Heterogenic origin of micro RNAs in Atlantic salmon (Salmo salar) seminal plasma

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Abstract

Background The origin and contribution of seminal plasma RNAs into the whole semen RNA repertoire are poorly known, frequently being overlooked or neglected. Virtually nothing is known about seminal plasma RNAs in fish, including small RNAs, which have regulatory functions in gonadal development. Results In this study, we profiled microRNA (miRNA) constituents in the whole semen, as well as in fractionated spermatozoa and seminal plasma of Atlantic salmon (Salmo salar). Among 306 conserved miRNAs, 85 were differentially accumulated (>2 log-fold change and p-value < 0.01) between spermatozoa and the seminal plasma. We identified a number of seminal plasma-enriched and spermatozoa-enriched miRNAs. We localized the expression of some miRNAs in juvenile and mature testes. Two abundant miRNAs, miR-92a-3p and miR-202-5p, localized to both spermatogonia and somatic supporting cells in immature testis, and they were also highly abundant in somatic cells in mature testis. miR-15c-5p, miR-30d-5p, miR-93a-5p, and miR-730-5p were detected only in mature testis. miRs 92a-3p, 202-5p, 15c-5p, and 30d-5p were detected also in a juvenile ovary in locations corresponding to these from the testis. Additional RT-qPCR experiment demonstrated lack of correlation in miRNA transcript levels in seminal plasma versus blood plasma. Conclusions Our results indicate that salmon semen is rich in miRNAs, which are present in both spermatozoa and seminal plasma. The latter ones have partially different profile indicating their heterogenic origin. Testicular supporting somatic cells are likely the source of seminal plasma enrichment, whereas blood plasma unlikely contributes to the seminal plasma miRNA repertoire.

Background

Small RNAs are involved in regulation of various genetic elements important for differentiation, proliferation and apoptosis. Mature gametes carry the parental
information, which regulates the early embryonic development [1]. This parental information includes paternal epigenetic patterns and elements [2-4], delivered through spermatozoa, which regulate embryonic transcription [5, 6], and thereby imprint the fate of an embryo. The sperm contains different classes of RNAs, but their functions are very little known. Sperm RNAs can enter the oocyte during the fertilization [7], and studies on *Xenopus* sp. and mammals suggest their functional relevance [3, 6]. Several hypotheses on the role of sperm RNAs in embryonic development have been formed [3]
yet these conjectures need further experimental validation. Teleost fishes are the largest group of vertebrates, and their predominant reproductive features, such as lack of accessory glands in semen production, external fertilization, or spermatozoa activation upon the contact with water [10], make them distinct from tetrapods. Virtually, nothing is known about a potential transfer and possible role of paternal RNAs in embryo formation in fish. The whole semen RNAs are found not only in germ cells (spermatozoa), but also in the seminal plasma, where they are bound in protein complexes and encapsulated in micro vesicles [11]. Seminal plasma is an important constituent of semen and has a vital role in spermatozoa metabolism, survival and motility. It contains inorganic and organic compounds, proteins, and RNAs [12].
In fish, seminal plasma has been implicated in osmotic balance, proteolytic activities and fertilization success [14-16]. However, the repertoire, abundance, origin and functions of seminal plasma RNAs remain unknown in fish. Micro RNAs (miRNAs) are among the RNAs that are present in the sperm of vertebrates. These non-coding RNAs, approximately 22 nucleotides-long, function mainly in translational suppression by binding at 3’ UTR of mRNA to regulate various biological processes, including spermatogenesis [17]. In mouse, ingestion of sperm miRNA to oocytes causes heritable epigenetic change in gene expression [18]. In addition, a sperm-borne miRNA miR-34c is essential for the first cell division [2]. miRNA expression has been characterized in testes of some teleost fish [19], such as Atlantic halibut Hippoglossus hippoglossus [20].
Atlantic cod *Gadus morhua* [20], yellow catfish *Pelteobagrus fulvidraco* [21], or Nile tilapia *Oreochromis niloticus* [22]; however, the testis is a heterogenic organ composed of germline and somatic cell lineages, and the cellular origin of miRNAs has not been determined. Whole semen [23] and spermatozoal [24] miRNA profiling has been performed in zebrafish (*Danio rerio*), confirming abundance of miRNAs in fish sperm.

Quite often, the reported studies on sperm RNA refer to germ cell/spermatozoa RNA in reasoning and conclusions, while they have actually been performed on the whole semen. This is confusing, because no account is taken on possible non-spermatozoal RNA contribution in the whole semen. The objective of the present study was to profile miRNA transcriptomes in fish semen, fractionated to spermatozoa and seminal plasma, in order to determine the contribution and content of non-spermatozal miRNAs in the semen. We
chose Atlantic salmon (*Salmo salar*) as a model because of its relatively large body size and resulting collectible volumes of semen and blood plasma. To elucidate possible origin of seminal plasma miRNAs, we performed *in situ* hybridization of chosen differentially accumulated miRNAs in testis, and another experiment comparing chosen miRNAs in semen fractions with blood plasma miRNAs.

**Results**

**Atlantic salmon sperm small RNA sequencing and annotation**

On average, we obtained 31.6 million sequences per library and the total number of sequences exceeded 284 million. Of these, 53.8 % mapped to Atlantic salmon genome without a mismatch. The length distribution showed that over 44 % of the sequences were in size range of 42 – 43 nucleotides (nts) (Fig. 1A). In seminal plasma, some enrichment was found for the size range 27 – 35 nts compared to spermatozoa (34.6 % versus 17.9 %), whereas the fraction of size range 15 – 26 nts constituted 12.1% and 25.2 %, in seminal plasma and spermatozoa, respectively (Table 1 and Fig. 1A). In total, we found 30 205 087 unique sequences comprising 2.9 % miRNAs, 9 % piRNAs, 25.8 % tRNA fragments (tRFs) and 17.4 % mRNA fragments (Fig. 1B).

**miRNA profiling in the whole sperm and its fractions**

In total, we identified 306 mature miRNAs representing 97 families (Additional file 1). The highest average number of reads and miRNA diversity was found in the seminal plasma (Fig. 1C and Additional files 1 and 2). There was a high degree of similarity in miRNA types and their abundances between the whole semen and spermatozoa (*r* = 0.97), whereas the correlation was considerably lower (*r* = 0.61) between the seminal plasma and spermatozoa, as well as seminal plasma and the whole sperm (Additional file 3). The cluster analysis confirmed the separate clustering of seminal plasma samples versus the
whole semen and spermatozoa samples (Fig. 2). Sixteen miRNA families contributed to over 94% of all miRNA reads; out of them, four families (miR-15, miR-17, miR-25, and miR-30) constituted approximately 68%, 50%, and 65% of the total read counts for spermatozoa, seminal plasma and the whole sperm, respectively (Fig. 3). Notably, miR-202 constituted approximately 16% of all miRNA reads in the seminal plasma, whereas it was on negligible levels in the spermatozoa fraction.

We observed variation in normalized read counts among individuals. Highly pronounced variability (> 50 000 reads-per-million, rpm) was found in miR-25-3p, miR-30b-5p, and miR-92a-3p in spermatozoa fraction. In the seminal plasma samples, miR-30b-5p, miR-92a-3p, and miR-202-5p accumulation was considerably variable among males (Additional file 4).

The differential expression was profound between the seminal plasma and spermatozoa with 85 differentially accumulated miRNAs (Fig. 4). They represented 25.2% and 25.5% of the total miRNA read counts in the spermatozoa and seminal plasma, respectively. Among the top 10 dominant miRNAs, five miRNAs (miR-15c-5p, miR-16a-5p, miR-26a-5p, miR-30b-5p, and miR-92a-3p) did not show any significant differences between the semen fraction samples (Fig. 4). Some miRNAs, such as miR-730-5p, showed relatively higher accumulation in spermatozoa compared to seminal plasma (Fig. 4).

**Spatial expression of selected miRNAs in gonads**

Localization of selected differentially accumulated miRNAs in the testis was performed in order to determine whether the differential abundance between the sperm fractions was associated with cellular origin of miRNAs. The positive control, U6 snRNA, showed signal in nuclei of germ cells and somatic supporting cells in a juvenile testis (Additional file 5), whereas in a mature testis the signal was strong in somatic supporting cells but poorly distinguishable in spermatozoa due to their small size (Additional file 5). No signal was
detected in negative controls (Additional file 5). In juvenile testis, the two dominant miRNAs, miR-92a-5p and miR-202-5p, were localized to spermatogonia and somatic supporting cells surrounding lobules (Fig. 5A). In addition, miR-202-5p was found in primary spermatocytes, and its concentration in the space surrounding spermatogonia was prevalent (Fig. 5A). No signal was detected for miR-15c-5p, miR-30d-5p and miR-93a-5p (Fig. 5A), whereas miR-730-5p produced a faint signal (Fig. 5A). In mature testis, lobules were broken and spermatozoa were released to lumens surrounded by somatic supporting cells forming ridge-like structures (Fig. 5B). Given the fact that U6 snRNA positive control signal was poorly distinguishable in spermatozoa (Additional file 5), ISH of sections of mature testes provided information primarily on signal intensity in somatic supporting cells, which was strong for miR-30d-5p, miR-202-5p and miR-92a-5p, rather weak for miR-15c-5p, and poorly detectable for miR-93a-5p and miR-730-5p (Fig. 5A).

To investigate cross-sex conservation of the regulation of gonad development, we localized miRNAs in a juvenile ovary of Atlantic salmon. U6 snRNA control was giving clear signals in nuclei of germ cells and somatic cells. Similar to the juvenile testis, the ISH showed expression of miR-92a-5p and miR-202-5p in both previtellogenic oocytes and somatic supporting cells; the latter one was massively abundant. Signals for miR-15c-5p and miR-30d-5p were clear in oocytes but poorly distinguishable in somatic supporting cells, while miR-93a-5p and miR-730-5p expression was not distinguishable (Additional file 6).

Comparison of sperm and blood plasma miRNAs

To check whether the seminal plasma-enriched miRNAs originated from blood, we performed additional qPCR experiment. We found, in accordance with RNA-seq data, clear enrichment of the tested miRNAs in seminal plasma compared to the whole semen or spermatozoa, with the exception for miR-192, which was present in spermatozoa but
nearly absent in the seminal plasma (Fig. 6). We tested the expression of miR-451a meant as blood-specific miRNA in this experimental context. Indeed, blood plasma had high accumulation of miR-451a; although it was not detected in our RNA-seq experiment, miR-451a was also moderately present in the seminal plasma. Aside from miR-451a, miR-204 was relatively abundant in the blood plasma, while miR-145-3p, miR-148a-3p, and miR-7 were accumulated on low levels. None of the tested seminal plasma-enriched miRNAs showed similar abundance levels in the blood plasma.

**GO terms annotation of the differentially expressed miRNA targets**

To inspect the function of the differentially expressed miRNAs between spermatozoa and seminal plasma, we collected Atlantic salmon 3’UTR sequences from Genebank, which is not exhaustive since the genome annotation is incomplete. Out of 127 differentially expressed miRNAs, 101 miRNAs had 721 predicted target genes with 1573 assigned GO term annotations (Additional file 7). The highest numbers of GO term annotation were cellular integral component of membrane, GTP binding, ATP binding, metal ion binding, and regulation of transcription. This suggests that spermatogenesis is subject to regulation by the miRNAs and miRNA targets.

**Discussion**

In general, Atlantic salmon sperm contains diverse types of small RNAs, including miRNAs, tRNA fragments and piRNAs. In mature sperm, miRNAs were less abundant than piRNAs and the highest accumulation was observed for tRFs (25 % of the total reads). Enrichment of tRFs and reduction of piRNA have been reported during sperm maturation in mammals [26, 27].

Similarly, piRNAs cover 9 % of our small RNA sequencing. In teleost, piRNAs are abundant in testis and ovary mainly to protect the germline from transposon
Given that germ cells carry the genetic information for future generations and the rapid evolution of piRNA pathway in teleost [28], the role of recent whole genome duplication in these pathway need further investigation for which Atlantic salmon can be a good model.

**miRNA heterogeneity in sperm fractions**

We demonstrated for the first time that miRNAs in teleost semen have heterogenic cellular origin. miRNAs are present in body fluids through several possible export mechanisms, including microvesicles, apoptotic bodies, or high-density lipoproteins; they can also be vesicle-free [30, 31]. Differential accumulation of a substantial number of miRNAs in spermatozoa versus seminal plasma (Fig. 4) suggests that aside from germ cells, a considerable portion of semen miRNAs originates from non-germ cells. Very high correlation of miRNA profiles between the whole semen and spermatozoa contrasted with considerably lower correlations between the seminal plasma and both the whole semen and spermatozoa (Fig. 2). This indicates that miRNA heterogeneity in the seminal plasma (Fig. 3), as well as its diversity (Additional file 2) are mostly hidden when non-fractionated semen is analysed. This effect can be due to lower concentration of miRNAs in the seminal plasma as compared to that in spermatozoa.

Fish testis has germinal and interstitial compartments, which are composed of connective
tissue and Leydig cells. The connective tissue contains fibroblasts, immunological cells, collagen fibers, myoid cells, blood vessels and nerve fibers [32].

If seminal plasma miRNAs originated from the blood, they would likely reflect blood plasma miRNA content. However, our qPCR experiment demonstrates the lack of concordance between miRNA abundance profiles in seminal versus blood plasmas, with only two out of 13 miRNAs showing relative abundance in the blood plasma similar to that in the seminal plasma (Fig. 6). It suggests that the blood plasma is not the major source of the enrichment in seminal plasma miRNAs, which is in line with the existence of a blood-testis barrier in fish [33].

Concordance of ISH signal intensity in supporting somatic cells (Fig. 5) with the abundance of seminal plasma miRNAs (Fig. 4), along with intensive apoptosis in testicular cells associated with the final maturation of spermatozoa, suggests that somatic supporting cells, along with the germ cells, are a considerable source of seminal plasma miRNAs.

Diversity of miRNAs in the seminal plasma was higher than that in the whole sperm and spermatozoa (Additional file 2). This demonstrates that a certain contingent of low abundance miRNAs is not captured in the whole-semen samples subjected to a standard-depth sequencing. Aside from sperm fraction-specific expression, the captured diversity in the seminal plasma samples could result from the freeze-drying step in the procedure, which enhanced the efficacy of RNA extraction from the seminal plasma, where RNAs are natively highly diluted.
miR-202-5p, a conserved gonad-dominant miRNA in teleosts [25].

In mammals, miR-202-5p is a Sertoli cell-specific miRNA [36-38]. In contrast, it co-localizes with germ plasm components in zebrafish, suggesting its germline specificity [35]. However, in the recent study on zebrafish, miR-202-5p was highly abundant in mature gonads, both testes and ovaries, but not in the released mature spermatozoa or eggs [25].

In the present study, miR-202-5p localized to both germ and somatic lineages in juvenile testes and ovaries of Atlantic salmon, and was highly abundant in somatic
supporting cells in mature testes (Fig. 5 and Additional file 6). In medaka, miR-202-5p is abundant in unfertilized oocytes and in the follicular cells of the ovary, and is essential for regulation of oogenesis [39]. Together, these results suggest a conserved role of miR-202-5p in reproductive processes in teleost gonad development during gamete maturation, and indicate supporting somatic cell origin of miR-202-5p in a mature gonad.

To investigate whether the extracellular seminal plasma miRNAs have regulatory functions, or alternatively, whether they are just left-overs of the past regulatory processes, we constructed a minigene containing 6 targets sites for miR-202-5p and *in vitro* transcribed the sense and anti-sense of the construct. We collected fresh sperm from Atlantic salmon and incubated *in vitro* transcribed RNAs in the seminal plasma or the whole semen. However, both sense and antisense RNAs were degraded within the first moments of the incubation by unknown factors, possibly nucleases.

**miRNA accumulation in Atlantic salmon sperm**

Conserved and specific miRNAs were previously characterized in Atlantic salmon [40-42], but not in gonads or gametes. Some of the dominant miRNAs in Atlantic salmon semen, such as let-7a, miR-21a, miR-25, miR-26a, miR-128, and miR-202, were found abundant in zebrafish whole sperm analysis [24], and let-7a was among the most abundant miRNAs in human semen exosome
samples [43], indicating some functional conservation among divergent species. Abundant miRNAs reported in mouse, including miR-15c-5p, miR-16a-5p, miR-20a-5p, miR-30b-5p, miR-92a-3p, and miR-93a-5p were also found in Atlantic salmon sperm in the present study, indicating the enrichment of sperm with diverse types of miRNAs. Among these, miR-20a-5p and miR-93a-5p were expressed highly in mouse spermatozoa compared to spermatogonia with concomitant decrease in their predicted targets, signal transducer and activator of transcription 3 (Stat3) and phosphatase and tensin homolog (Pten) [44]. miR-202-5p was highly enriched in seminal plasma compared to spermatozoa, which is in-line with Sertoli-cell accumulation of this miRNA in human [36].

In contrast to mammals, miR-34 family was absent in Atlantic salmon sperm. Out of 35 miRNAs identified in human sperm [45], 23 miRNAs had homologs in our dataset, including miR-19, let-7, and miR-30 families. Some of these miRNAs have been associated with male fertility [36].
Although all top 26 miRNAs identified in zebrafish whole sperm [24] were also found in our dataset, the level of accumulation was dissimilar for some of the miRNAs; for example, miR-22 and miR-122 were among the abundant miRNAs in zebrafish, while they were less abundant in salmon sperm. Thus, it is important to characterize and profile small RNA constituents of various teleost fish to obtain unifying and discriminatory features to understand the role of miRNAs in semen. Previous study showed that primary miRNAs (pri-mir-1181, pri-miR-3648, pri-miR-3687, pri-mir-663 and pri-mir-181c) are found in human testis and spermatozoa [4]. This indicates the enrichment of sperm not only in mature miRNAs, but also in the preceding forms, which may be transferred to the oocyte and further processed. The role of miRNAs beyond spermatogenesis has been illustrated by miR-34c, which contributes to the first cleavage by regulating B-cell leukemia/lymphoma 2 [2]. miR-34 family has been implicated in the development of bovine gametes and
embryos [48]. The role of sperm-borne miRNAs in aquatic species with external fertilization strategy is not known.

Spermatogenic cells strictly depend on their interaction with the somatic elements of the testis, which requires expression of many genes encoding proteins with a role in epithelial transport. Many of the predicted targets of differentially expressed miRNA include several cation, anion and solute transport-related processes; for example, Solute carrier family 41 member 2, Vesicle transport protein SFT2B, potassium channel subfamily K member 6, among others. Among several kinds of activating signals, ions play a major role in salmonid sperm activation [49].

Proteolysis pathways genes were predicted targets of differentially expressed miRNAs. In salmonids, proteasomes modulate the activity of outer arm dynein for activating dynein-driven microtubule sliding for sperm motility [50].

Genes that regulate sperm motility, such as cAMP-responsive element-binding protein-like 2, ATP binding proteins were among the predicted targets. Previously cAMP-dependent phosphorylations of axonemal proteins have been reported to regulate the motility of sperm in salmonid fish [51].
In general, miRNAs regulates several genes that are involved in regulation of transcription, membrane transport, ATP synthesis pathways indicating their importance in Atlantic salmon spermatogenesis.

**Plausible sources and functions of seminal plasma miRNAs**

So far, no study clarified the source and role of miRNAs in the seminal plasma. miRNAs can be present in the seminal plasma because they are leftovers after the spermatogenesis, which is an intricate process involving cell proliferation and differentiation, as well as expulsion of cytoplasm content, and cell membrane disintegration.

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Many miRNAs that have been implicated in mammalian spermatogenesis were found in Atlantic salmon seminal plasma in the present study. Thus, seminal plasma miRNAs could be leftovers of the preceding spermatogenesis process. Accumulation during the enrichment of semen with organic and inorganic components from the blood plasma could hypothetically be another source of miRNAs in the seminal plasma. A study on proteome in common carp (*Cyprinus carpio*) indicated that the majority of seminal plasma proteins was similar to blood plasma proteins.

However, in the present study, the profile of miRNAs in blood plasma versus seminal plasma was clearly dissimilar (Fig. 6), with only one (miR-204) of 13 miRNAs
showing concordant transcript levels, one miRNA (miR-451a) enriched in blood plasma, and six seminal plasma miRNAs absent in the blood plasma. This indicates that if the blood plasma is the source of seminal plasma miRNAs, the accumulation is rather selective than passive. However, no mechanism for such the selective accumulation is known.

Most teleost fish has immobile spermatozoa in the seminal plasma, external fertilization, and short fertilization window; all of this suggests that the seminal plasma RNAs unlikely establish an effective contact with an egg. Hence, the role of seminal plasma miRNAs may be restricted to maintaining conducive environment for spermatozoa before the spermiation. Seminal plasma contains inhibitory factors that prevent the internalization of foreign nucleic acids.

\[53-56\]

It has been demonstrated that DNA-binding proteins at sperm cell surface allow internalization of exogenous DNA in the absence of inhibitory factors in the seminal plasma.

\[57\]

Spermatozoa can be also transfected by RNA elements.

\[58\]

Exogenous DNA transfer to sperm has been shown in salmonids.

\[59\]
Seminal plasma miRNAs can protect spermatozoa against exogenous nucleic acid invasions, because they can exist in extra vesicular-free forms, associating with high-density lipoproteins or Argonaute proteins.

miRNAs have been demonstrated to act on targets outside the cell; for instance, intestinal epithelial cells release miRNAs to the gut to control the type and the level of microbiota.

Thus, it is possible that miRNAs bound to Argonaute proteins are among the inhibitory factors that facilitate degradation of exogenous RNAs in the seminal plasma.

Conclusions

In summary, we characterized miRNAs in Atlantic salmon sperm fractions: spermatozoa and seminal plasma. Differential expression of some miRNAs suggested that seminal plasma miRNA originated not only from spermatozoa. Testicular localization of selected miRNAs indicated distinctive expression in spermatozoa and germ cell supporting cells. As miRNA profile in blood plasma showed low coherence with that from the seminal plasma, testicular supporting somatic cells are likely the source of miRNA enrichment in the seminal plasma. Their functions are unknown; we hypothesize their role in inhibition of exogenous nucleic acids in the sperm.

Methods

Ethics
Animal care and handling were done according to the guideline stated in Norwegian law of Animals in Research (The Norwegian Animal Protection Act no. 73 of 20 December 1974, Section 20-22, amended 19 June 2009).

Animals, sperm collection and fractionation

For small RNA sequencing experiments, sperm samples were collected from three sexually mature males from AquaGen Atlantic salmon breeding facility (AquaGen AS, Trondheim, Norway) by applying a gentle abdominal massage. The first portion of the milt was avoided to reduce possibility of contamination with urine. Ziploc bags with sperm samples were placed on a crushed ice (0 – 2 °C) in an insulated polystyrene box and shipped overnight to Nord University, Bodø, Norway, for further processing. Upon arrival, sperm samples were checked for the quality under the microscope [63]. Special emphasis was given to determine whether there was a contamination with exogenous cells, such as blood or other somatic cells, or bacteria. Each sperm sample was thoroughly screened through visual observation under the microscope (minimum five sub-samples, each screened in multiple view areas) for presence of exogenous material. No visual contamination was found. Part of each sperm sample was fractionated to spermatozoa and seminal plasma. The separation was performed by centrifugation of 15 ml whole semen at 6,000 rpm for 30 min at 4 °C. After centrifugation, 6 ml of the upper clear layer of the supernatant was transferred to a new tube. To eliminate contamination of seminal plasma with spermatozoa, lower part of supernatant was avoided. Absence of spermatozoa in the seminal plasma was checked under the microscope. The interphase between seminal plasma and the spermatozoa pellet was discarded. For spermatozoa fraction, to remove seminal plasma leftovers, spermatozoa pellet was diluted in an
isotonic Hank’s balanced salt solution, centrifuged as above, and the supernatant was discarded. The procedure was repeated. The whole sperm samples, as well as corresponding fractions of spermatozoa and seminal plasma, were snap-frozen in liquid nitrogen and stored at – 80 °C until RNA extraction.

For quantitative reverse transcription PCR (RT-qPCR) experiment, sperm and blood samples were obtained from five adult males (>10 kg weight) of AquaGen origin. Seminal plasma and spermatozoa fractions were separated as described above. Two ml of blood was taken from caudal vein using a needle and syringe, centrifuged at 2000 rpm for 10 min to obtain blood plasma, which was snap frozen in liquid nitrogen and stored at – 80 °C until RNA extraction.

RNA extraction and small RNA sequencing

Total RNA was extracted from the whole semen, as well as its fractions: spermatozoa and freeze-dried seminal plasma, using Trizol (Invitrogen, Carlsbad, California, USA) followed by extraction with chloroform, and ethanol precipitation at – 80 °C overnight after adding glycogen (Life Technologies, Foster City, CA, USA) and 3M sodium acetate (Life Technologies). Precipitates were recovered by centrifugation at 12000 rpm/4 °C for 1 h, washed with 750 µl 70 % ethanol, and re-suspended in 20 µl RNase-free water. Quality of RNA was checked using bioanalyzer (Agilent Technologies, Waldbronn, Germany). Lack of contamination with exogenous cells in sperm samples was confirmed by the absence of 18S RNA and 28S RNA fractions [64]. RNA samples were stored at – 80 °C until sequencing library preparation.

Sequencing libraries were prepared using NEXTflex™ Illumina Small RNA Sequencing Kit v2
(Bioo Scientific, Austin, TX, USA) and sequenced on NextSeq 500 (Illumina, San Diego, CA, USA) at the Genomics & Cell Characterization Core Facility, University of Oregon.

**Data analysis**

Adapter sequences were trimmed using cutadapt [65] and sequence quality was checked using FastQC [66]. Sequences were mapped to Atlantic salmon miRNAs (www.miRBase.org) and to the salmon reference genome (ICSASG_v2 downloaded from www.SalmoBase.org) using Bowtie [67] with single mismatch and best alignment reporting. tRNAs were predicted using tRNAscan-SE [68] and conserved piRNAs were identified by mapping to zebrafish piRNA from piRBase [69].

Differential expression of miRNAs was determined for the whole semen, spermatozoa, and seminal plasma of Atlantic salmon by applying negative binomial generalized linear model
using DESeq2 [ ]

with the minimum threshold of 10 reads. We tested the differential accumulation for miRNAs that showed >2-log fold change between the groups. miRNA targets were predicted using Targetspy [ ] on 3’UTRs extracted from genebank [72] and GO-term analysis for those targets genes was performed using Blast2Go [ ].

In situ hybridization

To determine cellular origin of miRNAs in sperm, we performed in situ hybridization (ISH) on samples of mature (n = 3) and juvenile (2-year-old, weight 500 g, n = 3) testes fixed in Bouin’s solution (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and embedded in paraffin. We also performed ISH on juvenile (n = 3) ovary sections for cross-sex comparisons. We used double-DIG-labeled probe (5’-DIG and 3’-DIG) for miR-15-5p, miR-30d-5p, miR-92a-3p, miR-93a-5p, miR-202-5p, and miR-730-5p (miRCURY LNA™ Detection probe, Exiqon, Copenhagen, Denmark) according to manufacturer’s instructions. In brief, embedded gonad sections (2.5 µm for juvenile testis, 5 µm for mature testis and 5 µm for juvenile ovary) were incubated at 60 °C for 45 min to melt the paraffin, and stored overnight at 4 °C. On the next day, the sections were deparaffinized in xylene for 15 min
and then re-hydrated through serially decreasing ethanol solutions. Targets were demasked using proteinase K (12 µg/ml) for 10 min. Sections were dehydrated in gradual ethanol solutions and dried. Denatured probes were diluted in 1x hybridization solution to a final concentration as shown in Table S1. Sections were framed using Gene frame (Thermo Scientific, England, UK), and a total of 125 µl was applied on each slide. The slides were then covered with Gene frame plastic cover slip and hybridized for 60 min at ~30 °C below the melting temperature of each probe (Additional file 8). Slides were washed using 5X, 1X, and 0.2X SSC buffer (Sigma-Aldrich) at the hybridization temperature. Digoxin was recognized by a sheep anti-DIG-AP directly conjugated with alkaline phosphatase (AP) (Roche, Mannheim, Germany). The specimens were incubated for 60 min at room temperature. AP converted the applied substrate into a water and alcohol insoluble dark-blue precipitate that appeared on slides after 2 h of incubation at 30 °C in the dark. The reaction was stopped and slides were counterstained with 0.1 % Nuclear Fast Red™ (Vector laboratories, CA, USA). After washing in tap water for 10 min and dehydration with sequential ethanol solutions, the slides were mounted using PERTEX® (HistoLab, Goteborg, Sweden). The slides were examined under Olympus BX51 microscope (Olympus, Tokyo, Japan) on the subsequent day and images were obtained using the CellB 27, build 1224 imaging software (Olympus).

**Quantitative reverse transcription PCR (RT-qPCR)**

In total, we performed RT-qPCR for selected 14 miRNAs using miRCURY LNA Universal RT microRNA PCR assays (Exiqon, Vedbaek, Denmark). Ten µl reverse transcription reaction was performed using the Universal cDNA synthesis kit with 20 ng total RNA in accordance with the manufacturer protocol. Reverse transcription thermocycling parameters were 42°C for 60 min, 95°C for 5 min. cDNA was diluted 1 in 80 and PCR was performed using
Sybr Green mastermix (Exiqon) as per manufacturer instructions with 10 µl reactions volume using a Roche480 thermal cycler (Roche). PCR thermocycling conditions were as follows: 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 1 min, melt curve analysis performed between 60–95°C for 15 min at a ramp-rate of 1.6°C/s. Second derivatives methods was used to calculate the quantification cycle (Cq) value. miR-429a was used as an endogenous reference, because it was expressed stably throughout the sample types (Additional file 9). We performed pairwise comparisons using Wilcoxon rank sum test with Bonferroni correction, and p-value < 0.05 was considered as significant.

**Abbreviations**

3’ UTR – 3’ untranslated region  
cDNA – complementary DNA  
FC – fold-change  
FDR – false discovery rate  
GO – gene ontology  
ISH – *in situ* hybridization  
mRNA - messenger RNA  
miRNA - micro RNA  
nt - nucleotide  
piRNA – PIWI protein-interacting RNA  
RPM - reads per million  
RT-qPCR - real-time quantitative reverse transcription PCR  
tRF – tRNA-derived fragment

**Declarations**

**Ethics approval and consent to participate**
No human subjects were involved in this study. For the procedures used and material collected, no formal ethics approval is needed. All experimental procedures and animal handling complied with the guidelines of Norwegian regulation for laboratory animal experimentation (The Norwegian Animal Protection Act, No. 73 of 20 December 1974, Section 20–22, amended 19 June 2009).

Consent to publish
Not applicable

Availability of data and material
Data is available at SRA. Accession number: PRJNA501899

Competing interests
The authors declare no competing interests.

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Author Contributions
TTB and IB conceived, designed and performed the experiments. TTB analyzed the data.

Both authors wrote and approved the final version of the manuscript.

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**Table 1**

Table 1. Summary of sequencing depth (total reads, in millions), number of miRNA reads (in millions), and average read per size for three small RNA size groups (miRNAs, piRNAs and other small RNAs). The latter one is calculated using the number of total reads of a given size divided by a number of unique sequences in a given size.
| Sample type       | Total reads (min - max) | Total miRNA reads (min - max) | Average read/size |
|-------------------|-------------------------|-------------------------------|-------------------|
|                   |                         | 15 - 26 nt                   | 27 - 35 nt        | 36 - 40 nt |
| Whole sperm       | 31.2 - 37               | 0.6 - 0.9                    | 48681             | 70808      | 50636      |
| Spermatozoa       | 29.7 - 37.2             | 0.4 - 0.6                    | 52191             | 62011      | 51635      |
| Seminal plasma    | 20 - 35.5               | 0.4 - 2.7                    | 19026             | 17126      | 8529       |

Additional Files Legends

**Additional file 1.** Raw miRNA read counts in samples of the Atlantic salmon whole sperm and its fractions: spermatozoa and seminal plasma, obtained from three individuals.

**Additional file 2.** Saturation plot for the number of miRNAs detected in the Atlantic salmon whole sperm and its fractions: spermatozoa and seminal plasma, in relation to the sequencing depth. The full circles indicate the inflection point where no more additional type of miRNA can be detected with the given sequencing depth.

**Additional file 3.** Correlations of miRNA abundances among the replicates and samples of the Atlantic salmon whole sperm and its fractions: spermatozoa and seminal plasma. From the top-right corner, Pearson’s correlation coefficient r-value is given (the font size marks reflects the value of r). Asterisk indicates significance at p-value < 0.05. From the bottom-left corner, correlation plots are displayed (values in read-per-million).

**Additional file 4.** Individual variability in accumulation of some abundant miRNAs in fractionated spermatozoa (A) and seminal plasma (B) from semen samples obtained from the three Atlantic salmon males.

**Additional file 5.** *In situ* hybridization using LNA probes for U6 snRNA positive and scramble negative controls in juvenile and mature Atlantic salmon testis sections.
**Additional file 6.** *In situ* hybridization for 6 miRNAs and U6 snRNA LNA probes in juvenile Atlantic salmon ovary sections. Different stages of previtellogenic oocytes are visible. Scale bars in left column = 100 µm; in the middle column = 50 µm; in the right column = 200 µm. Abbreviations: Oc-previtellogenic oocyte; Nc-nuclues; Gc-granulosa cell; Tc-Theca cell.

**Additional file 7.** miRNA targets and GO-term annotation.

**Additional file 8.** Double DIG-labelled LNA probes for *in situ* hybridization. Name, sequence, melting temperature, concentration of probe and hybridization temperature used for *in situ* experiment.

**Additional file 9.** The stability of miR-429 expression used for normalization of RT-qPCR in Atlantic salmon whole sperm, seminal plasma, spermatozoa and blood plasma. Five individuals were used.

**Figures**
Figure 1

Major composition of small RNAs in Atlantic salmon sperm. A) Sequence length distribution for each small RNA library of whole sperm, spermatozoa and seminal plasma. B) Annotated RNA types. C) miRNA size distribution in the whole sperm, spermatozoa and seminal plasma.
Cluster analysis of consistency of miRNA contents and abundances among the samples of the whole sperm and its fractionated spermatozoa and seminal plasma, obtained from three Atlantic salmon individuals. The dendrogram represents the similarity between samples.
Figure 3

The most dominant miRNAs in the whole sperm and its fractions: spermatozoa and seminal plasma, in Atlantic salmon.
Figure 4

Differentially accumulated miRNAs between spermatozoa and seminal plasma fractions of Atlantic salmon sperm. Vertical and horizontal dashed-lines show fold change cut-off of 2 and adjusted p-value of 0.05, respectively. Red and labeled dots represent differentially accumulated miRNAs between the spermatozoa and seminal plasma fractions. FDR = false discovery rate; LogFC = logarithmic fold-change rate.
Localization of six miRNAs in sections of A) juvenile and B) mature Atlantic salmon testes, using in situ hybridization with LNA probes. Scale bars in the left column of each panel represent 100 µm, while the scale bars in the right column represent 50 µm. Abbreviations: C-cyst; LC-Leydig cell; ST- Sertoli cell; SG-spermatogonia; SC I- primary spermatocytes; Sz-spermatozoa.
Figure 6

RT-qPCR for selected miRNAs in the blood plasma versus the whole sperm and its fractions (spermatozoa and seminal plasma). Relative expression values were obtained after normalizing to miR-429 expression. Average values are given (n=6). Bars represent standard deviations. Letter indicates p-value < 0.05

Supplementary Files

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