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Differential Expression of Oar-Mir-133, Oar-Mir-433-3p Oar-Mir-150, and Oar-Mir-376d in Ram Sperm Associated with Fertility

Koc Spermasında Fertilite ile İlişkili Olan Oar-Mir-133, Oar-Mir-433-3p, Oar-Mir-150 ve Oar-Mir-376d'nin Farklılaşmış Gen İfadesi

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ABSTRACT

Objective: Ram fertility is a crucial determinant of reproductive efficiency in sheep production systems, yet the molecular mechanism of male fertility remains incompletely understood. Small non-coding RNAs (sncRNAs), particularly microRNAs (miRNAs), have been recognized as essential epigenetic regulators of spermatogenesis, sperm function, and early embryonic development. We aimed to investigate the differential expression and biological significance of four specific miRNAs; oar-miR-133 (log2FC = +3.30, p < .001), oar-miR-433-3p (log2FC = +5.72, p < .001), oar-miR-150 HF (log2FC = -3.49, p < .001), and oar-miR-376d (log2FC = -5.74, p < .001) in relation to ram sperm fertility phenotypes

Methods: We selected based on their statistically significant differential expression in a previously published small RNA sequencing dataset comparing spermatozoa from high-fertility (HF; n = 4; avg. 99.2% pregnancy) and low-fertility (LF; n = 4; avg. 73.6% pregnancy) rams.

Results: We showed that oar-miR-133 and oar-miR-433-3p were significantly upregulated in HF rams, while oar-miR-150 and oar-miR-376d were downregulated. Functional enrichment analysis of their predicted target genes revealed involvement in biological processes such as heterochromatin formation, cellular response to stress and cytokines, macromolecule metabolic regulation, and developmental processes. Protein–protein interaction (PPI) network analysis, using Maximal Clique Centrality (MCC), identified several central hub genes including DICER1, DROSHA, DDX10, TUFM, and EEF2. We showed that hub genes may be associated with RNA metabolism, mitochondrial function, and translational control processes critical for sperm integrity and fertilization capacity.

Conclusion: These results highlight the potential of specific miRNAs and their gene networks as biomarkers for ram fertility.

Keywords: Biomarker, fertility, miRNA, ram, sperm.

ÖZ

Amaç: Koç fertilitesi, koyun üretim sistemlerinde üreme verimliliğinin kritik bir belirleyicisidir; ancak erkek fertilitesinin moleküler mekanizması henüz tam olarak anlaşılamamıştır. Özellikle mikroRNA'lar (miRNA'lar) gibi küçük kodlamayan RNA'lar (sncRNA'lar), spermatogenez, sperm fonksiyonu ve erken embriyonik gelişimde temel epigenetik düzenleyiciler olarak kabul edilmektedir. Bu çalışmada, koç spermi fertilite fenotipleriyle ilişkili dört spesifik miRNA'nın; oar-miR-133 (log2FC = +3.30, p < ,001), oar-miR-433-3p (log2FC = +5.72, p < ,001), oar-miR-150 HF (log2FC = -3.49, p < ,001) ve oar-miR-376d (log2FC = -5.74, p < ,001) diferansiyel ekspresyonu ve biyolojik önemi araştırılmıştır.

Yöntem: miRNA'lar daha önceki sekanstan elde edilmiş küçük RNA dizileme veri setindeki istatistiksel olarak anlamlı diferansiyel ekspresyonlarına göre seçildi. Bu amaçla yüksek (YF; n = 4; ort. %99,2 gebelik) ve düşük doğurganlığa sahip (DF; n = 4; ort. %73,6 gebelik) koçlardan elde edilen spermatozoalar karşılaştırılmıştır.

Bulgular: Çalışmamızda oar-miR-133 ve oar-miR-433-3p'nin HF koçlarda anlamlı şekilde yukarı yönlü regüle edildiği, oar-miR-150 ve oar-miR-376d'nin ise aşağı yönlü regüle edildiği saptanmıştır. Tahmini hedef genlerinin fonksiyonel zenginleştirme analizi, heterokromatin oluşumu, hücresel stres ve sitokinlere yanıt, makromolekül metabolik regülasyonu ve gelişimsel süreçler gibi biyolojik süreçlerde rol oynadıklarını ortaya koymuştur. Maksimal Clique Merkeziliği (MCC) kullanılarak yapılan Protein-Protein Etkileşim (PPI) ağı analizi, DICER1, DROSHA, DDX10, TUFM ve EEF2 gibi çeşitli merkezi "hub" genleri tanımlamıştır. Bu hub genlerinin RNA metabolizması, mitokondriyal fonksiyon ve sperm bütünlüğü ile fertilizasyon kapasitesi için kritik olan translasyonel kontrol süreçleriyle ilişkili olabileceği gösterilmiştir.

Sonuç: Bu sonuçlar, spesifik miRNA'ların ve bunların gen ağlarının koç doğurganlığı için potansiyel biyobelirteçler olarak önemini vurgulamaktadır.

Anahtar Kelimeler: Biyobelirteç, doğurganlık, koç, miRNA, sperm.

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Introduction

Male fertility is crucial for reproductive success, particularly in sheep farming where one ram can significantly influence the genetic makeup of an entire flock. Although reproductive technologies have advanced, predicting male fertility remains difficult due to the intricate interaction between compensable and non-compensable factors. Standard semen analysis methods, while beneficial, often miss the finer molecular details that are key to understanding a ram's true fertilizing potential (Kastelic & Thundathil, 2008).

Among the non-compensable factors, small non-coding RNAs (sncRNAs) such as microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), and fragments derived from tRNAs have been identified as key players in regulating sperm development, function, and early embryo formation (Bodu et al., 2025a; Bodu et al., 2025b; Sharma, 2019). These RNA molecules are incorporated into sperm during their development in the testes and epididymis, and are thought to affect gene activity after fertilization by triggering epigenetic changes (Sendler et al., 2013).

MicroRNAs are essential regulators of cellular functions, primarily by degrading messenger RNAs or preventing their translation. In the context of male reproduction, these molecules are tied to vital processes like sperm motility, capacitation, programmed cell death, and chromatin remodeling (Abu-Halima et al., 2013; Capra et al., 2017). Rather than being residuals, the miRNAs found in sperm are thought to have active roles. Changes in their expression have been linked to differences in fertility across multiple species, including cattle, pigs, and humans (Salas-Huetos et al., 2019).

In livestock breeding, gaining insight into the miRNA profile of sperm cells is crucial because sire fertility directly affects both economic outcomes and genetic progress. Despite this importance, detailed profiling of small non-coding RNAs (sncRNAs) in ram sperm is still scarce. To bridge this gap, our earlier research employed small RNA sequencing on sperm samples from rams with clearly defined fertility levels (Hitit et al., 2024). That investigation identified both known and previously unreported sncRNAs, uncovering clear differences in their expression patterns between rams with high and low fertility.

In this study, we focus on four miRNAs such as oar-miR-133, oar-miR-433-3p, oar-miR-150, and oar-miR-376d that showed the most significant expression differences linked to fertility. Each of these miRNAs is associated with key biological functions: miR-133 is involved in germ cell differentiation, miR-433 plays a role in neuroendocrine regulation, miR-150 is tied to immune system modulation, and miR-376d is connected to the cellular stress response (Kasimanickam et al., 2022; Luo et al., 2016; Zhang et al., 2012). Our goal is to further characterize their potential as non-invasive biomarkers for ram fertility and to establish a foundation for functional studies that can validate their mechanistic roles in sperm physiology and embryo development. By integrating high-throughput sequencing with fertility phenotyping and functional annotation, this study contributes to the growing field of reproductive epigenetics and offers practical insights for improving sire selection in small ruminant breeding programs.

Methods

Ram Fertility Assessment and Experimental Design

Fertility data were obtained from Anatolian Merino rams housed at Bahri Dagdas International Agricultural Research Institute, Konya, Türkiye. Rams were evaluated over three breeding seasons based on the pregnancy outcomes of naturally mated ewes. Ewes' estrus behavior was monitored daily using teaser rams, and mating events were systematically recorded. Fertility scores were calculated based on confirmed pregnancies (high-fertility group [n = 31; 94.5 ± 2.8%] and the low-fertility group [n = 25; 83.1 ± 5.73%] and used to classify rams into high-fertility (HF) and low-fertility (LF) groups. Rams with the highest (n = 4; avg. 99.2% pregnancy) and lowest (n = 4; avg. 73.6% pregnancy) conception rates were selected for molecular analysis (Bodu et al., 2025c).

Semen Collection

Following ethical (Bahri-Dagdaş Research Center Ethical Committee, Turkey [Number: 22.12.2016/58] approval, semen was collected from mature rams using an artificial vagina (AV) after conditioning them with teaser ewes. To ensure consistency and minimize variation, initial ejaculates were discarded prior to the experimental collection. Semen samples with high motility and concentration were aliquoted and diluted to a standardized sperm count. Samples were then cryopreserved in liquid nitrogen and stored at −80 °C until RNA extraction.

RNA Isolation

Frozen sperm samples were thawed and passed through a 500-mesh filter to eliminate debris. Somatic cell contaminants were lysed using a Triton X-100 and SDS buffer, followed by washing and microscopic validation to confirm sperm cell purity. Total RNA was extracted using a modified column-based microRNA isolation kit. Lysis involved guanidine thiocyanate buffer enriched with DTT, and homogenization was performed by repeated needle shearing. RNA was purified with DNase treatment to eliminate residual genomic DNA. Concentration and integrity were measured using NanoDrop and Bioanalyzer systems to ensure suitability for small RNA sequencing (Hitit et al., 2024).

Library Preparation and Sequencing

For small RNA library construction, one microgram of total RNA per sample was used. RNA fragments corresponding to small RNAs (18–40 nt) were size-selected using polyacrylamide gel electrophoresis. Libraries were constructed using the TruSeq Small RNA Library Prep Kit (Illumina), which included adaptor ligation, reverse transcription, and PCR amplification. Library quality was validated via Qubit fluorometry and Bioanalyzer assessment. Pooled libraries were sequenced on the Illumina HiSeq 2500 platform with 50 bp single-end reads.

Bioinformatics and Functional Analysis

Sequencing data were processed to remove low-quality reads, adaptors, and contaminants. Clean reads were aligned to the Ovis aries reference genome using Bowtie. Known miRNAs were identified using miRBase annotations, while novel candidates were predicted via miREvo and miRDeep2 algorithms. Expression levels were normalized as TPM (transcripts per million). Differential expression analysis between HF and LF groups was conducted using DESeq2 with $p < .05$ and $|\log2FC| > 1$ considered significant. The four miRNAs highlighted in this study oar-miR-133, oar-miR-433-3p, oar-miR-150, and oar-miR-376d were selected from a broader dataset generated and analyzed in our previous publication (Bodu et al., 2025a). These candidates were chosen based on their statistically significant expression patterns and putative roles in fertility-related molecular functions.

Results

Differentially expression of oar-miR-133, oar-miR-433-3p oar-miR-150, and oar-miR-376d

Four miRNAs were identified as significantly differentially expressed between HF and LF sperm: oar-miR-133: Upregulated in HF (log2FC = +3.30, $p < .001$), oar-miR-433-3p: Upregulated in HF (log2FC = +5.72, $p < .001$), oar-miR-150: Downregulated in HF (log2FC = -3.49, $p < .001$), oar-miR-376d: Downregulated in HF (log2FC = -5.74, $p < .001$). These miRNAs are involved in key biological processes such as apoptosis regulation, differentiation, and epigenetic modulation. miR-133 has been implicated in muscle and germ cell development, while miR-433 and miR-376d are often associated with neurogenesis and stress response pathways. miR-150 is a known modulator of immune-related pathways and cellular differentiation.

Functional Enrichment Analysis of Target Genes

To ascertain the potential biological roles of the four fertility-associated miRNAs (oar-miR-133, oar-miR-433-3p, oar-miR-150, and oar-miR-376d), we performed functional enrichment analysis of their predicted target genes using the g:Profiler platform for Ovis aries. The results revealed significant enrichment in multiple Gene Ontology (GO) categories across the three primary domains: Molecular Function (MF), Biological Process (BP), and Cellular Component (CC). In the MF category, target genes were predominantly associated with catalytic activity (GO:0003824, p = 3.47×10^{-4}), small molecule binding (GO:0036094, p = 4.72×10^{-3}), and ion binding (GO:0043167, p = 6.24×10^{-3}), indicating their roles in enzymatic regulation and molecular interactions critical for sperm metabolism and function. In the BP category, notable terms included catabolic process (GO:0009056), positive regulation of biological process (GO:0048518), and developmental process (GO:0032502), highlighting the involvement of target genes in cellular remodeling and differentiation pathways. Importantly, genes involved in constitutive heterochromatin formation (GO:0140719) and heterochromatin formation (GO:0031507) were significantly enriched, suggesting a potential link between these miRNAs and sperm chromatin organization or epigenetic state. Additional enrichment in response to stress (GO:0006950), response to cytokine (GO:0034097), and positive regulation of macromolecule metabolic process (GO:0010604). Within the CC category, enriched terms such as cytoplasm (GO:0005737), catalytic complex (GO:1902494), endomembrane system (GO:0012505), and plasma membrane (GO:0005886) (Figure 1).

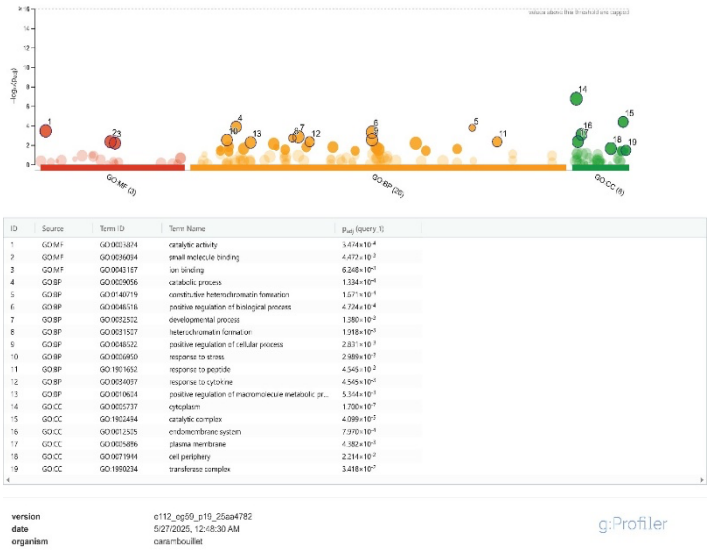

Figure 1.

Gene Ontology (GO) enrichment analysis of predicted target genes of differentially expressed miRNAs. GO terms were classified into three functional categories: Biological Process (BP), Molecular Function (MF), and Cellular Component (CC). The plot displays the top-ranked GO terms based on –log10 (p-value).

Hub Gene Analysis of target genes

To identify key regulatory elements among the oar-miR-133, oar-miR-433-3p oar-miR-150, and oar-miR-376d targets, we constructed a protein–protein interaction (PPI) network and performed hub gene analysis. The top 20 hub genes were visualized based on their connectivity and centrality scores. Among these, DDX10, DICER1, DROSHA, and RPL13A emerged as the most prominent nodes, highlighting their potential roles as regulatory nodes in post-transcriptional control, RNA helicase function, and ribosomal assembly. The presence of both DROSHA and DICER1, core enzymes in the miRNA biogenesis pathway. Other hub genes such as TUFM (involved in mitochondrial protein synthesis), EEF2 (elongation factor 2), and MRPL32 (mitochondrial ribosomal protein L32) underscore the importance of translational control and mitochondrial function in sperm biology aligning with known requirements for ATP generation and flagellar motility. Interestingly, a separate subnetwork consisting of CDK1, PBK, and KIF2C was identified (Figure 2).

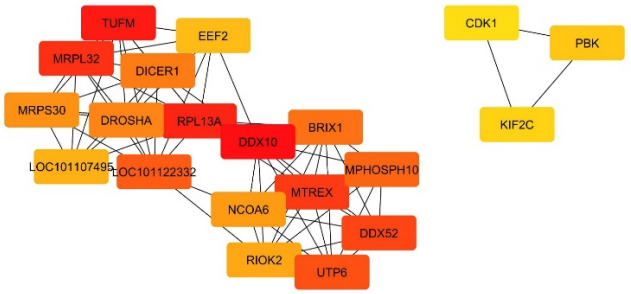

Figure 2.

Hub genes from target genes associated with fertility. Hub gene network constructed from predicted targets of four fertility-associated miRNAs. The top 20 hub genes were identified through protein–protein interaction (PPI). Node color intensity corresponds to MCC scores, with red indicating the highest centrality and yellow the lowest among the top-ranked nodes.

Discussion

This study provided new insights into the molecular mechanisms associated with ram fertility by focusing on four miRNAs oar-miR-133, oar-miR-433-3p, oar-miR-150, and oar-miR-376d whose expression patterns were found to be significantly different between high-fertility and low-fertility rams. The differential expression of these miRNAs supports their potential role in regulating post-transcriptional gene expression during spermatogenesis and sperm maturation.

Our finding that oar-miR-133 and oar-miR-433-3p are upregulated in high-fertility rams suggests that they may enhance sperm function or stability. miR-133 has previously been associated with cell differentiation and stress responses, including regulation of actin cytoskeleton dynamics, which is essential for sperm motility (Luo et al., 2023). Similarly, miR-433 is known to modulate genes involved in cell growth, hormonal signaling, and metabolic regulation (Carè et al., 2007), and may thus play a role in sperm energy metabolism or interaction with the female reproductive tract.

On the other hand, oar-miR-150 and oar-miR-376d were significantly downregulated in high-fertility rams, which could reflect reduced activation of immune or stress pathways. miR-150 is a known regulator of immune cell differentiation and inflammatory response (Song et al., 2023), and its lower expression in high-fertility rams may indicate a more stable testicular microenvironment conducive to spermatogenesis. miR-376d has been implicated in oxidative stress and apoptosis signaling (Xu et al., 2020), suggesting that its suppression might protect spermatozoa from damage during transit or storage.

The enrichment of gene targets in GO terms related to heterochromatin formation, response to cytokines, metabolic processes, and developmental regulation underscores the multifaceted roles these miRNAs likely play in maintaining sperm functionality and genomic integrity. Notably, the enrichment of targets within the catalytic complex, cytoplasm, and endomembrane system implies compartmentalized regulatory activity that might influence localized translation, mitochondrial activity, or vesicle-mediated signaling all of which are vital for sperm motility, capacitation, and fertilization (Jodar et al., 2018; Krawetz et al., 2011).

The identification of hub genes such as DICER1, DROSHA, and DDX10 suggests the possibility of autoregulatory feedback loops in which miRNAs modulate their own biogenesis machinery. DICER1 and DROSHA are essential enzymes for miRNA maturation, and alterations in their regulation could influence global miRNA expression profiles within sperm (Shen and Hung, 2015). Other key hub genes like TUFM, a mitochondrial translation factor, and EEF2, which controls ribosomal elongation during translation, indicate that energy metabolism and protein synthesis are crucial in defining sperm fertility potential. Furthermore, the presence of mitosis-related genes such as CDK1 and KIF2C in the hub network raises the possibility that miRNA-target interactions may also influence early events post-fertilization, including paternal genome remodeling and zygotic cell cycle regulation. This is supported by findings in other mammals, where paternal RNAs contribute to embryonic development beyond the fertilization event (Hamatani, 2012).

Taken together, the integration of expression profiling, GO enrichment, and PPI network analysis in this study reveals a coherent regulatory network involving fertility-associated miRNAs and their targets. These miRNAs not only serve as promising biomarkers for ram fertility but may also participate directly in the epigenetic and molecular programming of sperm. Future studies should focus on validating the predicted miRNA-target interactions through luciferase assays, in vitro transfection, or knockout models, and expanding these findings in larger animal cohorts to evaluate their utility in reproductive management and sire selection.

Conclusion

This study identifies a core group of differentially expressed sncRNAs in ram sperm that distinguish between HF and LF fertility phenotypes. These findings support the utility of miR-133 and miR-433-3p as positive fertility biomarkers, and miR-150 and miR-376d as potential indicators of stress or subfertility. Further validation in larger cohorts and functional studies are warranted to confirm their biological relevance and practical application in sheep breeding.

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