ovaries was the abnormal

12   increased number of pre-vitellogenic follicles. Along with this phenotype, we observed a

13   reduced expression of *gsdf* (a member of the TGFb family) and of the *foxl2* transcription

14   factor. Both *gsdf* and *foxl2* are expressed in granulosa and are well known for their role

15   as key regulators of early oogenesis/folliculogenesis (34)(51)(52). Downregulation of

16   these factors thus confirms that *miR-202* likely controls the early development of

17   granulosa cells (*i.e.* granulosa proliferation and/or differentiation) through the

18   regulation of *gsdf* and *foxl2*, and ultimately leads to an impaired folliculogenesis. Such

19   hypothesis is also supported by the down-regulation of downstream key genes,

20   including steroidogenic genes (*star*, *cyp19a1a*, and *cyp17*) and the TGF-b family member

21   *inhibin*. The formers are involved in estrogen synthesis in ovarian follicles, while

22   *inhibin* plays a critical role during follicular development in zebrafish (53). Further

17

1 analysis of the proliferation and differentiation states of granulosa cells are needed to

2 fully understanding of the role of *miR-202* during folliculogenesis.

3 Of particular interest is also the down-regulation of *gsdf* in *miR-202* mutant ovaries.

4 Along with its well-established key role in the male-determination in medaka, the GSDF

5 factor is also thought to be necessary for normal ovarian development (54)(31). Indeed,

6 *gsdf* inactivation not only impairs male differentiation, but also leads to a severely

7 reduced female fecundity up to a total infertility for the most severe cases. In such

8 mutants, the number of pre-vitellogenic follicles is abnormally high (31). Except the

9 male-determination phenotypic defect, many features of the *gsdf* mutant are very

10 similar to that observed for *miR-202* KO females, including the pre-vitellogenic follicles

11 accumulation. We hypothesize that *miR-202* promotes *gsdf* expression in the ovary and

12 boost its action on follicular development. In any case, such hypothesis remains to be

13 confirmed and molecular mechanisms underlying the interaction between miR-202-5p

14 and *gsdf* remain to be identified.

Mir-202 knock-out impairs female meiosis

16 The germinal cradle is composed somatic *sox9b*-expressing cells that surround mitotic

17 and meiotic germ cells, as well as primordial oogonia barely engage into folliculogenesis

18 (4). Our results show that *miR-202* is expressed in the germinal cradle, in a subset of

19 *sox9b*-expressing cells surrounding primordial oogonia, which together likely

20 correspond to primordial follicles. Interestingly, no modification of *sox9b* expression

21 was observed in mutant ovaries, and previous studies in mouse have shown that *sox9*

22 regulates the expression of *miR-202* in granulosa cells (55). It is thus possible that *miR-202*

23 *202* acts downstream of *sox9b* in medaka as well. However, molecular mechanisms that

18

1 regulate the expression of *miR-202* in follicles at later stages, in vitellogenic and post-

2 vitellogenic follicles, remain to be determined since *sox9b* is not expressed at these

3 stages.

4 We also observed, in juvenile ovaries, a down-regulation of the early meiotic oocyte

5 markers *foxl3* and, to a lesser extent *sycp3*, even though no *miR-202* expression was

6 detected in oocytes at this stage. Cellular interactions between germinal and somatic

7 cells are thought to be important and to be mediated by several secreted factors

8 (56)(57)(43). Consequently, any early defect of oocytes is likely to occur in the context

9 of granulosa dysfunction (discussed above), including the onset of meiosis. One

10 possibility is that *miR-202* depletion in the granulosa affects oocyte-somatic

11 communications, as suggested by the down-regulation of KELCH proteins that have

12 important functions in oocyte-somatic cell interactions in drosophila (43). This

13 hypothesis is also supported by the dysregulation of the *npr1b* receptor and the

14 intracellular effector *srgap3* of the SLIT/ROBO signaling pathway, which could both also

15 mediate cellular communications in the ovary (46)(47).

16 **_Mir-202_, a major player rather than a fine modulator**

17 As discussed above, a drastic reduction of the reproductive success was observed for

18 both male and female medaka lacking *miR-202,* including a reduced female fecundity

19 and the production of poor quality eggs that cannot be fertilized. This phenotype was

20 confirmed using two mutant lines bearing different INDELs obtained with the same

21 guide RNA. It is generally considered that the purview of miRNAs is more likely the

22 maintenance of regulatory networks, by fine-tuning gene expression, rather than the

23 establishment of key regulatory networks for developmental decisions or core

1   physiological processes. This concept is supported by the fact miRNAs KO are commonly

2   associated with "modest" phenotypic effects, which are strongly exacerbated only under

3   particular condition, as for example manipulations, stresses or disease conditions (28).

4   This is in contrast with the drastic reproductive phenotype observed here for *miR-202*

5   KO fishes. It is however possible that *miR-202* modulates a large networks of targets,

6   which would have a synergistic effect on key regulatory pathways for folliculogenesis,

7   such as *gsdf* (as discussed above), and ultimately leads to subfertile or sterile females.

8   Nevertheless, the phenotypes observed in *miR-202-/-* fish are among the most severe

9   observed after miRNA KO, and it would be very informative in the future to determine

10   whether the drastic *miR-202* KO phenotype is an exception in the ovary.

11   **A new look at oogenesis**

12   Surprisingly, the transcriptomic analysis performed in juvenile females, during the first

13   reproductive cycle and before the occurrence of the first spawning, did not result in the

14   identification of a large number of differentially expressed. It should however be

15   stressed that among de differentially expressed genes were many genes that are crucial

16   for steroidegenesis and reproduction, such as *star* and members of the *wnt* family (as

17   discussed above). Despite these usual suspects, the transcriptome analysis also shed

18   light on other genes such a genes of the *kelch* family that are less studied but believed to

19   play an important role in oogenesis based on existing data in other animal species (58).

20   Finally, the identification of genes that were previously not known to participate in

21   oogenesis, such as *setd4*, *npr1b* and *srgap3*, could shed a new light on our understanding

22   of this complex and coordinated biological process. This begs for further investigations

23   that will greatly benefit from *miR-202-/-* fishes as a novel biological model.

1 **Conclusion**

2 In summary, our results show that *mir-202* is a key miRNA involved in the regulation of

3 follicular recruitment and growth. This provides the first functional evidence that

4 microRNAs are necessary for the female reproductive success and in particular the

5 regulation of female fecundity. Furthermore, the present study shed new light on the

6 regulatory mechanisms that control the early steps of follicular development, which

7 remain poorly understood to date. A further systematic *in vivo* functional analysis of

8 other ovarian-predominant miRNAs should greatly increase our knowledge on the

9 overall role of miRNAs in oogenesis and female fecundity in fish.

10 # Materials and methods

11 **Ethics statement**

12 All experimental procedures were conducted in strict accordance with the French and

13 European regulations on animal welfare recommendations and were approved by the

14 INRA LPGP Animal Care and Use Committee.

15 **Medaka breeding and tissues collection**

16 Adult medaka (*Oryzias latipes*) from the CAB strain and adults of the *Tg(sox9b::EGFP)*

17 medaka line were raised at 26°C. Fishes were raised under a growing photoperiod

18 regime until 3 months post-fertilization (12h light/ 12h dark) and under a reproduction

19 photoperiod regime after 3 months post-fertilization (14h light/10h dark). For QPCR

20 analysis, eleven different tissues/organs (brain, eyes, fins, gills, heart, intestine, kidney,

21 liver, muscle, ovary and testis) were collected from wild-type and miR-202-/- fish.

22 Embryos were collected at different stages (1-cell, 8-cell, stage 11/12, stage 17/18, stage

1 25/26, stage 29/30 and stage 39), according to the developmental table described by

2 Iwamatsu *et al.* (59). For tissues/organs dissection, adult medaka fishes were

3 euthanized by immersion in a lethal dose of tricaine à 30-50mg/L. All tissues/organs

4 and embryos were immediately frozen in liquid nitrogen and subsequently stored at

5 −80°C until RNA extraction. For histological analyses, ovaries were collected from wild-

6 type females, fixed overnight in 4% paraformaldehyde (PFA) at 4°C and dehydrated in

7 100% methanol and stored at -20°C, before histological analyses.

8 **Establishment of the *miR-202* mutant medaka line**

9 For the CRISPR/Cas9 knock-out analysis, the target genomic sequence was identified

10 with the help of the ZiFiT online tool (http://zifit.partners.org/ZiFiT/) and using the

11 medaka genome reference available on the Ensembl genome database (Ensembl gene:

12 ENSORLG00000021212). A short sequence in the mature miR-202-3p was selected as

13 followed: GG-(N)18-NN. Two inverse-complementary primers (Forward 5'-

14 TAGGCATAAGGCATGGGAAAAT-3' and (Reverse 5'-AAACATTTTCCCATGCCTTATG-3')

15 were annealed and cloned into the pDR274 vector (Addgene plasmid 42250) in the BsaI

16 cloning site. The modified pDR274 vector was digested with DraI and the miR-202

17 specific guide RNA (mir202-sgRNA) was transcribed using the T7 RNA polymerase

18 (P207, Promega). For the Cas9-RNA *in vitro* synthesis, the pCS2-nCas9n vector (Addgene

19 plasmid 47929) was linearized with NotI and capped RNA encoding the Cas9 was

20 transcribed with the mMessage mMachine SP6 Kit (AM1340, Life Technologies)

21 following manufacturer's instructions. Cas9 and sgRNA were purified using

22 phenol/chloroform and precipitated by Ammonium acetate. *Cas9*-RNA (100 ng/µL) and

23 *mir202*-sgRNA (10 ng/µL) were co-injected into one-cell stage embryos. Injected

22

1   embryos were raised to sexual maturity and 10 fishes were genotyped to identified

2   founder fishes (F0). Fishes harboring the same INDEL mutation (-7+3) were selected

3   and outcrossed with wild-type fishes to obtain F1 heterozygous. Such outcrosses were

4   performed at each generation in order to maintain the line. Homozygous fishes were

5   produced for histological and molecular phenotyping analyses. A second line harboring

6   another INDEL mutation (-8) was used in order to confirm the reproductive phenotype,

7   but this line was not used for further histological and molecular analyses.

8   **Genotyping**

9   Genomic DNA was extracted from a small piece of the caudal fin sampled from

10   anesthetized adult fishes. Samples were lysed in 75mL of lysis buffer containing 1,25 M

11   NaOH and 10 mM EDTA (pH 12) incubated at 90C for 1h and were neutralized with

12   75mL of neutralization solution containing 2 M Tris-HCl (pH 5). To identify F0 founder

13   fishes, genomic DNA around the expected mutation site was sequenced. For systematic

14   genotyping of individuals of the established line, wild-type and mutant (INDEL -7+3)

15   alleles were specifically detected by HIDI-PCR using specific reverse primers for each

16   allele (S2 Table). The HIDI polymerase (Genaxxon bioscience, M3025.0250) was used

17   with the following PCR conditions: 95°C for 2min; and 40 cycles of 95°C for 20 sec, 57°C

18   for 15 sec and 72°C for 30 sec; and then 72°C for 7 min.

19   **Total RNA extraction**

20   Frozen tissues were lysed with Precellys Evolution Homogenizer (Ozyme, bertin

21   technologies) in TRI Reagent (TR118, Euromedex) and total RNA was extracted using

22   the "nucleospin RNA" kit (740955, Macherey Nagel).

1 **Quantitative PCR**

2 For expression analysis of mature miRNA forms, 20 ng of total RNA were reverse-

3 transcribed (RT) using the TaqMan advanced miRNA cDNA Synthesis Kit (A28007,

4 Applied Biosystems). Twenty fmol/µl of an external calibrator cel-miR-39-3p

5 (478293_miR, Life technologies) was added in the first step of the RT-Taqman PCR

6 (polyA step) for 20ng of RNA. The cDNA was diluted (1:5) and universal primers (20x

7 miR-Amp Primer Mix, 100029187, Applied Biosystems) were added in the last step of

8 the RT reaction. The TaqMan QPCR was performed using 5 µl of diluted cDNA, 1 µl

9 TaqMan Advanced miRNA Assay solution_(CCU001S, Special Product Custom designed

10 advanced miRNA assay, Life technologies) and 10 µl Fast Advanced Master Mix

11 (4444557, Applied Biosystems) in a total volume of 20 µl. Specific modified probes

12 complementary to *miR-202*-5p and -3p were designed as followed: mir-202-3p 5'$_{\text{FAM}}$-

13 AGAGGCATAAGGCATGGGAAAA-3'$_{\text{Quencher}}$ and mir-202-5p 5'$_{\text{FAM}}$-

14 TTCCTATGCATATACTTCTTTG-3'$_{\text{Quencher}}$ (Life technologies). QPCR was performed using

15 the Step One Plus system (Applied Biosystems, USA) with the following conditions: 95°C

16 for 20 sec; and 40 cycles of 95°C for 1 sec and 60°C for 20 sec. The relative expression of

17 miRNA within a sample set was calculated from standard curve using Applied Biosystem

18 StepOne V.2.0 software. All QPCR were performed in duplicates. MiR-26a (hsa-miR-26a-

19 5p, A25576, Thermofisher Scientific) or cel-miR-39-3p was used for normalization.

20 For mRNA and pri-miR-202 expression analysis, 2µg of total RNA was reverse-

21 transcribed using the Maxima First Strand cDNA Synthesis Kit (K1671, ThermoFisher

22 Scientific). The cDNA was diluted (1:20). The SyberGreen QPCR was performed using 4

23 µl of diluted cDNA, 5 µl of GoTaq QPCR Master Mix 2x (A600A, Promega) and 100 nM of

24 each primer (S3 Table), in a total volume of 10 µl. The QPCR was performed using the

24

1   Step One Plus system (Applied Biosystems, Foster City, USA) with the following

2   conditions: 95°C for 2 min; and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Standard

3   curves were generated using five serial cDNA dilutions (from 1:2 to 1:32) of a pool of all

4   samples. The relative abundance of target cDNA was calculated from standard curve

5   using Applied Biosystem StepOne V.2.0 software. All QPCR were performed in triplicates

6   and the *rpl7* gene was used for normalization.

Microarray analysis

8   Gene expression profiling was conducted using an Agilent 8x60K microarray as

9   previously described (60). Samples were randomly distributed on the microarray for

10   hybridization. The data were processed using the GeneSpring software (Agilent v.14.5)

11   using gMedianSignal values. The gene expression data was scale normalized and log(2)

12   transformed before the statistical analysis. Corresponding data were deposited in Gene

13   Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) database under the

14   reference GSE111388. The differences between the groups were analyzed with unpaired

15   t-test after application of minimum two-fold change filter with the significance level of 5

16   % (p < 0.05) after Benjamini-Hochberg correction.

Fluorescent *in situ* hybridization and immunostaining

18   For fluorescent *in situ* hybridization (FISH), fixed ovaries were embedded in paraffin

19   and sections (9 µm thickness) were performed with a microtome (HM355, microm). An

20   anti-sense Locked Nucleic Acid (LNA) oligonucleotide was designed and produced by

21   Exiqon A/S to detect the mature miR-202-3p form. Since medaka and human miR-202-

22   5p sequences are identical, we used the hsa-miR-202-5p miRCURY LNA™ miRNA

23   detection probe to detect the mature medaka miR-202-5p form. A LNA™ Scramble-miR

1 probe (5'-GTGTAACACGTCTATACGCCCA-3') was used as a negative control. All LNA™

2 probes were double-DIG labeled at both 5' and 3' ends. FISH was performed using the

3 microRNA ISH Buffer Set (FFPE) Hybridization Buffer (ref. 90000, Exiqon), following the

4 manufacturer's instructions with some modifications. Permeabilisation was performed

5 for 7 min at room temperature using of Proteinase-K (10 mg/ml, P2308 Sigma). LNA

6 probes were used at 20 nM at 53°C (30°C below the RNA Tm °C) for 2 hours. Samples

7 were then incubated overnight at 4°C with a rabbit anti-DIG HRP-conjugate antibody

8 (1:500, Roche). For the GFP detection, a chicken anti-GFP (1/500, ref. ab13970, Abcam)

9 was added at this step. The anti-GFP was first detected with a goat anti-chicken

10 AlexaFluor488-conjugate antibody (1/500, ref. A11039, Life Technologies) for 1 hour at

11 room temperature. Then, the anti-DIG-HRP antibody was detected with the TSA-Cy5

12 substrate (1:50, TSA™ PLUS Cy5 kit, NEL 745001KT, Perkin Elmer) for 15 min at room

13 temperature. All pictures were taken under SP8 confocal.

Nuclear staining and image analysis

15 Fixed ovaries were embedded in paraffin and median sections (7 μm thickness) were

16 performed with a microtome (HM355, microm). Nuclei were stained with DAPI (0,1

17 μg/ml) at room temperature for 15 minutes in the dark. Median sections were washed

18 1h in PBS at room temperature. Images of whole sections were acquired with a

19 nanozoomer (HAMAMATSU). For quantitative image analyses, individual oocyte's area

20 was measured using an image analysis software (Visilog 7.2 for Windows). Based on

21 DAPI staining intensity, the nucleus were segmented then the inner part surround by

22 the nucleus and corresponding to oocytes were individually measured. Pictures were

23 taken under a Nikon AZ100 microscope and DS-Ri1 digital camera.

1

ACKNOWLEDGMENTS

Author Contributions

SG performed histological and molecular analyses, participated in mutant fish phenotyping, data analysis and manuscript writing. JBu participated in histological analyses. AB participated in generating the mutant line and in tissue collection and RNA extraction. LH participated in reproductive phenotype analysis. ALC performed the microarray analysis. JM performed bioinformatics analyses. VT and JBo conceived the

27

1 study, participated in data analyses and wrote the manuscript. VT supervised and

2 coordinated the study. All authors read and approved the final manuscript.

Additional information

Supplementary information accompanies this paper

Competing financial interests

6 The authors declare that they have no competing interests.

Availability of data and material

8 The microarray expression data are available from the gene expression omnibus (GEO)

9 database (accession # GSE111388, http://www.ncbi.nlm.nih.gov/geo/).

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Supporting information

S1 Fig. FISH on ovarian section performed with a scramble control probe

S1 Table. Differentially expressed genes obtained through a microarray analysis

S2 Table. Primers used for HIDI-PCR

S3 Table. Primers used for QPCR

Figure captions

Figure 1: Expression profile of miR-202-5p in adult tissues and during embryonic development. (A) Sequence and secondary structure of the pre-miR-202. miR-202-5p is in red and miR-202-3p in blue. **(B)** Tissue distribution of miR-202-5p in eleven adult tissues obtained by TaqMan QPCR (Box plots, n=8, the ends of the boxes define the 25th and 75th percentiles; a line indicates the median and bars define the 5th and 95th percentiles). Cel-miR-39-3p was used as an external calibrator for normalization. **(C)** Expression profile of miR-202-5p during embryonic development. Pools of embryos at the same stage were used for TaqMan QPCR. *ns*, no significant difference (Student t-test). #expression levels not significantly different from the background signal.

Figure 2: Expression of miR-202-5p and miR-202-3p in the ovary. Fluorescent *in situ* hybridizations (FISH) were performed on sections of ovaries from juvenile and adult medaka females. MiR-202-5p **(A, B)** and miR-202-3p **(C, D)** were detected with specific LNA miRNAs detection probes (in red). Ovaries of adult females were dissected from the transgenic medaka line *Tg*(*sox9b*::EGFP). The somatic GFP+ cells of the germinal cradle, including early granulosa cells, were immunodetected (in green) **(B, D)**. Nuclei are stained with DAPI (in blue). **(A')** A magnified view of the juvenile ovarian

34

1　section. **(B', B" and B''')** Magnified views of the adult ovarian section. Mir-202-5p is

2　detected in granulosa cells of follicles at all stages in both juvenile and adult ovary

3　(arrowhead), but not in the theca cells (arrow). **(B''')** MiR-202-5p co-localize with GFP in

4　early granulosa cells surrounding pre-vitellogenic follicles. **(C, D)** Mir-202-3p is not

5　detected in ovaries from juvenile and adult fishes. PreV, pre-vitellogenic follicle; V,

6　vitellogenic follicle; PV, post-vitellogenic follicles. Scale bars: 500 μm (A-D), 50 μm (A',

7　B', B") and 20 μm (B''').

8

9　**Figure 3: Analysis of the reproductive phenotype of *miR-202-/-* adult fish. (A)**

10　Genomic regions of wild-type and mutant fishes showing the INDEL mutations (-7+3)

11　inserted in the miR-202-3p sequence using CRISPR/Cas9 genome engineering. **(B)**

12　Expression levels of the pri-miR-202 and miR-202-5p forms in the ovaries of wild-type

13　and homozygotes females (*miR-202-/-*) were measured by QPCR. **(C)** Spawning

14　frequency of mutant and wild-type females were monitored during ten days. Subfertile

15　females (irregular spawning) and sterile females (only one spawning) represent

16　respectively 85% and 15% of all analyzed females. **(D)** Number of eggs per clutch

17　spawned by wild-type or subfertile females when mated with wild-type or mutant

18　males. **(E)** Embryonic viability of spawn eggs measured by the percentage of eggs that

19　are fertilized and that develop correctly. **(F)** Representative pictures of eggs from the

20　different crosses of wild-type and mutant males and females. Mean values (± SEM) are

21　displayed on the graphs. * indicates expression levels that are significantly different

22　(Mann Whitney test, $p < 0.05$). Different letters indicate a significant difference, as

23　determined by a one-way ANOVA test (Tukey's post hoc test). #expression levels not

24　significantly different from the background signal.

35

1   **Figure 4: Quantitative image analysis of *miR-202-/- * juvenile ovaries. (A)** Median

2   sections of ovaries from juvenile wild-type and *miR-202-/-* fishes. All nuclei are stained

3   with DAPI (in blue). Image sections were automatically segmented. **(B)** Section area,

4   mean follicle diameter and follicle number automatically determined. **(C)** Size

5   distribution of follicles on sections. The number of small follicles (50-100 µm) is

6   significantly higher compared to wild-type, while the number of follicles in the larger

7   diameter class (200-350 µm) tends to decrease. Box plots are displayed on graphs for

8   wild-type (n=7) and *miR-202-/-* (n=8) juvenile females (in red and green, respectively).

9   The ends of the boxes define the 25th and 75th percentiles; a line indicates the median

10   and bars define the 5th and 95th percentiles. Individual values are shown for the graphs

11   of panel B. Asterisks indicate significant differences (* $p < 0.05$ and ** $p < 0.01$, Mann

12   Whitney test). Scale bar: 500 µm.

13

14   **Figure 5: Quantitative image analysis of *miR-202-/- * adult ovaries. (A)** Median

15   sections of ovaries from wild-type, sterile and subfertile mutant females. All nuclei are

16   stained with DAPI (in blue). Image sections were automatically segmented. **(B)** Section

17   area, mean follicle diameter and follicle number automatically determined. **(C)** Size

18   distribution of follicles on sections. In ovaries from sterile females, the number of large

19   follicles (>400 µm) is significantly reduced, while small follicles (<100 µm) tend to

20   accumulate. In the ovary from subfertile females, the number of medium follicles (100-

21   400 µm) is significantly reduced, but small size follicles (<100 µm) do not accumulate.

22   Box plots are displayed on graphs for wild-type (n=10), *miR-202-/-* subfertile (n=10)

23   and *miR-202-/-* sterile (n=4) adult females (in red, green and blue, respectively). The

24   ends of the boxes define the 25th and 75th percentiles; a line indicates the median and

36

1   bars define the 5th and 95th percentiles. Individual values are shown for graphs of panel

2   B. Asterisks indicate significant differences (* $p < 0.05$ and ** $p < 0.01$, Mann Whitney
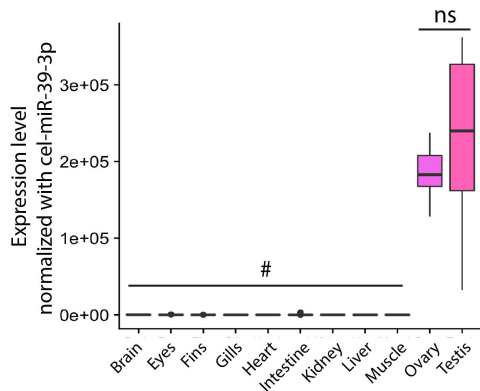
3   test). Scale bar: 500 μm.

4

5   **Figure 6: mRNA expression profiles of ovarian genes in *miR-202 -/- *females.** Gene

6   expression levels in wild-type and ovaries from juvenile females were obtained by

7   QPCR. (A) Genes expressed in the somatic germ-cell supporting cells. **(B)** Genes

8   expressed in primary oocytes within the germinal cradle. **(C)** Genes that belong to the

9   WNT and KELCH families. **(D)** Dysregulated genes that have never been previously

10 described as molecular players of oogenesis. Expression levels were measured in

11 triplicates. Box plots are displayed on graphs for wild-type (n=6) and *miR-202-/-* (n=5)

12 juvenile females. The ends of the boxes define the 25th and 75th percentiles; a line

13 indicates the median and bars define the 5th and 95th percentiles. Individual values are

14 shown. The *rpl7* gene was used for normalization. Asterisks indicate significant

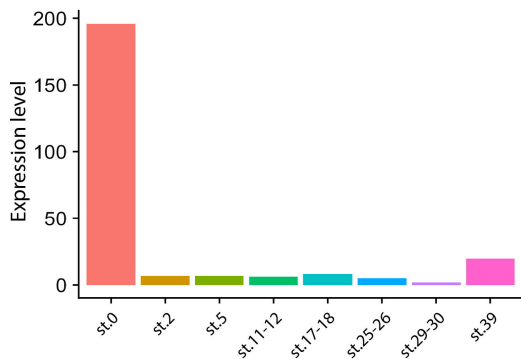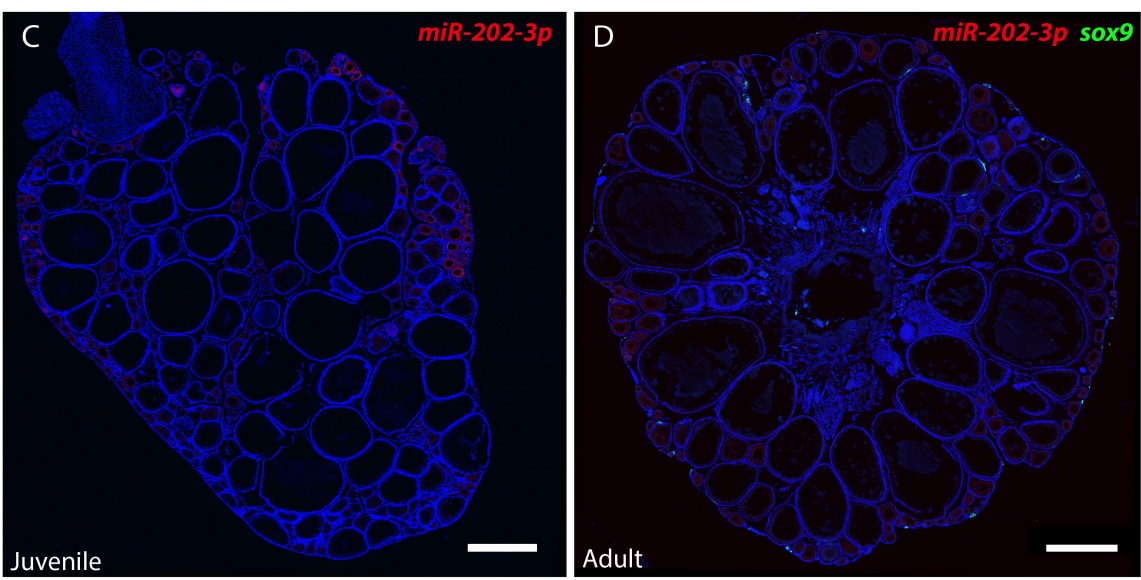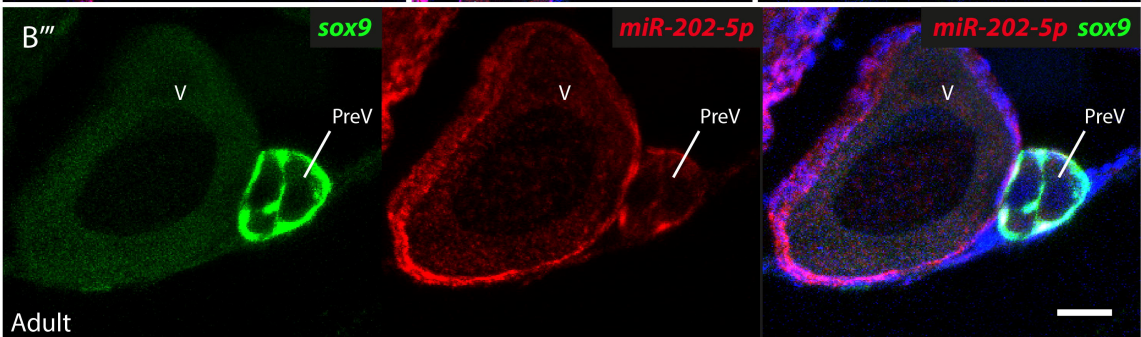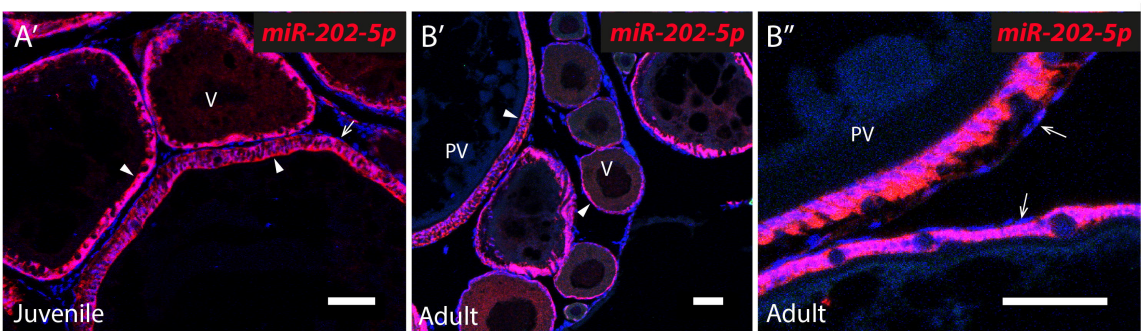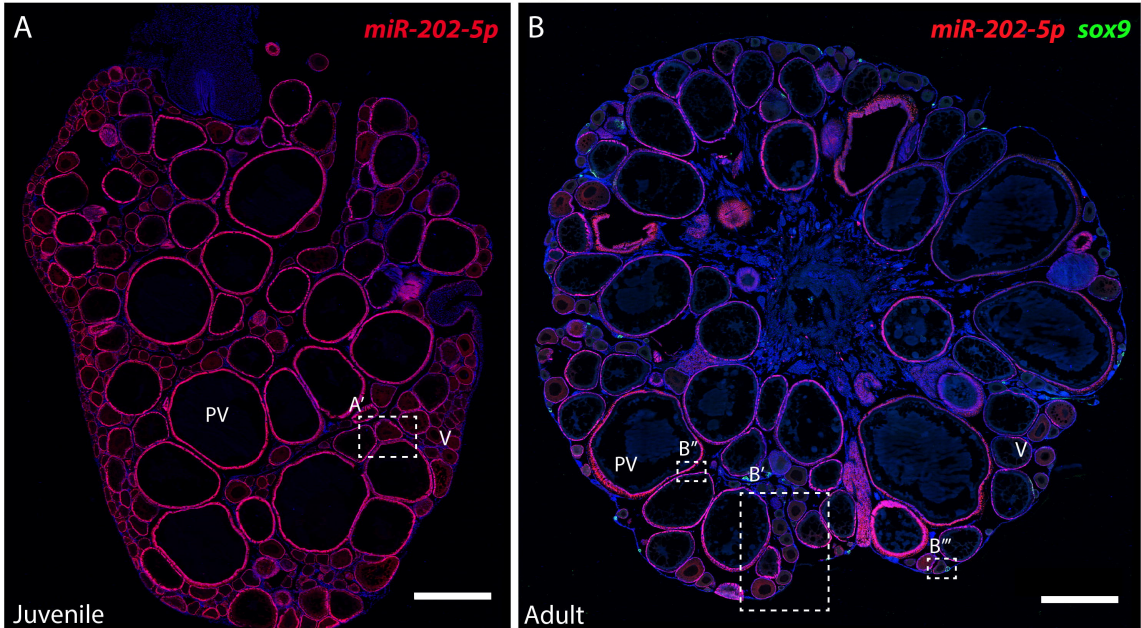15 differences (* $p < 0.05$ and ** $p < 0.01$, Mann Whitney test).

A
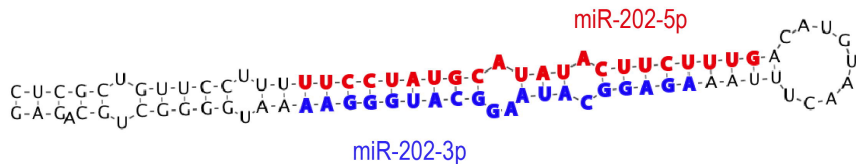
B

Expression of miR-202-5p
in various adult tissues
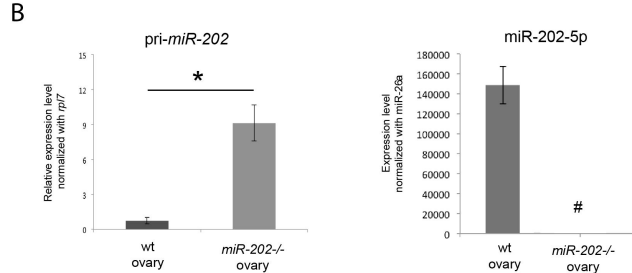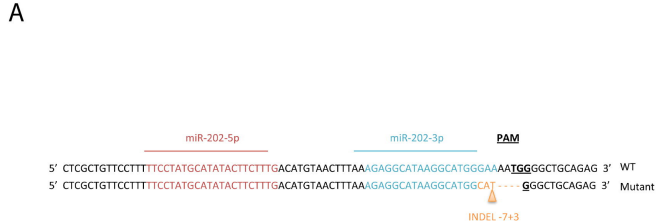
C

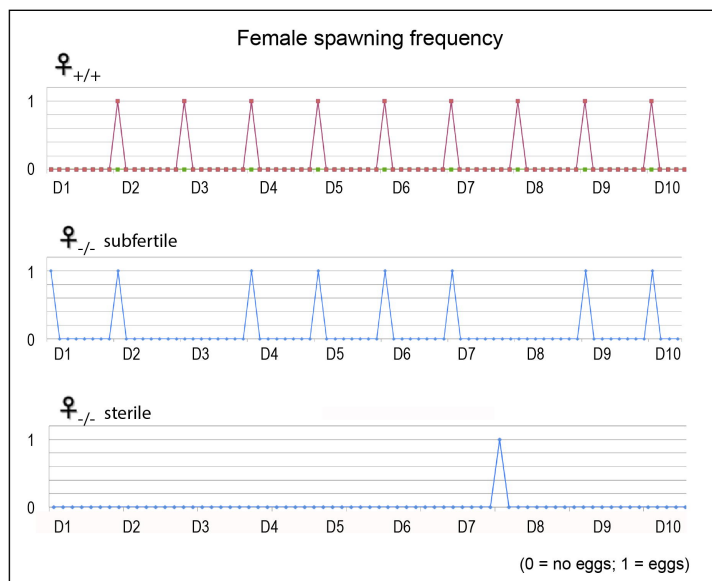Expression of miR-202-5p
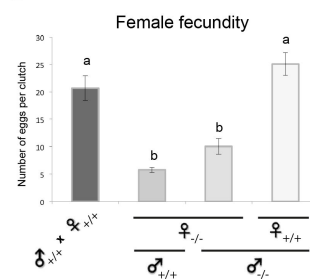during embryonic development

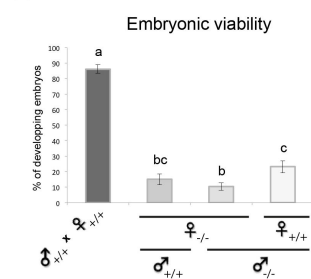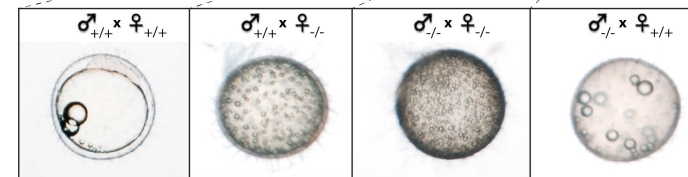A *miR-202-5p*
PV A' V
Juvenile

B *miR-202-5p* *sox9*
PV B'' B' V B'''
Adult

A' *miR-202-5p*
V
Juvenile

B' *miR-202-5p*
PV V
Adult

B'' *miR-202-5p*
PV
Adult

B''' *sox9* *miR-202-5p* *miR-202-5p* *sox9*
V PreV V PreV V PreV
Adult

C *miR-202-3p*
Juvenile

D *miR-202-3p* *sox9*
Adult

A

		miR-202-5p		miR-202-3p		PAM	

5' CTCGCTGTTCCTTT**TTCCTATGCATATACTTCTTTG**ACATGTAACTTTAAAGAGGCATAAGGCATGGGAAAAT**TGG**GGCTGCGAGAG 3' WT

5' CTCGCTGTTCCTTT**TTCCTATGCATATACTTCTTTG**ACATGTAACTTTAAAGAGGCATAAGGCATGGCAT- - - - -**G**GGCTGCGAGAG 3' Mutant

INDEL -7+3

B

pri-*miR-202*

Relative expression level normalized with *rpl7*

wt ovary — *miR-202-/-* ovary *

miR-202-5p

Expression level normalized with miR-26a

wt ovary — *miR-202-/-* ovary #

C

Female spawning frequency

♀+/+

D1 D2 D3 D4 D5 D6 D7 D8 D9 D10

♀-/- subfertile

D1 D2 D3 D4 D5 D6 D7 D8 D9 D10

♀-/- sterile

D1 D2 D3 D4 D5 D6 D7 D8 D9 D10

(0 = no eggs; 1 = eggs)

D

Female fecundity

Number of eggs per clutch

♂+/+ x ♀+/+ (a)

♀-/- / ♂+/+ (b)

♀-/- / ♂-/- (b)

♀+/+ / ♂-/- (a)

E

Embryonic viability

% of developing embryos

♂+/+ x ♀+/+ (a)

♀-/- / ♂+/+ (bc)

♀-/- / ♂-/- (b)

♀+/+ / ♂-/- (c)

F

♂+/+ x ♀+/+   ♂+/+ x ♀-/-   ♂-/- x ♀-/-   ♂-/- x ♀+/+

A

| Wild-type | miR-202-/- | miR-202 -/- | Segmented |

B

Ovarian section area

Mean follicular diameter

Follicle number

C

Distribution of follicles in different size classes

A

Wild-type | Subfertile *miR-202 -/-* | Sterile *miR-202 -/-*

B

Ovarian section area

Section surface (µm²)

ns

*

wild-type   subfertile   sterile

Mean follicular diameter

Mean diameter (µm)

*

*

wild-type   subfertile   sterile

Follicle number

Total number of oocytes

ns

*

wild-type   subfertile   sterile

C

Distribution of follicles in different size classes

Number of oocytes

ns

ns

ns

*

ns

**

wild-type
subfertile
sterile

<100   100-400   >400

Class of size (µm)

A
- **cyp19a1a** — mRNA abundance (AU); wt vs *miR-202-/-* (**)
- **cyp17** — mRNA abundance (AU); wt vs *miR-202-/-* (**)
- **gsdf** — mRNA abundance (AU); wt vs *miR-202-/-* (*)
- **inh** — mRNA abundance (AU); wt vs *miR-202-/-* (**)
- **foxl2b** — mRNA abundance (AU); wt vs *miR-202-/-* (**)

B
- **sycp3** — mRNA abundance (AU); wt vs *miR-202-/-* (ns)
- **foxl3** — mRNA abundance (AU); wt vs *miR-202-/-* (**)
- **olvas** — mRNA abundance (AU); wt vs *miR-202-/-* (ns)
- **sox9b** — mRNA abundance (AU); wt vs *miR-202-/-* (ns)

C
- **wnt2bb** — mRNA abundance (AU); wt vs *miR-202-/-* (**)
- **wnt4a** — mRNA abundance (AU); wt vs *miR-202-/-* (**)
- **klhl23** — mRNA abundance (AU); wt vs *miR-202-/-* (**)

D
- **setd4** — mRNA abundance (AU); wt vs *miR-202-/-* (**)
- **npr1b** — mRNA abundance (AU); wt (**) vs *miR-202-/-*
- **srgap3** — mRNA abundance (AU); wt (*) vs *miR-202-/-*
- **control gene up** — mRNA abundance (AU); wt (**) vs *miR-202-/-*