Long non-coding RNAs and Autophagy in Embryonic stem cells

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Contents

- Embryonic Stem Cells (ESCs)
- Long non-coding RNA in ESCs and paper
- Autophagy in ESCs and paper
Embryonic Stem Cells (ESCs)
**Embryonic stem cells (ES cells)** are pluripotent stem cells derived from the inner cell mass of the blastocyst.
Two special properties

- Pluripotency
- Self-Renewal

Self-Renewal

Stem Cell → Differentiation → Mature Cell
The core regulatory network governing ESC pluripotency is based on the key transcription factors Oct4, Sox2, and Nanog.
Pathway in mESCs
mES Media

- DMEM
- LIF
- Nucleotide
- Foetal Bovine Serum
- L-lutamine
- Non-Essential Amino Acids
- beta-Mercaptoethanol

Feeder cell (MEF) or Gelatin
Pathway in hESCs
# hES Media

## hESC Culture Media:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM-F12 Media</td>
<td>200ml</td>
</tr>
<tr>
<td>Knockout Serum Replacer</td>
<td>50ml</td>
</tr>
<tr>
<td>Store frozen in 25ml aliquots. Thaw immediately before use.</td>
<td></td>
</tr>
<tr>
<td>200mM L-Glutamine + 2-Mercaptoethanol Solution</td>
<td>1.25ml</td>
</tr>
<tr>
<td>Non-essential Amino Acids 100X Solution</td>
<td>2.5ml</td>
</tr>
<tr>
<td>B-FGF Solution</td>
<td>0.5ml</td>
</tr>
</tbody>
</table>

Feeder cell (MEF) or Matrigel
Differentiation systems of ESCs

- **Mesoderm (Middle Layer)**
  - Cardiac Muscle
  - Skeletal Muscle Cells
  - Tubule Cell of the Kidney
  - Red Blood Cells
  - Smooth Muscle (In Gut)

- **Endoderm (Internal Layer)**
  - Lung Cell (Aveolar Cell)
  - Thyroid Cell
  - Pancreatic Cell

- **Ectoderm (External Layer)**
  - Skin Cells of Epidermis
  - Neuron Cell
  - Pigment Cell
The formation of EBs from ESC triggers spontaneous differentiation of all cell types, it is an inefficient method for the generation of specific cell types because the microenvironment within the EB is difficult to control.
Long Non-coding RNA in ESC
What is long non-coding RNA?

- In the human genome, only 1.2% of transcriptional gene products encode proteins.
# What is Long non-coding RNA?

The list of Non-coding RNA

<table>
<thead>
<tr>
<th>Type</th>
<th>Abbr.</th>
<th>Function</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfer RNA</td>
<td>tRNA</td>
<td>Translation</td>
<td>All organisms</td>
</tr>
<tr>
<td>Ribosomal RNA</td>
<td>rRNA</td>
<td>Translation</td>
<td>All organisms</td>
</tr>
<tr>
<td>Small nucleolar RNA</td>
<td>snoRNA</td>
<td>Nucleotide modification of RNAs</td>
<td>Eukaryotes and archaea</td>
</tr>
<tr>
<td>MicroRNA</td>
<td>miRNA</td>
<td>Gene regulation</td>
<td>Most eukaryotes</td>
</tr>
<tr>
<td>Piwi-interacting RNA</td>
<td>piRNA</td>
<td>Transposon defense</td>
<td>Most animal</td>
</tr>
<tr>
<td>Small interfering RNA</td>
<td>siRNA</td>
<td>Gene regulation</td>
<td>Most Eukaryotes</td>
</tr>
<tr>
<td>Long noncoding RNA</td>
<td>IncRN</td>
<td>Various</td>
<td>Eukaryotes</td>
</tr>
</tbody>
</table>
Long non-coding RNA (long ncRNAs, lncRNA) are generally considered as non-protein coding transcripts longer than 200 nucleotides. This limit is due to practical considerations including the separation of RNA in common experimental protocols.
Inc RNA is Transcriptional noise?

Transcriptional noise

Tissue-specific

Differences in different developmental stages

Variation
Types of IncRNA

(1) sense
(2) antisense
(3) bidirectional
(4) intronic
(5) intergenic
Archetypes of Inc RNA

I. Signal

II. Decoy

III. Guide

IV. Scaffold
The functions of IncRNA?

1. **Long ncRNAs in the regulation of gene transcription**
   - gene-specific transcription
   - regulating basal transcription machinery

2. **Long ncRNAs in post-transcriptional regulation**
   - splicing
   - translation

3. **Long ncRNAs in epigenetic regulation**
   - Genomic Imprinting
   - X-chromosome inactivation (Xist)
The functions of lncRNA?

Long noncoding RNAs in mammalian cells

Inc RNA in ESCs

1. Expression of several Inc RNAs correlates with the expression of pluripotency markers.

   Sox2 overlapping transcript (Sox2ot), a long noncoding RNA, is transcribed in the same orientation with Sox2 gene. Similarly to Sox2, Sox2ot is expressed in pluripotent ES cells and is initially down-regulated upon EB differentiation.

2. IncRNAs are regulated by the core regulation factors in ESCs.

More than 100 IncRNA promoters are bound by stem cell factors such as OCT4 and Nanog.

Sox2 and Oct4 were each sufficient to drive expression of IncRNA-Sox2 promoter, and the expression of both Oct4 and Sox2 together caused synergistic increases in expression.

2. **Inc RNAs are regulated by the core regulation factors in ESCs.**

- IncRNA-RoR, was shown to be directly targeted by the key pluripotency factors Oct4, Sox2, and Nanog through colocalization of the three factors close its promoter region.

3. Expression of several lincRNAs correlates with the differentiation events.

945 lincRNAs expressed during EB differentiation, of which 174 were differentially expressed correlating with pluripotency or specific differentiation events.

**Table 1. Summary of microarray expression results**

<table>
<thead>
<tr>
<th>Probe class</th>
<th>Total</th>
<th>Expressed above background</th>
<th>Differentially expressed&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coding</td>
<td>14,827</td>
<td>8,625 (58%)</td>
<td>2,103 (24%)</td>
</tr>
<tr>
<td>Noncoding</td>
<td>3,659</td>
<td>945 (26%)</td>
<td>174 (18%)</td>
</tr>
<tr>
<td>Combined</td>
<td>18,486</td>
<td>9,570 (52%)</td>
<td>2,277 (24%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Significant differential expression was defined as probes with B-statistics > 3 and fold-change > 2.

Genome Res. 2008 18: 1433-1445
3. Expression of several lnc RNAs correlates with the differentiation events.

Genome Res. 2008 18: 1433-1445
4. Epigenetic regulation during ES cell differentiation.

Using chromatin immunoprecipitation, we provide evidence that both lncRNAs are associated with trimethylated H3K4 histones and histone methyltransferase MLL1.

Molecular Cell 2011, 43, 1040-1046
5. Assemble Paraspeckle

hNEAT1, long non-coding RNA, is not expressed in hESCs, but is induced upon differentiation.
What do we know about lncRNA in ESCs, now?

1. Expression in the different stage during differentiation.
2. lncRNAs are coordinately transcribed with their associated protein-coding transcripts.
Conserved long noncoding RNAs transcriptionally regulated by Oct4 and Nanog modulate pluripotency in mouse embryonic stem cells

Jameelah Sheik Mohamed, Philip Michael Gaughwin, Bing Lim, et al.

RNA 2010 16: 324-337 originally published online December 21, 2009
Access the most recent version at doi:10.1261/rna.1441510
Embryonic stem cell

Oct4 and Nanog are part of a core transcriptional regulatory network that is required for mESC gene expression regulation.

This paper focuses area in mESC genomics concerns Oct4- and Nanog-regulated RNA transcripts that do not encode protein but may modulate mESC pluripotency or differentiation at the RNA level.
Results

Identification of putative Oct4- and Nanog-targeted conserved IncRNAs

- We searched for candidate IncRNA genes throughout the entire catalog of genomic proximal target genes. Proximal targets were defined as genes that, based on their genomic position, mapped in close proximity to Oct4 and Nanog high-confidence binding sites.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>AK005651</th>
<th>AK028326</th>
<th>AK043754</th>
<th>AK141205</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcript length (bp)</td>
<td>565</td>
<td>3056</td>
<td>1731</td>
<td>2710</td>
</tr>
<tr>
<td>ChIP-PET (Loh et al. 2006)</td>
<td>Oct4</td>
<td>Oct4</td>
<td>Nanog</td>
<td>Nanog</td>
</tr>
<tr>
<td>Expression profile</td>
<td>Brain, testis</td>
<td>Brain, nerve, eye, embryonic tissue, inner ear, pituitary gland, skin</td>
<td>Brain, nerve, bone marrow</td>
<td>Brain, lung, embryonic tissue, intestine, muscle</td>
</tr>
</tbody>
</table>
LncRNA **AK028326** has been described as retinal noncoding RNA 2 (RNCR2), strongly expressed in the developing retina and widely expressed in central nervous system neurons.


Results

- Oct4 and Nanog IncRNA targets are differentially expressed during mESC differentiation.

Retinoic acid (RA) treatment simultaneously induces mESC differentiation and impacts IncRNA transcription.
Results

- Oct4 and Nanog RNAi modulates Inc RNA transcription

Differential expression of Inc RNA upon robust Oct4 and Nanog RNAi knockdown.
Results

- Directed knockdown of the IncRNA AK028326 promotes loss of pluripotency and co-activates Oct4.
Results:

mesodermal marker
down-regulation of meso- and endodermal markers

trophectoderm markers

AK028326 RNAi

AK141205 RNAi
Directed knockdown of the IncRNAs AK028326 and AK141205 affects cell proliferation and morphology.
Overexpression of the lncRNA gene AK028326 and AK141205 promotes mESC lineage-specific differentiation.

Overexpressed AK141205 RNA levels saw significant elevations of endogenous levels of Oct4 mRNA, whereas Nanog mRNA levels were not significantly perturbed.
Results

Overexpression of the IncRNA gene AK028326 and AK141205 promotes mESC lineage-specific differentiation

AK028326-OE increased transcription of the ectodermal markers Pax6, Pax7, and Sox11.
AK141205-OE enhanced meso- and ectodermal differentiation.
Results

Overexpression of the IncRNA gene AK028326 and AK141205 promotes mESC lineage-specific differentiation

Both AK028326-OE mESCs and AK141205-OE exhibited flattened, smaller colonies
Discussion

- RNAi and overexpression of even a partial fragment of IncRNA AK028326 are sufficient to promote mESC differentiation under self-renewing conditions, and to promote meso- and ectodermal gene transcription, respectively.
AK141205, a novel and potentially Nanog-repressed IncRNA, positively regulates Oct4.

LncRNAs may prove to be important intermediaries in the Oct4/Sox2/Nanog network.

Our findings should help elucidate mechanisms by which IncRNAs can modulate pluripotency.
Discussion

A model for AK028326 and AK141205 IncRNA placement into the Oct4 and Nanog regulatory network.
Autophagy in ESCs
What is autophagy?

Autophagy, is a catabolic process involving the degradation of a cell's own components through the lysosomal machinery.
Types of Autophagy
In mammals, fertilization induces **massive autophagy**, which plays an essential role in early embryogenesis. Nutrient availability may be limited in embryos until implantation, and autophagy functions as a major nutrient-providing system during this period.
The role of autophagy in development and differentiation in mammals.
# Table 2 Phenotypes of systemic knockout mice of ATG-related genes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atg3&lt;sup&gt;−/−&lt;/sup&gt;, Atg5&lt;sup&gt;−/−&lt;/sup&gt;, Atg7&lt;sup&gt;−/−&lt;/sup&gt;, Atg9&lt;sup&gt;−/−&lt;/sup&gt;, Atg16L1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Neonatal lethal with reduced amino acid levels, suckling defect (Atg9 has an additional role in innate immune responses induced by double-stranded DNA)</td>
</tr>
<tr>
<td>beclin 1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Early embryonic lethal (E7.5 or earlier) with defects in proamniotic canal closure (heterozygous mice show increased susceptibility to spontaneous tumours)</td>
</tr>
<tr>
<td>FIP200&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Embryonic lethal (E13.5–E16.5) due to defective heart and liver development</td>
</tr>
<tr>
<td>Ambra1st&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Embryonic lethal (~E14) with defects in neural tube development, and hyperproliferation of neural tissues</td>
</tr>
<tr>
<td>ULK1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Increased reticulocyte number with delayed mitochondrial clearance</td>
</tr>
<tr>
<td>Atg4C&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Viable, fertile, increased susceptibility to carcinogen-induced fibrosarcoma</td>
</tr>
<tr>
<td>LC3B&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Normal phenotype</td>
</tr>
<tr>
<td>GABARAP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Normal phenotype</td>
</tr>
</tbody>
</table>
Beclin 1 in development

- No homozygous mutant offspring were born from intercross of beclin 1-/- mice in 100 pups genotyped at weaning by Southern blot or PCR analysis, indicating that beclin 1 homozygous mice die during embryonic development.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Genotype</th>
<th>+/-</th>
<th>+/-</th>
<th>--/--</th>
</tr>
</thead>
<tbody>
<tr>
<td>At weaning</td>
<td>+/-</td>
<td>35</td>
<td>79</td>
<td>0</td>
</tr>
<tr>
<td>Embryo, E8.5</td>
<td>+/-</td>
<td>6</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Embryo, E7.5</td>
<td>+/-</td>
<td>7</td>
<td>14</td>
<td>5</td>
</tr>
</tbody>
</table>

Increase of cancer rate in beclin 1/- mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total</th>
<th>Normal</th>
<th>Tumor</th>
<th>% of Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/-</td>
<td>21</td>
<td>18</td>
<td>3</td>
<td>14.2%</td>
</tr>
<tr>
<td>+/-</td>
<td>27</td>
<td>11</td>
<td>16</td>
<td>59.2%</td>
</tr>
</tbody>
</table>

P=0.001
Beclin 1 in ESCs

The failure of beclin 1-/- ES culture to form expanded cystic EBs is presumably due to a reduction of cell death or cell clearance at the core of the EB.

It was an accumulation of apoptotic cells in 3 to 6 day old Beclin1 null murine embryoid bodies.
Murine ES cells (mESC) were found to display an increase in LC3I to LC3II conversion, indicative of autophagy, 3 to 6 days after differentiation as embryoid bodies.

J Cell Biol. 2001 Feb 19;152(4):657-68
Autophagy in Human Embryonic Stem Cells

Introduction

- Autophagy have essential role in preimplantation development of mouse embryos and cavitation of embryoid bodies.
- Little is known about the role of autophagy in early human development.
- Human embryonic stem cells (hESC) offer a unique window on the earliest differentiation events in early human development.
Introduction

Human Embryonic Stem Cell lines stably expressing GFP-LC3

HES3-GFP-LC3  HES4-GFP-LC3  pluripotency marker TG30

bright fluorescent puncta  autophagosomes  lysosomal membrane marker LAMP-1
Introduction

Oct4

TRA-181

DAPI

TRA-160

TG 30

Control cell
Six weeks after injection of HES3-GFP-LC3 into SCID mice teratomas were formed that showed evidence of differentiation into cell types representative of the three germ layers, including cartilage and muscle (mesoderm), glandular epithelium (endoderm) and keratinocytes, neuroepithelium and primitive neuronal cells (ectoderm).
Higher magnification autophagosomes could be clearly observed.
Regulation of autophagy in HES3-GFP-LC3

![Regulation of autophagy in HES3-GFP-LC3](image)

(200 nM)
Cells were maintained in either conditioned medium (A, C) or unconditioned medium (B, D).
Cells maintained in CM supplemented with SB431542 (10 mM) were imaged after 3 hours incubation.

Fluorescent puncta were counted in cells for three independent experiments after 2 hours and 7 days of incubation in the presence of SB431542.
To our knowledge an increase in autophagy in human embryonic stem cells during these earliest steps of differentiation has thus far not been reported.

Spontaneous and induced differentiation promotes autophagy.

This paper speculate that autophagy may play an upstream regulatory role in hESC differentiation through degradation of pluripotency regulating protein complexes.
It is largely unknown about the function of autophagy during ESC differentiation.

1. Autophagy and direction of differentiation

2. Autophagy and pluripotency

3. Which type autophagy in differentiation
Autophagy and IncRNA in ESCs
Thank you for your attention!