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"microRNA expression and function in Embryonic Stem Cells: miR-100, miR-137 and miR-34a are required for ESC differentiation"

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Abstract

Given their capacity to self-renew and differentiate efficiently into the different cell types, Embryonic Stem Cells (ESCs) provide a valid model to understand the complex network of signaling interactions in the mammalian embryo and open up new possibilities for cell therapy. So a deeper understanding of the molecular mechanisms that regulate generation, self-renewal and differentiation of ESCs is become crucial not only to fulfill their clinical promise but also to get insight into the molecular mechanisms controlling early events of mammalian development. The emergence of microRNAs (microRNAs) as potent regulators of gene expression at the post-transcriptional level has broad implications in all facets of biology, including ESCs and early development.

In recent years, the role of microRNAs in ESCs and mammalian embryogenesis has begun to be explored but specific roles of the microRNAs in the regulation of ESC specific fate are still largely unknown. In this context, our interest is to identify microRNAs regulating ESC functions. We performed a systematic comparison of microRNA expression in undifferentiated versus differentiating mouse ESCs. We report that different microRNAs are increased upon the induction of differentiation. We compared the entire list of candidate mRNA targets of upregulated microRNAs with that of mRNA dowregulated in ESCs upon the induction of differentiation. Among the candidate targets emerged from this analysis, we found three genes Smarca5, Jarid1b and Sirt1, previously demonstrated to be necessary to sustain the undifferentiated phenotype in ESCs. On this basis, we first demonstrated that Smarca5 is a direct target of miR-100, Jarid1b of miR-137 and confirmed previously published data demonstrating that Sirt1 is a direct target of miR-34a in a different context. The suppression of these three microRNAs by anti-miRs

caused block of ESC differentiation induced by LIF withdrawal. On the other hand, the overexpression of the three microRNAs resulted in an altered expression of differentiation markers. These results demonstrated that miR-100, miR-137 and miR-34a are required for proper differentiation of ESCs, and that they function by targeting, among the others, the mRNAs of Smarca5, Jarid1b and Sirt1.

In conclusion, we have characterized a subset of microRNAs that are necessary for proper differentiation of mouse ESCs. The identification of microRNAs that are up-regulated upon the induction of ESC differentiation suggests that they suppress, directly or indirectly, the expression of genes necessary to maintain ESCs in the undifferentiated state. The identification of targets of the microRNAs that we have studied may provide tools to drive ESC differentiation towards specific lineages.

Introduction

The discovery of mouse ESCs represented a major advance in biology and experimental medicine because it provided the basis for establishing an in vitro model of early mammalian development and represented a potential source of differentiated cell types for cell replacement therapy. However the application of ESC as a model for developmental biology has been limited and their use in cell replacement therapy remains a main goal in the field. So advances in our understanding of ESC differentiation are necessary to provide new insights essential for establishing ESC-based developmental models and for the generation of clinically relevant population for cell therapy.

Mouse ESCs were first derived from the inner cell mass (ICM) of blastocyst-stage embryos in 1981 (Evans and Kaufman 1981). Blastocyst is a vesicular structure comprising of a cluster of cells, the inner cell mass (ICM), adhering to one side of the vesicle and of an epithelial outer layer, the trophectoderm (TE), enclosing a fluid filled space (the blastocoel cavity). The ICM is composed of approximately 20 cells that give rise to all cells of the embryo, while TE cells give rise to extraembryonic tissues, such as placenta, critical for supporting embryonic development (Figure 1) (Ohtsuka and Dalton 2008).

These two morphologically distinct cell types in the blastocyst express different sets of genes, which owing to their specificity and their changes in expression level drive the events of embryogenesis.

Cdx2 and Eomes are two TE specifically genes, essential for the delineation of the cell lineage earlier in the compacting pre-implantation embryo (Strumpf, Mao et al. 2005) and for proper differentiation of the TE into trophoblast (Russ, Wattler et al. 2000). At the same time Oct3/4, Sox2, Nanog, Bmp4 and Nodal are the genes

specifically expressed in the ICM (Nichols, Zevnik et al. 1998; Niwa, Miyazaki et al. 2000; Avilion, Nicolis et al. 2003; Chambers, Colby et al. 2003; Mitsui, Tokuzawa et al. 2003). Concurrent with the implantation of the blastocyst, the ICM gives rise to a new epithelial layer, the primitive endoderm (PrEn), on its blastocoelic surface. Later this epithelium also lines the luminal surface of the mural trophectoderm away from the ICM and contributes to the endoderm of the visceral and parietal yolk sac (visceral and parietal endoderm: VE and PE). In addition to the PrEn, the ICM gives rise to the epiblast of the post-implantation embryo from which all tissues of the embryo are derived (Rossant, Chazaud et al. 2003).

Because some genes, such as Gata6, are expressed in the inner cell mass of the pre-implantation blastocyst and later shift their expression to the PrEn after this tissue is formed, it is postulated that the PrEn is formed by sorting of ICM cells that are committed to the PrEn lineage and by organizing them into an epithelial layer. So cells with divergent lineage fates may be present before the PrEn emerges (Pfister, Steiner et al. 2007). After the embryo has implanted in the uterus, the trophectoderm overlying the ICM (the polar TE) proliferates (Rossant and Cross 2001) and grows into a thick column of extraembryonic ectoderm (ExE), which extends into the blastocoel and carries a compact epiblast at its distal pole. Both the ExE and the epiblast are enveloped by the VE. The embryonic epiblast is composed of a single layer of pseudostratified epithelia called primitive ectoderm, which give rise to all three embryonic germ layers (ectoderm, mesoderm and definitive endoderm) and primordial germ cells (Pfister, Steiner et al. 2007).

All these steps of embryogenesis can be recapitulate by ESCs. In fact the importance of ESCs to modern biology and medicine derives from two unique characteristics that distinguish them from all other organ-specific stem cells identified so far. First, they can self-renew continuously if they are cultured under

conditions that prevent their differentiation, so they can be maintained and expanded as pure populations of undifferentiated cells for extended periods of time, possibly indefinitely, in culture. Second, they possess the remarkable property of pluripotency.

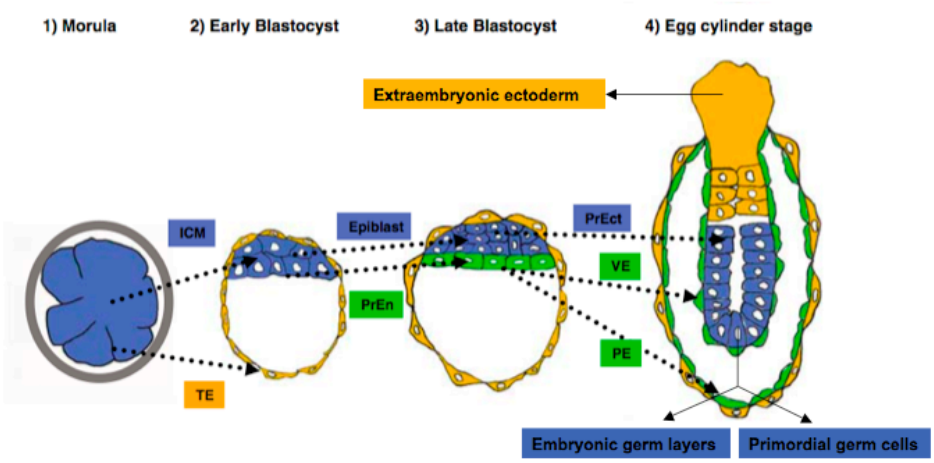


Figure 1. Overview of peri-implantation mouse development.

(1) Morula: around embryonic day 2.5. (2) Early blastocyst has two distinct cell types: ICM cells (blue) that gives rise to all cells of the embryo and TE cells (orange) which give rise to extraembryonic tissues. (3) At the late blastocyst stage of development the ICM is surrounded by a third cell type known as primitive endoderm (PrEn, green), that give rise to the yolk sac to support embryonic development. Formation of PrEn around the ICM denotes the epiblast stage of development. (4) Egg cylinder stage: PrEn differentiated toward visceral (VE) and parietal endoderm (PE) while a cavity forms resulting in the formation of a single layer of pseudostratified epithelia known as primitive ectoderm (PrEct). The cells of primitive ectoderm give rise to all three embryonic germ layers (ectoderm, mesoderm and definitive endoderm) and primordial germ cells. Adapted by (Ohtsuka and Dalton 2008).

Pluripotency is a property of the ICM from which ESCs are derived, so they have the ability to differentiate into various cell types of a vertebrate embryo. In fact under appropriate conditions and after removal of factors that maintain ESCs in undifferentiated state, they can generate progeny consisting of derivatives of the

three embryonic germ layers: ectoderm, endoderm and mesoderm (Figure 2), (Keller 1995).

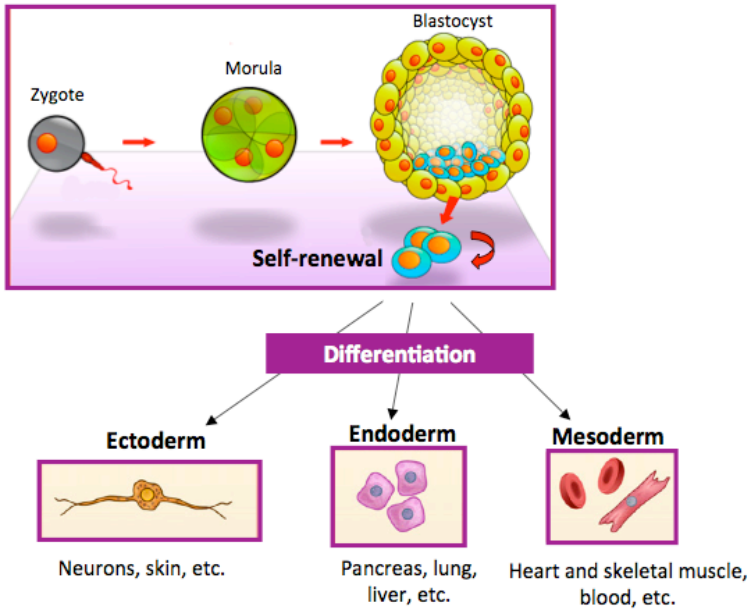


Figure 2. Differentiation of ESCs.

ESCs are able to differentiate into the derivatives of the three embryonic germ layers: ectoderm, endoderm and mesoderm.

The most extraordinary characteristic of ESCs is that, even after extended propagation and manipulation in vitro, they hold the ability to re-enter embryogenesis. Indeed when introduced into the pre-implantation mouse embryo they colonize all fetal lineages plus yolk sac mesoderm, allantois and amnion and produce functional differentiated progeny in all tissues and organs. ESCs are able to colonize also the germ cell lineage, generating functional gametes because they maintain a diploid karyotype necessary for meoisis (Bradley, Evans et al. 1984).

Traditionally, ESC pluripotency has often been defined as the ability to generate all cell types of an embryo apart from the trophectoderm. This is because an earlier

analysis of chimeric mouse embryos, produced by the injection ESCs into blastocysts, had shown that these cells are excluded from the trophectoderm lineage (Beddington and Robertson 1989). However, it has subsequently been found that the ICM and ESCs do still possess the ability to differentiate into the trophectoderm lineage (Pierce, Arechaga et al. 1988; Niwa, Toyooka et al. 2005).

The ability for multilineage differentiation of ESC is reproduced in culture where these cells can produce a wide range of well-defined cell types.

Three general approaches are used to initiate ESC differentiation. With the first method, ESCs are allowed to aggregate and form three-dimensional colonies known as embryoid bodies (EBs) (Doetschman, Eistetter et al. 1985; Keller 1995). This technique leads to formation of multidifferentiated structures in which the developmental program of ICM/epiblast cells is reactivated in the ESCs. Cellular differentiation proceeds on a schedule similar to that in the embryo but in the absence of proper axial organization or elaboration of a body plan (Doetschman, Eistetter et al. 1985). Each embryoid body develops multiple cell types, and further differentiation is elaborated on subsequent attachment and outgrowth. It is possible to bias the differentiation for or against specific cell types by addition of inductors, such as retinoic acid (Rohwedel, Guan et al. 1999). However, the final cultures are invariably a heterogeneous mixture (Keller 2005).

With the second method, ESCs are cultured directly on stromal cells, and differentiation takes place in contact with these cells (Nakano, Kodama et al. 1994). Another possibility is that ESCs are cultured in a medium, conditioned by other cell types. OP9 are the most commonly used stromal cell line for differentiation studies (Nakano, Kodama et al. 1994). They were originally isolated from CSF-1-deficient op/op mice (Yoshida, Hayashi et al. 1990). Coculture with stromal cells provides the beneficial growth promoting effects of the particular cell

line used. However, undefined factors produced by these supportive cells may influence the differentiation of the ESCs to undesired cell types. An additional problem with this method is the difficulty that can be encountered when attempting to separate the ESC-derived cells from the stromal cells (Keller 2005).

The third protocol involves differentiating ESCs in a monolayer on extracellular matrix proteins (Nishikawa, Nishikawa et al. 1998). Differentiation in monolayers on known substrates can minimize the influence of neighboring cells and supportive stromal cells and in this regard is one of the simplest protocols. With this protocol the matrix proteins are critical, and different proteins may dramatically influence the generation and survival of the developing cell types (Keller 2005).

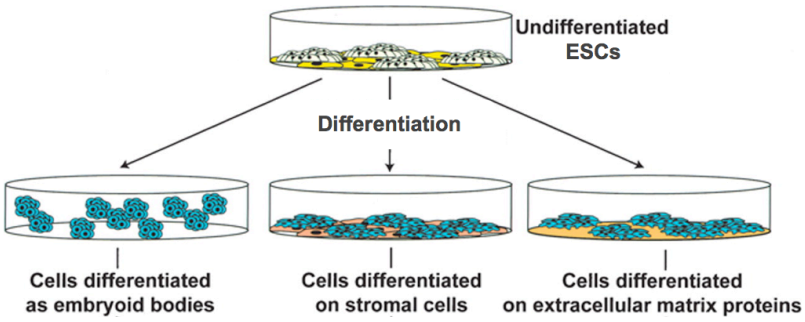


Figure 3. Three different protocols used for ESC differentiation.
Taken from Keller 2005.

It seems that there is no intrinsic limitation to the ability of ESCs to differentiate in vitro. But can cells generated in vitro really acquire the full specialization of differentiated cell types in vivo? Remarkably, the available data indicate that ESC derivatives can become specialized to a high degree in culture. ESC progeny in fact, can express appropriate markers and functional attributes of specific cell sub-types, such as neurons or cardiomyocytes, (Maltsev, Rohwedel et al. 1993; Strubing, Ahnert-Hilger et al. 1995; Lee, Hart et al. 2004). Accumulating evidence

from transplantation studies indicates that the specification of at least some ESC derivatives is sufficient to allow their integration into adult tissue.

The opportunity to observe and then to manipulate such complex differentiation processes opens unheralded opportunities for mammalian developmental biology and cell engineering (Figure 4).

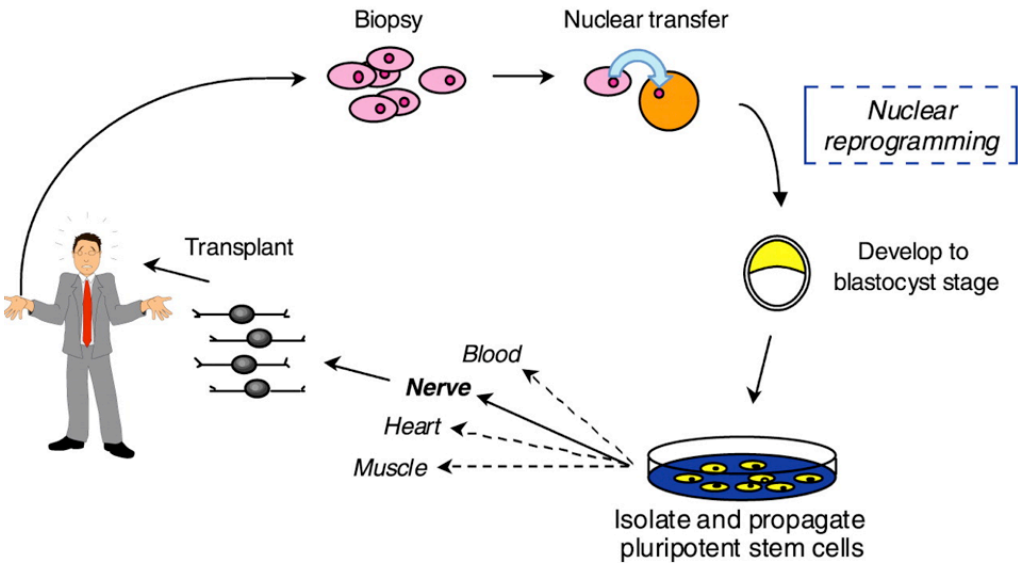


Figure 4: Stem cell therapy.

Outline of the isolation of human pluripotent stem cells, expansion in culture, differentiation into the cell type of relevance and transplantation. Stem cells may be derived from surplus preimplantation embryos generated in the course of in vitro fertilization treatment and donated for research, or possibly in future from embryonic entities produced by nuclear transfer. The second route would avoid any requirement for immunosuppression after transplantation (Smith 2001).

Cell transplantation to restore tissue function after disease or injury is in theory applicable to a huge variety of human ailments, spanning neurodegeneration (Svendsen and Smith 1999), diabetes (Soria, Roche et al. 2000), and myocardial infarction (Klug, Soonpaa et al. 1996).

Recent studies with mouse ESCs offer encouragement in this regard, provided that mouse cells are a valid model for human pluripotent stem cells (hPSCs). Thus ESC-derived neural cells have been demonstrated to survive and to exhibit at least some aspects of appropriate region-specific neuronal differentiation when introduced into the developing mouse brain (Brustle, Spiro et al. 1997). In adults, transplantation of glial derivatives of ESCs results in a degree of remyelination in a rat model of multiple sclerosis (Brustle, Jones et al. 1999) and, most dramatically, to a partial functional recovery in a spinal cord injury model (Liu, Qu et al. 2000).

In conclusion the possibility of propagating pluripotent stem cells from mammalian embryos is a gift from nature that has provided unparalleled research tools for investigating mammalian development, genetics and physiology. Now these cells offer the foundations for an entirely new form of human medicine.

Therefore, a detailed understanding of the mechanisms that enable propagation of ESCs in a pluripotent state, such as signaling trasduction pathways, transcription factors, cell-cycle and epigenetic regulators, is essential to realize their therapeutic potential.

Signaling trasduction pathways

ESC pluripotency is maintained during self-renewal by the prevention of differentiation and the promotion of proliferation. For mouse ESCs, leukemia inhibitory factor (LIF), a member of the IL-6 cytokine family, is a key factor that prevents differentiation (Smith, Heath et al. 1988). After withdrawal of LIF from ESC culture medium, proliferation continues but differentiation is induced, and stem cells do not persist beyond a few days (Figure 5). LIF stimulates ESCs through the gp130, which works as a heterodimer together with LIF-Receptor.

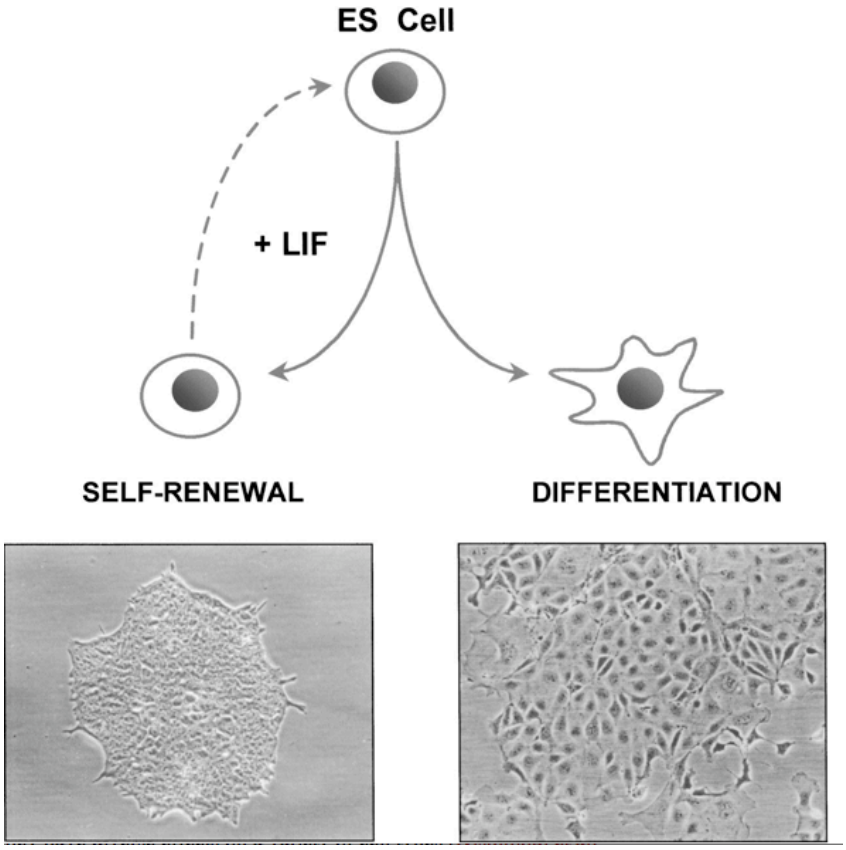


Figure 5: ESC self-renewal and differentiation.

Upper panel: diagram of alternative fates of an ESC and the effect of LIF. Lower panel: photographs of self-renewing ESC cultured in the presence of LIF (left) and differentiated derivatives 4 days after LIF withdrawal (right) (Smith 2001).

Activation of gp130 leads to the activation of the Janus-associated tyrosine kinase (JAK) and of signal transducer and activation of transcription (STAT), (Figure 6). The ability of LIF to maintain the ESC self-renewal is dependent upon activation of STAT3 (Niwa, Burdon et al. 1998), whose activation is sufficient to prevent ESCs differentiation in the presence of serum (Matsuda, Nakamura et al. 1999). A downstream candidate target of activated STAT3 is c-Myc (Wang, Xie et al. 1998). In addition to STAT3 activation, LIF also stimulates the activation of the mitogen-activated protein kinase (MAPK), which promotes differentiation (Burdon, Stracey et al. 1999). So ESCs can maintain their property in the presence of LIF due to the balance of the STAT3 activation and MAPK effect.A synergistic effect with LIF-

STAT3 pathways is given by Wnt pathway (Hao, Li et al. 2006). In fact Wnt signaling activation can upregulate STAT3 expression and elevate the level of c-Myc. Wnt signaling is endogenously activated in ESCs and is downregulated upon differentiation (Sato, Meijer et al. 2004).

Another important pathway for proliferation, survival and maintenance of pluripotency in ESCs is the Phosphoinositide 3-kinase (PI3K)/Akt pathway (Figure 6). Two modulators of PI3K/Akt pathway are specifically expressed in ESCs, ERas and Tcl1 (Takahashi, Murakami et al. 2005). ERas encodes a constitutively active form of a Ras-family small GTPase that activates PI3K to stimulate ESC proliferation and tumorigenicity after ectopic transplantation in vivo (Takahashi, Mitsui et al. 2003). The Tcl1 gene product augments Akt activation by forming a stable heterodimeric complex with Akt (Teitell 2005).

Also the members of TGF superfamily, including TGFβ, Activin, Nodal and Bone morphogenic proteins (BMPs) are involved in ESC self-renewal and differentiation (Figure 6). In particular it has been demonstrated that LIF-STAT3 cannot maintain mouse ESCs in serum-free medium, indicating that there are many unknown factors present in serum, which are required to co-affect with LIF-STAT3. This serum factor is likely to be BMP4 acting via activation of Smad1/5/8. Addition of BMP4 to the media enables LIF to maintain mouse ESCs in serum-free culture (Ying, Nichols et al. 2003). Smad1/5 activation results in the expression of inhibitor of differentiation (Id) protein, which functionally antagonizes neurogenic bHLH transcription factors and blocks the neural differentiation (Gerrard, Rodgers et al. 2005). Exogenous expression of Id mimics the effect of BMP4 in mouse ESCs (Ying, Nichols et al. 2003). Furthermore, BMP4 can maintain mouse ESCs in the presence of LIF, possibly by blocking the MAPK signaling cascade (Qi, Li et al. 2004).

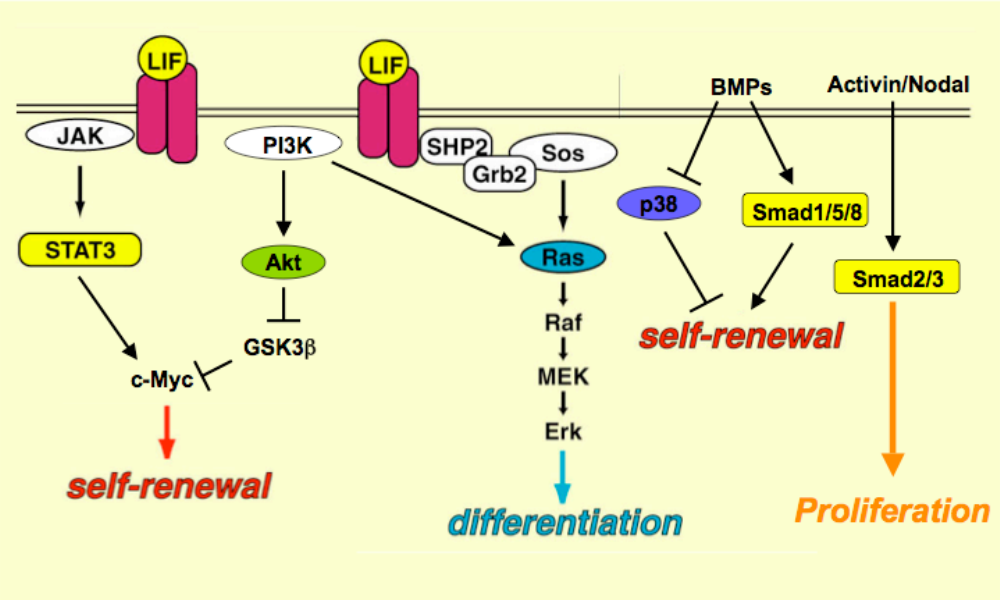


Figure 6. Key signaling pathways required for maintaining pluripotency of ESCs.

LIF signaling activates JAK-STAT3 to induce target genes essential for pluripotency, such as c-myc. C-myc is also regulated negatively by glycogen synthase kinase-3 (GSK3)β via inhibitory phosphorylation. LIF also induces MAP kinase activation, which antagonizes self-renewal and promotes differentiation. BMP signals potentially function in two ways: (i) activation of Smad1/5/8-Id gene and (ii) suppression of p38 MAP kinase. Activin A has been shown to contribute to ESC proliferation but not to pluripotency. (Taken from Ohtsuka 2008 and modified).

Transcription Factors

In order to maintain the stable self-renewal of ESCs, the mechanisms that prevent their differentiation and promote their proliferation must be transmitted to their daughter cells. Thus, the expression levels of the genes that are involved in these mechanisms need to be stably maintained.

A transcription factor network that is stabilized by positive and negative regulation between its components is a good mechanism for maintaining the stable gene

expression patterns that determine a particular cell phenotype (von Dassow, Meir et al. 2000).

Oct3/4, Nanog and Sox2 transcription factors are essential for ESC self-renewal and differentiation. Some of these factors are expressed specifically in pluripotent cells, such as Oct3/4 and Nanog, and are switched ON/OFF by input environmental signals. They are also regulated by themselves. When these genes are expressed, the self-renewal genes are activated, and the differentiated genes are repressed. So ESCs can maintain their pluripotency.

Oct3/4

Oct3/4 (encoded by Pou5f1 gene) is a POU domain-containing transcription factor that binds to an octamer sequence, ATGCAAAT. Oct3/4 acts as gatekeeper to prevent ESC differentiation. In fact Oct3/4 is highly expressed in ESCs, and its expression decreases when these cells differentiate and lose pluripotency. The precise levels of this gene are required to maintain the ESC state. Loss of Oct3/4 causes inappropriate differentiation of ESCs into trophectoderm.

Oct3/4 has been reported to directly prevent differentiation towards trophectoderm by interacting with Cdx2 (a trigger for trophectoderm differentiation), to form a repressor complex. This complex interferes with the autoregulation of these two factors, giving rise to a reciprocal inhibition system that establishes their mutually exclusive expression (Niwa, Toyooka et al. 2005).

LIF does not appear to regulate Oct3/4, and Oct3/4 does not appear to regulate Jak-STAT signaling, suggesting that the Oct3/4 pathway is a parallel pathway for maintaining ESC self-renewal. Many of Oct3/4 target genes also contain STAT-binding sites, suggesting that the two transcription factors may cooperate in ESCs. In contrast with its target genes (Fgf4, Utf1, Opn, Rex1/Zfp42, Fbx15, and Sox2

(Nishimoto, Fukushima et al. 1999; Tomioka, Nishimoto et al. 2002; Zeng, Miura et al. 2004), little is known about upstream regulators.

Nanog

Nanog is a homeobox-containing transcription factor with an essential role in maintaining the pluripotent cells of the ICM and ESCs. It is expressed in pluripotent cells, and is absent in differentiated cells. Nanog disruption in ESCs results in differentiation to primitive endoderm (Liu, Lu et al. 2007).

The mechanism through which Nanog regulates stem cell pluripotency remains entirely unknown. Based on the differences in gene expression between wild-type and Nanog null cells, it has been proposed that Nanog regulates pluripotency mainly as a transcription repressor for downstream genes that are important for cell differentiation such as Gata4 and Gata6 (Chambers, Colby et al. 2003; Mitsui, Tokuzawa et al. 2003). However, Nanog can also activate the genes necessary for self-renewal such as Rex1 and Oct3/4 (Pan, Li et al. 2006). Furthermore, Nanog promoter has been suggested as a direct target of the Oct3/4/Sox2 complex through ChIP analysis, in vitro-binding experiments and RNAi-mediated knockdowns (Boyer, Lee et al. 2005; Kuroda, Tada et al. 2005; Rodda, Chew et al. 2005).

The gatekeeper function of Nanog might not be restricted to preventing the differentiation of ESCs into primitive endoderm, as it has been reported that Nanog also blocks neuronal differentiation induced by the removal of LIF and BMPs from serum-free culture (Ying, Nichols et al. 2003). In addition, Nanog can also reverse mesoderm specification by repressing brachyury, which encodes the mesoderm-specific T-box transcription factor T. This factor directly activates Nanog expression, indicating that negative feedback is involved in the balance between

self-renewal and mesodermal differentiation (Suzuki, Raya et al. 2006). Thus, Nanog can block primitive endodermal differentiation, neuronal differentiation and mesodermal differentiation under different culture conditions.

Sox2

Sox2 (SRY-related HMG box2), a member of the HMG-domain DNA-binding protein family, transcription factor occupies an important position in the maintenance of the pluripotent transcription factor network. Sox2 is known to co-operate with Oct3/4 in activating Oct3/4 target genes (Yuan, Corbi et al. 1995). To date, ES-specific enhancers that contain binding sites for Oct3/4 and Sox2 have been identified in several genes, including Nanog (Boyer, Lee et al. 2005; Kuroda, Tada et al. 2005; Rodda, Chew et al. 2005) and Rex1, that is also directly regulated by Nanog (Shi, Wang et al. 2006). Interestingly, both Oct3/4 and Sox2 possess enhancers that are activated by the Oct3/4-Sox2 complex in a stem-cell-specific manner (Tomioka, Nishimoto et al. 2002; Chew, Loh et al. 2005; Okumura-Nakanishi, Saito et al. 2005). Knockdown of Sox2 in mouse ESCs induces differentiation into multiple lineages, including trophectoderm, indicating its functional importance in the maintenance of pluripotency (Ivanova, Dobrin et al. 2006).

Thus Oct3/4, Sox2, and Nanog may be the main transcriptional factors in regulating ESC pluripotency. Recent studies have enabled the construction of transcriptional regulatory networks in ESCs that provide a foundation for understanding how these factors control pluripotency and influence subsequent differentiation events. These key transcription factors have also been identified that form an intrinsic core-regulatory circuit that maintains ESCs in the pluripotent state in vitro (Figure 7).

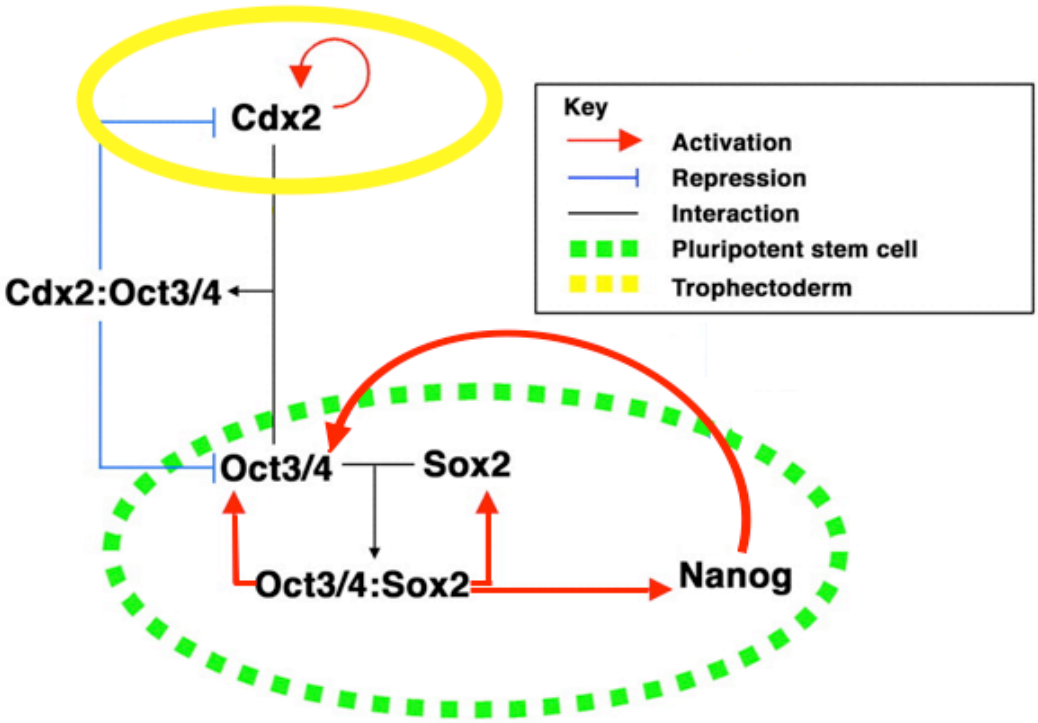


Figure 7. A trascription factor network to control ESC self-renewal and differentiation.

Transcription factor networks for pluripotent ESCs (green) and trophectoderm (yellow). Positive-feedback loops between Oct3/4, Sox2 and Nanog maintain their expression to promote continuous ESC self-renewal. Cdx2 is autoregulated and forms a reciprocal inhibitory loop with Oct3/4, which acts to establish their mutually exclusive expression patterns. A combination of positive-feedback loops and reciprocal inhibitory loops converts continuous input parameters into a bimodal probability distribution, resulting in a clear segregation of these cell lineages (Adapted by Niwa 2007).

Cell-cycle regulators

In contrast to somatic cells, ESCs have an abbreviated cell cycle. In fact, these cells divide very rapidly and transit the cell cycle once every 8-12 h, depending on the cell line and cultivation conditions. This feature, which may contributes to the self-renewal ability of these cells, depends on a very short G1 phase (only 1-2 h) (Stead, White et al. 2002; Becker, Ghule et al. 2006).

Somatic cells require mitogen activated cyclin dependent kinases (CDK) 4 and 6, cyclins (D and E) and members of the retinoblastoma (Rb) tumor suppressor protein family to transit from G1 into the S phase. Instead in ESCs a constitutively active CDK2-cyclin E activity drives cell division. Moreover, ESCs express very low levels of D-type cyclins, whose levels increase dramatically during in vitro and in vivo differentiation (Liu, Lu et al. 2007) and possess inactive forms of members of the retinoblastoma (Rb) tumor suppressor protein family. The only cell cycle regulators that show cell cycle-dependent expression in ESCs are CDK1 and cyclin B1, both of which show regulation during the G2 phase of the cell cycle (Stead, White et al. 2002).

After DNA damage, somatic cells undergo cell-cycle arrest in the G1 phase to get rid of the damaged DNA, while ESCs choose apoptosis or differentiation instead of going through the checkpoint between the G1 phase and S phase. Indeed the mechanisms involved in G1/S checkpoint in somatic cells cycle (chk1/chk2/Cdc25A and p53-p21) are not functional in ESCs (Aladjem, Spike et al. 1998; Bartek and Lukas 2001; Asahi, Otsu et al. 2004; Hong and Stambrook 2004).

Recent studies demonstrated that p53 plays an important role in ESC response to low levels of DNA damage that are normally introduced in rapidly dividing cells. It was proposed that p53 coordinate ESC self-renewal capability and DNA damage responses by directly suppressing Nanog expression and therefore inducing differentiation (Lin, Chao et al. 2005). So p53 is another mechanism for repressing Nanog expression when ESCs differentiate (Xu 2005).

When relatively high levels of DNA damage have been introduced ESCs undergo apoptosis. In fact the function of p53 in ESCs may also be to trigger apoptosis to eliminate the cells with damaged DNA (Fluckiger, Marcy et al. 2006). So p53 may

activate apoptosis or differentiation pathways depending on stress type and severity. For example in response to endogenous reactive oxygen species (ROS), ESCs undergo mitochondrial-dependent apoptosis, triggered by p53 translocation to mitochondrial instead of to nucleus. In this context Sirt1, a p53 deacetylase, plays a fundamental role in p53 subcellular localization because blocks nuclear translocation of p53, thus inhibiting p53-mediated suppression of Nanog expression observed in Sirt1-/- ESCs (Han, Song et al. 2008).

Epigenetic Modifications

Differentiation of ESCs from pluripotent to developmentally more restricted states is accompanied by global changes in genome expression patterns. Genes active in earlier progenitors are gradually silenced at developmentally later stages, and subsets of cell type-specific genes are turned on. This progression is the result of selectivity active expression of transcription factors in concert with epigenetic modification.

The idea that the epigenetic modifications contribute to determine pluripotency or differentiation of ESCs is supported by the evidence that the inactivation of components (Snf2b, Snf2h, Snf5, etc.) of the chromatin remodeling system affects the viability of F9 EC cells (Bultman, Gebuhr et al. 2000; Klochendler-Yeivin, Fiette et al. 2000; Stopka and Skoultchi 2003).

Epigenetic modifications refer to meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself (Levenson and Sweatt 2005). In mammals, epigenetic processes mainly include DNA methylation, histone modification and noncoding RNA-mediated processes. Epigenetic features in ESCs are different from the somatic cells: the pluripotent epigenome keeps the chromatin structure open to allow rapid genetic regulation (Figure 8).

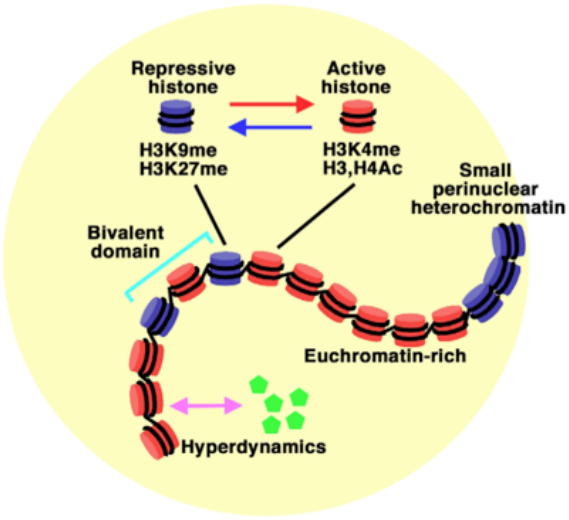


Figure 8. Characteristics of pluripotent epigenome.

Epigenetic features of the pluripotent cell nucleus. The volume of the nucleus is larger than that of a differentiated cell as a result of the relaxed chromatin structure. Small regions of perinuclear heterochromatin exist, but most of the chromatin exists as euchromatin, bearing histone marks associated with transcriptional activity. The hyperdynamics of chromatin proteins (green) might contribute to the maintenance of euchromatin. Bivalent domains are also a feature of the pluripotent epigenome, in which active histone marks (such as H3K4me) are flanked by transcriptionally repressive histone marks (such as H3K9me) (Niwa 2007).

In fact, ESCs chromatin displays characteristics of loosely euchromatin, such as an abundance of acetylated histone modifications and increased accessibility to nucleases (Meshorer and Misteli 2006).

ESCs undergo functionally important global and gene-specific remodeling of chromatin structure during in vitro differentiation. Increase in the heterochromatin marker 3methylated residue K9 of histone H3 (3meH3K9) and a decrease in the global levels of acetylated histones H3 and H4 (a euchromatin marker) were observed during RA-induced differentiation (Lee, Hart et al. 2004). Furthermore, histone deacetylase (HDACs) and methyl-CpG-binding protein (MECPs) are

expressed in ESCs and their levels are dynamically regulated as cells undergo differentiation (Rao 2004).

DNA methylation

DNA methylation is a covalent modification of cytosine at position C5 in CpG dinucleotides. In mammals, over 70% of CpG dinucleotides are methylated and nearly all DNA methylation occurs on CpG dinulceotides that are underrepresented in the genome with the exception of CpG islands (CpG clusters). Unmethylated CpG islands are usually found in the promoters and in the first exons (Jones and Takai 2001). DNA methylation is catalyzed by several DNA methyltransferase (DNMTs). The de novo establishment of DNA methylation relies on DNMT3a and 3b, whereas the maintenance of DNA methylation depends on DNMT1 that specifically recognizes semi-methylated DNA and methylates the remaining strand (Bestor 2000). DNMT2 does not have methyltransferase activity and its function is not clear (Okano, Xie et al. 1998). DNMT3L (DNMT3-like) lacks enzymatic activity, but could act as a cofactor for DNMT3a and 3b and modulate their enzymatic activity.

Mammalian DNA methylation has been implicated in a wide range of cellular functions, including tissue-specific gene expression, cell differentiation, cell fate determination, genomic imprinting, and X chromosome inactivation (Bird 2002).

DNA methylation is a fundamental process that ESCs use to silence genes required for induction of differentiation. This was demonstrated in experiments in which ESCs, deficient either in the DNA methyltransferases DNMT1, both DNMT3a and DNMT3b, or the CpG island-binding protein CGBP that binds to non-methylated DNA, showed severe DNA hypomethylation and a complete differentiation block (Jackson, Krassowska et al. 2004; Carlone, Lee et al. 2005).

Moreover recent exciting discoveries showed that exogenous expression of Oct3/4, Sox2, c-Myc- and Klf4 could induce pluripotency in fibroblasts derived from either embryonic or adult mice or humans (Takahashi, Tanabe et al. 2007; Wernig, Meissner et al. 2007). These induced pluripotent stem cells (iPS) exhibit many in vitro properties as ESCs but a different in vivo pluripotency ability depending on their DNA methylation state. The iPS lines that have high level of DNA methylation pattern at Oct 3/4 promoter exhibit low germ line transmission efficiency, whereas the iPSs that exhibit low Oct3/4 promoter methylation similar to that of ESCs have high germ line incorporation efficiency (Okita, Ichisaka et al. 2007).

Histone modifications

Histone modifications encompass a range of modifications, including acetylation, methylation, phosphorylation, ADP ribosylation and ubiquitylation, that extend the information content of the underlying DNA sequence and confer unique transcriptional potential. Histone modifications can have both repressive and activating functions. The best-understood modifications are the trimethylation of the Lys9 and Lys27 residues of histone H3 (3meH3K9 and 3meH3K27), which have repressive functions, and 3meH3K4 and H3K9 acetylation (H3K9ac), which are associated with active genes.

In addition, mono-, di-, and trimethylation at the same lysine residues lead to different levels of gene activation or repression and are involved in distinct cellular pathways.

The initial histone modification studies were focused largely on histone acetylation that is catalyzed by two opposing enzymes, histone acetyltransferease (HAT) and histone deacetylase (HDAC). To date, at least 6 HATs and HAT complexes and 18 HDACs have been identified in mammals (Lee and Workman 2007; Xu, Parmigiani

et al. 2007). Similarly, histone methylation and demethylation enzymes are also involved in cellular functions. One of the best studied histone methylation is the methylation of H3K9 by SUV39h, ESET and G9a. In ESCs, proper H3K9 methylation is critical for regulated expression of imprinted genes (Mikkelsen, Ku et al. 2007). Recently, the discovery of histone demethylases has greatly advanced our understanding in chromatin-mediated gene regulation (Klose and Zhang 2007).

A series of recent studies have reveled that ESCs possess certain novel epigenetic features consisting in a complex of proteins that act mainly to stabilize a repressive chromatin structure. These complex of proteins are Polycomb group (PcG) proteins required for early developmental gene expression (Pasini, Bracken et al. 2004). They can facilitate oligomerization, condensation of chromatin structure, and inhibition of chromatin remodeling activity. PcG proteins bind to a large set of genes composed of transcriptional regulators and signaling factors, which promote ESC differentiation. Many of the target genes were de-repressed in the absence of the PcG components. Thus, PcG proteins make an essential contribution to the ESC state by repressing the premature expression of differentiation genes. But when ESCs differentiate, these target genes were preferentially activated. That is to say PcG repress differentiation gene expression in a flexible manner, which can be reversed later by gene-specific and lineage-specific signals (Bernstein, Mikkelsen et al. 2006; Boyer, Plath et al. 2006; Buszczak and Spradling 2006; Lee, Jenner et al. 2006).

PcG proteins form three complexes: Polycomb repressive complex 2 (PRC2), PRC1, and PhoRC (Schuettengruber, Chourrout et al. 2007). PRC2 is involved in the initiation of silencing and contains histone deacetylase and histone H3K27

methyltransferase activities, and PRC1 is implicated in maintaining gene repression (Valk-Lingbeek, Bruggeman et al. 2004).

microRNAs

Transcription factors are essential players in regulating ESC functions but recently, post-transcriptional gene regulation is emerging as another essential regulator of ESC development (Cheng, Tavazoie et al. 2005). In particular it was demonstrated that a crucial role in the post-transcriptional regulation of gene expression is played by microRNAs (Bartel 2004).

microRNAs are 20–25 nucleotide (nt) non-coding RNAs that bind to the 3' untranslated region (UTR) of target mRNAs through an imperfect match to repress their translation and stability (Rana 2007). This is achieved by forming a ribonucleoprotein complex, called the RNA-induced silencing complex (RISC), that contains an Argonaute family member. microRNAs are derived from precursor transcripts, called primary microRNAs (pri-microRNAs), that are first processed in the nucleus into an intermediate form (pre-microRNAs) by the Microprocessor protein complex; this contains the Drosha and DiGeorge syndrome critical region gene 8 (DGCR8; also known as Pasha) proteins. The pre-microRNAs are then translocated by the exportin5 exportin 5–RanGTP shuttle system into the cytoplasm, in which they are further processed by Dicer, an RNase III-like enzyme, into mature microRNAs (Figure 9), (Gangaraju and Lin 2009). microRNAs are also produced by two non-canonical, Drosha- or Dicer-dependent pathways. In the first pathway, the early processing step is done by spliceosome and by a debranching enzyme that yields a short hairpin that is ready for further processing by Dicer. These non-canonical microRNAs have been termed mirtrons (Okamura, Hagen et al. 2007; Ruby, Jan et al. 2007).

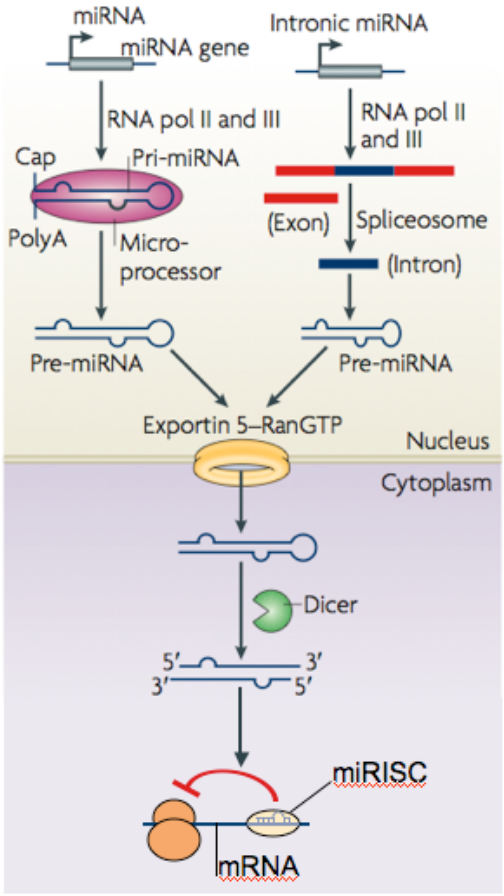


Figure 9. Biogenesis and regulatory features of the microRNA patways.

MicroRNA (microRNA) genes are transcribed by RNA polymerases II and III into primary transcripts called the pri-microRNAs. These are processed into pre-microRNAs in the nucleus by a Microprocessor complex, which contains the RNase III enzyme Drosha and the double-stranded RNA-binding protein DiGeorge syndrome critical region gene 8 (DGCR8; also known as Pasha). Some microRNAs, called mirtrons, have also been shown to be generated from introns that bypass Drosha requirement. Pre-microRNAs are then transported by exportin 5, which is a karyopherin, and RanGTP into the cytoplasm, where they are further processed by the RNAse III enzyme Dicer. This results in double-stranded 20–25 nucleotide (nt) intermediates with 2 nt overhangs on the 3' end. One of the RNA strands is then loaded by Dicer into an RNA-induced silencing complex (RISC), which contains an Argonaute-subfamily member, that then targets the 3' untranslated region of the target mRNAs by an imperfect match between the microRNA and the mRNA, to repress translation. However, it has been recently shown that microRNAs can activate translation of target mRNAs (Gangaraju and Lin 2009).

In the second pathway, short hairpin RNAs (shRNAs) are processed by unknown nucleases into pre-microRNAs and are further processed into microRNAs by Dicer. microRNAs that are derived in this way have been termed endogenous shRNA-derived microRNAs (Babiarz, Ruby et al. 2008).

Recently, the ESCs and microRNA fields have converged with the identification of ESC specific microRNAs (Houbaviy, Murray et al. 2003). In addition to canonical microRNAs, mirtrons and shRNA-derived microRNAs have also been identified in ESCs (Babiarz, Ruby et al. 2008). It is now clear that microRNAs provide a new dimension to the regulation of ESC functions. Based on their function in translational attenuation, microRNAs seem to regulate ESC fate and behavior by fine-tuning the protein levels of various factors that are required for stem cell or niche cell functions.

The overall function of the microRNA pathway in ESCs has been evaluated in humans and mice by analyzing the phenotypes of DGCR8 and Dicer mutants, respectively, as these two proteins have crucial roles in the production of mature microRNAs.

DGCR8-deficient ESCs exhibit either delayed or reduced expression of differentiation markers, as well as delayed kinetics of cell cycle progression. Most DGCR8-deficient ESCs are arrested in the G1 phase, which indicates that the main function of the microRNA pathway is to promote the ESC cycle at the G1–S-phase transition. In addition, DGCR8-null ESCs exhibit differentiation defects as they fail to stably silence the expression of self-renewal markers, such as Oct3/4, Rex1, Nanog and Sox2. In support of this, injected DGCR8-mutant ESCs into host mice fail to differentiate into different germ layers, in contrast with injected wild-type ESCs that can give rise to teratomas, tumours that can give rise to cell types of all three germ layers (Wang, Medvid et al. 2007).

The crucial role of microRNAs in ESCs was also demonstrated by Berstein and co-workers, which reported that Dicer is essential for early mouse development, as its absence leads to embryonic lethality (Bernstein, Kim et al. 2003). In fact, in their study Berstein and co-workers demonstrated that after Dicer +/- mice intercrossing, of all mice born none were homozygous mutants, suggesting that the Dicer1-deficient mice are not viable. Moreover they did not obtain Dicer -/- embryos older than E7.5, suggesting that Dicer is essential in early stage of development. Another important evidence was that at E7.5, the number of Dicer1-null embryos was 50% lower than expected from mendelian ratios, probably because a fraction of the embryos died at an even earlier stage of development. Finally they reported that Dicer1-deficient embryos had also defects in differentiation marker expression. Indeed they did not observe brachyury expression, suggesting that development of Dicer1 mutants is arrested before the body plan is configured during gastrulation.

Whereas the Dicer and DGCR8 ESC mutants provide an overall assessment of the function of the microRNA pathway in ESCs, cloning and deep sequencing of microRNAs from ESCs have revealed the identity of the specific microRNAs that are expressed in ESCs and might function in self-renewal and differentiation of ESCs. For example, in mice, the cloning and sequencing of small RNAs revealed that the miR-290–295 cluster is specific to ESCs and that its levels decreases as ESCs differentiate. In a simplified way, this indicates that the miR-290–295 cluster has specific roles in maintaining pluripotency (Houbaviy, Murray et al. 2003). However, more recent studies indicate that the real role of miR-290–295 is to induce differentiation. Indeed this cluster achieves this role by stably targeting repressors of DNMTs, such as retinoblastoma like-2 (RBL2), which methylate CpG islands, leading to stable epigenetic repression of Oct3/4 transcription (Benetti,

Gonzalo et al. 2008; Sinkkonen, Hugenschmidt et al. 2008). However, it remains to be addressed whether and how this epigenetic repression of Oct3/4 transcription is sufficient to promote differentiation, given that DNMTs are global regulators of CpG island methylation status in the entire genome.

By contrast, levels of miR-21 and miR-22 increase substantially following the induction of differentiation, which indicates that these microRNAs might have important roles in stem cell differentiation. A recent study shows that miR-21 has potential binding sites in the 3' UTRs of the mRNAs that encode for Nanog, Sox2 and possibly Oct3/4 and that the transcription of miR-21 is regulated in ESCs by a transcriptional repressor called the RE1-silencing transcription factor REST, which directly interacts with cis elements upstream of the miR-21 gene (Singh, Kagalwala et al. 2008).

Other differentiation-related microRNAs, miR-134, miR-296 and miR-470, were recently found to target coding regions of Nanog, Sox2 and Oct3/4 to promote differentiation (Tay, Zhang et al. 2008).

Although different studies have revealed the specific microRNAs that are expressed in ESCs only in few cases it was identify the function in self-renewal and differentiation of ESCs of the specific microRNAs. Indeed while it is becoming increasingly evident that microRNAs have crucial roles in the self-renewal and differentiation of ESCs, research on the role of microRNAs in ESCs is still in its infancy, in particular for what concerns the role of microRNAs in assisting ESC differentiation. In fact microRNAs exhibit a high degree of stage- and tissue-specificity and the expression profile of microRNAs strictly depends on the differentiation program occurring. As consequence specific roles of the microRNAs in the control of ESCs specific fate are still largely unknown and have to be investigated.

Result

microRNA expression profile

In order to identified microRNAs assisting ESC differentiation and to characterize their functional role we analyzed changes in the expression levels of microRNAs as a consequence of ESC differentiation, by collecting RNA samples from mouse E14 cells grown in undifferentiated conditions and after 48 and 96 hours in differentiation conditions (See Methods and Figure 10).

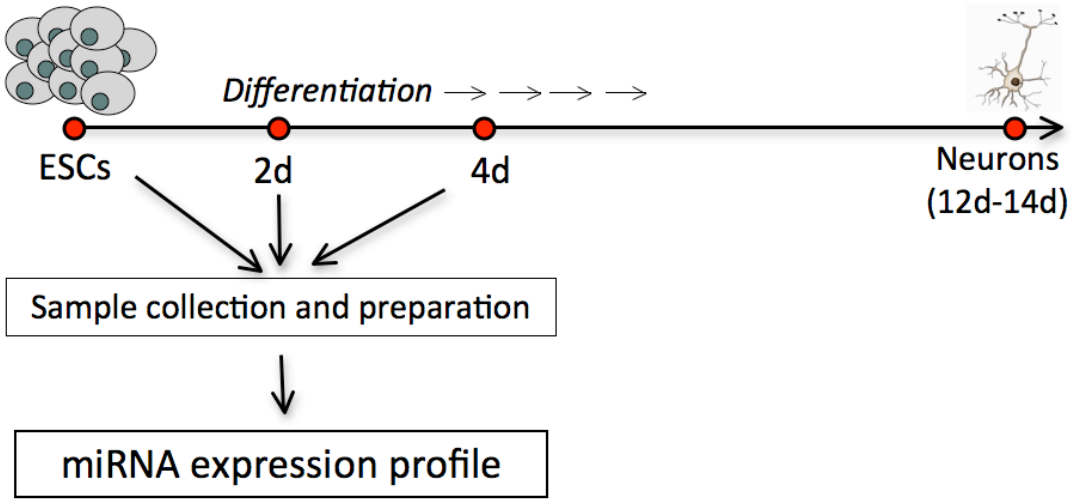


Figure 10. Profiling workflow.

ESCs were grown in undifferentiated conditions and in differentiation culture medium for two and four days. Then RNA samples were collected from these cells and used for microRNA expression analysis.

We chose to collect RNA samples at two early time points of the differentiation program because we were interested in the characterization of microRNAs controlling the first phases of differentiation decision.

The collected RNA samples were first validated and then used to measure the abundance of microRNAs by Taqman procedure. To confirm that at these two time points the cells have undergone a differentiation program we measured the abundance of stemness and differentiation markers by using real-time PCR procedure. As shown in figure 11, stemness markers, such as Oct4, Nanog and Rex1 decreased at 2 and 4 days of differentiation, while early differentiation markers, such as Fgf5 (Fibroblast growth factor 5, embryonic visceral endoderm marker) and Sox1 (neuroectodermal marker) appeared specifically upon differentiation induction.

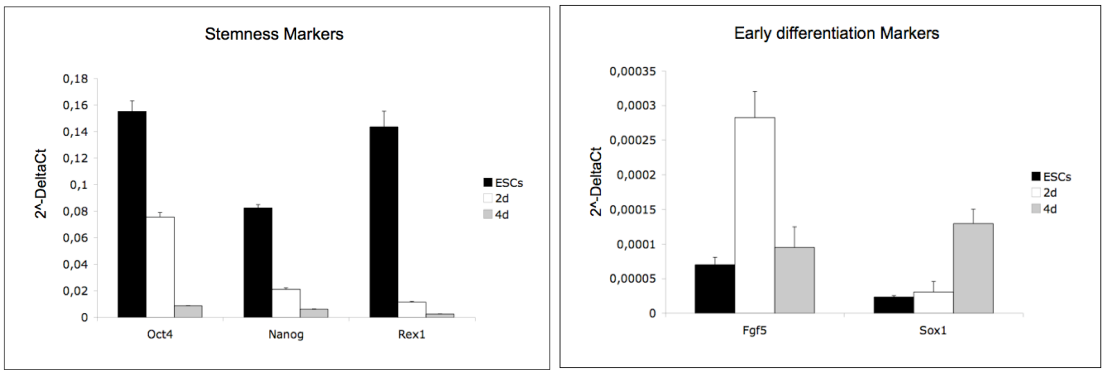


Figure 11. RNA sample validation.

Stemness and early differentiation marker evaluation in undifferentiated (ESCs) and in 2 and 4 days differentiating ESCs.

To perform TaqMan microRNA profiling we used two pre-configured microfluidic cards, each containing 384 pairs of dried TaqMan primers and a probe for the detection of each microRNA annotated in the Sanger miRBase version 10 database (Figure 12).

This procedure provided specificity for the mature form of microRNAs, a highly specific quantization and also a single-base discrimination among very similar microRNAs.

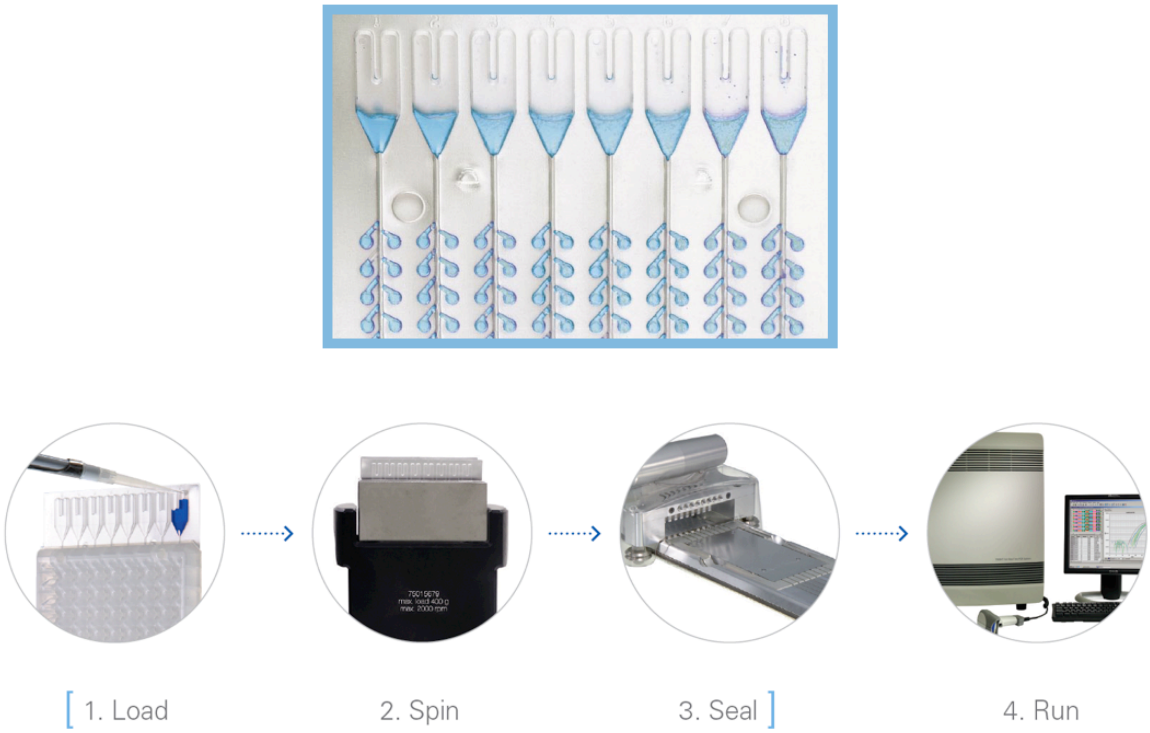


Figure 12. microRNA quantization by using TaqMan Arrays.

The TaqMan Array functions as an array of reaction vessels for the PCR step. Typically, the wells of the TaqMan Array contain dried TaqMan primers and probe for real-time amplification of user-specified microRNAs. Relative levels of microRNA expression are determined from the fluorescence data generated during PCR. 1) Synthesis of cDNA from total RNA samples and loading into the fill reservoirs. 2) Centrifugation of cards to distribute the cDNA samples to the reaction wells. 3) TaqMan Array sealing. 4) Run on the 7900HT Applied Biosystem instrument. (Taken from Applied Biosystem).

The results of TaqMan microRNA expression profile allowed us to cluster microRNAs in five groups. We reported: 78 microRNAs decreased upon the induction of ESC differentiation (Table1); 29 microRNAs undetectable in undifferentiated ESCs and expressed upon the induction of the differentiation program (Table 2); 162 microRNAs already expressed in ESCs and increased upon differentiation induction (Table 3, 4 and 5); 104 microRNAs undetectable

both in undifferentiated and differentiated ESCs (Table 6); 105 microRNAs unmodified upon the induction of differentiation (Table 7).

The analysis of groups of microRNAs whose profiling was already described in ESCs allows us to validate our data. In fact, we verified if there was coherence between our microRNA expression data and published microRNA expression profiles. In particular, in the group of microRNAs decreased upon the induction of ESC differentiation we found microRNAs belonging to the miR-290 cluster, already known to be specific of the undifferentiated state of ESCs. We found that this cluster of microRNAs quickly decreased upon ESC differentiation. Opposite to this group, we found that microRNAs belonging to the miR-302 family are strongly induced upon the activation of the differentiation program, as reported in the literature (Figure 13). So we can conclude that there was a good degree of correlation between our data and literature data.

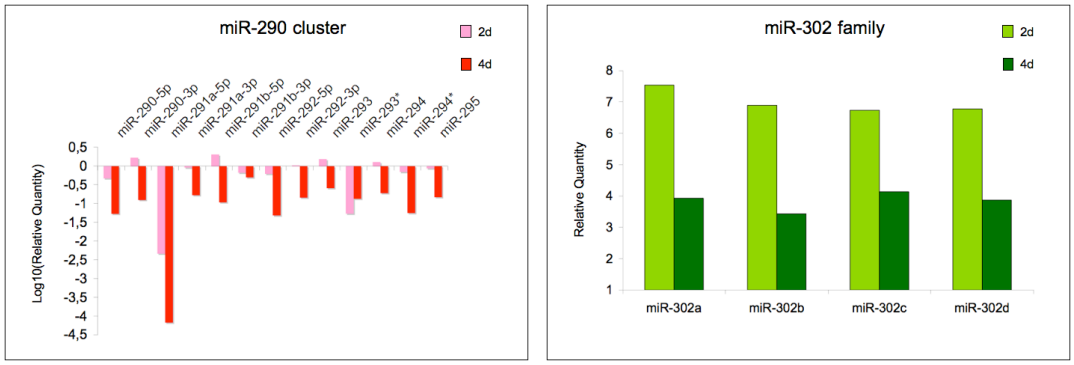


Figure 13: Expression profile of microRNAs, belonging to miR-290 cluster and miR-302 family.

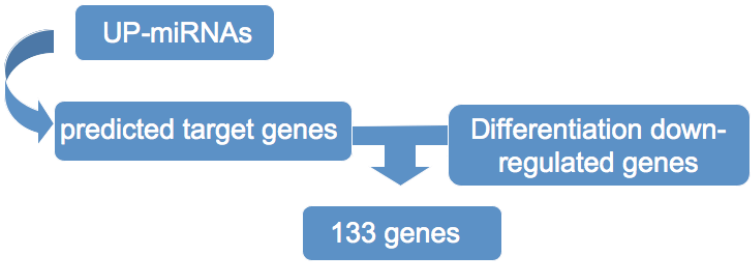
The level of each microRNA, at day 2 and 4 of differentiation, is expressed as relative quantity of its level (set to 1) in undifferentiated ESCs. Because the expression levels of microRNAs, belonging to miR-290 cluster, were plotted as $\log_{10}$(RelativeQuantity), microRNA expression levels in undifferentiated ESCs appear as 0. All fold-expression changes were calculated using the equation $2^{-\Delta ct}$.

microRNA target prediction

When ESCs are grown in undifferentiated conditions, stemness-related genes are activated while differentiation-related genes are repressed. When differentiation starts, stemness genes have to be repressed and differentiation genes activated. So we speculated that microRNAs induced after the induction of differentiation can work as integral component of the gene regulation circuitry and therefore can contribute to the down-regulation of stemness genes.

So we focused on the up-regulated microRNAs upon ESC differentiation induction and performed for them the target prediction by using TargetScan database, version 5.1. To select candidate targets, we compared this list of predicted targets with available list of mRNAs down-regulated upon ESCs differentiation (Loh, Wu et al. 2006), because we considered that RNAs targeted by a microRNA are generally degradated (Liu, Bezprozvannaya et al. 2008; Viswanathan, Daley et al. 2008). This approach allowed us to observe that many targets of up-regulated microRNAs are significantly downregulated in differentiating ESCs (Figure 14 and Table 8).

In this list of candidate targets, we noted some mRNAs encoding proteins involved in chromatin remodeling, a crucial event in ESC differentiation. Among them, we selected three candidates whose functions are particularly relevant for ESC differentiation. They are Smarca5, putative target of miR-100, Jarid1b, candidate target of miR-137, and Sirt1, possible target of miR-204 and miR-217. Sirt1 was previously demonstrated to be target of the miR-34a (Han, Song et al. 2008), a microRNA presents in our list of up-regulated microRNAs (see Table 3) but that did not appear as targeting Sirt1 in TargetScan database version 5.1.



ABCD4	EFHC2	JARID1B	NFYB	QDPR	SPRY2
ABCG4	EGLN3	JARID2	NME7	RARG	SPRY4
ADAM23	EIF4A2	JMJD1A	NOL8	RFX2	SRM
AES	ELAVL2	KLF4	NR5A2	RIF1	SSTR1
ARID5B	ENAH	KLF5	NRP2	RNF125	STMN1
B3GNT1	EPS8	LAPTM5	NUP35	RNF138	STOML1
BCAT2	FGF4	LRAT	OTX2	RNMT	SYCP3
BCLAF1	FN1	LRBA	PADI2	RPS16	SYNGR1
BRP44L	FOXD3	LRRC2	PARP16	SDAD1	TCEA3
BSPRY	FZD4	MAT2A	PAX6	SFRS3	TCF15
CACNB4	GCLM	MCF2	PCOLCE2	SFRS7	TFRC
CACYBP	GFPT2	MGA	PDCD10	SHMT1	TLE4
CALML4	GLDC	MKRN1	PECAM1	SIRT1	TNFSF11
CHCHD4	GNPNAT1	MRAS	PFKP	SLC25A5	TRIM2
CREB1	GSTM3	MSC	PHC1	SLC29A1	TRIM25
CTBP2	GSTT2	MTF2	PIGA	SMAD7	TWISTNB
CTH	GTF2H1	MYB	PLA2G10	SMARCA5	UBTF
DEF6	HAT1	MYBL2	PML	SNX10	UBXD4
DERL3	HELLS	MYNN	PMM1	SOCS2	VEGFC
DTX1	HK2	MYST4	POU5F1	SOCS3	WDR35
EED	ICAM1	NCL	PSAT1	SPOCK2	XBP1
EEF1E1	INA	NEFH	PTP4A3	SPP1	XRCC5
					ZFP42

Figure 14. Strategy used to identify putative targets of the up-regulated microRNAs and list of genes down-regulated in differentiating ESCs and candidate targets of the up-regulated microRNAs.

miR-100, miR-137, miR-204 and miR-217 belong to the group of microRNAs undetectable in undifferentiated ESCs and expressed upon the induction of the differentiation program (Figure 15), while miR-34a belongs to the group of microRNAs expressed in ESCs and increased upon the induction of differentiation (Table 3). For miR-100 and miR-137 we confirmed TaqMan expression profile by Northern blot Analysis (Figure 15).

The three putative targets of the microRNAs that we have selected are considered key molecules in ESC functions. Smarca5, also known as Snf2h, is a member of the SWI/SNF family of proteins and a component of the chromatin remodeling and spacing factor RSF (Babiarz, Ruby et al. 2008). Smarca5 is required for survival of blastocyst-derived stem cells *in vitro* and its knockout results in early arrest of embryonic development, before implantation, suggesting that it is required for the proper development of the ICM (LeRoy, Loyola et al. 2000).

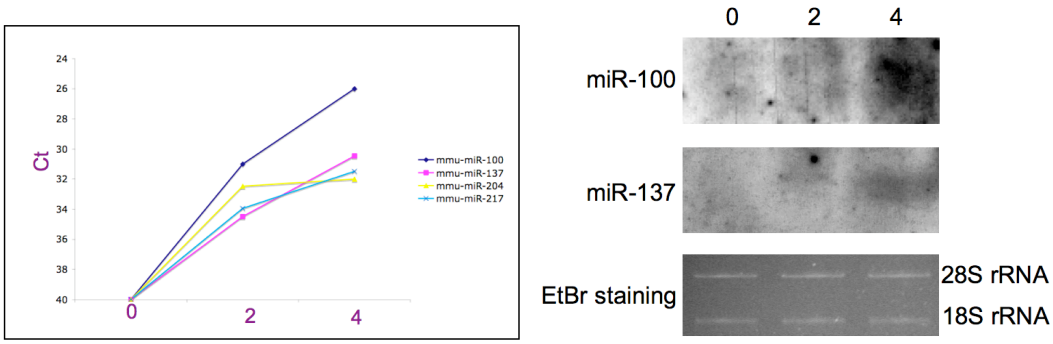


Figure 15. miR-100, miR-137, miR-204 and miR-217 expression profile in differentiating ESCs.

Left panel: TaqMan microRNA expression profile in ESCs and differentiating ESC derivatives. Right panel: Northern blot analysis in ESCs and differentiating ESC derivatives. 0, 2 and 4 are days after differentiation. Gel was stained with Ethidium bromide for loading control.

Similarly, Jarid1b, also known as KDM5b, is a histone H3K4 demethylase (Stopka and Skoultchi 2003) that is expressed in ESCs and in the very early steps of embryonic development. Its constitutive expression in ESCs prevents differentiation and sustains the expression of undifferentiated ESC markers (Seward, Cubberley et al. 2007). The third candidate target, SIRT1, a well known histone and p53 deacetylase, is required in ESCs to maintain high levels of responsiveness to endogenous reactive oxygen species. In particular Sirt1 regulates apoptosis and Nanog expression in ESCs by controlling p53 subcellular localization in response to reactive oxygen species (Babiarz, Ruby et al. 2008).

Smarca5, Jarid1b and Sirt1 are targets of miR-100, miR-137 and miR-34a, respectively

We confirmed by western blot analysis that the expression levels Sirt1, Smarca5 and Jarid1b are significantly reduced upon the induction of ESC differentiation (Figure 16).

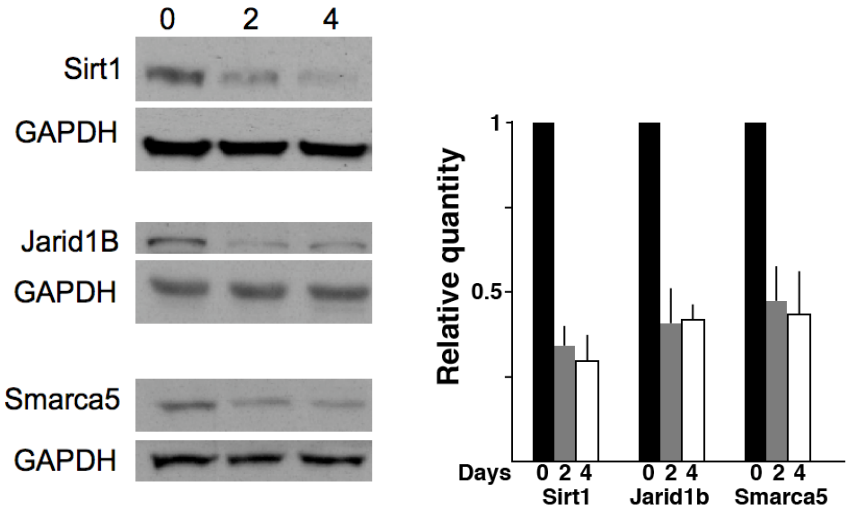


Figure 16. Sirt1, Jarid1b, and Smarca5 are decreased during ESC differentiation.

Protein levels of Sirt1, Jarid1b, and Smarca5 were measured in undifferentiated (ESCs) and 2 and 4 days differentiated ESCs. Significant decrease of protein levels was detected already at 2 days of differentiation. Right panel shows densitometric analysis of western blot. Relative quantity is calculated by assigning the arbitrary value 1 to the amount found in undifferentiated ESCs. Error bars represent SD of three separate experiments.

Thus we addressed whether the overexpression of our selected microRNAs in undifferentiated ESCs was able to induce a decrease of their putative targets at protein level. As shown in figure 17, the protein levels of Smarca5, Jarid1b and Sirt1 are significantly decreased upon overexpression of miR-100, miR-137 and miR-34a, respectively, while overexpression of miR-204 and miR-217 had no effects on Sirt1 protein.

Smarca5, Jarid1b and Sirt1 are direct targets of miR-100, miR-137 and 34a, respectively

We addressed whether miR-100, miR-137 and 34a were able to bind directly Smarca5, Jarid1b and Sirt1 3'UTR, respectively.

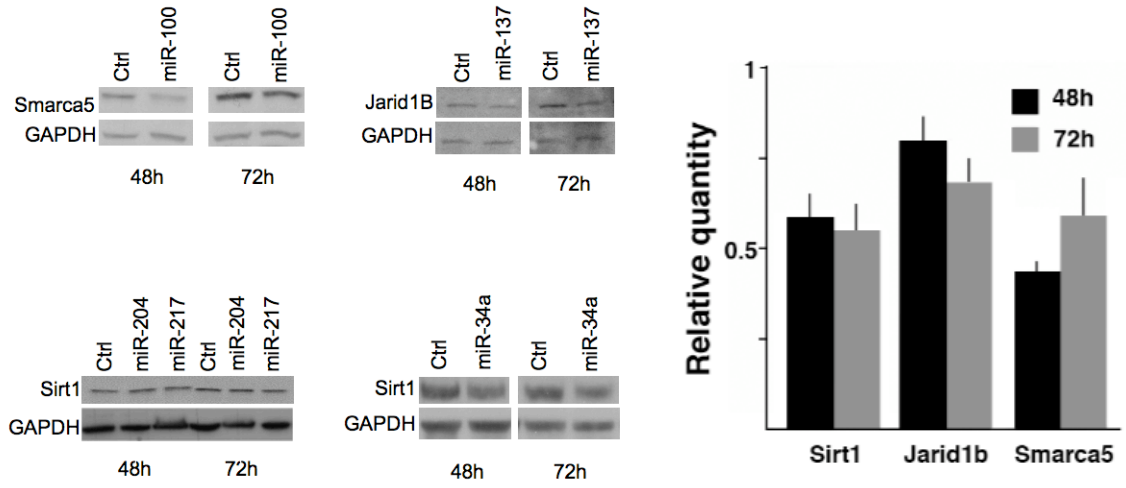


Figure 17. miR-100, miR-137 and miR-34a target Smarca5, Jarid1b and Sirt1 respectively.

Smarca5, Jarid1b and Sirt1 are suppressed by microRNA overexpression. The decrease of Smarca5, Jarid1b and Sirt1 induced by miR-100, miR-137 and miR-34a, respectively was measured by densitometric analysis of western blot. Relative quantity is calculated by assigning the arbitrary value 1 to the amount found in cells transfected with control microRNA. Standard deviations refer to the valued obtained in three separate experiments. In all the cases the differences vs control microRNA transfected cells is significant with $p < 0.001$.

To do this, we generated constructs encoding luciferase followed by the 3'UTR of the three target mRNAs or the corresponding antisense fragments.

We expected that if miR-100, miR-137 and 34a were able to directly bind the corresponding 3'UTR on luciferase transcript, there should be a luciferase activity inhibition after microRNA overexpression (Figure18).

After the transfection in 293 cells of all our three luciferase constructs, the Luciferase expression was affected by the overexpression of the corresponding microRNA, while in the all cases of Luc-antisense construct transfection the luciferase expression was unaffected (Figure 19).

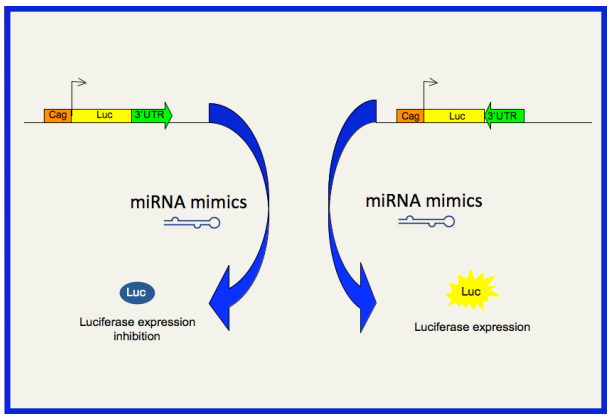


Figure 18. Luciferase assay strategy.

Luciferase constructs encoding the 3'UTR of Smarca5, Jarid1b and Sirt1 mRNAs or the corresponding antisense fragments were generated. We evaluated luciferase activity after miR-100, miR-137 and 34a overexpression.

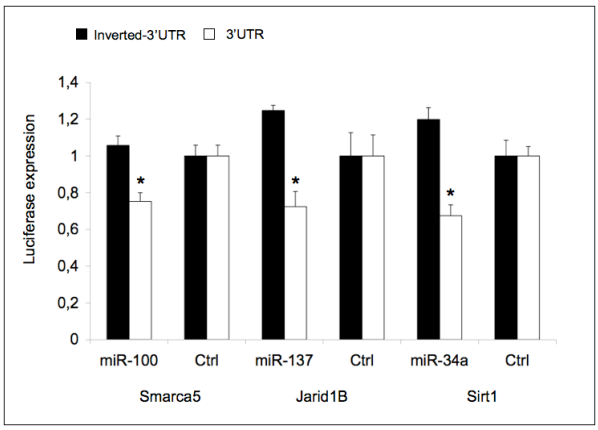


Figure 19. Smarca5, Jarid1b and Sirt1 are direct target of miR-100, miR-137 and miR-34a, respectively.

Luciferase constructs bearing the 3'UTRs or inverted 3'UTRs of the three mRNAs were transfected in HEK293 cells together with the cognate microRNA or control microRNA. Luciferase assays demonstrated a significant ($p<0,01$) downwregulation due to microRNA co-transfection. Constructs bearing the antisense 3'UTRs were unaffected by the microRNA expression. The data were normalized with Renilla luciferase activity.

We then explored whether physiologic increase of endogenous miR-100, miR-137 or 34a was able to induce a downregulation of Luc expression, similar to that observed with the cognate overexpressed microRNAs. To this aim, we generated stable ESC clones by transfection with Luc constructs bearing the direct or reverse

3’ UTR of Smarca5, Jarid1b and Sirt1. The resulting ESC clones were induced to differentiate in order to activate the physiologic microRNA expression. In undifferentiated ESC clones, bearing the 3’ UTR of Smarca5 and Jarid1b, the Luciferase expression was robust and decreased upon the induction of differentiation (Figure 20), thus demonstrating that endogenous microRNAs accumulated in differentiating cells to levels sufficient to target the two corresponding 3’UTR and to downregulate the reporter. As a control, we demonstrated that the Luc constructs bearing the antisense UTRs were transcribed to the same extent before and after the induction of differentiation.

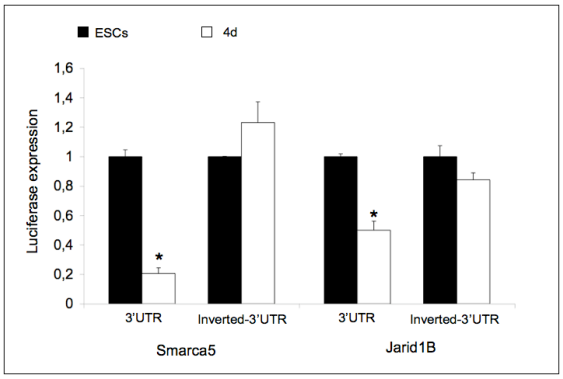


Figure 20. Smarca5 and Jarid1b 3’UTR is targeted by endogenous microRNAs.

ESCs stable clones for luciferase constructs bearing the 3’UTRs or inverted 3’UTRs of Smarca5 and Jarid1b were induced to differentiate. Luciferase expression measured at 4 day of differentiation is strongly ($p<0,01$) decreased specifically in clones with the 3’UTRs. The data are normalized with total protein amount.

The rate of luciferase inhibition in differentiating ESC clones, bearing the 3’ UTR Luc construct for miR-100 and miR-137, is higher than luciferese inhibition in 293 cells, transiently transfected with the same luc constructs and overexpressing the cognate microRNAs. We reasoned that this difference in luciferase inhibition was

due to the difference in luciferase expression in transiently transfected 293 and stable clones. In fact the latter had a much more lower level of luciferase expression.

The undifferentiated Luc-Sirt1 ESC clone showed very low levels of luciferase expression probably due to the presence of miR-34a also in undifferentiated conditions. In this case we could not test the effect of endogenous miR-34a on Luc construct.

miR-100 and miR-137 overexpression affected Smarca5 and Jarid1b signaling pathways respectively

We next analized for Smarca5, Jarid1b and Sirt1 signaling pathways to verify whether the overexpression of their cognate microRNAs affected their biological functions.

Smarca5 is the SWI/SNF-related matrix-associated actin-dependent regulator of chromatin, subfamily a, member 5. Three known families of ATP-dependent chromatin remodeling complexes exist: the SWI/SNF-family, the ISWI family and the Mi-2 family, distinguished by their catalytic ATPase subunit. Smarca5 is the ATPase subunit of the major mammalian ISWI ATP-dependent chromatin remodeling complex. Smarca5 interacts with a variety of DNA-binding factors. In several instances these interactions have been shown to result in transcriptional repression. For example, the NORC complex, containing the ISWI ATPase Smarca5 and the nucleolar protein Tip5, has been shown to repress ribosomal gene transcription by recruiting DNA methyltransferase and histone deacetylase activities to rDNA promoters, to establish structural characteristics of heterochromatin such as DNA methylation, histone hypoacetylation and methylation on lysine 9 (Santoro, Li et al. 2002).

Moreover, by immunoprecipitation and GST pull-down experiments, it was demonstrated that Smarca5 interacts with the de novo DNA methyltransferase, DNMT3b, critical for embryonic development. DNMT3b also interacts with HDAC1, HDAC2, HP1 proteins and Suv39h1. All these proteins co-localize extensively with each other and with DNMT3b and Smarca5 in heterochromatic regions (Geiman, Sankpal et al. 2004). Suv39h1 is believed to be important mediator of histone H3 methylation on lysine 9 (meH3K9) (Lehnertz, Ueda et al. 2003). This histone modification is recognized and bound by HP1 proteins (Eskeland, Eberharter et al. 2007).

There is clear evidence that DNA methylation, supported by DNMT3B interaction with Smarca5, is required to reinforce and maintain meH3K9 patterns (Fahrner, Eguchi et al. 2002; Nguyen, Weisenberger et al. 2002).

Therefore, Smarca5 knockdown may be associated with modifications of chromatin methylation state, resulting in decreased meH3K9.

Moreover it was previously demonstrated that the knockdown of Smarcc1, another member of the ATP-dependent chromatin remodeling complexes, significantly reduced 3meH3K9 in retinoic acid treated ESCs.

All these previous observation supported our idea to verify that Smarca5 is required for changes in ESC chromatin structure and in particular for 3meH3K9. As expected Smarca5 knockdown decreased global 3meH3K9 (Figure 21). Then we tested if overexpression of miR-100 interfered with Smarca5 function in chromatin methylation changes. As shown in figure 21 miR-100 overexpression inhibited the 3meH3K9 at the same level of the shRNA specific for Smarca5.

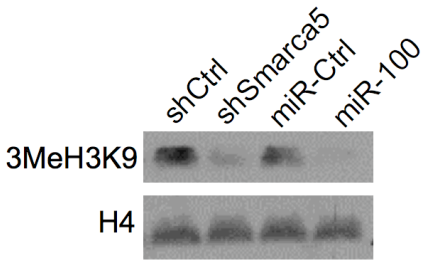


Figure 21. miR-100 down-regulates Smarca5 and inhibits 3meH3K9.

Protein levels of 3meH3K9 were measured in undifferentiated ESCs transfected with miR-100 or with a miR-Ctrl. We reported also 3meH3K9 protein level after transfection with a specific shRNA targeting Smarca5 with its cognate control. Protein levels were normalized for histone H4 protein total amount.

Jarid1b is a specific demethylase of lysine 4 of histone H3 (meH3K4). Dey and colleagues demonstrated that Jarid1b demethylates and thereby represses the promoters of different modulators of ESC fate decision, such as Egr1, p27 and BMI1 genes (Dey, Stalker et al. 2008). So we decided to assess whether miR-137 overexpression was able to affect Egr1 repression, as consequence of Jarid1b suppression. As shown in figure 22 after miR-137 overexpression the promoter of Egr1 gene showed a significant increase in 3meH3K4 levels with consequent increase in Egr1 transcript. So we concluded that miR-137 downregulated Jarid1b and interfered with the ability of this protein to repress Egr1 promoter.

Yamakuchi and colleagues demonstrated that miR-34a overexpression in HCT116 downregulated Sirt1, a class III histone deacetylases (HDACs), and consequently its signaling pathways. In fact they demonstrated that because Sirt1 can induce p53 deacetylation, miR-34a overexpression increased p53 acetylation as consequence of Sirt1 downregulation. Furthermore miR-34a overexpression increased expression of p21 and PUMA, both transcriptional targets of p53 (Yamakuchi, Ferlito et al. 2008).

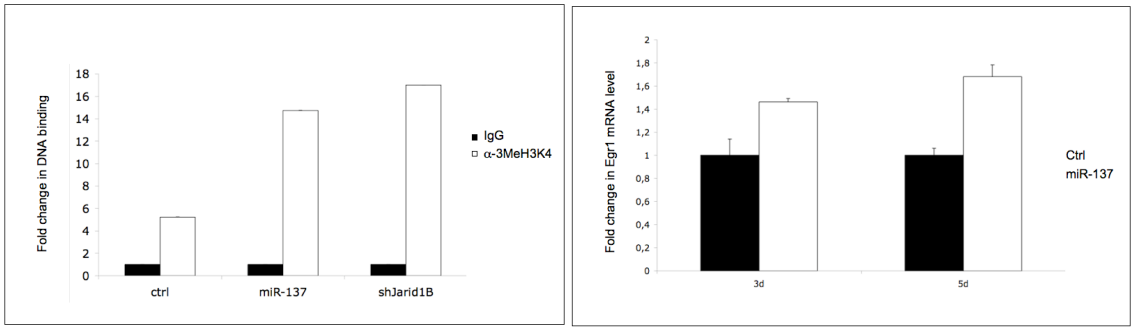


Figure 22. miR-137 overexpression affected Jarid1b signaling pathways.

Left panel: qChip analysis of 3MeH3K4 in Egr1 promoter after Jarid1b downregulation, induced by miR-137 overexpression or by a trasfection of cells with an shRNA against Jarid 1B. Right panel: mRNA expression level of Egr1 gene after miR-137 overexpression.

When we overexpressed miR-34a in ESCs we did not observe an increase in p53 acetylation also after damage induction by exposition to UV radiation (Figure 23).

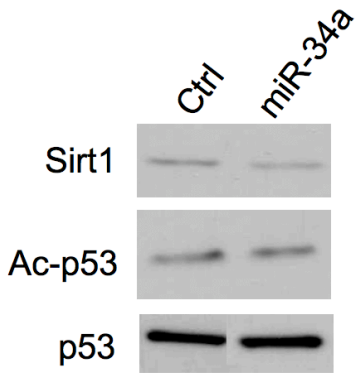


Figure 23. miR-34a does not increase p53 acetylation in ESCs.

ESCs transfected with miR-34a or with a control were exposed to 60 J/M^2 UV radiation and harvested 16 hour after treatment. Sirt1 and acetylated p53 protein level were measured by western Blot analysis. Protein amount was normalized by measure of total p53 level.

To explain our result we reasoned that ESCs probably possess a Sirt1-indipendent system to block p53 acetylation and therefore nuclear translocation of p53 after damage induction. This is because ESCs prefer the mitochondrial transcription-independent killing action of p53 to nuclear transcription-dependent apoptosis,

since the first is more rapid (Erster, Mihara et al. 2004). In fact it was demonstrated that SIRT1 blocks nuclear translocation of p53 induced by oxidative stress via its deacetylation, while induces mitochondrial translocation and consequently the activation of the trascription-indipendent killing activity of p53. As consequence, in damaged ESCs, the suppression of Sirt1 by mir-34a overexpression is not sufficient to elevate the level of acetylated-p53, which is too dangerous for damaged ESCs that prefer to die via apoptosis instead of being repaired.

Overexpression of miR-100, miR-137 and miR-34a is compatible with the persistence of the undifferentiated phenotype

The important role of Smarca5, Jarid1b and Sirt1 in the maintenance of the ESC undifferentiated phenotype suggests the possibility that downregulation of these genes driven by miR-100, miR-137 and miR-34a in undifferentiated cells may have effects on the undifferentiated phenotype or even may induce ESC differentiation (Figure 24).

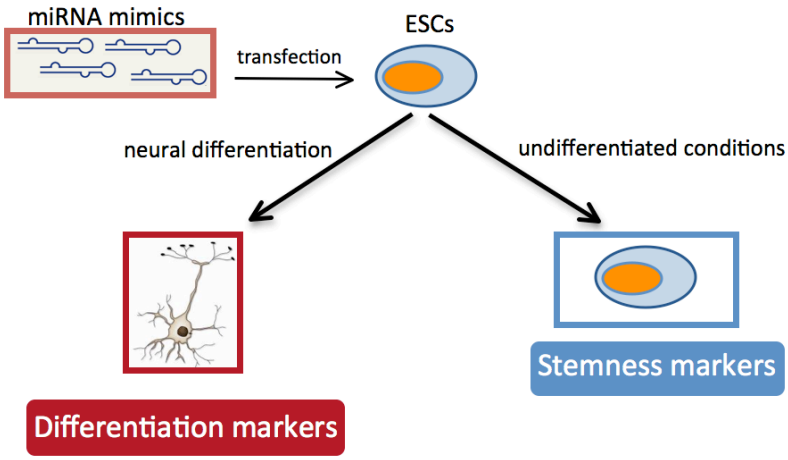


Figure 24. Workflow of experiments based on microRNA overexpression.
ESCs were transfected with microRNA mimics, grown in undifferentiating or differentiating conditions and then analyzed for stemness and differentiation marker expression.

Thus, to explore this possibility, ESCs grown in undifferentiated conditions were transfected with the single microRNA or with a mixture of the three microRNA mimics. 48 and 72 hours after transfection we analyzed the expression of stemness markers, such as Oct3/4 and Nanog mRNAs and alkaline phosphatase (AP), that resulted unmodified compared to that observed in cells transfected with control microRNA (Figure 25).

Accordingly, no inappropriate differentiation was observed, and markers of differentiation such as Sox1 (ectoderm), Sox17 (endoderm) and Brachyury (mesoderm) were undetectable both in control and miR-transfected cells.

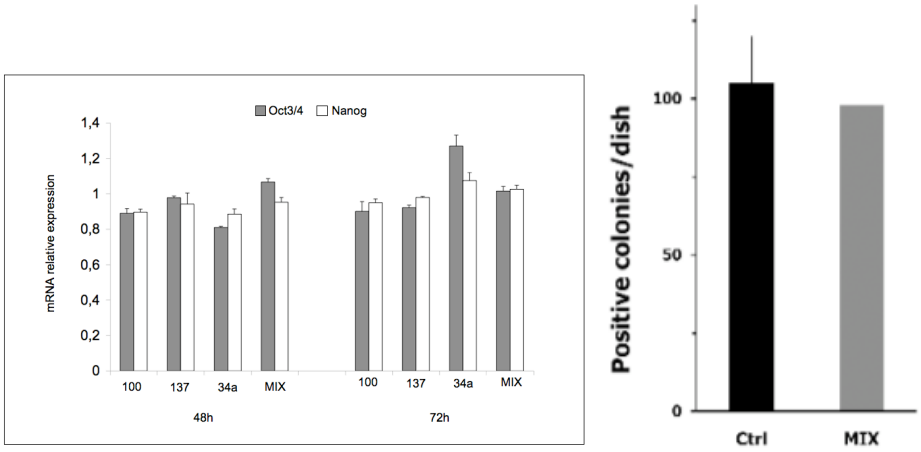


Figure 25. miR-100, miR-137 and miR-34a overexpression does not affect ESC phenotype in undifferentiating conditions.

Left panel: miR-100, miR-137 and miR-34a were transfected singly or as a mixture of three (MIX) in undifferentiated ESCs. Expression levels of stemness marker Oct3/4 and Nanog measured at 48 and 72 hours after trasfection were unchanged upon single microRNA overexpression as well as upon overexpression of the three microRNAs (MIX) at the same time. mRNA expression levels were reported as relative fold changes compared to the data obtained in control microRNA transfected cells. Right panel: we measured the number of alkaline phosphatase positive colonies obtained from microRNAs mixture and control transfected cells, grown 5 days at low density.

Another possibility that deserves to be examined is that the overexpression of these microRNAs in ESCs modifies the ability to proper differentiate. To address

this point, we transfected ESCs with the mixture of the three microRNA mimics, and then induced them to differentiate. As shown in figure 26, we did not observe any change in Oct3/4 and Nanog downregulation upon the induction of differentiation, thus indicating that overexpression of the microRNAs did not impair the ability of the cells to differentiate.

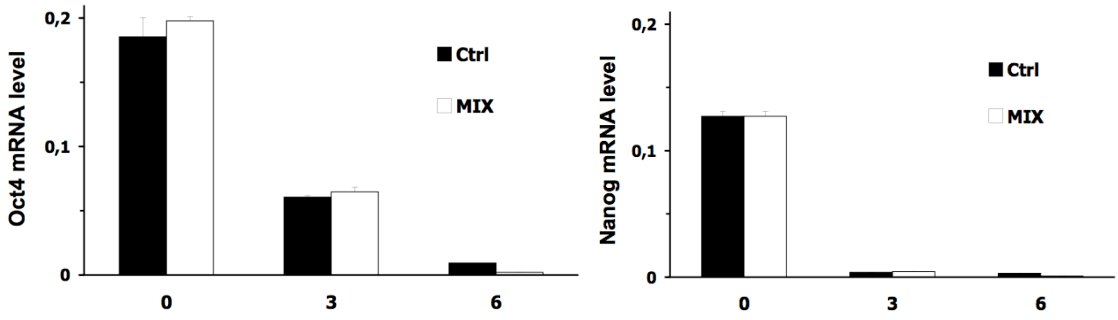


Figure 26. miR-100, miR-137 and miR-34a overexpression does not affect the decrease of stemness markers during ESC differentiation.

ESCs were transfected with a mixture of miR-100, miR-137 and miR-34a and induced to differentiate. Expression levels of stemness markers Oct3/4 and Nanog were measured at different time points (0, 3 and 6 day of differentiation). No differences were found in Oct3/4 and Nanog expression profile during differentiation in microRNA mixture (MIX) transfected cells compared to control microRNA transfected cells (Ctrl). The data were normalized to internal control.

However, early differentiation markers, examined at various days after the induction of differentiation, showed some significant differences between cells transfected with control microRNA and the mixture of the three upregulated microRNAs (Figure 27). The most relevant difference was observed in the case of the mRNA of Sox1, an early marker of neural differentiation, which resulted significantly more expressed in microRNA-transfected cells than in control cells, at day 6 of differentiation. A similar increase was observed in the case of Sox17, marker of endoderm. These results suggest that the main functional role of the

three microRNAs is in the regulation of ESC differentiation and not in the maintenance of the undifferentiated phenotype.

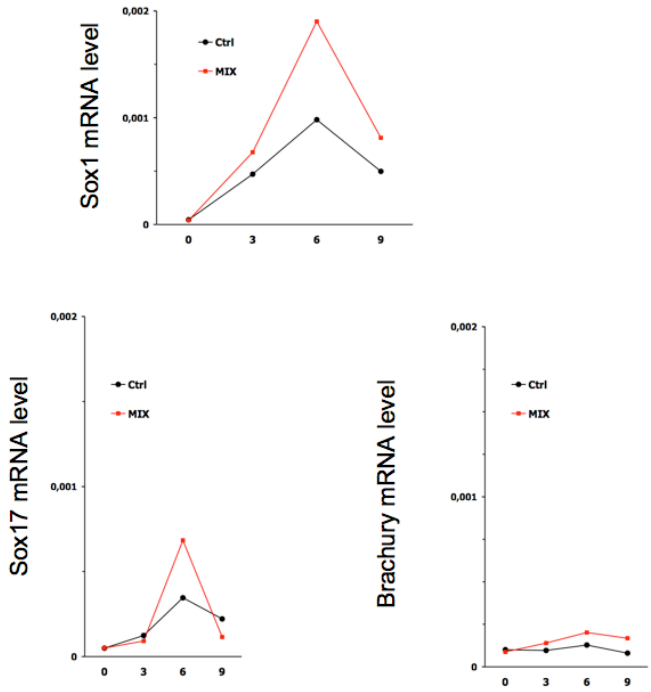


Figure 27. miR-100, miR-137 and miR-34a overexpression induces an early differentiation marker appearance in differentiating ESCs.

Expression levels of differentiation markers were measured in microRNA mixture and control microRNA transfected cells during differentiation (0, 3, 6 and 9 days of differentiation). The expression level of Sox1 (ectoderm) and Sox17 (endoderm) appeared slightly increased in microRNA overexpressing cells (MIX) compared to control cells (Ctrl).

Inhibition of miR-100, miR-137 and 34a results in the block of ESC differentiation

We explored whether the knockdown of miR-100, miR-137 and miR-34a hampers ESC differentiation. ESCs were transfected with the single anti-miRs or with mixtures of them. The cells were grown in the absence of LIF for 5 days to

promote spontaneous differentiation into multiple lineage and then the stemness markers were analyzed (Figure 28).

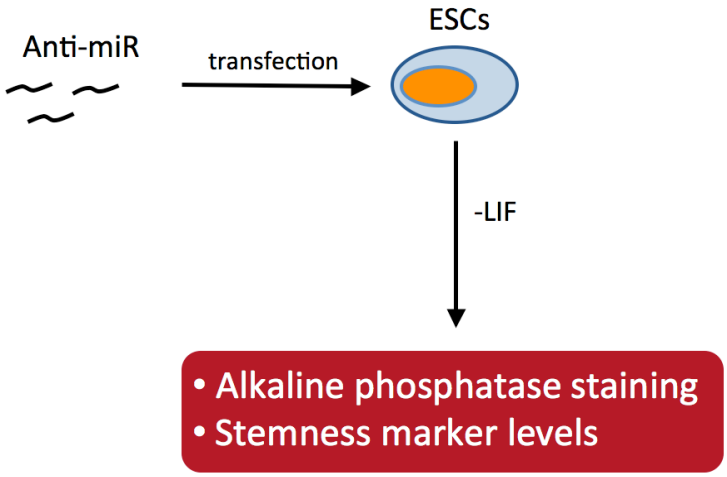


Figure 28. Schematic representation of anti-microRNA expression and consequent functional analysis.

ESCs were transfected with anti-microRNAs, grown in the absence of LIF for 5 days and then analyzed for stemness marker expression.

The suppression of single microRNAs had no effect on the expression of stemness markers Oct3/4, Nanog and Rex1, which decreased to the same extent in control and microRNA-transfected cells, when spontaneous differentiation occurred (data not shown). On the contrary, when the cells were transfected with the mixture of all three microRNAs, the expression of the stemness makers was maintained elevated even 5 days after LIF withdrawal (Figure 29). Accordingly, analysis of the expression of another marker of stemness, the alkaline phosphatase (AP), demonstrated that the cells transfected with the three microRNAs generated a significantly higher number of AP positive colonies than control microRNA transfected cells.

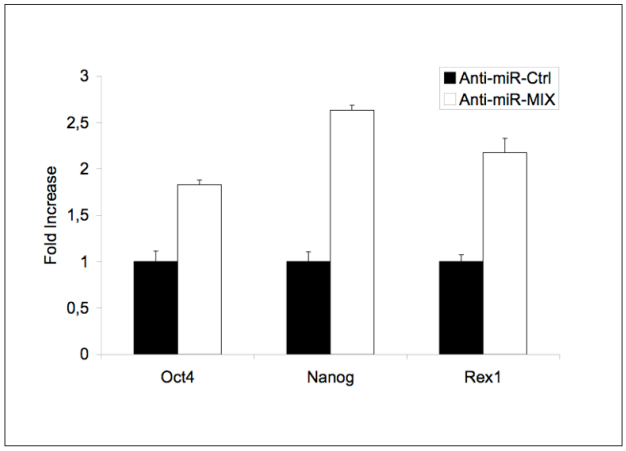


Figure 29. miR-100, miR-137 and miR-34a suppression induces maintenance of stemness marker expression in differentiating ESCs.

ESCs were transfected with a mixture of anti-miR-100, anti-miR-137 and anti-miR-34a and the levels of stemness markers (Oct3/4, Nanog and Rex1) were measured after 5 days of culture in absence of LIF. Expression of stemness markers appears significantly higher in cells transfected with anti-miR mixture compared to the control.

Furthermore, the shape of the colonies and the intensity of AP staining in microRNA-transfected cells were similar to that observed in the cells grown in undifferentiated conditions (Figure 30 and data not shown). In conclusion, suppression of miR-100, miR-137 and miR-34a clearly hampered ESC differentiation.

In vivo analysis of anti-miR transfected ESCs

When injected subcutaneously in nude mice, ESCs give rise to teratomas containing well-differentiated mesodermal, ectodermal, and endodermal tissue and cell types (Nishikawa, Jakt et al. 2007), (Figure 31). So we expected that ESCs, unable to correctly differentiate, could impair teratoma formation after injection in nude mice.

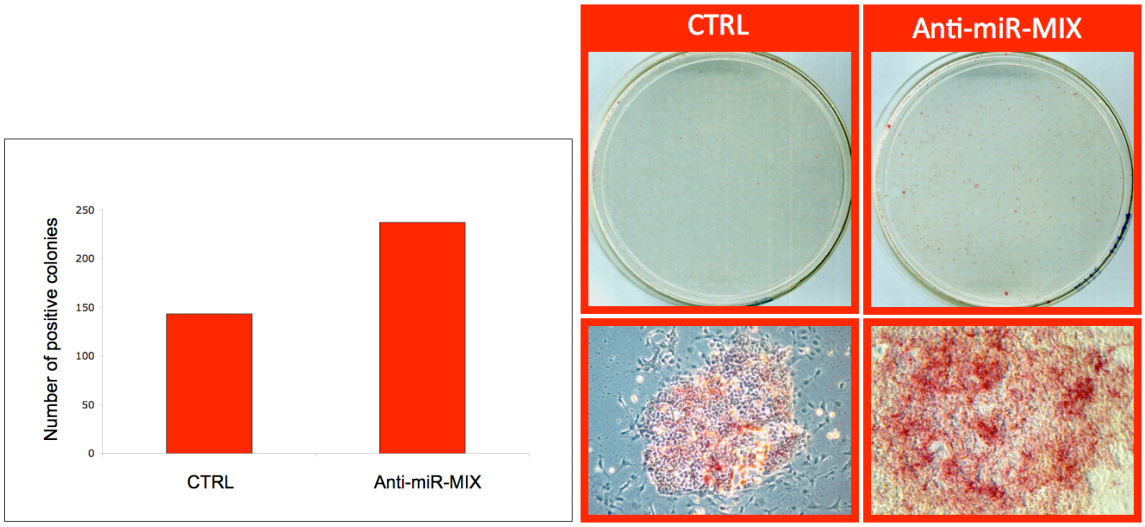


Figure 30. Effect of miR-100, miR-137 and miR-34a suppression impairs ESC differentiation.

Number of alkaline phosphatase positive colonies was measured after 5 days of culture in absence of LIF in both anti-miR mixture and control miR transfected cells. Anti-miR mixture transfected cells showed maintenance of alkaline phosphatase expression with colonies wider and more stained than the control. Error bars represent SD of triplicates.

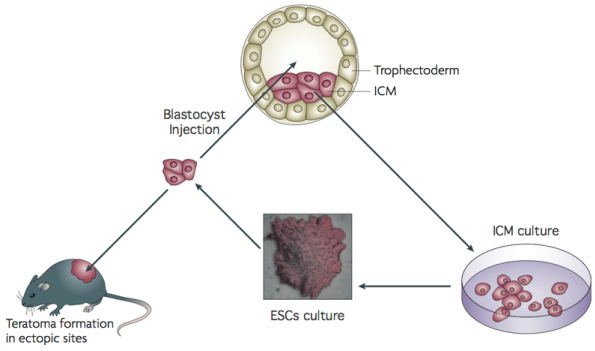


Figure 31. ESCs give rise teratomas when implantated in nude mice.

ESCs ability to give teratoma derived from their pluripotent state. The same cells can be injected into blastocyst to obtain chimeric mouse, in which ESCs are integrated into the embryo and give rise to all cell lineages (Nishikawa, Jakt et al. 2007).

To investigate this point we transfected ESCs with an anti-miR-Ctrl or with a mixture of anti-miR-100, anti-miR-137 and anti-miR-34a. Then the cells were

grown in a culture medium without LIF and after 5 days implanted subcutaneously in nude mice. We injected anti-miR mixture and anti-miR control transfected cells on the left and on the right side of the same mice, respectively (n=3). In all three mice the anti-miR mixture transfected ESCs formed very small teratomas compared with teratomas formed by control cells, but without any evident histological difference (Figure 32). These results indicated that the suppression of miR-100, miR-137 and miR-34a blocks ESC differentiation capacity and therefore their ability to generate fully developed teratoma. The formation of a small tumor by anti-miR transfected ESCs could be due to few cells that did not incorporate the anti-miR mixture during transfection.

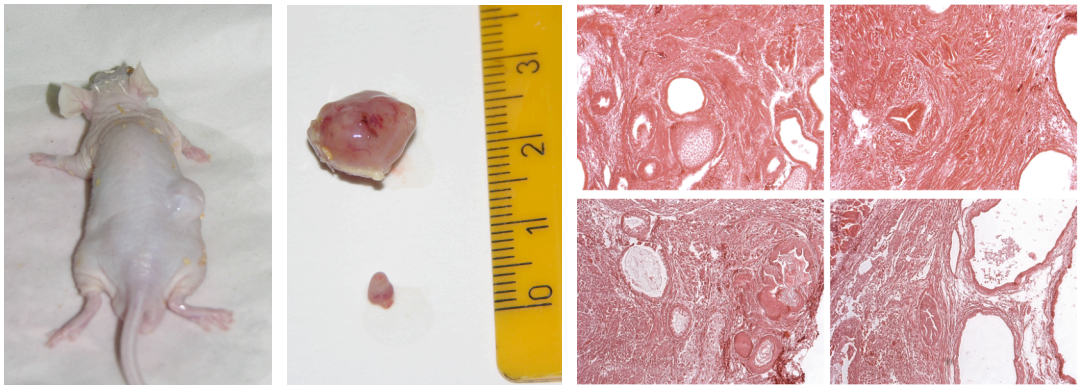


Figure 32. Teratoma formation in nude mice.

The suppression of miR-100, miR-137 and miR-34a blocks ESC differentiation capacity, reducing their teratoma formation ability. Left panel: Nude mouse injected with ESCs transfected with an anti-miR-Ctrl (right side) and with anti-miR-MIX (left side). Central panel: Examples of teratomas formed after injection in nude mice of ESCs transfected with anti-miR-Ctrl (upper tumor) and anti-miR-MIX (lower tumor). Right panel: Haematoxylin-eosin staining of teratomas formed by ESCs transfected with anti-miR-Ctrl (upper sections) and anti-miR-MIX (lower sections).

microRNA	2 days (fold change)	4 days (fold change)	Ct ESCs	Ct ESCs	Ct 2d	Ct 2d	Ct 4d	Ct 4d
let-7a*	0,976	0,401	35,984	35,982	36,143	35,989	35,222	35,975
miR-10a*	0,950	N.D.	35,980	34,991	35,801	35,982	40,000	40,000
miR-127*	1,199	0,129	33,564	34,981	34,599	34,180	33,797	35,993
miR-141	1,263	0,198	30,976	30,984	30,955	30,971	32,974	32,982
miR-141*	0,516	N.D.	32,471	31,043	31,974	33,994	40,000	40,000
miR-142-3p	2,337	0,237	29,980	29,974	28,960	28,957	31,988	31,973
miR-142-5p	3,521	0,423	34,966	34,982	33,973	32,964	34,976	35,918
miR-150	0,343	0,052	25,978	25,964	27,977	27,967	29,958	29,969
miR-154*	1,102	0,235	30,920	30,771	30,585	30,967	32,305	31,869
miR-182	1,840	0,359	23,954	24,989	23,947	23,959	25,993	25,971
miR-183	1,583	0,349	28,981	28,996	27,989	27,997	29,977	29,965
miR-183*	1,147	0,161	26,986	27,926	27,894	27,795	28,809	28,858
miR-196a*	0,961	0,251	35,099	34,954	34,984	34,975	35,919	34,307
miR-200a	0,800	0,281	26,946	27,969	27,955	27,951	28,951	28,965
miR-200a*	0,437	0,102	31,811	31,235	32,888	32,220	33,987	33,461
miR-200b	0,872	0,267	25,990	25,978	26,990	26,989	27,978	27,973
miR-200b*	0,269	0,085	32,445	32,917	34,215	33,223	36,042	33,408
miR-200c	0,807	0,157	25,981	25,972	26,973	26,958	27,960	27,971
miR-200c*	N.D.	N.D.	32,694	32,908	40,000	40,000	40,000	40,000
miR-205	0,547	0,496	30,974	31,986	31,019	31,973	32,011	32,013
miR-20b*	1,368	0,237	31,469	31,984	31,724	31,838	32,978	33,487
miR-210	0,217	0,467	26,991	26,987	28,977	28,989	26,959	26,976
miR-211	N.D.	N.D.	31,979	31,977	40,000	40,000	40,000	40,000
miR-24-2*	0,304	0,418	30,822	30,748	31,885	31,470	30,992	30,818
miR-27a*	0,355	0,180	30,872	30,944	31,833	31,894	32,332	31,951
miR-27b*	0,856	0,294	31,533	30,972	31,624	31,840	31,959	31,940
miR-290-3p	1,675	0,126	24,954	25,991	25,977	24,970	27,951	28,982
miR-290-5p	0,471	0,053	23,835	23,930	24,806	24,847	27,918	27,900
miR-291a-3p	0,911	0,168	21,969	21,962	22,974	22,961	23,944	24,981
miR-291a-5p	N.D.	N.D.	27,679	27,783	40,000	40,000	40,000	40,000
miR-291b-3p	0,663	0,499	35,899	34,998	34,514	33,982	35,989	35,328
miR-291b-5p	2,036	0,109	28,958	28,979	28,958	28,959	31,987	31,995
miR-292-3p	1,043	0,145	19,951	19,940	20,974	20,970	22,963	22,963
miR-292-5p	0,622	0,048	28,923	28,952	28,776	28,756	31,245	30,827
miR-293	1,534	0,259	19,970	19,971	19,952	19,960	21,971	21,970
miR-293*	0,054	0,135	32,447	32,993	40,000	33,185	34,977	34,987
miR-294	1,279	0,191	22,954	23,985	23,987	23,979	25,982	25,971
miR-294*	0,701	0,056	26,969	26,971	26,955	26,943	28,844	28,886
miR-295	0,873	0,149	19,987	19,984	20,981	20,985	21,969	21,984
miR-299*	0,899	0,342	30,887	30,137	30,931	30,930	31,024	30,836

miR-29a*	1,255	0,199	34,337	35,420	34,518	35,998	35,972	35,998
miR-300	1,621	0,258	30,647	31,203	30,614	30,916	31,261	31,600
miR-376b*	1,211	0,451	27,953	27,959	27,964	27,978	27,953	27,939
miR-378*	0,901	0,367	33,974	33,955	33,969	33,963	34,973	32,955
miR-429	0,488	0,153	25,970	25,969	27,982	28,000	28,988	28,989
miR-449a	2,514	0,435	30,965	30,963	29,952	29,945	31,969	31,967
miR-449c	1,067	0,410	34,337	32,870	33,284	33,634	33,807	35,989
miR-465a-3p	2,055	N.D.	34,964	33,974	33,986	34,986	40,000	40,000
miR-465b-5p	1,071	0,127	32,001	31,976	31,972	31,972	33,989	33,988
miR-466a-3p	0,975	0,210	28,695	28,843	28,593	28,878	29,838	29,886
miR-466b-3-3p	1,167	0,250	28,971	28,870	28,967	28,889	29,669	29,827
miR-466d-3p	1,148	0,394	23,986	23,836	23,901	23,975	23,900	23,863
miR-467a*	1,043	0,423	27,977	27,957	27,970	27,971	28,985	27,963
miR-467b*	0,700	0,306	31,001	30,983	30,951	30,980	30,972	30,977
miR-467e*	N.D.	N.D.	32,022	31,957	40,000	40,000	40,000	40,000
miR-485*	0,645	0,082	26,972	26,965	27,990	27,987	29,981	29,969
miR-489	0,916	0,183	29,969	29,961	30,983	30,975	31,956	31,946
miR-665	1,859	0,377	30,983	31,991	30,985	31,003	30,984	32,001
miR-672	0,649	0,133	26,967	26,952	27,953	27,951	28,961	28,954
miR-673-5p	1,244	0,300	30,715	30,633	30,928	30,779	31,331	31,381
miR-692	1,045	0,281	28,832	28,695	29,049	28,789	29,403	29,580
miR-697	0,564	0,322	29,956	30,962	31,010	31,009	30,942	30,995
miR-702	0,946	0,416	33,883	33,326	32,951	32,963	33,414	33,209
miR-706	1,102	0,447	27,866	27,889	27,875	27,936	27,952	27,967
miR-708	1,137	0,467	25,934	26,965	26,954	26,959	26,940	26,942
miR-708*	N.D.	N.D.	34,985	33,978	40,000	40,000	40,000	40,000
miR-715	N.D.	N.D.	27,974	27,952	40,000	40,000	40,000	40,000
miR-741	2,630	0,444	34,980	33,956	33,994	33,964	34,028	33,986
miR-743b-3p	N.D.	N.D.	35,004	35,012	40,000	40,000	40,000	40,000
miR-7a	0,922	0,345	29,963	30,968	30,974	30,966	31,978	31,972
miR-7b	0,640	0,210	32,955	32,988	33,967	33,975	34,983	35,002
miR-801	0,751	0,465	29,823	29,884	30,850	30,859	31,791	31,222
miR-875-5p	0,652	0,274	33,230	33,461	32,843	34,834	33,739	35,445
miR-878-3p	0,524	0,446	31,670	31,429	31,883	31,794	31,774	31,971
miR-878-5p	N.D.	N.D.	35,958	35,951	40,000	40,000	40,000	40,000
miR-881	0,547	N.D.	34,982	36,006	34,995	36,094	40,000	40,000
miR-881*	N.D.	N.D.	34,010	34,978	40,000	40,000	40,000	40,000
miR-92a*	1,821	0,230	29,982	29,989	28,969	28,966	30,989	30,982

Table 1. microRNAs decreased upon the induction of ESC differentiation.

Expression changes at 2 and 4 days of differentiation are calculated as fold change relative to undifferentiated ESCs. Ct are referred to two independent samples of undifferentiated (Ct ESCs) cells and two (Ct 2d) or four (Ct 4d) days differentiated ESCs. Cut off value was a fold change $\leq$ 0,50 at 4 days of differentiation.

microRNA	Ct ESCs	Ct ESCs	Ct 2d	Ct 2d	Ct 4d	Ct 4d
let-7c-1*	40,00	40,00	40,00	40,00	35,99	35,12
miR-100	40,00	40,00	31,00	31,00	25,99	25,99
miR-124*	40,00	40,00	30,75	31,40	29,88	29,48
miR-125b*	40,00	40,00	33,97	35,96	32,11	32,86
miR-125b-3p	40,00	40,00	40,00	40,00	34,96	33,97
miR-133b	40,00	40,00	40,00	40,00	30,94	32,03
miR-137	40,00	40,00	35,01	33,98	29,96	30,98
miR-153	40,00	40,00	34,97	34,98	31,97	32,97
miR-187	40,00	40,00	40,00	40,00	32,99	32,01
miR-204	40,00	40,00	31,99	32,96	32,00	31,99
miR-214*	40,00	40,00	40,00	40,00	34,07	34,00
miR-216a	40,00	40,00	32,96	34,00	32,00	33,02
miR-216b	40,00	40,00	31,98	32,98	29,96	29,95
miR-217	40,00	40,00	33,95	33,94	31,98	30,99
miR-219	40,00	40,00	32,00	31,96	27,95	27,97
miR-302b*	40,00	40,00	32,78	32,04	40,00	40,00
miR-325	40,00	40,00	40,00	40,00	31,97	30,97
miR-325*	40,00	40,00	40,00	40,00	33,00	32,99
miR-326	40,00	40,00	40,00	40,00	30,43	29,93
miR-345	40,00	40,00	40,00	40,00	34,00	34,04
miR-383	40,00	40,00	40,00	40,00	33,96	36,02
miR-384-3p	40,00	40,00	40,00	40,00	31,98	31,99
miR-486	40,00	40,00	40,00	40,00	29,97	30,00
miR-598	40,00	40,00	40,00	40,00	31,98	34,01
miR-684	40,00	40,00	40,00	40,00	35,01	35,97
miR-743a	40,00	40,00	31,01	31,05	31,01	29,90
miR-761	40,00	40,00	40,00	40,00	35,00	35,09
miR-879	40,00	40,00	40,00	40,00	35,95	35,97
miR-99a	40,00	40,00	30,96	31,99	25,00	25,98

Table 2. microRNAs undetectable in undifferentiated ESCs and expressed upon the induction of the differentiation program.

Ct are referred to two independent samples of undifferentiated (Ct ESCs) cells and two (Ct 2d) or four (Ct 4d) days differentiated ESCs.

microRNA	2 days (fold change)	4 days (fold change)	Ct ESCs	Ct ESCs	Ct 2d	Ct 2d	Ct 4d	Ct 4d
miR-1	5,544	5,750	35,922	35,994	34,968	33,976	32,961	33,988
miR-103	3,503	4,547	28,999	28,979	27,994	27,987	26,989	26,994
miR-107	3,135	4,198	34,002	32,973	32,937	32,000	32,036	30,958
miR-128a	5,037	2,383	33,971	32,982	31,972	31,962	31,967	31,974
miR-129-3p	3,462	4,029	30,968	30,969	29,976	29,984	28,969	28,966
miR-130a	2,599	2,576	25,958	25,967	25,971	25,972	24,966	24,960
miR-130b	2,183	3,348	26,977	26,971	25,963	25,951	24,955	24,962
miR-132	4,599	3,699	29,000	29,005	26,761	27,975	28,022	27,969
miR-133a	3,875	7,243	31,983	31,997	29,968	29,989	28,980	28,986
miR-135a	4,567	12,853	30,978	30,972	29,976	30,002	27,978	27,991
miR-138	2,348	2,488	29,959	30,985	29,970	29,973	28,958	28,963
miR-143	3,203	5,355	30,978	30,963	30,986	29,966	28,983	28,969
miR-148b	3,245	2,563	32,978	32,970	31,984	31,947	31,965	30,961
miR-149	2,241	2,001	27,929	27,930	26,932	26,951	25,854	25,899
miR-151-3p	2,137	2,083	28,938	28,975	29,017	28,977	27,968	27,998
miR-152	3,586	10,731	31,989	31,974	30,980	30,978	28,969	28,972
miR-15a	2,845	3,767	29,013	28,984	27,988	27,971	26,989	26,961
miR-15b	2,310	2,150	27,972	27,982	26,993	26,963	26,977	26,960
miR-16*	6,704	3,102	34,701	35,037	32,423	32,949	32,814	32,760
miR-181a-1*	5,921	21,844	34,205	33,492	32,635	32,649	30,821	30,225
miR-185	2,485	3,261	31,967	31,989	30,979	30,958	29,981	29,974
miR-18a	3,971	3,983	26,938	26,963	25,969	25,963	24,950	24,969
miR-197	3,161	3,863	31,967	32,056	30,960	30,965	30,967	31,991
miR-19b	2,918	2,300	18,977	19,985	18,991	18,966	17,945	18,070
miR-218	2,161	9,587	27,940	27,955	27,962	27,953	24,956	24,967
miR-218-2*	3,888	12,431	35,035	36,994	35,875	35,979	33,657	33,857
miR-223	4,051	6,474	35,974	36,027	33,997	35,983	34,989	33,967
miR-28	2,435	2,723	28,961	28,959	27,953	27,946	27,978	27,974
miR-296-3p	2,974	2,905	29,964	30,997	28,981	29,993	28,988	28,975
miR-298	3,782	4,031	27,966	28,982	26,964	26,949	26,966	26,988
miR-301b	2,002	3,753	25,968	26,988	25,959	25,963	23,964	23,960
miR-302a	7,537	3,926	24,956	25,962	22,959	22,958	22,953	22,957
miR-302b	6,890	3,428	22,962	23,967	20,965	20,973	21,982	21,983
miR-302c	6,734	4,133	28,018	27,982	26,990	26,997	25,965	26,994
miR-302d	6,776	3,865	24,972	24,972	22,967	22,960	22,975	22,953
miR-30b	2,146	2,828	26,980	26,982	25,972	25,981	24,974	24,968
miR-30c	2,235	2,703	25,977	25,971	24,954	24,949	23,954	23,945
miR-32	3,851	3,375	33,970	32,974	31,956	31,962	30,976	31,984
miR-322	2,743	11,354	30,981	30,987	29,977	29,985	26,945	26,949
miR-324-5p	3,231	5,210	30,941	30,940	29,950	29,946	28,963	28,970
miR-328	10,429	13,193	32,959	31,968	29,977	30,973	28,977	28,969
miR-330	2,325	3,836	32,016	32,947	36,023	30,977	30,998	30,993
miR-331-3p	2,774	3,836	27,977	27,996	26,954	26,956	25,985	25,955
miR-331-5p	7,329	4,346	32,972	33,955	31,985	32,993	31,995	31,970
miR-335-3p	2,952	4,035	28,981	28,977	27,979	27,970	26,976	26,987
miR-335-5p	5,634	15,634	29,953	29,980	27,953	28,967	25,958	25,985
miR-339-5p	3,437	3,269	30,952	31,986	29,982	30,982	29,978	29,987
miR-340-3p	2,929	6,898	31,958	33,970	31,960	31,974	29,959	29,963

miR-340-5p	3,751	7,799	29,967	30,999	29,988	28,970	27,967	27,965
miR-342-5p	2,752	8,228	35,989	36,040	33,011	34,005	32,033	31,976
miR-344	3,340	4,471	30,987	30,987	29,996	29,988	29,979	29,999
miR-345-5p	2,988	4,386	32,040	31,971	31,969	30,982	30,991	29,967
miR-34a	2,986	2,501	27,966	27,969	26,965	26,967	26,972	26,983
miR-34c	3,498	7,576	32,985	32,986	30,981	32,970	30,972	30,979
miR-350	3,537	5,012	32,980	32,990	31,979	31,970	30,981	30,968
miR-351	4,143	10,921	30,971	31,980	30,974	30,987	29,989	29,989
miR-362-3p	5,881	4,760	32,940	34,963	31,950	32,970	31,964	31,960
miR-365	2,043	3,462	32,990	31,999	31,974	31,982	29,962	30,981
miR-367	5,959	2,388	24,964	24,982	22,974	22,980	22,963	22,952
miR-369-5p	4,368	2,380	30,950	30,961	29,968	28,948	29,967	29,968
miR-370	5,852	2,815	27,973	27,964	26,977	26,970	26,977	26,990
miR-380-5p	4,703	3,020	29,984	29,989	27,965	27,971	27,966	27,969
miR-381	3,776	2,086	34,987	33,981	32,982	33,975	33,985	32,963
miR-384-5p	7,847	40,479	31,931	32,979	30,971	29,953	26,947	26,943
miR-409-5p	4,348	2,522	31,988	30,963	30,984	30,983	29,963	30,976
miR-450a-5p	3,374	3,463	32,953	34,003	32,967	32,981	31,976	31,973
miR-487b	4,385	2,008	28,976	29,992	27,970	27,973	27,961	27,966
miR-493	13,656	6,504	33,984	35,005	32,989	32,967	32,959	32,992
miR-500	2,071	3,775	31,977	31,963	31,990	30,972	30,976	30,972
miR-503	3,468	11,252	30,984	30,999	29,990	29,970	27,967	27,980
miR-503*	2,395	4,484	32,968	33,984	31,966	32,984	30,986	30,976
miR-532-3p	2,478	2,506	30,998	30,980	29,980	29,981	28,984	30,004
miR-532-5p	2,438	2,477	26,976	27,982	26,967	26,971	25,960	25,961
miR-542-3p	2,901	2,183	34,980	33,976	33,986	33,982	32,967	33,983
miR-574-3p	2,086	2,735	29,994	30,980	29,980	29,972	28,959	28,985
miR-592	3,070	2,980	34,814	34,845	32,811	33,237	32,975	32,525
miR-652	3,246	4,514	28,976	28,971	27,998	27,980	26,975	26,969
miR-670	19,571	39,961	35,927	35,971	36,006	35,984	33,002	33,983
miR-671-3p	28,445	14,740	35,971	35,997	32,988	32,998	32,988	32,999
miR-676	2,812	2,186	30,994	30,993	29,946	30,986	29,973	29,972
miR-701	5,245	11,319	35,923	36,037	35,994	33,964	33,851	33,355
miR-744	2,859	3,695	29,970	29,954	28,948	28,953	27,953	27,957
miR-744*	4,129	2,403	33,971	33,995	32,982	32,965	31,970	32,973
miR-872	2,566	3,170	29,965	29,953	28,942	28,945	27,947	27,948
miR-9	2,659	15,103	27,978	28,022	26,939	26,989	23,971	23,958

Table 3. microRNAs increased upon the induction of ESC differentiation.

Expression changes at 2 and 4 days of differentiation are calculated as fold change relative to undifferentiated ESCs. Ct in two independent samples of undifferentiated (Ct ESCs) cells and in cells grown for two (Ct 2d) or four (Ct 4d) days in differentiation conditions. Cut off value was an increase of at least two folds at 2 and 4 days of differentiation.

microRNA	2 days (fold change)	4 days (fold change)	Ct ESCs	Ct ESCs	Ct 2d	Ct 2d	Ct 4d	Ct 4d
miR-106a	2,198	1,250	20,961	20,965	19,888	19,946	19,943	19,943
miR-125a-3p	2,304	1,208	33,978	33,966	32,961	32,978	32,974	32,978
miR-127	2,879	0,975	24,950	24,947	24,975	24,992	24,937	24,954
miR-146a	2,087	1,731	29,983	29,983	28,968	28,970	28,982	28,972
miR-148a	3,113	1,808	28,938	28,949	27,949	28,971	27,937	28,983
miR-17	2,174	1,826	20,954	20,961	19,944	19,940	19,959	19,953
miR-186	2,357	1,993	26,959	26,953	26,970	26,962	25,960	25,946
miR-186*	2,952	1,616	31,465	30,938	29,734	29,840	29,843	29,849
miR-18b	4,455	1,546	31,966	31,978	29,969	31,005	30,957	30,990
miR-190	2,282	1,687	32,972	33,970	32,968	32,979	32,967	32,980
miR-192	2,183	1,918	30,966	30,962	30,984	30,973	30,973	29,968
miR-194	2,006	1,863	32,987	33,004	31,976	32,006	31,996	32,012
miR-20b	2,289	0,820	21,959	21,961	20,939	21,957	21,945	21,947
miR-21*	2,256	0,991	35,207	34,977	33,981	34,469	33,954	34,527
miR-22*	2,182	1,025	32,741	32,887	31,883	31,822	31,933	32,280
miR-29a	2,186	0,916	25,937	25,937	24,933	24,927	25,946	25,947
miR-302a*	4,501	1,148	29,920	29,187	26,965	27,901	28,944	28,081
miR-302c*	6,734	1,804	33,185	33,195	30,987	30,933	31,957	32,000
miR-323-3p	3,324	1,540	25,971	25,977	24,963	24,972	25,978	25,982
miR-337-3p	3,309	1,626	28,978	28,976	27,979	27,982	27,975	27,961
miR-337-5p	3,347	1,442	28,959	29,979	27,959	28,987	28,979	28,977
miR-34b-3p	2,355	1,730	29,967	29,959	29,970	29,976	29,985	29,979
miR-363	3,531	1,485	28,973	28,967	27,960	27,967	27,959	28,986
miR-369-3p	3,034	1,874	33,973	34,980	32,970	33,987	32,960	32,950
miR-376a	2,793	1,496	27,980	27,986	26,995	26,982	26,989	26,981
miR-376b	2,516	0,597	26,986	26,999	26,999	25,966	27,988	28,020
miR-379	2,415	1,207	25,931	25,943	25,958	25,957	25,950	25,947
miR-382	2,964	1,628	27,989	28,011	25,936	25,998	25,975	26,977
miR-409-3p	2,002	0,655	23,955	24,973	23,961	23,951	24,957	24,965
miR-410	2,599	1,188	26,969	26,962	25,960	25,945	25,939	25,938
miR-411	2,041	1,004	24,956	24,957	24,975	24,978	24,965	24,967
miR-431	2,815	1,300	24,975	24,969	23,969	23,974	23,958	23,964
miR-433	2,663	1,256	28,967	28,964	28,972	28,975	28,968	28,968
miR-434-3p	2,200	1,177	28,004	27,990	26,986	26,982	26,963	26,985
miR-434-5p	3,529	1,324	30,991	30,983	28,987	29,989	29,982	29,984
miR-467a	2,887	1,655	24,979	24,963	23,967	23,968	23,968	23,982
miR-467b	2,034	0,986	27,987	27,986	26,955	26,966	27,979	27,992
miR-467c	2,245	1,013	26,986	26,990	27,011	25,971	26,980	26,974
miR-467d	2,182	1,300	28,968	28,964	28,976	28,981	27,961	27,951
miR-467e	2,271	1,122	28,998	29,970	28,977	28,982	28,986	28,988
miR-494	2,352	1,327	25,986	26,984	25,981	25,989	25,981	25,964
miR-495	2,783	0,883	26,959	26,942	26,967	26,958	27,967	27,977
miR-496	4,886	1,784	33,983	33,975	32,974	32,981	32,971	33,987
miR-497	2,595	0,655	29,990	29,987	28,976	28,979	29,978	29,983
miR-539	2,588	1,085	26,930	26,944	26,953	26,956	26,948	26,941
miR-540-3p	3,142	0,772	29,995	29,993	28,966	28,972	29,982	29,992
miR-543	2,528	0,588	28,964	28,968	28,979	28,977	29,974	29,971
miR-544	2,771	1,583	31,980	31,968	30,972	30,949	30,959	31,975
miR-667	2,704	1,453	27,961	27,967	27,979	27,973	27,993	27,990
miR-669a	2,605	1,323	27,947	27,954	27,974	27,977	27,970	27,980

miR-673-3p	2,155	0,550	28,654	29,786	28,960	28,750	29,493	29,930
miR-679	3,566	0,945	33,051	32,991	31,965	31,980	31,940	31,975
miR-680	2,509	1,204	29,976	29,981	28,951	28,970	28,985	30,027
miR-682	2,315	1,707	32,007	31,996	30,979	30,934	30,950	31,985
miR-764-5p	2,672	0,737	33,831	33,897	32,876	32,987	33,996	33,134
miR-96	2,647	0,848	30,976	31,971	30,995	30,965	31,985	31,996

Table 4. microRNAs increased only at day 2 after the induction of ESC differentiation.

Expression changes at 2 and 4 days of differentiation are calculated as fold change relative to undifferentiated ESCs. Cts are referred to two independent samples of undifferentiated (Ct ESCs) cells and two (Ct 2d) or four (Ct 4d) days differentiated ESCs. Cut off value was an increase of at least two folds at 2 days.

microRNA	2 days (fold change)	4 days (fold change)	Ct ESCs	Ct ESCs	Ct 2d	Ct 2d	Ct 4d	Ct 4d
let-7c	1,521	2,144	34,985	33,977	33,970	34,972	32,956	32,946
let-7e	0,791	4,900	32,971	33,004	32,977	34,006	30,972	29,959
let-7i	1,969	2,086	32,004	32,985	31,982	31,999	32,009	31,997
miR-106b	1,895	2,236	25,967	25,957	25,966	25,975	24,971	24,976
miR-124	1,944	7,920	29,987	29,994	30,001	29,974	26,956	27,984
miR-125b-5p	0,815	11,232	29,986	29,967	30,976	30,975	26,981	25,963
miR-145	1,942	3,214	28,958	29,982	28,969	28,970	27,979	27,984
miR-181a	1,228	26,300	32,991	32,927	31,957	33,965	28,987	28,994
miR-190b	1,327	2,763	35,286	34,734	34,640	34,716	32,941	33,146
miR-199a-3p	1,431	2,186	31,984	31,970	30,974	32,004	30,983	30,979
miR-206	0,775	10,224	36,992	35,942	36,782	36,890	33,694	33,956
miR-21	1,641	2,010	24,972	25,963	25,971	25,986	24,960	24,999
miR-26b	1,816	2,212	27,969	27,973	27,955	27,972	26,967	26,966
miR-301a	1,857	3,677	25,978	25,972	25,980	25,985	23,979	23,943
miR-30d	1,629	2,119	28,973	28,991	28,986	28,981	27,960	27,981
miR-322*	1,407	8,223	33,990	33,994	32,985	33,045	29,992	29,996
miR-324-3p	1,452	2,684	29,982	30,039	30,015	30,003	28,976	28,990
miR-455*	1,278	2,750	32,990	32,993	32,995	32,979	30,981	30,969
miR-685	1,913	2,625	28,985	28,991	29,001	29,004	27,976	27,961
miR-9*	1,000	3,613	30,974	30,967	30,964	30,967	28,982	27,966
miR-93	1,915	2,419	24,967	24,964	23,946	23,944	22,942	22,950

Table 5. microRNAs increased upon the induction of ESC differentiation only at day 4 after the induction of differentiation.

Expression changes at 2 and 4 days of differentiation are calculated as fold change relative to undifferentiated ESCs. Ct are referred to two independent samples of undifferentiated (Ct ESCs) cells and two (Ct 2d) or four (Ct 4d) days differentiated ESCs. Cut off value was an increase of at least two folds at day 4.

microRNA	microRNA	microRNA	microRNA	microRNA	microRNA
let-7a	miR-199a-5p	miR-448	miR-488*	miR-683	miR-759
let-7b*	miR-199b*	miR-449b	miR-490	miR-686	miR-762
let-7d*	miR-201	miR-450a-3p	miR-499	miR-688	miR-764-3p
let-7f*	miR-202-5p	miR-450b-5p	miR-504	miR-691	miR-770-3p
let-7f	miR-207	miR-451	miR-505	miR-693-3p	miR-802
let-7i*	miR-208	miR-452	miR-509-5p	miR-693-5p	miR-871
miR-105	miR-208b	miR-453	miR-511	miR-695	miR-873
miR-10b*	miR-214	miR-455	miR-547	miR-707	miR-874
miR-10b	miR-215	miR-463*	miR-551b	miR-710	miR-875-3p
miR-122	miR-220	miR-464	miR-615-3p	miR-711	miR-876-3p
miR-133a*	miR-23a	miR-465a-5p	miR-615-5p	miR-712*	miR-876-5p
miR-139-3p	miR-30c-2*	miR-466c-5p	miR-654-3p	miR-713	miR-883a-3p
miR-145*	miR-330*	miR-466d-5p	miR-654-5p	miR-717	miR-883a-5p
miR-146b*	miR-343	miR-466h	miR-666-5p	miR-718	miR-98
miR-147	miR-346	miR-468	miR-668	miR-719	
miR-148a*	miR-374*	miR-469	miR-675-3p	miR-742	
miR-150*	miR-382*	miR-470	miR-675-5p	miR-743b-5p	
miR-188-3p	miR-433*	miR-483*	miR-681	miR-758	

Table 6. microRNAs undetectable both in undifferentiated and differentiated ESCs.

microRNA	2 days (fold change)	4 days (fold change)	Ct ESCs	Ct ESCs	Ct 2d	Ct 2d	Ct 4d	Ct 4d
let-7b	0,090	1,127	35,984	36,025	35,945	40,000	36,023	36,025
let-7g	0,469	0,558	30,969	31,966	32,978	31,988	31,988	32,004
miR-101a	1,904	1,889	29,973	29,974	28,938	29,986	28,985	28,984
miR-101b	1,154	0,986	27,938	27,914	27,897	27,970	26,943	26,860
miR-106b*	1,060	0,992	27,868	27,933	27,866	27,909	26,947	26,797
miR-125a-5p	1,288	1,932	27,979	27,959	28,984	28,975	26,978	26,984
miR-126-3p	1,585	0,858	25,961	25,965	27,001	26,986	26,992	26,981
miR-126-5p	1,732	0,855	29,978	29,961	29,973	29,972	29,977	29,970
miR-130b*	1,222	1,319	30,964	30,888	30,841	30,953	29,866	28,956
miR-134	1,714	0,741	24,961	25,986	24,978	24,994	25,990	25,969
miR-135a*	0,975	0,618	28,944	28,857	28,934	28,855	28,881	28,947
miR-135b	1,403	0,809	24,977	24,972	24,959	24,987	24,973	24,973
miR-136	1,588	0,647	26,983	26,954	26,958	27,996	27,967	27,985
miR-138*	0,960	1,058	35,093	34,692	34,617	35,158	35,500	34,333
miR-139-5p	0,982	0,541	29,967	30,973	30,966	30,975	30,984	30,970
miR-140	1,969	1,693	26,963	26,975	25,947	25,946	25,954	25,968
miR-146b	0,962	0,948	29,977	29,985	29,971	29,961	28,965	28,966
miR-155	0,892	0,641	28,946	28,947	29,946	29,961	28,939	28,948
miR-15a*	1,569	1,346	30,824	31,410	30,208	30,175	29,972	29,865
miR-15b*	1,242	0,845	28,731	28,795	28,814	28,974	28,811	28,848
miR-16	1,078	1,172	22,994	22,947	22,941	22,964	22,984	22,980
miR-17*	0,917	0,576	31,168	31,435	31,913	31,815	31,338	30,857
miR-181c	0,837	1,435	31,932	31,982	32,946	31,964	31,975	31,992
miR-184	1,566	0,830	33,933	34,954	33,970	34,961	34,955	34,967
miR-188-5p	1,867	1,238	30,945	30,933	30,956	30,948	30,945	29,928
miR-18a*	0,920	0,624	30,925	30,946	30,712	30,918	30,909	30,214
miR-191	1,180	1,099	24,000	23,975	23,962	23,972	24,005	23,986
miR-191*	1,511	0,810	34,814	36,272	35,405	34,906	35,378	35,867
miR-193*	0,850	1,091	34,124	35,314	35,417	34,696	33,586	33,889
miR-193b	0,951	1,123	29,998	29,970	29,958	29,964	28,951	28,970
miR-195	1,996	0,770	27,973	27,968	27,975	27,962	27,967	27,978
miR-19a	1,971	1,846	22,983	22,972	21,963	22,983	21,975	21,967
miR-203	1,271	0,650	29,975	29,974	29,957	29,968	29,962	30,976
miR-20a	1,636	1,509	21,971	21,955	21,973	21,963	20,955	20,956
miR-20a*	1,593	1,469	28,983	29,030	28,954	28,945	27,818	27,964
miR-218-1*	0,255	1,681	35,956	35,041	35,817	35,300	34,727	35,639
miR-22	1,091	1,070	28,904	28,924	28,986	28,984	28,980	28,991
miR-221	1,346	1,785	28,957	28,973	28,989	28,969	29,001	28,990
miR-222	0,772	0,747	26,943	26,944	27,946	27,956	26,938	26,941
miR-224	1,439	1,224	31,973	31,987	31,983	31,972	30,966	30,960
miR-23b	1,756	0,740	31,969	32,980	31,991	31,965	31,968	31,999
miR-24	0,870	0,984	22,960	22,961	23,975	24,988	23,985	23,988
miR-25	1,868	1,954	28,974	28,953	28,959	28,977	27,949	27,966
miR-26a	1,456	1,718	25,965	25,969	25,961	25,965	24,963	24,949
miR-26b*	1,357	1,246	33,392	32,564	33,423	32,928	32,181	31,566
miR-27a	0,705	1,071	28,952	28,955	29,957	29,963	28,977	28,969
miR-27b	1,478	1,158	28,942	28,946	29,962	29,957	28,952	28,958
miR-28*	0,821	0,831	29,110	29,107	29,487	28,884	28,789	28,957
miR-296-5p	1,641	1,002	27,995	27,941	28,016	27,975	28,000	27,964

miR-297a*	1,309	0,587	27,842	28,117	27,860	27,969	28,052	28,920
miR-297b-5p	1,863	0,943	30,973	30,975	29,972	29,946	29,966	30,966
miR-297c	1,747	1,060	31,052	30,984	30,016	29,995	29,955	29,971
miR-29b	1,989	1,034	32,958	34,993	33,999	32,973	33,973	32,963
miR-29c	1,969	1,072	29,988	29,978	29,975	29,982	28,979	29,974
miR-300*	0,246	1,242	35,624	35,444	37,180	35,971	35,147	34,972
miR-30a	1,495	1,806	27,928	28,970	28,962	28,961	27,952	27,956
miR-30a*	1,069	1,609	27,867	27,917	27,965	27,762	25,926	25,915
miR-30b*	0,993	1,339	33,475	33,319	32,688	32,178	32,422	32,763
miR-30e	1,827	1,875	26,922	27,953	26,921	26,925	26,952	26,950
miR-30e*	1,117	1,331	27,856	27,956	27,798	27,881	26,847	26,873
miR-31	1,338	1,477	26,965	26,970	26,969	26,967	25,974	25,960
miR-31*	1,238	1,099	28,983	28,992	28,992	28,972	27,975	27,967
miR-320	1,432	0,726	28,970	28,972	28,975	28,976	28,965	28,959
miR-329	1,986	1,912	32,969	32,955	32,971	31,946	31,971	31,966
miR-33*	1,299	1,012	32,981	32,981	31,970	33,000	31,966	31,983
miR-339-3p	0,959	0,687	29,001	29,989	29,055	29,988	30,013	29,016
miR-342-3p	1,301	1,343	26,961	26,956	27,981	27,982	26,960	26,980
miR-34c*	1,255	0,792	28,969	28,954	28,975	28,969	28,953	28,973
miR-361	1,268	1,533	31,977	31,968	31,973	31,989	30,985	30,979
miR-374	0,854	0,753	25,952	26,965	26,873	26,894	25,956	26,940
miR-376a*	1,459	0,645	31,983	30,946	30,981	30,963	30,974	30,991
miR-376c	1,939	1,055	25,963	25,965	25,976	25,985	25,974	25,972
miR-378	1,298	0,693	26,036	25,944	25,933	25,855	25,839	25,885
miR-380-3p	0,652	1,182	33,972	34,985	33,957	35,020	33,952	33,994
miR-411*	1,162	0,575	30,973	31,048	31,989	31,976	31,982	31,978
miR-425	1,385	0,906	27,946	27,984	28,035	27,973	27,972	27,946
miR-425*	0,378	0,934	32,973	32,981	33,978	33,962	32,974	32,978
miR-484	1,721	1,116	23,952	23,946	23,952	23,941	23,957	23,957
miR-491	1,373	1,619	30,936	31,980	30,984	30,982	30,959	30,978
miR-501-5p	0,003	0,528	33,984	33,979	40,000	40,000	32,983	32,962
miR-542-5p	1,493	1,981	33,001	31,978	32,985	31,972	30,975	30,975
miR-674	1,957	1,579	30,982	30,997	29,984	29,970	29,976	29,961
miR-674*	1,681	1,360	28,978	28,973	28,980	27,967	27,972	27,980
miR-676*	1,291	1,660	35,983	34,988	34,979	33,985	33,988	33,970
miR-678	1,167	0,605	31,732	31,683	31,710	31,974	31,365	31,955
miR-687	1,986	1,125	26,072	25,947	24,896	25,986	24,995	25,961
miR-690	0,967	0,897	23,982	23,955	23,942	23,967	23,967	23,932
miR-694	1,608	0,748	33,649	33,144	33,266	33,616	33,281	32,541
miR-696	1,359	0,589	25,802	26,972	26,689	26,956	26,725	26,913
miR-699	0,756	0,525	25,974	25,982	26,967	26,925	25,937	25,873
miR-700	1,100	0,644	29,778	29,774	29,347	29,782	29,764	29,701
miR-704	1,408	1,060	32,275	32,800	32,243	32,461	31,773	31,855
miR-709	1,283	0,633	22,914	22,945	22,963	22,953	22,925	22,939
miR-720	1,055	0,655	23,871	23,937	23,964	23,960	23,954	23,986
miR-760	1,660	1,360	30,920	30,920	29,813	30,928	29,607	29,865
miR-7a*	1,175	0,630	28,972	28,980	28,987	27,961	27,957	27,968
miR-804	0,939	0,866	33,046	32,961	33,121	33,934	32,483	32,312
miR-805	0,710	0,505	25,929	25,821	26,831	26,687	25,867	25,648
miR-872*	0,998	1,445	28,971	28,956	28,953	28,958	27,964	27,971
miR-877	1,108	0,722	31,235	30,960	31,859	30,957	30,849	30,734
miR-877*	1,295	0,566	29,987	28,980	29,000	29,006	28,989	29,004
miR-92a	1,821	0,804	23,939	23,950	23,951	23,947	24,962	24,963
miR-93*	1,348	1,142	26,955	26,949	26,962	26,960	25,955	25,956

miR-99b	1,758	1,995	26,983	26,958	26,943	26,992	25,972	25,995
miR-99b*	1,109	1,478	32,998	33,001	32,989	32,992	30,972	30,987

Table 7. microRNAs unchanged upon the induction of ESC differentiation.

Expression changes at 2 and 4 days of differentiation are calculated as fold change relative to undifferentiated ESCs. Cts are referred to two independent samples of undifferentiated (Ct ESCs) cells and two (Ct 2d) or four (Ct 4d) days differentiated ESCs. Cut off value was a fold change < 2 at day 2 of differentiation and < 2 but > 0,50 at 4 days of differentiation.

Target	microRNA
ABCD4	miR-340-5p
ABCG4	miR-9, miR-322, miR-218, miR-133a, miR-15a, miR-132, miR-32, miR-185, miR-145, miR-370, miR-93, miR-367, miR-106b, miR-15b, miR-25, miR-17, miR-20a, miR-137, miR-133b, miR-217
ADAM23	miR-32, miR-367, miR-25
AES	miR-124
ARID5B	miR-384-5p, miR-124, miR-302a, miR-302d, miR-301b, miR-301a, miR-302b, miR-32, miR-130b, miR-30b, miR-30c, miR-130a, miR-367, miR-30d, miR-25, miR-101a, miR-30e, miR-30a
B3GNT1	let-7e, let-7c, let-7i
BCAT2	miR-9, miR-135a, miR-32, miR-367, miR-25
BCLAF1	miR-384-5p, miR-181a, miR-9, miR-362-3p, miR-330, miR-339-5p, miR-30b, miR-30c, miR-30d, miR-329, miR-30e, miR-30a, miR-219, miR-326
BRP44L	miR-124, miR-302a, miR-302d, miR-302b, miR-137, miR-217
BSPRY	miR-32, miR-367, miR-25
CACNB4	miR-340-5p
CACYBP	miR-384-5p, miR-30b, miR-30c, miR-30d, miR-30e, miR-30a
CALML4	miR-135a, miR-218, miR-223, miR-296-3p, miR-138
CHCHD4	miR-152, miR-148b, miR-148a
CREB1	miR-181a
CTBP2	miR-206, miR-133a, miR-1, rno-miR-1, miR-101a, miR-133b
CTH	miR-384-5p, miR-322, miR-15a, miR-30b, miR-30c, miR-15b, miR-30d, miR-30e, miR-30a
DEF6	miR-124
DERL3	miR-330, miR-326
DTX1	miR-138, miR-204
EED	miR-384-5p, miR-181a, miR-30b, miR-30c, miR-30d, miR-101a, miR-30e, miR-30a
EEF1E1	miR-204
EFHC2	miR-137
EGLN3	miR-9, miR-218, miR-301b, miR-301a, miR-130b, miR-130a, miR-93, miR-106b, miR-17, miR-20a
EIF4A2	miR-181a, miR-132, miR-19b, miR-19a
ELAVL2	miR-181a, miR-9, miR-152, miR-340-5p, miR-302a, miR-302d, miR-302b, miR-148b, miR-19b, miR-26b, miR-542-3p, miR-19a, miR-148a, miR-26a, miR-140, miR-486, miR-216a, miR-153, miR-217, miR-383
ENAH	miR-384-5p, miR-181a, miR-335-5p, miR-9, miR-322, miR-124, miR-340-5p, miR-302a, miR-302d, miR-330, miR-15a, miR-301b, miR-301a, miR-365, miR-302b, miR-130b, miR-30b, miR-30c, miR-130a, miR-19b, miR-542-3p, miR-15b, miR-30d, miR-186, miR-101a, miR-30e, miR-194, miR-19a, miR-30a, miR-544, miR-216b, miR-204, miR-326, miR-384-3p, miR-153, miR-217, miR-383
EPS8	miR-218, miR-124, miR-365, miR-32, rno-miR-381, miR-367, miR-381, miR-25, miR-216b, miR-137, miR-204
FGF4	miR-93, miR-106b, miR-17, miR-20a
FN1	miR-101a
FOXD3	rno-miR-381, miR-381, miR-382
FZD4	miR-152, miR-218, miR-124, let-7e, miR-103, miR-107, miR-330, miR-301b, miR-301a, miR-130b, miR-145, miR-130a, miR-148b, miR-93, miR-106b, let-7c, let-7i, miR-101a, miR-194, miR-17, miR-148a, miR-496, miR-20a, miR-219, miR-326, miR-216a
GCLM	miR-9, miR-32, miR-145, miR-367, miR-186, miR-25
GFPT2	miR-384-5p, miR-124, miR-340-5p, miR-32, miR-30b, miR-30c, rno-miR-381, miR-367, miR-30d, miR-381, miR-25, miR-30e, miR-30a, miR-153
GLDC	miR-384-5p, miR-30b, miR-30c, miR-30d, miR-30e, miR-30a
GNPNAT1	miR-9, miR-206, miR-1, miR-103, miR-107, rno-miR-1, miR-26b, miR-26a
GSTM3	miR-145
GSTT2	miR-137
GTF2H1	miR-384-5p, miR-152, miR-301b, miR-301a, miR-130b, miR-30b, miR-30c, miR-130a, miR-148b, miR-30d, miR-30e, miR-148a, miR-30a
HAT1	miR-486
HELLS	miR-384-5p, miR-34c, miR-30b, miR-30c, miR-34a, miR-30d, miR-30e, miR-30a
HK2	miR-9, miR-125b-5p, miR-351, rno-miR-351, miR-143, let-7e, miR-19b, let-7c, let-7i, miR-125a-5p, miR-19a
ICAM1	miR-125b-5p, miR-351, rno-miR-351, miR-125a-5p
INA	miR-34c, miR-592, miR-34a, miR-138

JARID1B	miR-18a, miR-132, rno-miR-381, miR-381, miR-194, miR-18b, miR-137
JARID2	miR-384-5p, miR-181a, miR-322, miR-503, miR-152, miR-206, miR-1, miR-15a, miR-301b, miR-301a, miR-32, miR-130b, miR-30b, rno-miR-1, miR-30c, miR-130a, miR-148b, miR-138, miR-367, miR-19b, miR-26b, miR-15b, miR-30d, miR-25, miR-30e, miR-19a, miR-148a, miR-30a, miR-26a, miR-544, miR-204, miR-153
JMJD1A	miR-384-5p, miR-30b, miR-30c, miR-30d, miR-30e, miR-30a
KLF4	miR-135a, miR-152, miR-124, miR-103, miR-107, miR-32, miR-145, miR-148b, rno-miR-381, miR-367, miR-26b, miR-381, miR-25, miR-148a, miR-146a, miR-26a, miR-137
KLF5	miR-9, miR-152, miR-143, miR-145, miR-148b, miR-21, miR-148a, miR-153
LAPTM5	miR-219
LRAT	miR-206, miR-218, miR-1, miR-362-3p, rno-miR-1, miR-329
LRBA	miR-181a, miR-223
LRRC2	miR-133a, miR-26b, miR-26a, miR-133b
MAT2A	miR-384-5p, miR-124, miR-340-5p, miR-30b, miR-30c, miR-26b, miR-30d, miR-30e, miR-30a, miR-26a, miR-153
MCF2	miR-218, miR-124, miR-143
MGA	miR-181a, miR-9, let-7e, miR-103, miR-107, let-7c, let-7i, miR-216a, miR-383
MKRN1	miR-384-5p, miR-302a, miR-302d, miR-302b, miR-30b, miR-30c, miR-93, miR-19b, miR-106b, miR-30d, miR-30e, miR-19a, miR-17, miR-30a, miR-20a
MRAS	miR-135a, miR-152, miR-206, miR-34c, miR-1, miR-362-3p, rno-miR-1, miR-148b, miR-34a, miR-138, miR-26b, miR-329, miR-148a, miR-26a
MSC	miR-149
MTF2	miR-181a, miR-138, miR-186, miR-153
MYB	miR-322, miR-503, miR-15a, miR-301b, miR-301a, miR-130b, miR-130a, miR-15b, miR-153
MYBL2	miR-384-5p, miR-143, miR-30b, miR-30c, miR-30d, miR-30e, miR-30a
MYNN	miR-103, miR-107, miR-93, miR-106b, miR-17, miR-20a
MYST4	miR-328, miR-135a, miR-340-5p, rno-miR-381, miR-381, miR-101a
NCL	miR-206, miR-1, rno-miR-1
NEFH	miR-32, miR-367, miR-25, miR-137
NFYB	miR-384-5p, miR-335-5p, miR-340-5p, miR-197, miR-32, miR-30b, miR-30c, rno-miR-381, miR-367, miR-30d, miR-381, miR-25, miR-30e, miR-30a, miR-221, miR-544, miR-216b, miR-137
NME7	miR-301b, miR-301a, miR-130b, miR-130a, miR-19b, miR-19a
NOL8	miR-181a
NR5A2	miR-384-5p, miR-9, miR-30b, miR-30c, rno-miR-381, miR-30d, miR-381, miR-186, miR-30e, miR-30a, miR-216a
NRP2	miR-322, miR-15a, rno-miR-381, miR-15b, miR-381, miR-149
NUP35	miR-301b, miR-301a, miR-130b, miR-130a, rno-miR-381, miR-93, miR-106b, miR-381, miR-17, miR-20a
OTX2	miR-152, miR-206, miR-1, miR-301b, miR-301a, miR-130b, miR-145, rno-miR-1, miR-130a, miR-148b, miR-148a, miR-219, miR-153
PADI2	miR-330, miR-137, miR-326
PARP16	miR-384-5p, miR-328, miR-124, miR-30b, miR-30c, miR-30d, miR-30e, miR-30a, miR-761
PAX6	rno-miR-190b, miR-365, miR-190b, miR-190
PCOLCE2	miR-32, miR-367, miR-25
PDCD10	miR-384-5p, miR-206, miR-1, miR-103, miR-107, miR-30b, rno-miR-1, miR-30c, miR-26b, miR-30d, miR-186, miR-30e, miR-30a, miR-26a
PECAM1	miR-26b, miR-26a
PFKP	miR-302a, miR-302d, miR-302b, miR-93, miR-106b, miR-17, miR-20a
PHC1	miR-340-5p, miR-324-5p, miR-149, miR-186
PIGA	miR-384-5p, miR-328, miR-152, let-7e, miR-302a, miR-302d, miR-301b, miR-301a, miR-302b, miR-130b, miR-30b, miR-30c, miR-130a, miR-148b, let-7c, miR-30d, let-7i, miR-30e, miR-148a, miR-30a, miR-361, miR-486, miR-153
PLA2G10	miR-19b, miR-19a
PML	miR-124, miR-133a, miR-149, miR-133b
PMM1	miR-322, miR-15a, miR-15b
POU5F1	miR-335-5p, miR-491
PSAT1	miR-145
PTP4A3	miR-18a, miR-140, miR-18b, miR-137
QDPR	miR-124
RARG	miR-384-5p, miR-124, miR-143, miR-330, miR-30b, miR-28, miR-30c, miR-30d, miR-30e, miR-30a, miR-326

RFX2	miR-384-5p, miR-30b, miR-30c, miR-30d, miR-30e, miR-30a
RIF1	miR-340-5p, rno-miR-381, miR-381
RNF125	miR-181a, miR-322, miR-124, miR-340-5p, miR-103, miR-107, miR-15a, miR-15b, miR-186, miR-101a, miR-140, miR-486, miR-204, miR-384-3p
RNF138	miR-135a, miR-322, miR-503, miR-206, miR-1, miR-15a, rno-miR-1, miR-26b, miR-15b, miR-149, miR-186, miR-26a, miR-137, miR-204, miR-216a
RNMT	miR-384-5p, miR-181a, miR-223, miR-362-3p, miR-103, miR-107, miR-30b, miR-30c, miR-30d, miR-329, miR-30e, miR-30a
RPS16	miR-340-5p
SDAD1	miR-384-5p, miR-30b, miR-30c, miR-30d, miR-30e, miR-194, miR-30a, miR-761
SFRS3	miR-135a, miR-206, miR-1, rno-miR-1, miR-21, miR-486
SFRS7	miR-384-5p, miR-181a, miR-30b, miR-30c, miR-30d, miR-30e, miR-30a, miR-216b
SHMT1	miR-218, miR-330, miR-326
SIRT1	miR-384-5p, miR-181a, miR-9, miR-135a, miR-133a, miR-30b, miR-30c, miR-138, miR-30d, miR-30e, miR-30a, miR-204, miR-133b, miR-217
SLC25A5	miR-137
SLC29A1	miR-124
SMAD7	miR-181a, miR-322, miR-503, miR-340-5p, miR-15a, miR-32, miR-93, miR-367, miR-106b, miR-15b, miR-21, miR-25, miR-17, miR-20a, miR-216a
SMARCA5	miR-384-5p, miR-30b, miR-30c, miR-30d, miR-99b, miR-101a, miR-30e, miR-30a, miR-100, miR-99a
SNX10	miR-384-5p, miR-30b, miR-30c, miR-30d, miR-30e, miR-30a
SOCS2	miR-101a, miR-194, miR-153
SOCS3	miR-384-5p, miR-181a, miR-152, miR-218, miR-124, miR-340-5p, miR-30b, miR-30c, miR-148b, miR-19b, miR-30d, miR-30e, miR-19a, miR-148a, miR-30a, miR-221, miR-383
SPOCK2	miR-181a, miR-124, miR-340-5p, miR-32, miR-367, miR-26b, miR-25, miR-26a, miR-137
SPP1	miR-181a
SPRY2	miR-340-5p, miR-21, rno-miR-758
SPRY4	miR-181a, miR-322, miR-133a, miR-15a, rno-miR-381, miR-93, miR-19b, miR-106b, miR-15b, miR-381, miR-19a, miR-17, miR-140, miR-20a, miR-133b
SRM	miR-340-5p
SSTR1	miR-149
STMN1	miR-101a
STOML1	miR-384-5p, miR-30b, miR-30c, miR-30d, miR-30e, miR-30a
SYCP3	miR-137
SYNGR1	miR-218, miR-197, miR-370
TCEA3	miR-186
TCF15	miR-132
TFRC	miR-9, miR-152, miR-124, miR-34c, miR-145, miR-148b, miR-34a, miR-194, miR-148a, rno-miR-758, miR-544
TLE4	miR-322, miR-503, miR-124, miR-103, miR-107, miR-302a, miR-302d, miR-15a, miR-302b, miR-93, miR-106b, miR-15b, miR-17, miR-20a, miR-761
TNFSF11	miR-93, miR-106b, miR-17, miR-20a
TRIM2	miR-181a, miR-9, miR-322, miR-206, miR-1, miR-18a, miR-302a, miR-302d, miR-15a, miR-301b, miR-301a, miR-302b, miR-130b, miR-339-5p, miR-145, rno-miR-1, miR-130a, miR-19b, miR-15b, miR-19a, miR-18b, miR-204, miR-761
TRIM25	miR-9, miR-186, miR-544
TWISTNB	miR-340-5p
UBTF	miR-145, miR-370, miR-26b, miR-101a, miR-26a, miR-382
UBXD4	miR-93, miR-106b, miR-17, miR-20a
VEGFC	miR-137
WDR35	miR-221, miR-544
XBP1	miR-32, miR-367, miR-25
XRCC5	miR-340-5p
ZFP42	miR-186, miR-384-3p

Table 8. TargetScan prediction.

List of genes down-regulated during ESC differentiation and predicted target all UP-regulated microRN

Discussion

microRNAs function as fine regulators of gene expression and they have been implicated in the maintenance of ESC phenotype and in modulation of their differentiation program. Several microRNAs expressed in mammalian ESCs have been cloned (Calabrese, Seila et al. 2007; Babiarz, Ruby et al. 2008) and mouse ESC specific microRNAs have been described (Houbaviy, Murray et al. 2003). However their function, particularly at early stages of ESC differentiation, has not been reported so far, even if it was demonstrated that ESCs lacking Dicer or Drosha, and therefore most mature microRNAs do not differentiate into most lineages (Kanellopoulou, Muljo et al. 2005; Murchison, Partridge et al. 2005; Chang, Wentzel et al. 2007). Several factors complicate the identification and the functional characterization of microRNAs during ESC differentiation. In fact, the expression profile of microRNAs strictly depends on the differentiation program occurring. Moreover, considering that microRNAs exhibit a high degree of stage- and tissue-specificity, it is likely that those operating during narrow windows of differentiation, that is those controlling the balance between self-renewal and differentiation, may be under-represented in the current databases. So the complete picture of the microRNAs involved in ESC functions and especially their roles in the regulation of ESC fate have to be further investigated.

In this study we reported a systematic analysis of microRNA expression profile in undifferentiated and differentiating mouse ESCs. Briefly to perform our profiling experiments we collected RNA samples from undifferentiated and two and four day differentiated ESCs and then we performed a TaqMan analysis. The choice of time points at which to collect RNA samples during the course of differentiation is a crucial point for a microRNA profiling experiment. In fact, individual microRNAs

can show dynamic changes during ESC differentiation, and therefore the profiling of microRNA expression before and after differentiation would only allow assessment of ultimate changes, thereby precluding identification of microRNAs expression changes during differentiation. So to identify microRNAs controlling the balance between self-renewal and differentiation of ESCs we proceeded with the examination of the microRNA expression at two early differentiation time points.

We did not choose to use retinoic acid (RA) to induce differentiation but a different protocol that induced a slower and more physiological differentiation, mainly into neurons.

Another important issue needs to be addressed regarding microRNA expression profiling. The latter is an important tool for the identification of differentially expressed microRNAs but remains a technical challenge for high-throughput microRNA expression analysis, because the number of microRNAs continues to increase with in silico prediction and experimental verification. So the choice of an advanced microRNA expression profiling approach, as the quantitative RT-PCR array (qPCR-array), rather than oligonucleotide microchip (microarray), is fundamental. In fact, comparison between microarray and qPCR-array indicated superior sensitivity and specificity of qPCR-array, which has also higher reproducibility and a lower false positive rate of differential microRNA expression (Chen, Gelfond et al. 2009). These previous observations suggested us to use the quantitative RT-PCR approach for our microRNA profiling.

Comparison of the expression profiles between undifferentiated ESC and differentiating cells allowed us to classify microRNAs in five groups: (1) microRNAs decreased upon the induction of ESC differentiation; (2) microRNAs undetectable in undifferentiated ESCs and expressed upon the induction of the differentiation program; (3) microRNAs expressed in ESCs and increased upon

the induction of ESC differentiation; (4) microRNAs undetectable both in undifferentiated and differentiated ESCs; (5) microRNAs unmodified upon the induction of differentiation.

The analysis of groups of microRNAs whose profiling was already described in ESCs allows us to verify that there was a high degree of coherence between our microRNA expression data and published data. Indeed in the group of microRNAs decreased upon the induction of ESC differentiation we have found miR-290 cluster well known to be ESC-specific and to have key functions in stemness maintenance such as DNA methylation and proliferation control (Sinkkonen, Hugenschmidt et al. 2008). Also miR-200 family is an ESC specific family of microRNAs (Calabrese, Seila et al. 2007) that we found to be strongly decreased upon induction of ESC differentiation. These results were in agreement with Lin and co-workers, which demonstrated that overexpression of the members of this microRNA family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) was most effective in retarding stemness marker loss after LIF removal, while their knockdown facilitated differentiation (Lin, Jackson et al. 2009). In the group of microRNAs undetectable in undifferentiated ESCs and expressed upon the induction of the differentiation program we reported 29 microRNAs. Some of them (miR-100, miR-137, miR-204, miR-383 and miR-99a) were already described to be undetectable in ESCs (Calabrese, Seila et al. 2007). Earlier studies had reported that miR-135 and miR-124 regulate neurogenesis during central nervous system (CNS) development (Cao, Pfaff et al. 2007; Visvanathan, Lee et al. 2007). Accordingly we have classified these two microRNAs belong to our group of strongly increased microRNAs upon induction of ESC differentiation. In the same group we listed also miR-9, well known to modulate ESC-derived neurogenesis (Krichevsky, Sonntag et al. 2006).

Furthermore Smirnova and co-workers demonstrated that mir-23, miR-26 and miR-29 were more strongly expressed in astrocytes than neurons (Smirnova, Grafe et al. 2005).

In our differentiating ESCs miR-23b, miR-26a, miR-29b and miR-29c displayed a fold induction lower than our cut off value (fold increase<2), so we classified them in the group of microRNAs whose expression was unchanged during ESC differentiation. On the other hand, miR-26b and miR-29a were increased upon differentiation with a fold induction just a bit higher than the cut off value. These results are in agreement with Smirnova and co-workers. Indeed our differentiation protocol produced mainly neurons and only a small fraction is represented by astrocyte differentiated cells.

The let-7 family is comprised of 12 family members (let-7-a1, a2, a3, b, c, d, e, f1, f2, g, i and miR-98), known to be strongly induced during ESC neural differentiation and in brain (Wulczyn, Smirnova et al. 2007). However, we found only three members (let7-c, let-7e and let-7i) of this family in our up-regulated microRNAs and we observed an increase of the expression profile of these specific microRNAs only at day 4 of differentiation. Nevertheless, because we focused our analysis to the early phase of differentiation our data were consisting with the previous description of let-7 family as late expressed microRNA family in mammalian embryonic development.

We found a good degree of congruity between our data and published data also in the case of miR-302 cluster (all miR-302 family and miR-367), belonging to the group of microRNAs increased upon ESC differentiation induction (Ciaudo, Servant et al. 2009).

miR-17 family consists of 14 mature microRNAs (miR-17, miR-18a, miR-18b, miR-19a, miR-19b, miR-20a, miR-20b, miR-25, miR-92a and 92b, miR-93, miR-106a

and miR-106b), expressed as three polycistronic clusters. According to us Foshay and collaborators reported that some members of this family (miR-17, miR-20a, miR-93, and miR-106a) are expressed in ESCs and increased upon induction of differentiation (Foshay and Gallicano 2009). We found a very similar profile also for the other members of this family, which are significantly expressed in undifferentiated cells and their expression appreciably increased during differentiation. Previously, these microRNAs have been considered ESC-specific for their abundance in undifferentiated ESCs and in some cases for their reduction in differentiated ESCs (Houbaviy, Murray et al. 2003; Calabrese, Seila et al. 2007). However a very recent work demonstrated that their expression persisted or increased upon ESC differentiation according to our results (Ciaudo, Servant et al. 2009). Differences in the ESC line and in differentiation protocol used could explain these discrepancies.

In our data miR-21 followed the expression profile of miR-17 cluster microRNAs. miR-21 is expressed in ESCs and its expression appreciably increased in ESC differentiating derivatives. This is in agreement with the role of miR-21 recently described as inhibitor of self-renewal and pluripotency of ESCs through suppression of Nanog, Sox2 and possibly Oct3/4 stemness genes (Singh, Kagalwala et al. 2008).

Finally, in the group of microRNAs undetectable both in undifferentiated and differentiated ESCs we found some microRNAs already described to be absent in undifferentiated ESCs such as miR-122, miR-208, miR-214, miR-215, miR-451, miR-452, miR-551b, miR-675 and miR-693 (Calabrese, Seila et al. 2007).

Notably, in our study we reported that a large number of microRNAs are increased upon the induction of differentiation. For some of them we also performed a

systematically identification of the mRNA targets by integrated experimental and bioinformatics approaches.

We focused on the group of up-regulated microRNAs because the role of microRNAs in undifferentiated ESCs is enough established compared with their possible role in assisting ESC differentiation. We reasoned that for ESC-derived cell types it is equally critical to suppress genes necessary to maintain ESCs in the undifferentiated state as well as to suppress genes driving differentiation toward alternative fates. While this occurs at the transcriptional level, it is possible that microRNAs contribute to this process by clearing expressed mRNAs that antagonize newly adopted fate. Moreover, the identification of targets of these microRNAs will contribute to a more detailed knowledge of the molecular mechanisms of ESC differentiation and may provide tools to direct ESC differentiation towards specific lineages.

We focused on three of these up-regulated microRNAs, miR-100, miR-137 and miR-34a, whose candidate targets are downregulated upon ESC differentiation, and we provided evidence that these microRNAs are necessary for proper differentiation of ESCs.

Several groups associated miR-100 and cancer. miR-100 can act either as a tumor suppressor (Nagaraja, Creighton et al. 2010) or as oncogene (Ueda, Volinia et al. 2009). However, its role in cell differentiation is completely unknown. It belongs to miR-99 microRNA family that comprises other two more members: miR-99a and miR-99b. miR-100 and miR-99a are very similar in nucleotide sequence, indeed differ only for one nucleotide (Figure 33), so they also share candidate targets (http://www.mirbase.org/).

mmu-mir-99a
AACCCGUAGAUCCGAUCUUGUG
mmu-mir-99b
CACCCGUAGAACCGACCUUGCG
mmu-mir-100
AACCCGUAGAUCCGAACUUGUG

Figure 33. Nucleotide sequence of miR-99 family member.

In yellow are evidenced the differences in nucleotide sequences between the three microRNAs.

miR-100 and miR-99a are undetectable in undifferentiated ESCs, accumulate starting from day 2 after the induction of differentiation and reach robust levels of expression at day 4 of differentiation. Instead miR-99b is already present in undifferentiated ESCs and increases when differentiation occurs. We demonstrated that Smarca5 is a new direct target for miR-100. Of course, the biological effects observed in cells where this microRNA was silenced could be also due to the targeting of other still unknown mRNAs. Similarly to miR-100, miR-137 is undetectable in undifferentiated ESCs, appears at day 2 and significantly increases after 2 more days of differentiation. Also in this case we have identified a new target for this microRNA, Jarid1b. miR-137 is a tumor suppressor microRNA; it was downregulated in human oral cancers and its re-expression blocks tumor cell proliferation (Kozaki, Imoto et al. 2008). The possible role of miR-137 in the regulation of cell differentiation is also supported by the observations that its overexpression induces the differentiation of mouse neural stem cells and of other stem cells derived from oligodendroglioma or glioblastoma multiforme (Silber, Lim et al. 2008).

miR-34a is a well known microRNA, whose involvement in various cellular functions is well documented and whose role as tumor suppressor is widely accepted (Chang, Wentzel et al. 2007). It belongs to miR-34 family that comprises miR-34a, miR-34b and miR-34c members that are all microRNAs induced upon

ESC differentiation. It was previously demonstrated that miR-34a targets Sirt1 (Yamakuchi, Ferlito et al. 2008) and is regulated by p53 (Hermeking 2010), which, in turn, is activated by Sirt1 suppression. The induction of miR-34 upon ESC differentiation and the concomitant decrease of Sirt1 (Figure 15 and (Loh, Wu et al. 2006)), suggest that p53 could be activated through this mechanism and this hypothesis is in agreement with the demonstration that p53 represses Nanog gene transcription, thus favoring ESC differentiation (Lin, Chao et al. 2005). Another not alternative mechanism through which miR-34a may influence ESC differentiation could be based on the downregulation of Wnt1, which was recently demonstrated to be a target of miR-34a (Hashimi, Fulcher et al. 2009). Wnt1 is required to maintain long-term mouse ESC pluripotency (Miyabayashi, Teo et al. 2007) and differentiation of ESCs is accompanied by a significant downregulation of Wnt1 protein level (Sampath, Pritchard et al. 2008). Also in this case, miR-34a-dependent targeting of Wnt1 could contribute to drive differentiation of ESCs.

We obtained strong evidence that the suppression of these three microRNAs, we have characterized, is necessary for proper differentiation of mouse ESCs. In fact, their suppression impairs ESC differentiation in vitro (Figure 30) and in vivo (Figure 32). On the basis of these results we proposed a key role for microRNAs that overcomes their function as modulators. Indeed, it becomes increasingly evident that microRNAs play a fundamental and irreplaceable role in the regulation of the biological processes, including ESC fate and behavior. So it becomes indispensable also to study also the mechanisms that control their expression at transcriptional level. We identified Smarca5, Jarid1b and Sirt1 as target of miR-100, miR-137 and miR-34a, respectively, but it is important to identify other possible targets of these and other microRNAs to verify the regulating key molecules that define stem cell fate and behavior.

Methods

Plasmid construction

The firefly luciferase gene was derived by PCR from pGL3 basic vector (Promega) with a forward and reverse primer carrying EcoRI restriction site and inserted in pCAG-MS vector in EcoRI site. The 3'UTRs of Sirt1 (from nucleotide 2802 to 4341), Jarid1b (from nucleotide 4804 to 6311) and Smarca5 (from nucleotide 3519 to 3742) genes were obtained from ESCs by PCR with a sense and antisense primer carrying a KpnI restriction site. The 3'UTR and inverted 3'UTR of these three genes were cloned downstream to firefly luciferase gene in KpnI site in pCBA-GFP vector by replacing GFP (Parisi, Passaro et al. 2008). Sequences of oligonucleotides used for cloning are provided in Appendix 2.

Cell culture, transfection and luciferase assay

E14Tg2a (BayGenomics) mouse ESCs were maintained on feeder-free, gelatine coated plates in the following medium: GMEM (Sigma) supplemented with 2mM glutamine (Invitrogen), 100U/ml penicillin/streptomycin (Invitrogen), 1mM sodium pyruvate (Invitrogen), 1x non essential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), 10% FBS (Hyclone), and 103 U/ml leukemia inhibitory factor (LIF) (Millipore, Billerica, MA, USA). HEK293 cells were cultured in DMEM (Invitrogen) supplemented with 2mM glutamine (Invitrogen), 100U/ml penicillin/streptomycin (Invitrogen) and 10% FBS (Invitrogen). Both HEK293 cells and ESCs were plated at $6x10^4$ cells/cm^2 16 hours before transfection. Transfection of plasmids, shRNAs (Open Biosystems), pre-microRNAs (Ambion) and anti-microRNAs (Ambion) in ESCs were performed using Lipofectamine 2000 following manifacturer's instructions. To generate the stable cell lines, E14Tg2a

cells were transfected with luciferase expression plasmids (see above) and recombinant clones were selected with G418 (Invitrogen) or with shRNAs and silenced clones were selected with puromycin.

For luciferase assay HEK293 cells were co-transfected with 500ng of pCAG-MS reporter vector carrying 3'UTR or inverted 3'UTR, 10ng pRL-TK vector (Promega) and 100nM of pre-microRNAs or scrambled oligonucleotides (both from Ambion) by using Lipofectamine 2000 (Invitrogen). Firefly and renilla luciferase activities were measured 48h after transfection. For E14 ESCs stably transfected with reporter vector or inverted reporter vector we performed luciferase activities at day 4 of differentiation. Firefly and renilla luciferase activities were measured with the dual-luciferase reporter system (Promega) by Sirius Luminometer (Berthold Detection Sistems). The data generated were expressed as relative to scrambled oligonucleotide-transfected cells, after normalization to Renilla luciferase reading. All transfection experiments were repeated in triplicate.

ESC differentiation

ESCs were trypsinized into a single cells suspension, collected by centrifugation and resuspended in the following medium: Knockout Dulbecco's minimal essential medium supplemented with 10% Knockout Serum Replacement (both from Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), 2 mM glutamine (Invitrogen), 100 U/mL penicillin/streptomycin (Invitrogen). To induce efficient neural differentiation the cells were plated at low density ($3x10^3$ cells/cm^2) on gelatin-coated dishes and differentiation medium was changed on alternated days.

RNA isolation and TaqMan analysis

For TaqMan analysis total RNA was isolated from undifferentiated and two and four days differentiated ESCs with mirVana microRNA Isolation kit (Ambion). From

each sample, 10 ng of total RNA were used to synthesize single-stranded cDNA with TaqMan MicroRNA Reverse transcription kit (Applied Biosystems) combined with the two Rodent Megaplex RT Primer Pool (Pool A and Pool B). Expression level of all microRNAs of Sanger miRBase version 10 database were measured by using TaqMan MicroRNA Arrays (Applied Biosystems) with 7900HT instrument and the Sequence Detection Systems (SDS) Software version 2.1 (Applied Biosystems). The microRNA profiling data were analysed performing a comparative analysis by using the comparative Ct method ($2^{-\Delta Ct}$, RQ Manager 1.2 software, Applied Biosystems).

For Northern Blot analysis total RNA was isolated by using the TRI Reagent (Sigma) according to the manufacturer's instructions. For each sample 20 µg of total RNA were fractionated on 15% TBE-Urea gel (Criterion precast Gel, Bio-Rad), stained with EtBr (Sigma) for loading control, then transfered to Hybond membrane (Amersham Pharmacia) and finally fixed by UV cross-linking in a Stratalinker (Stratagene) according to manufacturer's instruction. The membrane was hybridizated with 10pmol of miRCURY LNA Detection Probe digoxigenin labeled (Exiqon) according to manufacturer's instruction. The signal detection was obtained by an anti-digoxigenin-Alkaline Phosphatase antibody (Roche) and a chemiluminescent substrate (CDP-Star, Roche) according to manufacturer's instruction.

Preparation of cell lysate and Western blot analysis

Undifferentiated and differentiated ESCs were lysed in a buffer containing 1mM EDTA, 50 mM Tris-HCl pH 7.5, 70 mM NaCl , 1% Triton, protease inhibitor cocktail (Sigma) and protein extracts were analyzed by Western Blot. The following primary antibodies are used: rabbit anti-Smarca5 (hSNF2H H-300; Santa Cruz

Biotechnology), rabbit anti-KDM5B/Jarid1b (Abcam), rabbit anti-Sirt1 (Abcam), rabbit anti-p53 (Upstate), rabbit anti-acetylated-p53 in Lys379 (Cell Signaling), mouse anti-Gapdh (Santa Cruz). Antibody-protein complexes was detected by HRP-coniugated antibodies and ECL (both from Amersham Pharmacia). To analyze acetylated p53 levels by western blot analysis, ESCs were exposed to 60J/M^2 UV radiation and harvest after 16 hours.

Chromatin Immuno Precipitation (ChIP) analysis

Cells were cross-linked with 1% formaldehyde for 10 minutes at 37% and formaldehyde was then inactivated by the addition of 125 mM glycine. The chromatin was then sonicated to an average DNA-fragment length of 200-1000 bp. Soluble chromatin extracts were immunoprecipitated using anti-H3K4 antibody (upstate). Supernatant obtained without antibody was used as input control. The amount of Egr1 promoter in precipitated DNA was calculated by real-time PCR relative to the total input chromatin, and expressed as percent of total chromatin according to the following formula: 2Ct x 10, where Ct represents the cycle threshold and Ct = Ct(input) – Ct(immunoprecipitation) (Frank, Heim et al. 2002).

Teratoma Formation

Nude mice were injected subcutaneously with $2x10^6$ ESC cells transfected with anti-miR mixture (left side) or anti-miR control (right side). Four weeks after the injection, tumors were surgically dissected from the mice. Samples were fixed in 4% paraformaldehyde, and embedded in paraffin. Sections were stained with heamatoxylin and eosin.

Real-Time PCR

Total RNA was extracted by using TRI-Reagent (Sigma). The first-strand cDNA was synthesized according to the manufacturer's instructions (M-MLV RT kit; Invitrogen). Real Time RT-PCR was carried out on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using Power SYBR Green PCR Master mix (Applied Biosystems). The housekeeping GAPDH mRNA was used as an internal standard for normalization, using $2^{-\Delta Ct}$ method. Gene specific primers used for amplification are listed in Appendix 3.

Alkaline phosphatase staining

For alkaline phosphatase staining, ESCs were cultured at clonal density (100 cells/10 cm^2). The cells were fixed in 10% cold Neutral Formalin Buffer (10% formalin, 110 mM Na2HPO4, 30mM NaH2PO4.H2O) for 15 minutes and then rinsed in distilled water for 15 minutes. The staining was obtained by incubation for 45 minutes at room temperature with the following staining solution: 0.1M Tris-HCl, 0.01% Naphthol AS MX-PO4 (Sigma), 0.4% N,N-Dimethylformamide (Sigma), 0.06% Red Violet LB salt (Sigma).

Bioinformatics analysis

We took advantage from available data reporting gene expression profiles associated with differentiation induced by three chemical treatments (retinoic acid (RA), dimethyl sulfoxide (DMSO) and hexymethyl-bis-acetamide (HMBA), (Loh, Wu et al. 2006). In these arrays for each treatment, molecular profiles of treated cells were contrasted to that of untreated cells using two-channel microarrays across three time points. In total 18 microarrays data were available, in which the expression data was log transformed and median-center normalized. These 18 log expression data were treated as replicates, treatment and time factors were

ignored. So we used the absolute of average log expression ratio across the 18 arrays and sorting them by ascending level of expression ratio after differentiation. We obtained a list of genes differentially expressed across differentiation and we selected only gene downregulated upon ESCs differentiation. The list of selected genes was compared with putative targets of our subset of microRNAs upregulated during ESC differentiation. Target prediction was performed with Targetscan version 5.1.

Appendix

microRNA	2 days (fold change)	4 days (fold change)	Ct ESCs	Ct ESCs	Ct 2d	Ct 2d	Ct 4d	Ct 4d
let-7a			40,000	40,000	40,000	40,000	40,000	40,000
let-7a*	0,976	0,401	35,984	35,982	36,143	35,989	35,222	35,975
let-7b	0,090	1,127	35,984	36,025	35,945	40,000	36,023	36,025
let-7b*			40,000	40,000	40,000	40,000	40,000	40,000
let-7c	1,521	2,144	34,985	33,977	33,970	34,972	32,956	32,946
let-7c-1*			40,000	40,000	40,000	40,000	35,988	35,119
let-7d*			40,000	40,000	40,000	40,000	40,000	40,000
let-7e	0,791	4,900	32,971	33,004	32,977	34,006	30,972	29,959
let-7f			40,000	40,000	40,000	40,000	40,000	40,000
let-7f*			40,000	40,000	40,000	40,000	40,000	40,000
let-7g	0,469	0,558	30,969	31,966	32,978	31,988	31,988	32,004
let-7i	1,969	2,086	32,004	32,985	31,982	31,999	32,009	31,997
let-7i*			40,000	40,000	40,000	40,000	40,000	40,000
miR-1	5,544	5,750	35,922	35,994	34,968	33,976	32,961	33,988
miR-100			40,000	40,000	31,002	30,996	25,994	25,994
miR-101a	1,904	1,889	29,973	29,974	28,938	29,986	28,985	28,984
miR-101b	1,154	0,986	27,938	27,914	27,897	27,970	26,943	26,860
miR-103	3,503	4,547	28,999	28,979	27,994	27,987	26,989	26,994
miR-105			40,000	40,000	40,000	40,000	40,000	40,000
miR-106a	2,198	1,250	20,961	20,965	19,888	19,946	19,943	19,943
miR-106b	1,895	2,236	25,967	25,957	25,966	25,975	24,971	24,976
miR-106b*	1,060	0,992	27,868	27,933	27,866	27,909	26,947	26,797
miR-107	3,135	4,198	34,002	32,973	32,937	32,000	32,036	30,958
miR-10a*	0,950		35,980	34,991	35,801	35,982	40,000	40,000
miR-10b			40,000	40,000	40,000	40,000	40,000	40,000
miR-10b*			40,000	40,000	40,000	40,000	40,000	40,000
miR-122			40,000	40,000	40,000	40,000	40,000	40,000
miR-124	1,944	7,920	29,987	29,994	30,001	29,974	26,956	27,984
miR-124*			40,000	40,000	30,748	31,402	29,878	29,483
miR-125a-3p	2,304	1,208	33,978	33,966	32,961	32,978	32,974	32,978
miR-125a-5p	1,288	1,932	27,979	27,959	28,984	28,975	26,978	26,984
miR-125b*			40,000	40,000	33,966	35,961	32,114	32,863
miR-125b-3p			40,000	40,000	40,000	40,000	34,962	33,965
miR-125b-5p	0,815	11,232	29,986	29,967	30,976	30,975	26,981	25,963
miR-126-3p	1,585	0,858	25,961	25,965	27,001	26,986	26,992	26,981
miR-126-5p	1,732	0,855	29,978	29,961	29,973	29,972	29,977	29,970
miR-127	2,879	0,975	24,950	24,947	24,975	24,992	24,937	24,954
miR-127*	1,199	0,129	33,564	34,981	34,599	34,180	33,797	35,993
miR-128a	5,037	2,383	33,971	32,982	31,972	31,962	31,967	31,974
miR-129-3p	3,462	4,029	30,968	30,969	29,976	29,984	28,969	28,966
miR-130a	2,599	2,576	25,958	25,967	25,971	25,972	24,966	24,960
miR-130b	2,183	3,348	26,977	26,971	25,963	25,951	24,955	24,962
miR-130b*	1,222	1,319	30,964	30,888	30,841	30,953	29,866	28,956
miR-132	4,599	3,699	29,000	29,005	26,761	27,975	28,022	27,969
miR-133a	3,875	7,243	31,983	31,997	29,968	29,989	28,980	28,986

miR-133a*			40,000	40,000	40,000	40,000	40,000	40,000
miR-133b			40,000	40,000	40,000	40,000	30,937	32,031
miR-134	1,714	0,741	24,961	25,986	24,978	24,994	25,990	25,969
miR-135a	4,567	12,853	30,978	30,972	29,976	30,002	27,978	27,991
miR-135a*	0,975	0,618	28,944	28,857	28,934	28,855	28,881	28,947
miR-135b	1,403	0,809	24,977	24,972	24,959	24,987	24,973	24,973
miR-136	1,588	0,647	26,983	26,954	26,958	27,996	27,967	27,985
miR-137			40,000	40,000	35,010	33,976	29,961	30,978
miR-138	2,348	2,488	29,959	30,985	29,970	29,973	28,958	28,963
miR-138*	0,960	1,058	35,093	34,692	34,617	35,158	35,500	34,333
miR-139-3p			40,000	40,000	40,000	40,000	40,000	40,000
miR-139-5p	0,982	0,541	29,967	30,973	30,966	30,975	30,984	30,970
miR-140	1,969	1,693	26,963	26,975	25,947	25,946	25,954	25,968
miR-141	1,263	0,198	30,976	30,984	30,955	30,971	32,974	32,982
miR-141*	0,516		32,471	31,043	31,974	33,994	40,000	40,000
miR-142-3p	2,337	0,237	29,980	29,974	28,960	28,957	31,988	31,973
miR-142-5p	3,521	0,423	34,966	34,982	33,973	32,964	34,976	35,918
miR-143	3,203	5,355	30,978	30,963	30,986	29,966	28,983	28,969
miR-145	1,942	3,214	28,958	29,982	28,969	28,970	27,979	27,984
miR-145*			40,000	40,000	40,000	40,000	40,000	40,000
miR-146a	2,087	1,731	29,983	29,983	28,968	28,970	28,982	28,972
miR-146b	0,962	0,948	29,977	29,985	29,971	29,961	28,965	28,966
miR-146b*			40,000	40,000	40,000	40,000	40,000	40,000
miR-147			40,000	40,000	40,000	40,000	40,000	40,000
miR-148a	3,113	1,808	28,938	28,949	27,949	28,971	27,937	28,983
miR-148a*			40,000	40,000	40,000	40,000	40,000	40,000
miR-148b	3,245	2,563	32,978	32,970	31,984	31,947	31,965	30,961
miR-149	2,241	2,001	27,929	27,930	26,932	26,951	25,854	25,899
miR-150	0,343	0,052	25,978	25,964	27,977	27,967	29,958	29,969
miR-150*			40,000	40,000	40,000	40,000	40,000	40,000
miR-151-3p	2,137	2,083	28,938	28,975	29,017	28,977	27,968	27,998
miR-152	3,586	10,731	31,989	31,974	30,980	30,978	28,969	28,972
miR-153			40,000	40,000	34,973	34,979	31,970	32,974
miR-154*	1,102	0,235	30,920	30,771	30,585	30,967	32,305	31,869
miR-155	0,892	0,641	28,946	28,947	29,946	29,961	28,939	28,948
miR-15a	2,845	3,767	29,013	28,984	27,988	27,971	26,989	26,961
miR-15a*	1,569	1,346	30,824	31,410	30,208	30,175	29,972	29,865
miR-15b	2,310	2,150	27,972	27,982	26,993	26,963	26,977	26,960
miR-15b*	1,242	0,845	28,731	28,795	28,814	28,974	28,811	28,848
miR-16	1,078	1,172	22,994	22,947	22,941	22,964	22,984	22,980
miR-16*	6,704	3,102	34,701	35,037	32,423	32,949	32,814	32,760
miR-17	2,174	1,826	20,954	20,961	19,944	19,940	19,959	19,953
miR-17*	0,917	0,576	31,168	31,435	31,913	31,815	31,338	30,857
miR-181a	1,228	26,300	32,991	32,927	31,957	33,965	28,987	28,994
miR-181a-1*	5,921	21,844	34,205	33,492	32,635	32,649	30,821	30,225
miR-181c	0,837	1,435	31,932	31,982	32,946	31,964	31,975	31,992
miR-182	1,840	0,359	23,954	24,989	23,947	23,959	25,993	25,971
miR-183	1,583	0,349	28,981	28,996	27,989	27,997	29,977	29,965
miR-183*	1,147	0,161	26,986	27,926	27,894	27,795	28,809	28,858
miR-184	1,566	0,830	33,933	34,954	33,970	34,961	34,955	34,967
miR-185	2,485	3,261	31,967	31,989	30,979	30,958	29,981	29,974

miR-186	2,357	1,993	26,959	26,953	26,970	26,962	25,960	25,946
miR-186*	2,952	1,616	31,465	30,938	29,734	29,840	29,843	29,849
miR-187			40,000	40,000	40,000	40,000	32,987	32,011
miR-188-3p			40,000	40,000	40,000	40,000	40,000	40,000
miR-188-5p	1,867	1,238	30,945	30,933	30,956	30,948	30,945	29,928
miR-18a	3,971	3,983	26,938	26,963	25,969	25,963	24,950	24,969
miR-18a*	0,920	0,624	30,925	30,946	30,712	30,918	30,909	30,214
miR-18b	4,455	1,546	31,966	31,978	29,969	31,005	30,957	30,990
miR-190	2,282	1,687	32,972	33,970	32,968	32,979	32,967	32,980
miR-190b	1,327	2,763	35,286	34,734	34,640	34,716	32,941	33,146
miR-191	1,180	1,099	24,000	23,975	23,962	23,972	24,005	23,986
miR-191*	1,511	0,810	34,814	36,272	35,405	34,906	35,378	35,867
miR-192	2,183	1,918	30,966	30,962	30,984	30,973	30,973	29,968
miR-193*	0,850	1,091	34,124	35,314	35,417	34,696	33,586	33,889
miR-193b	0,951	1,123	29,998	29,970	29,958	29,964	28,951	28,970
miR-194	2,006	1,863	32,987	33,004	31,976	32,006	31,996	32,012
miR-195	1,996	0,770	27,973	27,968	27,975	27,962	27,967	27,978
miR-196a*	0,961	0,251	35,099	34,954	34,984	34,975	35,919	34,307
miR-197	3,161	3,863	31,967	32,056	30,960	30,965	30,967	31,991
miR-199a-3p	1,431	2,186	31,984	31,970	30,974	32,004	30,983	30,979
miR-199a-5p			40,000	40,000	40,000	40,000	40,000	40,000
miR-199b*			40,000	40,000	40,000	40,000	40,000	40,000
miR-19a	1,971	1,846	22,983	22,972	21,963	22,983	21,975	21,967
miR-19b	2,918	2,300	18,977	19,985	18,991	18,966	17,945	18,070
miR-200a	0,800	0,281	26,946	27,969	27,955	27,951	28,951	28,965
miR-200a*	0,437	0,102	31,811	31,235	32,888	32,220	33,987	33,461
miR-200b	0,872	0,267	25,990	25,978	26,990	26,989	27,978	27,973
miR-200b*	0,269	0,085	32,445	32,917	34,215	33,223	36,042	33,408
miR-200c	0,807	0,157	25,981	25,972	26,973	26,958	27,960	27,971
miR-200c*			32,694	32,908	40,000	40,000	40,000	40,000
miR-201			40,000	40,000	40,000	40,000	40,000	40,000
miR-202-5p			40,000	40,000	40,000	40,000	40,000	40,000
miR-203	1,271	0,650	29,975	29,974	29,957	29,968	29,962	30,976
miR-204			40,000	40,000	31,991	32,963	32,000	31,993
miR-205	0,547	0,496	30,974	31,986	31,019	31,973	32,011	32,013
miR-206	0,775	10,224	36,992	35,942	36,782	36,890	33,694	33,956
miR-207			40,000	40,000	40,000	40,000	40,000	40,000
miR-208			40,000	40,000	40,000	40,000	40,000	40,000
miR-208b			40,000	40,000	40,000	40,000	40,000	40,000
miR-20a	1,636	1,509	21,971	21,955	21,973	21,963	20,955	20,956
miR-20a*	1,593	1,469	28,983	29,030	28,954	28,945	27,818	27,964
miR-20b	2,289	0,820	21,959	21,961	20,939	21,957	21,945	21,947
miR-20b*	1,368	0,237	31,469	31,984	31,724	31,838	32,978	33,487
miR-21	1,641	2,010	24,972	25,963	25,971	25,986	24,960	24,999
miR-21*	2,256	0,991	35,207	34,977	33,981	34,469	33,954	34,527
miR-210	0,217	0,467	26,991	26,987	28,977	28,989	26,959	26,976
miR-211			31,979	31,977	40,000	40,000	40,000	40,000
miR-214			40,000	40,000	40,000	40,000	40,000	40,000
miR-214*			40,000	40,000	40,000	40,000	34,067	34,000
miR-215			40,000	40,000	40,000	40,000	40,000	40,000
miR-216a			40,000	40,000	32,962	34,003	31,995	33,023

miR-216b			40,000	40,000	31,977	32,983	29,962	29,952
miR-217			40,000	40,000	33,947	33,937	31,975	30,988
miR-218	2,161	9,587	27,940	27,955	27,962	27,953	24,956	24,967
miR-218-1*	0,255	1,681	35,956	35,041	35,817	35,300	34,727	35,639
miR-218-2*	3,888	12,431	35,035	36,994	35,875	35,979	33,657	33,857
miR-219			40,000	40,000	32,002	31,964	27,953	27,970
miR-22	1,091	1,070	28,904	28,924	28,986	28,984	28,980	28,991
miR-22*	2,182	1,025	32,741	32,887	31,883	31,822	31,933	32,280
miR-220			40,000	40,000	40,000	40,000	40,000	40,000
miR-221	1,346	1,785	28,957	28,973	28,989	28,969	29,001	28,990
miR-222	0,772	0,747	26,943	26,944	27,946	27,956	26,938	26,941
miR-223	4,051	6,474	35,974	36,027	33,997	35,983	34,989	33,967
miR-224	1,439	1,224	31,973	31,987	31,983	31,972	30,966	30,960
miR-23a			40,000	40,000	40,000	40,000	40,000	40,000
miR-23b	1,756	0,740	31,969	32,980	31,991	31,965	31,968	31,999
miR-24	0,870	0,984	22,960	22,961	23,975	24,988	23,985	23,988
miR-24-2*	0,304	0,418	30,822	30,748	31,885	31,470	30,992	30,818
miR-25	1,868	1,954	28,974	28,953	28,959	28,977	27,949	27,966
miR-26a	1,456	1,718	25,965	25,969	25,961	25,965	24,963	24,949
miR-26b	1,816	2,212	27,969	27,973	27,955	27,972	26,967	26,966
miR-26b*	1,357	1,246	33,392	32,564	33,423	32,928	32,181	31,566
miR-27a	0,705	1,071	28,952	28,955	29,957	29,963	28,977	28,969
miR-27a*	0,355	0,180	30,872	30,944	31,833	31,894	32,332	31,951
miR-27b	1,478	1,158	28,942	28,946	29,962	29,957	28,952	28,958
miR-27b*	0,856	0,294	31,533	30,972	31,624	31,840	31,959	31,940
miR-28	2,435	2,723	28,961	28,959	27,953	27,946	27,978	27,974
miR-28*	0,821	0,831	29,110	29,107	29,487	28,884	28,789	28,957
miR-290-3p	1,675	0,126	24,954	25,991	25,977	24,970	27,951	28,982
miR-290-5p	0,471	0,053	23,835	23,930	24,806	24,847	27,918	27,900
miR-291a-3p	0,911	0,168	21,969	21,962	22,974	22,961	23,944	24,981
miR-291a-5p			27,679	27,783	40,000	40,000	40,000	40,000
miR-291b-3p	0,663	0,499	35,899	34,998	34,514	33,982	35,989	35,328
miR-291b-5p	2,036	0,109	28,958	28,979	28,958	28,959	31,987	31,995
miR-292-3p	1,043	0,145	19,951	19,940	20,974	20,970	22,963	22,963
miR-292-5p	0,622	0,048	28,923	28,952	28,776	28,756	31,245	30,827
miR-293	1,534	0,259	19,970	19,971	19,952	19,960	21,971	21,970
miR-293*	0,054	0,135	32,447	32,993	40,000	33,185	34,977	34,987
miR-294	1,279	0,191	22,954	23,985	23,987	23,979	25,982	25,971
miR-294*	0,701	0,056	26,969	26,971	26,955	26,943	28,844	28,886
miR-295	0,873	0,149	19,987	19,984	20,981	20,985	21,969	21,984
miR-296-3p	2,974	2,905	29,964	30,997	28,981	29,993	28,988	28,975
miR-296-5p	1,641	1,002	27,995	27,941	28,016	27,975	28,000	27,964
miR-297a*	1,309	0,587	27,842	28,117	27,860	27,969	28,052	28,920
miR-297b-5p	1,863	0,943	30,973	30,975	29,972	29,946	29,966	30,966
miR-297c	1,747	1,060	31,052	30,984	30,016	29,995	29,955	29,971
miR-298	3,782	4,031	27,966	28,982	26,964	26,949	26,966	26,988
miR-299*	0,899	0,342	30,887	30,137	30,931	30,930	31,024	30,836
miR-29a	2,186	0,916	25,937	25,937	24,933	24,927	25,946	25,947
miR-29a*	1,255	0,199	34,337	35,420	34,518	35,998	35,972	35,998
miR-29b	1,989	1,034	32,958	34,993	33,999	32,973	33,973	32,963
miR-29c	1,969	1,072	29,988	29,978	29,975	29,982	28,979	29,974

miR-300	1,621	0,258	30,647	31,203	30,614	30,916	31,261	31,600
miR-300*	0,246	1,242	35,624	35,444	37,180	35,971	35,147	34,972
miR-301a	1,857	3,677	25,978	25,972	25,980	25,985	23,979	23,943
miR-301b	2,002	3,753	25,968	26,988	25,959	25,963	23,964	23,960
miR-302a	7,537	3,926	24,956	25,962	22,959	22,958	22,953	22,957
miR-302a*	4,501	1,148	29,920	29,187	26,965	27,901	28,944	28,081
miR-302b	6,890	3,428	22,962	23,967	20,965	20,973	21,982	21,983
miR-302b*			40,000	40,000	32,775	32,043	40,000	40,000
miR-302c	6,734	4,133	28,018	27,982	26,990	26,997	25,965	26,994
miR-302c*	6,734	1,804	33,185	33,195	30,987	30,933	31,957	32,000
miR-302d	6,776	3,865	24,972	24,972	22,967	22,960	22,975	22,953
miR-30a	1,495	1,806	27,928	28,970	28,962	28,961	27,952	27,956
miR-30a*	1,069	1,609	27,867	27,917	27,965	27,762	25,926	25,915
miR-30b	2,146	2,828	26,980	26,982	25,972	25,981	24,974	24,968
miR-30b*	0,993	1,339	33,475	33,319	32,688	32,178	32,422	32,763
miR-30c	2,235	2,703	25,977	25,971	24,954	24,949	23,954	23,945
miR-30c-2*			40,000	40,000	40,000	40,000	40,000	40,000
miR-30d	1,629	2,119	28,973	28,991	28,986	28,981	27,960	27,981
miR-30e	1,827	1,875	26,922	27,953	26,921	26,925	26,952	26,950
miR-30e*	1,117	1,331	27,856	27,956	27,798	27,881	26,847	26,873
miR-31	1,338	1,477	26,965	26,970	26,969	26,967	25,974	25,960
miR-31*	1,238	1,099	28,983	28,992	28,992	28,972	27,975	27,967
miR-32	3,851	3,375	33,970	32,974	31,956	31,962	30,976	31,984
miR-320	1,432	0,726	28,970	28,972	28,975	28,976	28,965	28,959
miR-322	2,743	11,354	30,981	30,987	29,977	29,985	26,945	26,949
miR-322*	1,407	8,223	33,990	33,994	32,985	33,045	29,992	29,996
miR-323-3p	3,324	1,540	25,971	25,977	24,963	24,972	25,978	25,982
miR-324-3p	1,452	2,684	29,982	30,039	30,015	30,003	28,976	28,990
miR-324-5p	3,231	5,210	30,941	30,940	29,950	29,946	28,963	28,970
miR-325			40,000	40,000	40,000	40,000	31,971	30,965
miR-325*			40,000	40,000	40,000	40,000	33,000	32,987
miR-326			40,000	40,000	40,000	40,000	30,427	29,934
miR-328	10,429	13,193	32,959	31,968	29,977	30,973	28,977	28,969
miR-329	1,986	1,912	32,969	32,955	32,971	31,946	31,971	31,966
miR-33*	1,299	1,012	32,981	32,981	31,970	33,000	31,966	31,983
miR-330	2,325	3,836	32,016	32,947	36,023	30,977	30,998	30,993
miR-330*			40,000	40,000	40,000	40,000	40,000	40,000
miR-331-3p	2,774	3,836	27,977	27,996	26,954	26,956	25,985	25,955
miR-331-5p	7,329	4,346	32,972	33,955	31,985	32,993	31,995	31,970
miR-335-3p	2,952	4,035	28,981	28,977	27,979	27,970	26,976	26,987
miR-335-5p	5,634	15,634	29,953	29,980	27,953	28,967	25,958	25,985
miR-337-3p	3,309	1,626	28,978	28,976	27,979	27,982	27,975	27,961
miR-337-5p	3,347	1,442	28,959	29,979	27,959	28,987	28,979	28,977
miR-339-3p	0,959	0,687	29,001	29,989	29,055	29,988	30,013	29,016
miR-339-5p	3,437	3,269	30,952	31,986	29,982	30,982	29,978	29,987
miR-340-3p	2,929	6,898	31,958	33,970	31,960	31,974	29,959	29,963
miR-340-5p	3,751	7,799	29,967	30,999	29,988	28,970	27,967	27,965
miR-342-3p	1,301	1,343	26,961	26,956	27,981	27,982	26,960	26,980
miR-342-5p	2,752	8,228	35,989	36,040	33,011	34,005	32,033	31,976
miR-343			40,000	40,000	40,000	40,000	40,000	40,000
miR-344	3,340	4,471	30,987	30,987	29,996	29,988	29,979	29,999

miR-345			40,000	40,000	40,000	40,000	33,997	34,043
miR-345-5p	2,988	4,386	32,040	31,971	31,969	30,982	30,991	29,967
miR-346			40,000	40,000	40,000	40,000	40,000	40,000
miR-34a	2,986	2,501	27,966	27,969	26,965	26,967	26,972	26,983
miR-34b-3p	2,355	1,730	29,967	29,959	29,970	29,976	29,985	29,979
miR-34c	3,498	7,576	32,985	32,986	30,981	32,970	30,972	30,979
miR-34c*	1,255	0,792	28,969	28,954	28,975	28,969	28,953	28,973
miR-350	3,537	5,012	32,980	32,990	31,979	31,970	30,981	30,968
miR-351	4,143	10,921	30,971	31,980	30,974	30,987	29,989	29,989
miR-361	1,268	1,533	31,977	31,968	31,973	31,989	30,985	30,979
miR-362-3p	5,881	4,760	32,940	34,963	31,950	32,970	31,964	31,960
miR-363	3,531	1,485	28,973	28,967	27,960	27,967	27,959	28,986
miR-365	2,043	3,462	32,990	31,999	31,974	31,982	29,962	30,981
miR-367	5,959	2,388	24,964	24,982	22,974	22,980	22,963	22,952
miR-369-3p	3,034	1,874	33,973	34,980	32,970	33,987	32,960	32,950
miR-369-5p	4,368	2,380	30,950	30,961	29,968	28,948	29,967	29,968
miR-370	5,852	2,815	27,973	27,964	26,977	26,970	26,977	26,990
miR-374	0,854	0,753	25,952	26,965	26,873	26,894	25,956	26,940
miR-374*			40,000	40,000	40,000	40,000	40,000	40,000
miR-376a	2,793	1,496	27,980	27,986	26,995	26,982	26,989	26,981
miR-376a*	1,459	0,645	31,983	30,946	30,981	30,963	30,974	30,991
miR-376b	2,516	0,597	26,986	26,999	26,999	25,966	27,988	28,020
miR-376b*	1,211	0,451	27,953	27,959	27,964	27,978	27,953	27,939
miR-376c	1,939	1,055	25,963	25,965	25,976	25,985	25,974	25,972
miR-378	1,298	0,693	26,036	25,944	25,933	25,855	25,839	25,885
miR-378*	0,901	0,367	33,974	33,955	33,969	33,963	34,973	32,955
miR-379	2,415	1,207	25,931	25,943	25,958	25,957	25,950	25,947
miR-380-3p	0,652	1,182	33,972	34,985	33,957	35,020	33,952	33,994
miR-380-5p	4,703	3,020	29,984	29,989	27,965	27,971	27,966	27,969
miR-381	3,776	2,086	34,987	33,981	32,982	33,975	33,985	32,963
miR-382	2,964	1,628	27,989	28,011	25,936	25,998	25,975	26,977
miR-382*			40,000	40,000	40,000	40,000	40,000	40,000
miR-383			40,000	40,000	40,000	40,000	33,956	36,015
miR-384-3p			40,000	40,000	40,000	40,000	31,980	31,988
miR-384-5p	7,847	40,479	31,931	32,979	30,971	29,953	26,947	26,943
miR-409-3p	2,002	0,655	23,955	24,973	23,961	23,951	24,957	24,965
miR-409-5p	4,348	2,522	31,988	30,963	30,984	30,983	29,963	30,976
miR-410	2,599	1,188	26,969	26,962	25,960	25,945	25,939	25,938
miR-411	2,041	1,004	24,956	24,957	24,975	24,978	24,965	24,967
miR-411*	1,162	0,575	30,973	31,048	31,989	31,976	31,982	31,978
miR-425	1,385	0,906	27,946	27,984	28,035	27,973	27,972	27,946
miR-425*	0,378	0,934	32,973	32,981	33,978	33,962	32,974	32,978
miR-429	0,488	0,153	25,970	25,969	27,982	28,000	28,988	28,989
miR-431	2,815	1,300	24,975	24,969	23,969	23,974	23,958	23,964
miR-433	2,663	1,256	28,967	28,964	28,972	28,975	28,968	28,968
miR-433*			40,000	40,000	40,000	40,000	40,000	40,000
miR-434-3p	2,200	1,177	28,004	27,990	26,986	26,982	26,963	26,985
miR-434-5p	3,529	1,324	30,991	30,983	28,987	29,989	29,982	29,984
miR-448			40,000	40,000	40,000	40,000	40,000	40,000
miR-449a	2,514	0,435	30,965	30,963	29,952	29,945	31,969	31,967
miR-449b			40,000	40,000	40,000	40,000	40,000	40,000

miR-449c	1,067	0,410	34,337	32,870	33,284	33,634	33,807	35,989
miR-450a-3p			40,000	40,000	40,000	40,000	40,000	40,000
miR-450a-5p	3,374	3,463	32,953	34,003	32,967	32,981	31,976	31,973
miR-450b-5p			40,000	40,000	40,000	40,000	40,000	40,000
miR-451			40,000	40,000	40,000	40,000	40,000	40,000
miR-452			40,000	40,000	40,000	40,000	40,000	40,000
miR-453			40,000	40,000	40,000	40,000	40,000	40,000
miR-455			40,000	40,000	40,000	40,000	40,000	40,000
miR-455*	1,278	2,750	32,990	32,993	32,995	32,979	30,981	30,969
miR-463*			40,000	40,000	40,000	40,000	40,000	40,000
miR-464			40,000	40,000	40,000	40,000	40,000	40,000
miR-465a-3p	2,055		34,964	33,974	33,986	34,986	40,000	40,000
miR-465a-5p			40,000	40,000	40,000	40,000	40,000	40,000
miR-465b-5p	1,071	0,127	32,001	31,976	31,972	31,972	33,989	33,988
miR-466a-3p	0,975	0,210	28,695	28,843	28,593	28,878	29,838	29,886
miR-466b-3-3p	1,167	0,250	28,971	28,870	28,967	28,889	29,669	29,827
miR-466c-5p			40,000	40,000	40,000	40,000	40,000	40,000
miR-466d-3p	1,148	0,394	23,986	23,836	23,901	23,975	23,900	23,863
miR-466d-5p			40,000	40,000	40,000	40,000	40,000	40,000
miR-466h			40,000	40,000	40,000	40,000	40,000	40,000
miR-467a	2,887	1,655	24,979	24,963	23,967	23,968	23,968	23,982
miR-467a*	1,043	0,423	27,977	27,957	27,970	27,971	28,985	27,963
miR-467b	2,034	0,986	27,987	27,986	26,955	26,966	27,979	27,992
miR-467b*	0,700	0,306	31,001	30,983	30,951	30,980	30,972	30,977
miR-467c	2,245	1,013	26,986	26,990	27,011	25,971	26,980	26,974
miR-467d	2,182	1,300	28,968	28,964	28,976	28,981	27,961	27,951
miR-467e	2,271	1,122	28,998	29,970	28,977	28,982	28,986	28,988
miR-467e*			32,022	31,957	40,000	40,000	40,000	40,000
miR-468			40,000	40,000	40,000	40,000	40,000	40,000
miR-469			40,000	40,000	40,000	40,000	40,000	40,000
miR-470			40,000	40,000	40,000	40,000	40,000	40,000
miR-483*			40,000	40,000	40,000	40,000	40,000	40,000
miR-484	1,721	1,116	23,952	23,946	23,952	23,941	23,957	23,957
miR-485*	0,645	0,082	26,972	26,965	27,990	27,987	29,981	29,969
miR-486			40,000	40,000	40,000	40,000	29,968	30,003
miR-487b	4,385	2,008	28,976	29,992	27,970	27,973	27,961	27,966
miR-488*			40,000	40,000	40,000	40,000	40,000	40,000
miR-489	0,916	0,183	29,969	29,961	30,983	30,975	31,956	31,946
miR-490			40,000	40,000	40,000	40,000	40,000	40,000
miR-491	1,373	1,619	30,936	31,980	30,984	30,982	30,959	30,978
miR-493	13,656	6,504	33,984	35,005	32,989	32,967	32,959	32,992
miR-494	2,352	1,327	25,986	26,984	25,981	25,989	25,981	25,964
miR-495	2,783	0,883	26,959	26,942	26,967	26,958	27,967	27,977
miR-496	4,886	1,784	33,983	33,975	32,974	32,981	32,971	33,987
miR-497	2,595	0,655	29,990	29,987	28,976	28,979	29,978	29,983
miR-499			40,000	40,000	40,000	40,000	40,000	40,000
miR-500	2,071	3,775	31,977	31,963	31,990	30,972	30,976	30,972
miR-501-5p	0,003	0,528	33,984	33,979	40,000	40,000	32,983	32,962
miR-503	3,468	11,252	30,984	30,999	29,990	29,970	27,967	27,980
miR-503*	2,395	4,484	32,968	33,984	31,966	32,984	30,986	30,976
miR-504			40,000	40,000	40,000	40,000	40,000	40,000

miR-505			40,000	40,000	40,000	40,000	40,000	40,000
miR-509-5p			40,000	40,000	40,000	40,000	40,000	40,000
miR-511			40,000	40,000	40,000	40,000	40,000	40,000
miR-532-3p	2,478	2,506	30,998	30,980	29,980	29,981	28,984	30,004
miR-532-5p	2,438	2,477	26,976	27,982	26,967	26,971	25,960	25,961
miR-539	2,588	1,085	26,930	26,944	26,953	26,956	26,948	26,941
miR-540-3p	3,142	0,772	29,995	29,993	28,966	28,972	29,982	29,992
miR-542-3p	2,901	2,183	34,980	33,976	33,986	33,982	32,967	33,983
miR-542-5p	1,493	1,981	33,001	31,978	32,985	31,972	30,975	30,975
miR-543	2,528	0,588	28,964	28,968	28,979	28,977	29,974	29,971
miR-544	2,771	1,583	31,980	31,968	30,972	30,949	30,959	31,975
miR-547			40,000	40,000	40,000	40,000	40,000	40,000
miR-551b			40,000	40,000	40,000	40,000	40,000	40,000
miR-574-3p	2,086	2,735	29,994	30,980	29,980	29,972	28,959	28,985
miR-592	3,070	2,980	34,814	34,845	32,811	33,237	32,975	32,525
miR-598			40,000	40,000	40,000	40,000	31,984	34,009
miR-615-3p			40,000	40,000	40,000	40,000	40,000	40,000
miR-615-5p			40,000	40,000	40,000	40,000	40,000	40,000
miR-652	3,246	4,514	28,976	28,971	27,998	27,980	26,975	26,969
miR-654-3p			40,000	40,000	40,000	40,000	40,000	40,000
miR-654-5p			40,000	40,000	40,000	40,000	40,000	40,000
miR-665	1,859	0,377	30,983	31,991	30,985	31,003	30,984	32,001
miR-666-5p			40,000	40,000	40,000	40,000	40,000	40,000
miR-667	2,704	1,453	27,961	27,967	27,979	27,973	27,993	27,990
miR-668			40,000	40,000	40,000	40,000	40,000	40,000
miR-669a	2,605	1,323	27,947	27,954	27,974	27,977	27,970	27,980
miR-670	19,571	39,961	35,927	35,971	36,006	35,984	33,002	33,983
miR-671-3p	28,445	14,740	35,971	35,997	32,988	32,998	32,988	32,999
miR-672	0,649	0,133	26,967	26,952	27,953	27,951	28,961	28,954
miR-673-3p	2,155	0,550	28,654	29,786	28,960	28,750	29,493	29,930
miR-673-5p	1,244	0,300	30,715	30,633	30,928	30,779	31,331	31,381
miR-674	1,957	1,579	30,982	30,997	29,984	29,970	29,976	29,961
miR-674*	1,681	1,360	28,978	28,973	28,980	27,967	27,972	27,980
miR-675-3p			40,000	40,000	40,000	40,000	40,000	40,000
miR-675-5p			40,000	40,000	40,000	40,000	40,000	40,000
miR-676	2,812	2,186	30,994	30,993	29,946	30,986	29,973	29,972
miR-676*	1,291	1,660	35,983	34,988	34,979	33,985	33,988	33,970
miR-678	1,167	0,605	31,732	31,683	31,710	31,974	31,365	31,955
miR-679	3,566	0,945	33,051	32,991	31,965	31,980	31,940	31,975
miR-680	2,509	1,204	29,976	29,981	28,951	28,970	28,985	30,027
miR-681			40,000	40,000	40,000	40,000	40,000	40,000
miR-682	2,315	1,707	32,007	31,996	30,979	30,934	30,950	31,985
miR-683			40,000	40,000	40,000	40,000	40,000	40,000
miR-684			40,000	40,000	40,000	40,000	35,005	35,971
miR-685	1,913	2,625	28,985	28,991	29,001	29,004	27,976	27,961
miR-686			40,000	40,000	40,000	40,000	40,000	40,000
miR-687	1,986	1,125	26,072	25,947	24,896	25,986	24,995	25,961
miR-688			40,000	40,000	40,000	40,000	40,000	40,000
miR-690	0,967	0,897	23,982	23,955	23,942	23,967	23,967	23,932
miR-691			40,000	40,000	40,000	40,000	40,000	40,000
miR-692	1,045	0,281	28,832	28,695	29,049	28,789	29,403	29,580

miR-693-3p			40,000	40,000	40,000	40,000	40,000	40,000
miR-693-5p			40,000	40,000	40,000	40,000	40,000	40,000
miR-694	1,608	0,748	33,649	33,144	33,266	33,616	33,281	32,541
miR-695			40,000	40,000	40,000	40,000	40,000	40,000
miR-696	1,359	0,589	25,802	26,972	26,689	26,956	26,725	26,913
miR-697	0,564	0,322	29,956	30,962	31,010	31,009	30,942	30,995
miR-699	0,756	0,525	25,974	25,982	26,967	26,925	25,937	25,873
miR-700	1,100	0,644	29,778	29,774	29,347	29,782	29,764	29,701
miR-701	5,245	11,319	35,923	36,037	35,994	33,964	33,851	33,355
miR-702	0,946	0,416	33,883	33,326	32,951	32,963	33,414	33,209
miR-704	1,408	1,060	32,275	32,800	32,243	32,461	31,773	31,855
miR-706	1,102	0,447	27,866	27,889	27,875	27,936	27,952	27,967
miR-707			40,000	40,000	40,000	40,000	40,000	40,000
miR-708	1,137	0,467	25,934	26,965	26,954	26,959	26,940	26,942
miR-708*			34,985	33,978	40,000	40,000	40,000	40,000
miR-709	1,283	0,633	22,914	22,945	22,963	22,953	22,925	22,939
miR-710			40,000	40,000	40,000	40,000	40,000	40,000
miR-711			40,000	40,000	40,000	40,000	40,000	40,000
miR-712*			40,000	40,000	40,000	40,000	40,000	40,000
miR-713			40,000	40,000	40,000	40,000	40,000	40,000
miR-715			27,974	27,952	40,000	40,000	40,000	40,000
miR-717			40,000	40,000	40,000	40,000	40,000	40,000
miR-718			40,000	40,000	40,000	40,000	40,000	40,000
miR-719			40,000	40,000	40,000	40,000	40,000	40,000
miR-720	1,055	0,655	23,871	23,937	23,964	23,960	23,954	23,986
miR-741	2,630	0,444	34,980	33,956	33,994	33,964	34,028	33,986
miR-742			40,000	40,000	40,000	40,000	40,000	40,000
miR-743a			40,000	40,000	31,007	31,045	31,013	29,901
miR-743b-3p			35,004	35,012	40,000	40,000	40,000	40,000
miR-743b-5p			40,000	40,000	40,000	40,000	40,000	40,000
miR-744	2,859	3,695	29,970	29,954	28,948	28,953	27,953	27,957
miR-744*	4,129	2,403	33,971	33,995	32,982	32,965	31,970	32,973
miR-758			40,000	40,000	40,000	40,000	40,000	40,000
miR-759			40,000	40,000	40,000	40,000	40,000	40,000
miR-760	1,660	1,360	30,920	30,920	29,813	30,928	29,607	29,865
miR-761			40,000	40,000	40,000	40,000	34,997	35,089
miR-762			40,000	40,000	40,000	40,000	40,000	40,000
miR-764-3p			40,000	40,000	40,000	40,000	40,000	40,000
miR-764-5p	2,672	0,737	33,831	33,897	32,876	32,987	33,996	33,134
miR-770-3p			40,000	40,000	40,000	40,000	40,000	40,000
miR-7a	0,922	0,345	29,963	30,968	30,974	30,966	31,978	31,972
miR-7a*	1,175	0,630	28,972	28,980	28,987	27,961	27,957	27,968
miR-7b	0,640	0,210	32,955	32,988	33,967	33,975	34,983	35,002
miR-801	0,751	0,465	29,823	29,884	30,850	30,859	31,791	31,222
miR-802			40,000	40,000	40,000	40,000	40,000	40,000
miR-804	0,939	0,866	33,046	32,961	33,121	33,934	32,483	32,312
miR-805	0,710	0,505	25,929	25,821	26,831	26,687	25,867	25,648
miR-871			40,000	40,000	40,000	40,000	40,000	40,000
miR-872	2,566	3,170	29,965	29,953	28,942	28,945	27,947	27,948
miR-872*	0,998	1,445	28,971	28,956	28,953	28,958	27,964	27,971
miR-873			40,000	40,000	40,000	40,000	40,000	40,000

miR-874			40,000	40,000	40,000	40,000	40,000	40,000
miR-875-3p			40,000	40,000	40,000	40,000	40,000	40,000
miR-875-5p	0,652	0,274	33,230	33,461	32,843	34,834	33,739	35,445
miR-876-3p			40,000	40,000	40,000	40,000	40,000	40,000
miR-876-5p			40,000	40,000	40,000	40,000	40,000	40,000
miR-877	1,108	0,722	31,235	30,960	31,859	30,957	30,849	30,734
miR-877*	1,295	0,566	29,987	28,980	29,000	29,006	28,989	29,004
miR-878-3p	0,524	0,446	31,670	31,429	31,883	31,794	31,774	31,971
miR-878-5p			35,958	35,951	40,000	40,000	40,000	40,000
miR-879			40,000	40,000	40,000	40,000	35,954	35,974
miR-881	0,547		34,982	36,006	34,995	36,094	40,000	40,000
miR-881*			34,010	34,978	40,000	40,000	40,000	40,000
miR-883a-3p			40,000	40,000	40,000	40,000	40,000	40,000
miR-883a-5p			40,000	40,000	40,000	40,000	40,000	40,000
miR-9	2,659	15,103	27,978	28,022	26,939	26,989	23,971	23,958
miR-9*	1,000	3,613	30,974	30,967	30,964	30,967	28,982	27,966
miR-92a	1,821	0,804	23,939	23,950	23,951	23,947	24,962	24,963
miR-92a*	1,821	0,230	29,982	29,989	28,969	28,966	30,989	30,982
miR-93	1,915	2,419	24,967	24,964	23,946	23,944	22,942	22,950
miR-93*	1,348	1,142	26,955	26,949	26,962	26,960	25,955	25,956
miR-96	2,647	0,848	30,976	31,971	30,995	30,965	31,985	31,996
miR-98			40,000	40,000	40,000	40,000	40,000	40,000
miR-99a			40,000	40,000	30,958	31,991	25,004	25,977
miR-99b	1,758	1,995	26,983	26,958	26,943	26,992	25,972	25,995
miR-99b*	1,109	1,478	32,998	33,001	32,989	32,992	30,972	30,987

Appendix 1. Full report of microRNA expression profile in undifferentiated ESCs and in two and four days differentiating ESC derivatives.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
Smarca5	GGGGTACCGCATTTTTGTCTTATAATCACTAACTG	GGGGTACCCTACATACTATGTTTCTAGCTCAGTAAGC
Jarid1b	GGGGTACCTGTAATTCAGGACTCCAATAAAGAAG	GGGGTACCGCTTATGGCTACAGACAGTGAGG
Sirt1	GGGGTACCGATTCAGGAATTGCTCCACCAG	GGGGTACCGAAGATAATCCAGTCATTAAACGGTC
Luc-EcoRI	CCGGAATTCATGGAAGACGCCAAAAACATAAAG	CCGGAATTCTTACACGGCGATCTTTCCGC

Appendix 2. Primers used for cloning.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
Brachyury	AGAAGACGAGGACGTGGCAG	CATTACACACCACTGACGCACA
Egr1	GGGAGAGGCAGGAAAGACATAA	TCTGAGATCTTCCATCTGACCTAAGA
Gapdh	GTATGACTCCACTCACGGCAAA	TTCCCATTCTCGGCCTTG
Nanog	TCAGAAGGGCTCAGCACCA	GCGTTCACCAGATAGCCCTG
Oct3/4	AACCTTCAGGAGATATGCAAATCG	TTCTCAATGCTAGTTCGCTTTCTCT
Rex1	GCAGTTTCTTCTTGGGATTTCAG	CTAATGCCCACAGCGAT
Sox1	CATCTCCAACTCTCAGGGCT	ACTTGACCAGAGATCCGAGG
Sox17	GATGCGGGATACGCCAGTG	CCACCACCTCGCCTTTCAC

Appendix 3. Primers used for Real-Time PCR.

Gene	Forward primer (5'to3')	Reverse primer (5'to3')
Egr1	TTCACGTCACTCCGGGTCCTCC	AGTTCTGCGCGCTGGGATCTCTC

Appendix 4. Primers used for ChIP

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