Matoušek Award 2020
Final Report

Awardee:	Ahmed Gad
Title of the project:	Characterization and functional analysis of extracellular vesicles in follicular fluids: implications for porcine oocyte quality and development
Laboratory:	Developmental Biology
Job title:	Postdoc

Introduction:

In this project, we hypothesized that ovarian follicles with different oocyte capacities, differ in their fluid extracellular vesicles (EVs) characteristics and/or miRNA cargo. These EVs could regulate oocyte maturation and embryonic development and could be used as non-invasive biomarkers for oocyte developmental competence in pigs. Therefore, the main objectives of this project were to:

1- Isolate and characterize EVs of follicular fluids in correlation to oocyte quality.
2- Analyse EVs-enclosed miRNA as potential biomarkers for oocyte developmental competence.
3- Investigate the role of EVs during oocyte maturation, fertilization, and embryo development.

Principal results:

1- Oocyte staining and categorization of follicular fluids (FFs):

A total of approximately 600 antral follicles (2-6mm) were aspirated individually and each oocyte was stained individually with 0.5% Lissamine Green B stain (LB). Oocytes were classified according to the stain to LB+ (stained; low quality) and LB- (unstained; high quality) as shown in fig.1. FFs corresponding to each oocyte type were pooled together into FFL+ and FFL- for further analysis.

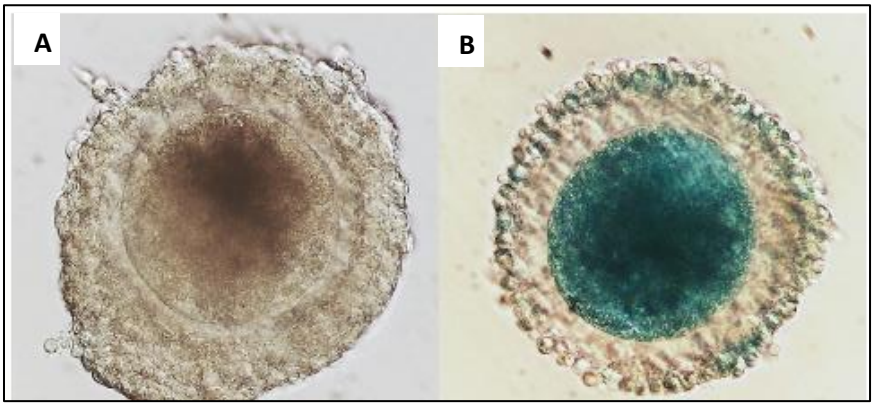

Fig.1: Classification of porcine oocytes after Lissamine Green B (LB) staining. **A)** High qulaity oocyte (LB-); **B)** Low quality oocyte (LB+)

2- Isolation and characterization of EVs from FFs:

We tested two different EVs isolation kits. The first was the ExoQuick isolation kit which was principally based on the precipitation of EVs. The second was the Exo-spin kit which was based on precipitation and Size-exclusion chromatography separation technology. The Exo-spin kit showed specific and better isolation quality. Therefore, it has been selected for further isolations. EVs were isolated from each FF group (FFL+ and FFL-) and then characterized using three different methods:

a- ***Western blot (WB) analysis*****:** WB showed the presence of EV-associated tetraspanin and protein markers (CD63 and ALIX). In addition, cellular specific protein markers (CytC and ATP5A) were detected only in cell lysates but not in the isolated EVs indicating the absence of other cellular membrane contamination in the EV preparations (Fig. 2A).

b- ***Transmission electron microscopy (TEM):*** Isolated EVs were evaluated morphologically by TEM and appeared as circular bilayer enclosed vesicles with a diameter range of approx. 20-200 nm (Fig. 2B).

c- ***Nanoparticle tracking analysis (NTA):*** Size distribution and concentration of the isolated EVs were analyzed in both groups. The median size of EVs was 135.6±1.9 and 135.7±5.3 nm in FFL+ and FFL-, respectively with no significant differences. Total EV concentrations was significantly higher in FFL- compared to FFL+ (9 x10^9 vs. 7.7x10^9 particles/mL, respectively; Fig.2C).

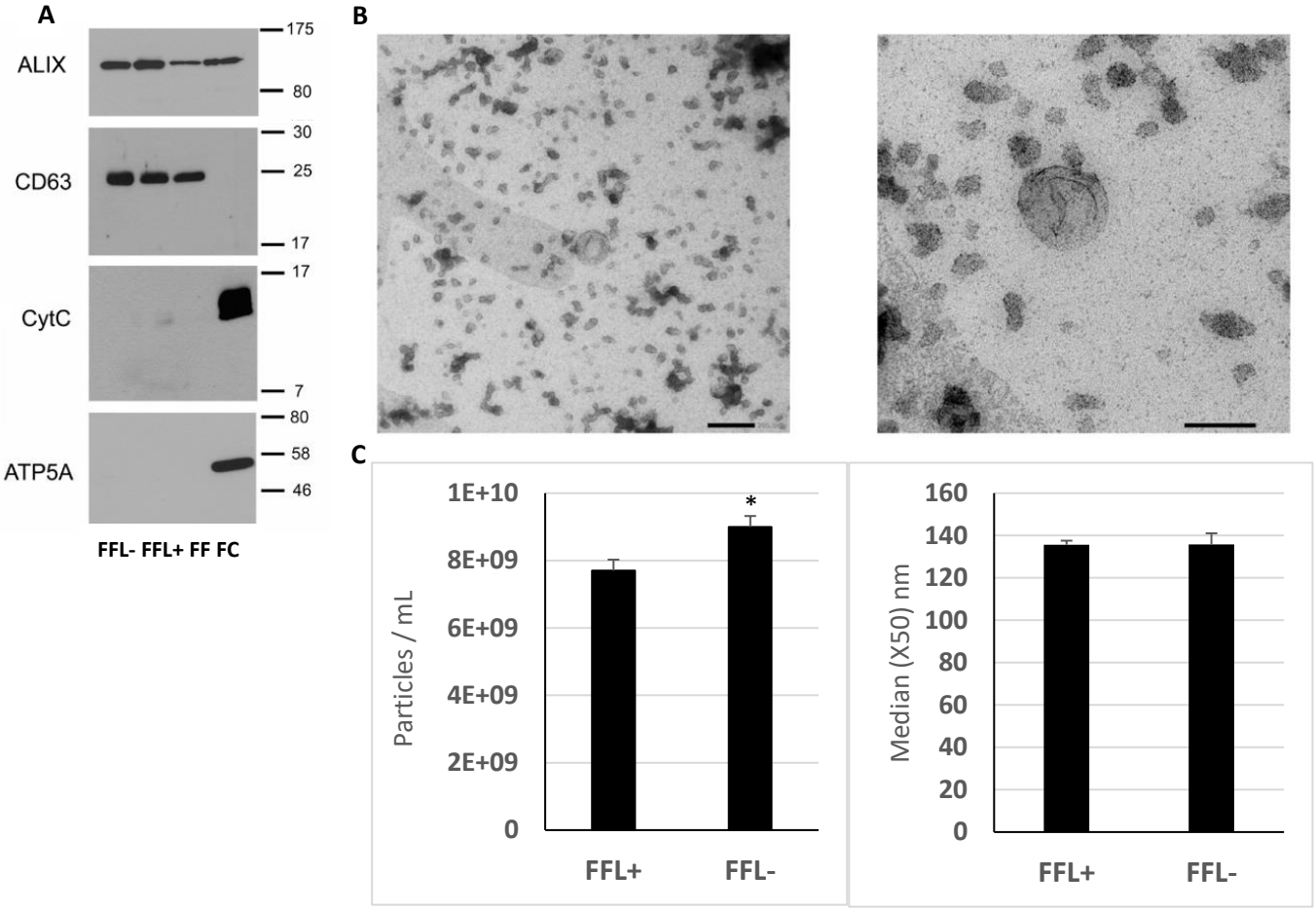

Fig.2: Characterization of EVs isolated from FFs. **A)** Western blot analysis of EV-associated tetraspanin and protein markers (CD63 and ALIX) and cellular specific protein markers (CytC and ATP5A) in FFL+, FFL- groups, filtered FF and follicular cell lysate (FC). **B)** Representative photos of TEM analysis. **C)** Analysis of concentration (particles/mL) and particle size (nm) of EVs in FFL+ and FFL- groups. * *P* valule < 0.05

3- RNA isolation and miRNA expression profiling:

- Total RNA was isolated from EVs samples of different groups using miRNeasy Micro Kit (Qiagen). RNA quality and quantity were evaluated using the Agilent bioanalyzer. As shown in Fig. 3, bioanalyzer analysis revealed that EV samples were free of ribosomal RNA (rRNA) and contain enough concentration of small RNA.

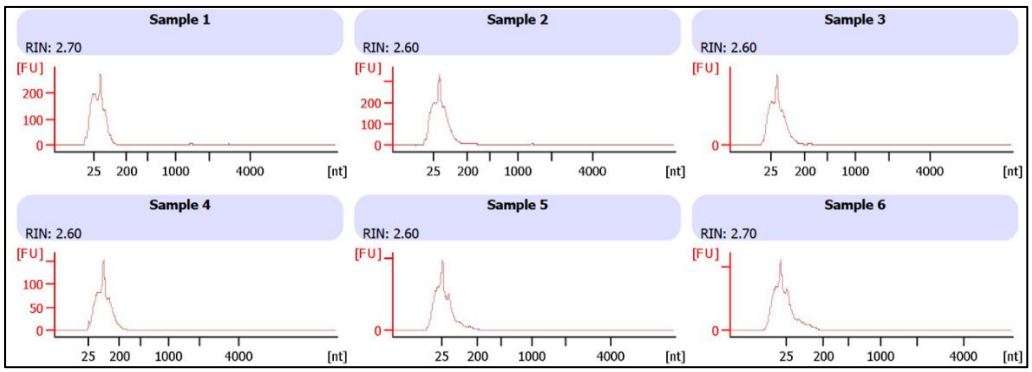

Fig.3: Bioanlyzer analysis of total RNA isolated from EV samples.

- Small-RNA libraries were prepared from the isolated RNA using QIAseq miRNA Library Kit (QIAGEN) and sequenced on an Illumina NovaSeq 6000 sequencer. Principal component analysis (PCA) and heatmap showed that the replicates of each group were clustered together and the two groups were separated from each other (Fig. 4).

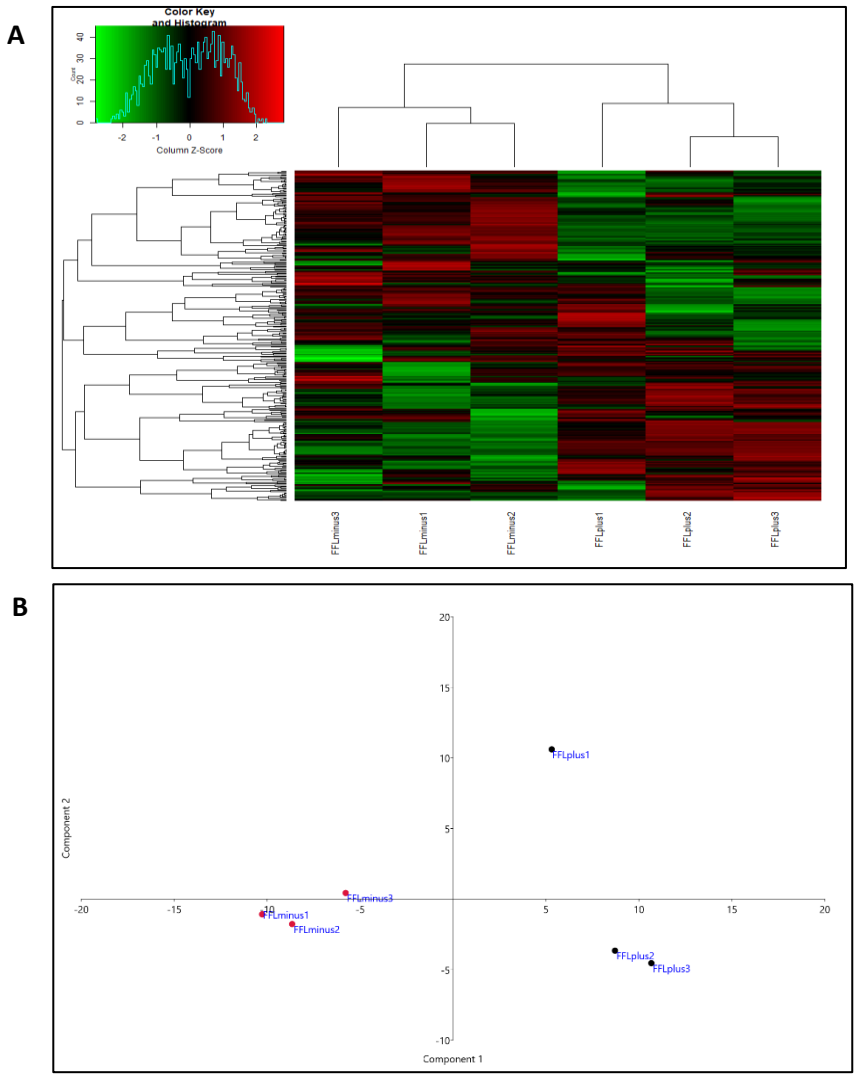

Fig.4: A) Heatmap for all expressed miRNAs in both groups (3 replicates each). **B)** Principal component analysis including all 3 replicates of the two FFL groups.

- MiRNA sequencing results revealed that out of 457 known miRNAs in porcine a total of 297 miRNAs were detected in both EV groups and 9 miRNAs were exclusively expressed in each group (Fig. 5). From the commonly expressed miRNAs, a total of 82 miRNAs were significantly differentially expressed (DE) with 50 up- and 32 down-regulated in FFL+ compared to FFL- group (Fold change > 1.5; Adj P-value < 0.05) (Fig. 5).

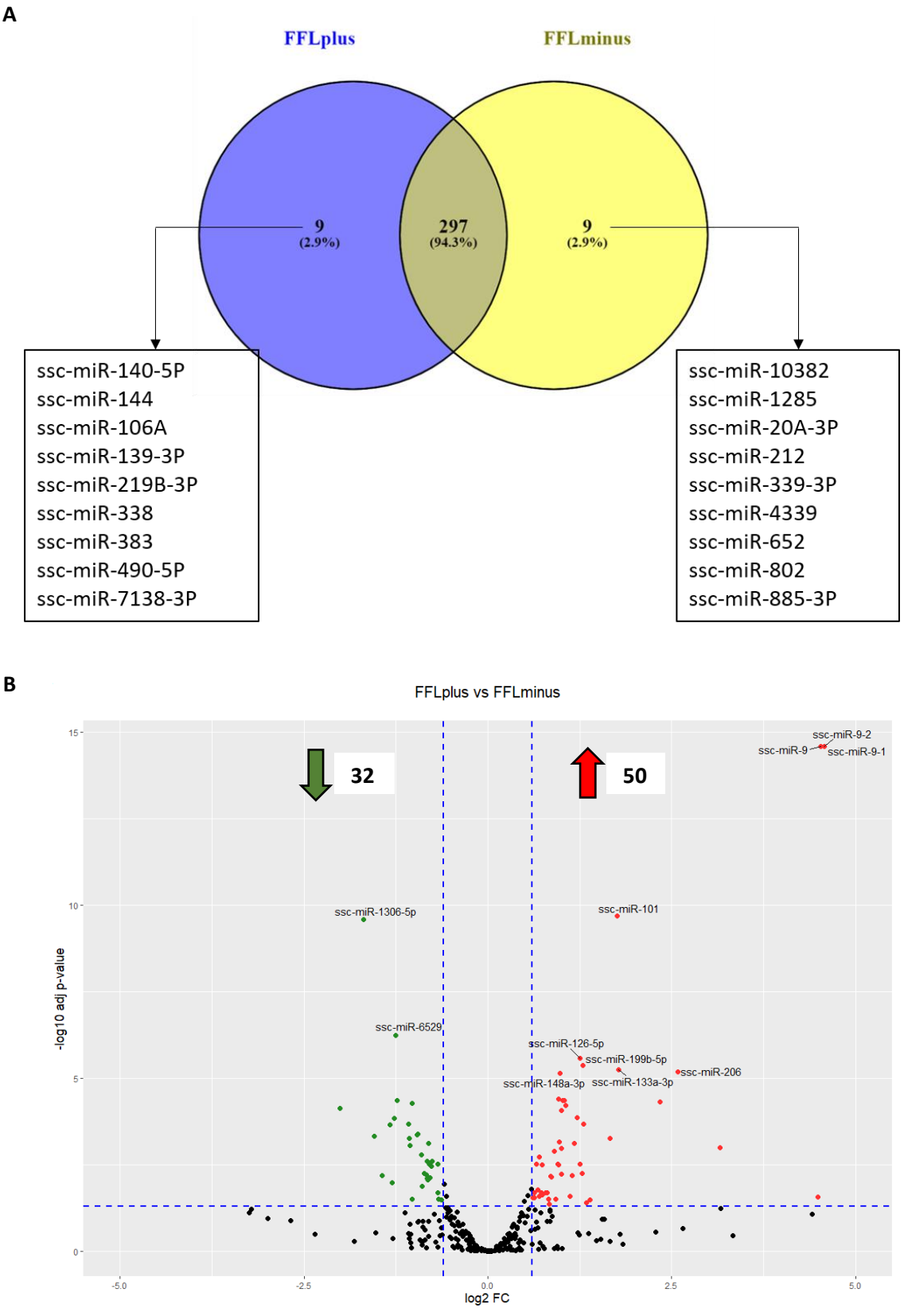

Fig.5: A) Venn diagram for commonly and exclusively expressed miRNAs in FFL+ compared to FFL- group. **B)** Volcano plot representing up (red) and down (green) regulated miRNAs in FFL+ compared to FFL- group.

Abstract and conference:

We are preparing an abstract to present the results of this project during the upcoming AETE2021 meeting (The Association of Embryo Technology in Europe, Dublin; September 8-9th 2021; https://www.aete.eu/). Meanwhile, the full paper will be prepared to be submitted this year.

Conclusion and applications:

From these principal results, we found that EVs isolated from FFs of follicles with different oocyte qualities contain different miRNA cargo. These miRNAs could be used as potential non-invasive molecular biomarkers for oocyte selection. Moreover, understanding the role of these specific miRNAs will give more insights and enhance our knowledge of oocyte developmental competence which will improve our current in vitro culture system.

Future work:

Based on the current results, we will go further for functional analysis to discover the role of isolated EVs from FFs of high and low-quality oocytes on the maturation and developmental competence of oocytes. Moreover, we will try to focus on and explore the origin and function of the differentially expressed miRNAs, detected in the current study, and their role during oocyte development.

Limitations:

Due to the COVID-19 pandemic during the year 2020, we faced some delays in purchasing and receiving materials, chemicals and services related to our project.

Financial and budget report:

Item	**Planned budget Kč**	**Spent budget Kč**
Materials and chemicals: including EVs isolation kits, chemicals for oocyte culturing, RNA isolation and Western blot related chemicals and antibodies.	**65,000.00**	**66,421.89**
Services: including miRNA sequencing and Transmission electron microscope service.	**75,000.00**	**72,774.68**
Total	**140,000.00**	**139,196.57**

Date: 23.02.2021 Awardee: Ahmed Gad …….

Date: 23.02.2021 Head of the Lab: Radek Prochazka ……..

Comments from the head of the lab:

Dr. Gad and his co-workers from LVB did a very good job on the identification of high and low-quality oocytes, collection of follicular fluid from individual follicles, isolation and characterization of extracellular vesicles, including analysis of their miRNA cargo and differential expression. The present data confirm the author´s hypothesis on that miRNA cargo of follicular fluid-derived exosomes is in correlation with the oocyte quality. They identified a number of exosomal miRNAs that are exclusively or differentially expressed in follicles bearing oocytes with high and low quality. This knowledge has become a ground for future work of our laboratory – the functional studies on the role of exosomes and their specific miRNAs in the regulation of oocyte maturation and early embryonic development. The report of Dr. Gad represents a solid base for a high-quality paper that will be submitted to a respected journal in the field of molecular reproduction. The budget of the project was spent as planned in the proposal.

In conclusion, I believe that the work of Dr. Gad met the principal ideas and aims of the Dr. J. Matoušek Award, i.e. to support original and distinctive projects of young talented scientists.

Abstract of Contribution 103

ID: 103
Folliculogenesis,Oogenesis and Superovulation
Poster presentation
Keywords: follicular fluid, extracellular vesicles, miRNA

Characterization and miRNA profiles of small extracellular vesicles originated from porcine follicular fluids and their association with oocyte quality

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[1]Institute of Animal Physiology and Genetics, Czech Academy of Sciences, Liběchov, Czech Republic; [2]Department of Animal Production, Faculty of Agriculture, Cairo University, Giza, Egypt; [3]Constantine the Philosopher University in Nitra, Nitra, Slovakia; gad@iapg.cas.cz

Follicular fluid (FF) provides the essential microenvironment needed for oocyte development and subsequently influences its quality. Extracellular vesicles (EVs) have been detected in the ovarian FFs and reported to play essential roles in regulating follicular and oocyte development through their cargo molecules including microRNAs (miRNAs). The objective of this study was to characterize and identify miRNA expression profiles of small EVs from porcine FFs in association with oocyte quality. Approximately 600 antral follicles (2-6 mm) were aspirated individually and cumulus-oocyte complexes (COCs) were stained with 0.5% Lissamine Green B stain (LB), vital synthetic staining for determining oocyte competence. Each COC was classified separately according to the stain into LB+ (stained; low quality) and LB- (unstained; high quality). FFs corresponding to each oocyte quality were pooled together into FFL+ (low-quality) and FFL- (high-quality). EVs were isolated from FFL groups (3 biological replicates/group; 0.5 mL FF/replicate) using the Exo-spin kit based on precipitation and Size-exclusion chromatography separation technology. Isolated EVs were characterized by western blot (WB), transmission electron microscopy (TEM), and nanoparticle tracking analysis (NTA). Total RNA was isolated from EV samples using Trizol and miRNeasy Micro Kit. Small-RNA libraries were prepared using QIAseq miRNA Library Kit and sequenced on an Illumina NovaSeq 6000. WB analysis proved the presence of specific EV-associated tetraspanin and protein markers (CD63 and ALIX) and the absence of cellular-specific protein markers (CytC and ATP5A). Morphological evaluation by TEM showed EVs as circular bilayer enclosed vesicles. The median size of EVs was 132.6 *vs.* 135.7 nm and the concentration was 9 $x10^9$ *vs.* $8.8x10^9$ particles/mL in the FFL+ *vs.* FFL- group respectively, with no significant differences between them. Sequencing analysis revealed that a total of 295 known miRNAs were commonly detected in the EVs of both FFL groups. MiR-27b-3p, miR-140-3p, miR-29a-3p, miR-202-5p, and miR-16 were the top highly abundant miRNAs in both groups representing around 45% of the total reads. Differentially expression (DE) analysis exhibited that 22 miRNAs (including miR-9, miR-6516, and miR-206) were up- while 19 (including miR-193a-5p, miR-125b, and miR-320) were down-regulated in FFL+ compared to FFL- group (FC > 2; FDR < 0.05). DE-miRNAs target gene analysis uncovered pathways associated with oocyte development including oocyte meiosis, ubiquitin-mediated proteolysis, and signaling pathways (MAPK, PI3K-Akt, FoxO, and AMPK). Our findings indicated that FF-EVs contain different miRNA cargo in association with oocyte quality. These miRNAs could be used as potential non-invasive biomarkers for oocyte selection. Further investigations of EV-miRNA functions during oocyte development will give more insights into oocyte developmental competence.

Supported by IAPG-Matoušek Award 2020 and Ministry of Education, Youth and Sports of the Czech Republic (CZ.02.1.01/0.0/0.0/15_003/0000460).

Re: Final Report to be submitted_dr. Matousek Prize

Ahmed Gad

čt 29. 4. 2021 15:29

Doručená pošta

Komu: Barbora Vesela <Vesela@iapg.cas.cz>;

1 příloha (264 kB)

A.Gad AETE2021 Abstract.pdf;

Dear Ms. Veselá,

Thank you for your reply. Please find attached the abstract of the upcoming AETE conference. The abstract has been submitted on the 13th of April 2021 with ID number 103. Regarding the paper, it is still under preparation and we will target the Journal of Extracellular Vesicles (ISSN: 2001-3078) under the similar topic of the submitted abstract.

Best regards,

Ahmed

Ahmed Gad (Ph.D.)

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From: Barbora Vesela
Sent: Thursday, April 29, 2021 3:13 PM
To: Ahmed Gad
Subject: Re: Final Report to be submitted_dr. Matousek Prize

Dear dr. Gad,

thank you very much for the clarification. Your report seems to be sufficient.

I would only need you to specify the paper you have mentioned in part "abstract and conference" in the report - do you have some details already - e.g. which journal and which topic will you choose? Could you, please, send

me the abstract for the conference, if already submitted?

Thank you in advance.

Best regards,

Barbora Veselá

Barbora Veselá
Manažerka a koordinátorka IGS

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Od: Ahmed Gad
Odesláno: 29. dubna 2021 14:59
Komu: Barbora Vesela
Předmět: Re: Final Report to be submitted_dr. Matousek Prize

Dear Ms. Veselá,

Thanks for your email. Regarding the final report of the Dr. J. Matousek Award 2020 project, I would like to inform you that I already submitted the final report on the 23rd of February 2021. I sent it via email to uzfg@iapg.cas.cz and I submitted it as a signed hard copy to the secretary office.

Please find attached a copy of my final report and please let me know if something else is needed.

Best regards,

Ahmed Gad

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