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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The PhD program

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2. Abbreviations

ASCs	Adult Stem Cells
AKT	Serine/Threonine Kinase 1
BMP4	Bone Morphogenetic Protein 4
cESCs	Chicken Embryonic Stem Cells
CHD1	Chromosome helicase DNA binding protein 1
cPGCs	Chicken Primordial Germ Cells
CRISPR/CAS9	Clustered regularly interspaced palindromic repeats/CRISPRassociated 9
CVH	Chicken Vasa Homologue
DPPA4	Development pluripotent associated 4
DGCR8	DiGeorge Critical Region 8
ESSRB	Estrogen related receptor β
ESCs	Embryonic Stem Cells
EpiSCs	Epiblast-like Stem Cells
ERK	Extracellular signal regulated kinases
FGF2	Fibroblast Growth Factor 2
FSCs	Fetal Stem Cells
FGF	Fibroblast Growth Factor
FGFR	Fibroblast growth factor receptor
GAPDH	Glyceraldehyde 3- phosphate dehydrogenase
GSCs	Gonadal Stem cells
GSK-3	Glycogen synthase kinase 3
HELLS	Lymphoid specific helicase
hESCs	Human Embryonic Stem cells
ICM	Inner Cell Mass
iPSCs	Induced Pluripotent Stem cells
2i	Dual inhibition of MEK and GSK3 receptors
KLF4	Kruppel–Like Factor 4

LIF	Leukemia Inhibitory Factor
LIN28	Protein lin-28 homolog A
MAPK	Mitogen activated protein kinase
MEK	MAP kinase-ERK kinase
mEpiSCs	Mouse Epiblast like Stem Cells
mESCs	Mouse Embryonic Stem Cells
MKK1	Mitogen-activated protein Kinase Kinase
MDM2	Mouse double minute homologue 2
MSCs	Mesenchymal Stem Cells
MYC	MYC proto-oncogene, bHLH transcription factor
NANOG	Homeobox transcription factor
NR5A2	Nuclear receptor sub-family 5 group A member 2
OCT4	Octamer-Binding Transcription Factor 4
PCH	Pericentromeric Heterochromatin
PGCs	Primordial Germ Cells
PRC2	Polycomb recessive complex 2
rabiPSCs	Rabbit Induced Pluripotent Stem Cells
REM2	GTP – binding protein 2
rESCs	Rabbit Embryonic Stem Cells
SALL4	Sal – like protein 4
SCNT	Somatic Cell Nuclear Transfer
SMARC6	Actin dependent regulators of chromatin 6
SMAD	SMAD family members are signal transducers and transcriptional modulators
SOX2	Sex Determining Region Y-Box 2
SSEA-1/4	Stage Specific Embryonic Antigen-1/4
STAT3	Signal Transducer and Activator of Transcription 3
SV40LT	S 40 large T antigen

TALEN	Transcription Activator-like Effector Nuclease
TBX3	T box transcription factor 3
TERT	Telomerase reverse transcriptase
TFG-β	Transforming growth factor β
USCs	Umbilical Cord Stem Cells
UTF1	Undifferentiated embryonic cell transcription factor 1

3. Introduction and objectives

3.1. Importance of the field

"Necessity is the mother of invention ". Stem cell research has revolutionized 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 animal model used is the rodents. However, with the discovery of genetic techniques the traditional animal models could be genetically modified and hence, the development of transgenic animals started. The most eminent was the development of the transgenic mice and rats especially the gene knock-out mice and rats based on the available gene knock out techniques. The same technique was used to develop transgenic animals in other species like birds, pigs and cattle.

But, as there are physiological differences between the humans and animals, the next step was to develop the "Humanization of these animal models ". Exploiting the genetic engineering techniques the human genes were introduced into mice and rats as models to mimic the human disease physiology using human derived immune system genes (Shultz et al., 2012).

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 (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 the nature they can be genetically modified and used to create germline chimeras (Nakamura et al., 2013). The most studied PGCs are cPGCS (chicken PGCs). 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 rate is not yet fully investigated. 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 in the nucleus. This is the classical or the canonical pathway by which a mature miRNA is generated (***Figure 1***).

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.

Drosha enzyme of the microprocessor complex is part of the RNAs III family of endonucleases. The amino terminal of Drosha recognizes the pri-miRNA. The Drosha along with DGCR8 as the DGCR8 enzyme is a protein that interacts with Drosha. The Drosha at its carboxyl end contains two RNAse III domains (RIIID) and the double stranded RNA binding domain (dsRBD). It is these RIIID that combine to form one and form an interface in which it processes the stem loop structure of the pri-miRNA. The carboxyl terminus of DGCR8 enzyme interacts with Drosha and helps in initializing the pri-processing. The combined actions of both DGCR8 and Drosha constitute the microprocessor complex and its function (***Figure 2***), (Ha and Kim, 2014).

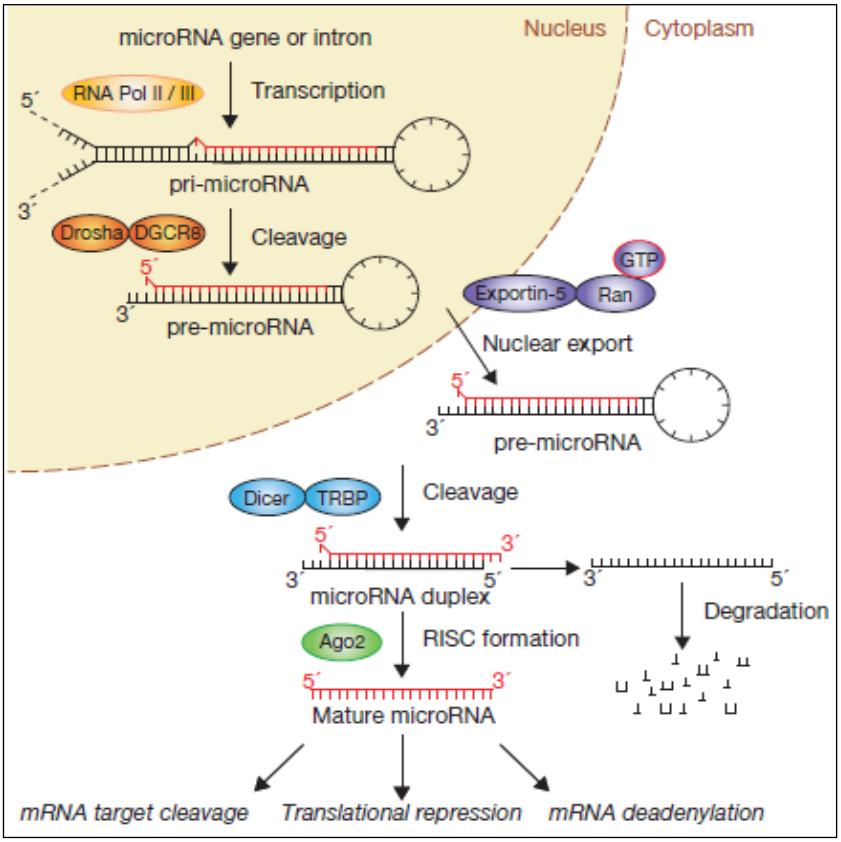


Figure 1: *miRNA Biogenesis – canonical pathway of miRNA maturation (Winter et al.,2009).*

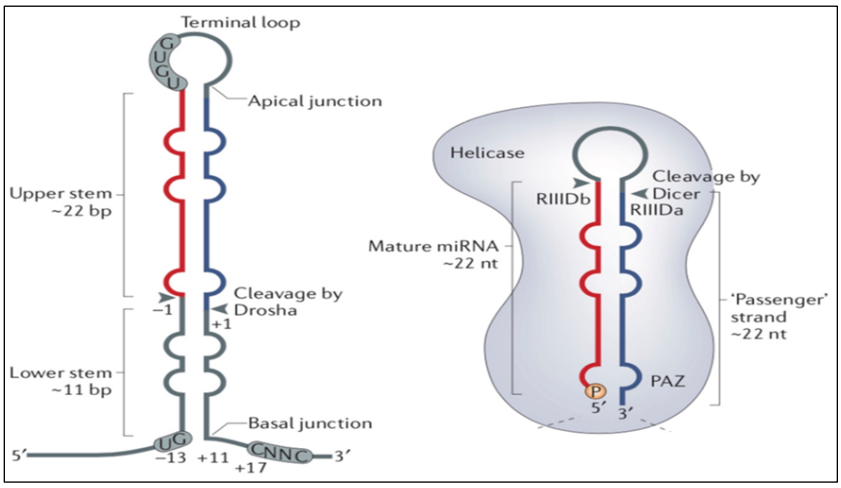


Figure 2: *Pri-miRNA processing by the microprocessor complex – The pri-miRNA is a stem loop structure of 35 nucleotides. It is divided into the upper stem and lower stem. The cleavage by Drosha takes place by calculating 22 nucleotides from the apical junction and 11 nucleotides bases from the basal junction. This generates a pre-miRNA consisting of the overhangs at the 5' end and the 3'end that contains both the guide strand (mature miRNA strand) and the passenger strand to be cleaved by Dicer (Ha and Kim, 2014).*

The pre-miRNA is then exported from the nucleus to the cytoplasm by exportin-5. Here, Dicer an another RNAse III endonuclease cleaves the pre-miRNA to a miRNA duplex consisting of both the 5p and the 3p arm (***Figure 1***). One of the two arms is usually destroyed and the guide strand (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 5p arm 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 is de-adenylated else if the pairing is imperfect the miRNA undergoes translation repression 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 the 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).

The phenotypic effects are different depending on which enzyme is knocked out. 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. (Cirera-Salinas et al., 2017) elucidated the independent important role of DGCR8 in mESCs. In the study, the mESCs that were knocked out for DGCR8 protein were complemented with the mutant phosphorylated DGCR8 protein. It was found that although this mutant form could restore the normal miRNA production but could not exit the mess 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 well characterized male and female chicken primordial germ cell (cPGC) lines as well as rabbit induced pluripotent stem cell lines (rabiPSCs) which cell lines I could characterise during my PhD work.

3.2. Objectives of my work

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.

4. Literature review

4.1. Basics of stem cells

The following chapter provides an overview about the basic history, terminology regarding stem cells as a general perspective.

4.2. Origin of stem cells

In the contemporary time, stem cells have revolutionized the field of molecular biology and transgenic technology. With the advent of PCR-based techniques and next generation sequencing machines stem cells hold immense application in the domain of therapeutics, regenerative medicine, animal model studies (Reik and Surani, 2015). The potential application of stem cells was discovered many years ago in the 19th century. The term stem cell was first coined by eminent German biologist Ernst Haeckel, 1868 (Ramalho-Santos and Willenbring, 2007). Ernst Haeckel was remarkably influenced by the works of Darwin, especially, his works on evolutionary theory. Darwin proposed about the existence of a common ancestor from all life-forms originated. Haeckel described a phylogenetic relationship between these ancestors and termed them as 'Stammbaume '(German word for trees or more likely to represent family of trees: stem cell trees). It was from this word that the terminology 'Stammzelle' arose for stem cells. 'Stammzelle' is used to describe a common unicellular ancestor form which all multicellular organisms have descended (Ramalho-Santos and Willenbring, 2007).

In this pre-context, the meaning of the word stem cells was used in two different directions one as a concept of a general unicellular ancestor and other as a fertilized egg which gave rise to cells of organism. In this concept, the stem cells were defined distinct as a germ cell capable of transmitting information from one generation to another, with regard to Weismann theory of germplasm.

Parallel to this, there was ongoing work about the origin of the blood cells. The term stem cell was used to define the presence of a common hematopoietic cell that gave rise to both white and red blood cell types. However, because of lack of experimental evidence there was long debate regarding the exact usage and terminology for the word stem cell (Ramalho-Santos and Willenbring, 2007).

In the present period, stem cell is defined as precursor cells that have unlimited self-renewal capacity and can grow for unlimited time and period. They have regenerating capacity and can also develop or give rise to any cell of the organism. They can be maintained in an

undifferentiated state but can later be differentiated to a particular type of cell (Bhattacharyya, 2012). The property to divide infinitely and self - renew is called potency.

Based upon the origin of the stem cell and its potency the stem cell can be classified into different types (***Figure 3, Table 1***).

4.3. Classification of stem cells

4.3.1. Developmental potency of stem cells

Potency is defined as the developmental potential of the given stem cell: whether it is able to give rise all cells of the body, or only specific cells restricted to a lineage (Singh et al., 2015) (***Table 1***).

Developmental Potential	Definition	Example
Totipotent	These cells have the ability to form entire organism.	Zygote up to the 8 stages of the morula
Pluripotent	These cells have the potential to give rise to any cell type found in the primary germinal layers of the embryo (endoderm, ectoderm and mesoderm).	Embryonic Stem Cells
Multipotent	These cells can give rise to all cell types within a given lineage.	Mesenchymal Stem Cells from adults.
Oligopotent	These cells have limited differentiation potential	Stem cells from adult lymphoid/myeloid tissues
Unipotent	These cells can differentiate only into specific defined lineage.	Cells from liver, lung etc.

Table 1: *Classification of stem cells based on developmental potency (Bhattacharyya, 2012)*

4.4. Origin of stem cells

4.4.1. Embryonic stem cells (ESCs)

ESCs are obtained from the blastocyst following five days after fertilization. The blastocyst is a hollow sphere of cells composing of outer of layer of cells called the trophoblast and an inner cell mass (ICM) attached to it. It is from this ICM that ESCs are isolated and cultivated.

The ICM give rise to the embryo (Chagastelles and Nardi, 2011). The ESCs are pluripotent in nature.

4.4.2. Fetal stem cells (FSCs)

FSCs are isolated from the foetal blood and from the embryonic annexes .They are multipotent in nature (Bhattacharyya, 2012)

4.4.3. Adult Stem cells (ASCs)

ASCs are multipotent in nature. They are located in specific stromal niches, which is in the adult tissues or organs, they have limited self - renewal and differentiation ability. They are normally responsible for the maintenance of organ i.e. repair of the tissue owning to pathophysiological processes (Chagastelles and Nardi, 2011).

One example of ASCs is mesenchymal stem cells (MSCs) that are isolated from the stroma of any tissue. These MSCs give rise to all stem cells mesodermal in nature.

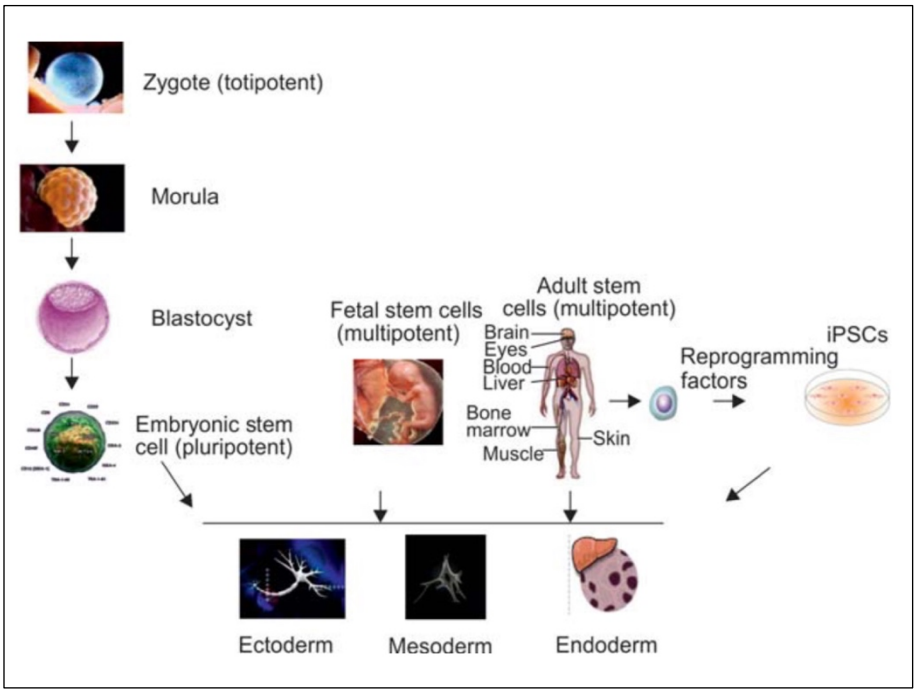


Figure 3: *Stem cell classification based upon source and potency (Bhattacharyya, 2012).*

4.4.4. Hematopoietic stem cells (HSCs)

HSCs that are originated form the bone marrow and give to rise all cell types of the blood.

4.4.5. Umbilical cord stem cells (UCSs)

Umbilical cord is a rich source of stem cells. There are two sources: one is the amniotic membrane which give rise to umbilical cord epithelium stem cells and other is the umbilical blood form which umbilical cord hematopoietic stem cell and mesenchymal stem cells can be isolated.

4.5. History of the stem cells

The establishment of ESCs can be traced back to the 1980's. The first ESCs was established in mice (Martin, 1981). The normal mouse blastocyst was isolated and cultured under *in vitro* conditions using the conditioned media generated from the mouse embryonic carcinoma cell lines (mECCs). The culturing of mouse blastocysts in this media derived the first ESC cell line in mouse. The mECCs are similar to mESCs in morphology, formation of colonies and express the stem cell markers like SSEA-3, SSEA-4. Evans and Kaufmann et al., 1981 also reported the establishment of mESCs from mouse embryos.

Following successful establishment of mESCs, there were attempts to establish hESCs also. Using, *in vitro* fertilized oocytes the first attempt to generate hESCs was done (Thomson et al., 1998). Human ESCs express mainly the SSEA-3 and SSEA-4, whereas mESCs express the SSEA-1 marker. In both species, the ESCs were developed on mitomycin-C treated mouse fibroblasts which were used as the feeder layer. Feeder layer culturing is the basics for culturing of ESC as well as iPSC lines in all species.

Successful establishment of these stem cell lines opened doors for future work on therapeutics, regenerative medicine. However, isolation of the above-mentioned human stem cells, especially the human ESCs face a lot of ethical restriction and consideration. The isolation of ESCs from blastocyst is ethically restricted due to destruction of the blastocyst for research purposes. But, in 2006 this problem was overcome by the advent of induced pluripotent stem cell technology developed by Takashi and Yamanaka. They reprogrammed adult cells to pluripotent stem cells via genetic reprogramming (Takahashi and Yamanaka, 2013).

The iPSCs technology and genetic reprogramming is discussed in the section below.

4.6. Induced pluripotent stem cell technology and genetic reprogramming

Genetic reprogramming is defined as the phenomenon by the virtue of which somatic cells can be reprogrammed to behave as ESCs like in nature (Buganim et al., 2013). The

epigenome of the somatic cells is completely changed. Initially genetic reprogramming was done by somatic cell nuclear transfer (SCNT) or by cell fusion. However, in 2006 Takashi and Yamanaka by the aid of transcription factors generated iPSCs.

The four transcription factors used to somatically reprogram the mouse embryonic fibroblast to iPSCs state are collectively called as OKSM or Yamanaka factors (O-OCT4, K-KLF4, S-SOX2 and M-c-MYC). Yamanaka cited that OCT4, SOX2 are core transcription factors (Takahashi and Yamanaka, 2006). C-MYC and KLF4 are tumour–related factors (Takahashi et al., 2007). They have downstream targets which enhances the proliferation and transformation efficiency. Although, Takahashi and Yamanaka were successfully able to reprogram the cells, but they found low efficiency in the transformation. Only a small portion of the cells were transformed. There could be many reasons for the low frequency. First of all was the right dosage i.e. the amount of the 4 factors needed to reprogram the cells. Secondly, they postulated that other than the expression of these 4 factors the cells must undergo several other changes; such as changes in the chromosomal arrangement i.e. chromatin modification via methylation and de-acetylating enzymes. These are changes in the epigenome configuration of cells which takes place during the process of reprogramming (Takahashi and Yamanaka, 2006).

Other than the reprogramming factors, the delivery system used to deliver these factors and the type of donor cell used for reprogramming play important roles (González et al., 2011). Hence, in order to define the appropriate conditions for reprogramming and the right transcription factors required more detailed analysis of the iPSCs technology (***Table2***).

4.7. Description of the transcription factors – OKSM

To express/overexpress	**To repress**
Important for embryonic development:	*Apoptosis, cell cycle and senescence:*
OCT4, SOX2, NANOG, UTF1, LIN28, SALL4, NR5A2, TBX3, ESSRB, DPPA4	p16INK4AA#, p53#, microRNA 21
Proliferation and cell cycle:	*Epigenetic Regulators:*
MYC*, KLF4*, SV40LT*, REM2, MDM2*, cyclin D1*	histone de-acetylase, histone de-methylase, G9a, DNMT1*
Epigenetic regulators	*Signalling pathways*
CHD1, PRC2	TGFβ, WNT, ERK-MAPK
vitamin C, hypoxia, E-cadherin, miR-294, TERT*	**: Potential oncogene, #: Potential tumour suppressor gene*

Table 2: *The main roles of the OKSM factors (González et al., 2011).*

4.8. Naive and primed pluripotency

There are two defined states of pluripotency: Naïve and Primed pluripotency. Naïve pluripotency is termed as the ground state pluripotency. The cells are defined as similar to ESCs in terms of transcriptional modification and signalling pathways required for the maintenance of the pluripotency (Tapponnier et al., 2017)(Coronado et al., 2013). Cell exhibiting naïve state of pluripotency rely on the LIF (Leukaemia inhibitory factor) and BMP4 (Bone morphogenetic protein 4) pathways for self-renewal and blocking differentiation. Other main characteristic of cells in naïve state of pluripotency is short G1 cell cycle stage. The shorter G1 cell cycle compared to other cells may be to acerbate the proliferation rate of the naïve state pluripotent stem cells. The LIF/STAT3 pathway STAT3 (Signal transducer and activator of transcription 3) pathway is responsible for the short G1 phase in naïve state pluripotent stem cells. Some of the examples of naïve state pluripotent stem cells is mouse embryonic stem cells (mESCs) (Savatier et al., 2017). The naïve pluripotency is characterized by the ability of these stem cells to form germline and somatic chimeras.

Primed pluripotency refers to state of pluripotency in which stem cells acquire features of epiblast like stem cells (EpiSCs). These stem cells are derived from pre-implantation blastocyst at epiblast embryonic day 6 to 7.5 (Tapponnier et al., 2017) . Unlike, the mESCs these EpiSCs lack the ability to be form germline and somatic chimeras. The EpiSCs can be converted to naïve state by overexpression of pluripotent factors like Klf4, Kfl2, Stat3, Nanog. Examples of primed pluripotency are rabbit induced pluripotent stem cells (rabiPSCs), mouse epiblast like stem cells (mEpiSCs), human embryonic stem cells (ESCs) and human induced pluripotent stem cells (hiPSCs). They rely on fibroblast growth factor 2 (FGF2)/Activin A or SMAD signalling pathway for self-renewal and pluripotency maintenance (***Table 3***).

Property	Ground Stage	Primed State
Embryonic Tissue	early epiblast	egg cylinder or embryonic disc
Culture stem cell	rodent ESCs	rodent EpiSCs; primate "ESCs"
Blastocyst chimaeras	yes	no
Teratomas	yes	yes
Differentiation bias	none	variable
Pluripotency factors	Oct4, Nanog, Sox2, Klf2, Klf4	Oct4, Sox2, Nanog
Naïve Markers	Rex1, NrOb1, Fgf4	absent
Specification Markers	absent	Fgf5, T
Response to Lif/Stat3	self-renewal	none
Response to Fgf/Erk	differentiation	self-renewal
Clonogenicity	high	low
XX Status	XaXa	XaXi
Response to 2i2i (dual inhibition of MEK and GSK3 receptors)	self-renewal	differentiation/death

Table 3: *The main differences between the naïve and primed state pluripotency (Table modified from (Nichols and Smith, 2009)*

4.9. Application of stem cells and induced pluripotent stem cells

The iPSCs hold promising applications in the fields of regenerative medicine, drug designing as well as genetic modification. Disease-specific iPSCs can be developed. Disease-specific iPSCs were generated in the case of spinal muscular atrophy (Takahashi and Yamanaka, 2013). Patient-specific iPSCs can be used for potential drug and therapeutic applications like testing the vaccines. The iPSCs hold immense importance in the field of neurological disorders like Parkinson's disease and haematology.

The iPSCs can also be used in replacement for animal model studies. The iPSCs can be differentiated into desired cell types (such as neurons, cardiomyocytes) which can be used for the study of the specific disorders (Singh et al., 2015).

The ***Figure 4*** below summarizes the applications of the iPSCs technology.

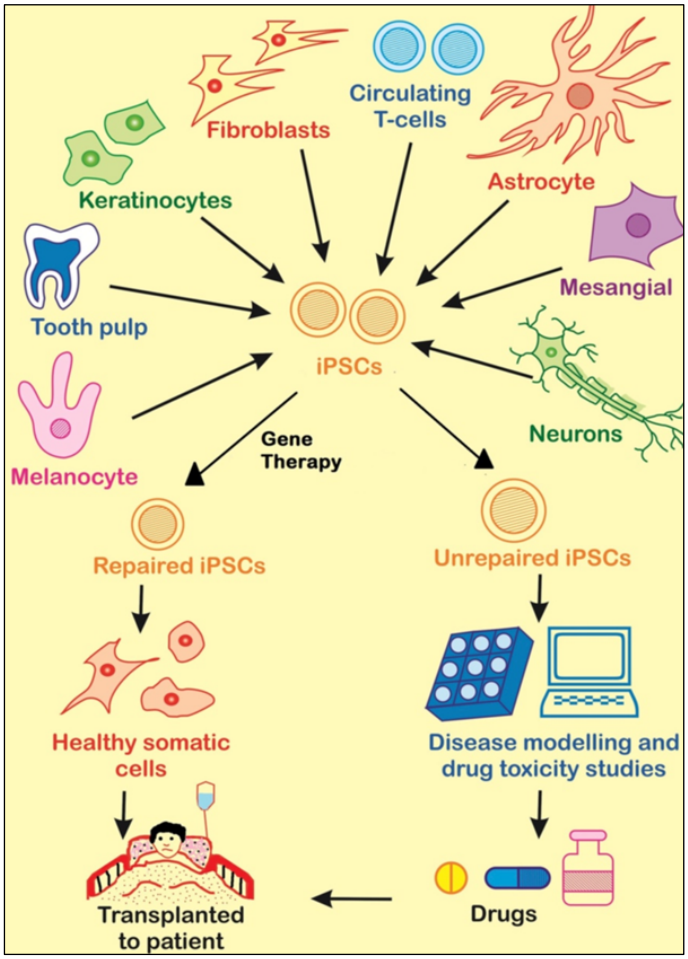


Figure 4.: *Applications of induced pluripotent stem cells.*
(Image modified from (Singh et al., 2015)

4.10. Chicken embryology

The embryology in chicken is classified into three stages: the early, middle and the late phases. The early and middle phases are the phases of organ development (organogenesis) takes place in the late phase, there is final maturation of the body axis and the body plan (Hamburger and Hamilton, 1951). Hamburger and Hamilton classified embryology into different stages called Hamilton and Hamburger stages based on the appearance of different classical morphological features as different hallmarks of development (***Figure 5***).

The first 7 stages are based on the development and the emergence of the primitive streak. The stages from 7 to 14 are based on the advent of the somite pairs. Stage 7 designates first somite; thereby, the stages following that are defined as addition of number of the pairs.

Stage 8 denotes stages with 4 somite's and hence , so on (Hamburger and Hamilton, 1951) (***Table 4***).

From stage 15, the main identifying criteria are the occurrence of the limb buds, visceral arches etc. as the advancement and development of the different stages. These criteria are used till stage 39. The final stages from 40 to 46, are defined on the measurement of the beak length and the formation of the toe. In chickens, there are 46 stages of embryonic development. Each stage is a representation of different hallmarks of development.

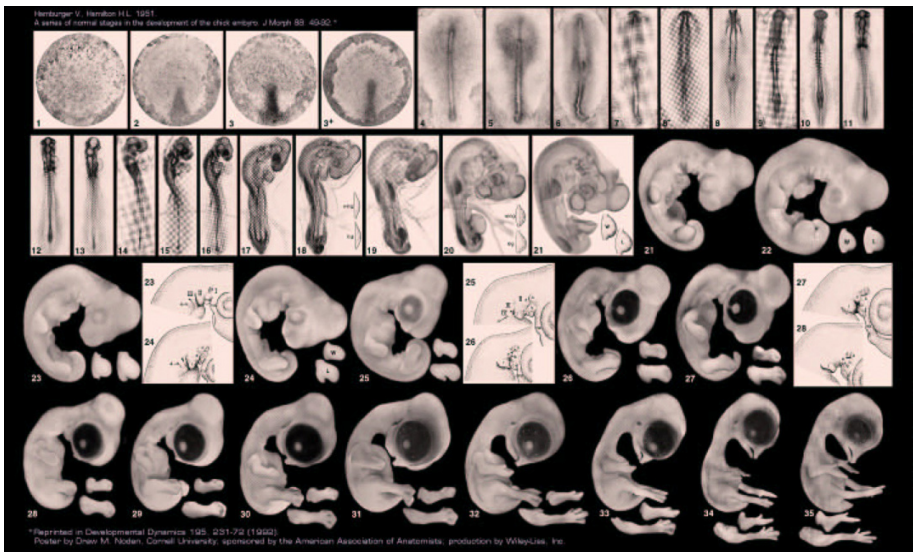


Figure 5: *The stages of embryonic development in chicken (Hamburger and Hamilton, 1951)*

Embryo development in chicken is an interplay of environmental and parental interactions. The parents, especially the mother plays an important role in providing the necessary nutrients, maternal determinants and other factors for the growth and proper development of the growing embryo. As well as, the embryo responds to signals from the environment, as environmental signals for development. There is interaction between the embryo on its own, the parents and these signals to determine the optimal time of hatching in chickens (Reed and Clark, 2011).

HH Stages	Age	Identification of Stages
Before Laying		
Early Cleavage	3.5-4.5 hr.	Shell membrane of egg formed in isthmus of oviduct
During Cleavage		Germ wall formed from marginal periblast
Late Cleavage	4.5-24.0 hr.	Shell of egg formed in uterus
After Laying		
1		Preprimitive streak (embryonic shield)
2	6-7 hr.	Initial primitive streak, 0.3-0.5 mm long
3	12-13 hr.	Intermediate primitive streak
4	18-19 hr.	Definitive primitive streak, +/- 1.88 mm long
5	19-22 hr	Head process (notochord)
6	23-25 hr	Head fold
7	23-26 hr	1 somite; neural folds
7 to 8-	ca. 23-26 hr	1-3 somites; coelom
8	26-29 hr	4 somites; blood islands
9	29-33 hr	7 somites; primary optic vesicles
9+ to 10-	ca.33 hr	8-9 somites; anterior amniotic fold
10	33-38 hr	10 somites;3 primary brain vesicles
11	40-45 hr	13 somites; 5 neuromeres of hindbrain
12	45-49 hr	16 somites; telencephalon
13	48-52 hr	19 somites; atrioventricular canal
13+ to 14-	ca. 50-52 hr	20-21 somites; tail bud
14	50-53 hr	22 somites; trunk flexure; visceral arches I and II, clefts 1 and 2
14+ to 15-	ca. 50-54 hr	23 somites; premandibular head cavities
15	50-55 hr	24-27 somites; visceral arch III, cleft 3
16	51-56 hr	26-28 somites; wing bud; posterior amniotic fluid
17	52-64 hr	29-32 somites; leg bud; epiphysis
18	3 da	30-36 somites extending beyond level of leg bud; allantois
19	3.0-3.5 da	37-40 somites extending into tail; maxillary process
20	3.0-3.5 da	40-43 somites; rotation completed; eye pigment

21	3.5 da	43-44 somites; visceral arch 1V, cleft 4
22	3.5-4.0 da	Somites extend to tip of tail
23	4 da	Dorsal contour from hindbrain to tail is a curved line
24	4.5 da	Toe plate
25	4.5-5.0 da	Elbow and knee joints
26	5 da	1st 3 toes
27	5.0-5.5 da	Beak
28	5.5-6.0 da	3 digits, 4 toes
29	6.0-6.5 da	Rudiment of 5th toe
30	6.5-7.0 da	Feather germs, scleral papillae; egg tooth
31	7.0-7.5 da	Web between 1st and 2nd digits
31	7.0-7.5 da	Web between 1st and 2nd digits
32	7.5 da	Anterior tip of mandible has reached beak
33	7.5-8.0 da	Web on radial margin of wing and 1st digit
34	8 da	Nictitating membrane
35	8.5 -9.0 da	Phalanges in toe
36	10 da	Length of 3rd toe from tip to middle of metatarsal joint =5.4 +/- 0.3 mm; length of beak from anterior angle of nostril to tip of bill=2.5 mm; primordium of comb; labial groove; uropygial gland
37	11 da	Length of 3rd toe=7.4 +/- 0.3 mm; length of beak =3.0 mm
38	12 da	Length of 3rd toe=8.4 +/- 0.3 mm; length of beak= 3.1 mm
39	13 da	Length of 3rd toe=9.8 +/- 0.3 mm; length of beak =3.5 mm
40	14 da	Length of beak =4.0 mm; length of 3rd toe=12.7 +/- 05.mm
41	15 da	Length of beak from anterior angle of nostril to tip of upper bill=4.5 mm; length of 3rd tow= 14.9 +/- 0.8 mm
42	16 da	Length of beak=4.8 mm; length of 3rd toe=16.7+/- 0.8 mm
43	17 da	Length of beak =5.0 mm; length of 3rd toe=18.6 +/- 0.8 mm
44	18 da	Length of beak =5.7 mm; length of the 3rd toe =20.4 +/- 0.8 mm
45	19-20 da	Yolk sac half enclosed in body cavity; chorio-allantoic membrane contains less blood and is "sticky" in living embryo
46	20-21 da	Newly - hatched chick

Table 4: *The 46 hamburger and Hamilton stages of classification* (Hamburger and Hamilton, 1951).

Cell type	Source	Confirmation Methods
ESCs	Stage X (EG &K)	EB formation, in vitro differentiation, somatic chimaeras
PGCs	Stages 14-17 (H&H)	Germline chimaeras
EGCs	Stage 28 (H&H)	EB formation, in vitro differentiation, somatic chimaeras
GSCs/s	Juvenile 6 wk. old and adult (24-wk old) male roosters	EB formation, in vitro differentiation, somatic chimaeras
iPSCs	Quail embryonic fibroblasts (embryonic day 11)	EB formation, in vitro differentiation, somatic chimaeras

Table 5: *Different sources of stem cells from avian embryos*
Image modified from (Intarapat and Stern, 2013a)

4.11. Chicken stem cells

Avian embryos (***Figure 6***) are upcoming pioneers in the field of non-mammalian biology and stem cell work. The avian embryos can serve as excellent model for studies related to developmental biology, drug designing, vaccine production , pharmaceutical production etc. (Farzaneh et al., 2017). The avian embryos have a small size, there can be easily collected due to their ease of availability all around the year and can be easily manipulated (Petitte et al., 2004).

The avian embryo is a rich reservoir of stem cells ***(Table 5).*** From, different embryonic stages different kinds of stem cells can be obtained and cultured under *in vitro* conditions. The ***Figure 6*** above depicts the different stages from which stem cells can be collected. The cESCs are similar to mEpiSCs and hESCs like. They are primed in nature. They have been successfully isolated and cultured *in vitro* condition. They can successfully contribute to somatic chimers but not germline chimeras. For germline chimeras, there is successful isolation of cPGCs. The cPGCs or chicken embryonic germ cells (cESCs) can be derived from the dorsal aorta, the vitelline artery, the heart of stage HH-14 embryos. The cPGCs during this stage migrate from the place of origin to the germinal crescent via the vasculature system; which is a striking feature of the cPGCs, hence the cPGCs can be also obtained from the blood.

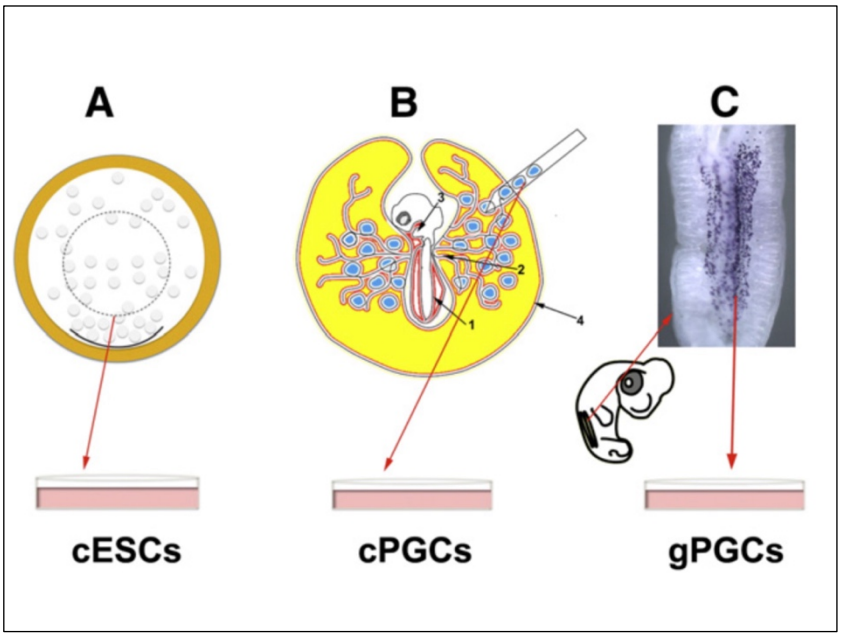


Figure 6: Different embryonic stages for stem cell derivation in chicken embryos. cESCs (chicken embryonic stem cells) can be isolated from stage X embryos blastodermal cells. The gonadal primordial germ cells (cPGCs) can be isolated from the vascular system or from the chicken gonads (Intarapat and Stern, 2013b).

4.11.1. Chicken primordial germ cells

PGCs are precursors of germ cells. Depending upon the chromosome complement of the embryo they develop into sperm or oocyte. As mentioned above, the chicken PGCs migrate from the epiblast (the place of origin) to the germinal crescent via the blood. This feature of cPGCs makes them easily accessible. The cPGCS can be collected from the blood and cultured *in vitro* (Bednarczyk, 2014). Chicken PGCs are potential pluripotent stem cells, as they express the pluripotency marker genes: *NANOG* , *SOX2* , *C-MYC* , *KLF4* (Jean et al., 2015; Macdonald et al., 2010).

In cPGCs, other than pluripotency specific genes, the germ cell specific markers also expressing. One of the most specific germ cell specific marker used for characterisation of cPGCS is the *CVH* (chicken vasa homologue) gene. The CVH protein expresses in the cytoplasm. It can be efficiently detected via immunostaining. CVH expression is specific to cPGCS (***Figure 7***).

The cPGCs also expresses the stem cell specific marker the SSEA-1 (stage-specific embryonic antigen-1) marker and another germ cell specific marker like DAZL (Deleted in azoospermia like).

Several attempts have been made to successfully isolate the cPGCs and grow them in *in vitro* conditions. The cPGCs can be used to create transgenic birds via genetic modification using CRISPR/CAS9 technology (Lee et al., 2017). The cPGCs can be also cryopreserved and used for successful establishment of cPGCs bio-banking for endangered bird species (Glover and McGrew, 2012; Nakamura, 2016).

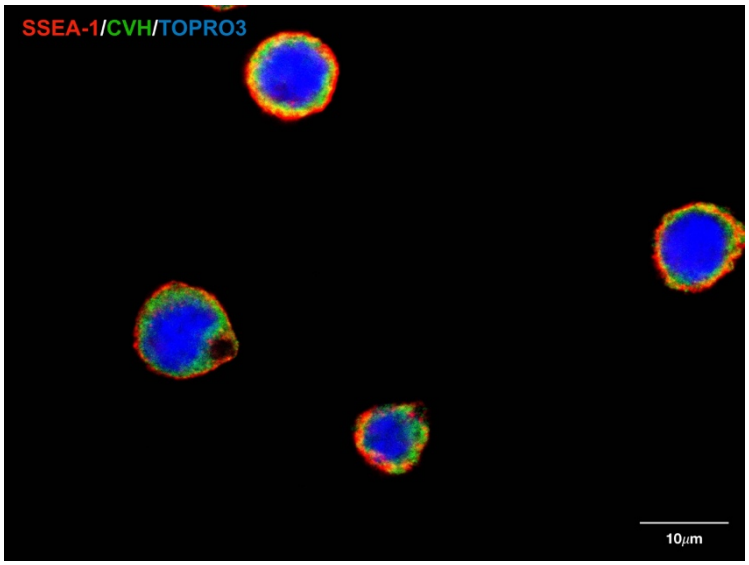


Figure 7: *Immunostaining of the cPGCs. CVH, the germ cell specific marker, stained with green fluorescence secondary antibody. SSEA-1, the stem cell specific marker stained with red fluorescence and blue for the nuclei staining (Image by Elen Gocza).*

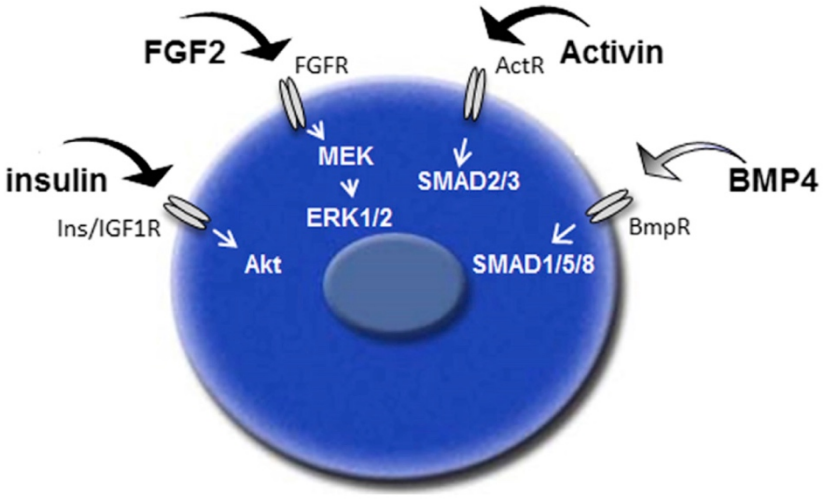


Figure 8: *Major signalling pathways control PGCs self-renewal and pluripotency: The PGCs rely on Activin A/SMAD receptor signalling along with BMP4 and ERK pathway for proliferation and continuous self-renewal capacity*

(Image copied from (Whyte et al., 2015).

McGrew et al devised a protocol and medium for *in vitro* cultivation of the cPGCS based on the above-mentioned molecular signalling pathways (***Figure 8***). A series of experimentation was conducted by McGrew et al to assess the media required for cPGCs *in vitro* cultivation. According to the experimental analysis, it was found that cPGCs are dependent both on FGF2, BMP4 and Activin/Nodal signalling via phosphorylation of SMAD2/3 and SMAD1/5/8 receptors. The Activin/Nodal signalling works via downstream activation of SMAD2/3 receptors ; there are part of the transforming growth factor β signalling (Pauklin and Vallier, 2015). Co-immunostaining analysis of the pSMAD2/3 and p SMAD1/5/8 in cPGCs confirmed the activation of the BMP4 and Activin/Nodal signalling in cPGCs. The Activin/Nodal signalling works via activation of Activin type I and II receptors. The mRNA analysis of the cPGCs confirmed the expression of both types of these receptors. The BMP4 also functions via BMP4R (Bone Morphogenetic Protein 4 Receptor) type 1 and 2 (Wagner, 2007). The expression of BMP4R type I and type II was also confirmed by Q-PCR analysis of mRNA in cPGCs. In cPGCs, FGF2 signalling acts via downstream activation of the ERK1 i.e. extra-cellular signal kinase receptor/mitogen – activated protein kinase (MAPK) signalling pathway (Ma et al., 2016). This was confirmed by using inhibitor for FGF receptor signalling. Inhibition of FGF2 receptor led to an ablation of ERK1 signalling. Also, the analysis confirmed the expression of insulin receptors like IGFR1 and 2 (Insulin growth factor receptor type 1 and INSR. In cPGCs insulin acts via downstream phosphorylation of AKT (Serine/Threonine Kinase1) receptor. Addition of the insulin inhibitor ablated the AKT receptor phosphorylation. Based on these experimental evidences the Activin/Nodal, BMP4 along with Insulin were categorized as the major signalling pathways governing cPGCs self-renewal and pluripotency as depicted in (***Figure 8***) above. Based on this protocol, the cPGCS isolated from the blood can be grown in the lab and later these cPGCS can be used for further experimental analysis. Hence, PGCs have immense capability and application in the field of germline cryopreservation and genetic modification of birds.

4.11.2. Application of chicken stem cells – general overview

The cPGCs hold immense application in the field of avian genetics. The ease of isolation of cPGCs along with their *in vitro* cultivation allows subsequent of modification of cPGCs using CRIPSR/CAS-9 technology for applications like therapeutics drug designing. Also, the isolated cPGCs can be cryo-preserved that helps in establishing bio-banks of cPGCs from endangered bird species (Kagami, 2016) . The applications summarized in the (***Figure 9***) below.

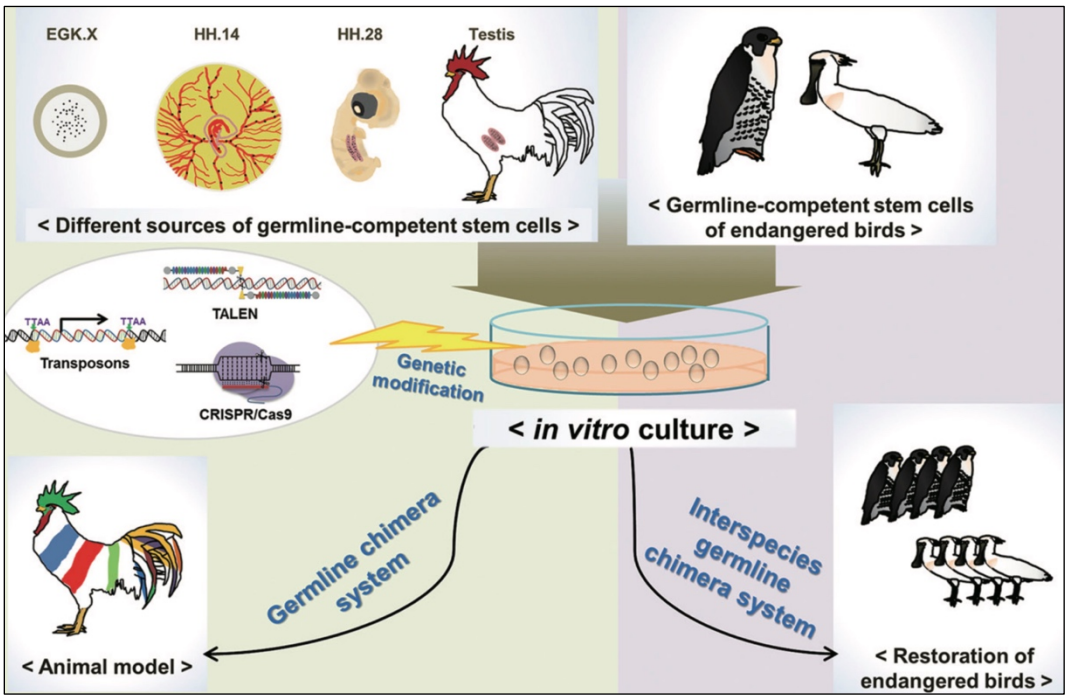


Figure 9: *Application of avian stem cells. The figure above summarized the main application of avian stem cells. In the current scenario, the main application is towards cryopreservation; especially, towards reservation of endangered bird species as well genetic modification via CRIPR/CAS9 technology. This can help create animal models that can be later used for studies on vaccine production, disease pathophysiology. (Image copied from (Han et al., 2015)*

Chicken is useful as model animal and cPGCs are good model as stem cells.

Chicken is a storehouse of different kinds of germline competent stem cells. Different stem cells can be isolated from the chicken at different developmental stages and these stem cells can be cultured *in vitro* and used for further *in vitro* studies and experimental work (***Figure 9***) (Han et al., 2015). The chicken embryo due to its following features such as ease in isolation of the cPGCs, cESCs from different developmental stages, easy incubation time for chicken eggs, short reproductive cycle is considered as the best animal model for studies for non-clinical trials regarding drug or vaccine testing as well as the ideal model for study of the CNS (central nervous system) and respiratory system (Bjornstad et al., 2015) as chicken is phylogenetically closer to mammals, is easily accessible, has a shorter incubation time. Also, the conditions for the growth of the chicken embryo are easier and manageable.

Hence, with the availability of the genome editing techniques and availability of the culture system for chicken stem cells; especially, the PGCs, the chicken proves to be an excellent animal model for study.

4.12. Rabbit embryo development and stem cells

4.12.1. Embryology

Rabbits (Oryctolagus cuniculus) are classical archetype of laboratory animals (Intawicha et al., 2009). They have a short gestation period of 31 days (Fang et al., 2006) . Rabbits are larger than mouse and can be housed indoors conveniently and managed well. They are larger in size and require low cost of maintenance; and are being used as animal models for studying various human diseases like cardiovascular, pulmonary, neuronal disorders , diabetes and also study congenital malformations (Beaudoin et al., 2003).

The embryonic developmental stages in rabbits is similar to humans and they can be staged using the same criteria except for the neuronal development (Beaudoin et al., 2003) . Rabbit has late implantation due to which it is easier to study its late developmental stages (Püschel et al., 2010) .In rabbits, the ovulation can be induced by mating or also artificially by the use of hormones and can take place within the time period of 10 to 13 hours ; allowing the timing of the pregnancy to be noted (Harper, 1961).

The rabbit embryonic development is divided into two phases; first rapid growth until the 15th day and later a slow growth phase from day 31 to 32 days onwards. The rabbit embryo is classified into 23 embryonic developmental stages, this classification uses organ wise description at each stage and is comparable to the human embryo. This classification is common for mammals, owning the fact all mammals have the similar stages in initial phase of development (***Table 6***).

Stages	Hallmarks of Development
Stage 10	Appearance of first somite
Stage 12	Development of rostral limb bud, appearance of pharyngeal arches along with development of complete optic vesicle
Stage 13	Formation of the caudal limb bud and further appearance of 4 more pharyngeal arches
Stage 14	Appearance of the optic plate, 3 cerebral vesicles and mesonephric ridges
Stage 15	Vertebrae formation with appearance of facial buds and hand plates
Stage 16	Development of the finger rays, prominent liver
Stage 19	Appearance of the visible auricle, straightening of the trunk, parallel limbs
Stage 20	Formation of elbow and fingers, with notches between toe rays
Stages 21-22	Free toes
Stages 22-23	Appearance of the knee, eyelids and the trunk
Stages 23	Joining of the hands and feet's in midline, eyelids closing the eyes
Final Stage	Complete appearance of the foetus and the complete organogenesis

Table 6: *Timeline of embryonic development in rabbits (Original).*

4.12.2. Stem cells

As described rabbits are important animal models for stem cell and other biology related work. In 2007, Wang *et al*., reported the generation of the first stable rabbit embryonic stem cells (rESCs). rESCs showed characteristics similar to the ESCs of other species like mouse and human. The proliferation rate of the rESCs was higher compared to the humans and also the rESCs were dependent on FGF signalling (Fibroblast growth factor) LIF (Leukaemia inhibitor factor) signalling and other pathways like WNT, TGFβ signalling (Intawicha et al., 2009; Wang et al., 2007) .

Although several groups attempted to create stable rESCs lines most of them were unsuccessful because of limited proliferative capacity of the rESCs. Honda *et al*. in 2008 reported an efficient method of creating stable rESCs by means of improving the efficiency of the feeder layer and an impoverished zona pellucida shielding method from the blastocyst (Honda et al., 2008). They secrete important nutrients and factors that support the *in vitro* culturing of the rESCs. However, an optimal density for feeder layer must be used as excess density can lead to damage in the growth of rESCs, due to contact inhibition mechanisms and availability of less surface area.

rESCs like human and mouse ESC expressed the pluripotency markers NANOG, OCT4 as well stem cell specific markers like SSEA-1, SSEA-4 (***Figure 10***).

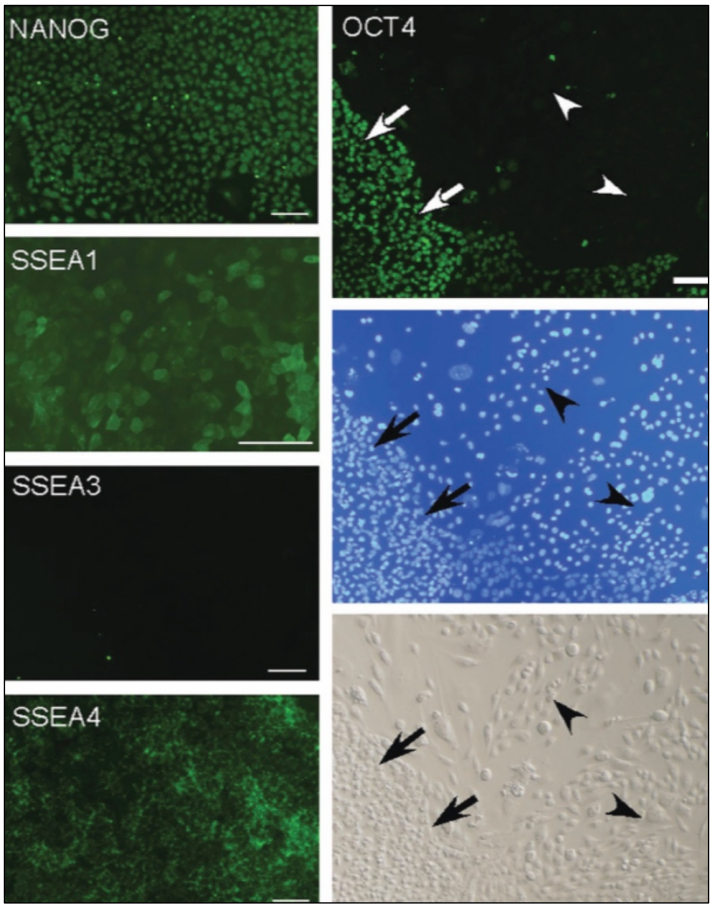


Figure 10: Expression of pluripotent and stem cell markers in rabbit embryonic stem cells: Immunostaining analysis was performed using antibodies against OCT4, NANOG, SSEA-1, SSEA-3 and SSEA-4. OCT4-positive undifferentiated cells are exclusively localized within the compacted colony (arrows), whereas OCT4-negative differentiating cells are sparsely distributed (arrowheads).

(Image from (Honda et al., 2008)

Using, the above-mentioned techniques and culture medium rESCs successfully have been generated.

4.12.3. Rabbit induced pluripotent stem cell

HiPSCs opened the doors for regenerative medicine and therapeutics. Following generation of hiPSCs, iPSCs were generated from other species like money , pigs and rabbits (Honda et al., 2010) . Honda *et al*., successfully generated rabiPSCs using lentiviral vector and the four reprogramming factors (OKSM). The rabiPSCs are primed in state and form flattened colonies similar in appearance to the hiPSCs, they have the ability to give rise to the three germ layers.

4.12.4. Stem cell inhibitors in stem cell research

Although, rESCs and rabiPSCs have been successfully established; but, they still need to be characterized fully at the functional and the molecular level (Osteil et al., 2013). The media conditions along with other factors that are needed for their *in vitro* culturing and *in vitro* differentiation still need to be properly defined in order for proper *in vitro* studies on the aforementioned stem cell lines after their establishment. There are key differences between the stem cell biology of the rESCs, rabiPSCs and ESCs of other species like mouse, monkey etc. It was found that rabiPSCs Osteil *et al*., 2013 that rabiPSCs do not exhibit all features of primed pluripotency (Osteil et al., 2013). They show same features of naïve pluripotency like single-cell dissociation using trypsin, naïve cell marker expression and expression of distal OCT4 enhancer (Osteil et al., 2016). Hence, a study conducted by Osteil *et al*., 2016 tend to identify and define the media conditions along with supplementary factors needed to derive stem cells from ICM of rabbit embryos in conditions similar to that of naïve mouse ESCs.

In order to define the optimal cultural conditions and supplementary factors different kind of stem cell inhibitors were used. The different kind of stem cell inhibitors used in stem cell research for rabbits is shown /listed in ***Table 7*** below, along with the major signalling pathway underlining the pluripotency state of stem cells in rabbits, i.e. rESCs and rabiPSCs.

Chemical Inhibitor	Targeting Pathway	Reference
GSK3 (glycogen synthase kinase 3) - inhibitor (indirubins BIO and kinase inactive analogue, 1-methyl-6 bromoindirubin -3'-oxime (MeBIO).	The canonical WNT pathway required for maintaining pluripotency in HESCs and MESCs. Its activation leads to β-catenin activation and GSK-3 inhibition needed for pluripotency.	(Sato et al., 2004)
PD 184352- protein kinase inhibitor (serine/threonine kinase)	This inhibits the Mitogen activated protein kinase (MAPK) pathway, the MKK1 kinase.	(Ying et al., 2008), (Davies et al., 2000)
PD 0325901- protein kinase inhibitor (serine/threonine kinases)	Inhibits the MKKL, MEK, ERK pathway	(Bain et al., 2007), (Ying et al., 2008)
3-[(3-(2-carboxyethyl)-4-methylpyrrol-2-yl) methylene]-2-indolinone (SU5402) –protein tyrosine kinase inhibitor	Inhibits the fibroblast growth factor receptors (FGFR) FGFR1 and FGFR4	(Mohammadi et al., 1997)
PD 173074- protein tyrosine kinase inhibitor	Inhibits the FGFR1	(Mohammadi et al., 1998)
CHIR99021- GSK-3 inhibitor	Inhibits the GSK-3 and activates β-catenin WNT pathway family	(Ying et al., 2008)
A-83-01	Inhibitor of Transforming growth factor beta receptor (TGF-β1)	(Kawamata and Ochiya, 2010)
Y-27632	Rho kinase inhibitor	(Kawamata and Ochiya, 2010)

Table 7: Stem Cell Inhibitors (Original)

4.12.5. Usefulness of rabbit as model animal and rabbit stem cells

Rabbit has several advantages as an animal model first of all, its embryonic development the developmental stages and kinetics of embryonic development resemble closely to the humans (Püschel et al., 2010) . The rESCs and rabiPSCs resemble the hESCs in behaviour. They rabiPSCs and rESCs can be cultured *in vitro* with defined media conditions and hence, therefore can be used for further animal model studies for a plethora of human diseases like cardiovascular, neuronal, blood borne diseases etc. The rabbit is frequently used for antibody

production in bio-chemical research. Also , the maintenance of rabbit as laboratory animals is easy and less expensive (Fischer and Meuser-Odenkirchen, 1988). Hence, the easiness of housing rabbit and its close similarity to human embryonic development and with the advent of iPSCs technology and genome modification technology, the combination of all the above factors make rabbit an excellent laboratory animal for *in vitro* studies.

4.12.6. Small non-coding RNAs and their role in developmental biology

Non-coding RNAs are an emerging class of RNAs controlling various aspects of embryogenesis and development biology. Recent studies have highlighted the importance of non-coding RNAs in regulating gene expression in various developmental stages, controlling cell fate maps as well as playing an important role in animal embryogenesis, pluripotency of stem cells.

There are various classes of non-coding RNA like miRNAs, piwi-interacting RNAs (Piwi-RNAs), siRNAs (small interfering RNAs), small nucleolar RNAs, transcription initiation RNA, long non-coding RNAs, circular RNAs, natural antisense transcripts, long intergenic non-coding RNAs etc. (Stingo et al., 2018). For the thesis, there will be an extensive discussion of miRNAs with a small mention of other RNA interference pathway like siRNA and a brief description of piwi-interacting RNAs.

Despite many classes of non-coding RNAs the most recognized are the three main ones-miRNA, siRNA and Piwi-RNAs.

4.13. MiRNAs in developmental biology

4.13.1. Overview of miRNA history

MiRNAs are small non-coding RNAs ; they are usually 22 nucleotides in size and associated with the Argonaute family protein (Ha and Kim, 2014). MiRNAs play an essential role in mRNA silencing. The miRNAs tend to bind to their target mRNAs and cause their translational repression. The miRNAs tend to bind to their target mRNAs and cause their translational repression, de-adenylation or target cleavage based upon the perfect complementarity match pair between miRNA seed sequence and the 3'UTR region of the mRNA. If the base pairing between the miRNA seed sequence and the sequence within the 3'UTR is perfect, the mRNA undergo translational repression else if there is imperfect base pairing they are de-adenylated or undergo target cleavage (Catalanotto et al., 2016).Recent studies have indicated that miRNA regulate gene expression post-transcriptionally via the aid of the RISC and Argonaute proteins. If the miRNA/mRNA duplex base pairing is partial then the miRNA undergo target cleavage via miRNA mediated decay (Xu et al., 2016) . Hence the effector functions of miRNAs depend upon the Watson and Crick base pairing

complementarity between the 3'UTR sequence and seed sequence of miRNA. If perfect it is translation repression else de-adenylation or target cleavage. The target cleavage can occur via miRNA mediated decay i.e. removal of the cap or the removal of the poly A tail. De-adenylation also occurs via removal of the cap or polyA tail leading to unstable mRNAs and subsequently their degradation via endo-nucleocytic cleavage (Eulalio et al., 2009). MiRNAs are noted to play important roles in many developmental processes and disease pathophysiology (Graves and Zeng, 2012)

The biogenesis of the miRNAs is tightly regulated by the cell. The miRNA biogenesis takes place in multiple steps; aided by different RNA binding proteins, modifying enzyme, transcriptional factors. But, the two main enzymes regulating the miRNA biogenesis is Drosha and the Dicer enzyme (***Figure 1***).

The transcription of miRNA is carried out by RNA polymerase II. Throughout the genome of plants, animals or humans there are abundant miRNA gene families. It is believed that some of these miRNA gene families arose by gene duplication events. But it is assumed that miRNAs having the same seed sequence from nucleotide position 2 to 8 belong to the same miRNA family. MiRNAs belonging to the same family may act upon same mRNA targets or may have alter mRNA targets as well.

Some of the miRNAs may have a common evolutionary origin but can be different or diverge from each other in their miRNA seed sequence. For example , the miRNA miR-141 and miR-200c belong to the miR-200 superfamily but have different mRNA targets because of difference in the seed sequence (Bartel, 2004). Apart from the canonical pathway (***Figure 1***), there are several other non-canonical pathways for the miRNA biogenesis. For example, in the case of mirtrons the miRNA is located in the intron of a mRNA coding genes and their biogenesis initiated by splicing and not mediated by Drosha (Graves and Zeng, 2012). In case of mirtrons the Drosha step is bypassed and the lariat generated by splicing is removed by a de-branching enzyme, then forms a hairpin structure having extra sequences at the 5' or 3' end that can be removed by the exonucleases.

Some of the small miRNAs that are derived from endogenous short hairpin RNAs also do not require the Drosha processing. The processing of the miR-451 bypasses the need for cleavage by Dicer. The formation of the mature miR-451 is instead dependent on the catalytic activity of the Argonaute protein 2. The Drosha-mediated cleavage of miR-451 generates a small hairpin overhang that cannot be cleaved by Dicer and hence, is directly loaded on the Argonaute protein 2 and the mature miRNA single stranded is generated (Ha and Kim, 2014).

Despite the existence of these non-canonical pathways, majority of the miRNAs are generated by the canonical pathways; only less than 1% are formed by non-canonical pathway. The miRNAs generated by the non-canonical pathway have low functional importance and abundance.

Thus, miRNA biogenesis is multi-enzyme mediated. The steps are regulated by the cells and aberrant miRNA generation has resulted in many developmental disorders like diabetes, hypertension and cancer as well as aberrant miRNA expression in stem cells is indicate of poor development of the stem cells; affecting its self-renewal capacity.

4.13.2. Stem cell specific miRNAs

MiRNAs are emerging players in the field of stem cell biology. In order to characterize , the role of miRNAs in maintaining the pluripotency of stem cells many studies have focussed on characterizing by knocking out the Dicer/Drosha enzymes in order to elucidate the role of miRNA biogenesis in stem cell self-renewal and pluripotency (Lichner et al., 2011). The experiments were conducted on mESCs and found mouse embryos in which the Dicer/Drosha enzymes were knocked out; the embryos showed severe developmental delay and poor proliferation rate. The embryos also lacked the ability to form teratoma and chimeras following the knock-out of these above mentioned enzymes (Li et al., 2017).

In order to dissect the main miRNAs involved in regulating the stem cell pluripotency; the mESCs lacking Dicer were screened for their miRNA library (Wang et al., 2010). Wang *et al*., determined a specific set of unique miRNAs signatures that was shown to enhance the proliferation rate of these Dicer knock-out mESCs as well as reduce the mESCs accumulated in the G1 phase. These miRNAs were denoted as embryonic stem cell specific miRNAs. In mice they were identified as the miR-290 cluster, in humans its orthologue is the miR-371 cluster.

The other main miRNA cluster identified is the miR-302 cluster (Ong et al., 2016). miR-302 cluster is an embryonic stem cell specific and evolutionary conserved in vertebrates (Gao et al., 2015).

The cluster comprises of 8 members having similar seed sequences and targeting the cyclin/cyclin dependent kinases responsible for the cell cycle regulation; especially the transition from the G1 to the S phase (Mathieu and Baker, 2013).

The above two mentioned miRNAs are the main miRNAs studied and analysed till date in the stem cells of most species.

4.13.3. Stem cell specific miRNA clusters in rabbit

The two main miRNA clusters identified in rabbits are the miR-302 cluster i.e. ocu-miR-302 cluster and ocu-miR-290 cluster (Tapponnier et al., 2017). Other than the miR-302 cluster, and the miR-290 cluster there is expression of the members of the human C19 MC cluster (ocu-miR-512, ocu-miR-498 and ocu-miR-520 e). The ocu-miR-290 and the homologue of human C19MC cluster in rabbits are located in the same coding region (Maraghechi et al., 2013).

The ocu-miR-290 and ocu-miR-302 are described in the section below.

4.13.4. MiR-290-295 cluster

The miR-290-295 cluster is an evolutionary conserved cluster, cited first found in mESCs (Wu et al., 2014). This cluster is reported to be origin from a common ancestor via gene duplication event. In mESCs, this cluster is found on chromosome 7 and has a single spliced transcript that is then sliced to produce 14 mature miRNAs (Houbaviy et al., 2005).

In rabbits, the ocu-miR-290 showed lower similarity to its mouse homologue. In rabbits, the ocu-miR-290 cluster is located on the reverse strand of short pseudo-chromosome chrUn0226. (Maraghechi et al., 2013) . Amongst the 14 mature sequences there were identified in rabbits the ocu-miR-290-5p, ocu-miR-292-3p and the ocu-miR-294 -3p were showing respectively high sequence homology to the mouse mature sequences. The expression of this miRNA like reported in mouse is high during the early embryogenesis. In, rabbits, this cluster is reported to play a role in regulating the early rabbit embryogenesis.

4.13.5. MiR-302 cluster

The miR-302 cluster in rabbits is located on chromosome 15 and shows high sequence similarity to the human miR-302 cluster. The ocu-miR-302 cluster is highly homologous to the human miR-302 cluster.

The main members of this cluster are ocu-miR-302a-3p, ocu-miR-302b-5p, ocu-miR-302b-5pocu-miR-302b-3p, ocu-miR-302c-5p, ocu-miR-302c-3p, ocu-miR-302d and ocu-miR-367. The target sequences i.e. seed sequence of these clusters are conserved (Maraghechi et al., 2013). The ocu-miR-302 has binding target sites for the Yamanaka factors (OKSM) needed for genetic reprograming. To the ocu-miR-302 promoter bind SOX2, NANOG and OCT4 (**Figure 11**) as well as trigger epigenetic changes that govern the architecture of the pluripotent stem cells. The epigenetic changes will be discussed later.

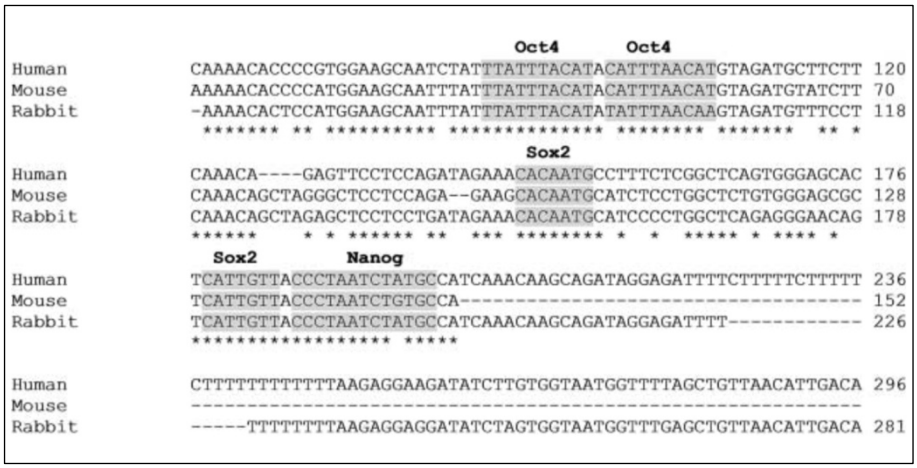


Figure 11: *Conservation and homology of the OCT4, SOX2 and NANOG binding sites between human, mouse and rabbits. The binding sites for the transcription factors OCT4, SOX2 and NANOG are conserved in the three species as indicated by the portion highlighted in grey (Image copied from (Maraghechi et al., 2013) .*

4.13.6. MiRNA inhibitors

MiRNAs have been described as potential players in stem cell biology and many disease pathophysiology (Graves and Zeng, 2012) . In order to study the loss or gain of function of the miRNAs; numerous technologies have been employed. They have been summarized in the ***Table 8*** below. In the present context, the main strategies used to perform loss of function for miRNAs will be discussed with particular focus on the miRNA inhibition technology using anti-miR oligonucleotides (Stenvang et al., 2012) .

Loss -of-function			
Technology	**Characteristics**	**In vitro**	**In vivo**
Genetic Knockout animals	Constitutive or Conditional	Primary Cells	Systemic or organ specific
miRNA sponges	Transient to long term inhibition	Transfection or viral delivery	Lentiviral or AAV mediated delivery
Anti-miR-oligonucleotides	Transient (in vitro) to long-lasting inhibition	Transfection or unassisted uptake	Unconjugated or 3'-cholestrol modified delivery
Target protectors	Transient	Transfection	Embryo injection (zebra fish)

Table 8: *Loss of function technology used to study miRNA function in in vitro (Image modified from (Stenvang et al., 2012)*

The anti-miR oligonucleotide strategy is used to perform inhibition experiments in rESCs, rabiPSCs and PGCs (Maraghechi et al., 2013). The anti-miR are chemical designed oligonucleotides that sequester the mature miRNA, along with competition from the *in vivo* mRNAs; leading to inhibition of the miRNA (***Figure 12***).

4.13.7. Chicken stem cells specific miRNAs

The most widely studied stem cell in chicken is PGCs. A study by (Jean et al., 2015) revealed that chicken PGCs express most of the pluripotent genes *i.e.* mainly the Yamanaka factors and also have a distinct germ cell marker profile. Another study (Shao et al., 2012) identified the novel miRNAs expressed in the chicken PGCs. According to their study, the most abundant cluster identified was miR-302 cluster, along with members of the let-7 family, gga-miR-30d, gga-miR-456, gga-miR-181a etc. However, the role of these miRNA needs

further characterization. The most abundantly miRNAs studied in chickens are the members of the miR-302 cluster.

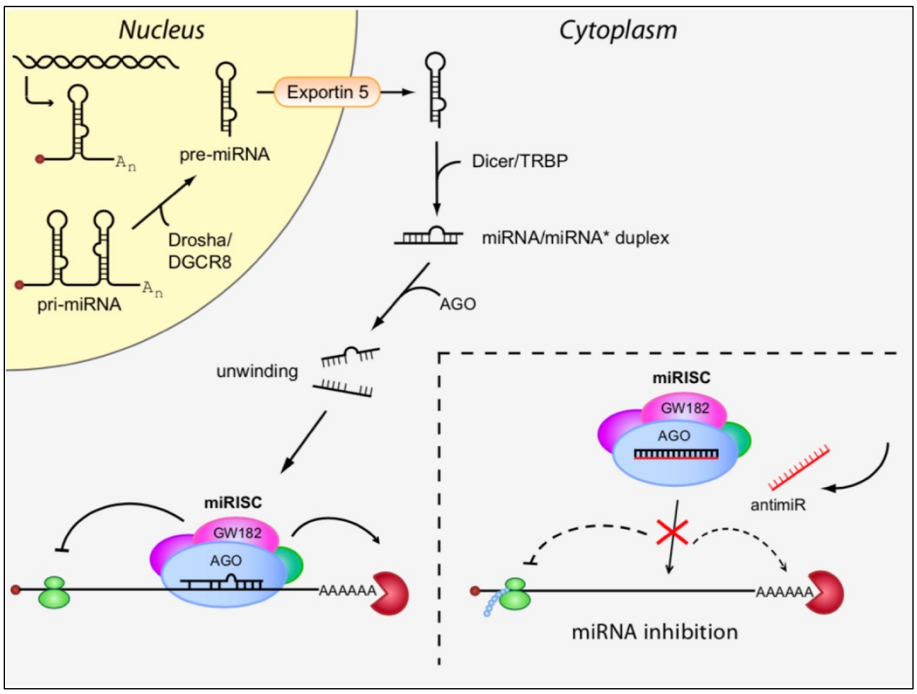


Figure 12: *MiRNA inhibition using the anti-miR technology – In the above picture in the normal cell the generated miRNA binds to its target mRNA and supresses its translation, but with the use of the anti-miR there is completion between the anti-miR and the endogenously expressing mRNAs. The binding of the anti-miR to the miRNA will cause its inhibition and preventing it to bind to its target mRNA. In the case of riPSCs, rESCs, the ocu-miR-302a was inhibited using the anti-miR for 302a.*

Image copied from (Stenvang et al., 2012)

4.13.8. MiR-302 cluster

The miR-302 cluster is an evolutionary conserved cluster (Gao et al., 2015). The cluster in chickens is located on chromosome 4. The cluster comprises of the following miRNAs: gga-miR-302a, gga-miR-302b-5p, gga-miR-302b-3p, gga-miR-302c-5p, gga-, miR-302c-3p, gga-miR-302d, and gga-miR-367. The cluster also harbours the miRNA gga-miR-1811. All the members of the cluster are expressed in the chicken PGCs (Lázár et al., 2018). In chickens, the cluster is involved in regulating the self-renewal and pluripotency of the PGCs via cell cycle regulation i.e. gga-miR-302a cluster via its action of cyclin kinases, cyclins proteins. However, the two arms of the gga-miR-302b miRNA tend to show concordant dysregulation. The arm gga-miR-302b-5p is proliferation promoter whereas the arm gga-miR-302b-3p attenuates proliferation (Anand et al. 2018).

4.14. MiRNA inhibition

The miRNA inhibition is performed in similar ways in chicken PGCs as described for the rabiPSCs. The anti-miR inhibitors were used for the gga-miR-302a, gga-miR-302b-5p and the gga-miR-302b-3p. The inhibition was performed for functional characterization of these miRNA in chickens (Lázár et al., 2017).

4.14.1. Small interfering RNAs (siRNAs) in developmental biology

Other than miRNAs, another aspect of non-coding RNAs regulating the gene expression post transcriptionally is siRNAs. SiRNAs are a group of 20 nucleotide long non-coding single stranded RNA that are generated by the Dicer enzyme. They are usually generated from double stranded RNA like transposable elements, hairpin RNA transcripts etc.(Stingo et al., 2018).

The main role of siRNAs is in RNA interference. The siRNA generated by the Dicer enzyme is incorporated into the RISC. Here, with the aid of Argonaute2 protein the siRNA is separated and the more stable strand i.e. with the 5' end is incorporated in the active RISC pocket and this guides the RISC to the target mRNA and it is cleaved (Dana et al., 2017)

4.14.2. Piwi-interacting RNAs (Piwi RNAs) in developmental biology

Piwi-RNAs are a small group of non-coding RNAs. They are about 23 to 24 nucleotides long and are usually expressed in the germ cells. The piwi protein is another type of Argonaute protein that is usually expressed in the germ cells. These piwi-RNAs interact with piwi to form piwi-inducing silencing complex the main role of this complex is to help in the repression of the transposable elements so as to maintain the genomic integrity (Iwasaki et al., 2015). In germ cells like PGCs the piwi-RNAs are involved in silencing the transposons as well as mediate expression of sex determining genes and help in differentiation of the gonads.

4.14.3. Epigenetic regulation of stem cell

Other than the Yamanaka factors, epigenetic changes also play an important role in determining the pluripotency status of the stem cells (Reik and Surani, 2015).

The main epigenetic factors s involved are the enzymes responsible for maintaining the DNA in hyper/hypo methylated status or the action of the histone acetylases. Once the stem cells have become differentiated, there is need for the pluripotent stem cell network such as the OCT4 gene to be silenced which is done via DNA methylation or the action of histones.

Modifying enzymes which repress the histone modifications (Feldman et al. 2006). There are many processes taking place during the maintenance of the pluripotency like chromatin

remodelling via its condensation or de-condensation (***Figure 13.***) (Lunyak and Rosenfeld, 2008).

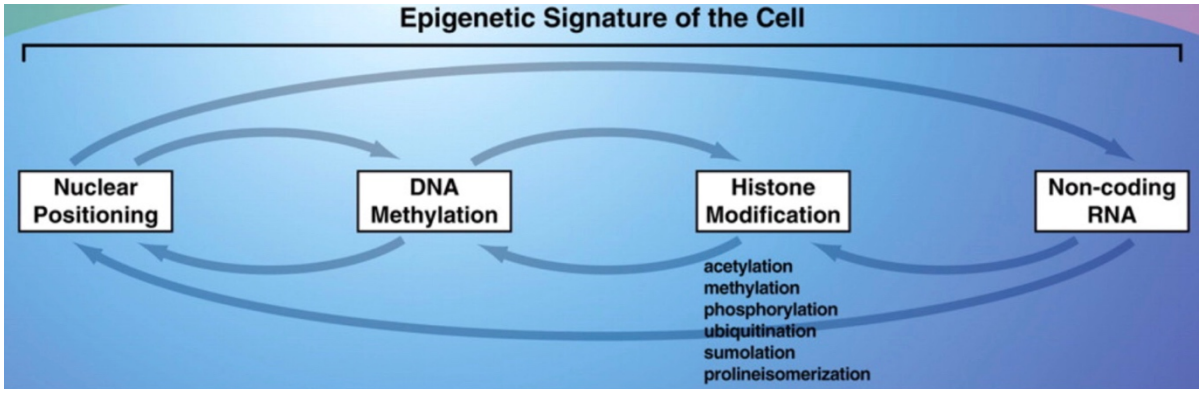


Figure 13: *The epigenetic markers of stem cell*
Image from (Lunyak and Rosenfeld, 2008)

In stem cells there is a concept called bivalent domains; according to this concept there are some domains which have some epigenetic marks like the methylation of the lysine 27 of H3 (H3K27me2/3), which is a marker of transcriptional repression. The other marker is methylation of lysine 4 on histone 3 (H3K4me3) which is a transcriptional activator. Based upon the dynamic state of the cell and other signalling network of the transcriptional factors along with the pluripotent genes either of these histone markers can occupy this bivalent domain and accordingly remodel the chromatin (Bernstein et al., 2006; Schübeler et al., 2004).

The well described essential gene involved in the epigenome setting of the stem cells is the polycomb proteins. The polycomb proteins complex are responsible for establishment of the H3K27me23 marks in the pluripotent stem cells for silencing of the genes responsible for differentiation (O'Carroll et al., 2001).

To conclude, the epigenome resetting is the interplay of transcriptional factors, the DNA methylating enzyme and histone de-acetylating and acetylating enzymes.

4.14.4. Chicken

To determine the epigenome setting of the chicken, a study was conducted by (Kress et al., 2016). In this study the epigenome profile of the chicken embryonic stem cells (cESCs), PGCs was compared with differentiated cESCs that were treated with retinoic acid. The main epigenetic marks H3K9me2/3, H3K27me2/me3, and H3K27ac etc.; were studied by immunoprobe detection. The H3K9me2 marks was present in the pericentromeric heterochromatin (PCH) in PGCs, but less dispersed compared to those found in the cESCs and BC (blastodermal cells). The H3K27me2/3 mark was found co-localized with H3K9me2

mark in BC, cESCs and differentiated cESCs. But, in case of PGCs there was only one dot of H3K27me2/3 observed in the PCH regions enriched with H3K9me2. This dot was observed in the 50 % of the nuclei, and could be corresponding the big W chromosome (Kress et al., 2016).

In case of active chromatin marks i.e. acetylation marks the distribution of these was similar in PGCs compared to other somatic cell types. In chicken PGCs, the level of H3K27me2/3 (Histone 3 lysine 27 tri/di methylation) mark was lower compared to the H3K9me2 (histone 2 lysine 9 di-methylation) marks.

The level of the genes encoding the enzymes responsible for these modifications were quantified by real – time PCR. It was found that the level of histone acetylases causing active chromatin remodelling and corresponding de-methylases was highest in PGCs, however, when the level of chromatin and nucleosome modifying genes were studied then only the *HELLS* (Helicase, Lymphoid specific) also known as *SMARC6 (chromatin remodelling specific gene-ATP dependent)* gene was observed.

4.14.5. Rabbit

The rabiPSCs have a primed state of pluripotency. The epigenetic configuration in rabiPSCs has a tighter chromatin organization and has the following epigenetic markers: two permissive H3K14 (Histone 3 lysine 14 acetylation), H3K9 (Histone 3 lysine 9 acetylation) and two H3K9 tri-methylation and the DNA 5' cytosine methylation causing gene inactivation. (Tapponnier et al., 2017) . Other than the above-mentioned markers the regions CR1 and CR4 (there are conserved regulatory elements present within the promoter region of *POUF51* (OCT4 homologue) gene in rabbits (Kobolak et al., 2009) are methylated at the CpG islands. The epigenetic mechanism controlling the epigenetic archetype of the rabbit stem cells is universal like for other stem cells via the expression of DNA methylation via methyltransferases, histone acetylases and de-acetylases (Osteil et al., 2013).

5. Materials and Methods

5.1. Investigations in chicken PGCs

5.1.1. Animals, permissions

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 protocols for animal management and embryo manipulation were conducted in regulation to that of NARIC Agricultural Biotechnology Institute.

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).

5.1.2. PGC medium

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. The main components of the Avian-KO-DMEM basal media comprises of 50x B-27 supplement, 100x GlutaMax, 100x NEAA, 50Mm beta-mercaptoethanol, 100mM nucleotide (DNA nucleotide), 100x pyruvate, 100x Penicillin/Streptomycin, 20% ovalbumin and 50mg/ml heparin sulphate along with additives like Human Activin A, 25ng/μl (PeproTech), Human FGF2, 10 ng/μl (R and D Biosystems, Sigma) and chicken serum. The stocks were prepared according to the instruction of the devised protocol by (Whyte et al., 2015). The final prepared medium was filtered through a 0.2 μm pore size syringe (Merck-Millipore) and the medium was aliquoted into 50ml tubes. These tubes are wrapped with aluminium foil to keep protected from the light. The medium is stored at 4·^{o}C.

5.1.3. PGC establishment

The cPGCs was isolated from the fertilized eggs of HH stage 13-17 (Hamburger and Hamilton, 1951) embryos (***Figure 14***). 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 300μl medium without feeder cells. After 1 or 2 weeks, the red blood cells had disappeared and only the cPGCs were clearly visible.

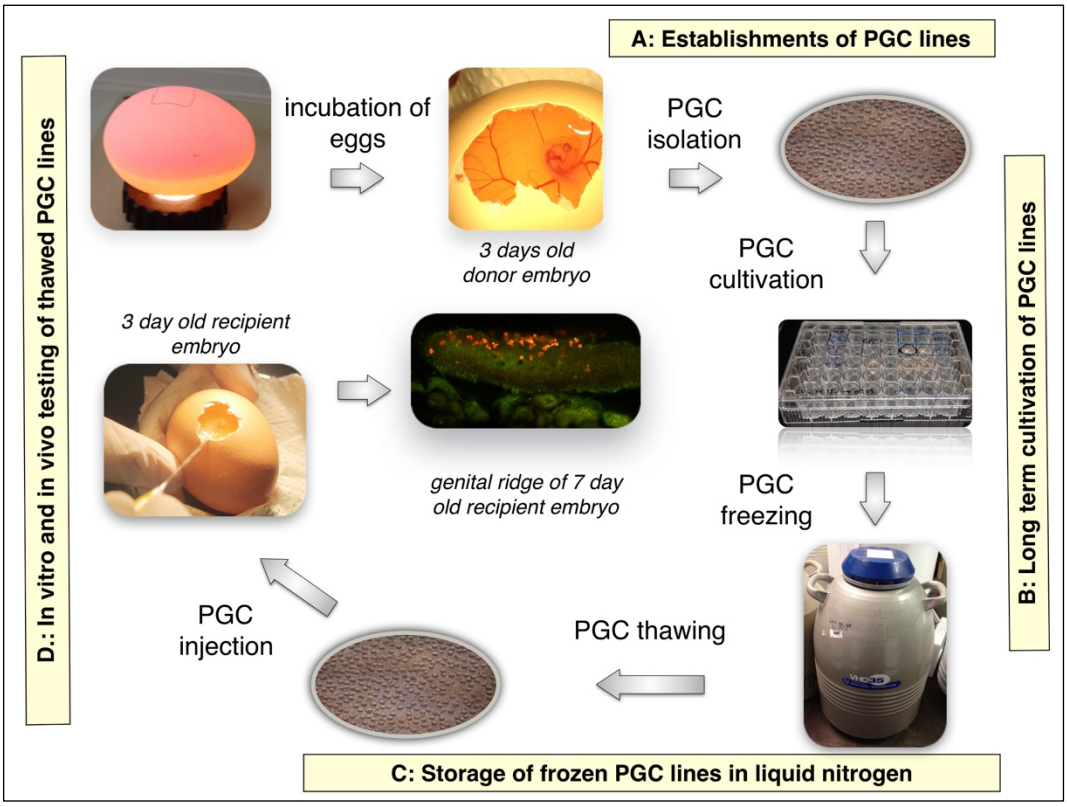


Figure 14: *The main experimental work flow followed by PGC cell line establishment, culturing and freezing (original)*

5.1.4. PGC culturing

Following the successful isolation and establishment of the cPGCs cell line they were cultured for 50 days. The cPGCs were checked daily and the one third of the media was changed every day. When the cPGCs had reached a cell number of 1.0 x 10^5, the cPGCs were collected and divided into two and further cultured at $2x10^4$ cells/ml in a 24 well culture plate. Once, the cell number reached confluence the PGCs were collected for RNA isolation and further analysis.

5.1.5. 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 freezing medium consists of the following reactants: 5 ml Avian-KO-DMEM, 400 μl of DMSO (8%), 500 μl of chicken serum (10%) and 37.5 μl of 20 mM CaCl2. After the freezing media has been prepared, the protocol of freezing was carried out. The PG 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 250μl of Avian-KO-DMEM medium. To this 250 μl of freezing media was slowly added. The contents from the Eppendorf tube are then moved to a labelled cryovial tube and the tube is immediately placed in -70° C. For further long–term cultivation the cells after one night should be moved to -150 C or liquid nitrogen.

5.1.6. 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°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). The three 96 well plates used as biological replicated with 6 by 6 parallel wells were prepared for studying each condition.

5.1.7. 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. This equipment allows the possibility of capturing time lapse videos. 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.

5.1.8. 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. The probes consisted of a coding sequence and a long spacer. The coding sequence is complementary to the mature miRNA sequence and contains chemical modification for enhancing the specificity and sensitivity of detection as well as for balancing the melting temperature of probes for hybridization. The spacer sequence is non–nucleotide specific and is to prevent non-complementary binding. The probe synthesis is *in situ* and based on the principle of light PGA lithography. 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).

5.2. Investigations in rabbit iPSCs (rabiPSCs)

5.2.1. Animals, Permissions

The New Zealand white rabbits were purchased from Hycole (Marcoing, France) or HyPharm (Roussay, France). The embryos were flushed from these rabbits using the superovulation protocol devised by (Salvetti et al., 2010) and subsequently cultured following artificial insemination using the protocol of Osteil et al., 2013. 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 INSERM, Lyon (Afanassieff et al., 2014). The rabiPSCs cell line EOS/B19 used in this study is transgenic as it is over-expressing the *OCT4* gene.

5.2.1. Rabbit embryos isolation and culturing

The rabbit embryos were isolated 1 or 3 days after the mating. The morula 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 morula 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. These obtained blastocysts were miRNA inhibited for ocu-miR-302a-3p and over – expressed for pre-cursor miR 302a then immunostained for OCT4.

5.2.2. RabiPSCs medium

The media for iPSCs culturing are subsequently prepared on the basis of protocol devised by (Afanassieff et al., 2014). The rabiPSCs require different kinds of media for different stages of culturing. The media along with their components and their usage are as follows: For 50 ml of total media, 38 ml of Knock out DMEM/F12 (no Hepes, no L glutamin) was added along with 0.5 ml of embryo MaxR nucleosides (100x), 0.5 ml of pencillin streptomycin glutamine liquid (100x)(G PSG), 0.5 ml of non-essential amino acids (100x), 50ml of 2-Mercaptoethanol, 50mM (1000x) (G-ME) and 10 ml knock–out serum replacement. For 100 ml of total media, 77 ml of knock out DMEMF12 (no Hepes , no L glutamin) was added long with 1 ml of embryoMaxR nucleosides (100x), 1 ml of G-PSG, 1ml of non-essential amino acids (100x), 100ml of G-ME and 20 ml of knock out serum replacement. For preparation of 650 ml, 500 ml of knock out DMEM/F12 (no Hepes, no L-glutamin) was added along with 6.5ml of each of the following – embryoMaxR nucleosides

(100x), G-PSG, non-essential amino acids (100x), 650 ml of G-ME and 130ml of knock-out serum replacement.

ANR-RES Culturing Medium contained the ANR-rabiPSCs Basic Medium in varying proportion depends upon the total sum required, to this the bFGF (FGF2) 13ng/ml (Invitrogen)(10μg/ml stock) in required μl amount was added . To 10 ml of ANR-rabiPSCs Basic medium 13 μl of bFGF was added. To 50 ml of ANR-rabiPSCs Basic medium 65μl of bFGF was added. To 100 and 200 ml of ANR-rabiPSCs Basic Medium 520 and 260μl 0f bFGF was added.

ANR RES puromycin media – This media contained the basic rabiPSCs media along with bFGF and puromycin and was used for rabiPSCs passaging. For 10μl of ANR-rabiPSCs Basic medium ,13μl of bFGF and 1 μl of puromycin 1μg /ml (10 mg/ml puromycin stock). For 50 ml of ANR-rabiPSCs-Basic medium, 65μl of bFGF and 5μl of puromycin was added. For 100 ml of ANR-rabiPSCs Basic medium, 130μl of bFGF and 10μl of puromycin.

5.2.3. 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. The MEF were passaged up-to 3 times, then were mitomycin C treated (60μl of Mitomycin C). The MEF were cultured on the Fibroblast medium (FM); which was used for culturing the MEF. The mitomycin C treated MEF were kept on gelatine coated 6 well culture plate (2ml gelatine per well). Following this the thawed rabiPSCs were cultured on these using the ANR-RES basic media. The media containing the puromycin resistance has been developed in order to select the rabiPSCs showing over-expression of *OCT4* gene (Osteil et al., 2013).

5.2.4. Protocol for preparation of mouse embryonic fibroblast medium

The main components for MEF media were as follows: DMEM (Gibson 31966-047) used in proportions of either 89 ml, 178 ml or 356 ml depending upon the total media volume of 100, 200 and 400 ml respectively. To this, streptomycin/penicillin G (PS stock)(Gibson 15140-122) was added in amounts of 1ml , 2ml or 4ml depending upon the total media prepared (100ml, 200ml and 40 ml) . The last added component was FBS (Fetal bovine serum) 10 %(Lot :07Q1613K.12.02.06)(Gibson) in varying amounts of 10ml for 100ml total media , 20ml for 200ml total media and 40ml for 400 total media sum.

5.2.5. RabiPSCs freezing

The rabiPSCs were frozen using the freshly prepared freezing media. The components of the medium were as described in the part above. The rabiPSCs were dissociated using trypsin, following this 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 cryotubes for storage at - 70^0C.

5.2.6. RabiPSCs proliferation test

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

5.3. Molecular biology techniques

5.3.1. 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.

5.3.2. PCR

5.3.2.1. Sex PCR

The sex of the established PGC cell lines were determined using the *CHD1* (Chromosome Helicase DNA Binding protein 1) primer set FW: 5'- TATCGTCAGTTTCCTTTTCAGGT-3'; RV: 5'- CCTTTTATTGATCCATCAAGCCT-3') 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 100V for 1.5-2.0 hours. The DNA bands were then visualized and photographed under UV illumination.

5.3.3. 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.

5.3.3.1. 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).

5.3.3.2. miRNA

The Q-PCR analysis was used for checking the expression of the PGC specific and stem cell specific markers in the rabiPSCs and cPGCs. The mRNA analysis was done for the following genes given in (**Table 9**) below. The miRNA analysis was done to check the expression of the members of the miR-302 cluster (**Table 10**).

5.3.1. 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-3pThe inhibition analysis on rabiPSCs was performed in the similar way.

Gene	NCBI number	Primers		Length of the product (bp)
cGAPDH	NM_204305.1	FW	GACGTGCAGCAGGAACACTA	112
		RV	CTTGGACTTTGCCAGCGAGG	
c*NANOG*	NM_001146142.1	FW	ATACCCCAGACTCTGCCACT	100
		RV	GCCTTCCTTGTCCCACTCTC	
c*POUV*	NM_01110178.1	FW	GAGGCAGAGAACACGGACAA	109
		RV	TTCCCTTCACGTTGGTCTCG	
CVH	NM_204708.1	FW	GAACCTACCATCCACCAGCA	113

		RV	ATGCTACCGAAGTTGCCACA	
c*DAZL*	NM_204218.1	FW	TGGTACTGTGAAGGAGGTGA	148
		RV	TGGTCCCAGTTTCAGCCTTT	

Table 9: *List of primer sequence used for Q-PCR analysis for the following genes.*

Name	Gene	Accession Number	Assay ID	Sequency
miR-92	hsa-miR-92	MI0000719	000430	UAUUGCACUUGUCCCGGCCUG
miR-302a	bta-miR-302a	MI0009791	005807_mat	AAGUGCUUCCAUGUUUUAGUGA
miR-302b-3p	hsa-miR-302b	MI0000772	000531	UAAGUGCUUCCAUGUUUUAGUAG
miR-302b-5p	gga-miR-302b*	MI0003700	008131_mat	ACUUUAACAUGGAGGUGCUUUCU
U6	U6 snRNA	NR_004394	1973.00	GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGATACAGAGAAGATTAGCATGGCCCCTGCGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTT

Table 10: *The list of primers used for the miRNA analysis*

5.4. 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, to minimize nonspecific binding of antibodies, 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 4ºC, 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.

5.5. 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, PGC marker expression along with the miR-302 cluster was done using the GenEx software. The expression or repression of the target gene relativeto the internal control gene in each sample was calculated with GenEx 6.0 program (Multiday, SE) using the formula $2-\Delta\Delta Ct$ where ΔCt = Ct target gene − Ct internal control and $\Delta\Delta Ct$ = ΔCt test sample − ΔCt control sample. Statistical differences between the examined groups were assessed by t-test using the GenEx 6.0 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 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.

6. Results

6.1. Chicken PGCs

For my thesis, I did a detailed study on the proliferation and the developmental properties of the two types of cPGCs cell lines established in my laboratory. Recently, we established 21 PGCs cell line from Partridge Colour Chicken breed PC and 10 PGCs cell lines GFP expressing from White Leghorn chicken breed.

6.1.1. Overview of the used PGC lines

Primordial germ cells are pre-cursor of functional gametes. In our lab, there are two types of established PGCs cell lines. One is the non-GFP expressing PGC cell line; established from PC and other is the GFP expressing PGCs cell line. For the establishment of the PC PGC cell line, 74 embryos were used. The derivation efficiency of the PC cell line was 28 % i.e. 21 PC PGC cell line were established and 21 of these PC PGC cell lines were cryo-preserved. Only 4 were female PC PGC cell line (derivation efficiency of female cell line was 19 %).

The GFP expressing PGC cell line was isolated from the While Leghorn chicken. There were 20 embryos used, the derivation efficiency was 50 %; with 10 established cell lines and 10 of them being cryopreserved. From the 10 established GFP expressing PGCs; only 2 i.e. (20 %) were female. Summary of the results as shown in (***Table 11***) below.

PC Chicken PGC line isolation	Number	%
Used embryos	74	100
Established PGC lines	21	28
Frozen PGC lines	21	28
Female cell lines	4	19

GFP Chicken PGC line isolation	Number	%
Used embryos	20	100
Established PGC lines	10	50
Frozen PGC lines	10	50
Female cell lines	2	20

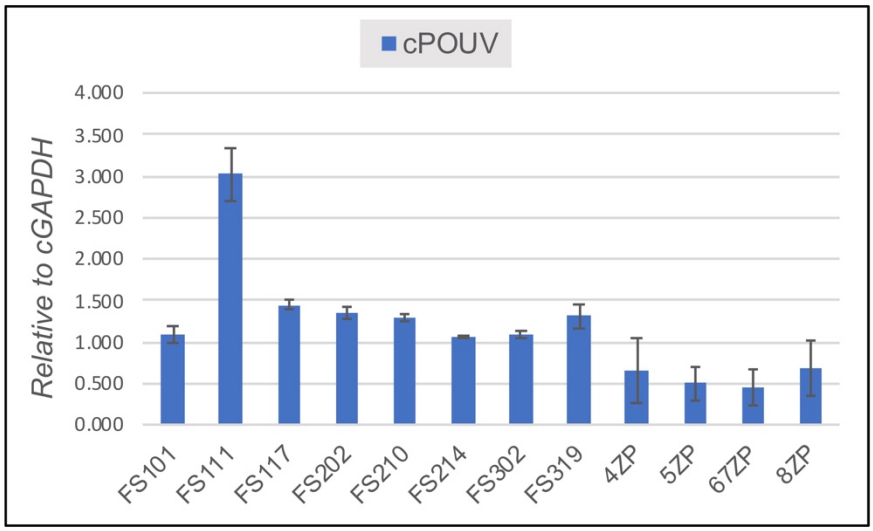
Table 11: *Summary of the GFP expressing and non-GFP expressing PGC cell lines.*

6.1.2. Stem cell specific marker expression

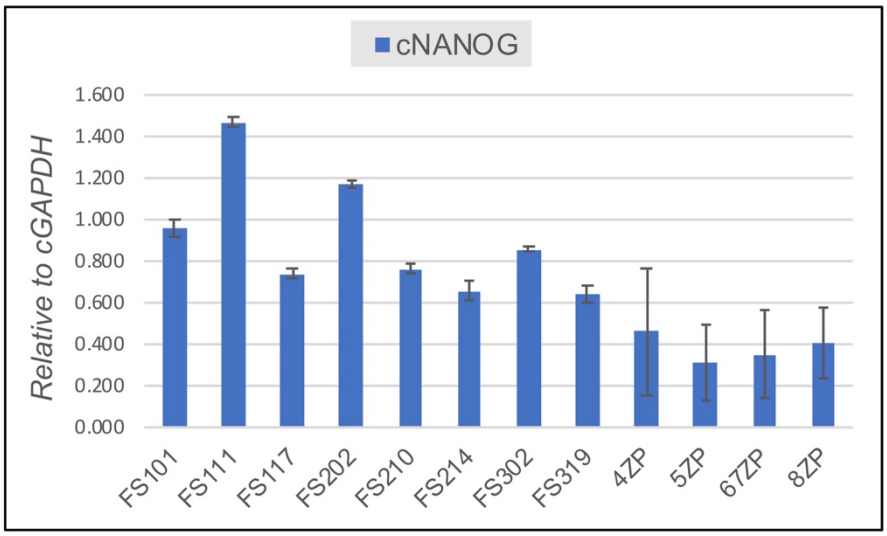
The GFP expressing and the non-GFP expressing PGCs cells were analysed for their pluripotent nature. In order to characterize, the pluripotency of these established PGC cell

lines the expression level of the stem cell specific marker c*POUV*, c*NANOG* and stem cell specific miRNA gga-miR-302a were analysed using the Q-PCR analysis.

A:



B:



C:

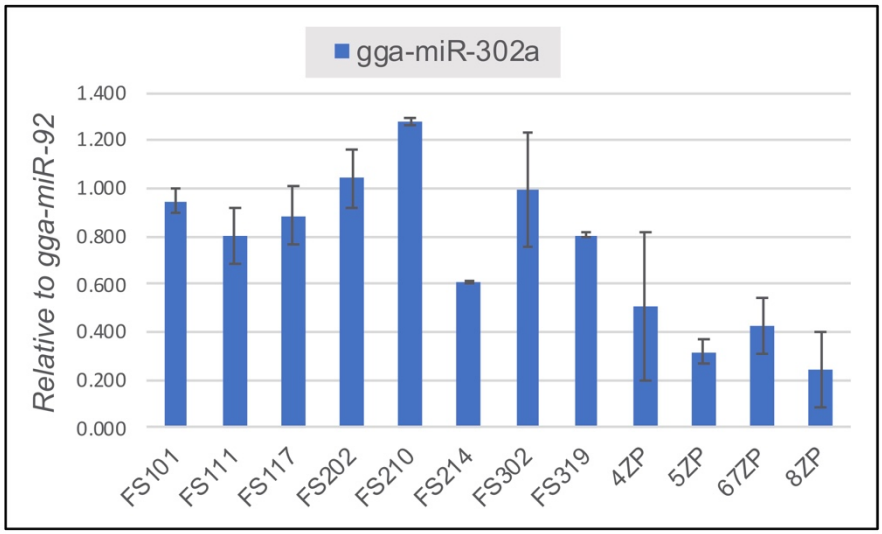


Figure 15: *The stem cell specific marker cPOUV (homologue of human OCT4 gene), cNANOG and gga-miR-302a were analysed in the non-GFP expressing PC 4 male PGC cell lines (FS101, FS117, FS202, FS210) ,4 female PC cell lines (FS111, FS214, FS302, FS319), 1 female GFP expressing cell lines (5ZP) and 3 male GFP expressing cell line (4ZP,67ZP and 8ZP) via Q-PC. The PC PGC cell line FS111 is a high proliferating cell line and hence; it showed the maximum expression level for the stem cell markers. The 5ZP (Female GFP expressing) cell line is lowest proliferating cell line and it showed the lowest expression level for the markers. Figure 15C displays the relative expression of the gga-miR-302a in the mentioned PGCs cell lines.*

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. **Figure 15 C** displays the relative expression of gga-miR-302a. The PC PGC cell line shows higher expression compared to the GFP expressing cell line.

The expression of the gga-miR-302a along with the stem cell markers affirms the pluripotent state of the established PGCs cell lines; both GFP and non-GFP expressing.

6.1.3. 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) via immunostaining. The PGC were stained using antibodies against the CVH gene to show expression (***Figure 16****)*

The FS101 cell line and FS111 derived from the PC were stained for the germ cell marker CVH and stem cell marker SSEA-1. The analysis as shown **in *Figure 17*** below. The nuclear staining was performed by ToPro3 (blue). The stem cell marker SSEA-1 stained in red and the germ cell marker CVH stained in green. CVH is a cytoplasmic marker and SSEA1 is a cell surface marker. The FS101 cell shown at lower magnification in ***Figure 1*****6 (A).** The same cell lines examined at higher magnification at ***Figure 16*** **(B).** The female PC cell line FS111 examined for the SSEA1 and CVH markers **in *Figure 16*** **(C)**. The same cell line examined at higher magnification in ***Figure 16*** **(D).** Using the same pairs of antibodies, similar immunostaining was performed on the GFP 4ZP and the GFP 5ZP male and female cell line. ***Figure 17.***

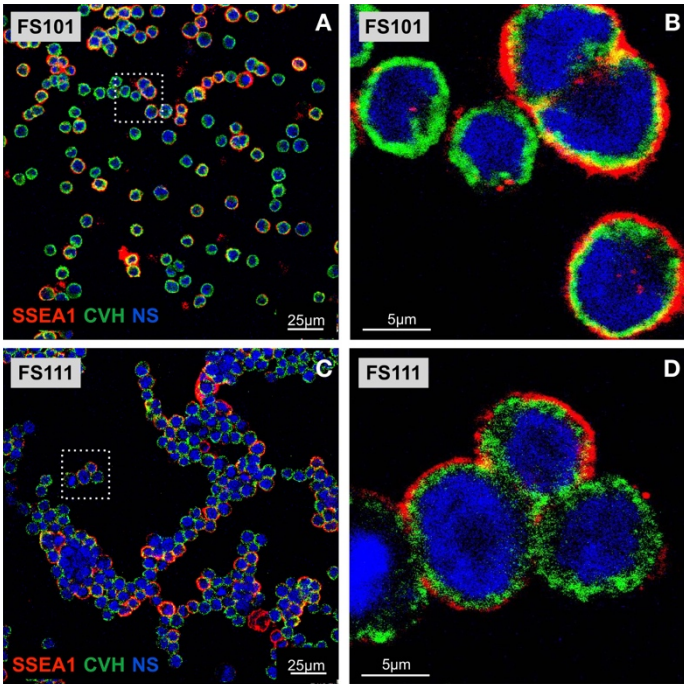


Figure 16: Immunostaining analysis of the male and female PC cell line FS101 and FS111 using the SSEA1 and CVH marker

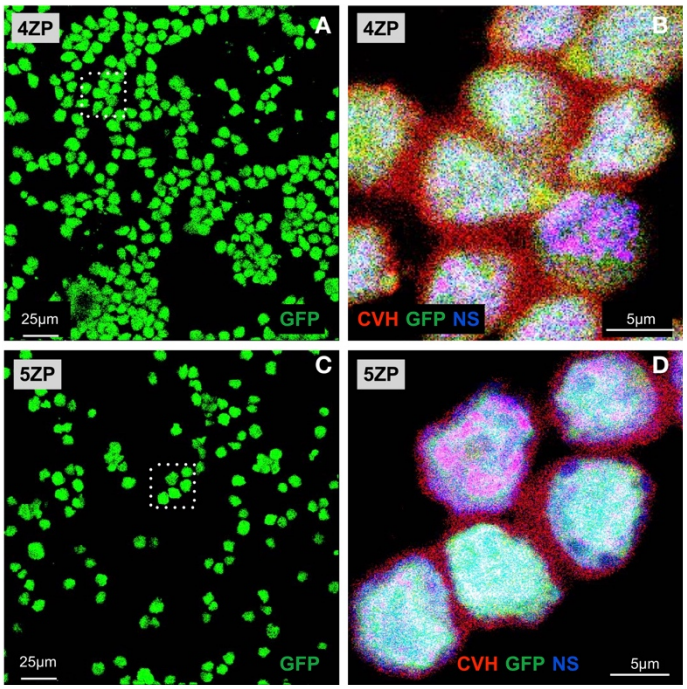


Figure 17: Immunostaining of the GFP expressing male 4ZP and 5ZP cell line

6.1.4. Doubling time measurement in PGCs

The doubling time analysis was done using time lapsed video time analyser. The doubling time was correlated with the PGC concentrations. The male and female GFP and non-GFP expressing PGC cell lines were measured in 12 small squares in a well in biological parallels of 2 at 3 different initial plating concentrations. 1x (1000 cells), 4x (4000 cells) and 8x (8000 cells).

It was observed that the doubling time is dependent on the initial cell. 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) (***Figure 18***). 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)) (***Figure18.***). 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) (***Figure 19***). 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).

Fig.18:

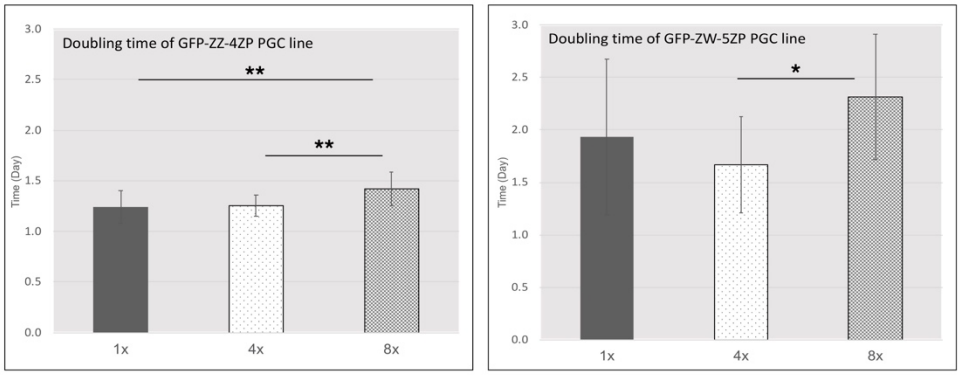


Fig.19:

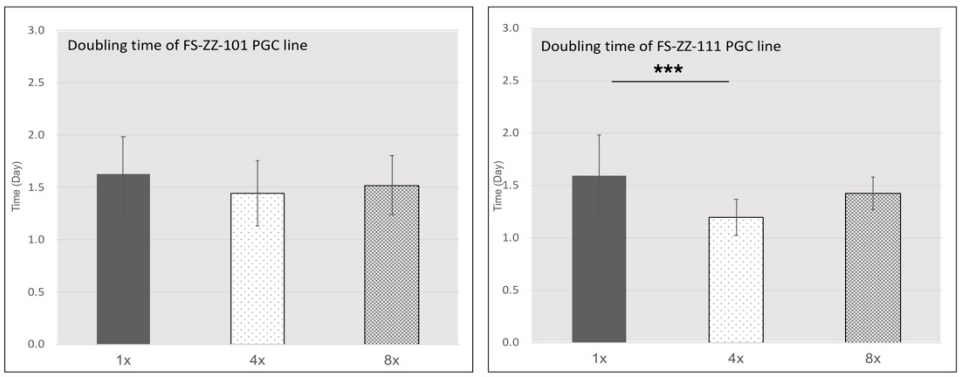


Figure 18: *Doubling time of GFP expressing male 4ZP and 5ZP cell line*

Figure 19: *Doubling time of the non-GFP expressing male and female PGC sample.*

6.1.5. 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.

6.1.6. MiRNA microarray

One male GFP expressing sample and one female GFP expressing sample (4ZP and 5ZP) along with male and female non-GFP expressing sample were sent for analysis (FS101 and FS111). The array plate consisted of 42 control plates and the samples were analysed in parallels of 3.

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 layout of the chip has shown in the ***Figure 20***.

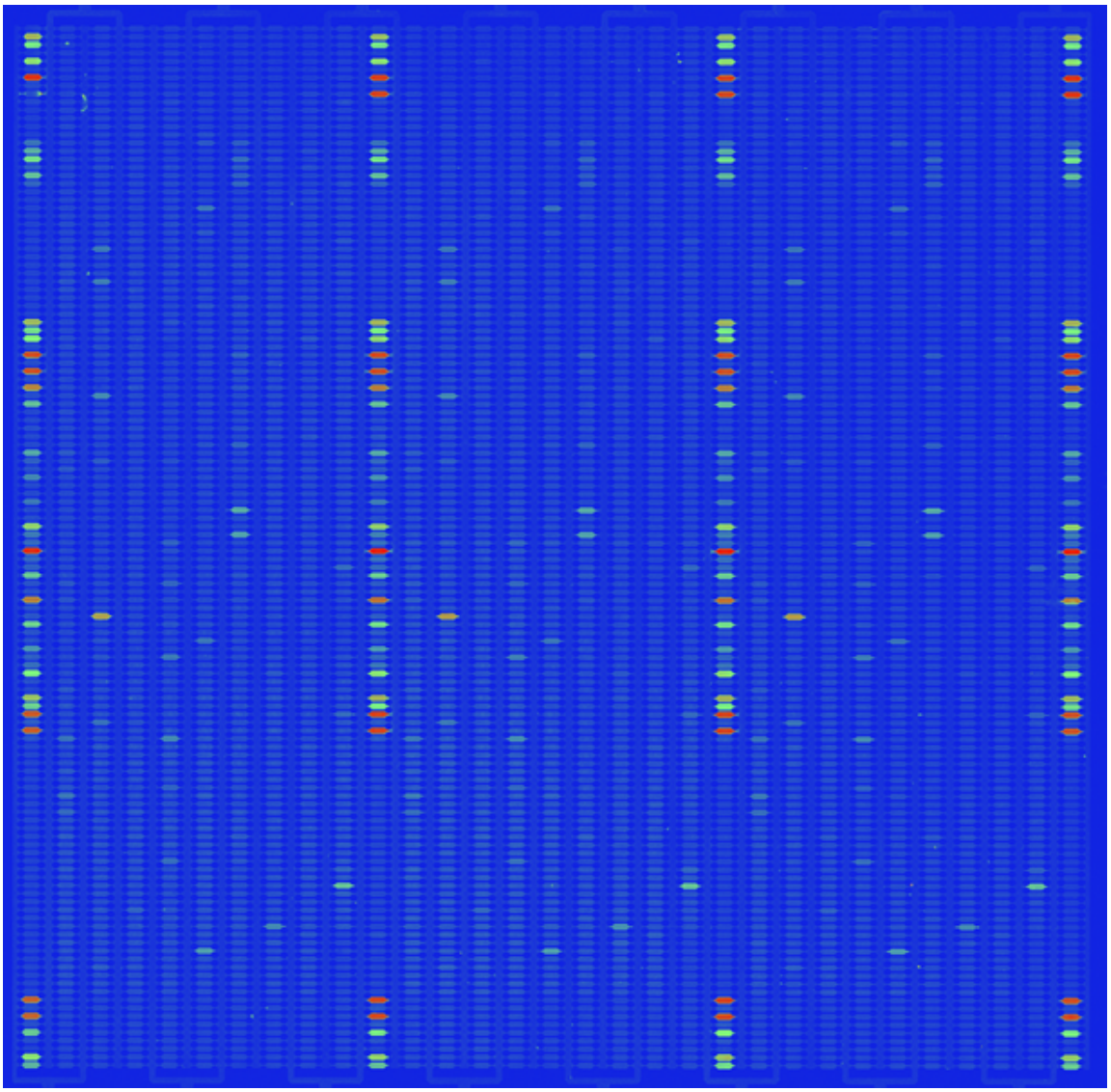


Figure 20: *The chip layout of the male GFP expressing sample with cy3 dye. The colours indicate the signal intensity for different probes. The intensity increases from 1 to 65,535. There is change in colour from blue to green then to yellow and red.*

The overview of the miRNA microarray summarized in the **Table12** 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

Table 12: *An overview of the miRNA array analysis*

6.1.7. Differential expression of miRNAs in female and male cell 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 results as shown below.

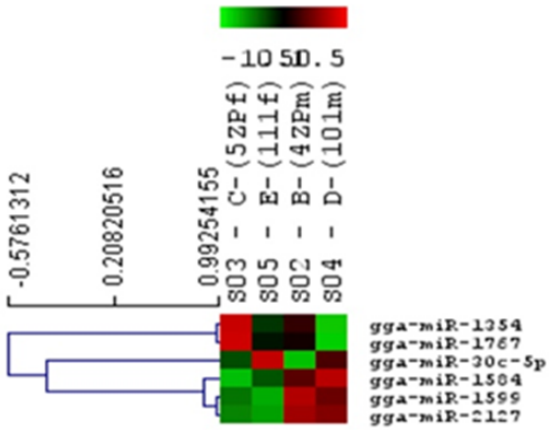


Figure 21: *Paired T test analysis between the male (FS101, 4ZP) and the female (5ZP, F111) cell line at the significance level of 0.05.*

The paired T (***Figure 21***) test analysis between the male and female identified at sex 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. The heatmap analysis was performed along with the clustering analysis.

According to the figure above, the cell lines 4ZPm (4ZP), FS101 (101m) are correlated together along with the female non-GFP FS111.

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.

6.1.8. Stem cell specific miRNA expression

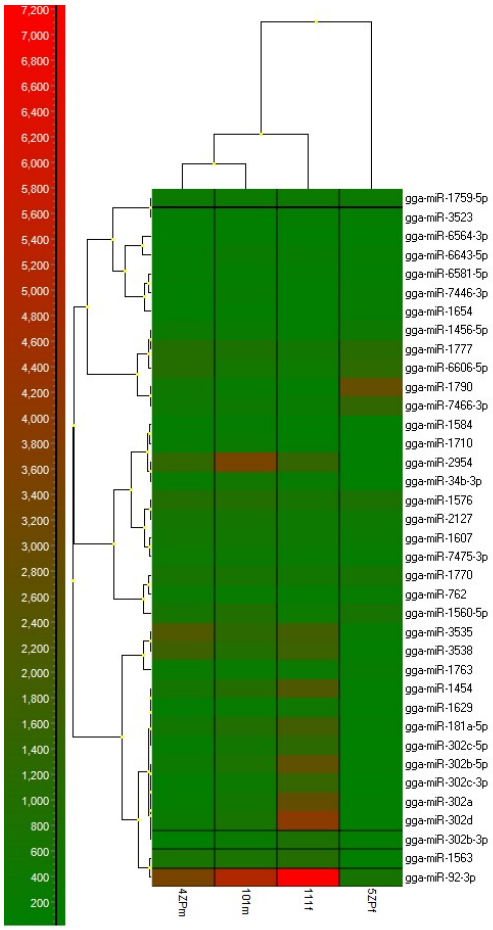


Figure 22: *A heatmap analysis was done for the miRNAs showing expressing in all the PGCs samples using (GenEx software).*

The heatmap (***Figure 22***) was performed using the expression data for the signal intensities of the 27 miRNAs that were expressing in all the 4 PGCs samples. The colour gradient of the heat map goes form light green to darker green then red and finally dark red. The colour gradient increases with the increase in the expression value. Despite, these 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 13***). 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 13: *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 PGC cell lines.

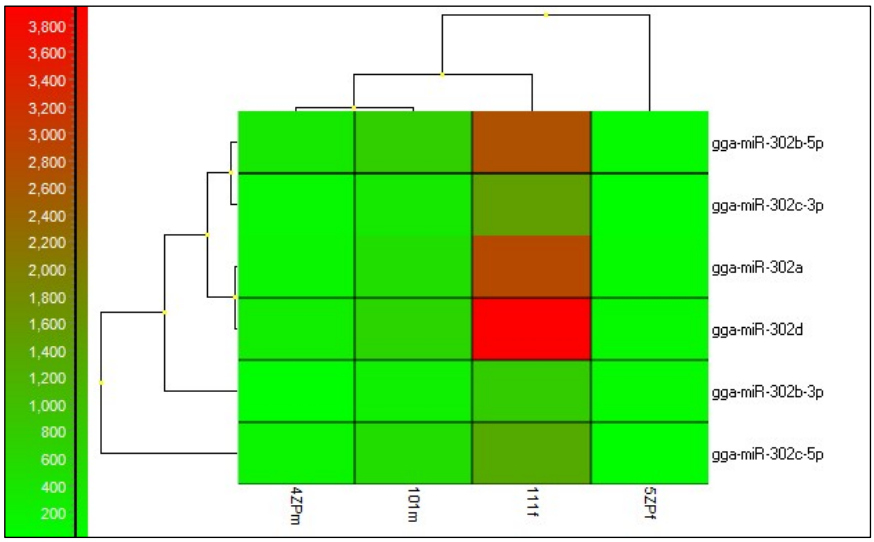


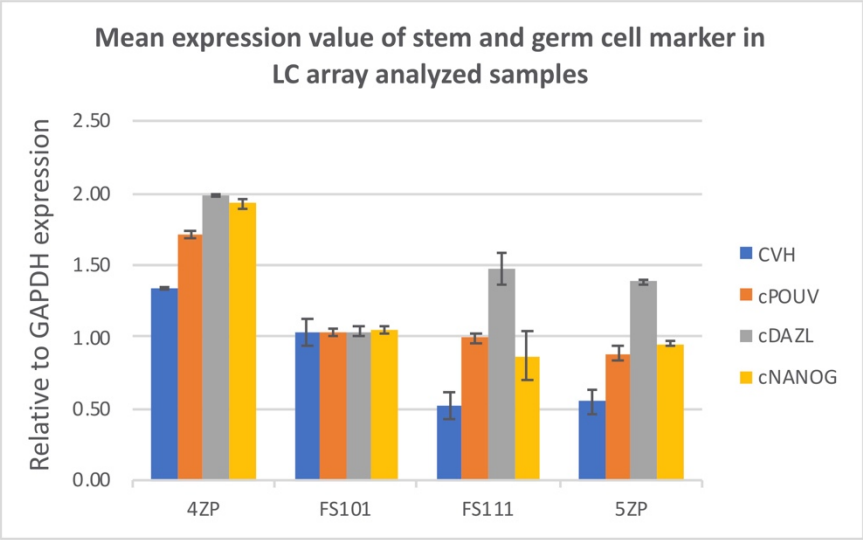
Figure 23: *Heatmap analysis of the gga-miR -302 cluster members (gga-miR-302a, gga-miR-302b-5p, gga-miR-302b-3p, gga-miR-302c-5p, gga-miR-302c-3p and gga-miR-302d).*

From the heat map above (***Figure 23***) it can be seen that, the members of the cluster miR-302 show expression in all the 4 PGCs samples. The expression gradient increases from light green to darker green than orangish red to red and finally extremely dark red. 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 expression were studied (***Figure 24***).

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. In chickens, the males are homogametic in nature and the females are heterogametic. The males have two ZZ chromosome and the female have one Z and W chromosome. The gene *CVH* is located on Z chromosome; due, to this reason the male PGC has higher expression of *CVH* gene than 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.

A:



B:

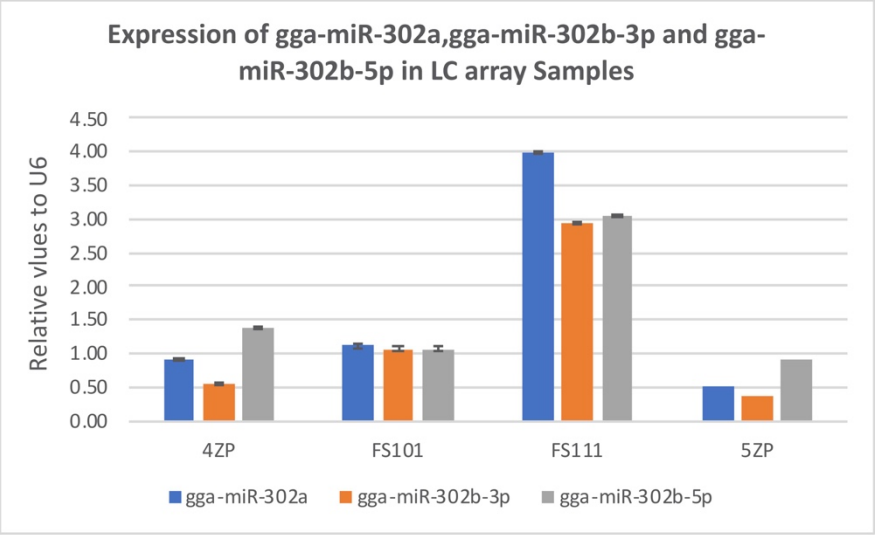


Figure 24: *Stem cell and germ cell marker expression along with expression of gga-miR-302 cluster members in the examined 4 PGCs of LC array data analysis by Q-PCR. However, despite the members showing expressing in all the examined 4 PGC cell lines, there was a discrepancy in expression of the member's ratio vice in the 4 samples. When the expression of the members of the cluster was compared ratio by ratio individually in the 4 PGCs samples, there was a disagreement in the ratio vice expression analysis.*

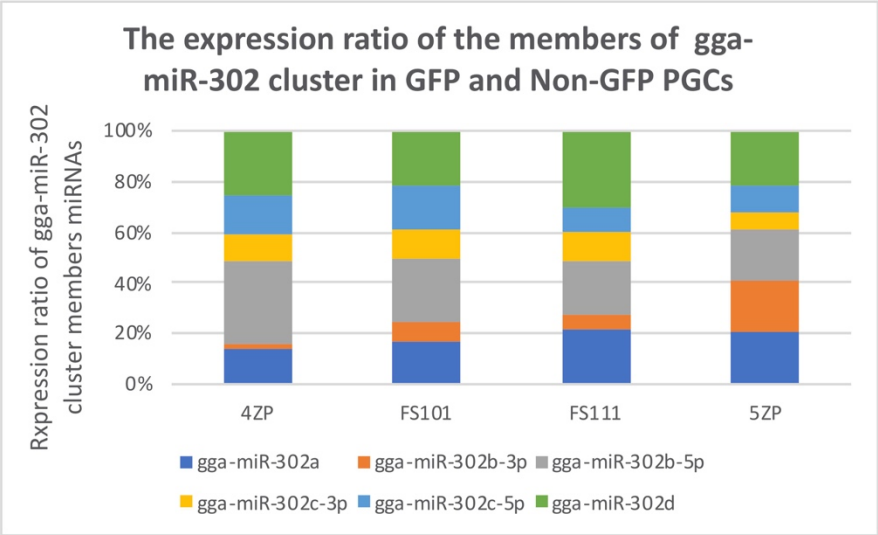


Figure 25: *Ratio vice expression analysis of the gga-miR-302 cluster member's individuals between the 4 PGCs cell lines. There is concordant dysregulation between the two arms of the gga-miR-302b. The arm 5p is highly expressed in the high proliferating PGCs cell lines 4ZP, FS101 and FS111 and vice versa for arm 3p which is showing high expression in the low proliferating cell line 5ZP. There is a discrepancy in arm ratio for two arms 5p and 3p. The arm ratio is high in high proliferating cell lines and low for low proliferating cell lines.*

It can be seen in the above (***Figure 25***) 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.

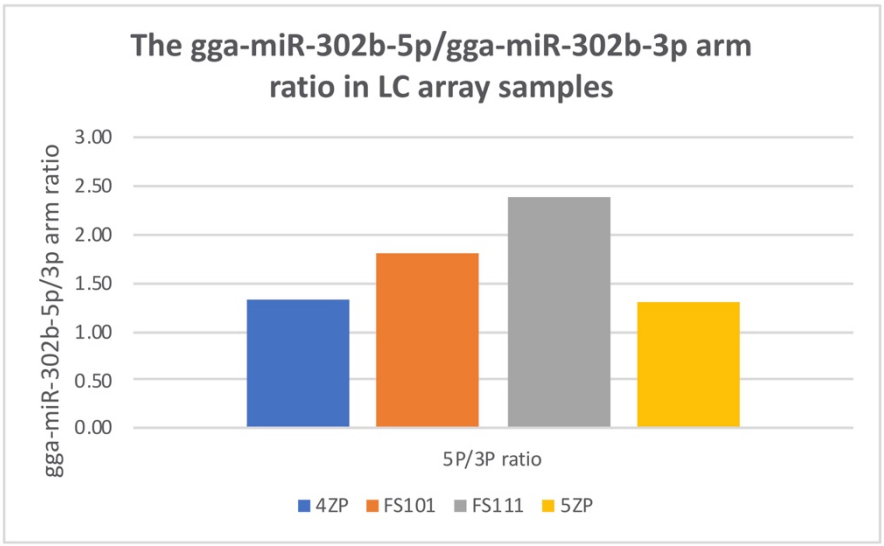


Figure 26: *Ratio analysis of gga-miR-302 cluster in the 4 PGC cell lines examined at LC array by Q-PCR*

The 4 PGC sample analysed by microarray analysis were analysed further for the 5p/3p arm ratio by Q-PCR (***Figure 26***). Following Q-PCR analysis, it was found that the array results were consistent with that of the Q-PCR analysis. The ratio of arm 5P/3P was low for the 5ZP line and high for the cell lines 4ZP, FS111 and FS101. (No repetition proliferation rate is a ratio calculated two samples).

6.1.9. 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.

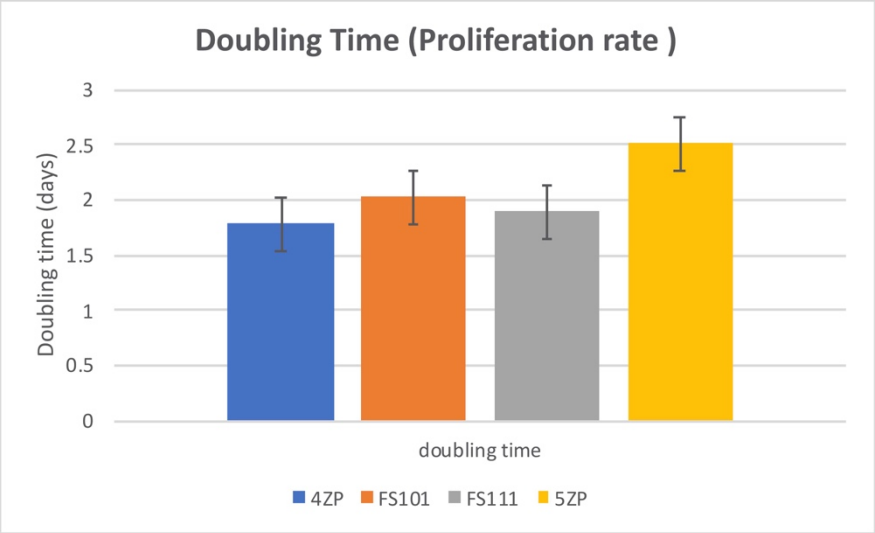


Figure 27: *The proliferation rate expressed as the doubling time.*

4ZP has the lowest doubling time and hence the highest proliferation rate. 5ZP has the highest doubling time and hence the lowest proliferation rate. The doubling time of FS101 is slightly higher than the FS111 cell line. Hence, compared to the FS101; F111 is faster proliferating cell line than FS101 *(**Figure 27**)*.

6.1.10. 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 and the apoptosis test were 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 28**)*.

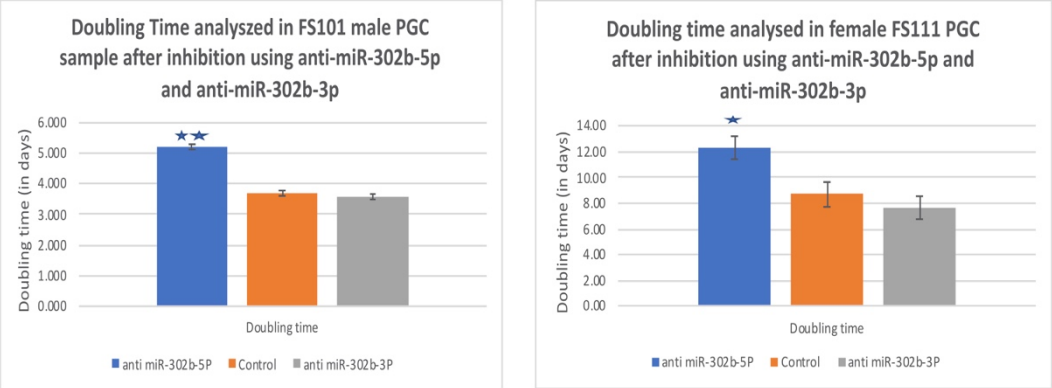


Figure 28: *The doubling time measurement and the gga-miR-302b-5p/gga-miR-302b-3p ratio was measured in the FS101 and F111 cell line prior to the inhibition test.*

From the figure 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.

Thus, the high proliferating PGC cell lines 4ZP, FS111 and FS101 had high 5p/3p arm ration compared to the low proliferating PGC cell line 5ZP.

The doubling time and the ratio was re-measured following the inhibition test (***Figure 29***).

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 29***).

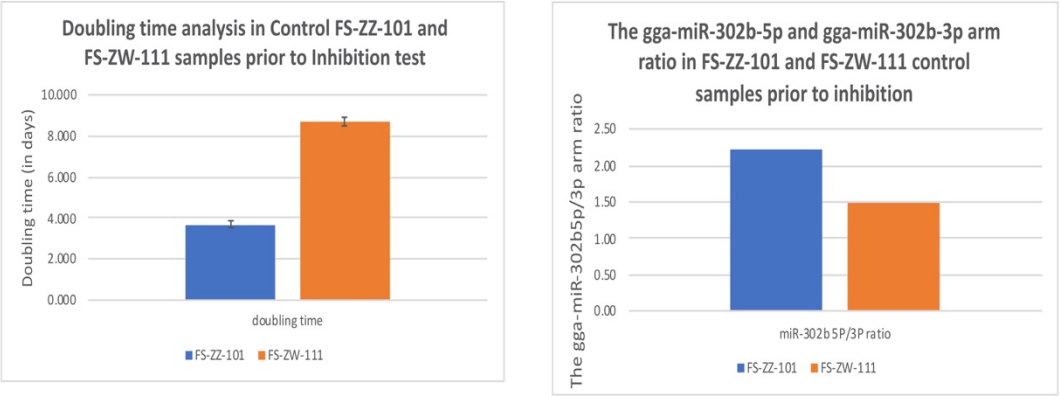


Figure 29: *Functional characterization of gga-miR-302b-5p and 3p using inhibition and doubling time analysis. In both biological samples FS101 and FS111, the doubling time increases significantly compared to control at gga-miR-302b-5p arm inhibition. For the 3p arm, compared to the control there is a decrease in the doubling time; thereby, slightly increasing the proliferation rate at gga-miR-302b-3p arm inhibition.*

6.2. Rabbit induced pluripotent stem cells and embryos

The rabiPSCs used in this study are the B19-EOS cell line (Tapponnier et al., 2017) . The rabbit ear fibroblast cell from New Zealand white rabbit breed were isolated and transferred to induced pluripotent stage using retroviral vectors expressing the human OKSM factors (Marielle Afanassieff, Yann Tapponnier, 2014).In my study, I characterized the miRNA expression in rabiPSCs B19-EOS cell line via Q-PCR, immunostaining and miRNA inhibition study.

6.2.1. 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 (***Figure 30***). The embryos were cultured for 24 hours to obtain the blastocyst. Following, this the embryos were transfected with miRNA mimic (Pre-has-miR-302a), the miRNA inhibitor (anti-miR-302a-3p) and the transfection control using the transfection agent siPORT-NeoFX (Thermo Scientific Fisher) for 72 hours (***Table14***).

Figure 30: *The experimental setup for rabbit embryo in vitro cultivation.*

Samples	# of morula 2.5 dpc	# of blastocyst 3.5 dpc	# of blastocyst 4.5 dpc	% of good embryos
Pre-miR-302a	10	8	8	80
Anti-miR-302a-3p	10	4	4	40
RDH control	5	2	2	40

Table 14: *The experimental setup for the rabbit embryo transfection*

6.2.2. Stem cell specific miRNA marker expression in rabbit embryos

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

6.2.3. 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) (***Figure 31***) 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 (***Figure 32*****).**

Fig.31A

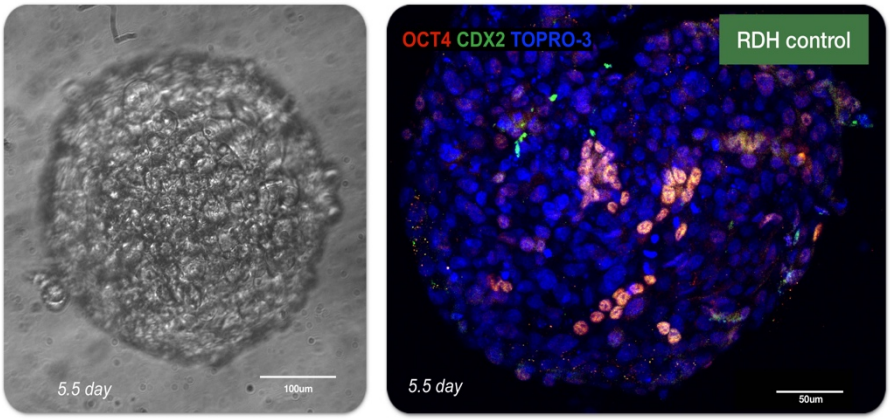


Fig.31B

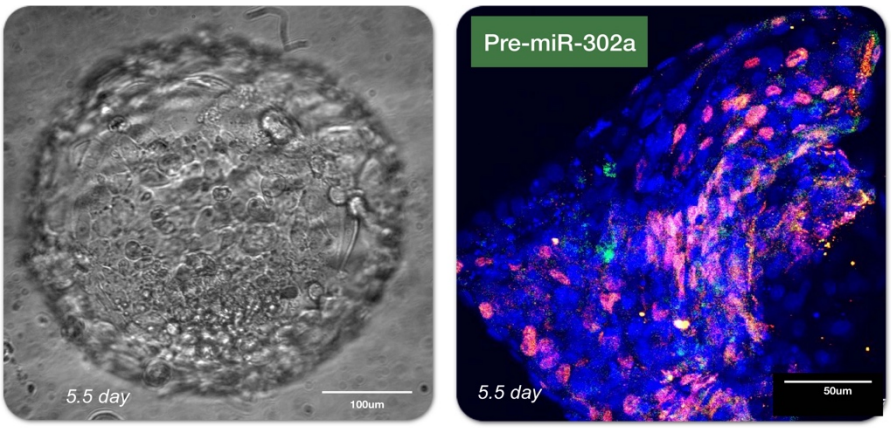


Fig.32

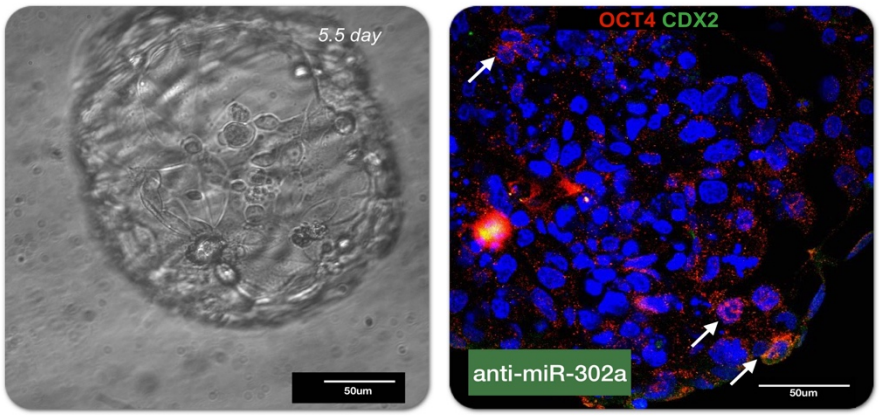


Figure 31: *The OCT4 expression analysed in rabbit blastocyst**: A:** Control embryo in RDH medium. **B:** Pre-miR-302a over expression.*

Figure 32: *The OCT4 expression analysed in rabbit blastocyst**. A:** rabbit embryos was cultured in anti-miR-302a-3P containing medium*

The Oct4 protein is shown in red (pinkish colour in picture), with the nuclear staining (blue in TOPRO-3). The immunostaining was performed in 5.5-day-old embryos. It was observed compared to the control there is high OCT4 expression in the pre-miR-302 treated embryos.

The results summarize the following observations: OCT4 expression is high in rabbit blastocyst in the case of pre-miR-302a over expressing condition .

6.2.4. Overview of used iPSCs line

The used rabiPSCs line was derived from somatic reprogramming of rabbit ear fibroblast using human OKSM factors (Afanassieff et al., 2014). The rabiPSCs showed cardinal features of primed pluripotency; as they are dependent on FGF2 for self–renewal and pluripotency. The rabiPSCs showed positive immunostaining for the gene *OCT4*. 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 (***Figure 33***). 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 over expression 2) miR-302a-3p inhibition and 3) control.

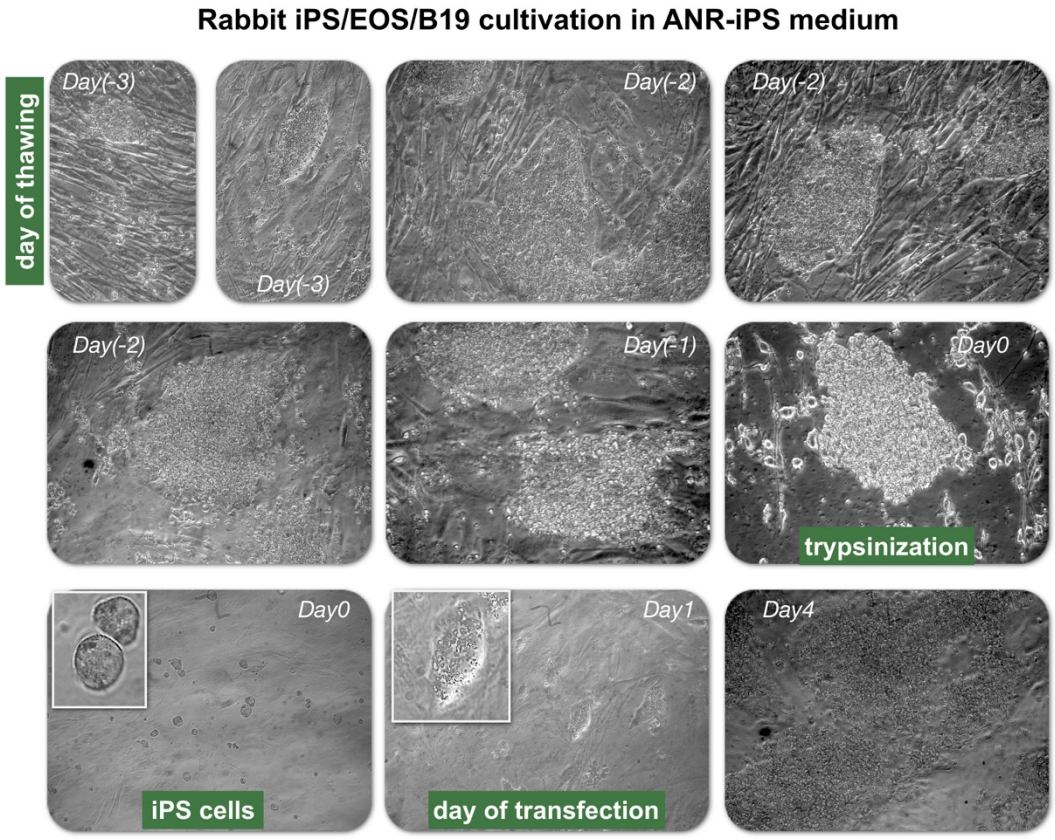


Figure 33: *The experimental procedure for rabiPSCs in vitro cultivation. The basic steps of thawing, passaging i.e. trypsinization followed by preparation of the rabiPSCs for transfection i.e. miRNA inhibition assay.*

6.2.5. 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.

6.2.6. 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 (***Figure 34***). The ocu-miR-302a-3p was functionally characterized using the CCK-8 proliferation kit.

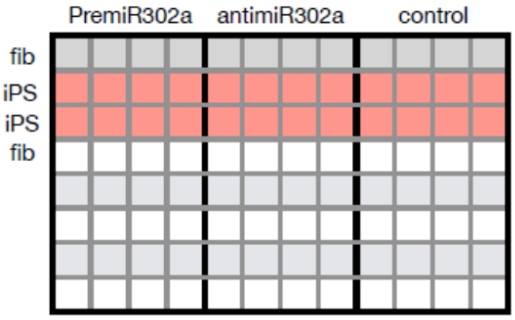


Figure 34: *An overview of the miRNA inhibition plate for rabiPSCs.*

6.2.7. 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 (***Figure 35***). The *OCT4* gene expression was related to the housekeeping gene *GAPDH* expression

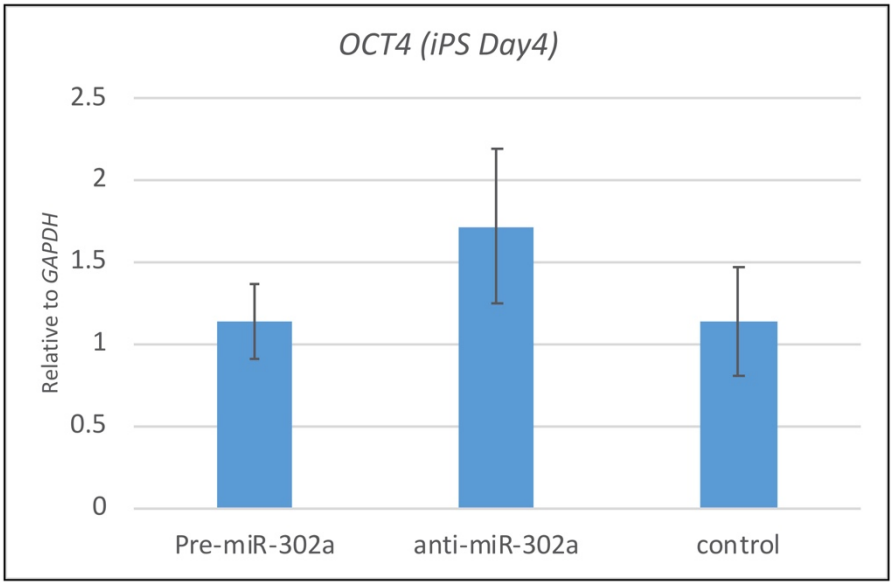


Figure 35: *The OCT4 gene expression relative to GAPDH analysed in rabiPSCs under three different conditions. Compared to the control there was slightly higher expression in the Pre-miR-302a-5p condition and high expression for the anti-miR-302a-3p. But, the result above revealed no significant difference.*

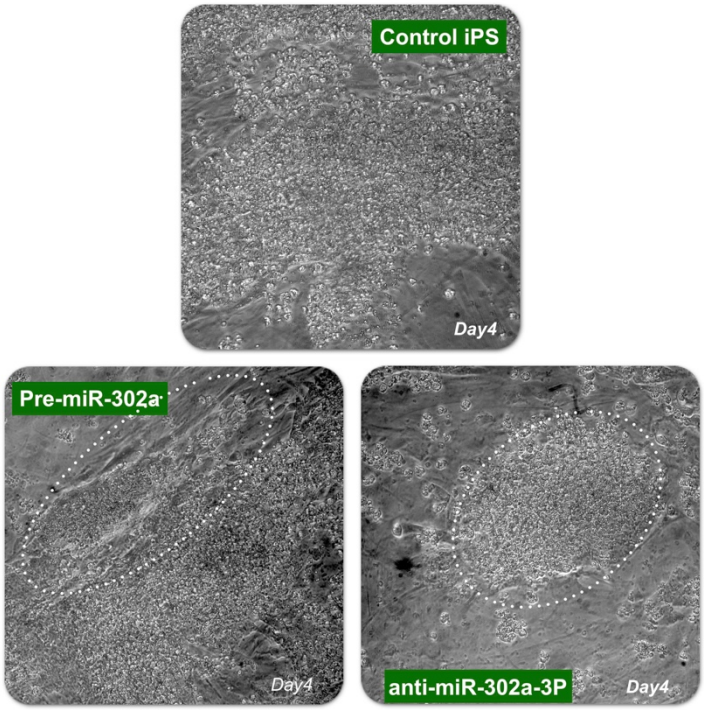


Figure 36: *The rabiPSCs culture after 4 days of culturing and inhibition in three different conditions. As, observed there is more naïve like structure of colony formation in the pre-miR-302a and anti-miR-302a-3p condition compared to the control*

6.2.8. 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 (***Figure 36***). On day 1, there was significant difference observed between the proliferation rate of the rabiPSCs between the anti-miR-302a-3p, pre-hsa-miR-302a and control. The inhibition the proliferation rate was highest in anti-miR-302a-3p condition on day 1. There was significant difference between the anti-miR-302a-3p and control ($p<0.05$), the pre-has-miR-302a and anti-miR-302a-3p ($p<0.05$).

On day 4, the difference between the control and the inhibition condition was still significant with the proliferation rate being high in the anti-miR-302a-3p condition for iPSCs2. However, on day 4 the pre-hsa-miR-302a was also significant high compared to the control and same level as the anti-miR-302a-3p. The increase in proliferation rate in the over - expression condition from day 1 to day 4 can be accounted to the *in vivo* processing of the mature miR-302a-5p arm in rabiPSCs cell lines (iPSCs2). For the 3rd cell line, iPSCs3, compared to the control there was a significant increase in the proliferation rate on day 4 for the cell line under both Pre and anti-miR conditions.

Hence, 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. The results as shown in ***Figure 37*** below.

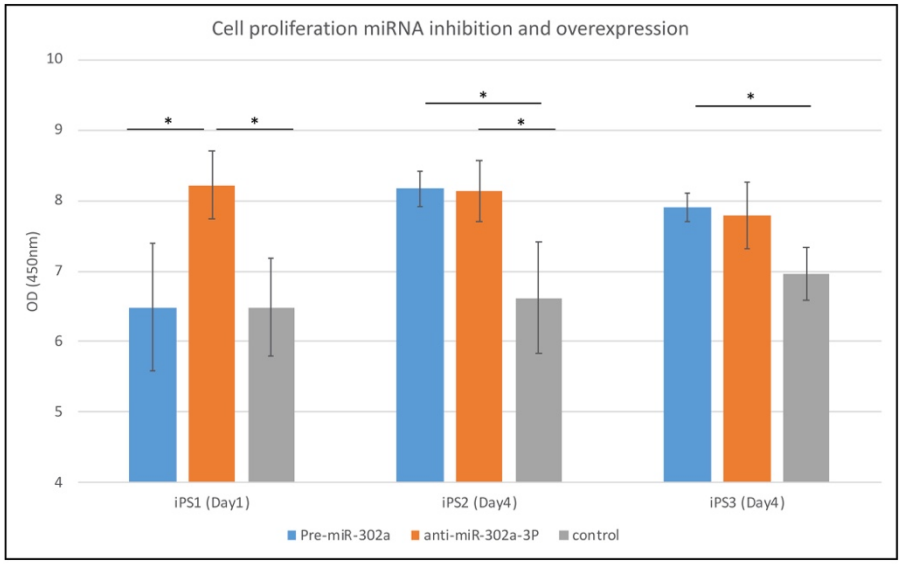


Figure 37: *Summarization of the proliferation test following inhibition and over-expression (*p‹0.05)*

7. 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.

8. Conclusions and recommendations

Stem cell technology has revolutionized the field of therapeutics and regenerative medicine. It has opened doors for work on *in vitro* drug designing, disease modelling. By the advent of the induced pluripotent stem cell technology; the ethical problems regarding isolation of ESC has been over-come. Now the iPSCs technology combined with gene knock out techniques like CRISPR/CAS9 has flagged the way for work on transgenic animals much more easily compared to the earlier time.

In our laboratory, there are established and well characterized cPGCs cell lines and rabiPSCs cell lines. We have 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 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.

8.1. MiRNA expression profile in chicken Primordial Germ Cells

This is the first study in which an overview of the global miRNA expression was performed in both male and female cPGCs using miRNA microarray (Lázár et al., 2018). In this study, total 148 expressed miRNAs (***Supplementary Table 1***) were identified in male and female PGCs. The microarray study revealed different novel miRNAs in chicken, as well as identified key members of know miRNA clusters identified in stem cells of other species. Some of the miRNA clusters are also identified in embryonic carcinoma stem cell (ECCs) (Rad et al., 2013). These clusters are cited to be either OncomiRs or tumour suppressor or cell cycle regulators in both ESC and ECC (Mens and Ghanbari, 2018) .

8.1.1. Concordant dysregulation of gga-miR-302b arms 5p and 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 cleaved from RNA induced silencing complex (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 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.

In my study, I obtained concordant dysregulation between the two arms of the gga-miR-302b. The ESCs and ECC tend to have a shorter G1 to S phase transition. Also, like the cancer stem cells the ESCs share some features of tumour genesis; like self-renewal capacity and unlimited cellular proliferation (Rad et al., 2013). In the study conducted by (Li et al., 2016) it was observed that miR-302 is endogenously highly expressed in human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs). The high expression of miR-302 helps in maintaining high levels of *OCT4* gene via *AKT* gene suppression. Inhibition of miR-302 using antagomirs; resulted in down- regulation of the self-renewal rate of hESCs, hiPSCs which was observed via cell colony formation assay. This result is consistent with our study in which inhibition of gga-miR-302b-5p resulted in increased doubling time and decreased proliferation rate.

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, hiPSCs 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-theronine 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 mechanism (**Figure 38**).

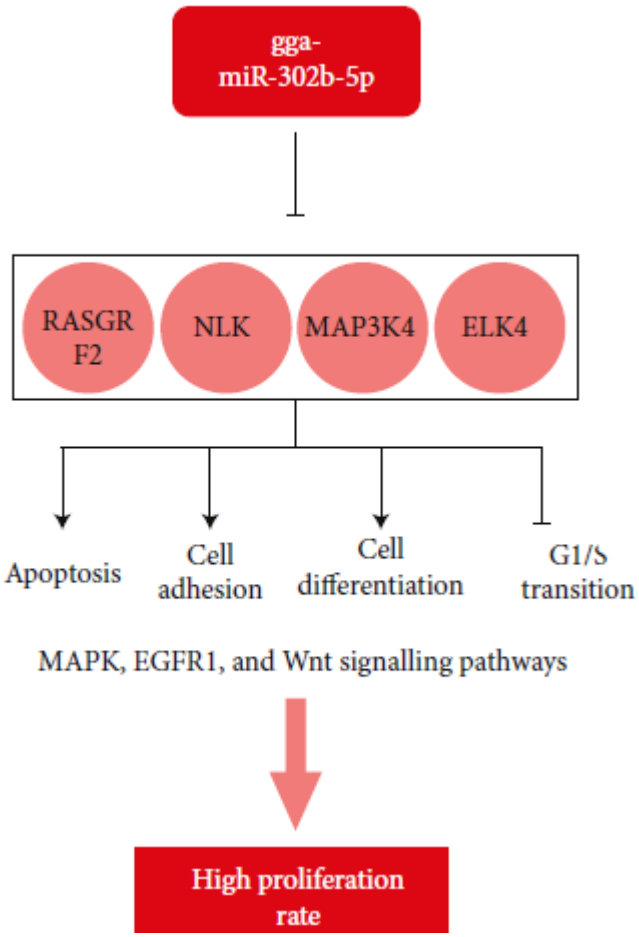


Figure 38: *The main targets of gga-miR-302b-5p in chickens as depicted above are involved in MAPK, WNT signalling pathways and controlling multiple pathways via a mirage of downstream targets. (Image modified from (Lázár et al., 2018)).*

8.2. MiRNA profile of rabbit induced Pluripotent Stem Cell

8.2.1. MiRNAs controlling proliferation rate of rabbit induced Pluripotent Stem Cell

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-5p were studied. In the previous study, conducted by Pouneh Maraghechi (Maraghechi et al., 2013), using solid sequencing analysis revealed high expression for the ocu-miR-302a-3p and lower expression for 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 effect in rabiPSCs but in opposite directions. The over expression of 5p arm increases proliferation and the inhibition of 3p arm increases proliferation.

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.

9. Keynotes of the study and future perspectives

This is the first study in which the miR-302 cluster members and their functional roles were characterized in cPGCs and rabiPSCs *in vitro* via proliferation and miRNA inhibition tests.

Other than miRNA characterization, my work also focussed on distinguishing the optimum culture conditions required for an efficient in vitro culturing protocol for cPGCS.

9.1. The main findings of my work as summarized below

7. A global miRNA expression profile in cPGCs: my study identified many miRNA clusters with distinct role on cPGCs proliferation and pluripotency.
8. Concordant dysregulation of the two arms of gga-miR-302b-5p and gga-miR-302b-3p.
9. Using a series of experimentation via Q-PCR, miRNA inhibition study and proliferation assay along with apoptosis the two arms were found to be concordantly dysregulated with arm 5p being a proliferation promoter (OncomiR) and arm 3p (tumour suppressor).
10. The proliferation rate was significantly reduced in cPGCs lines in which the miRNA gga-miR-302b-5p was inhibited.
11. For rabiPSCs it was found that inhibition of ocu-miR-302a-3p successfully increased the proliferation rate of rabiPSCs.
12. There was also a correlation found between cPGCs proliferation and cell concentration.
13. The optimal concentration for cPGCs cultivation was around 4000 cells (in one well of the 96 well cell culture plate) for most of the PGC line.

9.2. Future perspectives

Although, the study successfully characterized the functional role for the gga-miR-302b-3p / gga-miR-302b-3p in cPGCs and ocu-miR-302a-3p in rabiPSCs, there are still some unsolved aspects which need to be addressed in the future.

14. The main targets for the gga-miR-302b-3p and gga-miR-302b-5p still need to be discovered and characterized. Although, the study highlights some of the important targets still they need to be fully studied *in vitro*. Some of the targets that can be studied via western blotting or immunostaining studies are the signalling molecules like MAP3K4, TGFβ.
15. The effect of the inhibited miRNA needs to be fully exploited at the protein level. Many literature studies conducted on hESCs and hiPSCs cite *OCT4* gene and *AKT* gene to be important targets of the miR-302 cluster in human stem cells. This still needs to be fully elucidated in animals. Hence, it can be interesting to study the effect of gga-miR-302b-5p and 3p inhibition on protein level of these two genes.

16. The seed sequence of the miR-302 cluster in rabbits is found to be homologous to the miR-302 seed sequence of humans. Some of the identified targets for human miR-302 cluster are cell cycle inhibitors, cyclin dependent kinases. Therefore, it would be interesting to conduct *in vitro* studies using miRNA inhibition of ocu-miR-302a-3p and study the effect on these proteins via immunostaining or western blot.
17. The study also highlighted the importance of the 5p/3p arm of gga-miR-302b ratio. It would be interesting to decode the mechanism for dual existence for both arms from the same precursor miRNA. The enzymes Dicer, Argonaute, Drosha can be studied via immunochemical assays or at the molecular level to address this issue.
18. In chicken, there is no available information regarding the chicken Dicer gene. Hence, as a future recommendation the Dicer gene in chickens needs further characterization at both molecular and protein level. It can be studied via PCR-based approaches to identify potential hot spots or mutation in the gene that lead to various alternatively spliced forms of Dicer enzyme in chickens.
19. The study on cPGCs identified dysregulation of the two arms gga-miR-302b. The same study would be repeated in rabiPSCs in order to study the expression level and functional characterization of the ocu-miR-302b-3p/ 5p arms.
20. This is done to draw a parallel between cPGCs and rabiPSCs and to cite any differences between the expression level and effect of the miR-302 cluster and its members in cPGCs and rabiPSCs.

10. Összefoglaló

A tyúk primordiális csírasejtek (cPGC) a jövőben úttörő szerepet tölthetenek be az őssejtkutatás és a fejlődésbiológia területén. A tyúk embriók könnyen hozzáférhetők, a cPGC-k *in vitro* tenyészetekben könnyen fenntarthatók, genetikailag könnyen módosíthatók, így génbankok létrehozásának mellett, a PG sejtekből létrehozott genetikailag módosított állatmodellek tanulmányozásában is fontos szerepük van.

A nyúl indukált pluripotens őssejtek (rabiPSCs) szintén fontosak az őssejt-biológiai kutatások területén, mivel mind a cPG, mind pedig a rabiPS sejtek *in vitro* kísérletekben is jól alkalmazhatók.

Egyre nagyobb figyelem irányul a miRNS-ek felé. A miRNS-ek az őssejtek megújulásában és azok pluripotenciájának megőrzésében fontos szabályozó szerepet töltenek be.

PhD munkám során a tyúk őscsírasejtekben és a nyúl indukált pluripotens őssejtekben expresszáló miRNSek szerepét vizsgáltam. A cPGC-ben expresszáló miRNS-ek feltérképezése érdekében egy komplex miRNS microarray analízist végeztem LC microarray chipet alkalmazva. Csak kevés olyan miRNS-t találtam, ami a hím, illetve a női ivarú PGC vonalakban eltérően expresszált. Nagyobb eltérés csak a gyorsabban és lassabban osztódó, vagyis alacsony, illetve magas proliferációs rátával rendelkező cPGC vonalak között volt kimutatható a miRNS mintázatban.

Az őssejt-specifikus miRNS-ek közül a miR-302 klaszter tagjai magas expressziót mutattak a cPGC vonalakban. A miR-302 klaszter jól ismert őssejt-specifikus miRNS klaszter, aminek tagjai a miR-302a, miR-302b-5p, miR-302b-3p, miR-302c-5p, miR-302c-3p, miR-302d és miRNS-367. Ezen klaszter tagjainak expresszióját a nyúl iPS sejtekben is megtaláltuk.

Azt találtuk, hogy a tyúk gga-miR-302b-3p magasabb szinten expresszált a lassabban osztódó PGC vonalakban, míg a gga-miR-302b-5p expressziója alacsonyabb volt ezek esetében. A miR-302b-5p/miR-302b-3p arány erős diszregulációt mutatott. A 5p/3p arány kifejezetten magasabb volt a magas proliferációs rátát mutató PGC vonalakban és fordítva, alacsony volt ez az arány lassabban osztódó sejtvonalak esetében.

Hasonló jelenséget írtak le tumor őssejtek esetében is, vagyis a miRNS ellentétes karjainak diszregulációját lehetett megfigyelni néhány rákőssejt vonalban is. A miR-302b-3p tumor szupresszor hatású humán tüdőrákból származó tumor őssejt vonalak esetében, míg a miR-302b-5p „OncomiR”, azaz elősegíti a tumor sejtek proliferációját.

A gga-miR-302b-5p és a gga-miR-302b-3p esetében végzett funkcionális vizsgálatok igazolták, hogy a gga-miR-302b-5p gátlása növeli a duplikációs idejét a PG sejteknek. Eredményeinket Q-PCR alkalmazásával is igazolni tudtuk.

Hasonló eredményt figyeltünk meg a nyúl iPS sejtek esetében is. A gga-miR-302a-3p gátlása megnövelte a nyúl iPS sejtek proliferációs rátáját.

Eredményeink felhívják a figyelmet arra, hogy a miR-302 klaszter fontos szerepet tölt be a mind tyúk PG, mind a nyúl iPS sejtekben, az őssejtek proliferációs rátájának szabályozásán keresztül, azok pluripotenciájának megőrzésében, illetve a korai differenciálódási lépések szabályozásában. Továbbiakban szeretnénk többet megtudni a miRNS-ek által történő szabályozás molekuláris szintű folyamatairól is.

11. Summary

Chicken primordial germ cells (cPGCs) are upcoming pioneers in the field of stem and developmental biology. Chicken, because of an easy reproductive cycle and easy accessibility to collect chicken eggs and *in vitro* cultivation of the cPGCs; makes then an important animal model in the near future for bio-banking or animal model studies. The cPGCs can be easily cultured in the laboratory using a well–defined and available medium conditions. Other than cPGCs, rabbit induced pluripotent stem cells (rabiPSCs) are also important stem cells in field of stem cell biology. Both cPGCs and rabiPSCs can be grown under *in vitro* condition and used for further *in vitro* experiments. cPGCs and rabiPSCs are potential pluripotent stem cells. Recently, miRNA are emerging factors that govern the stem cell self-renewal and pluripotency. Many studies have identified miRNAs are factors controlling the pluripotency of the stem cells. Some of these miRNAs have been characterized as stem cell specific miRNAs. In my thesis work, I cultured cPGCs and rabiPSCs stem cells in the laboratory and characterized miRNA expression in them. In order to fully, exploit the relevant miRNAs expressed in cPGCs a complex microarray analysis was done using LC microRNA microarray. The microarray analysis revealed novel miRNA expression in the cPGCs. Some of these miRNAs tend to show male or female specific expression. Some miRNAs showing higher expression in the male cPGCs and some miRNAs showing higher expression in the female cPGCs. The miRNAs expression was different in male and female cPGCs and also showed different expression in slow proliferating cPG cell lines and high proliferating cPG cell lines.

From our results, we characterized the expression of the reported stem cell specific miRNA, the miR-302 cluster and its cluster members. The miR-302 cluster is well reported stem cell specific miRNA cluster, including the following members: miR-302a, miR-302b-5p, miR-302b-3p, miR-302c-5p, miR-302c-3p, miR-302d and miR-367. The expression of this cluster members was consistent with the reported literature. We also found the expression of this cluster members in the rabiPSCs.

From the miRNA complex array, we found that gga-miR-302b-3p highly expressed in slow proliferating PGC lines and low expression of gga-miR-302b-5p in slow proliferating lines. The arm 5p/3p arm ratio showed concordant dysregulation with the ratio being high in high proliferating PGCs cell lines and vice versa for low proliferating slow cell lines. Gga-miR-302b-5p was highly expressing in fast proliferating PGCs lines.

Concordant dysregulation of opposite arms of the same miRNA has been reported in cancer stem lines. MiR-302b-3p is tumour suppressor specific miRNA and attenuates proliferation as reported in the study of human lung cancer stem lines; whereas, miR-302b-5p is an

OncomiR i.e. it supports and promotes proliferation. These findings are in line with our obtained results. In order to further functionally validate the role of these miRNAs, we performed miRNA inhibition studies. By using anti-inhibitors for gga-miR-302b-5p and gga-miR-302b-3p we found that inhibition of gga-miR-302b-5p increased the doubling time (i.e. decreased the proliferation rate) in the cPGCs cell lines. The results were also validated by using Q-PCR.

Similar result was observed in the case of rabiPSCs. Inhibition of the gga-miR-302a-3p slightly increased the proliferation rate. The results of the miRNA inhibition studies were in close agreement with the reported rules of concordant dysregulation of the miR-302 cluster.

Our results are first conducted and cited results regarding the exact role of the miR-302 cluster in the cPGCs and the rabiPSCs. Till date, there has been evidence regarding the expression of miR-302a in rabbit and chicken stem cells, but their roles have yet not been functionally validated. In my thesis, I characterized the expression, as well as functionally validated the role of miR-302b-5p as an OncomiR for both PGCs and rabiPSCs, similar miR-302b-3p as a tumour suppressor miRNA causing a decrease in the proliferation rate. The above results provide directions for future studies on cPGCS and rabiPSCs work with the miR-302 cluster. Further functional characterization of the miRNAs at the protein level, would unravel the exact molecular mechanisms governing the pluripotency and proliferation rate at *in vivo* cellular level.

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13. Publications resulting from the doctoral work

13.1. International publications

1. Lázár, B., **Anand, M.**, Tóth, R., Patakiné Várkonyi, E., Liptói, K., Gócza, E. (2018): Comparison of the MicroRNA Expression Profiles of Male and Female Avian Primordial Germ Cell Lines. ***Stem Cells International,*** ID 1780679 (*shared first author*)(**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), pp. 518–529 *(shared first author* (**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*** 49(1):78-82. *(first author)*

13.2. 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 author)*

13.3. Abstracts and posters on international conferences

1. **Anand**, **M.**, Bence Lazar, Roland Toth, Eszter Patakine Varkonyi, Krisztina Liptoi, Elen Gocza (2018) Comparison the miRNA expression profile in chicken male and female primordial germ cells. Visegrad Conference on Development Biology, Brno, 2018.09.05-2018.09.09., poster
2. **Anand, M.**, Lázár, B., Tóth, R., Nagy, A., Eszter Patakiné Várkonyi E., Liptói, K., Gócza E. (2017): Investigation the heat shock related, and pluripotency associated miRNAs in chicken primordial germ cells. Microsymposium on small RNAs (IMP), Vienna, 2017.05.26 - 2017.05.28., poster
3. **Anand, M.,** Maraghechi, P., Németh, K., Lázár, B., Gócza, E. (2016): Investigation the role of microRNAs in early embryonic development and stem cells in rabbit and

chickens. *Transgenic Research*, 25(2): 220., 13th Transgenic Technology Meeting (TT2016), Prague, Czech Republic, 2016.03.20 - 2016.03.23., poster

4. Tóth, R., Lázár, B., Südy, Á., Nagy, A., Kidane, A., **Anand, M.,** Gócza, E. (2016): Left-right asymmetry of embryonic gonads in Transylvanian Naked neck chicken. *New Biotechnology* 33 (S): S212, 17th European Congress on Biotechnology, 2016.07.03 - 2016.07.06., Krakow, Poland
5. Gócza, E., Lázár, B., Maraghechi, M., **Anand, M**., Osteil, P., Tapponnier, Y., Afanassieff, M., Savatier, P. (2017): Exploration of rabbit microRNA expression profile in rabbit embryo and pluripotent stem cells. Final Conference of COST Action BM1308 Sharing Advances on Large Animal Models – SALAAM,2017.09.28. 2017.09.29., Halle, Germany, P-24
6. Tóth, R., Lázár, B., Nagy, A., **Anand, M**., Patakiné Várkonyi, E., Gócza, E. (2017): Comparison the integration efficiency of GFP expressing male and female chicken PGCs into the embryonic gonads. Final Conference of COST Action BM1308 Sharing Advances on Large Animal Models – SALAAM, 2017.09.28.2017.09.29., Halle, Germany, P-25.
7. Lázár, B., Tóth, R., Nagy, A., **Anand, M.,** Liptói, K., Patakiné Várkonyi, E., Gócza, E. (2017): Examination the combined effect of parental thermal stress and thermal treatment of the offspring on early embryonic development and on primordial germ cells. The Online Journal European Poultry Science, (IF: 0.296)
8. Lázár, B., Tóth, R., Nagy, A., **Anand, M.,** Liptói, K., Patakiné Várkonyi, E., Gócza, E. (2017): Primordial germ cell-based bio-banking of Hungarian indigenous chicken breeds. Poultry Science 96:(E Suppl.1.) p62 (IF: 1.908). Poultry Science Association 106th Annual Meeting, Orlando, USA, 2017.07.17 -2017.07.20. (World' s Poultry Science Association) OP-166
9. Lazar, B., Toth, R., **Anand**, **M.**, Molnar, M., Liptoi, K., Patakiné Várkonyi, E., Gocza, E. (2018): Germline chimera production from cryopreserved primordialgerm cell lines of a Hungarian indigenous chicken breed. The XVth European Poultry Conference, Dubrovnik, 2018.09.17 -2018.09.21.

13.4. Abstracts and posters in Hungary

1. Tóth, R., Lázár, B., Nagy, A., **Anand**, M., Patakiné Várkonyi, E., Gócza, E., (2017): Comparison the integration efficiency of the male or female chicken PGCs into the gonads. IDK 2017 Pécs, p176, 2017.05.19-21, ISBN: 978-963-429-113-8, Pécs
2. Tóth, R., Lázár, B., Südy, Á., Nagy, A., Kidane, A., **Anand, M**., Gócza, E. (2016): Left-right asymmetry during the development of embryonic gonads in chicken. Fiatal

Biotechnológusok Országos Konferenciája (FIBOK), 2016, Gödöllő, 2016.03.21 -2016. 03.22., p65, APE13

3. Tóth, R., Lázár, B., **Anand, M.,** Nagy, A., Patakiné Várkonyi, E., Gócza, E. (2017): Comparison the germ and stem cell specific marker expression in male and female embryo derived chicken PGCs. Hungarian Molecular Life Sciences 2017, Eger, 2017.03.31-2017.04.02., Eger, P-138, pp240-241. ISBN 978-615- 5270-34-5

13.5. Oral presentations

1. **Anand, M.,** Tóth, R., Kidane, A., Nagy, A., Lázár, B., Patakiné Várkonyi, E., Liptói, K., Gócza, E. (2017): Comparison the HSP70 and miR-138 expression in heat-treated and non-heat-treated chicken PGCs and genital ridges. Hungarian Molecular Life Sciences 2017, Eger, 2017.03.31-2017.04.02., Eger, O-49, pp88-89. ISBN 978-615-5270-34-5, oral presentation
2. **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. ISSBAR, Timisoara, Romania, 2016.05.26-2016.05.27. pp. 46-47., oral presentation
3. **Anand, M.,** Tóth, R., Kidane, A., Nagy, A., Lazar, B., Patakiné, Várkonyi E., Liptói, K., Gócza, E. (2016): A hősokk fehérjék és miRNS-ek expressziós mintázatának vizsgálata a hőkezelt és nem hőkezelt házi tyúk PGC tenyészetekben, valamint az ivarlécekben. Examination the expression pattern of heat shock related proteins and microRNAs in heat-treated and non-het treated chicken PGCs and genital ridges. XXXVI. Óvári Tudományos nap, p67, 2016.11.10., Mosonmagyaróvár, oral presentation

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15. Appendix

Sr.No	Name of the miRNA	Cluster/Family	Type of PGC expressed (Significant)	Role
1	gga-miR-15b-5p	miR-15	FS-ZW-111	tumour suppressor
2	gga-miR-15c-5p	miR-15	FS-ZW-111	OncomiR
3	gga-miR-16-5p	miR-15	FS-ZW-111, FS-ZZ-101	OncomiR
4	gga-miR-16c-5p	miR-15	FS-ZW-111	OncomiR
5	gga-miR-17-5p	miR-17	FS-ZW-111, FS-ZZ-101	OncomiR
6	gga-miR-18a-3p	miR-17	FS-ZW-111	OncomiR
7	gga-miR-18a-5p	miR-17	FS-ZW-111	OncomiR
8	gga-miR-18b-3p	miR-17	FS-ZW-111	OncomiR
9	gga-miR-18b-5p	miR-17	FS-ZW-111	OncomiR
10	gga-miR-19a-3p	miR-19	FS-ZW-111	novel miRNA
11	gga-miR-19b-3p	miR-19	FS-ZW-111	novel miRNA
12	gga-miR-20a-5p	miR-17	GFP-ZW-5ZP, FS-ZW-111	OncomiR
13	gga-miR-20b-5p	miR-17	FS-ZW-111	OncomiR
14	gga-miR-21-5p	miR-21	FS-ZW-111	OncomiR (ovarian cancer
15	gga-miR-23b-3p	miR-23	FS-ZW-111	OncomiR
16	gga-miR-24-3p	miR-24	FS-ZW-111	OncomiR
17	gga-miR-26a-5p	miR-26	FS-ZW-111, FS-ZZ-101	Viral Infection
18	gga-miR-30a-5p	miR-30	GFP-ZW-5ZP, FS-ZW-111, FS-ZZ-101	Breast Cancer
19	gga-miR-30b-3p	miR-30	GFP-ZW-5ZP, FS-ZZ-101	Breast Cancer
20	gga-miR-30b-5p	miR-30	FS-ZW-111	Breast Cancer
21	gga-miR-30c-5p	miR-30	FS-ZW-111, FS-ZZ-101	Breast Cancer
22	gga-miR-30d	miR-30	FS-ZW-111, FS-ZZ-101	Breast Cancer
23	gga-miR-30e-5p	miR-30	FS-ZW-111	Familial Breast Cancer
24	gga-miR-34a-5p	miR-34	FS-ZW-111	OncomiR

25	gga-miR-34b-3p	miR-34	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	OncomiR
26	gga-miR-92-3p	miR-17	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	OncomiR
27	gga-miR-92-5p	miR-25	FS-ZW-111	Cancer Serum biomarker
28	gga-miR-103-3p	miR-103	FS-ZZ-101, FS-ZW-111	Type 2 diabetes
29	gga-miR-106-5p	miR-17	GFP-ZW-5ZP, FS-ZW-111	Cancer
30	gga-miR-107-3p	homologous	FS-ZW-111	Type 2 diabetes
31	gga-miR-125b-5p	miR-10	FS-ZW-111	tumour suppresor
32	gga-miR-128-3p	miR-128	GFP-ZZ-4ZP, FS-ZZ-101, FS-ZW-111	Hepatocellular Carcinoma
33	gga-miR-130a-3p	miR-130	FS-ZW-111	OncomiR
34	gga-miR-130b-3p	miR-130	FS-ZW-111	Hepatocellular Carcinoma
35	gga-miR-130c-3p	miR-130	FS-ZW-111	Hepatocellular Carcinoma
36	gga-miR-135a-2-3p	miR-135	GFP-ZZ-4ZP	novel miRNA
37	gga-miR-140-3p	miR-140	GFP-ZZ-4ZP, FS-ZZ-101, FS-ZW-111	Viral Infection
38	gga-miR-146b-5p	miR-146	GFP-ZZ-4ZP	novel miRNA
39	gga-miR-146c-5p	miR-146	FS-ZW-111	Unregulated ortheoaritis
40	gga-miR-181a-5p	miR-181	GFP-ZZ-4ZP, FS-ZZ-101, FS-ZW-111	tumour suppressor
41	gga-miR-181b-5p	miR-181	GFP-ZW-5ZP, FS-ZZ-101, FS-ZW-111	tumour suppressor
42	gga-miR-205a	miR-205	FS-ZW-111	novel miRNA
43	gga-miR-214	miR-214	GFP-ZW-5ZP	Diabetic Marker
44	gga-miR-221-3p	miR-221	FS-ZW-111	Liver Cancer
45	gga-miR-222a	miR-221	FS-ZW-111	Liver Cancer
46	gga-miR-301a-3p	miR-130	FS-ZW-111	Hepatocellular Carcinoma
47	gga-miR-302a	miR-302	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	Pluripotency

48	gga-miR-302b-3p	miR-302	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-112	Pluripotency
49	gga-miR-302b-5p	miR-302	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 an d FS-ZW-113	Pluripotency
50	gga-miR-302c-3p	miR-302	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-114	Pluripotency
51	gga-miR-302c-5p	miR-302	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-115	Pluripotency
52	gga-miR-302d	miR-302	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	Pluripotency
53	gga-miR-367	miR-302	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	Pluripotency
54	gga-miR-383-5p	miR-383	FS-ZW-111	Tumour suppressor
55	gga-miR-454-3p	miR-454	FS-ZW-111	Tumour suppressor
56	gga-miR-456-3p	miR-456	FS-ZW-111	Blastodermal marker
57	gga-miR-466	miR-466	GFP-ZW-5ZP, FS-ZW-111	OncomiR
58	gga-miR-762	miR-762	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	novel miRNA
59	gga-miR-1306-5p	miR-1306	GFP-ZZ-4ZP, GFP-ZW-5ZP	novel miRNA
60	gga-miR-1354	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP	novel miRNA
61	gga-miR-1434	miR-1434	GFP-ZZ-4ZP, FS-ZZ-101, FS-ZW-111	High rate egg production
62	gga-miR-1451-5p	miR-1451	FS-ZW-111	Red blood cells
63	gga-miR-1454	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	Viral Infection
64	gga-miR-1456-5p	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	novel miRNA
65	gga-miR-1560-5p	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	novel miRNA

66	gga-miR-1562-5p	not defined	GFP-ZW-5ZP	novel miRNA
67	gga-miR-1563	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	novel miRNA
68	gga-miR-1575	not defined	GFP-ZW-5ZP	novel miRNA
69	gga-miR-1576	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	novel miRNA
70	gga-miR-1579	not defined	GFP-ZW-5ZP	novel miRNA
71	gga-miR-1583	clustered to miRNA 2954	GFP-ZW-5ZP	Heat Stress
72	gga-miR-1584	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	novel miRNA
73	gga-miR-1595-5p	not defined	GFP-ZW-111	novel miRNA
74	gga-miR-1599	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	novel miRNA
75	gga-miR-1607	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	novel miRNA
76	gga-miR-1610	not defined	GFP-FW-5ZP	novel miRNA
77	gga-miR-1612	not defined	GFP-ZW-5ZP, FS-ZW-111	Viral Infection
78	gga-miR-1629	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	novel miRNA
79	gga-miR-1636	not defined	GFP-ZW-5ZP	Viral Infection
80	gga-miR-1654	not defined	GFP-ZZ-4ZP, FS-ZZ-101, FS-ZW-111	novel miRNA
81	gga-miR-1657	not defined	GFP-ZW-5ZP	Polymorphism marker
82	gga-miR-1664-5p	not defined	GFP-ZW-5ZP	Biomarker
83	gga-miR-1677-3p	not defined	GFP-ZW-5ZP	novel miRNA
84	gga-miR-1678	not defined	GFP-ZW-5ZP, FS-ZW-111	novel miRNA
85	gga-miR-1690-5p	not defined	GFP-ZW-5ZP	novel miRNA
86	gga-miR-1702	not defined	GFP-ZW-5ZP	novel miRNA
87	gga-miR-1710	not defined	GFP-ZZ-4ZP, FS-ZZ-101, FS-ZW-111	Virus tolerant

88	gga-miR-1713	not defined	GFP-ZW-5ZP	novel miRNA
89	gga-miR-1718	not defined	GFP-ZW-5ZP	Viral Infection
90	gga-miR-1739	not defined	FS-ZZ-111	novel miRNA
91	gga-miR-1752	not defined	GFP-ZW-5ZP, FS-ZZ-101	novel miRNA
92	gga-miR-1759-5p	miR-1759	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	Ovarian development
93	gga-miR-1761	not defined	GFP-ZW-5ZP, FS-ZZ-101	novel miRNA
94	gga-miR-1762	not defined	GFP-ZW-5ZP	Ovarian cancer
95	gga-miR-1763	clustered with miR 1564	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	novel miRNA
96	gga-miR-1767	not defined	GFP-ZW-5ZP, FS-ZW-111	Viral Infection
97	gga-miR-1770	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	novel miRNA
98	gga-miR-1777	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-112	novel miRNA
99	gga-miR-1779	not defined	GFP-ZW-5ZP	Ovarian follicle development
100	gga-miR-1782	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP	novel miRNA
101	gga-miR-1783	not defined	GFP-ZW-5ZP	novel miRNA
102	gga-miR-1787	not defined	GFP-ZW-5ZP	novel miRNA
103	gga-miR-1790	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZW-111	Viral Infection
104	gga-miR-1796	not defined	GFP-ZW-5ZP	novel miRNA
105	gga-miR-1797	not defined	GFP-ZW-5ZP	novel miRNA
106	gga-miR-1809	not defined	GFP-ZW-5ZP, FS-ZZ-101, FS-ZW-111	novel miRNA
107	gga-miR-1811	clustered with miR 302	GFP-ZW-5ZP, FS-ZZ-101, FS-ZW-111	novel miRNA
108	gga-miR-2127	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	Tumour suppressor
109	gga-miR-2188-5p	miR-2188	GFP-ZW-5ZP	Viral Infection

110	gga-miR-2954	miR-2954	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	Viral Infection
111	gga-miR-3523	not defined	GFP-ZW-5ZP, FS-ZZ-101, FS-ZW-111	novel miRNA
112	gga-miR-3528	not defined	FS-ZW-111	Viral Infection
113	gga-miR-3530-5p	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	novel miRNA
114	gga-miR-3535	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-112	novel miRNA
115	gga-miR-3538	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-113	novel miRNA
116	gga-miR-3607-3p	miR-3607	FS-ZZ-101, FS-ZW-111	OncomiR
117	gga-miR-4732-5p	not defined	GFP-ZW-5ZP	novel miRNA
118	gga-miR-6547-5p	not defined	GFP-ZW-5ZP, FS-ZZ-101	SNP miRNA
119	gga-miR-6549-3p	not defined	GFP-ZW-5ZP	Egg production
120	gga-miR-6560-3p	not defined	GFP-ZW-5ZP	novel miRNA
121	gga-miR-6564-3p	not defined	GFP-ZW-5ZP, FS-ZZ-101, FS-ZW-111	novel miRNA
122	gga-miR-6581-5p	not defined	GFP-ZW-5ZP, FS-ZZ-101, FS-ZW-111	novel miRNA
123	gga-miR-6583-5p	not defined	GFP-ZW-5ZP	Ovarian cancer
124	gga-miR-6606-5p	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	Sexual dwarfism
125	gga-miR-6608-3p	miR-6608	GFP-ZW-5ZP, FS-ZZ-101	novel miRNA
126	gga-miR-6614-3p	not defined	GFP-ZW-5ZP, FS-ZW-111	novel miRNA
127	gga-miR-6626-5p	not defined	GFP-ZW-5ZP, FS-ZW-111	novel miRNA
128	gga-miR-6643-5p	not defined	GFP-ZW-5ZP, FS-ZZ-101, FS-ZW-111	novel miRNA
129	gga-miR-6653-3p	not defined	GFP-ZW-5ZP	novel miRNA
130	gga-miR-6657-3p	not defined	GFP-ZW-5ZP, FS-ZW-111	novel miRNA
131	gga-miR-6658-3p	not defined	GFP-ZW-5ZP	novel miRNA
132	gga-miR-6666-3p	not defined	GFP-ZW-5ZP	novel miRNA

133	gga-miR-6682-3p	not defined	GFP-ZW-5ZP	novel miRNA
134	gga-miR-6687-3p	not defined	GFP-ZW-5ZP	novel miRNA
135	gga-miR-6700-5p	not defined	GFP-ZW-5ZP	dwarfism
136	gga-miR-6708-5p	not defined	GFP-ZW-5ZP	novel miRNA
137	gga-miR-6711-5p	not defined	GFP-ZW-5ZP	novel miRNA
138	gga-miR-7439-5p	not defined	GFP-ZW-5ZP	novel miRNA
139	gga-miR-7446-3p	not defined	GFP-ZW-5ZP, FS-ZZ-101, FS-ZW-111	novel miRNA
140	gga-miR-7448-3p	not defined	GFP-ZW-5ZP, FS-ZW-111	novel miRNA
141	gga-miR-7450-5p	not defined	GFP-ZW-5ZP	novel miRNA
142	gga-miR-7455-5p	not defined	GFP-ZW-5ZP, FS-ZZ-101	novel miRNA
143	gga-miR-7466-3p	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	novel miRNA
144	gga-miR-7468-3p	not defined	GFP-ZW-5ZP	novel miRNA
145	gga-miR-7471-5p	not defined	GFP-ZW-5ZP	novel miRNA
146	gga-miR-7475-5p	not defined	GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111	novel miRNA
147	gga-miR-7478-3p	not defined	GFP-ZW-5ZP	novel miRNA
148	gga-miR-7483-5p	not defined	GFP-ZW-5ZP	novel miRNA

Supplementary Table 1: *An overview of the global miRNA expression profile in Chicken male and female primordial germ cells.*

ANR-RiPS-Basic-medium				Medium Components
500	77	38	ml	Knock-out DMEM/F12(no Hepes, no L-glutamine
6.5	1	0.5	ml	EmbryoMaxR Nucleosides (100x)
6.5	1	0.5	ml	Penicillin - Streptomycin-Glutamine, liquid (100x) (G-PSG)
6.5	1	0.5	ml	non-essential amino acids (100x)
0	0	0	ml	Na pyruvate 100mM (final 1mM)
650	100	50	µl	2-Mercaptoethanol, 50mM (1000x) (G-ME)
130	20	10	ml	Knock-out Serum Replacement
650	100	50	ml	SUM

Supplementary Table 2: *The basic rabiPSCs medium used for thawing.*

ANR-RES-medium-Culturing					
10	50	100	200	ml	ANR-RES-basic-medium
13	65	520	260	µl	bFGF (FGF2) 13ng/ml (Invitrogen)(10µg/ml stock)

Supplementary Table 3*: Concentration of the rabiPSCs + bFGF used for culturing.*

ANR-RES-puro-medium			Medium Composition	Cat. Number
10	50	ml	ANR-RES-basic-medium	
13	65	ml	bFGF 13ng/ml (Invitrogen) (10µg/ml stock)	13256-029
1	5	µl	Puromycin 1µg/ml (10mg/ml Puromycin stock)	P9620

Supplementary Table 4: *The rabiPSCs medium used for passaging.*

FM_medium + 10 % FBS (MEF medium)				Cat. Number	
89	178	356	ml	DMEM	Gibco 31966-047
1	2	4	ml	streptomycin/Penicillin G (PS stock)	Gibco 15140-122
10	20	40	ml	FBS (Lot: 07Q1613K.12.02.06)(G-FBS-2012)	Gibco
100	200	400	ml	SUM	

Supplementary Table 5: *The mouse embryonic fibroblast culture medium.*