The Role of RNA Interference in Stem Cell Biology: Beyond the Mutant Phenotypes

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Abstract

Complex gene regulation systems ensure the maintenance of cellular identity during early development in mammals. Eukaryotic small RNAs have emerged as critical players in RNA interference (RNAi) by mediating gene silencing during embryonic stem cell self-renewal. Most of the proteins involved in the biogenesis of small RNAs are essential for proliferation and differentiation into the three germ layers of mouse embryonic stem cells. In the last decade, new functions for some RNAi proteins, independent of their roles in RNAi pathways, have been demonstrated in different biological systems. In parallel, new concepts in stem cell biology have emerged. Here, we review and integrate the current understanding of how RNAi proteins regulate stem cell identity with the new advances in the stem cell field and the recent non-canonical functions of the RNAi proteins. Finally, we propose a reevaluation of all RNAi mutant phenotypes, as non-canonical (small non-coding RNA independent) functions may contribute to the molecular mechanisms governing mouse embryonic stem cells commitment.

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Introduction

The first report of RNA interference (RNAi) in 1998 and the discovery of its effector molecules, the small interfering RNAs (siRNAs) [1,2], have revolutionized our understanding of gene regulation. Since then, the biological importance of these silencing small RNAs (sRNAs) and other classes of non-coding sRNAs has been evidenced through a staggering array of publications implicating these molecules in virtually all aspects of cell biology. MicroRNAs (miRNAs), in particular, influence many biological processes including immune responses, hormonal regulation, phase transitioning and patterning, and embryonic and post-embryonic development [3]. Tight and stringent regulation of the sRNA pathways is a cellular necessity, as perturbations in the expression and/or activity of these small molecules have considerable deleterious effects including genetic instability, sterility, and loss of cellular identity. Recent research indicates that miRNAs have important roles in regulating stem cell self-renewal and differentiation by repressing the translation of selected mRNAs in stem cells and differentiating daughter cells [4]. More importantly, mouse embryonic stem cells (mESCs) deficient of Dicer or Dgcr8, two essential genes involved in miRNA biogenesis, fail to produce miRNAs and manifest defects in proliferation and differentiation, which confirms the essential role for the RNAi genes in early mouse development [5–7]. Unfortunately, the Dicer and Dgcr8 mutant mESCs reported by different laboratories have a distinct background, making their comparison difficult. Nevertheless, the aforementioned Dicer- and Dgcr8-deficient mESCs present differences...
in their phenotype (e.g., proliferation and cell cycle defects, impaired differentiation) [6–9], suggesting that the role of DICER in the siRNA pathway could have a specific function in mESCs homeostasis [7] or that DICER and/or DGCR8 proteins might have non-canonical functions other than their specific role in the miRNA pathway. Moreover, recent breakthroughs in stem cell biology now allow to distinguish between the exit from pluripotency (an event necessary for mESC commitment) and the differentiation of mESCs (their capacity to activate differentiation programs).

In this perspective, we present previous and emerging concepts in RNAi and stem cell biology fields. Finally, we propose that these new concepts should call for a reinvestigation of the RNAi mutant phenotypes in the light of their putative non-canonical functions in stem cell biology.

State-of-the-Art and New Advances in Stem Cell Biology

In 1981, two groups successfully isolated cells directly from mouse blastocyst for the first time [9,10]. Derived from the whole delayed blastocyst [9] or from the inner cell mass (ICM) isolated by immunosurgery [10], early embryonic cells exhibited the capacity to generate well-differentiated teratomas and presented a normal karyotype [9–11]. The ICM-isolated cells were named mESCs [10]. The first studies implemented the culture protocols of mESCs using feeder cells and serum-supplemented medium [9]. The establishment of these culture conditions highlighted either the presence (in the serum and in the conditioned medium) or the production (by the feeder cells) of factors favoring the survival of mESCs [9,10,12,13].

Despite these mESC isolation breakthroughs [9,10], the original empirically established protocols and culture conditions did not allow isolation of ESCs from other mammals. At the beginning, ESCs could be derived only from mouse embryos of inbred 129 strain and more rarely from the C57BL/6 strain [14]. Deconvolution of the mixture of the factors present in the serum and of the interactions between mESCs and the feeder cells allowed researchers to identify a key differentiation-inhibiting protein: the cytokine leukemia inhibitory factor (LIF) [15,16]. Addition of LIF to the serum allowed derived and long-term culture of mESCs in the absence of feeder cells [15,16]. However, this improvement was still limited to the 129 mouse strains. Briefly, LIF promotes self-renewal by repressing the differentiation programs via the stimulation of the signal transducer and activator of transcription 3 [17–20]. Moreover, LIF alone was not sufficient to sustain mESC culture, as serum withdrawal led to the differentiation of the cells [21]. Later, the bone morphogenetic factor-4 (BMP4) was found to also support mESC propagation [22]. In BMP4 plus LIF condition, mESCs from the 129 background were successfully derived and cultured in a feeder-free and serum-free environment [22]. Remarkably, LIF has also been shown to promote differentiation by stimulating the mitogen-activated protein kinase/Extracellular Signal-Regulated Kinase (ERK) pathway [23]. Indeed, suppression of the ERK pathway improved mESC self-renewal [24]. Finally, it was reported that mESCs produce significant amount of another potent activator of the ERK pathway: the fibroblast growth factor-4 [24,25]. Therefore, new approaches using ERK pathway repressors started to be employed. In conclusion, a new culture condition combining LIF, BMP4, and ERK pathway inhibitors allowed the reproducible derivation of mESCs from C57BL/6 and CBA strains in a feeder-free and serum-free environment [26].

In the early 2000s, studies revealed a new antagonist of mESC self-renewal, the glycogen synthase kinase-3 (GSK3), and further investigations showed that its inhibition enhanced mESC propagation [27–30]. GSK3 negatively regulates a range of cellular and intracellular pathways, including the canonical Wnt/ß-catenin pathway [31,32]. The big breakthrough happened in 2008, when Ying and colleagues defined a new medium combining three inhibitors: an FGF receptor inhibitor (SU5042), an ERK pathway inhibitor (PD184352), and a GSK3 inhibitor (CHIR99021) [33]. Mouse ESCs cultured in this new medium named 3i (for three inhibitors) displayed a stronger self-renewal compared to mESCs cultured in serum plus LIF or LIF and BMP4 conditions and even propagated in the absence of LIF or serum [33]. Afterwards, a single high potency and selectively MEK stands for MAP (Mitogen-Activated Protein) Kinase/ERK (Extracellular Signal-Regulated Kinase) Kinase (MEK) inhibitor (PD0325901) and a GSK3 inhibitor were shown to be sufficient to support the long-term culture of mESCs [34]; therefore, this new culture condition was coined 2i. Thus, it is now acknowledged that in 2i medium, mESCs reach a ground-state naïve pluripotency, resembling to the epiblast of the mature mouse blastocyst [35].

In addition, the 2i culture condition overcame the non-permissive strain barrier and enabled the derivation of ESCs from other mouse backgrounds [33,36–38] and from other rodent species [39,40]. More recently, the 2i media has permitted the derivation of haploid mESCs [41]. These cells are a tremendous valuable tool because they allow the usage of genetic screens, which will extend genome exploration into developmentally and medically relevant pathways [42].

Impact of these New Discoveries on the Pluripotency Network in mESCs

Several transcription factors are critical for the maintenance of the naïve pluripotent state of mESCs including OCT4, SOX2, and NANOG (OSN). Their
down-regulation is essential to exit from the pluripotency state and to differentiate into the three embryonic germ layers [43]. Indeed, when mESCs, derived from the E4.5 epiblast [44], are cultured in serum supplemented with LIF and/or on feeder cells, they present a mosaic of some pluripotent factors’ expression pattern [45–47]. Particularly, it has been shown that NANOG expression is heterogeneous in serum plus LIF cultures [48]. Similarly, the expression of STELLA and REX1, other important pluripotency transcription factors, presents dynamic heterogeneity in serum plus LIF culture condition [45, 49]. The fact that mESCs, cultured in serum plus LIF, express various lineage-specific genes in a heterogeneous manner indicates that a proportion of cells undergo priming and/or differentiation in response to the complex mix of serum components [45–47]. It has been proposed that the simultaneous heterogeneous expression of lineage-specific genes and pluripotency factors across the culture reflects an inherent fluidity of the pluripotent state and underpins pluripotency by preparing mESCs for lineage commitment [50, 51]. Importantly, mESCs cultured in 2i plus LIF media show substantially uniform expression of key pluripotency factors (e.g., NANOG and STELLA) [47]. Furthermore, transcription factors typically down-regulated at the onset of mESC differentiation such as KLF4, ESRRB, and REX1 are also homogenously expressed in 2i medium in contrast to serum plus LIF condition [35]. As a consequence of the homogeneity observed in the pluripotency-associated gene expression between the two growth conditions, cellular morphology also differs [47]. Mouse ESCs cultured in 2i media are morphologically uniform and present as homogenous clusters of small, tightly packed cells, whereas serum plus LIF cultured mESCs are rather flattened and exhibit heterogeneous morphologies [47].

The culture conditions (serum plus LIF or 2i plus LIF) also influence the epigenetic status of the mESCs [52]. The most highly expressed genes under both
conditions presented the trimethylation of the lysine 4 of the histone 3 associated with active promoters and the trimethylation of the lysine 36 of the histone H3 associated with coding body of transcribed genes. The levels of the trimethylation of the lysine 27 of the histone H3 (H3K27me3) have been shown to increase considerably in mESCs cultured in serum plus LIF media [53]. Further analysis demonstrated a global lack of H3K27me3 at promoter regions in 2i media, while in serum plus LIF condition, 60–65% of H3K27me3 was observed at promoters [53–55]. Indeed, in serum plus LIF conditions, many promoters could be distinguished and are called bivalent promoters (marked with the trimethylation of the lysine 4 of the histone 3 and H3K27me3), while only a few of them were found in 2i condition, mainly due to the previously described loss of H3K27me3 [54]. H3K27me3 mark is deposited on histone H3 by the histone lysine methyltransferase EZH2, a member of the polycomb II complex [56]. In serum plus LIF condition, genes targeted by polycomb II complex exhibited an important variability of their expression [57]. These targeted genes include many developmental regulators and lineage specification activators and are repressed with a unique chromatin signature in mESCs (highly enriched for the H3K27me3 mark) [58]. Taken together, the epigenomic and transcriptional profiles of mESCs cultured in 2i plus LIF condition resemble the epigenetic and transcriptional environment of the epiblast in the blastocyst, thereby allowing the direct capture and preservation of naïve pluripotency in vitro [47].

Besides, when 2i and LIF components are removed from the culture, naïve mESCs rapidly exit their pluripotent state and differentiate [47,59–61]. The exit from the pluripotent state requires the dissolution of the core pluripotency transcription factor circuit, but it still remains unknown how the differentiation machinery breaks down this network. Betschinger and colleagues took advantage of this rapid exit from the pluripotency of mESCs cultured in permissive media and developed an “exit from pluripotency assay”, in which the ability to self-renew in a 2i culture after a period of permissive culture is assessed by alkaline phosphatase staining [60]. Only the cells that will not commit to differentiation and will remain in a pluripotent state will survive and proliferate when reintroduced into the 2i culture condition. This new assay has enabled the study of new regulators of the commitment of mESCs to differentiation.

In conclusion, the 2i media rapidly emerged as a novel and very standard method to culture mESCs due to its well-defined composition, easily transposable from one laboratory to another and from one background of cells to another. Furthermore, it allows the exploration of the mechanisms involved in stem cell commitment using exit from pluripotency assays.

**Importance of Canonical RNAi Pathways in mESCs**

In mammals, two types of canonical RNAi pathways coexist: miRNA and siRNA pathways, which themselves could be divided into canonical or non-canonical miRNA pathways and into endogenous and exogenous siRNA pathways (Fig. 1).

MiRNAs have been identified as important post-transcriptional regulators of gene expression [62,63]. They are small and single-stranded non-coding RNAs, which control the expression of their target genes by primarily acting as sequence-specific inhibitors of the corresponding mRNA. This inhibitory effect can occur by transcript destabilization or translational inhibition or by both via deadenylation, decapping, and exonucleolysis [64]. As illustrated in Fig. 1, the production of a functional mature miRNA involves multiple processing steps. Most animal miRNAs are transcribed in the nucleus by RNA polymerase II as stem-loop long primary transcripts. Following transcription, they are processed sequentially in the nucleus and cytoplasm by two protein complexes containing RNase-III-type endonucleases, DROSHA and DICER. Specifically, DROSHA and its partner DGCR8, the so-called “Microprocessor complex”, trim the primary transcript (pri-miRNA) to a 70- to 100-nt stem-loop precursor miRNA (pre-miRNA). Subsequently, they are delivered to the cytoplasm by EXPORTIN 5, where it is cleaved by DICER, which forms a complex with TRBP, to produce a ~22-nt miRNA-3p:miRNA-5p duplex (canonical miRNA pathway). However, a subset of intronic miRNAs called “mirtrons” and intergenic short hairpin RNA-derived miRNAs can circumvent the Microprocessor complex. They are produced by splicing and the lariat debranching of short hairpin introns (mirtron pathway) or are directly transcribed into the precursor short hairpin RNA by RNA polymerase II [7-methylguanosine (m7G)-capped pathway] [65,66]. These Microprocessor-independent m7G-capped precursor miRNAs, whose 5’ ends coincide with transcription start sites and 3’ ends are most likely generated by transcription termination, are exported by EXPORTIN 1 to the cytoplasm. In summary, the biogenesis of miRNAs is achieved by at least three pathways: the canonical miRNA pathway and the two non-canonical mirtron and m7G-capped pathways (Fig. 1).

Once in the cytoplasm, one of the duplex strands (miRNA-3p or miRNA-5p) is preferentially incorporated into the RNA-induced silencing complex (RISC) in association with an Argonaute (AGO) family member. Within the RNA-induced silencing complex-AGO entity, the miRNA guides the complex to its RNA target, thereby mediating its repression. In animals, most miRNAs control gene expression by binding to the 3′ UTR of their target genes through Watson–Crick
base pairing between the target and the 5'-end of the miRNAs: the "seed" sequence. However, recent studies suggest that miRNAs might also repress mRNA targets by binding to other regions including the 5' UTR or protein-coding exons [64].

MiRNAs have an important role in regulating stem cell self-renewal and differentiation by repressing the translation of selected mRNAs in stem cells and differentiating daughter cells. The mESC miRNA signature is characterized by miRNAs inhibiting differentiation (e.g., miR-296, miR-134, miR-21, and let-7) [67,68]. Promoters of many miRNA genes are bound in mESCs by the core pluripotency factors (OSN) [69]. Interestingly, there is a reciprocal relationship; hence, several miRNAs that are up-regulated during mESCs differentiation have been shown to directly target the mRNAs coding for these pluripotency factors (OSN and KLF4) [70]. Indeed, various miRNAs have been employed to successfully increase the efficiency of reprogramming [71]. During the reprogramming of mouse embryonic fibroblasts (MEFs), a subset of miRNAs belonging to the miR-290-295 cluster was able to significantly enhance the colony number of induced pluripotent stem cells formation [71]. For a detailed description of the individual miRNAs and their function in stem cell biology and during differentiation we would like to refer to recent reviews [72–74].

In mammals, endogenous siRNAs (endo-siRNAs) have been only described in germ lines and in mESCs (endo-siRNA pathway) [75–78]. They are mainly derived from inverted repeat elements, convergent transcription, and pseudogenes/gene pairs [79] (Fig. 1). Endo-siRNAs are processed by DICER from double-stranded RNAs and may function like their artificial counterparts used in experimental RNAi. They can be loaded onto different AGO proteins, with the restriction that only AGO2 mediates the endonucleolytic cleavage of their target mRNAs [80]. However, examples of this mode of action are limited to mammals [81]. Nevertheless, exogenous siRNAs have been identified and can act as antiviral defense mechanisms [82,83].

In conclusion, the role of the RNAi protein core machinery (DGCR8, DROSHA, DICER, and AGO proteins) in miRNA and siRNA pathways is considered as a canonical function of these proteins, whereas an sRNA biogenesis-independent function is described as a non-canonical function of these proteins.

In vivo studies with RNAi mutants show embryonic lethality before the implantation stage [5,7,84]. As investigations during development have been done at 3.5 days post coïtum (dpc) and 6.5 dpc, the exact time frame and molecular mechanisms leading to this lethality have not been dissected so far (Fig. 2). Mouse ESCs that are deficient for RNAi genes fail to produce miRNAs and manifest defects in proliferation,
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EB: Embryoid body.
WT: Wild type.
NPC: Neuronal precursor cell.
Roles of RNAi pathways in Stem Cell Biology

Proteins in Mammals

Non-Canonical Functions of RNAi

Roles of RNAi pathways in Stem Cell Biology

Non-Canonical Functions of RNAi Proteins in Mammals

During the last 2 decades, all the RNAi members were thought to only function in the miRNA and siRNA pathways. Indeed, differences have been observed between Dgcr8−/− and Dicer−/− mutant mESCs (Table 1), which were explained by the potential role of DICER in the endo-siRNAs pathway [7]. However, recent evidence indicates the existence of additional non-canonical functions (small non-coding RNA independent) for the RNAi effector proteins in various biological systems. The earliest indications of miRNA-independent functions in mammals came from observations of differing phenotypes between Drosha- and Dicer-deficient cells [88,90,91]. Before these studies, it was assumed that both DROSHA and DICER RNAse-III proteins were only essential for miRNA biogenesis. Consequently, the deletion of both genes should result in similar phenotypes or weak differences due to the role of DICER in siRNA pathways. Nevertheless, DICER deficiency in the eye, but not DROSHA, leads to macular degeneration [88,91]. On the contrary, Drosha deletion in neuronal stem cells, but not Dicer, results in loss of stem cell identity and precocious differentiation [90]. Furthermore, DROSHA was found to be essential for T-cell development in a miRNA-independent manner [87]. Finally, a recent review nicely recapitulates all the non-canonical functions for DROSHA and DICER in different biological systems [92].

Interestingly, Macias et al. identified also new functions for the Microprocessor. A high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation experiment designed to identify novel substrates of the Microprocessor revealed that this complex binds and regulates a large variety of cellular RNAs [93]. The Microprocessor-mediated cleavage of several classes of RNAs not only regulates transcript levels but also modulates alternative splicing events independently of its miRNA function. DGCR8, alone, was also shown to bind cassette exons, suggesting a role in the regulation of alternative splicing [93]. Along these same lines, in the absence of DGCR8, more than 300 alternatively spliced events were misregulated [93]. A study also described a physiological DROSHA- and miRNA-independent function of DGCR8 in the control of fly neuronal morphogenesis; however, whether this is mechanistically linked to small nucleolar RNAs biogenesis is still unclear [94]. In addition, DGCR8 has been recently reported to directly interact with the exosome subunit RRP6 in a DROSHA-independent manner [95]. DGCR8 controls the stability of mature small nucleolar RNAs and human telomerase RNAs. This new non-canonical function identified DGCR8 as an adaptor to recruit the exosome complex to structured RNAs and induce their degradation [95].

In order to assess the canonical and non-canonical functions of the DGCR8 protein in stemness, exit from pluripotency, and differentiation of mESCs, our group complemented Dgcr8-deficient mESCs with wild-type and phosphomutant forms of the DGCR8 protein [86,96]. As previously described, all complemented mESCs were able to restore miRNA production [96]. Nevertheless, the phosphomutant-complemented DGCR8 form was not capable to exit from the pluripotency state and differentiate despite the presence of miRNAs. The integration of omics data and RNA immunoprecipitation experiments established DGCR8 as a direct interactor of the Tcf7l1 mRNA, a core component of the pluripotency network. Furthermore, we demonstrated that DGCR8 facilitates the splicing of Tcf7l1, an event necessary for the differentiation of mESCs. Our data revealed a new non-canonical function of DGCR8 in the modulation of the alternative splicing of Tcf7l1 mRNA in addition to its established function in miRNA biogenesis [86].

Finally, a nuclear localization of AGO proteins in human and mouse cell lines was also reported (reviewed in Refs. [81,97]). AGO2 is re-localized to the nucleus via Importins [98–100] and TNRC6A [101,102], indicating that these RNAi proteins could function in the nucleus as reported for plants, fungi, and invertebrates [103]. Moreover, several observations of robust transcriptional gene silencing, involving synthetic sRNAs complementary to the promoter region, have been described in mammals [104]. Striking evidences indicated the involvement of RNAi in the control of splicing in mammals by affecting the epigenetic modifications of the chromatin [105,106]. Notably, all of the aforementioned studies were performed using artificial sRNAs. Nevertheless, they clearly suggest a conserved function for the RNAi pathways in the control of transcription and splicing in mammals. However, until recently, there were no evidences for the existence of endogenous sRNAs involved in an RNAi-mediated control of these processes in mammals, although multiple reports suggest a role for AGO proteins in the nucleus. In addition to its nuclear localization, AGO2-
Cross-linking immunoprecipitation experiments show that 12% of the targets are mapped on the introns of mRNA [107]. Moreover, divergent transcription from active promoters has been observed in mESCs, generating both small 20- to 90-nt species and long noncoding RNA (lncRNAs) [108]. As endo-siRNAs have been thus far characterized in this cell type, they are consequently the most adapted model to study RNAi-mediated transcriptional gene silencing and alternative splicing.

Conclusions and Future Perspectives

The aforementioned recent developments in stem cell biology (2i culture conditions and exit from pluripotency assay) clearly demonstrate that two particular events are crucial for the commitment of stem cells to differentiation: repression of the pluripotent network (exit from pluripotency) and activation of the differentiation programs (toward the three germ layers) [20]. Nowadays, the establishment of chemically defined culture conditions for mESCs allowed the capture of the “ground state” or naïve state of pluripotency [35].

Most of RNAi mutant mice are lethal at the early implantation stage, which is a very difficult time-point to experimentally address in vivo (Fig. 2). Moreover, these proteins are essential for proliferation and differentiation into the three embryonic germ layers of mESCs. Previously, it was assumed that this early embryonic lethality and the stem cells phenotypes were caused by an impairment of the miRNA biogenesis. However, recently, the question arose as to whether the function of RNAi proteins in the miRNA or siRNA pathways or other biological processes is responsible for the developmental/differentiation defects observed. Furthermore, is this differentiation defect the result of an impaired activation of the cell-lineage-specific programs or rather a problem in the exit from the pluripotent state? In the light of the novel non-canonical functions recently discovered, we think it is necessary to reassess all RNAi mutant mESCs in the same genetic background and identify the underlying mechanism leading to the impaired exit from pluripotency and differentiation phenotypes, similar to our recent study in Dgcr8-deficient mESCs [86]. Taken together, these experiments will help advance and broaden our view on the functions of the RNAi proteins in stem cell biology and in early mammalian development.

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Abbreviations used:
RNAi, RNA interference; siRNA, small interfering RNA; sRNA, small RNA; miRNA, microRNA; mESCs, mouse embryonic stem cells; ICM, inner cell mass; LIF, leukemia inhibitory factor; BMP4, bone morphogenetic factor-4; GSK3, glycogen synthase kinase-3; OSN, OCT4, SOX2, and NANOG; H3K27me3, trimethylation of the lysine 27 of the histone H3; m7G, 7-methylguanosine; AGO, Argonaute; MEFs, mouse embryonic fibroblasts; endo-siRNAs, endogenous siRNAs; dpc, days post coitum; EB, embryoid body; MEK, MAP (Mitogen-Activated Protein) Kinase/ERK (Extracellular Signal-Regulated Kinase) Kinase; ERK, Extracellular Signal-Regulated Kinase.

References

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