SZENT ISTVÁN UNIVERSITY
Animal Husbandry Science PhD School

Investigation the role of microRNAs in early embryonic development and stem cells in rabbit and chicken

Thesis for Doctoral degree (PhD)

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1   Introduction

"Necessity is the mother of invention ". Stem cell research has revolutionised the field of regenerative medicine and therapeutics (Gearhart and Addis, 2009). However, prior to the advent of the stem cell technology most of the *in vitro* lab based studies regarding the study of disease pathophysiology and drug designing were done using animal models. The use of animals as a model to study the human physiology can be traced back to the ancient times the time of Aristotle (Ericsson et al., 2013). By the dawn of the nineteenth century, the usage of the animal models in the biomedical field had acerbated. The most prominent model animal is rodents. However, with the discovery of genetic techniques the traditional animal models could be genetically modified and hence, the development of transgenic animals started.

Despite the development of genetic engineering, gene editing and genome manipulating techniques and the availability of the rodent and non – rodent animal model system; there were shortcomings to the usage of animal models; hence, there was a need to look for alternative methods for studying human disease, therapeutics and drug designing (Bracken, 2009). The experiments conducted on animals cannot be used with maximum efficiency to extrapolate the data to human use. There can be many reasons for the failure, first of all poorly designed experimentation and execution.

This problem was overcome by the development of *in vitro* cell culture systems. The development of the culture conditions, cell culture systems has allowed the isolation of stem cells from different animal species and allowed the *in vitro* cultivation. The ability of the stem cells to develop into any potential cell type combined with today's modern techniques of genome editing can be used to generate differentiated cell types such as neuronal stem cells, cardiomyocytes and can be used to study neurological disorders like Parkinson disease, coronary artery disease etc. (Balzano et al., 2018; Reik and Surani, 2015). The emergence of the somatic reprogramming technique by (Takahashi and Yamanaka, 2006) further supported the stem cell work and seem to overcame the ethical problems regarding the isolation of hESCs (human embryonic stem cells) from human embryos. As, this technique could be used to generate the iPSCs (induced pluripotent stem cells). The iPSCs opened doors for further work on regenerative medicine as well as clinical drug-based studies.

Other than the iPSCs technology, and other existing stem cells, like ESCs (embryonic stem cells), the PGCs (Primordial germ cells) also played an

important role in re-boosting the stem cell biology. PGCs are up-coming potential stem cells, being germline in nature they can be genetically modified and used to create germline chimeras (Nakamura et al., 2013). The most studied PGCs are cPGCs. The cPGCs can be isolated form the chicken embryos and be maintained in *in vitro* conditions using the protocol established earlier (Whyte et al., 2015).

However, despite the existence of *in vitro* culture systems and media formulation the knowledge regarding the factors governing the pluripotency and self-renewal is not yet fully investigated. One of the most emerging players in stem cell biology are miRNAs (Lee et al., 2016).

MiRNAs are short non-coding RNAs. They regulate gene expression post-transcriptionally (Winter et al., 2009)

In mammals, the miRNA processing i.e. biogenesis begins from the nucleus. This is the classical or the canonical pathway by which a mature miRNA is generated. The miRNA gene is transcribed by RNA polymerase II or III into a primary miRNA (pri-miRNA). This pri-miRNA then is cleaved by the Drosha and the DGCR8 (DiGeorge Critical Region 8) enzyme as the pre-cursor miRNA (pre-miRNA). The Drosha and the DGCR8 constitute the microprocessor complex. The pre-miRNA is then exported from the nucleus to the cytoplasm by exportin-5. Here, Dicer another RNAse III endonuclease cleaves the pre-miRNA to a miRNA duplex consisting of both the 5p and the 3p arm. One of the two arms is usually degraded and the guide strand i.e. 5p arm is retained and incorporated into the RNA induced silencing complex (RISC) with the help of Argonaute protein. Argonaute proteins (AGO) are a group of proteins that interact with small non-coding RNAs and help in their processing. In case of miRNAs and most mammals AGO2 along with RISC helps in incorporation of the mature arm 5p in the silencing complex.

The miRNA mature arm contains a seed sequence that is complementary to the 3'UTR of the mRNA. If the base pairing between the seed and the 3'UTR is perfect, the mRNA undergoes translation repression else if the pairing is imperfect the mRNA undergoes de-adenylation or miRNA target cleavage (Graves and Zeng, 2012).

Studies conducted on mouse embryos and mESCs (Mouse Embryonic Stem Cells) , in which the enzymes Dicer and Drosha were knocked out highlighted the importance of the miRNAs in embryonic development and stemness (Lichner et al., 2011).

These studies indicate that other than playing role in the canonical pathway of generating miRNAs; these enzymes have also been implicated in

playing a role in the embryonic developmental pathways and stemness of ESCs.

It was earlier believed that since both Dicer and Drosha are involved in regulating the biogenesis of miRNAs and other non-coding RNAs; but, recent literature cites different roles and phenotypic effects observed when either Dicer, Drosha or DGCR8 protein are knocked out (Bodak et al., 2017).

But the phenotypic effects are different; depending on which enzyme is knocked. Thus, implicating independent function of both Dicer and Drosha in governing stem cell pluripotency independent of their role in miRNA biogenesis. A study by (Cirera-Salinas et al., 2017) elucidated the independent important role of DGCR8 in mESCs (Mouse Embryonic Stem Cells). In the study, the mESCs that were knocked out for DGCR8 protein were complemented with the mutant phosphorylated form of the DGCR8 protein. It was found that although this mutant form could restore the normal miRNA production but could not exit the mESCs from the pluripotent to the differentiation state; thus, indicating the role of DGCR8 in controlling the differentiation of ESCs.

From the above studies, the independent role of the different enzymes in miRNA biogenesis indicate miRNAs to be an emerging player controlling the stemness of the ESCs as well as miRNA have reported to induce genetic reprograming of somatic cells to produce iPSCs (Zeng et al., 2018).

The main miRNA cluster reported to play a role in regulating the stemness of the stem cells and iPSCs reprograming is the miRNA-302 cluster (Gao et al., 2015). In our laboratory, there are established and characterized male and female cPGCs cell lines as well as rabiPSCs (Rabbit Induced Pluripotent Stem cells) stem cell lines.

2   Objectives

1. To elucidate and define the optimum media conditions and cell concentration for in vitro PGC cultivation.
2. To characterize the role of miR-302 cluster in controlling the proliferation rate of cPGCs.
3. Functional characterization of miR-302-cluster members (302b-5p and 302b-3p arm) via miRNA inhibition assay.
4. To investigate the role of ocu-miR-302a-3p expression and its role in rabbit induced pluripotent stem cells via OCT4 immunostaining and miRNA inhibition.

3 Material and Methods

3.1 Investigations in Chicken PGCs

3.1.1 Animals, Permission

The animals were maintained according to the rules and regulations of Hungarian Animal Protection Law (1998. XXV111). Permission for experimental work on animals at the Research Centre for Farm Animal Gene Conservation (Godollo, Hungary) was granted by the National Food Safety Online, Animal Health and Welfare Directorate, Budapest. The Partridge Colour chicken breed was kept in Research Centre for Farm Animal Gene Conservation (Godollo, Hungary). The GFP expressing chicken breed White Leghorn was identical to one established by McGrew and colleagues (Whyte et al., 2015).

3.1.2 PGC Media

The PGC medium used was the Avian-KO-DMEM basal media (FACs medium) which is a modification of the knock-out DMEM manufactured by the Life Technologies.

3.1.3 PGC Establishment

The cPGCs was isolated from the fertilized eggs of HH stage 13-17 (Hamburger and Hamilton, 1951) embryos. About 1 to 2 μl of blood was taken up in glass micro-pipette from the dorsal aorta of the embryo under the stereomicroscope. The isolated cPGCs were then transferred to a 48 well plate containing 300 μl of medium without feeder cells. Following the staging of the embryos. After 1 or 2 weeks, the red blood cells had disappeared and only the cPGCs were clearly visible.

3.1.4 PGCs Culturing

Following the successful isolation and establishment of the cPGCs cell line they were cultured for 23, 30 and 50 days. Once, the cell number reached confluence the PGCs were collected for RNA isolation and further analysis.

3.1.5 PGC Freezing

The long term cultivated cPGCs can be cryopreserved. The protocol for freezing required the preparation of the freezing medium which was usually freshly prepared. The PGCs cells were suspended and collected from the wells and transferred to a 1.5ml Eppendorf tube. The cells were then centrifuged at 2000 rpm for 3 minutes. After centrifugation, the

supernatant was removed, and the pellet obtained was re-suspended in 250 μl of Avian-KO-DMEM medium. To this 250 μl of freezing media was slowly added. The contents from the Eppendorf tube were then moved to a labelled cryovial tube and the tube was immediately placed in -70^{0}C. For further long–term cultivation the cells after one night should be moved to -150^{0}C or liquid nitrogen.

3.1.6   PGC Proliferation

After 1 day of culturing, half of the PGC culture medium was replaced with fresh medium containing the diluted CCK-8 reagent (1:10 final concentration, Dojindo Laboratories, Japan) and was incubated for 3 hours at 37^{0}C. The product of the CCK-8 reagent was measured as the optical density absorbance at 450 nm using the CLARIOstarR Microplate Reader (BMG, Labtech, US).

3.1.7   Time –Lapse Video Recording Analysis

The ImageXpress Micro XLS Imaging system (Molecular Device) having a built-in incubator was used for high-content screening and image analysis. The device captures 12 views for each well of a 96 well culturing plate for 64 hours. The cell number was measured in every 4 hours.

3.2   Investigations in Rabbit iPSCs (rabiPSCs)

3.2.1   Animals, Permissions

The New Zealand white rabbits were purchased from Hycole (Marcoing, France) or HyPharm (Roussay, France). The rabbit fibroblasts were isolated from the ear of these New Zealand white rabbits and were subsequently reprogrammed to generate the rabiPSCs cell line (B19-EOS) bought from French group of (Tapponnier et al., 2017). The rabiPSCs cell line EOS/B19 used in this study is transgenic as it is over-expressing the a puromycin gene drive by the distal promoter region of *OCT4*.

3.2.2   RabiPSCs Media

The media for iPSCs culturing are subsequently prepared on the basis of protocol devised by (Schmaltz-Panneau et al., 2014). The rabiPSCs require different kinds of media for different stages of culturing. The media with their usage is as follows: ANR-rabiPSCs-Basic Medium for Thawing, ANR-RES Culturing Medium and ANR RES puromycin for passaging.

3.2.3   RabiPSCs Culturing

The rabiPSCs culturing was performed on feeder layer. Prior to the rabiPSCs culturing, the mouse embryonic fibroblasts (MEF) were passaged and cultured. MEF were used as feeder layers for the rabiPSCs. After mitomycin treatment.

3.2.4   RabiPSCs Freezing

The rabiPSCs were frozen using the freshly prepared freezing media. The rabiPSCs were dissociated using trypsin, following the cell suspension was collected and centrifuged. Following centrifugation, the cells were re-suspended in the basic culture medium and an equal volume of freezing medium was added drop-wise. The contents were then aliquoted to the cryo tubes for storage at - 70^0C.

3.2.5   RabiPSCs Proliferation Test

The rabiPSCs proliferation test was performed in the same way as for cPGCs using the CCK8 proliferation kit.

3.2.6   DNA Isolation

The DNA isolation was performed using the High Pure PCR Template kit (Roche Diagnostics, USA). The protocol was performed according to the instructions of the manufacture. The isolated DNA was stored at - 20^0C.

3.2.7   Sex PCR

The sex of the established PGC cell lines were determined using the *CHD1* (Chromosome Helicase DNA Binding protein 1) primer sets, as described before by Lee and colleagues (Lee et al., 2010).The extracted DNA was diluted to 25ng/µl concentration for PCR reaction and gel electrophoresis. MyTaq Red Mix was used for the reaction (Bioline, Cat. No. BIO-25043). The PCR products were then separated by electrophoresis, using 1% agarose gel stained with ethidium bromide, at 100 V for 1.5-2.0 hours.

3.2.8   RNA Isolation

The total RNA was extracted using the TRI Reagent kit (MRC, UK) using the instructions of the manufacturer. The concentration of the extracted RNA was determined using the Nano Drop Spectrophotometer. The isolated RNA was stored in - 70^0C.

3.2.9 MiRNA microarray

For analysing the miRNA expression patterns, the samples were sent to LC Sciences, Houston, TX, USA. Microarray assay kit was performed by LC Science Company. This assay is based on µParafloR microchip technology. The process started with the 3'-extension with a poly A-tail of 4 to 8 µg of total RNA using poly (A) polymerase. An oligonucleotide tag was later ligated to the poly (A) tail for fluorescent staining. Following this, hybridization was performed overnight on the µParaflo microfluidic chip using a micro-circulation pump (Actatic Technologies, Houston, TX, USA). The probes were designed based upon the miRBase 21 database. Following hybridization, the tag-conjugating dye Cy3 was circulated throughout the microfluidic chip. The fluorescent images were obtained using the Axon GenePix 4000B Microarray Scanner (Molecular Devices, Sunnyvale, CA) and digitized by the Array-Pro image analysis software (Media Cybernetics, Rockville, MD). The data were analysed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression).

3.2.10 cDNA writing, qPCR

The extracted RNA samples were reverse transcribed into cDNA with High Capacity cDNA reverse transcription Kit following the instructions of the manufacturer (Applied Bio systems, Life Technologies, Carlsbad, US). RT master mix was used for cDNA writing. The cDNA was stored at $20^{0.}$C. The synthesized cDNA was then used for quantitative real-time PCR. SYBR Green PCR master mix was applied for the qPCR as a double-stranded fluorescent DNA-specific dye according to the manufacturer's instructions (Applied Bio systems, Life Technologies, Carlsbad, US). For each gene examined, three parallels were analysed, fluorescence emission was detected and relative quantification was calculated with the GenEx program (MultiD, SE).

3.2.11 MiRNA qPCR analysis

The Q-PCR analysis was used for checking the expression of the PGC specific and stem cell specific markers in the cPGCs and rabiPSCs as well as expression of miR-302 cluster.

3.2.12 Immunostaining

Isolated PGCs were fixed with 4% PFA for 10 minutes. After washing with PBS (three times, five minutes each), cells were permeabilized with 0.5%

Triton X-100 (Merck-Millipore, US) for 5 minutes. After washing with PBS, the fixed cells were blocked for 45 minutes with a blocking buffer containing PBS with 5% (v/v) BSA. Then, cells were washed three times with PBS and were incubated with each of the primary antibodies including mouse anti-SSEA-1 (1:10, Developmental Studies Hybridoma Bank, US), rabbit anti-VASA (1:1000; kindly provided by Bertrand Pain, Lyon, France). After incubation overnight in the primary antibody solution in a humid chamber at 4°C, the cells were washed three times with PBS. Then, cells were incubated with the secondary antibodies, donkey anti-mouse IgM FITC Cy3 (1:400, Jackson ImmunoResearch, USA) donkey anti-rabbit IgG FITC (1:400, Jackson ImmunoResearch, USA), donkey anti-rabbit IgG conjugated to Alexa555 (1:400, Molecular Probes Inc., USA) in a dark humid chamber for 1 hour at room temperature. After washing with PBS, the nucleus was stained with TO-PRO®-3 stain (1:500, Molecular Probes Inc., US), which is a far red-fluorescent (642/661) nuclear and chromosome counterstain. Coverslips were mounted on the slide with the application of 20μl VECTASHIELD® Mounting Media (Vector Laboratories, Inc., US) and analysed by confocal microscopy (TCS SP8, Leica). Negative controls were stained only with the secondary antibody.

3.2.13 Rabbit Embryo Isolation and Culturing

The rabbit embryos were isolated 1 or 3 days after the mating. The morulas were flushed out from the rabbit 2.5 days' p.c. oviducts. The zona pellucida and the mucin coat was removed using the pronase treatment. The isolated morulas for then cultivated *in vitro* on culture plate to obtain blastocyst. The RDH embryo medium was used. The plates were prepared containing the medium under an oil droplet.

3.2.14 Statistical Analysis

All the data analysis was conducted by the GenEx (version 6.0) and the R studio (version 1.0.136) and R (version R-3.2.2). The heatmap, Venn diagram clustering analysis for the microRNA microarray data was performed using the GenEx software. The data for the Q-PCR analysis for the stem cell marker, PGCs marker expression along with the miR-302 cluster was done using the GenEx software. The ΔCT values of the expressed markers in these PGC cell lines were studied using the GenEx software and the expression was measured relative to the FS101 sample in each case. For the expression analysis of *cPOUV*, *cNANOG* and *OCT4 GAPDH* was used as the reference gene; for the studying the expression of

gga-miR-302a gga-miR-92 was used as the reference. There were two biological repeats used and analysed in 3 parallel samples at Q-PCR.

The doubling time analysis calculation and proliferation rate calculation was done using the R studio and R.

3.2.15 miRNA Inhibition

cPGCs FS101 and FS111 were transfected with inhibitors against the gga-miR-302b-5p and gga-miR-302b-3p at 100nM final concentration using the transfection agent siPORT (Applied Bio systems, Life Technologies, Carlsbad, US). The proliferation rate was measured for 3 days following inhibition for control and inhibited samples. The doubling time was measured using the OD values measured using the proliferation test. 48 following transfections, the cPGCs were collected for RNA isolation using RNA aqueous lysis buffer micro kit (Applied Bio systems). The isolated RNA was then used for Q-PCR analysis for stem cell, germ cell marker as well as for the miRNAs gga-miR-302a, gga-miR-302b-5p and gga--miR-302b-3p. The inhibition analysis on rabiPSCs was performed in the similar way.

4   Results

4.1   Overview of The Used PGC Lines

We used two types of established PGCs cell lines. One is the non-GFP expressing PGC cell line; established from PC (Partridge Colour Chicken) and other is the GFP expressing PGCs cell line from White Leghorn chicken.

4.1.1   Stem cell specific marker expression

The GFP expressing and the non-GFP expressing PGCs cells were analysed for the expression level of the stem cell specific marker c*POUV*, c*NANOG* and stem cell specific miRNA gga-miR-302a using the Q-PCR analysis. It was observed that the marker expression level is correlated to the proliferating rate of the PGCs. The cell lines showing high expression of stem cell markers are faster growing cell lines.

The miR-302a has been cited in literature as a stem cell and vertebrate specific miRNA. The PC PGC cell line shows higher expression compared to the GFP expressing cell line.

4.1.2   Immunostaining

The PC male (FS101) and female (F111) and GFP PGCs cell lines male (4ZP) and female (5ZP) were characterized for the PGC germ cell specific marker expression i.e. *CVH* (chicken vasa homologue) and stem cell marker SSEA-1 (Stage specific embryonic) via immunostaining. The PGCs were stained using antibodies against the *CVH* gene to show expression.

The nuclear staining was performed by ToPro3 (blue). The stem cell marker SSEA1 stained in red and the germ cell marker CVH stained in green.

4.1.3   Doubling time Measurement in PGCs

The doubling time analysis was done using time lapse video time analyser. The doubling time was correlated with the PGC concentrations.

It was observed that the doubling time is dependent on the initial cell concentration. The doubling time was expressed in days. The GFP-ZZ-4ZP PGC line showed the lowest doubling time (the maximum proliferation rate) with a value of 1.19 (4x). The highest doubling time was calculated in the case of GFP-ZW-5ZP PGC line (with 2.31 (8x), 1.94 (1x), 1.67 (4x). It was followed by FS-ZZ-101 PGC line with 1.63 (1x) and FS-ZW-111 PGC line with the value of 1.59 (1x) .Interestingly, the doubling time of

the GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111 was the lowest at 4x initial concentration (GFP-ZW-5ZP: 1.67 (4x); FS-ZZ-101: 1.45 (4x); FS-ZW-111: 1.19 (4x).

4.1.4   Stem cell specific miRNA expression

In order to fully characterize the miRNA expression in PGCs, a miRNA microarray analysis was performed on 2 male (FS101 and 4ZP) and 2 females (FS111 and 5ZP) PGCs samples using the µParafloR microfluidic chip technology (Houston, Texas, USA). The microarray analysis consisted of 991 chicken specific probes. The main aim was to identify the potential miRNAs controlling the pluripotency and proliferation rate of PGCs.

4.1.5   MiRNA microarray

The signal intensity was cited to be statistically significant and detectable, if it was greater than 32 and also the average signal intensity across the 3 parallels for each sample had p value <0.01 and also a p value of less than 0.01 considered the intensity to be detectable after the background subtraction and normalization. The overview of the miRNA microarray summarized in the **Table 1** below.

Analysis of the result of LC Science microarray	#
Number of examined gga-miRNAs	991
Number of examined gga-5S-b rRNAs	6
Number of used plate controls	42
Number of parallels of samples on plate	3
Number of examined chicken PGC cell lines	4
Number of examined female PGC lines (5ZP, FS111)	2
Number of examined male PGC lines (4ZP, FS101)	2
Number of expressing miRNAs in chicken PGC samples	153
Number of expressing miRNAs in all chicken PGC samples	27

Table1: An overview of the miRNA array analysis

4.1.6   miRNAs expression in female and male PGC Lines

For characterization of the differential expression of miRNA between the male and female samples, a paired T test analysis was done at the level of ($p < 0.05$). The paired T test analysis between the male and female identified a set of 6 differentially expressing miRNAs. These are gga-miR-1354, gga-miR-1757, gga-miR-30c-5p, gga-miR-1584, gga-miR-1599 and gga-miR-2127.

Amongst the set of miRNAs differentially expressing, gga-miR-30c-5p is an oncomiR and gga-miR-2127 is tumour suppressor miRNA. The other miRNAs gga-miR-1757, gga-miR-1584 and gga-miR-1599 are novel miRNAs; but they have been identified to be involved in pathways controlling viral infections in chicken. The gga-miR-1354 is a new miRNA and needs further studies for functional classification.

4.1.7   Stem Cell Specific miRNA Expression

The heatmap was performed using the expression data for the signal intensities of the 27 miRNAs that were expressing in all the 4 PGCs samples. Despite, 27 miRNAs found to be expressive in all the 4 PGCs they tend to show variability in the expression level between the 4 PGCs samples.   A Spemann coefficient and complete linkage analysis was

performed using the same software to identify the differences in miRNA expression between the 4 PGCs cell lines. It was found that the GFP expressing female cell line 5ZP behaved differently compared to the other three PGCs cell lines. Based on the miRNA data, the miRNAs were classified into two clusters 1 and 2 (**Table 2**). Cluster 1 are miRNA up regulated in PGC cell line 5ZP and cluster 2 are miRNAs up regulated in the other PGCs cell lines.

Cluster 2 Up-regulated miRNAs in high proliferation rate PGC lines (FS111,FS101,4ZP)
gga-miR-92-3p
gga-miR-3538
gga-miR-3535
gga-miR-302d
gga-miR-302c-5p
gga-miR-302c-3p
gga-miR-302b-5p
gga-miR-302a
gga-miR-2954
gga-miR-181a-5p
gga-miR-1563
gga-miR-1454

Cluster 1 Up-regulated miRNAs in 5ZP PGC line
gga-miR-1790
gga-miR-6606-5p
gga-miR-7466-3p

***Table 2**: MiRNAs up-regulated in the high and low proliferating cell line. The miRNA microarray analysis is made in accordance to the proliferating rate of the PGCs cell lines. 5ZP is a low proliferating cell lines and 4ZP, FS101 and F111 are high proliferating cell line.*

From the obtained data above, further analysis has been made on the gga-miR-302 cluster.

A heatmap analysis was performed using the GenEx software for the members of the gga-miR 302 cluster in all the 4 PGCs cell lines examined at LC array analysis. The data was supported by q-PCR analysis of the members of the gga-miR-302 cluster and the analysis of the germ cell marker and stem cell specific marker was also performed in the four PGCs cell lines.

From the heat map above it can be seen that, the members of the cluster miR-302 show expression in all the 4 PGCs samples. Compared to the other 4 PGCs, the lowest expression for all the members of the cluster are in cell line 5ZP and the highest expression was observed in the high proliferating FS111 cell line. Co -relation analysis showed that the FS111, FS101 and 4ZP cell lines are more correlated to each other and the cell line 5ZP behaves differentially.

The expression of the members of the clusters were studied in the 4 PGCs samples via Q-PCR analysis. Also, the stem cell and the germ cell marker expressions were studied.

The stem cell marker expression was highest in the highest proliferating cell line 4ZP and low in the lowest proliferating cell line 5ZP. The germ cell marker CVH showed highest expression in the male PGC cell lines compared to the females. The members of the gga-miR-302 cluster was expressed in all the 4 PGC lines with highest expression in F111 and low expression in 5ZP consistent with the obtained microarray data analysis.

It was observed that the ratio of the two arms of miRNA gga-miR-302; the ratio of the arm 5p/3p is low for cell line 5ZP i.e. expression of the arm 3p is higher in ratio proportion to arm 5p for this low proliferating cell line. For the other high proliferating cell line, the ratio of the arm 5p to the arm 3p is high.
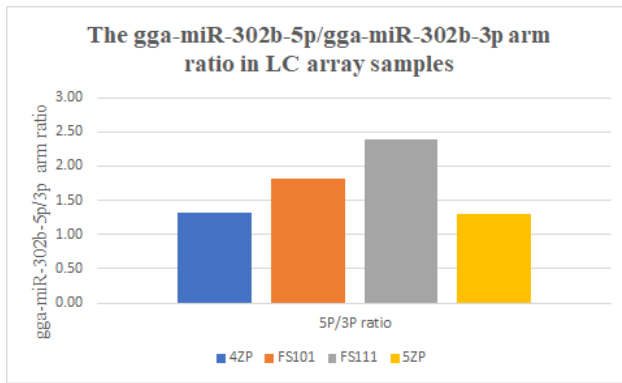
Figure 1: *Ratio analysis of gga-miR-302 cluster in the 4 PGC cell lines examined at LC array by Q-PCR*

The 4 PGC sample analysed by microarray analysis were analysed further for the 5p/3p arm ratio by Q-PCR (**Figure 1**). Following Q-PCR analysis, it was found that the array results were consistent with that of the Q-PCR analysis.

4.1.8 Proliferation Test

The proliferation test was performed using the CCK-8 reagent as described in the Material and Methods section. The proliferation rate is expressed as the doubling time. The doubling time is inversely proportional to the proliferation rate.

4ZP has the lowest doubling time and hence the highest proliferation rate. 5ZP has the highest doubling time and hence the lowest proliferation rate.

4.1.9   Inhibition Analysis

In order to functionally validate the role of gga-miR-302b-5p and gga-miR-302b-3p an inhibition analysis was performed using the miRNA inhibition test. Anti-miR against the gga-miR-302b-5p arm and gga-miR-302b-3p arm was used and subsequently the proliferation test was repeated.

The FS101 and FS111 PGCs cell line were selected for the inhibition analysis. The proliferation rate was measured in these two cell lines prior to inhibition (**Figure 2**)
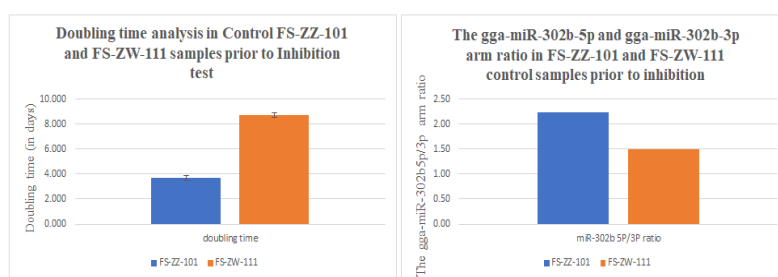
Figure2: *The doubling time measurement and the gga-miR-302b-5p/gga-miR-302b-3p ratio was measured in the FS101 and F111 cell line prior to the inhibition test.*

From the **Figure2** above, it can be inferred that there is a correlation between the doubling times i.e. proliferation rate and the 5p/3p ratio. The doubling time of the FS111 cell line is higher than the FS101 cell line. This mean that the proliferation rate of FS111 cell line is lower compared to the FS101 cell line. This goes with agreement for the 5p/3p ratio. The 5p/3p ratio for the FS111 cell is low compared to the FS101 cell line. Hence, the ratio 5P/3P is higher in cell lines showing high proliferation rate and was low in cell lines that show a low proliferation rate. The high 5P/3P ratio was due to the high expression of the gga-miR-302b-5p arm compared to the gga-miR-302b-3p arm. The low 5P/3P ratio is because of the high expression of the 3p arm compared to the 5p arm.

The doubling time and the ratio was re-measured following the inhibition test (**Figure 3**).

Following, the inhibition test it was observed that the doubling time significantly increased compared to the control in both the male FS-ZZ-101 and FS-ZW-101 female sample that were inhibited using the anti-miR

against the gga-miR-302b-5p. An increase in doubling time means a decrease in the proliferation rate.

For the gga-miR-302-3p arm, following inhibition using its anti-miR there was a decrease in the doubling time observed compared to the control, however the decrease was not significant. But a decrease in doubling time implies increase in proliferation rate compared to the control.

The results as displayed in graphs below (**Figure 3**).
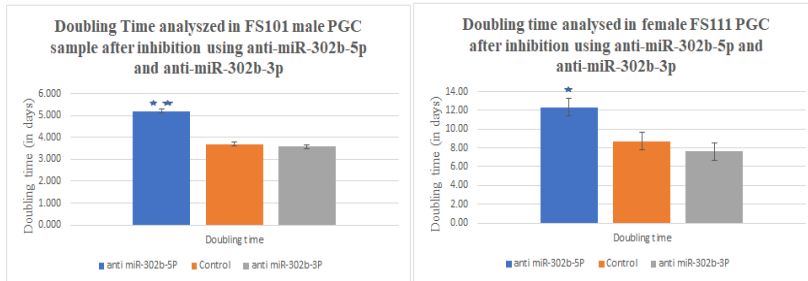
Figure3: *Functional characterization of gga-miR-302b-5p and 3p using inhibition and doubling time analysis. In both biological samples FS101 and FS111, the doubling time increases significantly compared to control at gga-miR-302b-5p arm inhibition. For the 3p arm, compared to the control there is a decrease in the doubling time; thereby, slightly increasing the proliferation rate at gga-miR-302b-3p arm inhibition.*

4.2   Rabbit Embryos

In my study, I characterized the miRNA expression in rabiPSCs B19-EOS cell line via Q-PCR, immunostaining and miRNA inhibition study.

4.2.1   Overview of Used Rabbit Embryos and Culturing

The embryonic culture dishes containing droplets of media under the oil layer were prepared. These were used for *in vitro* culturing of morula stage embryos. The embryos were cultured for 24 hours to obtain the blastocyst. Following, this the embryos were transfected with miRNA mimic (Pre-has-miR-302a-3p), the miRNA inhibitor (anti-miR-302a-3p) and the transfection control using the transfection agent siPORT-NeoFX (Thermo Scientific Fisher) for 72 hours

4.2.2 Stem cell specific miRNA expression in rabbit embryos

The stem specific marker OCT4 were analysed via immunostaining in rabbit embryos under three different experimental conditions.

4.2.3 Immunostaining

Immunostaining was performed on rabbit embryos using antibodies against OCT4 protein. The embryo shown is a 5.5-day-old embryo there was a difference observed in the immunostaining pattern for the stem cell marker OCT4 in rabbit blastocysts between the hsa-miR-302a-5p over expression and the hsa-miR-302a-3p anti–miR condition. In case of over expression (mimic) there was high OCT 4 expression observed but for the miR -302a -3p anti-miR there were few rabbit colonies expressing the OCT4 protein. As, rabbit ocu-miR-302 cluster is highly homologous to the human miR-302 cluster (Maraghechi et al., 2013). The *OCT4* gene is a target of the miR-302-3p in both rabbits and humans. Hence, inhibition of miR-302a-3p lead to down regulation of OCT4 expression.

4.3 Rabbit Induced Pluripotent Stem cells

4.3.1 Overview of Used iPSCs Line

In this study, the rabiPSCs were characterized for the expression of miR-302 cluster member expression ocu-miR-302a-3p via immunostaining and Q-PCR study and miRNA inhibition.

The rabiPSCs cell line EOS/B19 was subsequently thawed, passaged and prepared for rabiPSCs inhibition for miR-302a-3p. The inhibition was performed for 4 days. Following inhibition, the rabiPSCs cell line was characterized for the *OCT4* gene expression via Q-PCR and immunostaining under three different conditions: 1) Pre-miR-302a-3p over expression 2) miR-302a-3p inhibition and 3) control.

4.3.2 Stem Cell Specific miRNA Marker Expression

The main miRNA cluster identified in rabbit embryos and rabiPSCs was miR-302 cluster which has been cited to be stem cell and vertebrate specific cluster. In the study performed by (Maraghechi et al., 2013) it was identified the miR-302 cluster and its members were highly expressing in the rESCs and rabbit pre-implantation embryo.

In this study, the ocu-miR-302a-3p was characterized in the rabiPSCs using Q-PCR, and miRNA inhibition study. The inhibition as described below.

4.3.3 Inhibition Test

The inhibition was performed on the rabiPSCs by using the anti – miR against the ocu-miR-302a-3p. The rabiPSCs were characterized in three different conditions; one was using the pre-hsa – miR-302a mimic and the other was anti-miR against the ocu-miR-302-3p along with the transfection control for 96 hours using the transfection agent the SiPORT NeoFX .The ocu-miR-302a-3p was functionally characterized using the CCK-8 proliferation kit.

4.3.4 Q-PCR

The Q-PCR analysis was performed following the inhibition assay. The *OCT4* gene expression was measured under three different conditions. However, no significant difference was observed between the three conditions The *OCT4* gene expression was related to the housekeeping gene *GAPDH* expression.

4.3.5 Proliferation Test

To functionally validate the role of ocu-miR-302a-3p a proliferation test was performed following the miRNA inhibition. The inhibition began on Day 0. Following the inhibition, each day beginning from day 1 till day 4 the proliferation test was performed on the rabiPSCs under the three treatment conditions. Despite the inhibition of the miR-302a-3p arm the proliferation rate was high also the over-expression of the miR-302a-5p arm supported the growth of the rabiPSCs cell line *in vitro* and increased the proliferation rate.

5   Discussion and Conclusion

In our laboratory, there are established and well characterized cPGCs cell lines and rabiPSCs cell lines. We are using well defined media and conditions for the *in vitro* culturing of these above-mentioned stem cell lines. Both cPGCs and rabiPSCs are emerging players in the field of stem cell biology. In order, to fully explore the potential of the aforementioned stem cells; there is a need to properly characterize the factors that govern the self-renewal capacity and pluripotency of these stem cells. The main aim of my work was to characterize the miRNA expression pattern in these stem cells as miRNAs tend to be important factors governing the stemness of both rabiPSCs and cPGCs.

In my thesis, I could characterize the expression of miR-302 cluster; which explained in the literature section above is stem cell and vertebrate specific cluster. In my study, I have successfully demonstrated the expression of this cluster along with its members in both cPGCs and rabiPSCs and also functionally distinguished the role of two of cluster members, the miR-302b-5p and miR-302b-3p. MiRNA biogenesis is controlled by different enzyme at different steps of processing. The final steps involves processing of the mature miRNA arm i.e. either the 5p arm or the 3p arm that can be then incorporated into the RISC (Ha and Kim, 2014). The pre-miRNA is cleaved by Dicer; either the 5p arm (mature strand) or the 3p arm (passenger strand) depending upon the thermodynamic stability, physiological conditions of the cell can be alternatively selected (Mitra et al., 2015). But it has been observed in various cancer cells; for example, lung cancer, colon cancer that there is existence of both arm (5p and 3p) pair and this pair is showing concordant dysregulation. The pairs are either co-regulated up or down or in the opposite directions. Both the arms are expressed and are involved in various pathways controlling the cancer tumour carcinogenesis; cancer pathophysiology and other pathways like proliferation, apoptosis (Choo et al., 2014; Huang et al., 2014) .These 5p/3p pairs cross-target signalling molecules involved in different signalling pathways or co-regulate the molecules involved in same pathway either in the   same direction or reverse. Due to being metabolically and physiologically distinct; the miR-302b have distinguished targets in humans and chickens. The main targets of miR-302 in humans are cell cycle inhibitors (Greer Card et al., 2008). In hESCs, via *OCT4* gene regulation the miR-302s regulates the expression of cell cycle inhibitors and thus, promotes faster G1 to S phase transition; thereby increase the

self-renewal rate i.e. proliferation. It can assume that like in hESCs and hiPSCs. In cPGCs there is high endogenous expression of gga-miR-302b-5p which is causing up-regulation of these signalling molecules. The up-regulation of these signalling molecules is causing a downstream inhibition effect or down-regulation of other signalling molecules in other pathways. In hESCs and hiPSCs *OCT4* gene is up-regulated which causes suppression of tumour suppressor gene *AKT (serine-threonine kinase gene)*; thereby, indirectly silencing the negative cell cycle inhibitors and promoting fasting G1 to S phase transition and high proliferation rate. Similar, mechanism is observed in chicken but via different regulatory mechanisms.

As, the miR-302 cluster in rabbits is highly homologous to humans, the humans and rabbits share potentially same set of targets. Hence, it can be assumed the ocu-miR-302a cluster regulates the pluripotency and self-renewal of rabiPSCs via down-stream regulation of cell cycle kinase's and inhibitors. The main miRNA cluster cited to be pluripotent specific in rabbits is ocu-miR-302 cluster. In this study, the ocu-miR-302a-3p and ocu-miR-302a- 5p5p were studied. In the previous study, conducted by (Maraghechi et al., 2013) in which solid sequencing analysis revealed high expression for the ocu-miR-302a-3p and ocu-miR-302a-5p arm in rabbit stem cells.

Therefore, the first step was to functionally characterize the role of these two miRNAs in rabiPSCs. From the proliferation test it can be inferred that the above two miRNAs are proliferation promoter and help in increasing the proliferation of rabiPSCs but in opposite directions. The over expression of arm 5p increases proliferation and the inhibition of arm 3p increases proliferation.

6   New Scientific achievements

In this study*, in vitro* miRNA inhibition assays and cell proliferation assays were performed to characterize the roles of gga-miR-302 and ocu-miR-302 cluster members in chicken PGCs and rabbit iPSCs. Based on this experimentation the following new scientific results were obtained:

1. This was the first study where expression of gga-miRs in chicken primordial germ cells were characterized using μParafloR microchip technology. The results revealed novel miRNA signatures in cPGC lines. These miRNAs were identified to be involved in main cell cycle regulations pathways.
2. Based on the results of μParafloR microchip analysis, I conclude that in all examined male and female chicken PGC lines, the gga-miR-302 cluster members are highly expressing.
3. I could identify concordant dysregulation between the two arms of the gga-miR-302b miRNA. Mature gga-miR-302b-5p tend to show high expression in the highly proliferating PGC lines and low expression in the low proliferating cell lines. I verified high 5p /3p ratio in highly proliferating cPGCs.
4. This is the first study, in which the functional characterization of the gga-miR-302b-5p and gga-miR-302b-3p was performed in cPGCs using miRNA inhibition assay. Inhibition of gga-miR-302b-5p using anti-miR-302b-5p significantly increased the doubling time of cPGCs compared to the control, meanwhile the inhibition of gga-miR-302b-3p decreased the doubling time.
5. In this study I also investigated the optimal conditions for *in vitro* PGCs culturing. I found that the optimal PGC growth (optimal proliferation rate), depend on the initial cell concentrations, but there is significant difference between the individual PGC lines, too.
6. It is the first study, in which ocu-miR-302a were functional studied via miRNA inhibition and proliferation test. This study revealed that both ocu-miR-302a overexpression and inhibition of ocu-miR-302a-3p decreased the doubling time of rabbit iPSCs.

7   References

Balzano, F., Cruciani, S., Basoli, V., Santaniello, S., Facchin, F., Ventura, C., Maioli, M., 2018. MiR200 and MiR302: Two big families influencing stem cell behavior. Molecules 23.

Bodak, M., Cirera-Salinas, D., Luitz, J., Ciaudo, C., 2017. The Role of RNA Interference in Stem Cell Biology: Beyond the Mutant Phenotypes. J. Mol. Biol. 429, 1532–1543.

Bracken, M.B., 2009. Why animal studies are often poor predictors of human reactions to exposure. J. R. Soc. Med. 102, 120–122.

Choo, K.B., Soon, Y.L., Nguyen, P., Nguyen, N., Sook, M., Hiew, Y., 2014. Cross-Targeting in Colon Cancer Cells 1–14.

Cirera-Salinas, D., Yu, J., Bodak, M., Ngondo, R.P., Herbert, K.M., Ciaudo, C., 2017. Noncanonical function of DGCR8 controls mESC exit from pluripotency. J. Cell Biol. 216, 355–366.

Ericsson, A.C., Crim, M.J., Franklin, C.L., 2013. A Brief History of Animal Modeling. Mol. Med.

Gao, Z., Zhu, X., Dou, Y., 2015. The miR-302/367 cluster: a comprehensive update on its evolution and functions. Open Biol. 5, 150138.

Gearhart, J.D., Addis, R.C., 2009. The use of animals in human stem cell research: past, present, and future. ILAR J. 51, 1–2.

Graves, P., Zeng, Y., 2012. Biogenesis of Mammalian MicroRNAs: A Global View. Genomics, Proteomics Bioinforma. 10, 239–245.

Greer Card, D.A., Hebbar, P.B., Li, L., Trotter, K.W., Komatsu, Y., Mishina, Y., Archer, T.K., 2008. Oct4/Sox2-Regulated miR-302 Targets Cyclin D1 in Human Embryonic Stem Cells. Mol. Cell. Biol. 28, 6426–6438.

Ha, M., Kim, V.N., 2014. Regulation of microRNA biogenesis. Nat. Rev. Mol. Cell Biol. 15, 509–524.

Hamburger, V., Hamilton, H.L., 1951. A series of normal stages in the development of the chick embryo. Dev. Dyn. 88, 49–92.

Huang, C.J., Nhi Nguyen, P.N., Choo, K.B., Sugii, S., Wee, K., Cheong, S.K., Kamarul, T., 2014. Frequent co-expression of miRNA-5p and -3p species and cross-targeting in induced pluripotent stem cells. Int. J. Med. Sci. 11, 824–833.

Lee, J.C.I., Tsai, L.C., Hwa, P.Y., Chan, C.L., Huang, A., Chin, S.C., Wang, L.C., Lin, J.T., Linacre, A., Hsieh, H.M., 2010. A novel strategy for avian species and gender identification using the CHD gene. Mol. Cell. Probes 24, 27–31.

Lee, Y.J., Ramakrishna, S., Chauhan, H., Park, W.S., Hong, S.H., Kim, K.S., 2016. Dissecting microRNA-mediated regulation of stemness, reprogramming, and pluripotency. Cell Regen. 5, 1–10.

Lichner, Z., Páll, E.A., Kerekes, A., Pállinger, É., Maraghechi, P., Bodouble acutesze, Z., Gócza, E., 2011. The miR-290-295 cluster promotes pluripotency maintenance by regulating cell cycle phase distribution in mouse embryonic stem cells. Differentiation 81, 11–24.

Maraghechi, P., Hiripi, L., Tóth, G., Bontovics, B., Bosze, Z., Gócza, E., 2013. Discovery of pluripotency-associated microRNAs in rabbit preimplantation embryos and embryonic stem-like cells. Reproduction 145, 421–437.

Mitra, R., Lin, C.-C., Eischen, C.M., Bandyopadhyay, S., Zhao, Z., 2015. Concordant dysregulation of miR-5p and miR-3p arms of the same precursor microRNA may be a mechanism in inducing cell proliferation and tumorigenesis: a lung cancer study. Rna 21, 1055–1065.

Nakamura, Y., Kagami, H., Tagami, T., 2013. Development, differentiation and manipulation of chicken germ cells. Dev. Growth Differ. 55, 20–40.

Reik, W., Surani, M.A., 2015. Germline and Pluripotent Stem Cells 1–24.

Schmaltz-Panneau, B., Jouneau, L., Osteil, P., Tapponnier, Y., Afanassieff, M., Moroldo, M., Jouneau, A., Daniel, N., Archilla, C., Savatier, P., Duranthon, V., 2014. Contrasting transcriptome landscapes of rabbit pluripotent stem cells in vitro and in vivo. Anim. Reprod. Sci.

Takahashi, K., Yamanaka, S., 2006. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors 2, 663–676.

Tapponnier, Y., Afanassieff, M., Aksoy, I., Aubry, M., Moulin, A., Medjani, L., Bouchereau, W., Mayère, C., Osteil, P., Nurse-Francis, J., Oikonomakos, I., Joly, T., Jouneau, L., Archilla, C., Schmaltz-Panneau, B., Peynot, N., Barasc, H., Pinton, A., Lecardonnel, J., Gocza, E., Beaujean, N., Duranthon, V., Savatier, P., 2017. Reprogramming of rabbit induced pluripotent stem cells toward epiblast and chimeric competency using Krüppel-like factors. Stem Cell Res. 24, 106–117.

Whyte, J., Glover, J.D., Woodcock, M., Brzeszczynska, J., Taylor, L., Sherman, A., Kaiser, P., McGrew, M.J., 2015. FGF, Insulin, and SMAD Signaling Cooperate for Avian Primordial Germ Cell Self-Renewal. Stem Cell Reports 5, 1171–1182.

Winter, J., Jung, S., Keller, S., Gregory, R.I., Diederichs, S., 2009. Many roads to maturity: MicroRNA biogenesis pathways and their regulation. Nat. Cell Biol.

Zeng, Z.L., Lin, X. long, Tan, L.L., Liu, Y.M., Qu, K., Wang, Z., 2018. MicroRNAs: Important Regulators of Induced Pluripotent Stem Cell Generation and Differentiation. Stem Cell Rev. Reports 14, 71–81.

8   Publications of Mahek Anand related to the topic

1. B. Lázár, **M. Anand**, R. Tóth, E. Patakiné Várkonyi, K. Liptói, E. Gócza (2018): Comparison of the MicroRNA Expression Profiles of Male and Female Avian Primordial Germ Cell Lines. *Stem Cells International*, ID 1780679 (*shared first author publication*) (**IF: 3.563**)

2. **Anand**, **M**., Lázár, B., Tóth, R., Páll, E., Patakiné Várkonyi, E., Liptói, K., Homolya, L., Hegyi, Z., Hidas, A., Gócza, E. (2018): Enhancement the chicken primordial germ cell in vitro maintenance using automated cell image analyser. *Acta Veterinaria Hungarica* 66(4) (*shared first author publication*) (**IF:1.042**)

3. **Anand, M**., Tóth, R., Kidane, A., Nagy, A., Lazar, B., Patakiné, Várkonyi E., Liptói, K., Gócza, E. (2016): Examination the expression pattern of HSP70 heat shock protein in chicken PGCs and developing genital ridge. *Scientific Papers: Animal Science and Biotechnologies*, 2016, 49(1):78-82. (*first author publication*)

9   Publications in Preparations

1. Bontovics, B., Maraghechi, P., Slamecka, J., Lázár, B., **Anand, M.,** Németh, K., Fábián, R., Hiripi, L., Vašiček, J., Makarevich, A.V., Gócza, E., Chrenek, P.: Examination the effect of combined inhibition on development of *in vitro* cultured rabbit embryos. *Theriogenology (IF: 1.986)*

2. **Anand, M.,** Bontovics, B., Maraghechi, Lázár, B., Németh, K., Fábián, R., Tóth R., Gócza, E.: Functional analysis of stem cell specific ocu-miR-302 cluster in rabbit embryos and induced pluripotent stem cells. *Journal of Genetics (IF: 0.995) (First authors publication)*