Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Circ Res. 2012;110:1162-1165
doi: 10.1161/RES.0b013e318257c489
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7330. Online ISSN: 1524-4571

Noncoding RNA Scaffolds in Pluripotency

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LincRNAs Act in the Circuitry Controlling Pluripotency and Differentiation
Guttman et al
Nature. 2011;477:295–300.

Human Long Non-Coding RNAs Promote Pluripotency and Neuronal Differentiation by Association With Chromatin Modifiers and Transcription Factors
Ng et al
EMBOJ. 2012;31:522–533.

The molecular circuitry that maintains pluripotency of mouse and human embryonic stem cells has been protein-centric. Two recent reports now add long noncoding RNAs as partners alongside the transcription factors in the maintenance of pluripotency.

Understanding of the biological pathways that maintain pluripotency is one of the most striking developments in modern biology and has the potential to revolutionize the field of regenerative medicine. In the recent past, these pathways have been successfully employed to turn the lineage-committed somatic cell into functional pluripotent stem cell. Until recently research in this area was restricted to protein factors whose role in different biological pathways have been relatively well characterized. Pervasive transcription across mammalian genomes generates thousands of long noncoding transcripts and have been shown to involve diverse biological functions that have impact on development and differentiation. These recent developments generated intense scientific interest in finding new RNAs and mechanisms that could have a potential role in maintaining pluripotency.

Previously, small and long RNAs, which are under the control of pluripotent transcription factors, have been implicated in the maintenance of embryonic stem cell (ESC) state and it has been shown that their downregulation promotes ESC differentiation. For example, it has been reported that miRNAs like mir-141, mir-200, and mir-145 regulate the

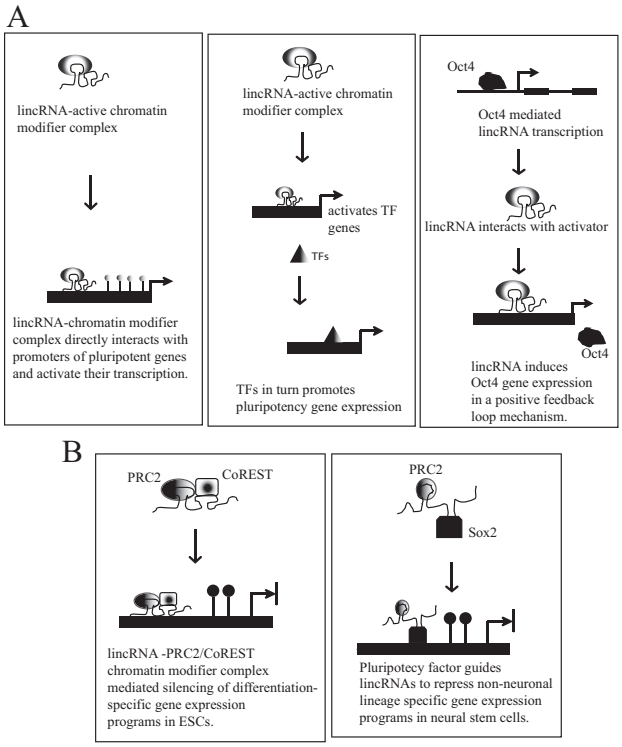

Figure. A, Models proposing the mode of action of pluripotent long noncoding RNAs. B, Long noncoding RNA mediated repression of differentiation-specific gene expression programs. **Left panel** shows long noncoding RNA-PRC2/CoREST complex mediate silencing of genes that promote differentiation in embryonic stem cells (ESCs). **Right panel** shows Sox2 guides long noncoding RNA-PRC2 complex to silence genes of non-neuronal lineages in neural stem cells. lincRNA indicates long intergenic noncoding RNA; TF, transcription factors.

ESC pluripotency program and that their expression is controlled by pluripotent transcription factors like c-Myc and Oct4.[1,2] Recent evidence also implicates long noncoding RNAs (lncRNAs), range in size from 200 nt to several hundred kb long in the ESC pluripotency.[3] Using custom-designed microarrays, a previous study identified a couple hundred lncRNAs, which are differentially expressed between ESCs and differentiated embryoid bodies. Some of these lncRNAs have been shown to interact with chromatin modifiers and regulate gene expression in cis by controlling local chromatin structure.[4] Like small noncoding RNAs, some lncRNAs are also under control of the pluripotency factors like Oct4 and Nanog, and interestingly, these lncRNAs seem to activate the transcription of pluripotent transcription factors in a regulatory positive feedback loop.[3] Though these reports point toward a functional role of lncRNA in maintaining pluripotency on a smaller scale, comprehensive investigations on the functional role of lncRNAs on a global perspective are lacking. Addressing this

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(*Circ Res*. 2012;110:1162-1165.)

***Circulation Research* is available at http://circres.ahajournals.org**
DOI: 10.1161/RES.0b013e318257c489

need, 2 recent investigations[5,6] provide in depth analyses of lncRNAs in the maintenance of pluripotency in ESCs using mouse and human as model systems.

Using mouse ESCs as a model system, Eric Lander and colleagues[5] performed an extensive and well-controlled investigation to explore the role of long intergenic noncoding RNA (lincRNA), identified using chromatin marks specific to active genes, in the complex molecular circuitry of ESCs. Previously, these authors have identified 226 lincRNAs that are differentially expressed in mouse ESCs. One hundred forty-seven of 226 lincRNAs were successfully downregulated using shRNA and their effects on global transcription were analyzed using genome-wide microarrays. The majority of the lincRNAs affect the global transcriptional profiles, and in each case the affected target genes range from 20 to 1000. The target genes are often located in trans, whereas only fewer candidates were noted affecting target gene expression in cis, indicating that the lncRNAs characterized using active chromatin maps have gene targets preferentially located in trans. Another interesting observation that Gutmann et al noted is more than 75% of 227 ES cell specific lincRNAs have binding sites for at least 1 of 9 of the well-characterized pluripotency associated transcription factors. This observation is consistent with previous data and also with a recent finding in the human ESCs (hESCs) that several lncRNA gene promoters are also enriched with binding sites for pluripotent transcription factors.[3,6] More importantly, knockdown of the pluripotency associated transcription factors using shRNA affected about 50% of the ES cell-specific lincRNAs. Collectively, these observations emphasize a strong and consistent link between pluripotency and lncRNAs.

Next, Guttman et al have addressed the functional role of lincRNAs in the pathways that maintain ESC state. The authors have characterized the lincRNA role in ESC state based on their effects on maintenance of pluripotency and repression of the lineage-specific gene expression programs in ESCs on their downregulation using shRNAs. To identify lincRNAs involved in the maintenance of pluripotency, the RNAs were downregulated in an ESC line expressing the luciferase gene under the endogenous *Nanog* promoter. By scoring the *Nanog* promoter activity in the loss of function experiments, they identified 26 lincRNAs with possible role in the maintenance of pluripotency. This indicates that lincRNAs could execute their functions via regulating key pluripotency fators like Nanog. This is not surprising considering the earlier observation that *Oct4* transcription can be modulated by lncRNA (AK141205) in a regulatory positive feedback loop.[3] Of note, this observation implicates lincRNAs operating upstream of the key pluripotency factors. Though the molecular mechanisms underlying the lincRNA mediated maintenance pluripotency are far from clear, the following models could explain the effects of lincRNA on pluripotency. (1) Association of the lincRNAs with active chromatin modifiers as documented by Guttman et al, and also by earlier studies,[4,7] indicate that lincRNAs may directly regulate pluripotency factors by recruiting the active chromatin modifiers to their gene promoters (Figure A, left panel). (2) lincRNAs activate transcription factors, which in turn regulate the expression of the pluripotency factors (Figure A, middle panel). (3) Alternatively, downregulation of *Nanog* promoter could also occur as a consequence of exit from the pluripotency state on downregulation of lincRNAs. In the latter scenario, the lincRNA could be maintaining nonredundant pluripotency pathways in parallel to that of protein factor controlled pluripotency pathways.

Repression of lineage-specific differentiation programs is another important requisite in the maintenance of ESC state. Several protein factors including polycomb proteins have been implicated in the repression of lineage-specific differentiation programs. To identify the lincRNAs that may have a role in the repression of genes responsible for differentiation programs, the authors compared the gene expression data sets from the shRNA-mediated knowndown of the lincRNAs with the gene expression patterns obtained after induced differentiation of mouse ESCs into different lineages. These comparisons yielded nearly 30 lincRNAs whose downregulation led to specific activation of gene expression programs that are critical for maintaining different lineages, suggesting that this subset of lincRNAs act as a repressor of the differentiation programs in mouse ESCs. However, their downregulation did not result in exit from the pluripotent state, indicating the existence of several redundant players in the repression of lineage-specific differentiation programs.

Guttman et al also addressed the lincRNA–protein interactions crucial for the maintenance of pluripotency and suppression of lineage-specific differentiation programs. They found that about 30% of 227 ESC-specific lincRNAs interact with the chromatin modifiers capable of writing, reading, and erasing the chromatin marks, indicating that lncRNAs like protein counterparts can program chromatin landscapes across the genome, and this is consistent with several lines of previous evidence on the role of noncoding RNA in chromatin organization.[8–10] In the shRNA knockdown experiments of lincRNA and their associated chromatin modifiers, more than 40% of 74 ESC-specific lincRNAs had overlapping gene expression programs with their associated chromatin modifiers, thus presenting a synergistic picture where lincRNA and protein partners act together on common gene targets.

In another interesting investigation, Stanton and colleagues[6] addressed the functional role of lncRNA in the maintenance of pluripotency and differentiation programs using hESCs as a model system. They used a customized microarray system containing 6671 transcripts with at least 6 to 8 probes covering each transcript. Of the 6671 lncRNAs, 36 lncRNAs showed hESC-specific differential expression on arrays. Further validation by qPCR identified 3 lncRNAs with exclusive expression to hESCs. All 3 lncRNA promoters are occupied with binding sites for pluripotency factors like Oct4 or Nanog and downregulation of these factors affected the expression of the lncRNAs, indicating that these lncRNA molecules could be direct targets of pluripotency transcription factors. However, downregulation of pluripotent transcription factors and upregulation of lineage-specific gene expression programs on downregulation of lncRNAs indicate that there is a tight interplay between lncRNA and pluripotency factors, where lncRNA and pluripotency factors regulate each other in a positive feedback loop mechanism (Figure

A, right panel). From the data, it is difficult to conclude whether lncRNAs act upstream or downstream of pluripotent transcription factors. Interestingly, pluripotency factors (Sox2) not only regulate the transcriptional activity of lncRNAs but also interact with lncRNAs, indicating that lncRNAs and pluripotency factors together with PRC2 complex members could be involved in lineage-specific repression programs.

The authors have also characterized lncRNA required for neuronal differentiation and identified about 35 lncRNAs that showed significant expression in neurons in comparison to hESCs and neuroprogenitor cells. Functional characterization of 4 lncRNAs revealed that their expression is crucial for differentiation to neurons from neural stem cells and this act of lncRNAs in neuronal differentiation also involves interactions with nuclear proteins like SUZ12 and REST. lncRNA's interaction with PRC2 in ESCs is required for maintaining pluripotency in ESCs through silencing lineage-specific differentiation genes (Figure B, left panel), whereas their interaction with PRC2 complex in neuronal stem cells is required to promote neuronal differentiation probably through repressing genes of nonneuronal lineages (Figure B, right panel). These contrasting observations highlight the cell type-specific plasticity of lncRNA functions with common protein interacting partners. We presume that this plasticity could be achieved in part through cell type-specific secondary structures of lncRNAs that could allosterically change the conformation of the interacting chromatin modifiers or transcription factors such that they could execute lineage-dependent gene expression programs.

Conclusions and Outlook

There have been several missing links in understanding how transcription factors alone can maintain ESC pluripotency and lineage-specific commitments of differentiating cells. Identification of genome-wide transcription encoding thousands of small noncoding RNAs and lncRNAs and uncovering of their role in differentiation and development has started providing more information on hitherto uncovered mechanisms involved in the maintenance of pluripotency and lineage commitment. Studies from Guttman et al and Ng et al along with previous studies have begun to provide insights into the intricate mechanisms that establish and maintain pluripotency. The common theme emerging from these studies is that there is a strong nexus between pluripotency factors and lncRNAs. Characterization of Oct4, Nanog, and Sox2 binding sites in the lncRNA promoter regions and the similarities in the lineage repression programs executed by lincRNAs and pluripotent transcription factors as indicated by Guttman et al suggest that lincRNAs take cues from pluripotency-specific transcription factors in maintaining pluripotency. On the other hand, their downregulation in lincRNA knockdown experiments suggest that lincRNA could act upstream of pluripotency factors. These studies have highlighted the intricate nature of the communication between pluripotent transcription factors and the so-called pluripotent lncRNAs and further studies in this direction would uncover the hidden communication between pluripotent transcription factors and pluripotent lncRNAs. It seems that pluripotency factors not only regulate the transcription of lncRNA promoters but also seem to interact with their encoded products. For example, interaction of the lncRNAs with both pluripotency factors like SOX2 and along with the chromatin repressor proteins like REST, indicate that Sox2 probably guide these lncRNAs to their target sites to mediate lineage-dependent repression function (Figure B, right panel). Given a wide reach of lncRNAs in the execution of various biological functions, one can envisage lncRNA regulating biological pathways independent of pluripotency factors. This contention is supported by the observation that knockdown of several lincRNAs responsible for maintenance of pluripotency resulted in downregulation of several pluripotent factors. Future research in this direction will help us in understanding whether lncRNAs carryout a primary role or a supporting role to pluripotency protein factors in the maintenance of pluripotency.

Deeper understanding of maintenance of pluripotency and various lineage-specific differentiation events hold a great promise for cell-based therapies in regenerative medicine. So far, no studies have been conducted to identify lncRNAs that are required for lineage-commitment of differentiating ES cells. Identification lncRNAs by Ng et al in neuronal differentiation is the first such investigation that could stimulate more studies in other differentiation events. Genome-wide association studies have identified a few hotspots encoding lncRNAs in cardiovascular diseases. An antisense RNA, *CDKN2B-AS1*, expressed from a gene desert region in 9p21 locus, is strongly associated with various cardiovascular diseases including coronary atherosclerosis.[11] Likewise, MIAT lncRNA on human chromosome 22 with pluripotency functions is linked to heart disease like myocardial infarction.[12] hESCs have been used as ex vivo source for cardiomyocytes for cell-based heart therapies. Given that the majority of lincRNAs interact with chromatin modifiers and their potential role in the maintenance of pluripotency and repression of lineage-specific gene expression programs, characterization of lincRNA involved in the cardiomyocyte differentiation would help significantly in improving cell-based therapies for cardiovascular disease.

Though the genome-wide association studies implicated several SNPs, mapping to gene desert and intergenic regions, in human diseases, only a few of them have been functionally characterized. With the availability of transcriptional landscapes across the human genome in normal and disease conditions, it is now possible to explore the functional link between disease-associated SNPs and lncRNA regulation and their significance to pathogenesis of various diseases, including cancer and cardiac diseases.

Sources of Funding

This work was supported by the grants from the Swedish Cancer Research foundation (Cancerfonden: Kontrakt no. 100422), Swedish Research Council (VR-M:K2011-66X-20781-04-3; VR-NT: 621-2011-4996), SciLife Lab Uppsala, and Barncancerfonden (PROJ11/067) to CK. CK is a Senior Research Fellow supported by VR-M.

Disclosures

None.

References

1. Xu N, Papagiannakopoulos T, Pan G, Thomson JA, Kosik KS. Microrna-145 regulates oct4, sox2, and klf4 and represses pluripotency in human embryonic stem cells. *Cell*. 2009;137:647–658.
2. Lin CH, Jackson AL, Guo J, Linsley PS, Eisenman RN. Myc-regulated microrna attenuate embryonic stem cell differentiation. *Embo J*. 2009; 28:3157–3170.
3. Sheik Mohamed J, Gaughwin PM, Lim B, Robson P, Lipovich L. Conserved long noncoding rnas transcriptionally regulated by oct4 and nanog modulate pluripotency in mouse embryonic stem cells. *RNA*. 2010;16: 324–337.
4. Dinger ME, Amaral PP, Mercer TR, Pang KC, Bruce SJ, Gardiner BB, Askarian-Amiri ME, Ru K, Solda G, Simons C, Sunkin SM, Crowe ML, Grimmond SM, Perkins AC, Mattick JS. Long noncoding rnas in mouse embryonic stem cell pluripotency and differentiation. *Genome Res*. 2008; 18:1433–1445.
5. Guttman M, Donaghey J, Carey BW, et al. Lincrnas act in the circuitry controlling pluripotency and differentiation. *Nature*. 2011;477:295–300.
6. Ng SY, Johnson R, Stanton LW. Human long non-coding rnas promote pluripotency and neuronal differentiation by association with chromatin modifiers and transcription factors. *The EMBO Journal*. 2012;31: 522–533.
7. Bertani S, Sauer S, Bolotin E, Sauer F. The noncoding RNA mistral activates hoxa6 and hoxa7 expression and stem cell differentiation by recruiting mll1 to chromatin. *Molecular Cell*. 2011;43:1040–1046.
8. Mondal T, Rasmussen M, Pandey GK, Isaksson A, Kanduri C. Characterization of the RNA content of chromatin. *Genome Res*. 2010;20: 899–907.
9. Pandey RR, Mondal T, Mohammad F, Enroth S, Redrup L, Komorowski J, Nagano T, Mancini-Dinardo D, Kanduri C. Kcnq1ot1 antisense noncoding rna mediates lineage-specific transcriptional silencing through chromatin-level regulation. *Mol Cell*. 2008;32:232–246.
10. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E, Chang HY. Functional demarcation of active and silent chromatin domains in human hox loci by noncoding rnas. *Cell*. 2007;129:1311–1323.
11. Harismendy O, Notani D, Song X, Rahim NG, Tanasa B, Heintzman N, Ren B, Fu XD, Topol EJ, Rosenfeld MG, and Frazer KA. 9p21 DNA variants associated with coronary artery disease impair interferon-gamma signalling response. *Nature*. 2011;470:264–268.
12. Ishii N, Ozaki K, Sato H, Mizuno H, Saito S, Takahashi A, Miyamoto Y, Ikegawa S, Kamatani N, Hori M, Nakamura Y, Tanaka T. Identification of a novel non-coding RNA, miat, that confers risk of myocardial infarction. *Journal of Human Genetics*. 2006;51:1087–1099.