Perspective

The miR-302-367 cluster as a potential stemness regulator in ESCs

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Abbreviations: miRNA, microRNA; ESC, embryonic stem cell; ECC, embryonic carcinoma cell; nt, nucleotide; qRTPCR, quantitative real-time-PCR; BMP, bone morphogenic proteins; TU, transcriptional units; Pol-II, DNA polymerase II; Pol-III, DNA polymerase III; ncRNA, non-coding RNA; mRNA, messenger RNA; CHIP, chromatin immunoprecipitation; mirPS, miRNA-induced pluripotent stem cell

Key words: miR-302-367 cluster, stem cells, human ESCs, miRNA promoter

Increasing experimental evidence suggests an important role of miRNAs in embryonic stem cell (ESC) biology. The miR-302-367 cluster is exclusively expressed at high levels in ESCs but not in either somatic stem cells or adult/embryonic differentiated cells. The human miR-302-367 gene structure has been recently described and its promoter has been identified, characterized and functionally validated in human stem cells. The miR-302-367 promoter activity depends on the ontogeny and hierarchical cellular stage. The miR-302-367 promoter is transcriptionally regulated by the ESC-specific transcription factors Oct3/4, Sox2 and Nanog and, its activity restricted to the ESC compartment. Functionally, this cluster regulates cell cycle in ESCs promoting self-renewal and pluripotency, therefore representing a master regulator in the maintenance of hESC stemness. We envision this data may open up new avenues to investigate the transcriptional regulators upstream miR-302-367 cluster and to dissect the complex interplay by which this miR-302-367 cluster integrates in the molecular network conferring pluripotency to ESCs. In this perspective, we summarize recent progress in the genomic and functional characterization of the miR-302-367 cluster and discuss its potential as a stemness determinant.

Linking ESC and MicroRNAs

Human ESCs are pluripotent cells derived from the inner cell mass of pre-implantation embryos.1,2 They are endowed with two unique properties that distinguish them from all other organ-specific stem cells identified thus far. First, they self-renew continuously in culture and can be expanded for extended periods of time while maintaining their undifferentiated status. Second, they are capable of retaining their developmental potential to differentiate into tissues representing the three germ layers.3-5 A considerable wealth of data concerning characterization, differentiation and manipulation of hESCs has been accumulated over the past few years, boosting the scientific interest of hESCs in biomedicine. Human ESCs have been hailed as a unique tool for a range of biomedical applications such as cell replacement therapy, drug discovery, disease modeling and developmental biology by providing a model to unravel molecular determinants and networks controlling stem cell maintenance, cell proliferation and lineage specification.6-8

MiRNAs are ~22 nt long non-coding RNAs that function as widespread transcriptional regulators in a variety of biological processes including proliferation, differentiation and maintenance of "stemness".9,10 Importantly, an increasing body of evidence suggests a strong link of miRNAs with ESC biology and early embryo development.11-17 In mammals, for instance, Dicer-/- embryos which lack the miRNA processing and regulation pathway display embryo lethality and complete loss of the stem cell compartment.17 Similarly, Dicer-/- mESCs evidence a pronounced impairment in proliferation and differentiation.18 Few miRNAs have been found to be differentially expressed between ESCs and differentiated derivatives or somatic cells. Worth discussing is the miR-302-367 cluster which is specifically expressed in ESCs but it is silenced upon differentiation, highlighting a potential role in self-renewal and pluripotency.

The miR-302-367 Cluster

Recent efforts have focused on defining the miRNA expression profiles in undifferentiated hESC as compared to their differentiated progeny.19-21 Among the so-called ES-specific miRNAs, the miR-302-367 cluster stands out due to its intracellular abundance and high cell type specificity. This miRNA-302-367 cluster was initially identified from cDNA libraries generated by directional cloning using size-fractionated RNA (17–26 nt) from undifferentiated hESCs.22 This cluster is codified in the human chromosome 4 and consists of nine different miRNAs co-transcribed in a polycistronic manner: miR-302a, miR-302a*, miR-302b, miR-302b*, miR-302c, miR-302c*, miR-302d, miR-367 and mir-367* (Fig. 1).22,23 The miR-302 family contains seven miRNAs with a highly conserved 5' region (Fig. 1). Recently, a miRNA homologue to the miR-302 family has been reported and registered as miR-302e; however it does not belong to the miR-302-367 cluster and it is codified in the chromosome 11.21

The miR-302-367 cluster was first identified to be expressed in mESC, hESC and in their malignant counterparts hECCs.22
The miR-302a was initially cloned from mESCs but was not detectable in other adult cell lines confirming its specificity at embryonic developmental stages. Higher-resolution methodologies such as microarray and qRT-PCR assays further supported the high level expression of this miR-302-367 cluster in mESCs, hESCs and hECCs. Regarding cancer biology, recent elegant work indicates that cancer may arise from stem cells, and that many cell signaling pathways essential for normal development (i.e., Notch, Wnt, BMPs, etc.) are involved in cancer initiation and progression, supporting a strong link between embryonic cells and cancer cells. Similarly, miRNAs also play a pivotal role in the initiation and progression of human cancer via deregulation of fundamental processes such as differentiation, proliferation and cell death through transcriptional repression of key tumor suppressors. Accordingly, the miR-302-367 family members are not expressed in normal somatic hematopoietic cells whereas certain acute immature leukemia cell lines express some members of this miR-302-367 cluster. If the target cell for transformation is an immature stem/progenitor cell the expression of this miR-302-367 would likely suggest a failure of the miR-302-367 to silence due to the differentiation blockade. In contrast, if the transformed cell lines originated from a mature hematopoietic cell which regains stem-cell properties and functions the expression of this miR-302-367 would likely suggest the presence of a leukemic stem cell, the expression (reactivation) of the miR-302-367 to silence due to the differentiation blockade.

miR-302-367 Gene Structure

It was initially thought that most miRNA-coding genes were located in intergenic genomic regions. However, extensive miRNA genomic analyses have shown that many miRNA-encoding genes lay within defined transcriptional units (TUs) being mostly intronic (~80%). Thus, miRNAs can be found as: (i) intronic miRNAs in protein-coding TUs; (ii) intronic miRNAs in non-coding TUs and (iii) exonic miRNAs in non-coding TUs. Additionally, miRNAs have also been found interspersed with Alu repeats.

The human miR-302-367 coding gene is located in an intergenic region of chromosome 4 and contains three exons and two introns with alternative splicing, which may or may not include exon 2 (Fig. 1). All miR-302-367 cluster members are codified within intron 1 (Fig. 1). The two spliced transcripts do not contain any significant open reading frame and therefore, this cluster of miRNAs may be considered as intronic miRNAs in a non-coding TU. However, the poor phylogenetic conservation of exonic regions suggests that the only biologically relevant gene product is the miRNA cluster. In protein-coding messenger RNAs (mRNAs), the balance of the interactions between the processing/ribonucleoprotein assembly of intronic miRNAs and the splicing process may regulate the levels of ncRNA and host mRNA. The reason why the miR-302-367 cluster is spliced out from the pri-miRNA, and the possible interactions between the miRNA processing machinery and the spliceosome during the miR-302-367 maturation, remain to be elucidated. The primary transcript of the human miR-302-367 gene is a 1974 nt long RNA with the 5' end located 153 nt upstream from the first encoded miRNA (miR-302b*), and the 3' end sited approximately 12 nt downstream a canonical polyA signal (Fig. 1). Although PolIII-driven transcription has been reported for miRNAs interspersed with Alu repeats, experimental evidence suggests that miRNAs are mostly class-II genes. Accordingly, the pri-miRNA of the miR-302-367 gene is a Pol-II capped and polyadenylated transcript.

The miR-302-367 Promoter Activity Is Ontogenically and Hierarchically Regulated

Over the last few years, a considerable wealth of data has been gathered concerning the identification and clustering of multiple miRNAs. However, to better understand how miRNAs integrate into complex molecular regulatory networks, an extended knowledge of their transcriptional regulation should be pursued. This approach demands an exhaustive characterization of the miRNA promoters and transcriptional enhancers. Unfortunately, to date, very little is known about the genomic and functional characterization of miRNA promoters and transcriptional units.
Interestingly, the silencing of the promoter activity during ontogeny and hierarchical cellular stage. From an ontogeny standpoint, the core promoter specifically drives the transcription and expression of ESC transcription factors. The wide black arrows indicate the suggested overall effect of the miR-302-367 cluster in the maintenance of self-renewal and pluripotency of hESCs.

Very recently, we have identified and characterized the putative promoter of the miR-302-367 gene which, to the best of our knowledge, represents the first human miRNA promoter characterized and functionally validated in human stem cells. This promoter was identified from multiple sequence alignment among loci from distinct mammal species. The core promoter constitutes a strongly conserved region of approximately 500 bp immediately upstream of the transcriptional start that contains a conventional TATA box (Fig. 1).

Functionally, the miR-302-367 promoter activity depends on the ontogeny and hierarchical cellular stage. From an ontogeny standpoint, the core promoter specifically drives the transcription of a reporter gene in ESCs but not in somatic cells (either stem cells or differentiated cells). This indicates that the miR-302-367 promoter activity is restricted to an embryonic stage of development being turned off later in development. From a hierarchical point of view, the miR-302-367 promoter is active on ESCs but its activity decays upon differentiation of ESCs. The miR-302-367 promoter is silenced in transformed embryonic cell lines lacking stem cell phenotype/properties, further confirming its hierarchical regulation. Interestingly, the silencing of the promoter activity during hESCs differentiation parallels the intracellular decrease of mature miR-302-367 miRNAs. Taken together, these data support that the ESC-specific expression of the miR-302-367 cluster is fully conferred by its promoter transcriptional activity.

Regarding the transcriptional regulation of the miR-302-367 gene, the timing and cell specificity of the promoter activity correlates very well with a potential transcriptional regulation exerted by the DNA-damage- and/or additional cell cycle regulators. A major phenotypic consequence associated with the depletion of mature miRNAs on ESCs is an impaired proliferation. It was recently reported that the specific inhibition of miR-302 family members in hESCs alters the cell cycle profile promoting a G1 arrest, resembling the cell cycle profile of more differentiated cell types. Conversely, exogenous overexpression of miR-302 in primary and transformed adult cells induces the exit from G1 phase. These results pinpoint the role of the miR-302 family as one of the putative cell cycle regulators in ESCs. Actually, cyclin D1 and Cdk4 have been recently found to be post-transcriptionally regulated by miR-302 in hESCs (Fig. 2). In addition to cell cycle regulation, the miR-302-367 cluster also seems to play a role in maintaining the undifferentiated ESC status by controlling the balance between cell specification and pluripotency (our own unpublished observations). Preliminary data from our laboratory suggests that the TGFB/Nodal/Activin pathway may be induced by the miR-302-367 through the inhibition of intermediate negative effectors of the TGFB/Nodal/Activin pathway (our own unpublished observations) (Fig. 2). The importance of the TGFB/Nodal/Activin family members in the maintenance of pluripotency of hESCs is widely established. As compared to other model systems, they display specific cell cycle features: a shorter G1 phase, an accumulation during S phase and lack of DNA-damage induced G1-checkpoints, therefore involving a G1 arrest, resembling the cell cycle profile of more differentiated cell types. Conversely, exogenous overexpression of miR-302 in primary and transformed adult cells induces the exit from G1 phase. These results pinpoint the role of the miR-302 family as one of the putative cell cycle regulators in ESCs. Actually, cyclin D1 and Cdk4 have been recently found to be post-transcriptionally regulated by miR-302 in hESCs (Fig. 2).

Relevance of the miR-302-367 Cluster in Stem Cell Biology

ESCs self-renew continuously in culture and can be maintained and expanded for extended periods of time while maintaining their undifferentiated status. As compared to other model systems, they display specific cell cycle features: a shorter G1 phase, an accumulation during S phase and lack of DNA-damage induced G1-checkpoints, therefore involving a G1 arrest, resembling the cell cycle profile of more differentiated cell types. Conversely, exogenous overexpression of miR-302 in primary and transformed adult cells induces the exit from G1 phase. These results pinpoint the role of the miR-302 family as one of the putative cell cycle regulators in ESCs. Actually, cyclin D1 and Cdk4 have been recently found to be post-transcriptionally regulated by miR-302 in hESCs (Fig. 2). In addition to cell cycle regulation, the miR-302-367 cluster also seems to play a role in maintaining the undifferentiated ESC status by controlling the balance between cell specification and pluripotency (our own unpublished observations). Preliminary data from our laboratory suggests that the TGFB/Nodal/Activin pathway may be induced by the miR-302-367 through the inhibition of intermediate negative effectors of the TGFB/Nodal/Activin pathway (our own unpublished observations) (Fig. 2). The importance of the TGFB/Nodal/Activin family members in the maintenance of pluripotency of hESCs is widely established. As compared to other model systems, they display specific cell cycle features: a shorter G1 phase, an accumulation during S phase and lack of DNA-damage induced G1-checkpoints, therefore involving a G1 arrest, resembling the cell cycle profile of more differentiated cell types. Conversely, exogenous overexpression of miR-302 in primary and transformed adult cells induces the exit from G1 phase. These results pinpoint the role of the miR-302 family as one of the putative cell cycle regulators in ESCs. Actually, cyclin D1 and Cdk4 have been recently found to be post-transcriptionally regulated by miR-302 in hESCs (Fig. 2). In addition to cell cycle regulation, the miR-302-367 cluster also seems to play a role in maintaining the undifferentiated ESC status by controlling the balance between cell specification and pluripotency (our own unpublished observations). Preliminary data from our laboratory suggests that the TGFB/Nodal/Activin pathway may be induced by the miR-302-367 through the inhibition of intermediate negative effectors of the TGFB/Nodal/Activin pathway (our own unpublished observations) (Fig. 2).
signaling pathways. The miR-302-367 rather than being at the top of the stem cell hierarchy, is likely to function as a crucial intermediate regulator since it is a downstream transcriptional target of Oct3/4, Sox2 and Nanog.27,31 Surprisingly, however, the ectopic expression of the complete human miR-302-367 cluster into several human cancer cell lines is sufficient to reprogram them into a pluripotent ES-cell-like state. These miRNA-induced pluripotent stem (mirPS) cells, gain the expression of the ES cell markers Oct3/4, SSEA-3, SSEA-4, Sox2 and Nanog while displaying a highly demethylated genome with a wide gene expression pattern similar to that observed in hESCs.32 These mirPS cells are able to differentiate into several tissue cell types.32 Based on the potential deregulation of miRNA profiling in transformed cancer cell lines, whether mirPS cells can be induced from healthy adult primary cells still needs to be investigated.

Concluding Remarks

MicroRNAs contribute to the regulation of stem cell pluripotency. Recently, several breakthroughs have been accomplished in this field: (i) the miRNA cluster miR-302-367 has been shown to be expressed in mouse and hESCs; (ii) the structure of the gene coding for the human miR-302-367 cluster has been identified; (iii) the functional miR-302-367 gene promoter region and transcriptional units have been identified, characterized and functionally validated in human stem cells; (iv) the promoter activity depends on the ontogeny and hierarchical cell stage; (v) cell biology studies, knockdown strategies and in silico models have evidenced that the miR-302-367 gene is downstream of the Nanog, Oct3/4, Rex1 and Sox2 regulation network; (vi) cyclin D1 and Cdk4 have been recently found to be post-transcriptionally regulated by miR-302 in hESCs and, (vii) the ectopic expression of the complete human miR-302-367 cluster into several human cell lines proved sufficient to reprogram them into a pluripotent ES-cell-like state. These mirPS cells, gained the expression of the ES cell markers while displaying a highly demethylated genome with a wide gene expression pattern similar to that observed in hESCs. Together, we envision how the multiple miRNAs fit in the exciting ESC puzzle.

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