Lamin A/C is expressed in pluripotent mouse embryonic stem cells

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Introduction

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass of the pre-implantation blastocyst, which are unique in their ability to self-renew indefinitely as well as differentiate into essentially all cell types of the adult organism. ESC pluripotency is accompanied by a unique dynamic chromatin organization and nuclear morphology (reviewed in refs. 1-4). The irregular nuclear shape of ESCs contains many invaginations and protrusions which are highly malleable, and the inter-membrane space of the nuclear envelope is wider and unevenly spaced compared with differentiated cells. Upon differentiation, this irregular nuclear shape transitions to a smooth and rigid form. Differentiation is further paralleled by changes in the association of blocks of chromatin with the nuclear periphery, including pluripotency genes such as Nanog, suggesting a tight connection between nuclear organization and the differentiation program. Globally, the chromatin in ESCs is dispersed, loosely packed, with large, poorly defined heterochromatin domains and an increased mobility of heterochromatin proteins, such as HP1. The chromatin is plastic and hyperdynamic, compared with large, poorly defined heterochromatin domains and an increased mobility of heterochromatin proteins, such as HP1. The chromatin is plastic and hyperdynamic, compared with differentiated cell types. This openness of the chromatin is correlated with high DNase I accessibility, and widespread transcription of both coding regions as well as repetitive elements of the genome. Furthermore, ESCs contain extensive regions with a bivalent chromatin signature in which the transcription-associated H3K4me3 histone modification co-exists with the polycomb-group repressive H3K27me3 modification. These “poised” domains are largely enriched over lineage-specific genes and become resolved to either an active (H3K4me3) or inactive (H3K27me3) state during differentiation. The resolution of bivalent domains to an inactive state correlates with an increase in DNA methylation at these promoters. The interconnection between the unique chromatin features and distinctive nuclear structure is thought to have an essential role in ESC pluripotency. The major structural component of the nucleus is the nuclear lamina, which forms directly below the inner nuclear membrane. The nuclear lamina is composed predominantly of a meshwork of type V intermediate filament proteins called lamins, which interact with chromatin, nuclear pore complexes and lamin-associated proteins, and have important functions in gene regulation and chromatin organization. The nuclear lamins can be subdivided into A- and B-types based on sequence homology and structural features. There are two major B-type lamins in most vertebrates, lamin B1 and lamin B2, which are expressed throughout development and are required for organogenesis and proper neural development. The A-type lamins are encoded by a single gene which is alternatively spliced yielding two isoforms, lamin A and lamin C, which differ in length transcripts of both isoforms were readily detected by q-PCR and deep RNA sequencing. Additionally, protein expression was validated in multiple primary and established ESC lines by immunoblotting using several independent antibodies. Immunofluorescence labeling showed localization of lamin A/C at the nuclear periphery of all Oct4/Nanog double-positive ESC lines examined, as well as in the inner cell mass of blastocysts. Our results demonstrate ESCs do express low levels of lamin A/C, thus models linking pluripotency and nuclear dynamics with the absence of lamin A/C need to be revisited.

Keywords: embryonic stem cell, nucleus, lamin A/C, lamina

Abbreviations: ESC, embryonic stem cell; Lmna, lamin A; MEF, mouse embryonic fibroblast; NPC, neural progenitor cell; LIF, leukemia inhibitory factor

The pluripotent nature of embryonic stem cells (ESC) is associated with a dynamic open chromatin state and an irregular nuclear shape. It has been postulated that the absence of lamin A/C contributes to these features. However, we show that mouse ESCs express low, yet readily detectable, amounts of lamin A/C at both the RNA and protein levels. Fully-length transcripts of both isoforms were readily detected by q-PCR and deep RNA sequencing. Additionally, protein expression was validated in multiple primary and established ESC lines by immunoblotting using several independent antibodies. Immunofluorescence labeling showed localization of lamin A/C at the nuclear periphery of all Oct4/Nanog double-positive ESC lines examined, as well as in the inner cell mass of blastocysts. Our results demonstrate ESCs do express low levels of lamin A/C, thus models linking pluripotency and nuclear dynamics with the absence of lamin A/C need to be revisited.

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used to define active gene transcription (Fig. 1B). Together, these data demonstrate that the *Lmna* gene is actively transcribed to yield full-length mRNA in ESCs.

To confirm that lamin A/C mRNA transcripts are being translated into protein, we performed immunoblotting experiments using a series of well characterized antibodies recognizing specifically either lamin A/C 28 or lamin A alone. All antibodies examined showed a clear signal in AB2.2 ESCs (Fig. 2A).

Both monoclonal and polyclonal lamin A/C antibodies showed a doublet band, which corresponds to the two protein isoforms, whereas the lamin A antibody specifically detected the larger lamin A isoform. Importantly, no signal was seen in an identically prepared lamin knockout (*Lmna* -/-) ESC line, confirming antibody specificity and the purity of the ESCs from MEF feeder cells (Fig. 2A, lane 2). Although this knockout ESC line has been reported to expresses a truncated Lmna protein, the epitopes recognized by the antibodies are not contained within this expressed portion. Both AB2.2 and *Lmna* -/- ESCs expressed the pluripotency marker Oct4 (Fig. 2A). Lamin A/C protein was also detected in 3 early-passage ESC lines, 4 additional well-established ESC lines and an iPS cell line (Fig. 2B), confirming that lamin A/C expression is not limited to the AB2.2 ESC line, nor an adaptation of long-term culturing, and is therefore a general feature of pluripotency. Furthermore, examination of previously published proteomics screens of ESCs revealed lamin A/C peptides. Combined, these results clearly demonstrate that lamin A/C is expressed at both the RNA and protein levels in multiple primary and well-established ESC lines.

Lamin A/C is correctly localized to the nuclear periphery in all cells within an ESC colony. We next performed immunofluorescence to determine expression patterns throughout during embryogenesis. This purported absence of lamin A/C in ESCs has served as a landmark for models and hypotheses explaining pluripotency, chromatin dynamics and ESC nuclear plasticity.

We sought to systematically re-investigate whether ESCs express lamin A/C. Interestingly, we show that lamin A/C is expressed in ESCs at both the RNA and protein levels, although at lower levels than in differentiated mouse embryonic fibroblasts (MEFs). Furthermore we show that lamin A/C is expressed in the inner cell mass of early blastocysts, from which ESCs are derived. Our results show conclusively that ESCs express lamin A/C and that its absence is not a marker of the undifferentiated pluripotent state.

**Results and Discussion**

Lamin A/C is expressed in embryonic stem cells. In order to examine lamin expression levels, we performed real-time quantitative PCR on RNA derived from ESCs, neural progenitor cells (NPCs) and mouse embryonic fibroblasts (MEFs) (Fig. 1A). ESCs were separated from the MEF feeder cells (see materials and methods) and, as expected, expressed the pluripotency markers Oct4 and Nanog (Fig. 1A). Importantly, we also detected lamin A and lamin C isoforms in ESCs at a similar level to NPCs, yet at a lower level than MEFs (Fig. 1A). The Lmna promoter is marked by the active-transcription associated histone H3 lysine 4 trimethylation (H3K4me3) mark supporting gene transcription. Examination of whole genome polyA+ RNA-sequencing data (J.H.B., M.A.E-M., D.L.S., unpublished data), as well as published data sets from mouse 27 and human ESCs, 27 confirmed full length lamin A/C mRNA was generated above thresholds to define active gene transcription (Fig. 1B). Together, these data demonstrate that the Lmna gene is actively transcribed to yield full-length mRNA in ESCs.

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individual cells in ESC colonies cultured either with MEF feeder cells in medium supplemented with 10% serum and leukemia inhibitory factor (LIF), or in serum-free, feeder-free 3i medium which maintains ESCs in a more uniform basal state of pluripotency. To ensure that the ESCs remained pluripotent, the ESCs grown on MEF feeders were not separated from the MEFs as above, but fixed on MEF feeder cells, which also serve as an internal control for immunofluorescence labeling. ESCs can be easily distinguished morphologically from MEFs as they grow in tight colonies, have a high nuclear to cytoplasmic ratio and have smaller and irregularly shaped nuclei (see for example Fig. 2C, fourth panel). Lamin A/C immunofluorescence demonstrated correct localization to the nuclear periphery in both LIF (Fig. 2C, top row) and 3i medium (Fig. 2C bottom row). Importantly, all cells within the colony displayed lamin A/C labeling, including the cells in the center of the colonies, confirming

Figure 2. Lamin A/C is expressed in mouse ESCs and localizes to the nuclear periphery of Oct4 positive ESCs. (A) Lamin A/C protein expression is detected in AB2.2 ESCs by immunoblotting using three independent antibodies. Monoclonal (first row) and polyclonal (second row) lamin A/C antibodies detect lamin A (70 kDa) and lamin C (55 kDa) isoforms. Lamin A/C knockout ESCs confirm purity of ESC protein preparation and immunoblotting specificity. All cell types express lamin B1. Oct4 is used to verify pluripotency and histone H3 is used as a loading control. (B) Extension of the immunoblotting analysis as above, in 9 independent ESC lines including AB2.2 (lane 1), three primary ESC lines (WB6.1, CH5, MK6, lanes 2–4 respectively), established ESC lines (R1, ZBTB4, v6.5, BlotCast, lanes 5–8 respectively) and an IPS cell line (lane 9). (C) Immunofluorescence was performed on ESCs grown on irradiated MEF feeder cells (top row) or in serum-free, feeder-free 3i medium (bottom row) for lamin A/C (first column, green), nanog (second column, red) and Oct3/4 (third column, orange) and counterstained with DAPI (fourth column, blue). Representative images showing a colony of AB2.2 ESCs, which label for the pluripotency markers Nanog and Oct4 and have lamin A/C localization at the nuclear periphery (arrows). Arrowheads indicate the top of MEF nuclei. Images were taken on a Zeiss LSM710 confocal microscope. A single z-section through the center of the ESC colony is shown. Scale bar represents 10 μm.
that lamin A/C expression is not limited to the peripheral cells, which may occur in some but not all partially differentiated cells. The lamin A/C positive ESCs co-expressed Oct4 and Nanog confirming their pluripotency (Fig. 2C, arrows). ESCs grown in 3i medium show uniform Nanog expression throughout all cells in the colony, consistent with this culture condition maintaining the cells in a ground-state of pluripotency. However, as a control, Lmna−/− ESCs did not show any lamin A/C labeling (Fig. 3, fifth row). Notably, ESCs have lower levels of lamin A/C when compared with MEFs. Therefore why previous reports have failed to detect lamin A/C in ESCs,15 as ESC staining is very faint and could be mistaken for background staining when optimal exposures for MEF nuclei are used. However when compared with the negative control staining, in which the primary antibodies were omitted (Fig. 3, last row), it is clear that the lamin A/C signal observed is a bona fide localization signal. The localization of lamin A/C to the nuclear periphery of all cells in the ESC colony was further confirmed in other established ESC lines (Fig. 3). Immunofluorescence using a different antibody specifically against lamin A also showed clear signal at the nuclear periphery in all cells in the colony in 5 separate ESC lines tested (Fig. 4). Our results convincingly show that lamin A/C is correctly localized to the nuclear periphery in all pluripotent ESCs examined. Therefore, absence of lamin A/C should not be used as a marker of the undifferentiated state.

Lamin A/C is expressed in blastocysts. As lamin A/C is expressed in embryonic stem cells, we tested whether we could detect lamin A/C in the developing blastocyst. Examination of published single cell RNA sequencing data sets of 2-cell, 4-cell, 8-cell and inner cell mass of blastocysts, in addition to ESCs, revealed Lmna transcript was present at all developmental stages, above the significant expression threshold cut-offs used.36 We isolated fresh blastocysts at E3.5 and performed immunofluorescence labeling for lamin A/C protein. We clearly detected lamin A/C protein at the nuclear periphery of both Nanog positive and Nanog negative cells in the developing blastocyst (Fig. 5). The Nanog positive cells represent the inner cell mass of the blastocyst from which ESCs are derived, demonstrating that the expression of lamin A/C is not acquired upon ESC derivation, nor is it a cell-culture phenomenon.

Conclusion

In summary, we demonstrate that the dynamic nuclear structure of ESCs is not due to an absence of lamin A/C. Our results show that lamin A/C is in fact expressed at both the RNA and protein levels in mouse ESCs as well as in the inner cell mass of the developing blastocyst. Additionally, immunofluorescence labeling clearly shows lamin A/C expression in pluripotent Oct4/Nanog double positive ESC colonies, eliminating any concerns that the lamin A/C detected by other assays is due to contamination of partially differentiated cells or MEF feeder cells.

A recent report has implicated lamin A/C in ESC chromatin mobility,14 however the effects seen were very moderate and limited to heterochromatin, with euchromatin remaining unchanged. Furthermore, overexpression of lamin A/C did not result in any alterations in pluripotency gene expression nor ESC colony morphology,15 consistent with our observations that lamin A/C does not contribute to the link between nuclear structure and pluripotency. It remains to be determined whether the lowered levels of lamin A/C compared with more differentiated cell types have a role in regulating the unique fluid chromatin and pluripotency of ESCs.

Importantly, our results provide insight into the absence of a phenotype in lamin B1/B2 knockout ESCs,17 as the presence of a low level of lamin A/C would be sufficient to maintain cell viability and pluripotency. However, we do not rule out that total levels and, in particular, the ratio between the different lamin proteins, may play an important developmental role in either cell differentiation or cell fate decisions. The strong developmental defects seen in mice upon removal of either both lamin B1 and B2,17 or lamin A/C,20 clearly demonstrate the importance of lamin proteins in coupling nuclear architecture to the gene expression program.

It remains to be determined how the highly irregular nuclear structure of ESCs is maintained in the presence of low levels of lamin A/C, and how this relates to the pluripotent nature of the cells. While the lower levels of lamin A/C in ESCs may contribute to their pluripotency, other nuclear envelope-associated factors may also be involved, and many are differentially expressed between ESCs and differentiated cells (Bergmann JH, Eckerley- Maslin MA and Spector DL, unpublished data). In particular, Syn-1, a lamin-associated protein, has been implicated in regulating the changes in nuclear envelope spacing that accompany ESC differentiation.4 Additionally, ESC differentiation can be inhibited by preventing expression of the nuclear pore protein Nup210.31 How these, in addition to other protein(s) or factors, contribute to the dynamic nuclear structure of ESCs and their pluripotency will be an exciting area of research not only for the fields of chromatin, nuclear organization and stem cell biology, but also for cellular reprogramming and its clinical applications.

Materials and Methods

Cell culture and blastocyst collection. ESCs were cultured using standard protocols in medium containing 1,000 U/ml Leukemia Inhibitory Factor (Millipore) with irradiated MEF feeders (GlobStem) on gelatin-coated plates. ESCs cultured in iSTEM 3i media (Stem Cells Inc.) were passaged at least six times in serum-free, feeder-free conditions on gelatin-coated plates before being analyzed. For immunofluorescence experiments, cells were grown either on gelatinized glass coverslips pre-seeded with MEF feeders for LIF experiments, or gelatinized glass coverslips alone for 3i experiments. For RNA and protein isolation, ESCs were soaked twice for one hour on gelatin-coated plates to remove MEFs, then immediately processed. AB2.2 (129/SvEvBrd-Hprtb-m2) ESC line was kindly provided by A. Mills, CSHL; R1 (129X1 × 129S1) ESC and v6.5 (C57/B6 × 129Sv) by S. Kim, CSHL; C57/B6 × Castaneus ESCs by C. Vakoc, CSHL; ZHBTc4 ESCs by A. Smith, Centre for Stem Cell Research, University of Cambridge;26 Imma17 ESCs by C. Stewart, Institute of Molecular Biology, A*STAR;26 mGFP2 iPSCs by R. Jaenisch, Whitehead Institute.
Figure 3. Lamin A/C localizes to the nuclear periphery in Oct4 positive ESCs. Immunofluorescence against Oct4 and lamin A/C was performed in R1 (top), v6.5 (second row), ZBTB44 (third row) and C57BL6/Castevous hybrid (fourth row) ESCs. The ESC colonies stain positively for Oct4 and lamin A/C. Lmna-/- knockout ESCs (fifth row) stain positively for Oct4 but not lamin A/C which is present in MEFs (arrow), confirming antibody specificity. The bottom row shows a control immunofluorescence in ABL2 ESCs in which primary antibodies were omitted. Scale bar represents 10 μm. Images were taken on a Zeiss LSM710 confocal microscope. A single z-section through the center of the ESC colony is shown. Scale bars represent 10 μm.
derived from AB2.2 ESCs using a protocol adapted from reference 38. E3.5 blastocysts from normal female mice were kindly isolated and provided by S. Kim, CSHL and P. Jiang, CSHL. Blastocysts were fixed in 4% formaldehyde made fresh from paraformaldehyde and processed for immunofluorescence within two hours of collection.

RNA-sequencing. Ten micrograms of total RNA was isolated using TriZol reagent (Ambion). PolyA+ RNA was isolated (Oligotex kit, Qiagen) and depleted of rRNA (ribominus kit). Stranded libraries were prepared using a protocol adapted from reference 39 for paired-end sequencing on the Illumina GA IIx platform. Reads were mapped to the mouse mm9 reference genome using the TopHat spliced-read aligner and coverage computed using BedGraph2 (bedtools suite). Coverage tracks were uploaded to and visualized in the UCSC genome browser in BigWig format.

Real-time RT PCR. Total RNA was isolated using TriZol reagent (Ambion), treated with amplification grade RNase-free DNase I (Invitrogen) and converted to cDNA using random hexamer primers (Applied Biosystems RT reagents) using manufacturer’s protocols. Quantitative real-time PCR was performed using SYBR green reagents (Applied Biosystems). Primer sequences are available upon request. Error bars represent standard deviation from three biological replicates normalized to the geometric mean of β-actin, CycloB1 and Pabpc1.

Antibodies, western blotting and immunofluorescence. Rabbit anti-lamin A (323–11, 291:2,000) and rabbit-anti-lamin A/C (266, 28:1:2,000) were kindly provided by R. Goldman, Northwestern University, Feinberg School of Medicine; mouse anti-lamin A/C (Active Motif 39287, 1:1,000); rabbit anti-laminB1 (abcam ab16048, 1:2,000); rabbit anti-Oct4 (Santa-Cruz sc9081, 1:2,000); rabbit anti-histone H3 (abcam ab1791, 1:10,000) were used. Western Blots were performed using anti-mouse or anti-rabbit secondary antibodies conjugated to HRP (1:10,000) and detected by ECL (Perkin-Elmer). For immunofluorescence, coverslips were fixed in 4% PFA for one hour in 3% BSA. Blastocysts were fixed in 4% PFA for 30 min at room-temperature, permeabilised in 0.25% Triton X-100 for 15 min at room-temperature, then blocked in 10% FBS with in 0.5% Triton X-100 for 6 degrees for five minutes and blocked for one hour in 3% BSA. Blastocysts were fixed in 4% PFA for 20 min at room-temperature, permeabilised in 0.25% Triton X-100 for 15 min at room-temperature; then blocked in 10% FBS with
0.1% Triton X-100 for 1–3 h at room-temperature. Cells were incubated with primary antibodies containing 3% BSA for either one hour at room temperature (Ox Eck 1:400, lamin B 1:4000) or overnight at four degrees (lamin A 1:200, lamin A/C 1:200).

All primary antibody incubations for blastocysts were performed overnight. Overnight incubations are necessary to ensure efficient antibody accessibility into the tightly packed ESC colonies. Anti-mouse, anti-rabbit and anti-rat secondary antibodies conjugated to Alexa-488, Alexa-594 or Alexa-647 (Invitrogen) were used, and DAPI was used to counterstain DNA.

Immunofluorescence imaging of single sections was performed using a Zeiss LSM710 laser scanning confocal microscope using a 63 × 1.4N.A. oil-immersion objective using 405 nm, 488 nm, 594 nm and 647 nm lasers. Slides were mounted in antifade containing 10% glycerol and 1 mg/ml p-Phenylendiamine (Sigma). Images represent single 0.1 μm sections through the center of ESC colonies or individual blastocysts. No post-acquisition image processing was performed. Figures show representative single z-sections.

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Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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