RISC in Development; A Review on RNA Induced Silencing Complex and its Contribution to Cellular Differentiation

Davoudpour A[1]

[1]Institute of Prophylaxy and Epidemiology of Cardiovascular Diseases (IPEK), Ludwig-Maximilians university, Munich-Germany

Corresponding author: Amirali Davoupour,
Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten, Klinikum der LMU München, Pettenkoferstrasse 9, 80336 München
Tel: +49-(0)89-5160-4371
Handy: +49-(0)174-1718942
E-mail: amirali.davoudpour@med.uni-muenchen.de

Abstract
Messenger RNAs may be targeted by short 19-27 nt RNAs generally called Small none-coding RNAs (snRNAs), the role of miRNAs among other snRNAs has been more studied and is well known. Many researches show that all compartments of RISC, Proteins and miRNAs take part in this wide range of regulatory impacts. Ago protein homologs plus miRNAs and target mRNAs form a silencing complex in P-bodies which lead to either cleavage, conservation or surprisingly amplification of target mRNA or gene product. This article reviews conceptions which contribute directly or implicate this important post transcriptional mechanism's function to differentiation or fate of pluripotent cells.

Keywords: Development, Embryo, RNAi

Introduction
Pluripotency is the ability of cells to differentiate into any fetal or adult cell type. Pluripotency is formed during early development and decoration of pluripotent cells. The OCT4 (POU5F1), SOX2 and NANOG transcription factors form the core of a network responsible for the transcriptional control of Embryonic Stem Cells (ESC) renewal and pluripotency.(1, 2)
The cytoplasm of an enucleated oocyte can induce pluripotency in the nuclei of somatic cells during nuclear transfer.(3)
Surprisingly, a high-throughput fluorescent in situ hybridization (FISH) screen developed by Eric Lécuyer and colleagues in the Krause lab. revealed that the majority (71%) of mRNAs expressed during embryonic development exhibit specific subcellular localization. Which suggest both a high regulation and potential of mRNAs in differentiation process . In cytoplasm of developing germ cells of many organisms RNA and proteins localize in germ-cell-specific cytoplasmic structures called P granules.(4)
In C. elegans, PGL-1 , GLH-1 and DEPS-1 are identified as critical components of P granules and are required for proper germ cell development.(5, 6) DEPS-1 is required for RNAi (RNA interference) of germline-expressed genes, possibly because DEPS-1 promotes the accumulation of RDE-4, a dsRNA binding protein required for RNAi.(7)
When ESCs differentiate, they must both silence the ESC self-renewal program and activate new tissue-specific programs. In the absence of DGCR8 (Dgcr8(-/-)) - DiGeorge syndrome critical region gene 8- a protein required for microRNA (miRNA) biogenesis, mouse ESCs are unable to silence self-renewal.(8)
In Drosophila the genes zucchini (zuc) and squash (squ) are required early during oogenesis for the translational silencing of osk mRNA and at later stages for proper expression of the Grk protein. Establishment of dorsal-ventral (DV) and anterior-posterior (AP) axes is achieved through the localized translation of protein products of gurken (grk) and oskar (osk) genes.(9) Zuc encodes a member of the phospholipase-D/nuclease family(10, 11) while squ encodes a protein with limited similarity to RNAase HII.(12) Zuc and Squ localize to nuage, an electron-dense structure

surrounding the nurse cell nuclei implicated in RNAi and RNA processing and transport.(13, 14)
Zuc and Squ physically interact with Aub (aubergin, one of the Piwi subfamily of Argonautes in Drosophila), thus pointing to a direct role for these proteins in the RNAi mechanisms and are required for the biogenesis of Repeat associated small interfering RNA (rasiRNAs) in ovaries and testis. Accordingly, mutations in these genes abolish the production of this class of siRNAs and lead to the deregulation of transposable elements and tandem repeats in the Drosophila germline.(15) Nuage granules disturbing, resulting in a displacement of the RISC components Ago2 and Dcr1.(16)
In male genetic content comes in imprints, meaning that pre-coded modifications take place during gametogenesis. In female as there is no mRNA synthesis between the end of the mouse oocyte growth phase and the first zygotic cleavage, post transcriptional mechanisms are essential for the natural formation of pluripotency. Ago2 the catalytic core of RISC has been shown to have a vital impact in normal development in both pre-implantation and post-implantion stages, it is also shown that Ago 2 is essential in gastrulation and mesoderm formation.(17) MicroRNAs are endogenous small RNAs which target mRNA through a mechanism involving members of Ago protein family. Argonaute associated with miRNA binds to the 3'-untranslated region (3'-UTR) of mRNA, the Argonaute-miRNA complex can also affect the formation of functional ribosomes at the 5' end of the mRNA by competing with translation initiation factors and or abrogating ribosome assembly (Initiation).(18)
In addition, the Argonaute-miRNA complex can also alter protein production by recruiting cellular factors(peptidases, post-translational modifying enzymes) that will target the degradation of the growing polypeptides (Elongation).(19) P-bodies are suggested as either storage or degradation sites for mRNAs stocked in.(20, 21, 22) In this review, we would like to discuss and link progresses in RNAi as an important post transcriptional regulator of gene product to processes which later lead to development of a pluripotent cell into a differentiated cell.

RNA interference: Noncoding RNAs (ncRNAs) have key roles in the regulation of complex genome functions and plasticity in multicellular organisms. In vertebrates, long dsRNA activates the interferon response and yields nonspecific degradation of mRNA but they also participate in some regulations at gene level.(23)
In mouse embryo, paternally expressed long ncRNA Kcnq1ot1 regulates epigenetic gene silencing in an imprinted gene cluster in cis over a distance of 400 kb. Gene silencing by the Kcnq1ot1 RNA involves repressive histone modifications, including H3K9me2 and H3K27me3, which are partly brought about by the G9a and Ezh2 histone methyltransferases. Analysis of conditional Dicer mutants reveals that the RNAi pathway is not involved in gene silencing in the Kcnq1ot1 cluster. RNA/DNA FISH shows that the Kcnq1ot1 RNA establishes a nuclear domain within which the genes that are epigenetically inactivated in cis are frequently found.(24)
In contrast small RNA (snRNA) duplexes with a length of 21-23 nucleotides trigger specific gene silencing and thus are widely used in gene function studies. The pathway of RNAi consists of nuclear processing of the pri-miRNA by the microprocessor complex Pasha/DGCR8 and Drosha(25) generating pre-miRNA, while DGCR8 is involved in producing both siRNAs and miRNAs. After transcription, silencing the drosha cofactor pasha in Meloidogyne incognita, inhibits normal embryonic development within the eggs similar to that of drosha-silenced eggs, eventually leading to embryonic lethality.(26)
MiRNAs are then exported to the cytoplasm through Exportin-5,(27) where dicer cuts the stem loop region producing small double stranded RNA(dsRNA). Dicer, is essential for meiotic maturation of mouse oocyte. While Dicer deficient ES cells show defects in differentiation and pluripotency(28) loss of Dcr-1 in mouse ESCs results in the depletion of miRNAs and causes slower proliferation and differentiation defects in vivo and in vitro,(29, 28) Using conditional allele of dicer-1 (dcr-1) in the mouse, specific deletion of dcr-1 in the T cell lineage results in impaired T cell development and aberrant T helper cell differentiation and cytokine production. A severe block in peripheral CD8(+) T cell development was observed upon dcr-1 deletion in the thymus. However, Dicer-deficient CD4(+) T cells, although reduced in numbers, are viable and can be analyzed further. These cells are defective in microRNA processing, and upon stimulation they proliferate poorly and undergo increased apoptosis.(30) Removal of Dicer in limb mesoderm phenotypically results developmental delays, in part due to massive cell death as well as disregulation of specific gene expression and finally formation of a much smaller

limb,(31) other data show expression of discrete set of microRNAs are expressed in hair follicles and epidermis, while dcr1 gene ablation in embryonic skin progenitors results not markedly differentiated cell without an increase in apoptosis.(32) Analysis of Dcr 1 -/- ESCs has also revealed defects in the centromeric chromatin, manifested as a loss of DNA methylation and histone H3K9 trimethylation, and an increased abundance of RNAs derived from centromeric repeats.(33, 29) In Dicer$^{-/-}$ ES cells, expressing Dicer at very low levels (~5%) the xi RNA levels were found to be significantly reduced upon differentiation and more importantly Dicer$^{-/-}$ ES cells showed a lack of Xist and H3K27 trimethylation foci characteristics of Xi chromosome suggesting direct role for dicer in X chromosome inactivation.(33) In contrast, there are papers which suggest no direct role for Dicer in Xist and H3K27 recruitment on to Xi.(34,35)

Back to RNAi pathway, dsRNA is then loaded onto the RNA induced silencing complex containing the RNA endonuclease Ago1, and unwound. Animal mRNAs typically base-pair imperfectly with the 3'-UTR of target mRNAs, one of miRNA strands would then act as guide strand, the guide strand confers specificity to the RISC complex that now recognizes mRNA targets that are in turn either degraded or translationally repressed.(36) MiRNAs can induce substantial mRNA degradation even in the absence of extensive base-pairing to their targets.(37) There are increasing evidences that miRNAs have important roles in differentiation of tissues, proliferating cells have altered patterns of microRNA expression, which can be used to identify the cell of origin and to subtype cancers.(38) Recently it has been shown that Tooth morphogenesis and ameloblast differentiation are regulated by micro-RNAs.(39) Antisense transcripts may also contribute to developmental regulation of key transcription factor genes by similar Dicer-promoted mechanisms, in an experiment within the developing CNS, Emx2 antisense RNA contributes to post-transcriptional down-regulation of its sense partner.(40)

Surprisingly, specific cellular conditions can turn miRNAs from silencers to translational activators. Vasudevan et al., surprisingly found that human Ago2 activates translation of target mRNAs on cell cycle arrest caused by serum starvation or contact inhibition, while it normally represses translation of the same target mRNAs in proliferating cells.(41) Lund and colleagues showed that miR-10a enhances translation of the reporter mRNA harboring a target site in the 5' UTR, although a regulatory 5' UTR motif, named "5'TOP motif", is necessary for this enhancement.(42) Sarnow's group reported that endogenous liver specific miR-122 activates translation of hepatitis C virus (HCV) RNA which has two miR-122 target sites and an IRES in its 5' UTR.40.(43) These exciting new findings, however, have made it even more difficult to explain how miRNAs regulate post transcriptional events.

Ago2, the catalytic core of RISC is involved in gastrulation and mesoderm formation.(17) Eliminating zygotic expression of Ago2, indicated thatthere was no requirement for Ago2 until only after implantation.(44, 45) Systematic knockdown of maternal Ago2, 3, and 4, individually and in combination, it is found that Ago2 is required for development beyond the two-cell stage. Knockdown of Ago2 stabilizes one set of maternal mRNAs and reduces zygotic transcripts of another set of genes.(46) Hannon's group generated a catalytically inactive mouse in which they replaced the endogenous allele by a carrying mutation in the DDH motif (Ago2ADH).(47) They observed that the animal underwent a normal embryogenesis but died within a few hours after birth and displayed severe sign of anemia. These embryos have an important reduction in red blood cell caused by a defect in the maturation of erythroid cells. These results represent the first evidence that the catalytic domain of Ago2 is essential for the survival of mammals. Intriguing studies by the Lei lab (NIDDK, NIH) provide evidence of a previously unknown role for the RNA silencing machinery in the regulation of gypsy insulator function and higher order chromatin organization in the nucleus. The gypsy insulator is thought to recruit a number of protein factors, including centrosomal protein 190 (CP190), in order to establish nuclear bodies responsible for forming distinct chromatin loops.(48) The functional role for these chromatin structures may be to physically isolate regulatory modules for different genes into specific chromosomal domains. The RNA silencing proteins Piwi and Argonaute2 (AGO2) interact physically with the gypsy insulator in an RNA-independent manner. Piwi also co-localizes with gypsy nuclear bodies during larval stages.(49) In flies carrying mutations of Piwi or AGO2, gypsy insulator function is decreased, suggesting that these two factors are critical components of the insulator complex. Oocyte endogenous siRNAs derived from processed pseudogenes suggest that mammalian RNAi, in addition to roles in the suppression of mobile and repetitive sequences known from

invertebrates, might also regulate endogenous genes.(50, 51) This hypothesis is now supported by the defective spindle phenotype of Dcr 1-/- and Ago2-/- oocytes, which is absent in Dgcr8-/-. Bioinformatic analysis of the Dcr 1-/- transcriptome show that many upregulated transcripts have complementary sequences to endo-siRNAs found in the oocyte.(52) They suggest a model in which the miRNA pathway becomes disengaged early during oocyte growth and RNAi becomes the dominant RNA silencing pathway essential for OZT (oocyte-to-zygote transition), RNAi has been shown to be involved in axial polarization in the Drosophila germline.(53, 54) In this species, establishment of dorsal-ventral (DV) and anterior-posterior (AP) axes is achieved through the localized translation of specific mRNAs. The protein products of gurken (grk) and oskar (osk) genes are essential for this process.(55, 56, 57)

Studies have reported that RNAs complementary to promoter DNA also inhibit gene expression. Human homologs of AGO1 and AGO2, EIF2C1 and EIF2C2 link the silencing pathways that target mRNA with pathways mediating recognition of DNA. There have been conflicting reports on whether Antigene RNAs (agRNAs) may induce DNA methylation.(58, 59, 60, 61, 62)

RNAi and Fertilization of oocyte: Fertilization- union of sperm and egg- is an event that triggers the development of a new organism. Of note, male and female haploid complements of chromosomes are distributed as separate entities for some time prior to their incorporation into first diploid embryonic cells.(63) At the meantime post transcriptional regulations play an important role in formation of pluripotency. Major zygotic gene expression occurs at two-cell stage, corresponding to the time at which mRNA for the majority of maternal transcripts are degraded by a less known mechanism.(64, 46)

A small subset of genes present in mammals is expressed exclusively from chromosome of maternal or paternal origin. This mono-allelic gene expression is termed imprinting. In sperms key developmentally regulated genes (including HOX gene) are pre-coded during spermatogenesis.(65) HOXA1 is a direct target of miR-10a and miR-10a expression in differentiated megakaryocytes is inverse to that of HOXA1,(66) along with miR-10a, miR-196a is expressed in patterns that are markedly reminiscent of those of Hox genes.(67) MicroRNAs located in and/or targeting HOX gene clusters were already discussed in details.(68, 69, 70, 71)

Maternal genome may also contain epigenetic pre-coding of developmental significance but since oocytes cannot be obtained in quantity it is not proven yet. Imprints are established during gametogenesis by placing symmetric 5-methylcytosine modifications in CpG dinucleotides of cis-acting control regions near imprinted genes.(72) These differentially methylated regions (DMRs) are methylated in either sperm or egg and are present in mature gametes. It is now also clear that post translational modifications of lysine and arginine residues present within nucleosomal histones also play major roles in epigenetic coding.(73) Mutation of H3.3 K27, but not of H3.1 K27, results in aberrant accumulation of pericentromeric transcripts, HP1 (Heterochromatin protein 1) mislocalization, dysfunctional chromosome segregation and developmental arrest. This phenotype is rescued by injection of dsRNA derived from pericentromeric transcripts, indicating a functional link between H3.3K27 and the silencing of such regions by means of an RNA-interference (RNAi) pathway (74). The role of RNAi in X-chromosome inactivation is reviewed by(75) chromosome inactivation results equivalent expression of X-linked genes and is mediated by cis coating of a long non-coading RNA termed Xist onto the future inactive X chromosome (Xi). Xist RNA is transcribed by RNA polymerase II (Pol II), is spliced and polyadenylated and located in the nucleus in a 3D domain along with genes which are silenced in X-inactivation procedure. RNAi machinery is intricately involved in the silencing of yeast centromeric chromatin via small RNA generated from the pericentric sense and antisense non-coding RNA and specialized protein complex named RNA-induced transcriptional silencing (RITS).(76) Ogawa et al., were able to detect distinctly sized small RNA of 24–42 nucleotides corresponding to Repeat A, Exon-7 and promoter regions of Xist upon differentiation of ES cells which shows inverse correlation to Xi RNA.(77) In C. elegans a complex of proteins composed of ERI-1/3/5/9, RRF-3, and DICER (the ERI/DICER complex) mediates RNAi processes , eri mutant animals (including eri-1, rrf-3, eri-3, and dcr-1) exhibit temperature-sensitive, sperm-specific sterility and defects in X chromosome segregation.(78)

Maintenance of heterochromatin domains by dsRNA binding proteins and small RNA has also been reported in plants and Drosophila (Kota,s., 2009).

Translational Repression mediated by miRNAs: Biochemically, translational repression is best understood in Drosophila, which possess at least two distinct RISCs that each mediate repression by different mechanisms. The first mechanism involves inhibition of translation initiation. Specifically, RISC formed from Drosophila Ago2 can block protein-protein interactions between eIF4E and eIF4G, which are required to form a competent pre-initiation complex on the target mRNA.(79) Unlike the slicing reaction, translational repression does not require extensive sequence complementarity between guide and target RNAs. As a general rule, only bases 2-7 of the guide RNA are required to match a target to initiate translational repression.(80) Drosophila Ago1, on the other hand, represses translation by promoting target mRNA deadenylation and degradation. Ago1-RISC contains the protein GW182, which recruits the poly(A) deadenylation complex Ccr4-Not and the mRNA decapping complex DCP1- DCP2 to target messages.(81) GW182 is also involved in directing target mRNAs to cytoplasmic foci called P-bodies, which are translationally inactive structures that function as sites of mRNA storage and/or degradation.(82) Mammalian RISCs employ similar mechanisms of translational repression(83) however the relevant circumstances and exact mechanism(s) used by specific RISCs, have yet to be determined.

Early studies implicated miR181, whose expression is increased in thymus, lymphoid tissues, and bone marrow, in promoting B-cell differentiation. Ectopic expression of miR181 in mouse hematopoietic precursor cells leads to a dramatic increase in B lineage cells.(84)

A well characterized ESC miRNome is dominatied by miRNAs sharing a 5'-proximal AAGUGC motif.(85, 86, 87) These miRNAs can be divided into three groups and may also serve as molecular markers for the early embryonic stage and for undifferentiated ESC cells (I) EEmiRC miRNAs, found in placental mammals,(88) (II) the miR-17-92 cluster -which are encoded as polycistrons from a single common transcript and its paralogues, which is conserved across verebrates and carries onco-miRs, this cluster can promote cell proliferation,(89) and (III) the miR-302/miR-467 group, including the miR-302 family in tetrapods and the miR-467 family in mouse. Let-7 miRNA family is expressed in adult and differentiated animal tissues accumulation of let-7 can be preveneted by LIN28 a promoter of pluripotency.(90) Interestingly the opposing activities of let-7 and pluripotent miRNAs represent one of the features that distinguish pluripotent and differentiated cells.(8)

There are also multiple factors such as Tudor staphylococcal nuclease (Tudor-SN) that are considered as components of RISC in humans, flies and nematodes and is therefore implicated in the RNAi pathway, but apparently have no significant role in differentiation of Trypanosoma brucei.(91)

Transcriptional Silencing and Formation of Heterochromatin: Beyond targeting message RNAs, some RISCs act directly on the genome. The best studied of these assemblies is the fission yeast RITS (RNA Induced Transcriptional Silencing) complex, which contains Ago1 with an associated siRNA, a protein called Tas3 and the chromodomain protein Chp1.(76) The RITS complex interrogates nascent transcripts as they are generated by RNA polymerase II in the nucleus. Upon target recognition the complex recruits histone methyltransferases, which modify histones associated with the DNA locus, forming heterochromatin.(92) The Chp1 subunit of RITS specifically recognizes histone-3 proteins bearing methylation on lysine-9, further reinforcing the association of the RITS complex with heterochromatin.(93)

The RITS complex also physically interacts with an RNA-directed RNA polymerase complex, which converts the targeted transcripts into dsRNA. Dicer then cleaves the dsRNA into new siRNAs, which can be loaded into new RITS complexes, thereby establishing a self-perpetuating silencing loop. Although the level of molecular detail is less well understood in other systems, plants and animals contain analogous systems for small RNA-guided formation of heterochromatin.(94) In particular, the Piwi clade appears to function in transcriptional silencing and formation of heterochromatin.(95)

Reversal of microRNA repression and mRNA localization in P-bodies in human cells: P-bodies are suggested as either storage or degradation sites for mRNAs. mRNA reporters repressed by miRNAs were found to localize in P bodies,(20) Intracellular localization of the endogenous CAT-1 mRNA and RL-cat reporters in cells grown under different conditions reveals that in nonstarved Huh7 cells, CAT-1 mRNA is concentrated in P-bodies dependent on miR-122. Most importantly, in Huh7 cells grown for 2 hours under amino acid deficiency CAT 1 protein increases without an increase on the mRNA level. CAT-1 mRNA was no longer detectable in P-bodies Starvation did not produce an

appreciable decrease in the miR-122 signal in P-bodies(36) arguing for an effect specific for the CAT-1 mRNA and possibly only a limited number of other mRNAs among the many regulated by miR-122 in liver cells.(96) Bhattacharyya et al., suggest that metazoan P-bodies are not only a site for mRNA turnover but also of storage of translationally repressed mRNAs. Interestingly, the same evidence is available for baker's yeast, an organism lacking miRNA regulation,(22) other examples of reversible action of miRNAs have been identified in neuronal cells. In neurons, many mRNAs are transported along the dendrites as repressed mRNPs to become translated at the final destination, dendritic spines, upon synaptic activation such local translation is important for spine development, learning, and memory.(97)

Discussion

The process of cell differentiation by mechanisms such as heterochromatin formation can be fully reversed and does not require irreversible nuclear changes. When Xenopus nuclei were transplanted from fully differentiated cells, in this case from the intestinal epithelium of feeding tadpoles, entirely normal and fertile male and female frogs were obtained,(98) it involves changes in nuclear gene expression but not in gene content, if egg proteins can be exchanged in seconds or minutes for those in transplanted somatic nuclei, complete reprogramming should always take place.(99) Reversal of mRNA from repression,(36) indeed is a process which may stimulate re-activation of a genetic material by physical and environmental factors such as pressure to provide cell with a signal to either stop, amplify or regulate a code, which may alter cellular function and would finally lead to differentiation. This also would suggest a role for accumulation of mRNAs in repressed form in P-bodies or similar compartments, to save some genetic transcripts while at the same time destroying unwanted transcripts. This hypothesis is supported by experiments regarding attenuated expression of RISC members, which results not markedly differentiated cell without an increase in apoptosis.(32) Although it is still early to find an ultimate goal for RNAi mechanism, but it will not be surprising to implicate a memory function for RISC.

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