miRNA functions in pluripotency and spermatogenesis

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Dedicated to my parents and my wife

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1. Zusammenfassung

Nicht-kodierende RNAs (ncRNAs – non-coding RNAs) wurden lange Zeit als Artefakte angesehen. Daher war die Entdeckung von kleinen ncRNAs, insbesondere von miRNAs, überraschend. Diese können die Genexpression post-transkriptionell regulieren. Sowohl Computer-gestützte als auch experimentelle Ansätze lassen das Vorkommen von miRNAs in allen lebenden Organismen vermuten – auch in Pflanzen und Homo sapiens. Eine immer größer werdende Anzahl an Ergebnissen weisen darauf hin, dass miRNAs eine wichtige Rolle in verschiedenen Aspekten des Lebens spielen – angefangen vom einfachen Zellmetabolismus, über die Kontrolle des Zellschicksals bis hin zur Entwicklung von multizellulären Organismen. Ziel der vorliegenden Arbeit war es, die Funktion von miRNAs in pluripotenten Stammzellen und während der Spermatogenese zu untersuchen und zu verstehen.

Im ersten Teil der vorliegenden Arbeit haben wir die Rolle der Pluripotenz-spezifischen miRNA-290-Gruppe während der Differenzierung von embryonalen Stammzellen (ESCs – embryonic stem cells) untersucht. Unsere Ergebnisse haben gezeigt, dass die Mitglieder der miRNA-290 Familie alleine nicht in der Lage sind, den Verlust der Pluripotenz während der Differenzierung von ESCs zu verhindern. Die Inhibition dieser miRNAs hingegen unterstützt den Differenzierungsprozess. Zusätzlich resultiert in ESCs die Überexpression der miR-290-Gruppe in der Unterdrückung der Differenzierung in Richtung Mesoderm und Keimzellen – möglicherweise über die Regulierung des Wnt-Signalweges. Zusammenfassend weisen diese Ergebnisse auf eine wichtige Funktion von miRNAs bei der Regulation der Differenzierung von ESCs hin.

Im zweiten Teil der vorliegenden Arbeit konnten wir zeigen, dass die ebenfalls Pluripotenz-assoziierte miRNA-302-Gruppe - im Gegensatz zu pluripotenten Zellen mit Keimbahn-Ursprung - in ESCs hoch exprimiert wird. Weiterhin identifizierten wir feine Unterschiede in den Expressionslevel von Keimzellmarker-Genen zwischen ESCs und den Keimbahn-entstammenden pluripotenten Zelltypen maGSCs (multipotent adult germline stem cells) und EGCs (embryonic germ cells). Interessanterweise war die Expression der Keimzellmarker-Gene negativ korreliert mit der Expression der miRNA-302 Familienmitglieder. Dementsprechend habe wir gefunden, dass die Überexpression der miRNA-302-Gruppe in maGSCs in der Herunterregulation der Keimzellmarker-Gene

resultiert. Zusammenfassend lässt sich sagen, dass unsere Ergebnisse zeigen, dass die miRNA302-Gruppe die Differenzierung von ESCs in Richtung Keimbahn reguliert.

Im dritten Teil der vorliegenden Arbeit war es unser Anliegen, die miRNA-Signatur verschiedener Keimzellstadien zu etablieren. Um dieses zu erreichen, haben wir eine doppelt-transgene Maus (Stra8/EGFP und Sycp3/DsRed) generiert, mit deren Hilfe wir reine Keimzellpopulationen aus dem Hoden der Maus isolieren konnten. Wir haben die miRNA-Expressionsprofile von spermatogonialen Stammzellen (SSCs – spermatogonial stem cells), premeiotischen (grünen) und meiotischen (roten) Keimzellen miteinander verglichen. Mit Hilfe dieses Ansatzes war es uns möglich, Stadien-spezifische miRNAs zu identifizieren, und wir konnten zeigen, dass z.B. miRNA-221, -203 und -34b-5q ihre Zielgene c-Kit, Rbm44 und Cdk66 regulieren und so die Spermatogenese beeinflussen. Zusammenfassend haben wir in dieser Studie das räumliche und zeitliche Expressionsprofil von miRNAs sowie deren möglichen Funktionen in der Spermatogenese beschrieben.

Zusätzlich konnten wir zwei neue Pluripotenz-spezifische miRNAs identifizieren: miRNA-135b und miRNA-363. Die Funktion dieser neuen Pluripotenz-spezifischen miRNAs wird zurzeit untersucht. Desweiteren haben wir über die Verwendung unserer doppelt-transgenen Maus eine Reihe bisher nicht charakterisierter Meiose-spezifischer Gene identifiziert. Im Moment werden diese Meiose-spezifischen Gene auf molekularer und biochemischer Ebene charakterisiert und bezüglich ihrer Funktionen während der Meiose untersucht.

1. Summary

For quite a long time, non-coding RNAs (ncRNAs) were discriminated and considered as an evolutionary error. In light of this opinion, discovery of small ncRNAs, in particular microRNA (miRNA), which can regulate the gene expression post-transcriptionally, was a surprise. Computational and experimental approaches revealed the presence of miRNA in almost all living organisms, including plants and *Homo sapiens*. Growing body of evidence suggests that miRNAs play a crucial role in different aspects of life, ranging from regulation of single cell metabolism through controlling of cell fate and development of multicellular organisms. The present thesis is aimed at understanding the function of miRNAs in pluripotent stem cells and in spermatogenesis.

In the first part of this thesis, we studied the role of miRNA-290 cluster, a pluripotency-related miRNA cluster, during differentiation of embryonic stem cells (ESCs). Our results show that miRNA-290 family members are not sufficient to prevent the loss-of-pluripotency during induced differentiation of ESCs. However, inhibition of these miRNAs was found to facilitate the differentiation process. In addition, overexpression of miR-290 cluster in ESCs resulted in prevention of differentiation towards mesoderm and germ cells, possibly through modulation of Wnt-signaling pathway. Collectively, these results support the assumption that miRNA-290 members are included in regulation of differentiation fate of ESCs.

In the second part of this thesis, we identified that ESCs but not germline derived pluripotent stem cells retain high expression levels of miRNA-302 cluster, another pluripotency-related miRNA clusters. Further, we found out subtle differences in expression levels of germ cell marker genes between ESCs and germline derived pluripotent cell types such as multipotent adult germline stem cells (maGSCs) and embryonic germ cells (EGCs). Interestingly expression of germ cell marker genes was negatively correlated with expression of miRNA-302 family members. In agreement with these observations overexpression of miRNA-302 cluster in maGSCs resulted in downregulation of germ cell marker genes. Taken together, our results highlight that miRNA-302 cluster regulates differentiation of ESCs to the germ cell lineage.

In the third part of this thesis, we made an attempt to establish the miRNA signature of various stage-specific germ cells. Towards this end, we generated a double transgenic mouse model (*Stra8*/EGFP and *Sycp3*/DsRed), which helped us to obtain pure germ cell populations from mouse testis. Next, we compared miRNA expression profiles between spermatogonial

stem cells (SSCs), pre-meiotic (green) and meiotic (red) cells. Through this study, we identified that stage-specific miRNAs, i.e., miRNA-221, -203 and -34b-5p regulate their corresponding targets such as *c-Kit*, *Rbm44* and *Cdk6* to orchestrate the spermatogenesis process. To sum up this study, we described spatiotemporal expression pattern of miRNAs and their possible functions during spermatogenesis.

In the present study, we also identified two novel pluripotent cell-specific miRNAs: miR-135b and miR-363. The function of these novel pluripotency-related miRNAs is under investigation. Furthermore, using our double transgenic mouse model, we identified several uncharacterized meiotic cell-specific genes. Currently, the molecular and biochemical characterization of these genes and their functions during meiosis is under the way.

2. Introduction

Gene expression is a process by which the information encoded in gene sequences is transcribed and mostly translated into gene product and is common for all living organisms. The course of gene expression is regulated in a simple way in *Prokaryotes* and becomes more complicated in *Eukaryotes*. Several steps during this process can be regulated, including remodeling of chromatin structure, generation and transport of transcripts, post-transcriptional modification and finally protein translation and post-translational modifications. Regulation of gene expression gives the cells possibilities to control their behavior and function, and defines cells fate. This regulatory mechanism explains how cells sharing the same genome can be so different from each other. For quite a long time, only proteins were considered as regulatory tools of cellular identity. Due to this fact, screening for new regulatory molecules was focused on protein level. Although the function of some regulatory genes was well documented, the protein products of these genes have never been identified, but rather led to the discovery of non-coding RNAs (ncRNAs) (Eddy, 2001). Interaction of ncRNA with either DNA or mRNA results in regulation of gene expression (mostly repression). MicroRNA (miRNA or miR) is one of the most abundant ncRNA families regulating gene expression in a post-transcriptional manner (Bartel, 2004). The goal of this thesis is the identification and functional characterization of miRNAs which are specific for pluripotent stem cells and for various cell types of mouse spermatogenesis. Hence, the regulation and function of miRNAs, in general, as well as their crucial role in maintenance of pluripotency and regulation of spermatogenesis will be emphasized in the following sections.

2.1. miRNA biogenesis

The first miRNA was described independently by two groups in 1993 (Lee *et al.*, 1993; Wightman *et al.*, 1993). Both research groups have found that the product of the *lin-4* gene, which negatively regulates the *lin-14* gene expression in *Caenorhabditis elegans* (*C. elegans*), is not a protein but two small RNAs derived from it (Lee *et al.*, 1993; Wightman *et al.*, 1993). Forward genetic approach identified that *lin-4* RNAs interact with 3´ untranslated region (3´ UTR) of *lin-14* gene and led to propose a new mechanism for the regulation of gene expression (Wightman *et al.*, 1991; Wightman *et al.*, 1993). This innovative hypothesis was rather considered as an exception until the year 2000 when *let-7*, the second example of

such transcription regulatory-RNA was discovered (Reinhart *et al.*, 2000). *Let-7* represses *lin-41* expression and, similar to previously described *lin-4*, regulates developmental timing in *C. elegans* (Reinhart *et al.*, 2000). Due to their functions in temporal development in worms, these small RNAs were initially named as small temporal RNAs (stRNAs) (Pasquinelli *et al.*, 2000). This name was later changed to microRNA when several research groups discovered that stRNA is not only limited to worm world, but is generally expressed in invertebrates as well as in mammals and plants (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001; Reinhart *et al.*, 2002). Together with the large number of newly discovered miRNAs in almost all known organisms, it became evident that miRNAs represent a conserved pathway for the regulation of gene expression (Ambros, 2004; He and Hannon, 2004).

The mechanisms underlying miRNA biosynthesis are highly conserved and involve two sequential steps: (1) generation of ~70 nucleotides (nt) precursor miRNA (pre-miRNA) originating from a longer primary miRNA transcript (termed pri-miRNA), (2) processing of the pre-miRNA into mature ~22 nt miRNA. **Figure 2.1**. presents an overview of the canonical pathway of miRNA synthesis in animals. Typically, the pri-miRNA transcript is generated by a RNA polymerase II-mediated process and includes one or more internal stem-loop hairpin structures containing the miRNA sequences (Cai *et al.*, 2004; Lee *et al.*, 2004). In the first step, the stem-loop structure is recognized and cleaved in the nucleus by microprocessor complex consisting of RNaseIII endonuclease Drosha and its cofactor DGCR8 (known as Pasha in *Drosophila melanogaster* (*D. melanogaster*) and *C. elegans*) which executes enzymatic function and the cleavage site identification, respectively (Lee *et al.*, 2003; Denli *et al.*, 2004; Han *et al.*, 2004; Han *et al.*, 2006). Drosha cleaves at the base of the stem and releases pre-miRNAs which are exported from the nucleus to the cytoplasm by Exportin-5 in the presence of Ran-GTP cofactor (Yi *et al.*, 2003). In the cytoplasm, the hairpin structure of pre-miRNA is processed by another RNaseIII endonuclease Dicer (Hutvagner *et al.*, 2001; Ketting *et al.*, 2001). Dicer cooperates with double strand RNA (dsRNA) binding protein TRBP (RDE-4 in *C. elegans* and Loquacious in *D. melanogaster*) to cleave the loop and thereby generates miRNA duplex (Tabara *et al.*, 2002; Haase *et al.*, 2005). Generally, only one miRNA from the duplex, termed major or guide, is functional, another one (minor, miRNA* or passenger miRNA) has no known function and becomes degraded (Schwarz *et al.*, 2003). The Dicer product (major miRNA) together with Argonaute proteins form miRNA-induced silencing complex (miRISC) which mediates post-transcriptional gene suppression (MacRae *et al.*, 2008). In principle, miRNA recognizes

complementary sequences in the 3′ UTR of the targeted genes. Mechanisms by which major/mature miRNA executes its function depend on the degree of complementarity between miRNA and its target mRNA. Full complementarity causes mRNA degradation while partial complementarity results in translation inhibition. Degradation of mRNA targets in context of full complementarity is mostly restricted to plants (Bartel, 2004). In animals, miRNAs bind to their target mRNAs through imperfect complementarity at multiple sites leading to translational repression rather than target degradation. Therefore one miRNA can regulate the expression of more than one target gene, and individual target genes can be simultaneously targeted by several miRNAs (Doench and Sharp, 2004; Lim *et al.*, 2005). Through the repression of target genes expression, miRNAs are involved in a wide variety of biological processes including developmental timing, cell proliferation and differentiation, cell cycle regulation, cell death and metabolism. Aberrant expression of many miRNAs has already been linked with developmental abnormalities and human diseases (Osman, 2012).

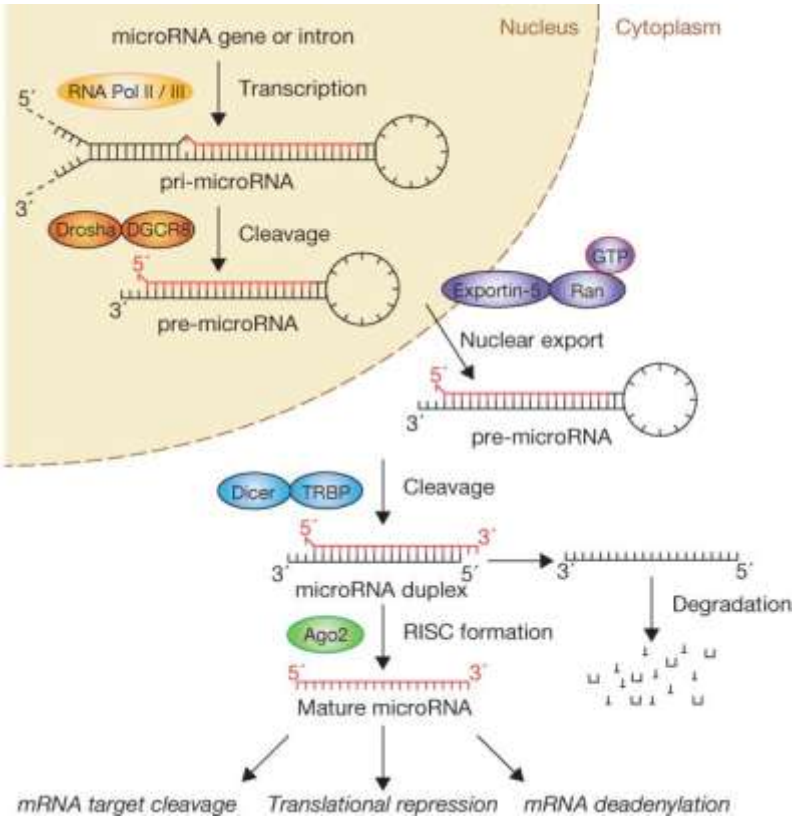

Figure 2.1. Schematic representation of canonical miRNA biosynthesis pathway. Canonical pathway of miRNA processing includes production of primary transcript (pri-miRNA) either by RNA polymerase II or occasionally by RNA polymerase III. Nascent pri-miRNA transcript is further processed by microprocessor complex (Drosha-DGCR8) in the nucleus. The resulting precursor (pre-miRNA) is exported from the nucleus to the cytoplasm by Exportin-5-RanGTP complex. Dicer together with TRBP cleaves pre-miRNA to give rise to miRNA duplex. Finally only one strand from the duplex (termed mature or major) is loaded onto miRISC and

regulates the gene expression either by mRNA cleavage or translation repression. The other strand has no known function and is mostly degraded (figure adopted fromWinter *et al.*, 2009).

2.2. Regulation of miRNA expression

Although the mechanism of miRNA biogenesis is well described, only a few studies illustrate the regulation of this process. Till now, no universal mechanism for miRNA maturation is proposed and most of our knowledge comes from examples of biosynthesis regulation of single miRNAs, miRNA clusters or only a subset of miRNAs (Krol *et al.*, 2010). Regulation of miRNA genesis can occur throughout each step of miRNA expression and maturation including pri-miRNA transcription, processing of pri-miRNA to pre-miRNA, export of pre-miRNA from nucleus to the cytoplasm, miRNA duplex formation, assembling of miRISC and finally modulation of mature miRNA.

Mechanisms controlling the gene expression at the transcriptional level are common for protein-coding genes as well as for miRNAs. During transcription many DNA-binding proteins like c-Myc, p53 or cell-specific transcription factors can bind to miRNA promoter region and regulate its expression in a tissues-specific or developmental-specific manner (O'Donnell *et al.*, 2005; Lin *et al.*, 2009; Boominathan, 2010). Moreover, miRNA genes can be regulated by epigenetic modifications (DNA methylation and histone modifications) occurring at their promoter region or at neighbouring *locus* (Saito *et al.*, 2006; Lujambio *et al.*, 2008; Lujambio and Esteller, 2009).

Once the transcriptional process is finished, different mechanisms regulate the maturation of mRNAs and miRNAs. The pri-miRNA transcript processing into mature miRNA involves two-step digestion mediated by Drosha and Dicer. Regulation of this stage entails post-transcriptional modification of enzymes or RNA template structure and control of interaction between both RNA and protein molecules. Interaction of Drosha with DEAD box RNA helicases p68 and p72 enables recruitment of other proteins (i.e. R-Smad, p53 or estrogen receptor-α) and regulates miRNA maturation (Davis *et al.*, 2008; Suzuki *et al.*, 2009; Yamagata *et al.*, 2009). The well-known example of RNA structure modification includes interaction between Lin28 or complex of nuclear factor 90 and nuclear factor 45 (NF90/NF45) with members of the let-7 family (Newman *et al.*, 2008; Rybak *et al.*, 2008; Sakamoto *et al.*, 2009). Lin28 binds to a specific sequence in the let-7 stem-loop structure and inhibits its maturation by changing the pri-miRNA conformation (Nam *et al.*, 2011). Unlike Lin28, interaction of heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) and KH-type splicing regulatory protein (KSRP) with the loop of pri-miR-18a and pri-miR-206,

respectively, promote pri-miRNA processing (Michlewski *et al.*, 2008; Trabucchi *et al.*, 2009). In addition, the maturation process can be regulated during export of pre-miRNA from the nucleus as well as during the incorporation of mature miRNA into the RISC complex. However, both mechanisms are poorly understood. A recent study has identified ~12nt semi-microRNAs (smiRNA) which are generated along the miRNA pathway and are capable of regulating the activity of corresponding mature miRNAs (Plante *et al.*, 2012).

2.3. miRNA annotation and registry

Together with the increasing number of newly discovered microRNAs it was necessary to develop a uniform system for miRNA annotation. In the year 2003, Victor Ambros and colleagues described a set of rules for the experimental verification of newly discovered miRNAs based on the expression and biogenesis criteria (Ambros *et al.*, 2003). Moreover, they proposed also a convention for nomenclature of miRNAs. To improve the communication between miRNA research groups, Sanger Institute created a miRNA registry (miRBase) (Griffiths-Jones, 2004). This registry serves as a platform to submit new miRNAs on one hand and provides detailed information about each published miRNA, on the other hand. The 19th release of miRBase is available since August 2012 and contains information about 25141 mature miRNAs in 193 species.

2.4. miRNA functions in animal development

Together with the discovery of *lin-4* and *let-7* functions, miRNAs have been classified as regulators of developmental processes (Lee *et al.*, 1993; Wightman *et al.*, 1993). The miRNA loss-of-function models displayed abnormalities during *C. elegans* larval maturation due to precocious or retarded development (Chalfie *et al.*, 1981; Ambros and Horvitz, 1984). Due to their high evolutionary conservation, *lin-4* and *let-7* miRNAs are suggested to perform the same function in other species (Pasquinelli *et al.*, 2000; Lagos-Quintana *et al.*, 2002). This hypothesis was further supported by the results of knock out (KO) experiments, where organisms with defective miRNA biogenesis were used as a tool for characterization of miRNA function. Depletion of Dicer, Drosha or Argonaute proteins was found to result in embryonic lethality in all tested animal models (Grishok *et al.*, 2001; Bernstein *et al.*, 2003; Wienholds *et al.*, 2003; Alisch *et al.*, 2007; Park *et al.*, 2010). Classical Dicer-KO mouse models displayed embryonic lethality at 7.5 day post coitum (dpc) due to the lack of stem cells, whereas conditional-KO resulted in defective proliferation and differentiation of

embryonic stem cells (ESCs) (Bernstein *et al.*, 2003; Kanellopoulou *et al.*, 2005). These results suggest that miRNAs are necessary for maintenance of stemness as well as for differentiation of pluripotent stem cells, where stage-specific miRNA might regulate the expression of target genes important for tissues development and/or their proper function. Following this observation, many research groups have started to analyze miRNA profiles of specific cell-types, tissues and organs in an attempt to elucidate their biological functions (Stefani and Slack, 2008). Upon the identification of a specific miRNA, its target mRNAs can be predicted using several web-based tools (Xia *et al.*, 2009). To verify the interaction between a miRNA of interest and its corresponding target, a simple assay was established (Nicolas, 2011). This assay is based on the expression of chimeric gene (e.g. luciferase ORF fused with 3´UTR of target of interest) upon miRNA overexpression. Finally, the interaction and regulation of target gene expression at protein level must be confirmed *in vivo* in a physiological condition. Development of miRNA microarray and deep sequencing technologies paved the way for the current golden age of miRNA research by replacing complicated and time consuming approaches for miRNA identification (Barad *et al.*, 2004; Liu *et al.*, 2004; Creighton *et al.*, 2009). Data obtained from the comparison of miRNA profiles of normal or defective tissues thus illustrate the importance of a specific miRNAs. The well-known example includes miRNA miss-expression in many types of human cancer (Kong *et al.*, 2012).

2.5. Role of miRNAs in pluripotent stem cells

Pluripotent stem cells are defined by two main characteristics: they are able to self-renew and can differentiate towards derivatives of all three germ layers and to germ cells (Bradley *et al.*, 1984). Since 1981, when Evans and Kaufman first established a protocol for the isolation and culture of mouse ESCs, these ESCs became the gold standard of pluripotency and model for further characterization (Evans and Kaufman, 1981). Understanding the molecular basis of ESCs self-renewal and differentiation properties would facilitate their use in future regenerative medicine applications. Among many identified factors, Oct4, Sox2, Klf4, c-Myc and Nanog compose a core transcription factor network regulating the stemness of pluripotent stem cells (Niwa, 2007). Interestingly, forced expression of these transcription factors allows the reprogramming of somatic cells into pluripotent cells, widely known as induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007; Yu *et al.*, 2007).

Since *Dicer* and *Dgcr8*-knockouts resulted in embryonic lethality due to depletion of stem cells, it signified a need for identification of individual pluripotency-specific miRNAs and to elucidate their role in pluripotency. Till now, members of miR-290 family (miR-371 family is human homolog) and miR-302 family were identified and characterized as specific for both mouse and human ESCs (Houbaviy *et al.*, 2003; Suh *et al.*, 2004). Moreover, miR-290 family is expressed highly in the other pluripotent cell lines including embryonic germ cells (EGCs), multipotent adult stem cell (maGSCs) and embryonic carcinoma cells (ECCs) (Zovoilis *et al.*, 2008; Zovoilis *et al.*, 2010). Interestingly, the expression of these pluripotency-specific miRNAs is regulated by the core pluripotency transcription factor network, suggesting their involvement in a common molecular pathway of pluripotency maintenance (Marson *et al.*, 2008). Although, ESC-specific miRNAs have a crucial role in maintenance of stemness, only a few studies enlightened their precise function. In general, members of ESC-expressed miRNAs promote pluripotency by regulating the cell cycle (Card *et al.*, 2008; Wang *et al.*, 2008; Lichner *et al.*, 2011). In addition, the NFκB signalling pathway was reported as a target of miR-290 family to promote the pluripotency by suppressing differentiation and epithelial-to-mesenchymal transition (Luningschror *et al.*, 2012). Recent studies have shown that ESC-specific miRNAs not only regulate the stemness but also facilitate somatic cells reprogramming towards iPSCs (Judson *et al.*, 2009; Lin *et al.*, 2011). Besides ESC-specific and cell cycle–regulating miRNAs, several miRNAs including miR-200 family, miR-205 and three miRNA clusters, miR-17~92, miR-106b~25 and miR-106a~363 have been shown to regulate the reprogramming process by facilitating mesenchymal-to-epithelial transition (Gregory *et al.*, 2008; Gregory *et al.*, 2011; Li *et al.*, 2011). Despite the well-known influence of miRNAs in pluripotency regulation, many challenges remain to elucidate the underlying mechanisms.

2.6. miRNA functions during male germ cells specification

Germ cells are the only cells in the body capable of transmitting genetic information through the generations by either oogenesis or spermatogenesis. Spermatogenesis is the process which gives rise to haploid male gametes and is common for all sexually reproducing animals. In mice, the process of spermatogenesis consists of three major phases: a) self-renewal and proliferation, b) meiotic division, and c) spermiogenesis. Spermatogenesis is strictly regulated by transcriptional as well as post-transcriptional mechanisms (Cooke and Saunders, 2002; Bettegowda and Wilkinson, 2010). The transcriptional mechanisms are well

described, however, the post-transcriptional regulation is still largely unknown (Pang *et al.*, 2003; Wu *et al.*, 2004). The support for the role of post-transcriptional mechanisms came from conditional knock out mouse models in which Dicer or Drosha were specifically depleted in primordial germ cells (PGCs) or spermatogonia (Hayashi *et al.*, 2008; Maatouk *et al.*, 2008; Korhonen *et al.*, 2011). In all cases, the lack of miRNAs resulted in infertility due to disruption of spermatogenesis. To unravel the role of miRNAs in spermatogenesis, several research groups have performed expression analysis using purified germ cells or cells from the whole testis (Yu *et al.*, 2005; Ro *et al.*, 2007; Yan *et al.*, 2007; Song *et al.*, 2009). Although they were able to identify many miRNAs that are specific for certain germ cell populations, no functional studies were performed to explain the molecular function of these miRNAs. Till now there are only few examples of miRNAs with well documented function in spermatogenesis: a) miRNA-122a is expressed in late germ cells and targets *Transition protein 2* gene (*Tnp2*) thus allowing the loading of protamines and subsequent chromatin compaction (Yu *et al.*, 2005), b) miRNA-21 which is highly expressed in spermatogonial stem cells (SSCs) and c) spermatocyte and spermatid-specific miRNA-34c are crucial for regulating apoptosis during spermatogenesis (Niu *et al.*, 2011; Liang *et al.*, 2012). In order to establish a comprehensive list of miRNAs and their functions during spermatogenesis, it is necessary to develop efficient strategies to enrich pure populations of various spermatogenic cell types. miRNA expression data obtained from such pure germ cell populations might help us to establish a spatiotemporal expression profile of miRNAs and to elucidate their function.

2.7. Objectives

To further expand our knowledge on the expression and function of miRNAs in pluripotent cells as well as in germ cells, the current study was undertaken with the following objectives:

1. Comparative expression and functional analysis of pluripotency-related miRNAs in various pluripotent cell types.
2. Identification of cell type-specific miRNAs and elucidation of their function during the process of spermatogenesis.

3. Results

microRNAs (miRNAs) are a class of small non-coding RNAs known to function in post-transcriptional regulation of gene expression. The goal of this thesis was to characterize the function of known pluripotency-specific miRNAs. Moreover, we extended this study to germ cells and established the miRNA signature of various cell types of mouse spermatogenesis and showed functional relevance for some of the stage-specific miRNAs. Collectively, we revealed the miRNA profiles of pluripotent cells as well as germ cells and uncovered their function in regulation of pluripotency and spermatogenesis, respectively. The results of this thesis are summarized in the following manuscripts:

3.1. Members of the miR-290 cluster modulate *in vitro* differentiation of mouse embryonic stem cells.

3.2. Embryonic stem cell-related miRNAs are involved in differentiation of pluripotent cells originating from the germ line.

3.3. MicroRNA signature in various cell types of mouse spermatogenesis: Evidence for stage-specifically expressed miRNA-221, -203, and -34b-5p mediated spermatogenesis regulation.

Each paragraph within the following results section contains a brief description of the aim of the study in context of the complete thesis, the status of each manuscript as well as the author contributions.

3.1 Members of the miR-290 cluster modulate *in vitro* differentiation of mouse embryonic stem cells

Recent studies have highlighted the importance of miRNAs, especially miR-290 family members, in maintenance of self-renewal and proliferation properties of pluripotent stem cells. In the first part of this thesis, we tested the possible function of miRNA-290 members during embryonic stem cell (ESC) differentiation. Towards this end, we performed miRNA gain-of-function and loss-of-function experiments in ESCs. The overexpression of miR-290 cluster in ESCs could not prevent the downregulation of *Oct4*, a pluripotency marker gene, and also failed to sustain the stemness during induced differentiation. However, the suppression of miRNA-290 members expression resulted in an immediate downregulation of *Oct4* and cells showed precocious differentiation. Additionally, differentiation towards mesoderm and germ cell lineage was found to be significantly affected in miR-290 cluster overexpressing cells. Reciprocally, the miR-290 cluster suppression resulted in preferential derivation of mesoderm and germ cell lineage cell types. Further studies revealed that miRNA-290 members target *Dkk1*, a Wnt-signaling inhibitor, to modulate the mesoderm and germ cells differentiation of ESCs. Collectively, our results demonstrate for the first time that pluripotent-specific miRNAs regulate the differentiation of ESCs.

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*** equal contribution to the work**

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3.2. Embryonic stem cell-related miRNAs are involved in differentiation of pluripotent cells originating from the germ line.

We have identified that the expression of miR-290 as well as miR-302 clusters is identical between undifferentiated pluripotent cells originating either from inner cell mass of the blastocyst (ESCs) or from germ cells (multipotent adult germline stem cells (maGSCs)). However, we noted that maGSCs retain high expression levels of miR-290 cluster, which is associated with high *Oct4* expression, during induced differentiation. In the second part of this thesis, we investigated the potential differences between ESCs and maGSCs as well as embryonic germ cells (EGCs) during differentiation and the relevance of miRNAs in this process. We found that undifferentiated maGSCs and EGCs express high levels of germ cell specific marker genes such as *Dppa3* and *Stra8* compared to ESCs. These expression levels were highly persistent even during the differentiation of maGSCs and EGCs. In light of these findings, we identified that ESCs but not maGSCs retains high expression levels of miRNA-302 cluster during differentiation and inversely correlates with the levels of early-germ cell marker genes. Finally as a proof of concept, we show that overexpression of miR-302 in maGSCs can suppress the preferential differentiation into germ cell lineage during differentiation.

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3.3. MicroRNA signature in various cell types of mouse spermatogenesis: Evidence for stage-specifically expressed miRNA-221, -203, and -34b-5p mediated spermatogenesis regulation.

Spermatogenesis, the process of haploid male gametes generation, is a complex process regulated by both transcriptional and post-transcriptional mechanisms. To better understand how post-transcriptional mechanisms mediated by miRNAs regulate the self-renewal as well as differentiation process of spermatogonial stem cells (SSCs), we analyzed the miRNA expression profile of various cell types of mouse spermatogenesis. To achieve this goal, we generated a transgenic mouse model (*Stra8*/EGFP and *Sycp3*/DsRed) in which pre-meiotic (PrM) and meiotic cells were marked by EGFP and DsRed, respectively. The isolation of pure germ cell populations, i.e., SSC, PrM and meiotic cells and the subsequent miRNA microarray expression analysis led us to establish the miRNA signature of individual cell type. Through functional studies, we show that miRNA-221 regulates the self-renewal of SSCs by targeting *c-Kit*, while miRNA-203 and -34b-5p function in preventing the precocious activation or repression of germ cell differentiation by targeting *Rbm44* and *Cdk6*, respectively. Taken together, through our data we suggest a working model where spatiotemporal expression of miRNA functions in the regulation of spermatogenesis.

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4. Discussion

Since the first discovery of miRNAs in *C. elegans* about 20 years ago, now they are identified in almost all living organisms. As much as 60% of human protein-coding genes might be regulated by miRNAs (Lewis *et al.*, 2005; Friedman *et al.*, 2009). Deregulation in miRNAs expression has been already reported to be associated with many human diseases, in particular with cancer. Moreover, miRNAs were shown to be essential for maintenance of stem cells as well as for normal embryogenesis (Bernstein *et al.*, 2003). In the present study, we investigated the expression and function of microRNAs in pluripotent stem cells as well as in germ cells. We have shown that miRNAs belonging to miR-290- and 302-clusters regulate the fate of pluripotent stem cells by suppression of differentiation pathways towards mesoderm and germ cell lineage. Moreover, we established miRNA expression profile of spermatogonial stem cells (SSCs), premeiotic and meiotic cells. Further, we have shown that germ cell specific miRNA-221, -203 and 34-5p regulate their corresponding target genes (*c-Kit*, *Rbm44* and *Cdk6*, respectively) to control the spermatogenesis. Taken together, our current studies revealed novel functions of pluripotent stem cells- and germ cell-specific miRNAs in the context of pluripotency and spermatogenesis.

4.1 Pluripotent stem cell-specific miRNAs and their functions

ESC specific miRNAs, the members of miR-290 and miR-302 clusters, have been shown to regulate pluripotency (Houbaviy *et al.*, 2003; Suh *et al.*, 2004; Lakshmipathy *et al.*, 2007; Morin *et al.*, 2008). Moreover, these ESC-specific miRNAs are highly expressed in other pluripotent cell lines such as multipotent adult germline stem cells (maGSCs), embryonic carcinoma cells (ECCs), embryonic germ cells (EGCs) and induced pluripotent stem cells (iPSCs) (Zovoilis *et al.*, 2008; Chin *et al.*, 2009; Zovoilis *et al.*, 2010). Similarly, members of miR-371 cluster (homolog of murine miRNA-290 family) and miRNA-302 cluster represent the majority of human ESC-specific miRNAs (Suh *et al.*, 2004). Our current knowledge concerning the function of ESC-specific miRNAs comes from analysis of phenotypes caused by genetic ablation of components of miRNA biosynthesis pathway. *Dicer* knockout embryos dye during embryonic development and *Dicer*-deficient (*Dicer*$^{-/-}$) ESCs are viable but manifest proliferation and differentiation defects (Bernstein *et al.*, 2003; Kanellopoulou *et al.*, 2005; Murchison *et al.*, 2005). Moreover, the *Dicer*$^{-/-}$ ESCs failed to form teratoma after injection into immunodeficient mice and showed incompetence to

contribute to chimera formation after injection into the blastocyst (Kanellopoulou *et al.*, 2005). Interestingly, *Dgcr8,* a co-factor of Drosha, deficient ESCs displayed a similar phenotype (Wang *et al.*, 2007).

Under physiological conditions, ESCs display a unique cell cycle pattern. As compared to somatic cells, ESCs have extremely short G1 phase and a high proportion of the cells in S-phase (White and Dalton, 2005). Analysis of cell cycle in *Dicer*$^{-/-}$ or *Dgcr8*$^{-/-}$ revealed that many cells accumulate in G1 cell cycle phase and show cell proliferation and growth defects (Murchison *et al.*, 2005; Wang *et al.*, 2008). Transfection of single miRNAs into *Dgcr8*$^{-/-}$ ESCs has shown that members of miR-290 cluster are sufficient to rescue the proliferation defect (Wang *et al.*, 2008). The Cyclin E-Cdk2 complex has been reported to positively regulate G1/S transition in ESCs (Kato, 1999; Burdon *et al.*, 2002). p21CIP, the protein product of *Cdkn1a* gene, is a well-known inhibitor of cyclin E-Cdk2 complex (Harper *et al.*, 1993). Simultaneously, *Cdkn1a* is post-transcriptionally regulated by miRNA-290 cluster and in the absence of miRNA-290 it can efficiently inhibit cyclin E-Cdk2 function resulting in G1 arrest (Wang *et al.*, 2008). Additionally, two members of cyclin E-Cdk2 pathway, *Rbl2* (*p107*) and *Lats2* were identified as direct targets of miRNA-290 cluster (Wang *et al.*, 2008). In consistence with these results, Lichner and colleagues have shown that miR-290 cluster regulate not only G1/S but also G2/M transition (Lichner *et al.*, 2011). Further, this study has identified Fbx15 and Wee1 as targets of miR-290 cluster. Fbx15 interacts with dynactin-1 and regulates the cell entry into S phase, while Wee1 inactivates Cdk1 protein and regulate G2/M transition (Tominaga *et al.*, 2006; Zhang *et al.*, 2007). The function of miRNA-290 cluster in cell cycle regulation is depicted in **Figure. 4.1**.

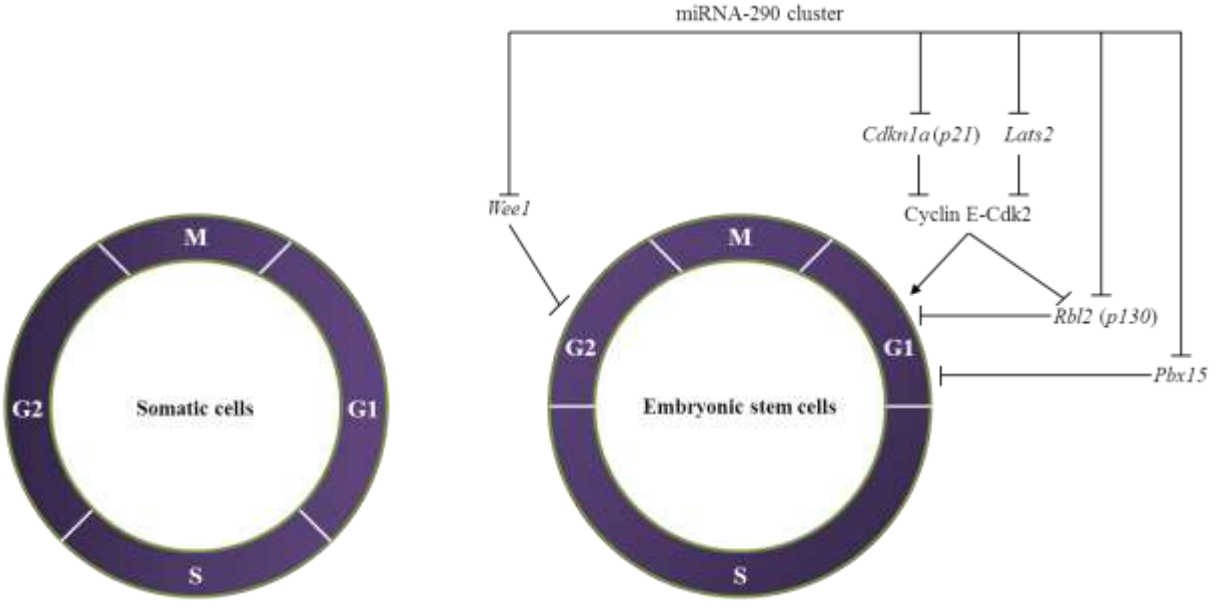

Figure 4.1. Cell cycle of somatic cells and ESCs. ESCs display unique cell cycle lacking fully formed gap phases G1 and G2. Moreover, most of the time (50-60%) is dedicated for S phase in ESCs. Members of miRNA-290 cluster repress the expression of *Cdkn1a* and *Lats2* to allow Cyclin E-Cdk2 mediated G1-S transition. Similarly, *Rbl2*, *Pbx15* and *Wee1* are inhibited by miR-290 members for G1-S and G2-M progression.

Although proliferation defects seen in *Dicer*$^{-/-}$ ESCs could be partially rescued by miR-290 cluster, it was not sufficient to overcome differentiation defects (Sinkkonen *et al.*, 2008; Wang *et al.*, 2008). In line with these results, two research groups have shown that miRNA depletion results in inactivation of *de novo* methyltransferases (Dnmts) (Benetti *et al.*, 2008; Sinkkonen *et al.*, 2008). Due to the inactivation of DNA methylation machinery, core pluripotent transcription factors such as *Oct4*, *Sox2* and *Nanog* showed persistent expression during differentiation and cells retained the pluripotent state (Benetti *et al.*, 2008; Sinkkonen *et al.*, 2008). However, these results cannot rule out that other miRNAs depleted in *Dicer*$^{-/-}$ and *Dgcr8*$^{-/-}$ cells are necessary for differentiation. This hypothesis is strengthened by the observation that the let-7 family members are highly expressed in ESCs during differentiation (Viswanathan *et al.*, 2008).

In the present study (Zovoilis *et al.*, 2009), we showed that ESC-specific miRNAs regulate stemness by suppression of genes involved in differentiation pathway. By employing gain-of-function and loss-of-function experiments in ESCs, for the first time we have shown that members of miRNA-290 family are sufficient to inhibit differentiation towards mesoderm and germ cells. We identified *Dkk1*, a well-known antagonist of Wnt signaling

pathway as a direct downstream target of miRNAs of 290 cluster. In contrast to these observations, activation of Wnt signaling pathway was mostly correlated with activation of mesoderm formation (ten Berge *et al.*, 2008). Since our knowledge about Wnt action in ESCs remains incomplete it is difficult to explain this discrepancy. In accordance to our study, Lichner *et al.*(2011) have shown that stable overexpression of miRNA-290 cluster in ESCs is sufficient to suppress expression of the early differentiation markers of all three germ layers. However, in our study we were not able to show any influence on differentiation towards endoderm and ectoderm lineage. This discrepancy most probably comes from technical limitation of transient transfections used in our study. In light of above findings, many research groups have tried to test the influence of miRNA-290 family on the reprogramming process. However, members of miRNA-290 cluster alone were not sufficient to reprogram somatic cells, but in combination with *Oct4*, *Sox2* and *Klf4*, the efficiency of reprogramming was shown to be increased ten times (Judson *et al.*, 2009). Since *c-Myc* is one of the target genes of Wnt signaling, it is obvious that adding miRNA-290 cluster to the reprogramming cocktail can activate *c-Myc* expression *via* Wnt pathway **(Fig. 4.2)**. Indeed our results could show that overexpression of miR-290 cluster leads to the activation of *c-Myc* (Zovoilis *et al.*, 2009). Collectively, these results strongly support the indispensable function of miRNAs in pluripotent stem cells as well as in normal animal development.

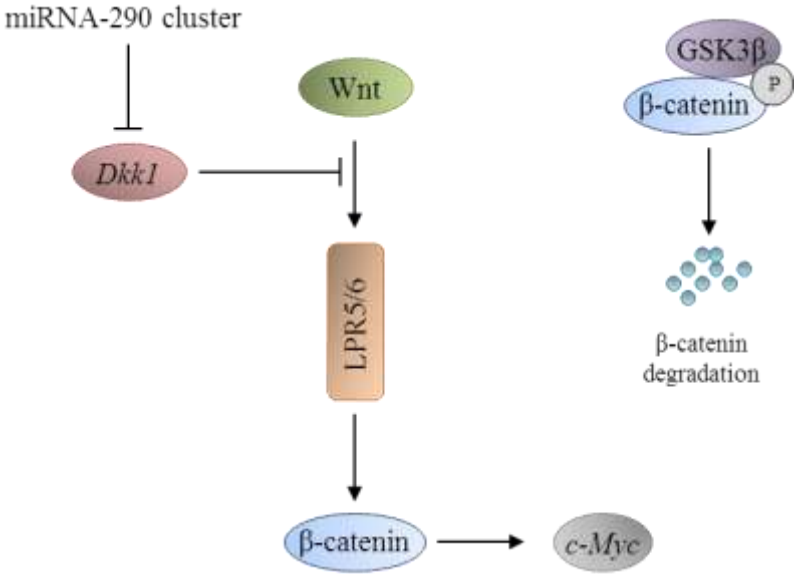

Figure 4.2. Schematic representation of the role of miRNA-290 cluster in Wnt signaling pathway. Suppression of *Dkk1* by miRNA-290 cluster enables binding of Wnts to the LPR5/6 receptor and prevents phosphorylation of β-catenin by GSK3β. Next, the stabilized β-catenin translocates into the nucleus and activates the transcription of *c-Myc*.

4.2. Identification of novel pluripotency-related miRNAs

In order to identify novel miRNAs which can play a crucial role in pluripotent stem cells, we compared miRNA expression profiles between pluripotent ESCs and embryonic fibroblasts (MEFs). This analysis led us to identify miR-130b, -124, -363-3p, and -135b as novel miRNAs expressed specifically in ESCs but not in MEFs **(Fig. 4.3A)**. We observed a specific expression of miR-290 family members in ESCs **(Fig. 4.3A)**, further validating our microarray analysis. We analyzed the expression of these novel miRNAs in several adult mouse tissues as well as in ESCs and found that miR-135b and -363 are preferentially expressed in ESCs **(Fig. 4.3B),** whereas miR-124 and -130b are found to be ubiquitously expressed **(Fig. 4.3C)**, thus excluding them as pluripotent cell-specific miRNAs. To confirm the specific expression of miR-135b and -363 in pluripotent cells, we analyzed their expression in various pluripotent stem cell types and found them to be highly expressed in all analyzed cell types **(Fig. 4.4A)**. Recently, several miRNAs were shown to play a major role during the reprogramming of somatic cell towards induced pluripotent stem cells (iPSCs) (Anokye-Danso *et al.*, 2011; Kuo and Ying, 2012). To analyze the expression profile of novel miR-135b and -363 during reprogramming, we checked their expression during the time course of iPSCs generation. This analysis indicated that miR-135b and -363 start expressing at day7 of reprogramming along with the expression of pluripotency-related and mesenchymal-to-epithilial-transition inducing miRNAs, miR-294 and -200b, respectively **(Fig. 4.4B).**

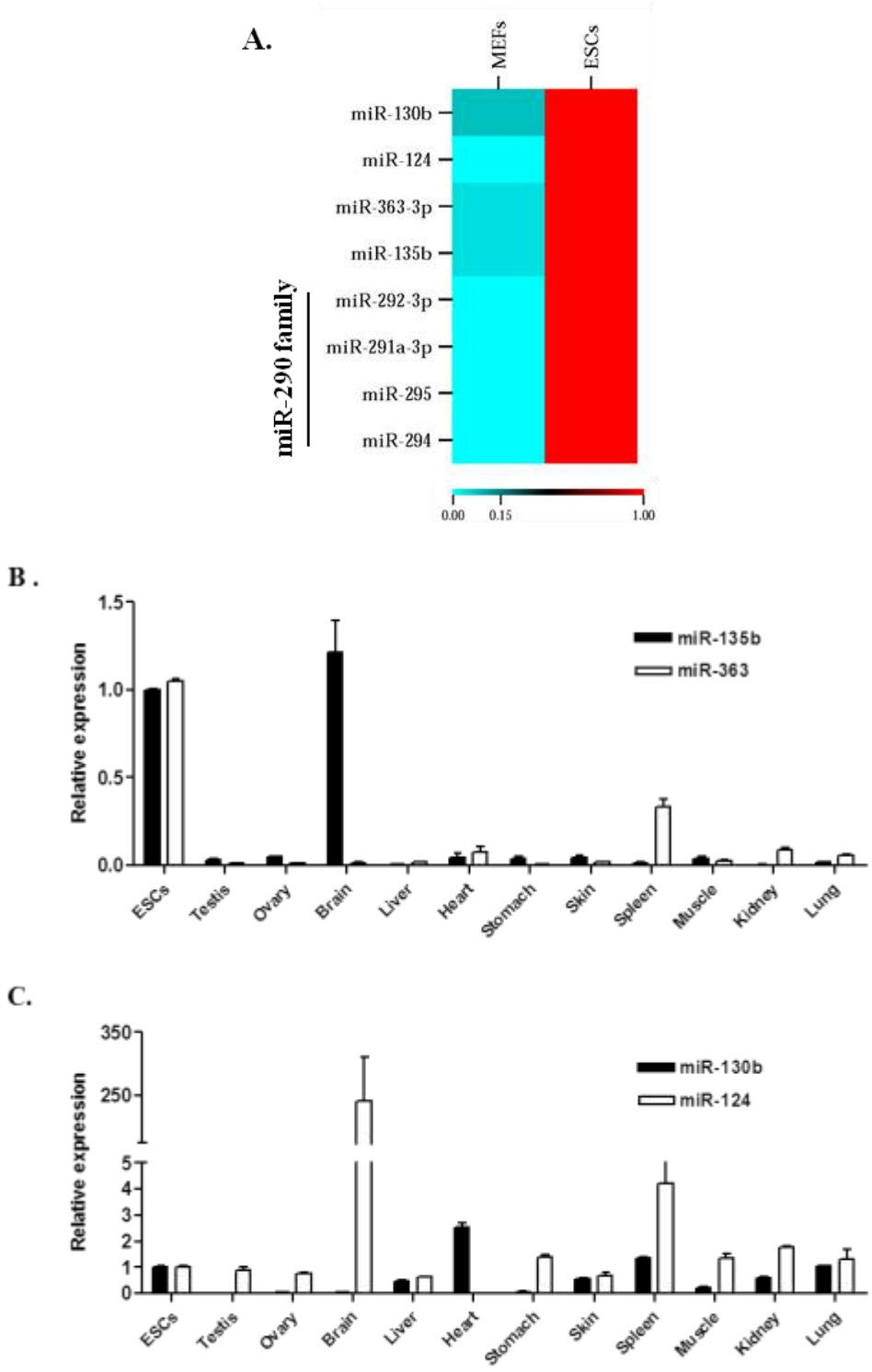

Figure 4.3. Identification and characterization of novel pluripotent cell-specific miRNAs. (A) Heat map representation of miRNA microarray data. The scale denotes an approximation of extent of miRNA relative upregulation in red and downregulation in cyan. Bar graph representation of qRT-PCR data showing the expression of miR-135b and miR-363 **(B)** as well as miRNA-130b and -124 **(C)** in various adult mouse tissues.

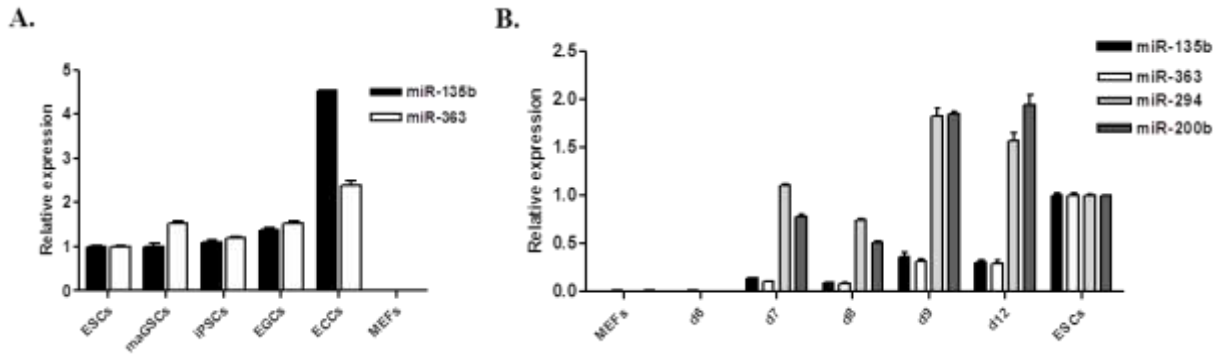

Figure 4.4 miR-135b and miR-363 are expressed in all pluripotent cell types. (A) Bar graph representation of qRT-PCR data of miR-135b and miR-363 in different pluripotent stem cell lines and in MEFs, a somatic cell type. **(B)** Bar graph showing the expression levels of miR-135b and miR-363 during somatic cell reprogramming time course.

4.2.1. miRNA-135b and -363 are transcriptional targets of pluripotency-related factors

The core pluripotency transcription factor network composed of Oct4 (O), Sox2 (S) and Nanog (N) regulates the expression of several pluripotency-related genes to maintain the pluripotency (Loh *et al.*, 2006; Chen *et al.*, 2008; Marson *et al.*, 2008). The transcriptional regulation by these core transcription factors thus indicates a gene as important for pluripotency maintenance. To identify whether miR-135b and -363 are transcriptionally regulated by the pluripotency network, we analyzed the publicly available chromatin immunoprecipitation-sequencing (ChIP-Seq) data of OSN binding sites across the mouse genome (Marson *et al.*, 2008). This analysis indicated that OSN bind ~4kb proximal to miR-135b and adjacent to -363 transcriptional start sites **(Fig. 4.5A)**. To validate these observations, we performed ChIP using OSN antibodies on the chromatin prepared from ESCs and found a specific binding of OSN to the putative promoter regions of miR-135b and -363, albeit at various levels **(Fig. 4.5B and 4.5C)**

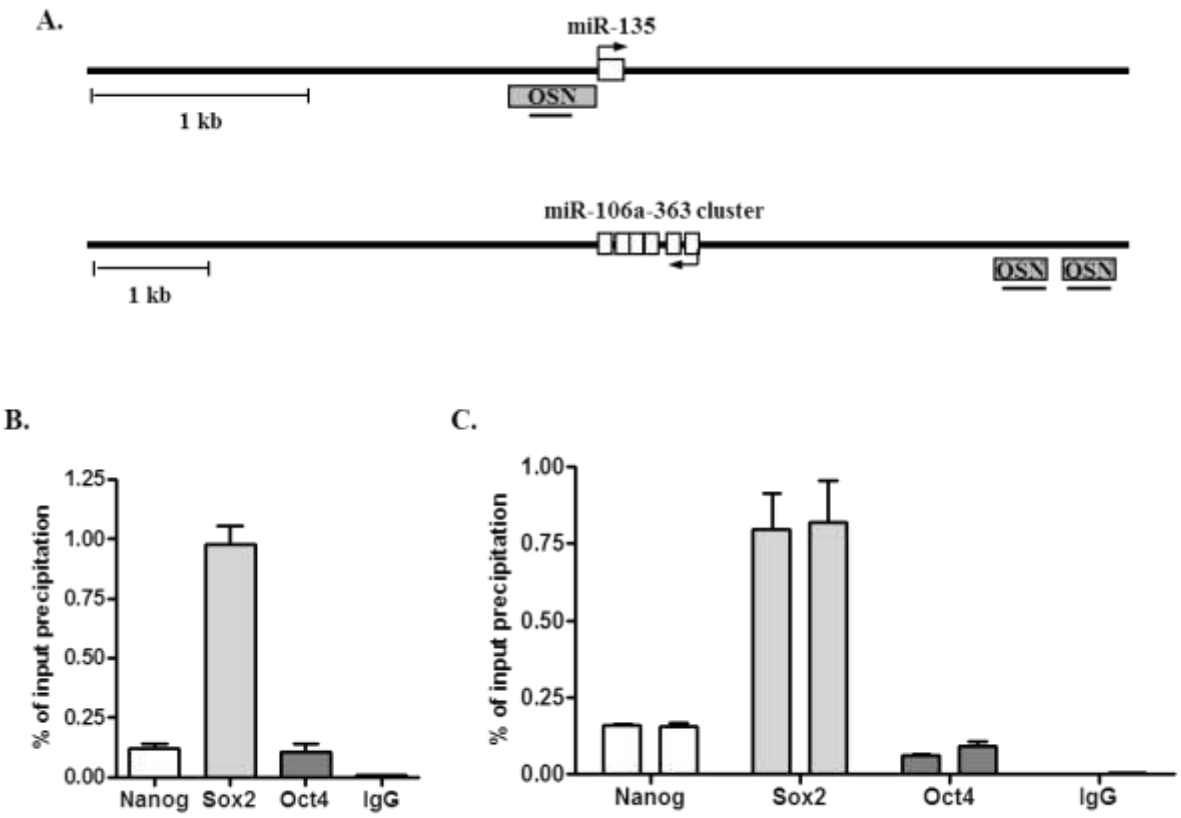

Figure 4.5. **miR-135b and -363 are transcriptional targets of core pluripotency transcription factors. (A)** The genomic architecture of miR-135b and miR-363 clusters along with the reported Oct4 (O), Sox2 (S) and Nanog (N) binding sites (Marson et al., 2008). The region analyzed for OSN binding using qRT-PCR is indicated as thick bar. Bar graph representation of OSN enrichment at the putative promoter regions of miR-135b **(B)** and miR-363 **(C).** The ChIP with IgG served as a negative control.

4.2.2. Identification of miR-135b and -363 target genes

In order to identify mRNA targets of miR-135b and -363, we used miRNA body map software to predict the targets of these two novel pluripotent cell-specific miRNAs. This analysis led us to identify cell cycle regulation and tumor suppressor genes as targets of miR-135b **(Fig. 4.6A)**. Similarly, miR-363 was predicted to target cell cycle regulators and differentiation-related genes **(Fig. 4.6B)**. To experimentally validate these putative targets, we constructed luciferase reporters using the 3′-UTRs of two selected targets for each miRNA. ESCs transfected with cell cycle negative regulator (*Ccng2*), a miR-135b target, reporter construct and miR-135b-mimic showed ~50% reduction in the luciferase reporter activity **(Fig. 4.6C)**. However, tumor suppressor gene *Rbl1*, another target of miR-135, showed no significant difference in luciferase reporter activity **(Fig. 4.6C)**. Similarly, transfection of ESCs with cardiomyocyte differentiation factor (*Nox4*) reporter construct and

miR-363-mimic showed ~30% reduction in luciferase activity **(Fig. 4.6D),** whereas neurogenesis inducer (*Myo1b*) showed no difference **(Fig. 4.6D)**. Based on these results, we selected Ccng2 and Nox4 for further studies. Interestingly, we observed an inverse correlation in expression between miRNAs and their targets in undifferentiated ESCs as well as during induced differentiation **(Fig. 4.6E, F)**. Further experiments aimed at understanding the function of these novel miRNAs and their targets during induced differentiation will uncover their role in pluripotency.

A.

No.	Target Gene	Known/predicted Function
1	Ccng2 (Cyclin G2)	Negative cell cycle regulation
2	*Cdca7l* (Cell division cycle associated 7-like)	Role in apoptosis
3	*Exo1* (Exonuclease 1)	DNA mismatch repair
4	*Hoxa10a* (HomeoboxA10)	Developmental processes
5	*Rad50* (Rad50 homolog)	Double strand breaks repair
6	*Rbl1* (Retinoblastoma like-1)	Tumor suppressor gene
7	*Sash* (Sam and Sh3 domain containing 1)	Tumor suppressor gene
8	*Zmat3* (Zinc finger, matrin-type 3)	Tumor suppressor gene

B.

No.	Target Gene	Known/predicted Function
1	*Nox4* (NADPH oxidase 4)	ESCs differentiation into smooth muscle cells
2	*Myo1b* (Myosin 1b)	Neuronal development
3	*Btg2* (Btg family, member 2)	Antiproliferative, p53 dependent component of DNA damage cellular response pathway
4	*Cdkn1c* (Cyclin-dependent kinase inhibitor 1C)	Inhibitor of several G1 cyclin/cdk complexes
5	*Hnf1b* (HNF1 homeobox B)	Differentiation towards visceral endoderm

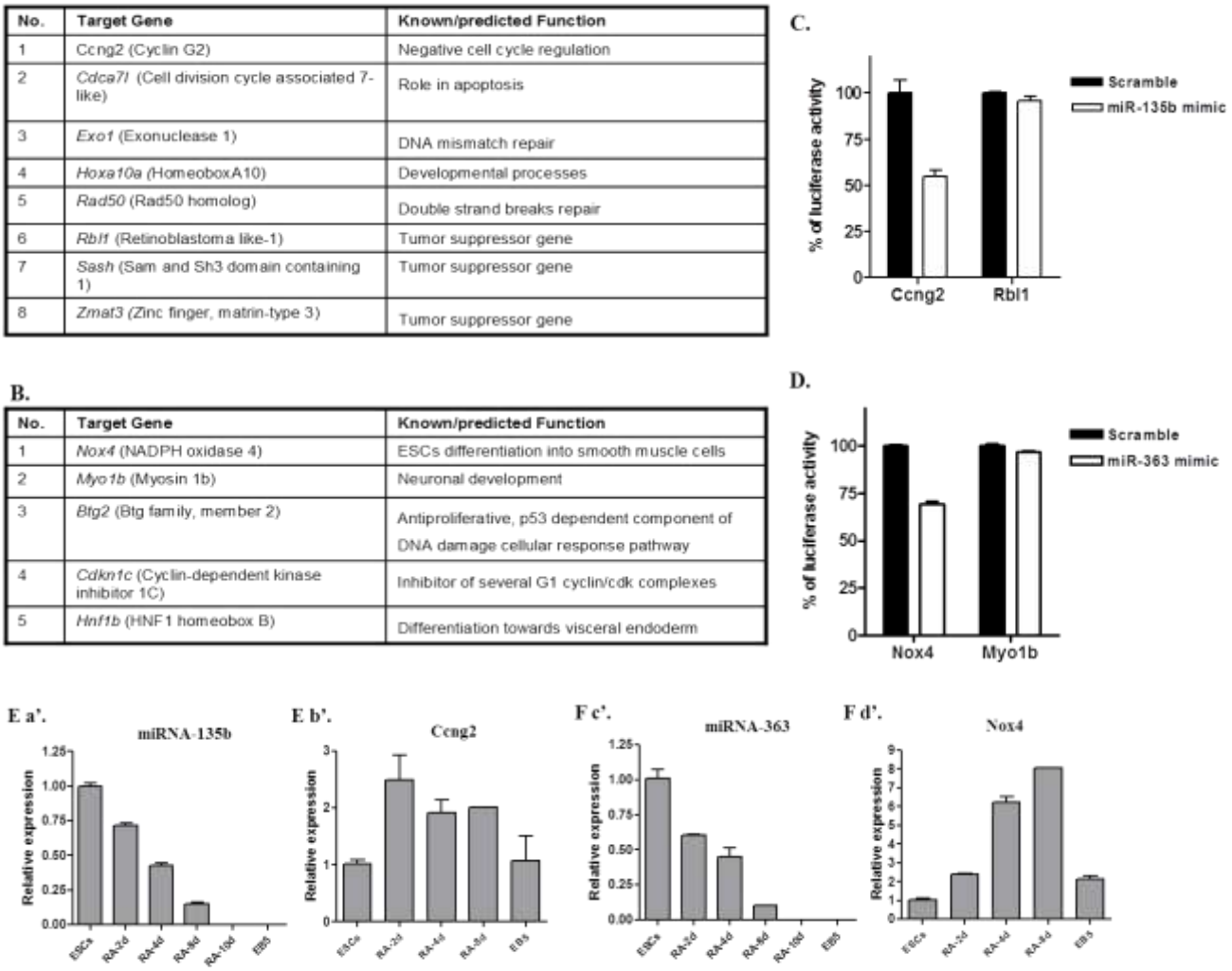

Figure 4.6. Identification and validation of putative target genes of miR-135b and -363. List of predicted target genes of miR-135b **(A)** and miR-363 **(B)**. Bar graph showing the luciferase reporter activities of miR-135b targets *Ccng2* and *Rbl1* **(C)**, and miR-363 targets *Nox4* and *Myo1b* **(D). (E)** Bar graph representation of qRT-PCR data showing the expression of miR-135b **(Ea')** and its target *Ccng2* **(Eb')** as well as miR-363 **(Fc')** and *Nox4* **(Fd')** during the time course of retinoic acid (RA) induced differentiation of ESCs.

4.3. miRNAs in somatic cell reprogramming

Generation of iPSCs by Takahashi and Yamanaka in 2006 opened up a new era in pluripotent stem cell research (Takahashi and Yamanaka, 2006). On the one hand iPSCs can overcome ethical issues associated with human ESCs and on the other hand they make it possible to generate patient specific stem cells and their use in cell replacement therapy by avoiding immune response. miRNAs are one of the factors which were shown to significantly enhance the efficiency of reprogramming process (Gregory *et al.*, 2008; Li *et al.*, 2011; Lin *et al.*, 2011). The members of miR-290 cluster, miR-302, miR-17~92, miR-106b~25, miR-106a~363 clusters as well as miR-200 cluster and miR-205 were reported to enhance the reprogramming process (Gregory *et al.*, 2008; Li *et al.*, 2011; Lin *et al.*, 2011). Interestingly, majority of these miRNAs were proposed to regulate mesenchymal-to-epithelial transition (MET). Generation of iPSCs requires suppression of mesenchymal program and activation of epithelial program in somatic cells which is highly facilitated by miRNAs **(Fig. 4.7)** (Li *et al.*, 2010).

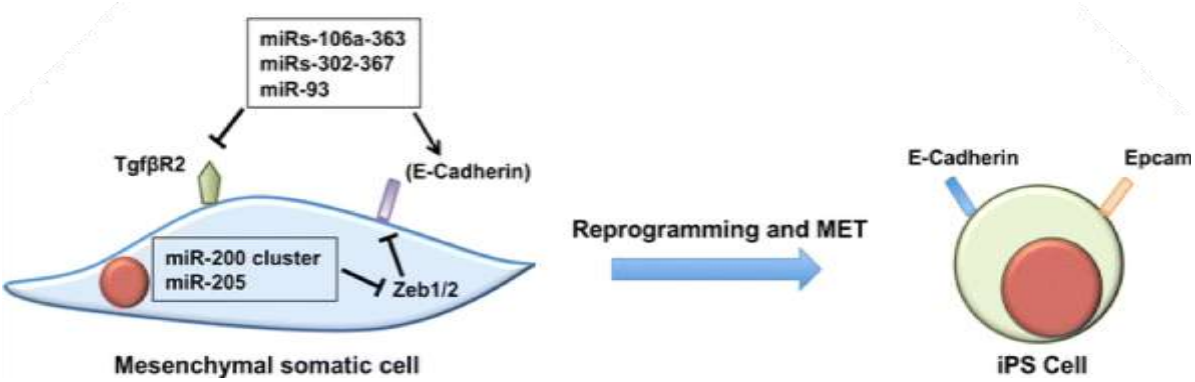

Figure 4.7. Schematic representation of miRNAs functions in the process of mesenchymal-to-epithelial transition. The members of miR-106~363, miR-302 cluster and miR-93 (member of miR-106b~25 cluster) repress *Tgfβ* receptor and inhibit anti-epithelial stimuli. Further, members of miR-106~363 and miR-302 clusters facilitate the expression of epithelial marker *E-Cadherin*. In addition, member of miR-200 cluster together with miR-205 repress the E-Cadherin antagonists *Zeb1/2* (figure adopted from Pfaff *et al.*, 2012).

4.4. Function of miRNAs in gametogenesis

In sexually reproducing organisms, gametes are the only cells capable of transmitting genetic information to the next generation. Many transcriptional as well as posttranscriptional mechanisms have been reported to regulate the process of gametogenesis (Pangas and Rajkovic, 2006; Bettegowda and Wilkinson, 2010). One of the recently discovered mechanisms governing gametogenesis includes miRNAs (Tang *et al.*, 2007; Meng *et al.*,

2011). By taking advantage of conditional knockout mouse models, many research groups have proven the importance of miRNAs in gametogenesis. The loss of total miRNA caused by depletion of Drosha or Dicer specifically in PGCs or spermatogonia resulted in male infertility (Hayashi *et al.*, 2008; Maatouk *et al.*, 2008; Korhonen *et al.*, 2011; Wu *et al.*, 2012). Similarly, lack of *Dicer* in mouse oocytes resulted in female infertility (Murchison *et al.*, 2007). Surprisingly, depletion of *Dgcr8* in oocytes did not affect female fertility (Suh *et al.*, 2010). Moreover *Dgcr8*$^{-/-}$ oocytes can be fertilized by wild type sperm and the resulting zygotes develop and give rise to healthy pups (Suh *et al.*, 2010). These results are in contradiction with previous reports and led to a suggestion that phenotypes observed in *Dicer*$^{-/-}$ females and zygotes are probably caused by a lack of endogenous small interfering RNAs (siRNAs) rather than miRNAs (Tang *et al.*, 2007; Ma *et al.*, 2010). In line with these assumptions, importance of endogenous siRNA, generated by Dicer, was reported in the regulation of gene expression in mouse oocytes (Tam *et al.*, 2008; Watanabe *et al.*, 2008). Taken together, miRNAs seems to have uneven contribution to gametes formation in male and female.

4.4.1. Functions of miRNAs in the process of spermatogenesis

Spermatogenesis is a process common for all sexually reproducing animals through mitotic proliferation, meiotic divisions and maturation phases to finally give rise to functional sperm (Cooke and Saunders, 2002). Spermatogonial stem cells (SSCs), the unipotent stem cells, assure the source for generation of male gametes. In an attempt to identify miRNAs with a function in spermatogenesis, many research groups have characterized miRNA expression profiles of various germ cell populations or testicular cells isolated form prepubertal and adult animals (Yu *et al.*, 2005; Ro *et al.*, 2007; Yan *et al.*, 2007; Song *et al.*, 2009). In the present study (Smorag *et al.*, 2012), we developed an efficient system for isolation of SSCs, pre-meiotic (PrM) and meiotic cells. Generation of double transgenic mouse model (*Stra8*/EGFP and *Sycp3*/DsRed) led us to isolate pre-meiotic spermatogonia (green) and primary spermatocytes (red) using FACS approach. Comparison of miRNA profiles between SSCs, PrM and meiotic cells led us to identify miRNAs specific for only one, two or all three cell types. Through our study, for the first time, dynamic changes in miRNAs expression during spermatogenesis can be shown. In agreement with recent studies, we identified many miRNAs expressed in a stage-specific manner (Yu *et al.*, 2005; Yan *et al.*, 2007; Song *et al.*, 2009; Buchold *et al.*, 2010; Niu *et al.*, 2011). Our results indicate that

miR-221, 203 and miR-34b-5p play an important role in spermatogenesis regulation by targeting *c-Kit*, *Rbm44* and *Cdk6* genes, respectively. Interestingly $Kit^{W/W}$, $Cdk6^{-/-}$ as well as its downstream target, $Cdk2^{-/-}$ mice are subfertile or infertile (Yoshinaga *et al.*, 1991; Ortega *et al.*, 2003; Malumbres *et al.*, 2004). Our knowledge about miRNA in later stages of spermatogenesis is rather poor and mostly based on analysis of miRNA profiles from whole testis. Till now only miR-122a and miRNA-34c were characterized in spermatids (Yu *et al.*, 2005; Liang *et al.*, 2012). miR-122a has been reported to regulate histone-protamine exchange by targeting transition protein 2 (Tnp2) (Yu *et al.*, 2005). However $Tnp2^{-/-}$ animals were fertile, although they show abnormalities during chromatin condensation in spermatids (Zhao *et al.*, 2001). Similarly, miR-34c has been shown to regulate apoptosis by targeting *Atf1* gene (Liang *et al.*, 2012). Atf1 has been reported to maintain cell viability during embryo development, however its function in germ cells is unknown (Bleckmann *et al.*, 2002). Interestingly, miRNA-34c was also detected in mature sperm and is known to regulate first cleavage division of the mouse zygote after fertilization. (Liu *et al.*, 2011). These results highlighted that sperm contributes more than just a delivery of paternal genome during zygote development. Functions of miRNAs during spermatogenesis and early embryonic development are summarized in **Figure. 4.8.**

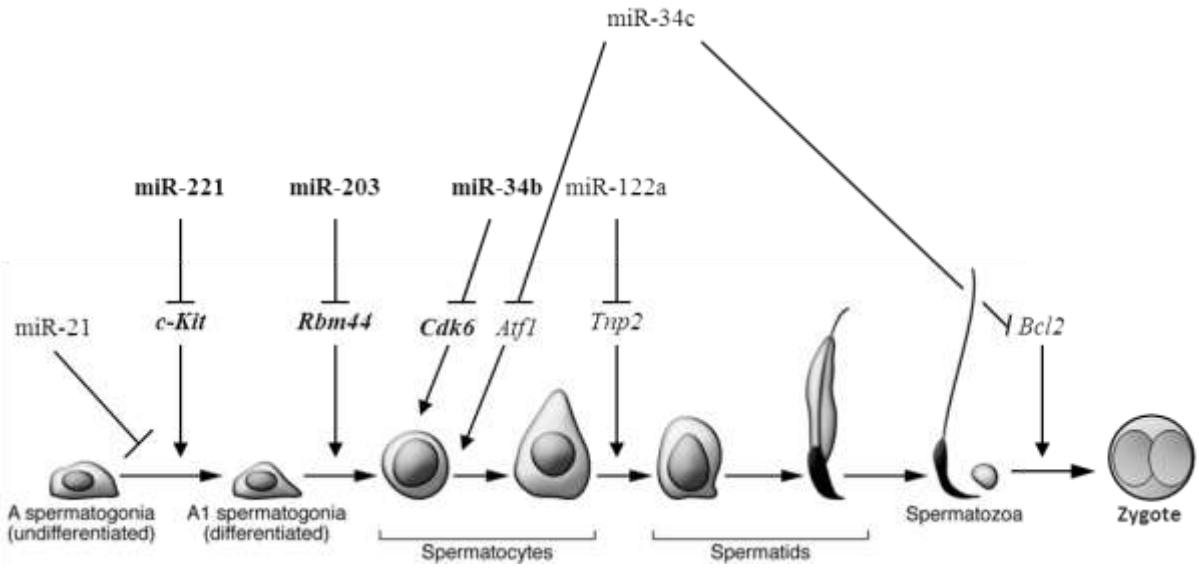

Figure 4.8. Working model for the function of miRNAs during spermatogenesis. Spatiotemporal expression of miRNAs regulates sequential steps of male germ cells maturation. miRNAs along with their corresponding targets, which were identified in the present study, are indicated in bold (Smorag *et al.*, 2012) (figure adopted and modified from Hogarth and Griswold, 2010).

4.5. miRNAs and human diseases

Growing body of evidence suggests that miRNAs play a crucial role in health and disease. According to miRNA-disease data base in the year 2010, there are 174 described diseases associated with dys-regulated expression of miRNAs (www.mir2disease.org) (Jiang *et al.*, 2009). Interestingly, 83 out of 174 diseases are cancer-related diseases. Due to relatively small size of mature miRNA sequences and their corresponding binding motifs in 3′-UTR of target genes, diseases caused by direct mutation in these regions are extremely rare. Till now, there is only one evidence for each case of mutation in miRNA and its target sequence. Point mutation (substitution of A to G) in the seed sequence of miRNA-96 was reported to result in hearing loss (Mencia *et al.*, 2009), while disruption of miRNA-189 binding site caused by substitution of G to A in 3′-UTR of *SLITRK1* was described as responsible for Tourette's syndrome (Abelson *et al.*, 2005). Since a single miRNA can regulate many targets and *vice versa* a single target can be regulated by several miRNAs, phenotype due to mutations occurring in either miRNAs sequence or their binding sites can be obscured by compensation mechanisms. This hypothesis can explain why diseases caused by mutations in the most abundant RNA family are so rare.

4.6. miRNAs as therapeutic drugs

Recent advances in understanding the function of miRNAs have led to use these small RNA molecules as a therapeutic drug. In theory, suppression of particular miRNA linked to disease might eliminate the block against the expression of corresponding therapeutic protein and conversely, delivery of mimic miRNAs can repress the expression of harmful gene. Although this new branch of pharmacology is quite attractive, the multiple functions of a single miRNA must be taken into consideration before any attempts of therapeutic usage. The data presented by Pharmaprojects (online data base of advances in pharmacological technology) summarizes the status of miRNA therapeutic approaches **(Table 4.1.)**. The first ever miRNA-based therapy against Hepatitis C virus (HCV) is already in second phase of clinical trials. Collectively, the current pace of miRNA research holds great promise for future development of novel miRNA-based therapeutics.

Generic name	Originator	Status	Pharmacology	Target	Aplication
SPC-3649	Santaris Pharma	Phase II	miRNA Inhibitor	miRNA-122	Infection, HCV, Hypercholesterolemia
anti-miR-122 oligo, Regulus	Alnylam	Preclinical	miRNA Inhibitor	miRNA-122	Infection, HCV
miRNA inhibitors, Miragen	Miragen Therapeutics	Preclinical	miRNA Inhibitor	miRNA-208a	Heart failure
miRNA mimetics, Miragen	Miragen Therapeutics	Preclinical	miRNA stimulant	Unspecific	Heart failure
prostate cancer miRNAs, Mirna	Mirna Therapeutics	Preclinical	miRNA stimulant	Unspecific	Cancer, prostate
AML miRNA therapy, Mirna	Mirna Therapeutics	Preclinical	miRNA stimulant	Unspecific	Cancer, leukaemia, acute myelogenous
nsclc miRNA therapy, Mirna	Mirna Therapeutics	Preclinical	miRNA stimulant	microRNA let-7a-1	Cancer, lung, non-small cell
herpes virus therapy, Rosetta	Rosetta Genomics	Preclinical	miRNA Inhibitor	Unspecific	Infection, Epstein-Barr virus, herpes simplex virus
miR-34a mimetics, Rosetta	Rosetta Genomics	Preclinical	miRNA stimulant p53 stimulant Apoptosis agonist	miRNA-34a tumor protein p53	Cancer, liver
hepatitis C therapy, Rosseta	Rosetta Genomics	Preclinical	miRNA Inhibitor	Unspecific	Infection, HCV
HIV therapy, Rosseta	Rosetta Genomics	Preclinical	miRNA Inhibitor	Unspecific	Infection, HIV/AIDS

Table 4.1. List of miRNA-based therapeutics which are in clinical and preclinical trials (table adapted and modified from http://www.pharmaprojects.com)

4.7. Identification and characterization of novel meiotic genes

Meiosis is the crucial process occurring during gametogenesis, leading to formation of haploid germ cells. However, our knowledge regarding meiotic processes is limited owing to only a few genes involved in this process. In order to identify new germ cells specific markers including meiotic genes, many research groups have performed transcriptome analysis of purified germ cells (Pang *et al.*, 2003; Yu *et al.*, 2003; Ma *et al.*, 2012). Although these studies were able to identify genes specific for germ cells, no functional characterization of identified genes was done. In the present study, we took advantage of our double transgenic mouse model (*Stra8*/EGFP, *Sycp3*/DsRed) to identify and to characterize

novel meiotic-specific genes. After isolation and characterization of pre-meiotic and meiotic cells from double transgenic mouse testis using FACS, we performed mRNA expression profiling using Agilent Technologies 44K Mouse Whole Genome Microarray. Hierarchical clustering of transcriptome results revealed distant clustering of pre-meiotic (green cells) and meiotic (red cells) cells, while their biological replicates were closely related **(Fig. 4.9A)**. Then, we applied a stringent selection criterion that is 7-fold expression difference between green and red cells to identify meiotic-specific genes. This analysis led us to identify 31 genes as pre-meiotic specific, while 142 genes were identified as meiotic-specific **(Fig. 4.9B.)**. Further, we selected 10 meiotic-specific candidate genes (named as Meio1-10) with unknown function, for further characterization. The selected candidates displayed highest expression in red cells compared to green cells, and have been reported as testis-specific with unknown function in gene expression data base (www.ebi.ac.uk/gxa/). RT-PCR analysis confirmed the expression of nine of them in testis, while Meio4 could not be amplified by RT-PCR **(Fig. 4.10)**. To confirm the testis specific expression of these novel Meio genes, we analyzed their expression in various adult mouse tissues. Seven out of nine Meio genes displayed testis-specific expression **(Fig. 4.11)**. Meio2 and Meio6 showed ubiquitous expression and were excluded from further characterization. Next, we confirmed that none of these testis-specific Meio genes are expressed in $Kit^{W/Wv}$ mouse testis (data not shown) indicating the germ cell-specificity. We checked the expression of these seven Meio genes during different mouse testicular developmental stages *i.e.* 5dpp till 20dpp **(Fig. 4.12.)**. Apart from Meio3, all other Meio genes expression was first detected around day 15 **(Fig. 4.12.)**, which correlates well with the appearance of primary spermatocytes in mouse testicular development. Taken together, these results led us to identify six novel meiosis-specific genes. The results of Meio genes expression analysis are summarized in **Table 4.2**. Further characterization of these selected Meio genes might help us to better understand their function in meiosis as well as to strengthen our knowledge about meiosis regulation.

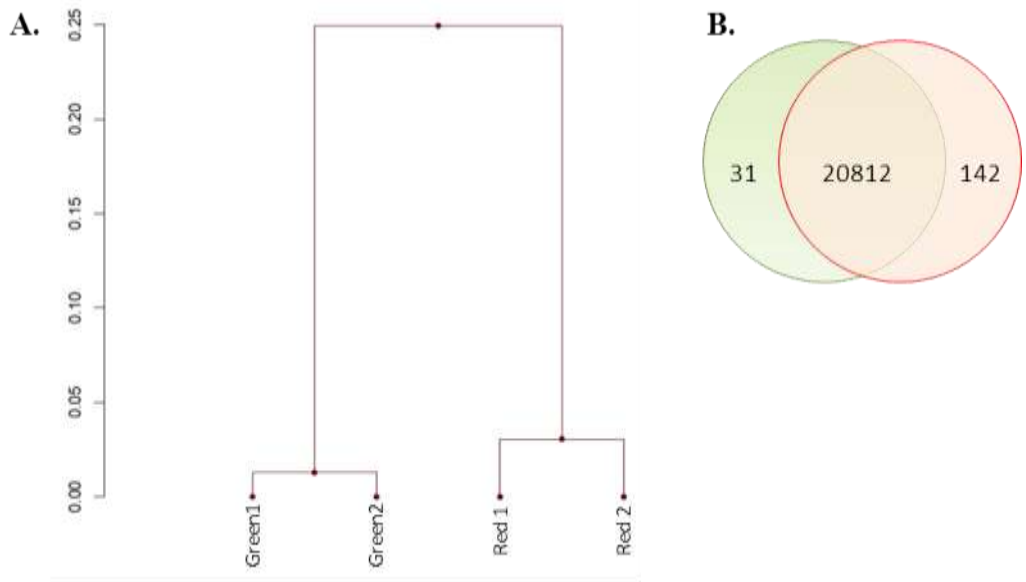

Figure 4.9. Transcriptome analysis of pre-meiotic (green) and meiotic (red) cells isolated from *Stra8*/EGFP and *Sycp3*/DsRed transgenic mouse testis. (A) Hierarchical clustering of transcriptome data. **(B)** Venn diagram illustrating number of green and red-specific genes.

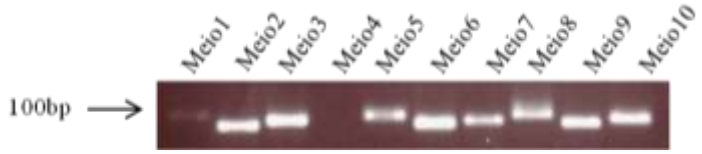

Figure. 4.10. Expression analysis of Meio1-10 genes in mouse testis. RT-PCR analysis for Meio1-10 genes expression in adult mouse testis.

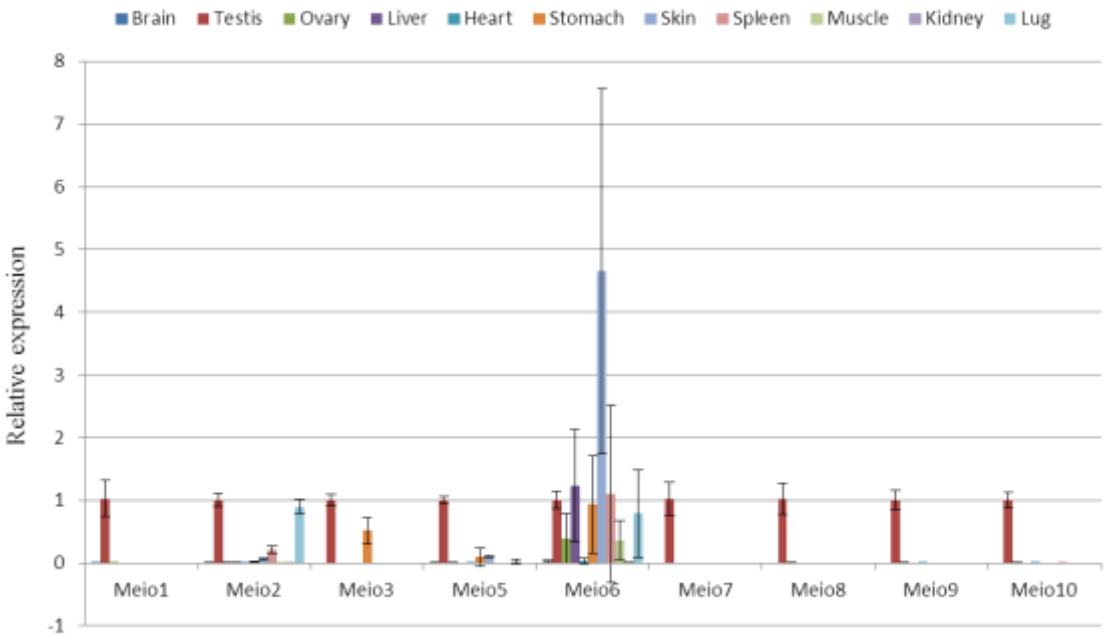

Figure 4.11. Expression analysis of novel Meio genes in different adult mouse tissues. Bar graph showing the expression levels of Meio genes in adult mouse tissues (combined qRT-PCR data of male and female tissues were normalized against testis expression).

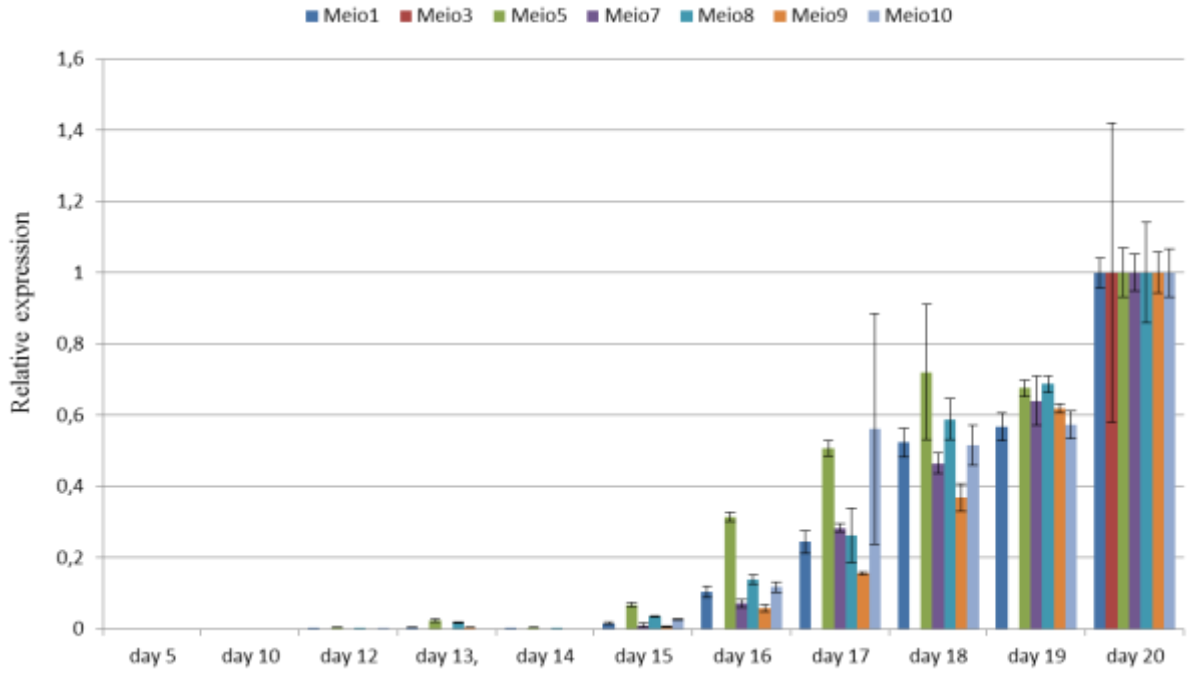

Figure 4.12. Expression analysis of novel Meio genes during mouse testis development. Bar graph showing the expression of Meio genes at various testicular developmental stages.

Name	Symbol	Testis expression	Testis specificity	Meiotic character	Absence in W/Wv
1700017D01Rik	Meio1	✓	✓	✓	✓
Pom121l2	Meio2	✓	X	n/a	n/a
1700017G19Rik	Meio3	✓	✓	X	n/a
4933415F23Rik	Meio4	X	n/a	n/a	n/a
Poteg	Meio5	✓	✓	✓	✓
Abca15	Meio6	✓	X	n/a	n/a
4933409D19Rik	Meio7	✓	✓	✓	✓
Fam170a	Meio8	✓	✓	✓	✓
1700008F21Rik	Meio9	✓	✓	✓	✓
4930403N07Rik	Meio10	✓	✓	✓	✓

Table 4.2 Characterization of novel meiotic-specific genes. The first column displays the official name of Meio genes followed by name given in the present study. V-indicates positive results, X-negative results and N/A –not analyzed.

4.8. Future endeavors and perspectives

In the present study, we identified two novel pluripotent cell-specific miRNAs (miR-135b and miR-363) and their targets (*Ccng2* and *Nox4*, respectively). Further studies using stable overexpression and downregulation of these miRNAs and their role during differentiation of ESCs would shed light on their function in pluripotent cells. Moreover, the functional characterization of their target genes *Ccng2* and *Nox4* during differentiation would help us to understand the differentiation potential of ESCs. It is interesting to note that miR-135b overexpression was reported in several cancer cell types. In line with these observations, our preliminary results also showed an overexpression of miR-135b in one prostate and two colorectal cancer cell lines. Hence, studies on how miR-135b is involved in cell cycle regulation of cancer cells as well as of pluripotent stem cells would help us to dissect the mechanism of cell cycle regulation in these cells. It is also interesting to test whether miR-135b can initiate the tumorgenesis. Additionally, generation of loss-of-function and gain-of-function mouse models will help us to understand their function during development.

The identification of stage-specific miRNAs during the process of spermatogenesis indicates the spatiotemporal control of this process by miRNAs. Interestingly, our *in silico* analysis indicated the presence of these stage-specific miRNAs in human genome, thus highlighting their possible conserved role in spermatogenesis. Further studies aimed at generation of loss-of-function mouse models and analysis of their phenotypes would help us to identify the functional significance of these miRNAs. The knowledge obtained through these mouse models might help us to identify the potential cause of infertility in idiopathic patients and development of possible therapies.

The transcriptome analysis of pre-meiotic and meiotic cells led us to identify several meiosis-specific genes with unknown functions. The identification of protein interaction partners of these novel genes and their functional characterization might help us to understand their physiological function during meiosis. Furthermore, generation of antibodies against protein products of these novel meiotic genes would facilitate cellular, molecular and biochemical studies. The higher expression of these genes in meiotic cells led us to speculate that the overexpression of these genes in pluripotent cells might result in successful progression of meiosis and thereby the generation of haploid gametes. Finally, the generation of knockout and transgenic mouse models for these genes will uncover their function during gametogenesis.

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6 Abbreviations

°C	degree Celsius
Ago-2	Argonaute-2
AP	Alkaline phosphatase
bFGF	Basic fibroblast growth factor
Bmp-4	Bone morphogenetic protein 4
Boll	Boule-like
bp	base pair
Cdk6	Cyclin-dependent kinase 6
cDNA	complementary DNA
ChIP	Chromatin Immunoprecipitation
DAPI	Diamidino-2-phenylindole dihydrochloride
Dazl	Deleted in azoospermia-like
Dgcr8	DiGeorge syndrome critical region gene 8
Dkk1	Dickkopf-related protein 1
DNA	Deoxyribonucleic acid
dpc	day post coitum
dpp	day postpartum
Dppa3	Developmental pluripotency associated 3
DsRed	*Discosoma sp*. Red Fluorescent Protein
dsRNA	double stranded RNA
dTg	double transgenic
ECCs	Embryonic Carcinoma Cells
EGCs	Embryonic Germ Cells
EGF	Epidermal growth factor
EGFP	Enhanced Green Fluorescent Protein
ESCs	Embryonic Stem Cells
FACS	Fluorescence-Activated Cell Sorting
FCS	Fetal Calf Serum
Fgf8	Fibroblast growth factor 8
FL	feeder layer
Fndc3a	Fibronectin type III domain containing 3A

GDNF	Glial cell-derived neurotrophic factor
GO	Gene Ontology
gPSs	germline-derived Pluripotent Stem Cells
GSK3β	Glycogen synthase kinase-3 beta
hnRNP A1	Heterogeneous nuclear ribonucleoprotein A1
HRP	Horseradish peroxidase
ICR	Imprinting Control Regions
Igf2r	Insulin-like growth factor 2 receptor
kb	kilobase pairs
kDa	kilo Dalton
Klf4	Kruppel-like factors 4
KO	Knock-out
KSRP	KH-type splicing regulatory protein
LIF	Leukemia Inhibitory Factor
LPR5/6	Lipoprotein receptor-related 5/6
M	Molarity
MACS	Magnetic Activated Cell Sorting
maGSCs	multipotent adult Germline Stem Cells
MEFs	Mouse embryonic fibroblasts
Meg3	Maternally expressed 3
mGSCs	multipotent Germline Stem Cells
miRISC	microRNA Induced Silencing Complex
miRNA or miR	microRNA
mRNA	messenger RNA
MSCI	Meiotic sex chromosome inactivation
ncRNA	non-coding RNA
NF45	Nuclear factor 45
NF90	Nuclear factor 90
nt	nucleotide
Oct4	Octamer-binding transcription factor 4
OG-2	Oct4/EGFP
ORF	Open reading frame
Ovol1	Ovo-like 1
PAGE	Polyacrylamide Gel Electrophoresis

Pasha	Partner of Drosha
PCR	Polymerase Chain Reaction
PGCs	Primordial Germ Cells
Piwil-2	Piwi-like 2
Plzf	Promyelocytic leukaemia zinc finger
PoM	post-meiotic
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
PrM	pre-meiotic
Prm1	Protamine 1
qRT-PCR	quantitative RT-PCR
RA	Retinoic acid
Rbl2	Retinoblastoma-like protein 2
Rbm44	RNA binding motif protein 44
RDE-4	RNAi Defective-4
RNA Pol	RNA polymerase
RNA	ribonucleic acid
Rnf-17	Ring finger protein 17
Rpl13	Ribosomal protein L13
Rsbn1	Round spermatid basic protein 1
R-Smads	receptor-regulated Smads
RT-PCR	Reverse Transcription PCR
Sall4	Sal-like protein 4
SD	standard deviation
Sdha	Succinate dehydrogenase complex, subunit A
SDS	Sodium Dodecylsulfate
siRNA	small interfering RNA
smiRNA	semiRNA
Snrpn	Small nuclear ribonucleoprotein-associated protein N
Sox2	Sex determining region Y-box 2
SSCs	Spermatogonial stem cells
Stag3	Stromal antigen 3
Stra8	Stimulated by retinoic acid gene 8
stRNA	Small temporal RNA

Sycp3	Synaptonemal complex protein 3
TBST	Tris-Buffered Saline and Tween 20
Tnp2	Transition protein 2
Tox	Thymocyte selection-associated high mobility group box
Tp2	Transition protein 2
TRBP	HIV-1 TAR RNA binding protein
UTR	Untranslated Region
β-Gal	β-Galactosidase
μm	micrometer

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9. List of publications

1. Zovoilis A, **Smorag L**, Pantazi A, Engel W: **Members of the miR-290 cluster modulate in vitro differentiation of mouse embryonic stem cells**. *Differentiation* 2009, **78**(2-3):69-78.
2. Zovoilis A, Pantazi A, **Smorag L**, Opitz L, Riester GS, Wolf M, Zechner U, Holubowska A, Stewart CL, Engel W: **Embryonic stem cell-related miRNAs are involved in differentiation of pluripotent cells originating from the germ line**. *Mol Hum Reprod* 2010, **16**(11):793-803.
3. **Smorag L**, Zheng Y, Nolte J, Zechner U, Engel W, Pantakani DV: **MicroRNA signature in various cell types of mouse spermatogenesis: Evidence for stage-specifically expressed miRNA-221, -203, and -34b-5p mediated spermatogenesis regulation**. *Biol Cell* 2012 Nov;104(11):677-92.

10. List of manuscripts in submission stage

1 Xu X, NakamuraT, **Smorag L**, Dressel R, Fitzner A, Linke M, Nolte J, Zechner U, Engel W, Pantakani DV. **Dppa3 binds to the IG-DMR of the *Dlk1*-Dio3 imprinting cluster and prevents its imprinting loss during the iPS cells generation.** (Manuscipt under review)