Role of stem cell proteins and microRNAs in embryogenesis and germ cell cancer

RONAK EINI, LAMBERT C. J. DORSSERS and LEENDERT H. J. LOOIJENGA*

Erasmus MC, University Medical Center Rotterdam, Department of Pathology, The Netherlands

ABSTRACT Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the blastocyst. These cells can proliferate indefinitely and differentiate into all cell lineages. Germ cell cancers (GCC) mimic embryonic development to a certain extent. The origin of GCC trace back to primordial germ cells/gonocytes in the embryo, which determines their specific characteristics such as totipotency and overall (exceptional) sensitivity to DNA damaging agents. Thus GCC provide a useful model system for the study of gene regulation involved in oncogenesis as well as development. Several reports have demonstrated the role of specific proteins and microRNAs (miRs) in the control of pluripotency and thus early development. miRs are small non-coding RNA molecules that post-transcriptionally regulate gene expression by base-paring to protein encoding mRNAs. miRs are predicted to regulate up to 30% of the protein-encoding genes within the human genome. They are expressed in a tissue-specific and developmentally regulated manner. Aberrant miR expression and its correlation with development and progression of cancers is an emerging field. Important evidences have shown that knock-down by synthetic anti-sense oligonucleotides or re-expression of specific miRs by pre-miR can induce drug sensitivity, leading to increased inhibition of cancer cell growth, invasion, and metastasis. In addition, miRs have been found in body fluids of patients with different types of diseases, including cancer. Therefore, investigation of miRs can shed light on the process of pathogenesis, and may provide biomarkers for diagnosis and prognosis. A subset of miRs is specifically expressed in ES cells and GCC, suggesting their critical role in early embryogenesis and development. In this review we discuss the current view of the biology of embryonic stem cell proteins and miRs in GCC, and their potential clinical impact.

KEY WORDS: *embryonic stem cell, embryogenesis, microRNA, germ cell cancer*

Introduction

During development of a multi-cellular organism processes of growth and differentiation are kept well-controlled, although not always in balance. Post-transcriptional control of gene regulation plays a key role in maintenance of this process by coordinating the functional effect of selected genes at specific moments in time and place. In early mammalian development from maturation of the germ cell lineage to initiation of gastrulation, the role of post-transcriptional regulation is particularly apparent since the rapid growth and the subsequent switch from unspecialized cells into specific cell types requires control on mRNA localization, stability and translation which all provide fundamental ways of gene regulation (Suh *et al.*, 2004).

The first cell fate decision is initiated by cell polarization at the 8- to 16-cell stage, where inner and outer cells are differentiated from each other. Outer cells adopt the trophoblast lineage, whereas the inner cell population give rise to the inner cell mass (ICM, the collection of cells that eventually will generate the fetus) forming the embryonic tissues and the primitive endoderm. The process of gastrulation converts the epiblast into the three definitive germ layers of the embryo, as well as generating the extra-embryonic tissues. The trophectoderm represents the extra-embryonic trophoblast lineage which participates in the formation of visceral yolk sac and envelops the inner cell mass. The early stem cells within the ICM begin to proliferate rapidly, ensuring that stocks of unspecialized cells are established for future differentiation into the three germ layers (Kuckenberg *et al.*, 2011, Lu *et al.*, 2009, Tremblay *et al.*, 2001).

Address correspondence to: L.H.J. Looijenga. Department of Pathology, Erasmus MC-University Medical Center Rotterdam, Josephine Nefkens Institute, Building Be, Room 432, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands. Tel: +31-10-7044-329; Fax: +31-10-7044-365. E-mail: l.looijenga@erasmusmc.nl

In fact, from the fully grown oocyte stage until the moment of Zygotic Genome Activation (ZGA), the genome is transcriptionally silent (Suh *et al.*, 2004). Therefore, all mRNA functionality regulation must occur post-transcriptionally (Lu *et al.*, 2009). One group of post-transcriptional regulators are miRs. These small non-coding RNAs range in size from 18 to 32 nucleotides in length and have emerged in the past decades as major players in posttranscriptional regulation in many multi-cellular organisms (Alvarez-Garcia and Miska, 2005). Hundreds of miRs have been identified in worms, flies, fish, frogs, mammals and flowering plants using molecular cloning and bioinformatics prediction strategies (Lim *et al.*, 2003, Reinhart *et al.*, 2002, Watanabe *et al.*, 2005) The first two miRs, lin-4 and let-7, were both initially identified based on their mutant phenotypes in forward genetic screens in the nematode *Caenorhabditis elegans* (Lee *et al.*, 1993, Reinhart *et al.*, 2000). Mutations causing loss of function in either lin-4 and let-7 result in defective development but at different developmental stages (Wienholds and Plasterk, 2005). After this observation, it has been reported that proper embryonic development, indeed requires a well-organised temporal and spatial expression of miR besides expression of cell-type specific genes (Chen, 2005, Karp and Ambros, 2005, Mineno *et al.*, 2006). In general, it can be concluded that miRs are involved in the regulation of all different processes, including cell division, cell differentiation and cell death. Therefore, based on the fact that miRs interfere with the translation of mRNAs or proteins, it was predicted that aberrant miR expression might be found in cancer (Alvarez-Garcia and Miska, 2005, Wienholds and Plasterk, 2005).

miRNA biogenesis and mechanism

The miRs are transcribed as long RNA precursors (pri-miR) that contain a stem-loop structure of about 80 bases. These pri-miRs are processed in the nucleus by the RNase III enzyme Drosha and DGCR8/Pasha, which form the microprocessor complex and process long pri- miRs into short hairpins called precursor miRs (pre-miR). The pre-miRs are exported from the nucleus by Exportin-5 (Landthaler *et al.*, 2004, Lee *et al.*, 2003, Lund *et al.*, 2004). In the cytoplasm, another RNase III enzyme, Dicer, cuts the pre-miR to generate the mature miR as part of a short RNA duplex. The RNA is subsequently unwound by a helicase activity and incorporated into a RNA-induced silencing complex (RISC). At this stage, miR can mediate down-regulation of target gene activity by two modes: translational inhibition or target mRNA cleavage. The choice is made based on the degree of complimentarity between the miR and target gene in combination with an Argonaute family protein. Near-perfect complementarity results in cleavage, followed by general RNA degradation of the targets, whereas partial complementarity causes translational inhibition (Wienholds and Plasterk, 2005).

Embryonic stem/germ cells and pluripotency

ES cells are capable of indefinite self-renewal as well as generating all cell-types within the body (pluripotency). These capacities of ES cells require unique transcriptional and cell cycle regulations (Jaenisch and Young, 2008). Gene expression analysis in the early embryo has led to the identification of several factors that may be involved in the regulation of early developmental events. These include genes encoding growth factors, their receptors and numerous transcription factors, among which pluripotency factors seem to play major role (Ovitt and Scholer, 1998). In this review, we will discuss predominantly three master pluripotency factors: OCT3/4, NANOG and SOX2. They function in combinatorial complexes to regulate the pathways involved in ES cell pluripotency and cellular differentiation: OCT3/4 (also known as POU5F1) is a POU-domain, octamer binding transcription factor, expressed in both mouse and human ES cells. OCT3/4 is critical for the maintenance of pluripotency in ES cells and is down-regulated in all differentiated somatic cell types *in vitro* as well as *in vivo* (Pesce and Scholer, 2001). Oct3/4 expression starts at the morula stage and becomes restricted at first to the inner cell mass during blastocyst formation, subsequently to the epiblast during gastrulation and finally to primordial germ cells (PGC) (Rosner *et al.*, 1990, Scholer *et al.*, 1989). PGC lacking Oct3/4 expression has been shown to undergo apoptosis rather than differentiation (Boiani *et al.*, 2004).

However, OCT3/4 is not the only key gene regulating ES cell and germ cell pluripotency. The homeodomain protein Nanog is also required for the maintenance of pluripotency in the inner cell mass *in vivo* (Mitsui *et al.*, 2003). Nanog plays a critical role in regulating the cell fate of the pluripotent epiblast and preventing differentiation to primitive endoderm. In addition, down-regulation of Nanog can induce both mouse and human ES cell differentiation to extra-embryonic lineages (Pan and Thomson, 2007). Nanog mRNA is also enriched in pluripotent germ cells. Similarly, Nanog deficiency in PGC triggers apoptosis but not cell differentiation (Yamaguchi *et al.*, 2009). Another key pluripotency regulating gene is SOX2. The SOX2 expression factor is a member of the SRY-related HMG box transcription factor family. SOX2 is expressed in pluripotent cells and multi-potent embryonic and extra-embryonic cells. Sox2 cooperates with Oct3/4 to activate the expression of a number of genes that regulate pluripotency including Nanog (Masui *et al.*, 2007). This appears essential for the derivation of induced pluripotent stem cells from primary human fibroblasts (Huangfu *et al.*, 2008). Interestingly, priming by Nanog results in improved capacity to generate iPS cells.

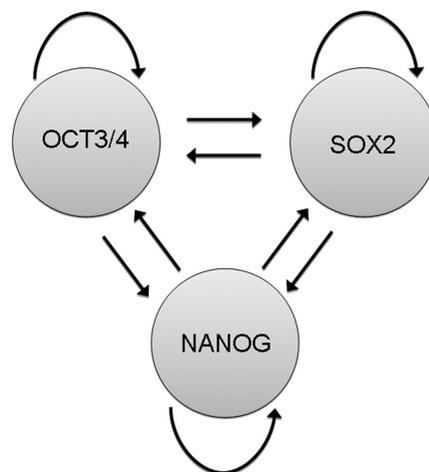We demonstrated that SOX2 is not present in human germ

Fig. 1. Schematic interconnections between OCT3/4, SOX2 and NANOG. *The core ES cell regulators form intrinsic self-regulatory and feed-back loops at the transcriptional and translational level to insure high expression levels and self-stabilizing system for the maintenance of pluripotency in mouse and human ES cells.*

cells, in contrast to mouse (see below), but instead another member of the SOX family, SOX17, is expressed in human germ cells and their malignant counterparts (de Jong *et al.,* 2008). Knock-out studies and expression data of SOX17 in primitive endoderm, fetal human stem cells, and early germ cells indicate a crucial role for this transcription factor in a number of lineages during embryonic and fetal development (Kanai-Azuma *et al.,* 2002). Sox2 has shown to be present in PGC and gonocytes (testis and ovary) of mouse whereas it is absent in human embryonic gonads. (Perrett *et al.,* 2008). An interesting hypothesis to be tested is that the protein complex containing OCT3/4 and SOX17 is predominantly involved in regulation of cell death and OCT3/4 and SOX2 in differentiation.

These three transcription factors interact physically with each other and occupy regulatory regions in many target genes towards the regulation of pluripotency. Oct3/4, Sox2 and Nanog co-occupy several hundred genes, often at overlapping genomic sites suggesting that these pluripotency factors generally do not control their target genes independently, but rather act co-ordinately to maintain the transcriptional program required for pluripotency. Furthermore, Oct3/4, Sox2 and Nanog are also bound to their own promoters forming an inter-connected auto-regulation loop to maintain the ES cell identity (Boyer *et al.,* 2005, Loh *et al.,* 2006). This is schematically illustrated in Fig. 1.

Induced pluripotent stem cells

Regarding the mentioned facts about the critical role of pluripotency factors in ES cells, in 2006, Takahashi and Yamanaka demonstrated that differentiated cells can be converted into so-called induced pluripotent stem cells (iPS cells) by forced expression of four transcription factors Oct3/4, Sox2, Klf4 and c-Myc, which have been termed Yamanaka factors (Takahashi and Yamanaka, 2006). Recently, in a similar approach towards converting somatic cells into pluripotent cells two independent groups (Miyoshi *et al.,* 2011; Anokye-Danso *et al.,* 2011) have reported that mouse and human somatic cells can be reprogrammed to pluripotency by introducing embryonic stem cell miR, miR-302 family and miR-367. It is also reported that introduction of another group of embryonic stem cell specific miR, miR-291-5, enhances production of mouse iPS cells. The miR-291-3p, miR-294 and miR-295, a dominant miR cluster in mouse ES cells with a role in promoting cell proliferation and maintaining pluripotency, increase the efficiency of reprogramming by Oct3/4, Sox2, Nanog and Klf4 (Judson *et al.,* 2009).

These evidences emphasis on the crucial role of ES cell miR in cell cycle, pluripotency, differentiation and early development of a multicellular organism.

Embryonic stem cell microRNAs

So far, a total of 678 miRs within human genome and 472 miRs within the mouse genome have been discovered, but only a subset of these miRs is expressed in ES cells. So far, 31 miRs have been reported to be ES cell specific (Morin *et al.,* 2008, Suh *et al.,* 2004, Wang *et al.,* 2009). In this review, we focus on two ES miR clusters: miR-371-3 cluster (the human counterpart of miR-290-295) and miR302a-d/367 cluster.

The overall roles of miRs in both mouse and human ES cells have been evaluated by analyzing the phenotypes of Dicer and Dgcr8 mouse mutants (Liu *et al.,* 2011, Liu and Tang, 2011). *Dicer*-deficient mouse ES cells exhibit defects in differentiation and G_1 cell-cycle arrest (Kanellopoulou *et al.,* 2005). Moreover, deletion of Dicer causes embryonic lethality (Bernstein *et al.,* 2003). Similarly, *Dgcr8*-deficient mouse ES cells show problems in cell-cycle progression and differentiation, evidenced by failing to silence self-renewal genes, such as Oct3/4, Nanog and Sox2, as well as delayed expression of differentiation markers (Liu *et al.,* 2011). The *Dgcr8* knockout ES cell phenotype differs from *Dicer1* knockout. Both knockouts are defective in differentiation. *Dicer1* knockout ES cells have more severe initial proliferation defect that is overcome over time possibly due to additional genetic events, however, *Dgcr8* knock-out ES cells show a stable and more subtle proliferative defect (Kanellopoulou *et al.,* 2005, Wang *et al.,* 2007). These overall miR-deficient cells showed a relative increase in the number of cells in the G_1 phase of the cell cycle. This finding suggests that miR suppress inhibitors of the G_1-S transition, allowing the rapid transition from mitosis to S phase (Wang *et al.,* 2008). These evidences demonstrate the crucial role of miR in ES cell self-renewal and differentiation. How miR regulates gene expression in ES cells is described recently (Marson *et al.,* 2008), indicating that the key ES cell transcription factors are associated with promoters for miRs that are expressed in ES cells. In fact, miR promoters are occupied by master ES cell transcription factors, including OCT3/4, SOX2 and NANOG. This suggests an important role of ES cell miRs in the maintenance of the pluripotent cell state, as summarized in Fig. 2.

On the other hand, miRs can regulate expression of pluripotency genes by binding to their coding regions. A good example

Fig. 2. Embryonic stem (ES) cell self-renewal and differentiation. *ES cells have the ability to self-renew or differentiate into cells of all three germ layers (endoderm, ectoderm, and mesoderm). In ES cells, OCT3/4, SOX2, and NANOG form a core transcriptional network influencing the stem cell self-renewal machinery. The interaction of downstream miRs and protein coded genes with these master ES cell transcriptional factors and their opposing functions play major role in the properties of ES cells.*
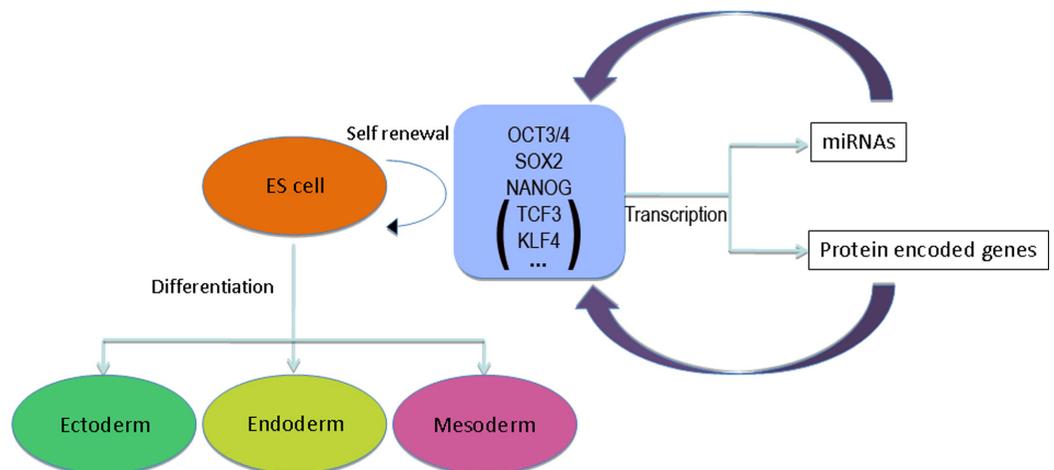
for regulatory circuitry between miRs and pluripotency genes is the one between Lin28 and let-7 miR. Lin-28, a marker of ES cells, also forms a negative feedback loop with the let-7 family miR to control each others levels. Lin-28 regulates expression of let-7 by binding to the precursors and blocking their maturation, whereas in differentiated cells where let-7 levels are increased, let-7 miR targets the Lin-28 mRNA (Liu *et al.*, 2011).

Germ cell tumor biology in summary

Malignant germ cell tumors are the most frequent malignancy in Caucasian males between 20 and 40 years of age, and the first cause of cancer death in this age group with increasing incidence (Huyghe *et al.*, 2007, Shah *et al.*, 2007). Human germ cell tumors are a heterogeneous group of neoplasm occurring in gonads, both testes and ovaries, and in different extra-gonadal sites along the midline of the body and the midline of the brain. The specific characteristics can either be due to the process of tumorigenesis or just a reflection of normal embryonal development, which contributes to the complexity of these tumors. Based on epidemiology, phenotypic characterization, chromosomal constitution and genomic imprinting, the group of testicular germ cell tumors comprises three types mainly: Type I, II and III. In the context of the role of ES cell miRs, predominantly the Type I and II will be discussed. Type I germ cell tumors are the teratomas and yolk sac tumors occurring in newborn and infants. The type II germ cell tumors comprise the seminomatous tumors (seminoma (SE)/dysgerminoma (DG) /germinoma) and nonseminomatous tumor, referred to as the malignant germ cell tumors, i.e., germ cell cancers (GCC). The non-seminomas are subdivided into embryonal carcinoma (EC, undifferentiated stem cell component, similar to ES) and the various derivatives: extra-embryonic lineages (yolk sac tumor (YS) and choriocarcinoma) and along the somatic lineage (teratoma) (Looijenga *et al.*, 2007,

Oosterhuis and Looijenga, 2005). The precursor cells of GCC are known as carcinoma *in situ* (CIS) or intratubular germ cell neoplasia unclassified (IGCNU) of the testis or gonadoblastoma (GB) of the dysgenetic gonad. They originate from an embryonic germ cell, either PGC or gonocyte, blocked in its physiological maturation process (Looijenga *et al.*, 2007, Skakkebaek, 1972). During normal germ cell development genomic imprinting plays a role. Genomic imprinting is the phenomenon that the paternal and maternal sets of chromosomes have different functionality in mammals, due to parental-specific epigenetic modification. This modification is established somewhere between the stage of a PGC and a spermatozoa (male) and an oocyte (female) (McLaren, 2003, Oosterhuis and Looijenga, 2005). The functional difference between the paternal and maternal haploid set of chromosomes comes from so-called imprinted genes, which are expressed from the paternal or maternal allele only, sometimes in a tissue-specific manner (Hajkova *et al.*, 2002). We demonstrated biallelic expression of imprintined genes in testicular GCC, in line with their erased embryonic germ cell origin (van Gurp *et al.*, 1994).

Although the exact mechanistic basis of monoallelic versus biallelic expression of imprinted genes is not solved yet, it is clear that it is related to epigenetic modifications of the genome, including DNA methylation and histone modification (Hajkova *et al.*, 2002). This issue is discussed in detail in Van der Zwan *et al.* (2013). Besides GCC, within the testis there are so-called type III germ cell tumors, known as spermatocytic seminomas (SS). They have shown to have a different cell of origin, i.e., spermatogonia/spermatocyte. These are characterized by a different pattern of chromosomal anomalies, different gene and protein expression, as well as genomic imprinting (Looijenga *et al.*, 2006). Interestingly, these two types of germ cell tumors represent two stages of normal germ cell development: PGC/gonocyte and the spermatogonia/spermatocyte.

OCT3/4 is the most informative diagnostic marker in recognizing CIS/GB, as well as SE/DG and EC (Looijenga *et al.*, 2003). In contrast, SOX2 is expressed in EC, but not the precursor lesions and SE and normal germ cells, while it is more heterogeneous in the differentiated nonseminomatous elements (de Jong *et al.*, 2008). On the other hand, SOX17 is a suitable marker to distinguish SE (positive) from EC (negative) (de Jong *et al.*, 2008, Korkola *et al.*, 2006). Moreover, LIN-28 is expressed in precursor lesions (both CIS and GB), as well as SE/DG, EC and yolk sac tumor (YS). In addition, elevated level of TSPY has been observed in a variety of tumor tissues, including GCC (Oram *et al.*, 2006). Testis-specific- protein Y-encoded (TSPY) is a multi-copy gene mapped to the critical region harbouring the gonadoblastoma locus on the Y chromosome, the only proven oncogenic locus on the male-specific chromosome(Oram *et al.*, 2006). The genetic interaction of TSPY and GB has been clinically established (Page, 1987). In testicular GCC patients as well as in model cell lines, co-expression of TSPY and androgen receptor (AR) is observed whereas such co-expression was not seen in normal testis of human or mouse (Akimoto *et al.*, 2010). In addition, TSPY is suggested to induce an

ES cell like
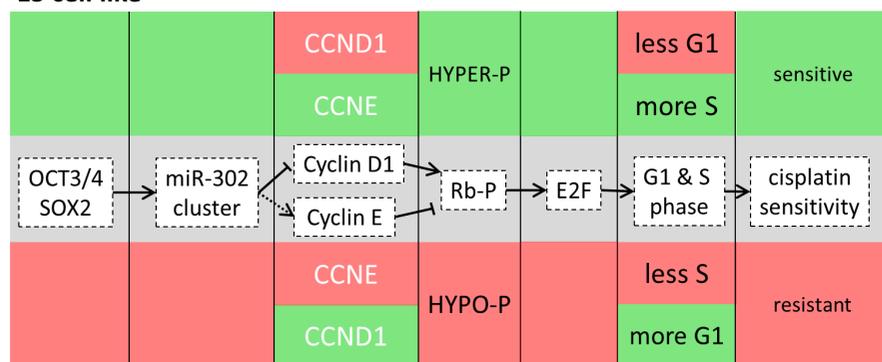

Differentiated cells

Fig. 3. Schematic presentation of G_1-S transition in ES cells and somatic cells. *In ES cells, high expression of OCT3/4 and SOX2 maintains high expression level of miR-302 cluster causing hyper-phosphorylation of Rb via directly suppression of Cyclin D1 directly and activation of Cycin E resulting in high expression of E2F protein. This causes short G_1-S phase by accumulating more cells in S phase and less in G_1. This will lead to sensitivity of ES cells to cisplatin. In differentiated (somatic) cells low expression of main pluripotency factors results in lower expression level of miR-302 cluster leads to hypo-phosphrylation of Rb via up-regulation of cyclin D1 (green) and down-regulation of cyclin E. Rb then inhibits E2F protein. This will cause long G_1-S due to accumulation of more cells in G_1 and less cells in S phase, and consequently result in a more resistant type of differentiated cell to cisplatin. Abbreviations and colors used: Red=low protein/ miR; green=high protein/miR; hyper-P = hyper phosphorylation; Hypo-P = hypophosphorylation.*

anti-androgenic microenvironment, possibly leading to a block in maturation of germ cells. This indicates that co-expression of TSPY and OCT3/4 is informative to detect CIS cells from delayed matured germ cells.

p53 in ES cells and GCC

Since extensive proliferation and differentiation of stem cells can contribute to hyper-proliferative disorders, a coordinated control of stem cell self-renewal and differentiation is fundamental for maintaining tissue and organ homeostasis. p53 appears to contribute to this process by controlling the proliferation, self-renewal and differentiation of both embryonic and adult stem cells. The first observation about a potential role of p53 in ES cells was reported in 1980 (Mora *et al.*, 1980) in which they observed that p53 was highly expressed in primary cell cultures obtained from 12-14 day old mouse embryos but not in cells from 16 day old embryos. In differentiated cells, p53 is an important regulator of cell proliferation. By controlling expression of the p21 gene, which encodes the inhibitor of cyclin-dependent kinases, p53 induces transition from G_1 into S-phase of the cell cycle. In addition, p53 is able to initiate apoptosis by both the extrinsic and intrinsic pathways. In concordance with these activities in differentiated cells, p53 also controls proliferation and cell death in ES cells. Treatment of human ES cells with nutlin, an inhibitor of p53 degradation, leads to the rapid accumulation of p21 and to cell cycle arrest at the G_1/S boundary (reviewed in Solozobova and Blattner, 2011). Interestingly, it is shown that knock-down of p53 in human adult fibroblast has dramatically increased the efficiency of iPS cell generation (Zhao *et al.*, 2008).

The presence of functional p53 in undifferentiated ES cells makes them hypersensitive to UV irradiation, whereas the differentiated cells were resistant to UV treatment. Wild-type ES cells undergoing apoptosis expressed functional p53 whereas ES cells lacking p53 showed enhanced proliferation in both undifferentiated and differentiated state and apoptosis was reduced. Undifferentiated ES cells with a functional p53 protein proliferate slower than p53-/-
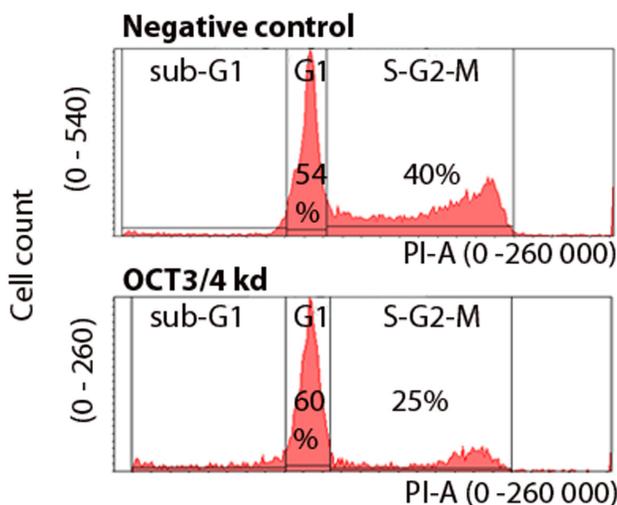

Fig. 4. Result of FACS analysis. NTera-2 cells were subjected to OCT3/4 siRNA and scrambled siRNA (negative control). *As it is indicated in OCT3/4 kd situation, more cells are accumulated in G_1 phase and less cells are in S phase compare to negative control.*

ES cells. This indicates that functional p53 is involved in regulating proliferation of these cells *in vitro* (Sabapathy *et al.*, 1997). This is related amongst others, to induction of pre-mature senescence after oncogenic stress. These observations could explain why genes that are involved in the establishment of oncogene-induced senescence such as p53 are amongst the most tumor suppressor mutated genes in tumor formation. However, GCC are an exception to this rule. They have been shown to express wild-type p53 and mutations in this protein are rare while they proliferate fast and respond to DNA damaging agents, such as cisplatin (in most cases) (Kersemaekers *et al.*, 2002 , Masters and Koberle, 2003).

ES cell division

ES cells have a unique cell cycle characterized by lack of G_0, absence of G_1 checkpoint and subsequently a short G_1/S transition which facilitates quick cell movement through G_1 to S phase allowing the cells to proliferate rapidly leading to rapid growth of the early embryo (He *et al.*, 2009). This specific characteristic of ES cells makes these cells hypersensitive to DNA damaging agents. In recent studies, miRs have shown to play a central role in achieving this unique cell cycle regulation (Hong and Stambrook, 2004, Wang *et al.*, 2009). In somatic cells, where the G_1/S checkpoint exists, the G_1 phase is a gap period between cytokinesis and DNA replication. During the early G_1 phase, upon stimulation of the mitogenic factors, D-type cyclins are expressed. These cyclins are required during G_1 restriction point to activate the Cdk4/6 and the Cdk2 kinases which are expressed throughout cell cycle. The Cdk4/6-Cyclin D complex then phosphorylates proteins of the retinoblastoma (pRB) family. This leads to the partial inhibition of RB and release of its target, the E2F transcription factor. Upon elevation of E2F, the expression of E-type Cyclin increases resulting in activation of Cdk2 which further phosphorylates RB. This event leads to the transcription of genes required for progression through the S phase. This will result in the elongation of G_1 phase and slower proliferation of the resulting somatic cells (Wang *et al.*, 2009, White *et al.*, 2005) (Fig. 3). Aberration in the expression of cell cycle regulatory factors can lead to uncontrolled proliferation which is one of the hallmarks of cancer (Hanahan and Weinberg, 2000). In conclusion, ES cells characterized by a very short G_1 phase and lack of a check point at the G_1/S transition, are very similar to many cancer cells (Wang *et al.*, 2009). In this context, it is shown that ectopic expression of TSPY increases cell proliferation *in vitro* and tumorigenesis *in vivo* (Oram *et al.*, 2006). Expression of TSPY facilitates the transition of the cells through the G_2/M phase of the cell cycle, indirectly up-regulates pro-growth genes and down-regulates apoptosis inducing molecules and growth inhibitory genes, thereby promoting cell proliferation in both *in vitro* and *in vivo*. TSPY harbours a SET/NAP domain represented by the SET oncoprotein and nucleosome assembly protein-1 (NAP-1) respectively. Several evidences have shown that SET/NAP-containing proteins are cell cycle regulators. They regulate the G_2/M transition by modulating cyclin-B-cyclin-dependent kinase 1 (CDK1) activity (Canela *et al.*, 2003).

Potential role of miR-290 and miR-302 clusters in embryonic stem cell division

In a study in which identification of the specific miR and targets involved in the process of rapid transition from mitosis to S phase

was aimed, it was found that introducing specific miR mimics in the *Dgcr8* knockout ES cells, could promote growth and partially rescued the proliferation defect in these cells. In that study, miR-290 cluster which is the most highly expressed miR in mouse ES cells and rapidly down-regulated upon differentiation, was selected (Calabrese *et al.*, 2007). Moreover, in another study, by adding back individual miR into *Dgcr8* knockout ES cells, many miRs, including members of the miR-290 and miR-302 clusters, were found to rescue the prolonged G_1 phenotype by reducing cells accumulation in S phase or G_2-M phase. These data show that ES cell-specific miRs promote ES cell proliferation at least in part by promoting the transition of cells from G_1 to S phase. These miRs are called ES cell-specific cell cycle-regulating miRs or ESCC miRs (Wang *et al.*, 2008).

It has been reported that expression of miR-302a caused decrease in the number of cells in G_1 phase and increased the population of cells in S-phase and G_2/M-phase in HeLa cells transfected with Pre-miR-302a compared to the cells transfected with negative control pre-miR. Therefore, consistent with the previous reports, in pluripotent human ES cells, the majority of cells were in S-phase and as cells progressively differentiate, the cell cycle profile shifts from a short G_1/high S-phase population to a high G_1-phase cell cycle and this shift is mediated through miR-302 decrease of cell cycle regulator level in pluripotent cells (Card *et al.*, 2008). In the same study, similar increase in the population of G_1-phase of NTera2 (an EC cell line of GCC origin) cells which have been differentiated with retinoic acid is detected. Interestingly, in an experiment done in our lab, we observed that accumulation of differentiated NTera2 cells in S phase was reduced when subjected to OCT3/4 siRNA followed by increasing cell population in G_1-phase analyzed by Fluorescence Activated Cell Sorting (FACS) confirming their ES characteristics (Fig. 4). When ES cells differentiate, G_1 phase lengthens and the rate of cell division slows (He *et al.*, 2009). We tested the combined down-regulation of OCT3/4 and SOX2 in NT2,

and observed massive induction of apoptosis in these cells. This also confirms the major role of these core pluripotency factors in proliferation and cell cycle of EC cells.

microRNAs in germ cell cancers

We demonstrated that specific miRs are able to mimic the effect of mutated p53 and thereby bypass pre-mature senescence after oncogenic exposure (Voorhoeve *et al.*, 2006). Using a high throughput unbiased functional screen, a cluster of miR mapped to chromosome 19, known as miR-371-3, was found to be responsible for this phenomenon, subsequently found to be expressed in almost all SE, EC as well as YS. Interestingly, a small number of SE with p53 mutation lacked expression of this set of miRs, and two GCC-derived cell lines with a low or no expression of these miRs showed either a mutated or non-expressed p53 supporting the proposed model. Therefore, miR-371-3 was considered a potential oncogene, able to mimic the effect of mutated p53 by preventing Ras-induced oncogenic senescence in GCC. This process is most likely occurring via suppression of CDK inhibitor LATS2 (Large tumor suppressor) which is the target of miR-371-3 (Voorhoeve *et al.*, 2006).

Based on these findings, a high-throughput screen of 157 miRs using a quantitative RT-PCR based approach in GCC was performed in which only the mature miRs were detected. First, indeed expression of the miR cluster 371-373 was confirmed. Secondly, unsupervised hierarchical clustering showed different expression of the cluster in different groups of tumors. miR-371-3 was highly expressed in SE, EC and YS, but not expressed in teratoma and normal testicular parenchyma (NT) (Fig. 5). In the same study, miR-302a-d cluster were differentially expressed between the clusters of GCC; highly expressed in SE and EC as well as YS and absent in teratoma (Gillis *et al.*, 2007).

Additionally, in an independent study (Novotny *et al.*, 2012) it was demonstrated that the miR-371-3 and miR-302/367 clusters were enriched in CIS samples while they were very low expressed or absent in the normal testis. This indicates that high expression of these miRs is not restricted to the invasive components, but intrinsic to the early pathogenetic stage. In addition, we have shown that no mutation, deletion or amplification was present in the loci of miR-371-3 and miR-302 clusters in the DNA of 242 GCC revealing that no genetic changes affect on the expression levels of these miR clusters (De Boer *et al.,* submitted for publication).

Expression levels of miR-371-3 and miR-302 clusters

We measured expression of the individual members of the miR-371-3 and miR-302 clusters in a series of GCC cell lines and primary GCC (EC, SE and YS, teratoma, normal testis as well as SS). Comparing the patterns of expression, it is of interest that in almost all samples (cell lines and tumors), within the different clusters, the same variant was showing the highest level of expression: miR-372 and miR-302b and miR-302d, respectively (Fig. 6). Since all members of the two clusters are generated from a single primary transcript (see above), this observation can not be explained by differences in transcription, but are likely due to variation in maturation and/or stability of the mature transcript. This needs further investigation.
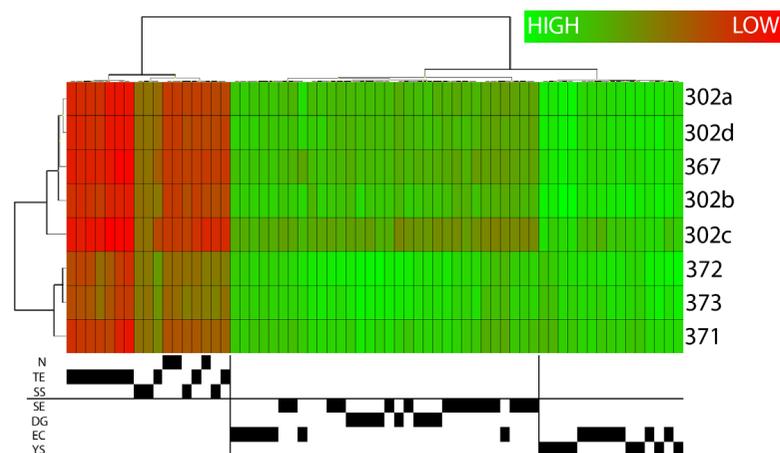

Fig. 5. Results of the unsupervised hierarchial clustering. *miR clusters miR-371-3 and miR-302/367 are differentially expressed between different subtypes of GCC; highly expressed in SE/DG and EC and absent in teratoma (TE), SS and normal testis parenchyma (NT). The numbers of each histological subtype are indicated: (SE $_{n=15}$), (EC $_{n=13}$), (YS $_{n=8}$), (TE $_{n=10}$), (SS $_{n=4}$), (DG $_{n=10}$) and (NT $_{n=3}$). For normalization, all miRs on the plate were used using mean expression of all genes (threshold C40) according to (Mestdagh* et al., *2009). Clustering was performed using Ward's algorithm; both dendrograms were formatted according to euclidean distance. GenEx 5.3.7.332 was used to analyze the data.*

miR-371-3

The miR-371–3 cluster and its murine ortholog, the ES cell-specific cell cycle regulating miR-290–5 cluster, are over-expressed in ES cells and down-regulated upon differentiation. These miRs play a crucial role in the maintenance of ES cell renewal (Stadler *et al.*, 2010, Suh *et al.*, 2004). They promote tumor invasion and metastasis in response to hypoxia (Huang *et al.*, 2008, Voorhoeve *et al.*, 2006). Recently, a direct regulation of the miR-371–3 cluster by Myc is reported (Cairo *et al.*, 2010). This is of interest in the context of iPS cells because c-Myc in combination with other core pluripotency factors was used to generate iPS cells from a differentiated fibroblast (Takahashi and Yamanaka, 2006). Lack of c-MYC in this set up results in a significantly lower efficiency in the ability to generate iPS cells (Nakagawa *et al.*, 2008, Wernig *et al.*, 2008, Zhao *et al.*, 2009). Moreover, as recently found for the murine ortholog miR-294 in ES cells (Melton *et al.*, 2010), it is suggested that Myc is up-regulated by the miR-371–3 cluster. This might be due to the direct binding of the miR to the *MYC* genomic region or inhibition of an intermediate repressor or direct activation through binding. Moreover, miR-290-295 cluster is reported to be highly enriched in the germ cell population of day 6 testis when compared to the somatic cell population (Medeiros *et al.*, 2011). This cluster is a target of pluripotency factors, and in turn targets the pluripotency factors Oct3/4, Sox2 and Nanog (Zheng *et al.*, 2011). It is shown that suppression of the miR-290 cluster, besides embryonic lethality, leads to the defective migration of PGC numbers in the gonads. In addition, they are differentially expressed between gonocytes and spermatogonia pointing to their role in the differentiation of gonocytes. These data demonstrate the role of the miR-290 cluster in the maturation of germ cells, indicating that this miR cluster plays an important role in transition from gonocyte to spermatogonia and in the pre-invasive lesion CIS, which has been disrupted and failed to successfully differentiate into a spermatogonium (McIver *et al.*, 2012).

miR-302/367

The cluster of miR miR-302/367 represents the first human miR promoter characterized in human ES cells and their malignant counterpart EC cells (Barroso-delJesus *et al.*, 2008, Suh *et al.*,

2004). The cluster miR-302-367 is located on chromosome 4 and contains eight different miRs: miR-302a, miR-302a*, miR-302b, miR-302b*, miR-302c, miR-302c*, miR-302d and miR-367. The first seven constitute the miR-302 family with a highly conserved sequence (Barroso-delJesus *et al.*, 2008). The major members of the miR-302 family (302a, 302b, 302c and 302d) share a high sequence homology, differing only in the 3'hexanucleotides. All four family members display a cytoplasmatic localization with no significant differences among them, meaning that the 3'hexanucleotides does not promote nuclear localization for any of these miRs.

miR profiling has confirmed cell type specificity for miR-302/367 expression; it is highly expressed in human ES and EC cells but not in adult human mesenchymal stem cells (hMSCs) and normal tissues. The miR-302/367 promoter activity depends on the hierarchical cellular stage. The promoter activity is functional in human and mouse ES cells and human EC cells, but it is turned off later in development (Barroso-delJesus *et al.*, 2008). miR-302/367 can target over 445 human genes and most of these targets are developmental signals involving the initiation and facilitation of lineage-specific cell differentiation during early human embryogenesis (Lin *et al.*, 2008). miR-302/367 promoter activity decays upon differentiation of both mouse and human ES cells, demonstrating that its activity is restricted to the ES cell compartment and that the ES cell-specific expression of the miR-302/367 cluster is fully conferred by its core promoter transcriptional activity. Importantly, these data are further supported by the fact that endogenous miR-302/367 expression is also down-regulated during human ES cell differentiation (Barroso-delJesus *et al.*, 2008). The important point is that the key ES cell-specific factors such as, OCT3/4, SOX2 and NANOG act as upstream regulators of the miR-302/367 cluster meaning that potential binding sites for these pluripotency factors in the promoter sequence of these miRs are present (Barroso-delJesus *et al.*, 2008, Card *et al.*, 2008). It has been demonstrated that in human ES cells the expression of miR-302a under OCT3/4/SOX2-depleted conditions was reduced by more than 50% in comparison with the level in cells transfected with non-targeting siRNAs. These data support the model that the expression of miR-302a is dependent on OCT3/4-SOX2 in human ES cells and suggest that OCT3/4 and SOX2 function as transcriptional activators (Card *et*
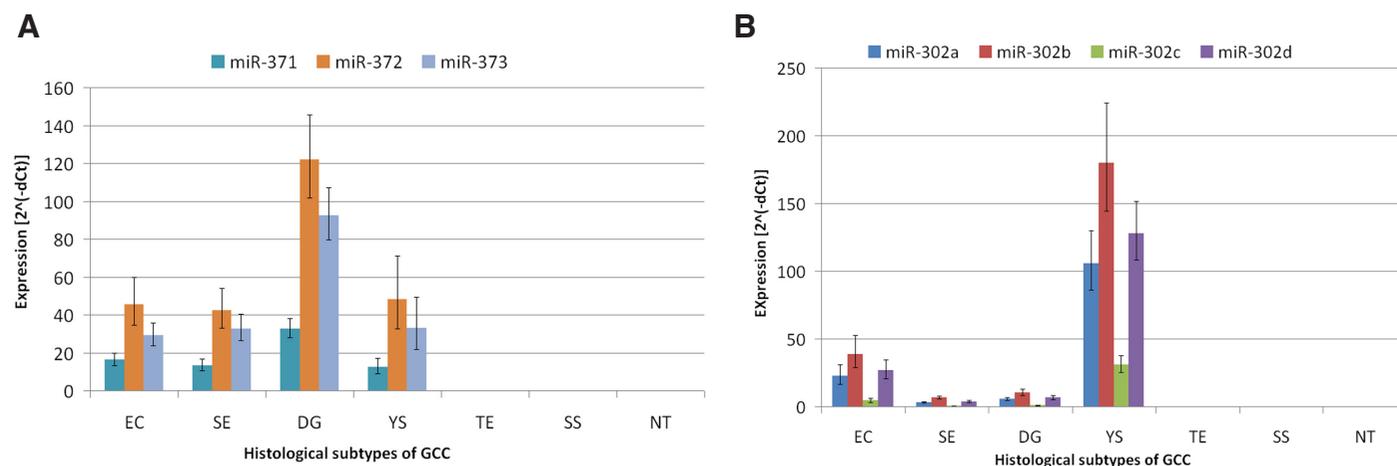

Fig. 6. Expression pattern of miR-371-3 and miR-302 clusters in germ cell cancer. *These include EC $_{n=14}$, SE $_{n=15}$, DG $_{n=10}$, YS $_{n=8}$, TE $_{n=10}$, SS $_{n=4}$ and NT $_{n=4}$.* **(A)** *Within cluster miR-371-3 miR-372 shows the higher level of expression in all samples;* **(B)** *Within the miR-302 cluster miR-302b and miR-302d show the highest level of expression. (The CT values of miRs are normalized as described before (Mestdagh* et al.*, 2009).*

al., 2008). Interestingly, siRNAs directed against OCT3/4 and SOX2 in a human EC cell line (NTera2) showed down-regulation of miR-302/367 cluster up to 50% in our experiment confirming the relationship between pluripotency and miR-302/367 cluster (shown in Fig. 7). This also confirms that initiation of differentiation reduces the level of miR-302/367 cluster in EC.

miR-371-3 and 302/367 in other cancer types

miR-371-3 and miR-302/367 have been reported to be involved in other malignancies than GCC. In a study regarding expression of miRs in oesophageal squamous cell carcinoma, it has been shown that miR-373 participates in the carcinogenesis of human oesophageal cancer by directly suppressing LATS2 expression (Lee *et al.*, 2009). Additionally, in a study related to human hepatocellular carcinoma (HCC), it is found that miR-373 is up-regulated in HCC tissues as compared with adjacent normal tissues, and promotes the proliferation of HCC cell lines by regulating the G_1-S transition (Wu *et al.*, 2011). In a number of studies it is reported that miR-373 can promote tumor invasion and metastasis such as breast and prostate cancer (Huang *et al.*, 2008, Negrini and Calin, 2008, Yang *et al.*, 2009). Additionally, up-regulation of miR-373 in retinoblastoma has been reported (Zhao *et al.*, 2009). Furthermore, miR-371-3 cluster has shown to be up-regulated in thyroid adenomas (Rippe *et al.*, 2010). Up-regulation of miR-302/367 has been reported in glioblastoma multiforme. It is shown that this miR cluster is rapidly and strongly induced in glioma-initiating cells (Fareh *et al.*, 2012). In addition, miR-302 is reported to be enriched in human leukaemia cell lines (Yu *et al.*, 2006), large B cell lymphoma (Lawrie *et al.*, 2009). miR-367 is also found to be increased in non-small-cell lung cancer (Campayo *et al.*, 2012). Furthermore, miR-302 has been shown to reprogram human skin cancer cells into a pluripotent ES cell like state (Lin *et al.*, 2008).

Functional read-out system in GCC cell lines

Considering all the mentioned facts about miR-371-3, we initiated a study in order to investigate the role of suppression of
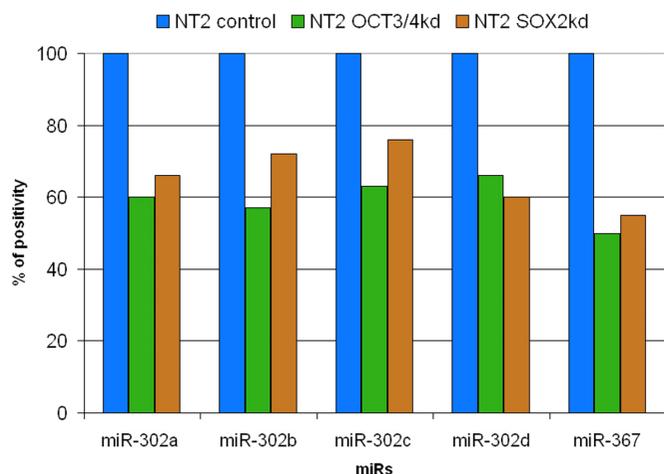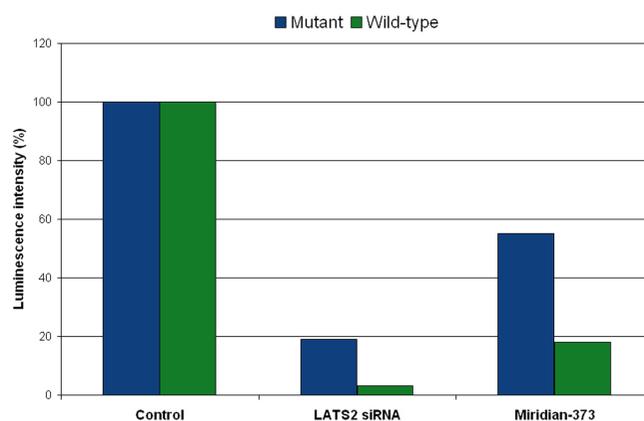
Fig. 8. Result of luciferase measurement assay. *It is shown that luciferase activity is down-regulated in both NTera2 cells subjected to LATS2 siRNA and miridian-373. Luciferase activity is also partly down-regulated in cells with a mutant construct of LATS2, which may be due to the partial homology between the siRNA or miridian and LATS2 sequence in the construct. The results are based on six parallel experiments (96 wells) for each condition tested. Standard deviations were: for controls: (0, 015 and 0, 014), LATS2 siRNA (0,079 and 0,058), miridian-373 (0,057 and 0,025) mutant and wild-type respectively.*

miR-371-3 in cell cycle and cisplatin sensitivity of GCC cell lines. As a consequence of the poor transfectability of the GCC cell lines, a lentiviral construct was designed to monitor the manipulation of miR levels. In the lentiviral construct, the luciferase reporter is linked to the 3' untranslated region (UTR) of LATS2. In addition, the construct contains a selection marker to allow for stable integration in the GCC cell genome. Its functionality was demonstrated using oligonucleotides for a miR-373 mimic (meridian-373) and a siRNA directed to the 3'UTR. These oligos were introduced into the cells by transient transfection and their consequence on luciferase activity was measured. Both the meridian-373 and the LATS2 siRNA caused strong reduction of the luciferase activity (Fig. 8), and thus demonstrate the sensitivity of the reporter system. Recently an alternative reporter system based on a retroviral construct (miR-Sens) was published (Beillard *et al.*, 2012). In this vector, the Renilla luciferase gene is also linked to the LATS2 3'UTR and the Firefly luciferase is needed for normalization in the absence of a selection marker. Following introduction of a retroviral vector causing expression of miR-373, the authors could demonstrate reduction of the luciferase activity as a result of the targeting of the LATS2 3"UTR (Beillard *et al.*, 2012). Both approaches are well suited to monitor the consequence of introduction of exogenous miR, but it remains to be established whether these reporters can monitor changes in endogenous levels of miR following cell differentiation or other treatments.

Cisplatin sensitivity in GCC and proposed model for involvement of microRNA clusters 371-3 and 302/367

Recently, involvement of some miRs (miR-193b and miR-320) in resistancy/sensitivity to platinum agents in ovarian cancer cell lines has been reported (Ziliak *et al.*, 2012). Platinum agents are main chemotherapeutic agents used in the treatment of cancers (Williams and Whitehouse, 1979). As mentioned, ES cell-specific miRs have a central role in G_1-S transition and promotion of cel-

Fig. 7. High throughput results show down-regulation of expression of the members of cluster miR-302/367 due to OCT3/4 and SOX2kd in NTera2 cells. *All miR members in the cluster show down-regulation (~ 50%) in cells subjected to OCT3/4 and SOX2 siRNA compare to cells transfected with scrambled siRNA.*

lular proliferation. EC cells, similarly to ES cells, have a short G_1-S transition and are therefore highly responsive to cisplatin-based chemotherapy. Indeed, NTera-2 cells respond to cisplatin as well. The level of expression of the miR-302 cluster in NTera-2 cells is relatively high (Gillis *et al.*, 2007). This causes sensitivity to DNA-damaging agents such as cisplatin (Fig. 3). Our model predicts that suppression of the two ES cell miRs, miR-302 and miR-371-3 cluster in GCC cell lines will cause longer G_1-S transition due to higher level of Cyclin D1 and LATS2 expression respectively. This will leads to suppression of CDK inhibitors and consequently will induce resistance to cisplatin in these cells.

Detection of miR-373, miR-302c and 367 clusters in serum of patients with GCC

Recently, there has been an enormous interest in investigating circulating miRs in blood plasma/serum as potential biomarkers for early disease, including cancer. In multiple reports, detection of cancer-specific miRs in serum of cancer patients has been reported (Chen *et al.*, 2008, Morimura *et al.*, 2011, Zahm *et al.*, 2011). Currently, serum markers such as alpha feto-protein (AFP) and human Chorionic Gonadotropine (hCG) are predominantly informative for initial diagnosis as well as follow up of GCC, predominalty related to YS and choriocarcinoma components, respectively (Horwich *et al.*, 2006). However, no informative serum marker for SE and EC exist, which is highly relevant, because of the distribution of these components in GCC overall, and their stem cell characteristics and behavior. Therefore, the necessity for new serum markers in order to detect and follow-up the other histological component of GCC is apparent. Interestingly, a case report (Murray *et al.*, 2011) showed the feasibility of measuring serum levels of the miR-371-3 and miR-302 clusters in a single patient. In addition, a decline of these miR level in serum of GCC patients after surgical removal of the (stage I) GCC was shown (Belge *et al.*, 2012). A subsequent study (Murray and Coleman, 2012) demonstrated elevation of these miR clusters in serum of multiple patients at the time of initial diagnosis with different histological subtypes. These findings open new avenues for diagnosis and follow-up the other mentioned cancer patients as well.

The finding of stable extracellular miRs in serum/plasma and other body fluid types suggests the possibility of their involvement in mediating cell-cell communication. This could imply that miRs convey specific information and therefore only some cellular miRs are exported or released from cells in response to biological stimuli. In addition, it is shown that cells release a significant number of RNA-binding proteins into the culture medium and one of them, nucleophosmin 1 (NPM1), can protect miRs from degradation. There is no evidence yet that miR containing complexes are taken up by other cells, although vesicles containing miRs are reported to be taken up by cells. It is possible that miRs outside vesicles, complexed with proteins, could be targeted to specific cell surface receptors (Wang *et al.*, 2010).

It is controversial whether miRs circulate freely or are encapsulated in micro-vesicles, particularly exosomes; in other words if their transport is active or passive. It is suggested that extracellular miRs are bound to Ago proteins and Ago2 in particular. It is shown that miRs remained stable in the cell lysates for at least 2 month meaning that Ago2/miR complexes are highly nuclease/protease resistant. The miR/Ago2 complexes are released from the cells upon necrosis or apoptosis (Turchinovich *et al.*, 2011). This has been confirmed by another study in which it is shown that although some circulating miRs are vesicle-associated, 90% of miRs are present in a non-membrane-bound form consistent with a ribonucleoprotein complex which is identified as Ago2-miR complex (Arroyo *et al.*, 2011). However, it is reported that the majority of miRs detectable in serum and saliva is concentrated in exosomes (Gallo *et al.*, 2012). In this study it is shown that the concentration of miRs is consistently higher in the exosome pellet compared to the exosome-depleted supernatant. The reason for this discrepancy is unclear but could be due to differences in isolation exosomes or differences between plasma and serum.

To confirm the findings in GCC patients, we aimed to detect the serum level of the mentioned miR clusters in a series of GCC patients at stage I and higher (i.e., metastatic disease). As a pilot study, we tested the serum level of miRs 373, 302c and 200c (as control) in a limited number of cases. The miR-200c is reported to be involved in metastasis of epithelial cancers (Hurteau *et al.*, 2007) and is not specific for GCC. Serum samples included in our study were high stage (stage II and higher) GCC serum samples, stage I GCC serum samples and inflammatory serum samples as controls (10 sample of each). Indeed a higher level of miRs was detected in serum of GCC patients compared to control samples, correlated with stage of presentation of the disease (Fig. 9). However, there are technical limitations in all studies reported, related to low RNA recovery from serum, and issues of normalization (done based on volume of *RNU6B*). To solve these problems, we developed together with Applied Biosystems (part of Life Technologies) a complete pipeline for detection of miRs in body fluids based on a magnetic-bead based purification and qPCR quantitation.
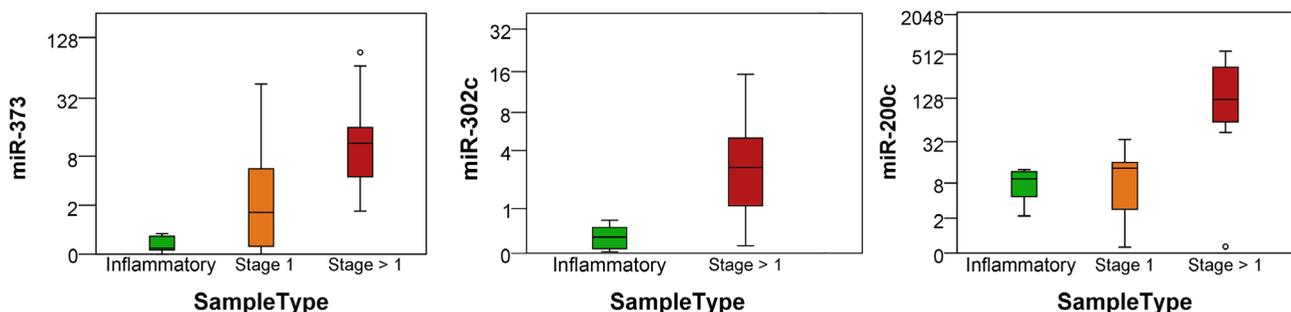

Fig. 9. Results of level of microRNA (miR) in serum of patients with stage I and higher stage germ cell cancer (GCC). *miR-373 and miR-200c show the highest levels in higher stage GCC and less expression level in stage I samples while the expression levels are very low in case of inflammation. The miR-302c was measured in inflammatory samples and high stage tumours showing higher expression level in high stage GCC.*

Correlation between the level of miR-302/367 and miR-371-3 cluster between the tumors and the serum of the same GCC

To investigate whether the pattern of miRs is serum correlates with the pattern observed in the matched primary tumors, a series of 10 GCC cases (including stage I and higher) were investigated. Statistical analysis showed no correlation between the level of miRs within the tumor and matched serum samples (statistical analysis not shown). This suggests that specific (active) mechanisms are involved in this process.

Methylation status of the promoter of miR-371-3 in GCC:

Gene function in cancer cells can be disrupted either through genetic alteration, directly by mutation, deletion or amplification (Baylin *et al.*, 2001). However, no such anomalies have been found so far in a series of 242 GCC regarding the miR-371-3 and miR-302/367 clusters. Alternatively, this can be the result of epigenetic changes, including DNA methylation. This is a major epigenetic modification of the genome that regulates crucial aspects of its function and allows cells to lock genes in the "off" position. Disruption of DNA methylation associated with specific imprinted genes is a common feature of human cancers (Plass and Soloway, 2002). Hypermethylation of promoter regions has been observed for several anti-oncogenes and hypomethylation has been reported for some proto-oncogenes. A genome-wide survey has revealed that the overall degree of methylation in SE is lower than in non-seminoma tissues (Smiraglia *et al.*, 2002). Methylation of genomic DNA is performed by DNA methyltransferases that transfer methyl groups to cytosine residues. The five genes known to encode DNA

methyltransferases (DNMT) include DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L. Here we mainly discuss DNMT1 which maintains methylation of genomic DNA during DNA replication, thus contributing to stability of gene expression from parental to daughter cells. It has been reported that in the DNMT1-/- mice in which loss of imprinting has been transiently induced, the imprint free stem cells formed *in vivo* various neoplasms, including a single tumor mimicking seminoma (Holm *et al.*, 2005). Therefore, one hypothesis for the high expression level of miR-371-3 (and possibly miR-302a/367) in GCC, could be that the overall retained demethylation pattern in the precursor cells is involved.

Indeed, changes in miR expression can occur through various mechanisms including chromosomal abnormalities, transcription factor binding and epigenetic alterations. Recent studies have shown that, in cancer, expression of some miR is silenced in association with CpG island hypermethylation (Lujambio *et al.*, 2008, Sharma *et al.*, 2010, Toyota *et al.*, 2008). In order to determine whether the expression pattern of miR-371-3 in GCC (EC, SE and YS) is due to the methylation status of the supposed promoter region, we examined the methylation status of 12 CpG sites within the CpG island of the promoter of miR-371-3 cluster. The CpG island is located 400 bp upstream miR-371 (Fig. 10). Firstly, the methylation status of the promoter of this miR cluster was tested in GCC cell lines using sequencing of bisulfite-treated DNA. A correlation between expression level of miR-371-3 cluster and methylation percentage of the promoter of this cluster was found). The correlation between miR-373 and methylation percentage was significant, however, this correlation for miR-371 and miR-372 was not significant. The reason for this is unclear and needs further investigations. Analysis of micro-dissected GCC samples (EC, SE, YS, and teratoma) showed a different pattern, which needs further investigation (data not shown).
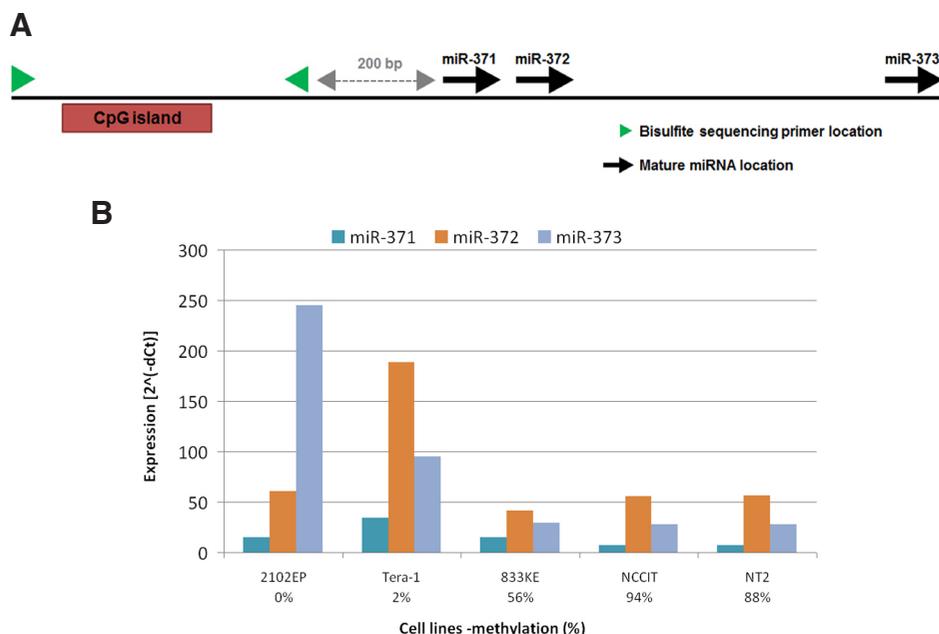
Concluding remarks

Emerging evidences have revealed the importance of miRs for normal development and maintenance of a multi-cellular organism. It is now proven that formation of ES cells as well as various differentiation lineages is dependent on miRs. The core transcriptional factors and ES cell miRs are linked in a regulatory circuitry that critically regulates both pluripotency and differentiation in human ES cells. These processes are represented in human GCC. In fact the most important reason to study the role of ES cell miRs in GCC is the fact that these cancers mimic embryonic development to a certain extent.

One of the core pluripotency marker in ES cells, OCT3/4, is an informative diagnostic marker for this type of GCC. Detailed investigation on the expression profile of miRs in GCC revealed expression of specific miRs differentially expressed in various histological components. Two of the most informative clusters of miRs were again miR-371-3 and miR-302/367. Interestingly, miR cluster 371-

Fig. 10. (A) miR-371-3 locus. The location of sequencing primers within the promoter of miR-371-3 and the location of mature miRs are shown. (B) Expression pattern of miR-371-3 in comparison to methylation. *Percentage of the total number of methylated CpG sites is shown for the cell lines. Expression of miR-373 was strongly, significantly negatively correlated with increasing percentages of methylation (Spearman's ρ -0.90, p=0.037). The correlation between expressions of miR-371/2 with methylation was not significant.*

3 is involved in inhibition of cellular senescence after oncogenic stress. This explained the presence of wild type P53 in GCC, in contrast to most other solid cancers. High level of miR-371-3 cluster in GCC leads to short G_1-S transition (via suppression of LATS2) which in turn increases the sensitivity of these tumors to chemotherapy. miR-302 cluster is highly expressed in SE, EC as well as YS. Functional analysis has shown that down-regulation of core pluripotency factors such as OCT3/4 and SOX2, induces reduction of the level of miR-302/367 cluster in an EC cell line NTera-2. Interestingly, OCT3/4 down-regulation in NTera2 induced longer G_1-S transition by accumulating more cells in G_1 phase and less cells is S phase. On the other hand, it is known that differentiated NTera2 cells in which pluripotency factors are down-regulated, show resistance to DNA-damaging agents such as cisplatin. All these together confirm that the link between ES cell miRs and pluripotency markers plays a major role in the pathogenesis of GCC as well as their exceptional sensitivity to DNA damaging agents. Currently, to improve knowledge about the insight roles of these sets of miRs in pathogenesis of GCC, functional analysis on the effect of suppression of these miRs on cisplatin sensitivity as well as cell cycle regulation of GCC cell lines is in process. These findings will shed novel light on normal and malignant germ cell development leading to diagnostic and prognostic implications.

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5 yr ISI Impact Factor (2011) = 2.959

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