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Changing Nuclear Landscape and Unique PML Structures During Early Epigenetic Transitions of Human Embryonic Stem Cells

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Abstract

The complex nuclear structure of somatic cells is important to epigenomic regulation, yet little is known about nuclear organization of human embryonic stem cells (hESC). Here we surveyed several nuclear structures in pluripotent and transitioning hESC. Observations of centromeres, telomeres, SC35 speckles, Cajal Bodies, lamin A/C and emerin, nuclear shape and size demonstrate a very different "nuclear landscape" in hESC. This landscape is remodeled during a brief transitional window, concomitant with or just prior to differentiation onset. Notably, hESC initially contain abundant signal for spliceosome assembly factor, SC35, but lack discrete SC35 domains; these form as cells begin to specialize, likely reflecting cell-type specific genomic organization. Concomitantly, nuclear size increases and shape changes as lamin A/C and emerin incorporate into the lamina. During this brief window, hESC exhibit dramatically different PML-defined structures, which in somatic cells are linked to gene regulation and cancer. Unlike the numerous, spherical somatic PML bodies, hES cells often display ~1–3 large PML structures of two morphological types: long linear "rods" or elaborate "rosettes", which lack substantial SUMO-1, Daxx, and Sp100.These occur primarily between Day 0–2 of differentiation and become rare thereafter. PML rods may be "taut" between other structures, such as centromeres, but clearly show some relationship with the lamina, where PML often abuts or fills a "gap" in early lamin A/C staining. Findings demonstrate that pluripotent hES cells have a markedly different overall nuclear architecture, remodeling of which is linked to early epigenomic programming and involves formation of unique PML-defined structures.

Keywords

human embryonic stem cells; PML; nuclear structure; lamina; SC35 domains; Speckles; Differentiation; pluripotency; nuclear shape

Although human embryonic stem cells (hESCs) have become an intense topic of biomedical research, a great deal about their basic biology is unknown. Embryonic stem cells are derived from the inner cell mass of blastocyst stage embryos and have two essential characteristics: an apparently unlimited ability to self renew and the capacity to differentiate

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into any cell in the adult body. While this developmental plasticity promises enormous clinical potential for cell replacement therapies and drug screening, the epigenetic properties and molecular mechanisms that confer these very special properties are poorly understood.

Nuclear structure and organization is increasingly recognized as a fundamental parameter of gene regulation. Numerous distinct nuclear compartments and bodies have been identified and studied in somatic cells, but much less is known about these structures in embryonic stem cells. Although the radial organization of chromosome territories does not appear to change substantially during differentiation of hESCs (Bartova et al., 2008a; Wiblin et al., 2005), greater chromatin factor mobility and genome-wide expression is seen in mouse ESC nuclei, suggesting a less fixed and more "plastic" nuclear structure (Meshorer et al., 2006b). A few observations in human ESC suggest some limited differences from somatic chromosome organization (Bartova et al., 2008b; Wiblin et al., 2005), however, the investigation of nuclear structure in relation to early embryonic programming has barely begun. Most aspects of somatic nuclear compartmentalization have yet to be examined in ES cells, particularly human ES cells.

We have examined several known somatic nuclear structures in human embryonic stem cells at different stages of differentiation: in undifferentiated pluripotent cells, in cells undergoing the earliest transition to commitment, and in more differentiated hESC cultures. We report significant differences in the presence of discrete nuclear compartments, such as SC-35 domains and Cajal bodies, and find that these changes in internal nuclear structure are concomitant with changes in nuclear envelope proteins, nuclear size, and shape. SC-35 domains or "speckles" are discrete regions highly enriched in RNA metabolic factors that are characteristic of essentially all somatic cell types, for which we find an unexpected difference in human ES cells. While this and other observations provide evidence for a multi-faceted remodeling of the nuclear landscape in hESC, the major focus of our results is on the most dramatic and intriguing difference seen for PML-defined nuclear structures.

Promyelocytic leukemia Bodies (PML NBs) have been the subject of intense study in somatic nuclei, in part because the breakdown of these structures is a key event in the genesis of Acute Promyelocytic Leukemia and some solid tumors (Bernardi et al., 2007) . The hallmark of PML bodies is PML protein, a member of the tripartite motif (TRIM) family. Normal somatic cells have between 5-30 PML NBs (on average ~10/cell) earlier identified as Nuclear Dot 10 (ND10) (Ascoli et al., 1991). These bodies are implicated in many cellular pathways, including, chromatin organization (Boisvert et al., 2001; Seeler et al., 1999), viral response (Ishov et al., 1996), DNA replication and repair (Dellaire et al., 2006; Eskiw et al., 2004; Zhong et al., 1999), transcriptional regulation (Boisvert et al., 2000; Wang et al., 2004; Wu et al., 2001) tumor suppression and apoptosis (Bernardi et al., 2008; Bischof et al., 2002) Consistent with their multi-faceted role, somatic PML bodies can contain a variety of regulatory proteins, such as Sumo-1, Sp100, p53, pRB, HP1 and Daxx. For somatic PML NBs, Sumo-1 modification of the PML protein is necessary for the recruitment of other associated factors and formation of the PML NBs (Zhong et al., 2000).

Of the limited number of studies examining nuclear structure in ES cells (Meshorer et al., 2006a; Wiblin et al., 2005), most compare undifferentiated ES cells to more mature somatic nuclei or ES cells after differentiation has progressed. An important aspect of this study is in the investigation of the earliest transition phase, when a fully uncommitted ES cell undergoes initial epigenetic changes that lead to cell-type commitment. During this short developmental window, it is likely that more marked or transient rearrangements of nuclear structure, genome organization and chromatin modification occur. Such changes may become evident just prior to the overt expression of standard differentiation markers, or the loss of pluripotency specific markers. We first examined the distribution of nuclear factors

which delineate several of the major nuclear subcompartments to determine the extent to which these are similar or distinct in very early embryonic cells. Results demonstrate major differences in the structural landscape of the human ES cell nucleus, particularly PML-defined nuclear structures.

METHODS

Maintenance of Undifferentiated hES Cell Culture

Three NIH approved hES cell lines were used in this study: H1, H7 and H9. Cultures were primarily grown in the Lawrence lab (H1 and H9), however separate subcultures (H1 and H9) grown at the University of Massachusetts Stem Cell Core (UMassSCC) facility were included. Additionally fixed samples of other subcultures (H1, H7 and H9), obtained from the University of Washington Stem Cell Core (courtesy of Carol Ware) were also examined. The Ware Lab and UMassSCC hESCs were cultured, characterized and fixed as previously described (Hall et al., 2008)

Most human ES cells were cultured in the Lawrence lab primarily using WiCell protocols (www.wicell.org), however, our lab uses mechanical passaging (see Supplement for further information) instead of enzymatic (collagenase). Cells were cultured on gamma-irradiated (5,000 rads) P3 primary CF-1 mouse embryonic fibroblasts (MEFs), in Dulbecco's modified Eagle's medium/Ham's F-12 medium containing GlutaMax supplemented with 20% KO-Serum (Invitrogen), 1mM sodium pyruvate, 0.1mM nonessential amino acids, 0.1 mM beta-mercaptoethanol (Sigma), and 2 ng/ml basic fibroblast growth factor (Invitrogen). Standard G-band karyotyping was carried out by the Cytogenetics Laboratory at UMass Medical School. Pluripotency of cultures is assessed on a routine basis using immunoflourescence with antibodies against Oct-4 (Santa Cruz), SSEA-4, SSEA-3, Tra-1-61 and Tra-1-81(Millipore) as well as Alkaline Phosphatase (Vector Labs) (Fig. S1) or differentiation markers such as SSEA-1 (Millipore), Pancytokeratin (Sigma-Aldrich), Lamin A (Millipore) and Nestin (Stem Cell Technologies) (Fig. S2.).

For microscopic analysis, undifferentiated hESC (defined as "Day 0") samples were mechanically passaged as above and plated onto Matrigel (BD Biosciences) coated cover-glass with MEF Feeders and ES media (see above). They were allowed to grow on the cover-glass for 2-3 days prior to fixation.

Differentiation of hES Cell Cultures

Human ES cells removed from MEF feeder cells and given differentiation promoting media (see below) begin to differentiate into multiple cell types.

Non-directed differentiation—suspended colonies, from mechanically passaged pluripotent cultures, were broken up into very small colonies and single cells. The cells were plated without feeders onto Matrigel coated cover-glass, and cultured in general differentiation media (Dulbecco's modified Eagle's medium (DMEM), 20% fetal bovine serum, 1mM sodium pyruvate and 0.1mM nonessential amino acids (Invitrogen)). Half-volume of media was changed daily until the desired time point.

Neuroectodermal differentiation—small colonies, from mechanically passaged pluripotent cultures were allowed to grow overnight in suspension as embryoid bodies in a supplemented induction media (see below). The cells were then plated onto Matrigel or laminin (BD Biosciences) coated cover-glass without feeders. Once small colonies adhered to the coated surface an additional cover-glass was placed over the cells to form a chamber. This chambering promoted a more uniform and rapid neuronal differentiation throughout the

culture. The chambered cultures exhibited a noticeable increase in morphological maturation by 14 days to non chambered cultures observed in parallel. The cells were grown in an induction media, containing DMEM/Ham's F-12 medium, 1% N2 Supplement (Gibco; 17502-048), .1mM nonessential amino acids, 2ug/ml Heparin (Sullivan et al., 2007).

Cell Preparation, Immunohistochemistry and FISH

All hESC samples analyzed by microscope (undifferentiated and differentiated) were grown on Matrigel coated cover-glass (see above). Fixation and extraction of differentiation time points were taken at 24 hour intervals beginning 24 hours after the initial plating and media change. For most nuclear molecular cytology, nuclei were permeabilized by our standard protocol using a brief triton extraction prior to paraformaldehyde fixation, as described in detail elsewhere (Johnson et al., 1991b; Tam et al., 2002). Since triton extraction is not compatible with most cytoplasmic and cell membrane markers, visualization of these markers had to be done on separate samples first fixed in paraformaldehyde prior to triton extraction, as previously described (Tam et al., 2002) (Fig. S1).

Our standard protocols for immunohistochemistry and FISH and for simultaneous protein/DNA detection have also been described (Johnson et al., 1991a; Tam et al., 2002). Most antibodies were used prior to DNA hybridization, with the antibody signal fixed in 4% paraformaldehyde for 10 min prior to hybridization. Probes used for in situ hybridization (FISH) were a 60mer human alpha satellite oligo (BioSource International), a 45mer human telomere (ttaggg) repeat oligo, and a BAC to the 4q35.1 region of chromosome 4 supplied by M. Rocchi (University of Bari, Bari, Italy) (Tam, 2004). Antibodies used were anti-HP1 gamma (Chemicon), anti-Sc35 (Sigma-Aldrich), and anti-coilin (A gift from Edward Chan), anti-Lamin A/C (Millipore), anti-Emerin (Immuquest), anti-Lamin B (Abcam), anti-PML (Millipore & N-19 from Santa Cruz), anti-SUMO-1 (Zymed), anti-Daxx (Santa Cruz), and anti-PCNA (Santa Cruz).

Image Analysis

Images were captured with either an Axiovert 200 or an Axiophot microscope. Multi-plane Z-Stacks for extended plane and 3D images were gathered using the Axiovert 200 coupled with Axiovision deconvolution software (to remove out of focus light) using a constrained iterative algorithm to render images (Carl Zeiss, Thornwood, NY). These scopes are equipped with 100X PlanApo objectives (NA 1.4) and Chroma 83000 multi bandpass dichroic and emission filter sets (Brattleboro, VT), set up in a wheel to prevent optical shift. Images were captured with an Orca-ER camera (Hamamatsu, Bridgewater, NJ) or an AxioCam MmR camera (Carl Zeiss, Thornwood, NY).

Most experiments were carried out a minimum of 3 times, with typically 100-200 cells scored in each experiment. Key results were confirmed by at least two independent investigators. Images were minimally enhanced for brightness and contrast to more closely resemble what was seen by eye through the microscope.

RESULTS

Human ES cell lines H9 and H1 were maintained on irradiated mouse feeder layers or on Matrigel under conditions which discourage or promote differentiation, as described in Methods. Cultured hESCs have an inherent propensity to differentiate, and thus, even without differentiation-promoting conditions, typically "undifferentiated" cultures contain some partially committed cells. To investigate potential changes in nuclear structure during the earliest transition of hESC epigenetic commitment, it was useful to examine "Day 0" cultures of largely undifferentiated cells (see Methods), in which a sub-population had just

begun to differentiate. Pluripotent human ES cells tend to grow in large clusters (colonies) and, as shown in Figure 1A and Supplemental Figures 1 & 2, colony morphology and cell/nuclear size and shape provide useful information, as cells typically begin to differentiate at the outer perimeter of the colony. For example, the largely undifferentiated hESC colonies stain brightly with pluripotency markers (e.g. Alkaline Phosphatase or Oct-4)(Fig. 1B and S1), but as cells at the colony edge begin to specialize, they lose pluripotency markers and begin to express lineage or differentiation specific markers, as illustrated in Figure S2. As seen in Figure 1A, the cells that comprise most of the colony interior have the uniformly small, round, tightly packed appearance characteristic of fully undifferentiated ES cells, however the cells at the colony edge show morphological differentiation, with nuclei that are clearly larger and more variable in shape (Fig. 1A). In addition, more outlying cells (in sparse regions outside of colonies) typically show even more marked morphological differences and are most likely to express standard differentiation markers, such as pancytokeratin (Fig. S1 and S2). Thus, we utilized these differences to compare nuclear structure of fully undifferentiated and early "differentiating" cells within the same "Day 0" culture, to best capture potentially transient changes during a very early window of epigenetic commitment. We also tested hESC cultures placed in differentiation promoting conditions for 2-14 days, to assess progressively more differentiated and synchronous cultures.

Undifferentiated hESC lack SC-35 domains and Cajal Bodies which form with the onset of differentiation

As an initial assessment of the "nuclear landscape" of human ESC, we surveyed several well-known hallmarks of somatic nuclear structure. Some structural features appear similar between pluripotent hESC and more committed or somatic nuclei. For example, consistent with previous observations of pluripotent ES cells (Wiblin et al., 2005), we saw no obvious difference in the overall distribution of telomeres (Fig. S3B), which, as in somatic cells, were widely distributed throughout the internal nucleoplasm in pluripotent and differentiating ES cells, . We had previously shown that the 4q telomere of somatic nuclei showed a distinctive localization at the nuclear periphery (Tam, 2004). Interestingly, this distinctive organization was already established in the undifferentiated hESC nuclei (Fig. S3D), although the 4q telomere may be slightly closer to the nuclear envelope in somatic cells. On the other hand, changes in centromere distribution were clearly apparent upon differentiation. Using an alpha satellite oligo hybridization to mark the centromeres (of all chromosomes), we found that the vast majority of centromeres localized more centrally in the nucleus of undifferentiated (Day 0) cells. However, in differentiated cells of Day 2-4 cultures, many more alpha satellite signals become repositioned to the nuclear periphery, a position more commonly seen in somatic cells. Representative examples are shown in Figure S3A, and further analysis of the organizational changes to centromeres will be presented elsewhere (Hall et al., in progress.) Just the observation that there are changes in overall distribution of centromeres (between pluripotent and more committed nuclei) indicates that major chromosomal rearrangements occur, as also suggested by other evidence that centromeres of specific chromosomes reposition (Bartova et al., 2008b).

Importantly, we found other unanticipated differences in major nuclear structures, which collectively indicate that sweeping changes to internal nuclear architecture occur during the early stages of differentiation. A prominent structural hallmark of somatic cell nuclei is the presence of ~10–30 prominent domains (referred to as SC-35 domains or speckles) containing high concentrations of numerous mRNA metabolic factors, which several studies have shown are surrounded by numerous specific active genes (Brown et al., 2008; reviewed in Hall et al., 2006; Shopland et al., 2003; Xing et al., 1995). The spliceosome assembly factor SC-35 readily identifies these discrete domains in differentiated hESC, and in somatic

cells (Fig. 1C4). Pluripotent hESC nuclei still stain (brightly) for the SC-35 protein, however it is not concentrated in defined domains, but distributes diffusely throughout the nucleoplasm (Fig. 1C1-2). As summarized in Figure 1C, after just 2 days of differentiation, most cells already have established this nuclear compartment. In contrast, in Day 0 cultures the undifferentiated cells within colonies lack well-defined SC-35 domains, and the SC-35 protein only begins to coalesce into discretely bordered domains in the subset of cells at the colony edge and outside the colony most of which clearly show morphological differentiation. The lack of well-defined domains apparent in undifferentiated hES cells was not due to their smaller, rounder morphology, as confirmed both by optical sectioning and in cells grown on Matrigel alone (without mouse feeders), which causes undifferentiated hESCs cells to flatten.

Another feature of some somatic cells is the Cajal Body, which are most prominent in highly metabolically active cells such as neurons and cancers cells, and are implicated in assembly or modification of the transcriptional and splicing machinery (Morris, 2008). Thus, it was of interest to determine if early human embryonic cells contain these structures at all. Similar to SC-35 domain formation, in Day 0 cultures the vast majority of undifferentiated cells within colonies lack any suggestion of a Cajal Body, although the coilin protein is weakly present and diffuse throughout the nucleoplasm (Fig. 1D). Coilin becomes clearly localized into typically 1-2 Cajal Bodies in nuclei of morphologically distinct cells at the colony edge and after several days of differentiation, most cells contain bright, well-formed Cajal Bodies, as seen in neuronal differentiated cultures in Figure 1D (left).

This analysis of SC-35 domains and Cajal Bodies in human ES cells suggest a common theme: that nuclear structure of pluripotent ES cells is much less compartmentalized than that of somatic cells, and a more structured nuclear interior evolves during the earliest transitions to cell specialization, as further considered in the Discussion. These observations indicate that there is a brief window of perhaps just a day or less (in an individual cell) when internal nuclear structure undergoes rapid developmental transitions.

Concomitant changes to NE/lamina composition and nuclear size and shape

During this transition period, major structural changes are also seen in the protein composition of the nuclear envelope (NE), concomitant with dramatic changes in the shape and size of nuclei. Using antibodies against lamin B and A/C we confirm a study by Constantinescu et al., (2006) (Constantinescu et al., 2006) demonstrating that uncommitted hESC cells consistently have lamin B protein, but lack lamin A/C (Fig 2A and S4A). Upon induction of differentiation, cells begin to acquire lamin A/C primarily between Days 2-5. In addition, we show for the first time that the lamina-associated protein, emerin, first becomes detectable in the nuclear envelope of most cells only during this same transitional window, 2-5 days of differentiation (Fig. 2). Both lamin A/C and emerin often first have a distinctly patchy appearance, which was particularly striking for emerin (Fig. 2 A3, C7 and D). Figure 2D shows a cluster of outer-colony cells in a Day 2 culture that have just begun to incorporate emerin in patches of the NE; note that these same cells have only begun to form defined SC35 domains, which become more defined as cells fully express lamin A/C (Fig. 2E). Thus, these changes in NE composition are also markers of a more committed population of hESC. Simultaneous staining of lamin A/C and emerin indicated that the incorporation of these two proteins into the NE was essentially contemporaneous. Since emerin was primarily detected in the cytoplasm in cells lacking clear lamin A/C staining, our results are consistent with other evidence that lamin A/C is required for localization of emerin to the nuclear envelope (Tulac et al., 2004).

Another prevalent difference, concomitant with the onset of changes to lamina-associated proteins, are marked alterations in nuclear shape and size, which appear to occur during a

brief (~1 day) window of early differentiation. Pluripotent hESC nuclei are usually about 5uM in diameter and uniformly round in shape. This is in contrast to the larger (typically 10-20uM diameter), more oval or irregular nuclei in the differentiating cells at the colony edge or in 2-7 day hESC cultures (Fig. 1). For any individual cell this represents a remarkable change in nuclear size and contour. And, while the precise timing varied, in some cultures whole areas of cells appeared to be undergoing shape changes (Fig. 1B, 2B and 3) often linked to early expression of lamin A/C or emerin. For example, lobular nuclei sometimes had just a patch of these proteins that corresponded to bends in the NE. Even in some cells that initially appear more uniform in shape (based on DAPI stain) lamin A/C staining often delineated nuclear invaginations or lobes. In some cultures between Day 0-2, groups of cells, which could comprise a substantial portion of the population, synchronously underwent shape changes that appeared to occur in waves (Fig. 3). These groups of cells in all other respects appeared viable and stained negatively for the apoptotic marker caspase (data not shown).

These findings highlight that the period when hES cells first begin to transition towards a more committed state represents a brief window of marked structural change throughout the nucleus. The formation of SC-35 domains, differences in NE protein composition, and changes in nuclear shape and size reflect large scale changes in internal organization and overall structure of embryonic nuclei. As these changes may occur shortly before the expression of overt differentiation markers or full loss of pluripotency markers, these changes in nuclear structure may reflect a very early manifestation of a changing or “poised” epigenetic state.

Dramatic linear and rosette PML structures are unique to early hESC cultures

Of the various nuclear structures examined here, differences between somatic-type PML bodies and PML-defined structures in the human embryonic cells were especially striking. In somatic cells, PML protein concentrates in typically about 10 uniformly spherical structures (~.3 microns diameter) (shown in the Tig-1 fibroblast cell in Fig. 4C), variably termed PML bodies or ND10 (Nuclear Dot 10) (Ascoli and Maul, 1991;Bernardi and Pandolfi, 2007) . Since these heavily studied structures consistently appear round (or as doughnuts or hollow-spheres at higher resolution), we were surprised to see radically different PML-defined structures in early human ES cell cultures. While in Day 0-2 hES cultures some cells contain a few typical round PML NBs, a substantial fraction of cells (~10-40%) contain unique PML-defined structures with striking, elaborate morphology. These structures can be classified in two types: long linear PML “rods” or large (>2 microns) “rosettes” (Fig. 4A, 4H-J and S4). The linear PML rods are not just slightly elongated PML bodies, but often extend 3 to as much as 10 microns (across most of the nucleus) (see Fig. S4). These PML rods are fairly straight, but typically exhibit a slight curvature towards one or both ends. The “rosette” PML structures appear to comprise a large configuration of “looping” threads, reminiscent of a coiled rope or a honey-comb, ranging in size from 2-8um. A cell may contain multiple linear PML rods or multiple PML rosettes, but most often were not both present in the same nucleus. 3-D reconstruction of these structures is illustrated in Figure S8.

The PML structures in hES cells are distinct from somatic PML bodies not only in morphology, but also in composition. While the PML protein (and its SUMO modification) is required to form the body (Zhong et al., 2000), somatic PML bodies typically contain other markers, such as SUMO, Sp100, and Daxx. As shown in Figure S6, the PML-defined structures of undifferentiated hESC were not clearly enriched for any of these proteins, using antibodies confirmed to detect them robustly in somatic PML bodies and in hESC days after differentiation. As will be further considered below, unique PML structures frequently appear associated with the nuclear envelope or nucleolar edge, which is also not

noted with somatic PML bodies. Care was taken to determine that these unusual PML structures were not an aberrant property of an hESC line or a few cultures, but were consistently present in a significant subpopulation of cells in several different hESC lines and numerous subcultures, grown on either Matrigel or mouse feeders, and using either enzymatic or mechanical passage. They were also observed in cultures grown and fixed in other core facilities and labs (see Methods), strongly indicating that they are not unique to our culture conditions or hESC sublines.

Unique PML structures are no longer seen after the first few days of differentiation

The frequency of unique PML structures in human ES cultures was examined as a function of pluripotency, and with varying times in differentiation-promoting conditions. Cultures were plated under conditions that favor maintenance of pluripotency and fixed (Day 0 of differentiation) or switched to differentiation-permissive conditions (Materials and Methods), and fixed at intervals thereafter. Results of dozens of experiments consistently showed that these unusual PML structures were typically seen in a significant subset of cells only between Day 0 and Day 2 of differentiation. Although the timing and frequency varied from 10-44% of cells in very early (Day 0-2) cultures, PML rod and rosette structures were rarely seen at later days of differentiation (Days 4-7) and were absent in more mature differentiated (Day 14-50) cultures. This is illustrated by quantification of a representative experiment in Figure 4B. Note that Day 2-7 cultures also contain round (doughnut-shaped) structures that are larger than somatic PML bodies; we refer to these as “torus” structures to distinguish them from the smaller somatic-type PML bodies (Fig. 4A).

Consistent with linear or rosette PML structures being linked to early epigenetic transition, they were often more prevalent in the cells at the outer rim of an otherwise undifferentiated colony (Fig 4E), in cells that began to undergo shape changes and/or express lamin A/C or show more defined SC35 domains (Fig. 1C, 2A and 4B). As illustrated in Figure 4, in Day 0 cultures the small uniform cells of tightly-packed undifferentiated colonies typically had one or more small round PML bodies, but these were often far fewer (1-3) than in somatic cells (10-30) (Fig. 4D). However, since unique PML structures (especially the rods) could be seen in some cells that still stained for the pluripotency markers alkaline phosphatase (Fig. 4F) and SSEA-4, we do not rule out that they can form prior to the onset of overt cell differentiation (as defined by these markers).

Due to the inevitable lack of synchrony in hES cultures, we could not definitively determine a consistent pattern as to whether one type of structure (rod or rosette) preceded the other, although there was some suggestion that linear structures precede rosettes (i.e. the latter were more common in the outlying cells that were generally more differentiated). Interestingly, numerous examples were seen in which the large rosette or linear structures appeared to be forming a multitude of small round PML bodies, as illustrated in figures 4H and S4(C-D), suggesting that these singular large structures may give rise to smaller more numerous somatic-type PML bodies.

We also considered whether the presence of unique PML structures was linked to cell-cycle phase. As illustrated in Figure S5E, unique PML structures were seen in cells irrespective of whether they stained positive or negative for PCNA, a marker of S-phase cells. Since pluripotent hES cells have a highly attenuated G1 phase (Becker et al., 2006), most non-S-phase (PCNA negative) cells are in G2, and unique PML structures were also noted in early G1 daughter cells pairs (Fig. S5G). Surprisingly, linear PML structures were also occasionally observed in mitotic cells, associated with condensed chromosomes (Fig. 5 and S5F). Thus, results indicate that cell-cycle differences do not explain the heterogeneity of PML structures within different cells of a culture.

Finally, in addition to differentiation state, cell-cell contacts and clustering appeared to be another factor which impacted the prevalence of unique PML structures, and likely contributed to the variability in their number between experiments. As illustrated in Supplemental Figure 7, we found that embryoid bodies (in which cells had extensive cell contacts in 3-dimensions) were more likely to show a very high frequency of cells with linear PML structures. For example, in the embryoid body shown, most nuclei contain at least one linear PML structure.

In sum, we conclude that unique PML structures arise transiently during very early transitions of human ES cells towards cell-type commitment and are absent after differentiation is established; they are seen in various stages of the cell-cycle, and the frequency of linear PML structures may be impacted by cell-cell contact.

Relationship of Unique PML structures to other Chromatin or Nuclear Structures

The typically long, straight nature of the linear PML structures suggests they may be relatively rigid or under tension, possibly tethered at the ends to other nuclear structures. While the PML rods had slight curvature, they did not show the numerous bends that would be expected if a flexible fibrillar structure (extending several microns) encountered the various structural obstacles within nuclei. Rather, their morphology and other observations suggest the PML rods may be extending to or be taught between other nuclear structures. In staining for PCNA in S-phase cells (above) we noted that PML structures sometimes associated with PCNA-labeled foci in cells with a late S-phase pattern (Fig. 5F), or in labeled regions at the nuclear envelope. Thus, we examined the spatial relationship of PML rods relative to centromeres (which label in late S-phase), using oligonucleotide hybridization to centric alpha-satellite DNA. As illustrated in Figure 5, in a given population of early transitioning human ES cells, many examples are seen of linear PML domains that show apparent contact or very close proximity to centromere signals. While this was not always the case, approximately 20% of linear PML structures (Figure 5C) contacted or slightly overlapped a centromere signal. Given that there were typically ~20-30 separate alpha-satellite DNA signals detected in a nucleus, it was not clear a priori whether the observed degree of association with PML rods was random. We initially attempted to evaluate this by comparing the degree of association with telomeres and SC-35 domains. While the frequency of telomere association appeared roughly similar (Fig. S3C), the smaller, more numerous telomeric signals made scoring difficult and the comparison to centromeres less than ideal. Visualization of PML and SC-35, in those cells that had formed defined SC-35 domains, did not suggest a preferential association of PML rods to these relatively large domains (which occupy more area of the nucleoplasm). Nonetheless, neither comparison provided a sufficiently clear control for random spatial association

If the associations between the PML rods and centromeres were random, they would be expected to occur anywhere along the length of the linear PML structure. However, we noted that the centomeres often appeared to be at or close to one (or both) ends of the PML rods. Therefore, as shown in Figure 5D, we scored the placement of the centromere signal relative to individual PML structures (i.e. end, middle, and two sections in-between). Based on scoring by three different individuals, results show a clear propensity for centromere signals to fall at or close to the end of the long linear structure. This strongly suggests a non-random relationship, whereby a subset of linear PML structures extends to or towards centric heterochromatin.

Rosette PML structures actually showed a slightly higher association with centromeric and telomeric signals, sometimes encircling these structures (Fig. 5B). This may be a significant interaction, but given their large size and irregular shapes, it was more difficult to discern whether this was a non-random association. We also scored the 1-3 more typical round PML

NBs that appear in many fully undifferentiated hES cells. Interestingly, in pluripotent hESC even these more "somatic type" PML structures show a higher association to centromeres (30%) and telomeres (30%) than was previously reported for somatic cells (Everett, 1999). As shown in Figure 5C, we confirm that in somatic cells (TIG-1 fibroblasts), such interactions are much less frequent. Thus, results support that in undifferentiated hESC there is significantly increased association of PML structures, of varying morphology, to centric heterochromatin.

Unique PML structures exhibit a striking relationship with the nuclear lamina

The clearest evidence for a non-random interaction between PML and differentiating nuclear architecture was with the nuclear lamina and lamina proteins. This was first suggested by the association of some unique PML structures with the nuclear envelope; linear PMLs often aligned at the extreme nuclear periphery (Fig. 5F and Fig 6B), and rosette structures often abutted the nucleolus or nuclear envelope (Fig. S4D and 6D). As detailed above, the nuclear envelope (NE) undergoes marked changes in shape, size, and composition, with lamin A/C and emerin emerging by about Day 2 of differentiation. It is during these first two days when the unique PML structures are most commonly seen. While unique PML structures subside in most cells by the time full incorporation of lamin A/C is evident (as bright uniform staining throughout the NE), in the population of transitional cells exhibiting patchy or weak lamin A/C stain that also contain unique PML structures, there was an unambiguous relationship between the two. Remarkably, as quantified in Figure 6C, about half of the unique PML structures in these cells showed clear structural relationships with lamin A/C. The nature of the spatial relationships made it more evident in even just single cells that there was a non-random relationship (Fig. 6D). As illustrated in Figure 6A and 6B, linear PML rods were repeatedly seen to completely align with the NE, and appeared in distinct gaps of lamin A/C (or lamin B) protein. The addition of lamin A/C staining made it apparent that some linear PML structures that had appeared to simply traverse the nuclear interior were often actually in a nuclear envelope fold, delineated by lamin A/C protein (Fig. 6B). Surprisingly, these PML structures appear integrated with the lamina structure itself, as they reside in gaps in lamin A/C staining (Fig. 6, see insets). The larger rosette structures also appear to associate with, but not overlap, lamin A/C stained regions when localized to the nuclear periphery (Fig. 6D and S5C).

In a subset of early differentiating hES cells, lamin A/C is present as one or two accumulations at the nuclear periphery (Fig. 6D), and these lamin A/C accumulations often associate with a PML structure. As illustrated in Figure 6D and 6A, and quantified in 6E, in cells that had just one or two accumulations of lamin A/C most (~75%) of the lamin A/C foci were, surprisingly, tightly abutting (but non-overlapping) a PML defined structure. These findings, most commonly seen for rosette or large "torus" PML structures (Fig 6E), and was often coincident with folds or changes to nuclear shape at that same site (Figure 6A and 6B). These results further support a relationship and raise the possibility that the PML structures may mark a site at which lamin proteins nucleate.

DISCUSSION

It is now well established that the nucleus of somatic cells has a complex, compartmentalized architecture that is intimately related to nuclear function and gene expression. In contrast, very little is known about nuclear structure and genome organization in pluripotent human embryonic stem cells, in which the genome-wide epigenetic programming is yet to take shape. This study significantly advances knowledge of nuclear structure in these unique and important cells. Findings demonstrate that the "nuclear landscape" of undifferentiated human ES cells is fundamentally different from more differentiated ES cells and somatic cells. In uncommitted ES cells certain nuclear major

structural “compartments” characteristic of somatic cells are either absent or markedly different. We further identify the time course, during the earliest onset of differentiation, in which these nuclear structures form. Results support a general principle that the epigenomic “openness” of the human ES cell is reflected in a nucleus in which nuclear factors are minimally compartmentalized. This is demonstrated by the absence of defined “SC-35 domains”, structures rich in RNA metabolic factors that are consistently present in somatic cell nuclei and surrounded by many active genes expressed in that cell type. These SC-35 domains only begin to form as pluripotent ES cells begin to commit to specific lineages. During this same brief time frame, Cajal Bodies first form in most cells, the nuclear envelope matures with incorporation of lamin A/C and emerin, and the shape and size of nuclei changes markedly. Most surprisingly, this remodeling of the nuclear landscape is concomitant with the transient formation of large, elaborate PML-defined structures, which ultimately resolve to the small spherical PML bodies of somatic cells.

Structural differences in human ES cells are of interest because of the relevance to their unique epigenetic programming, but also because any such differences may provide insight into the functional significance of the compartments themselves. For example, while SC-35 speckles are an essentially ubiquitous feature of somatic nuclei, yet findings here show that the concentration of RNA metabolic factors into these discrete domains is not necessary for basal nuclear function. Our lab and others have shown that in somatic cells SC-35 domains are surrounded by specific genes, often those highly-expressed in that cell type (e.g. Moen et al., 2004; Smith et al., 1999), leading to the suggestion that each domain represents a hub of actively used factors at the center of a “euchromatic neighborhood” (Shopland et al., 2003; reviewed in Hall et al., 2006). Thus, from this perspective the SC-35 domain organization would arise as part of the cell-type specific regulation and organization of the genome within the nucleus, and it is understandable that undifferentiated hESC would lack this organization. While hESC may have less “stored” factors than some cell types, it is not clear that they would have less stored factors than other highly metabolically active cells; thus the postulate of merely “storage sites” does not readily explain the lack of organization. Findings here support that SC35 speckles are linked to cell-type specific genomic organization that develops as the genome is segregated into more active versus inactive neighborhoods in somatic cells, rather than to the basal nuclear functions present in all cells.

Our findings collectively indicate that the most sweeping and interesting changes to hESC nuclear organization occurs in cultures during a ~24 hour window at the onset of differentiation. This may actually begin before overt differentiation (expression of cell type specific proteins), but structural reorganization of the nucleus may reflect nucleus-wide “epigenetic” programming in which cells enact, or become poised for, cell-type commitment. While the emergence of SC-35 speckles reflects establishment of internal nuclear organization and lamin A/C and emerin changes reflect maturation of the nuclear envelope, much of our study focused on a more intriguing but puzzling observation, regarding changes to PML-defined nuclear bodies. This goes beyond a case of somatic structures first appearing as cells begin to differentiate, but rather the discovery of a dramatically different PML-defined structure than any known in somatic (or cancer) cell types. We consistently find unusual PML structures in three commonly studied hES cell lines, H1, H7, and H9, in a significant subset of cells under a variety of culture conditions. However, because our results indicate that these are transient structures that arise during a brief transitional window, they may be missed by simply comparing undifferentiated hESC to somatic cells or more differentiated hES cells. We cannot rule out the possibility that such structures could form as an outcome somehow of culture, however the fact that they are found in cultures generated in other labs indicated that this is a more general feature of hES cells, at least in vitro. The unique PML-defined structures in hESC may also be related to the developmental expression of particular isoforms of PML. For example, it has been

shown that PML isoform II is capable of forming fibrillar structures and PML isoform IV is capable of forming irregular nuclear bodies when ectopically expressed in cells lacking other PML isoforms (Condemine et al., 2006).

Despite hundreds of studies, the role of PML bodies in somatic cells is not well understood (reviewed in: Okuno et al., 2004; Bernardi and Pandolfi, 2007), thus it is not surprising that any role for this novel hES cell PML structures remains unclear. The PML bodies of somatic cells are compositionally and structurally distinct, and are not thought to interact with the nuclear envelope or lamina. Although often discussed as a potential "depot" for storage or sequestration of regulatory factors (Yeager et al., 1999), there is evidence that they interact with chromatin, particularly centromeres and telomeres (Everett, 1999; Eskiw et al., 2003; Eskiw et al., 2004; Sutherland et al., 2001). Our findings provide several clues regarding the potential role(s) of these unusual hES cell PML structures. 1) They are prevalent only during a brief period when the whole internal and peripheral nuclear landscape is undergoing major organizational changes, including shape changes. 2) Many interact with lamin A/C accumulations or the nuclear lamina as it is changing in shape, size and composition. 3) They appear to have a spatial relationship to centromeres, during a period of time when our findings indicate many centromeres are being repositioned within nuclei. 4) The "taut" appearance of linear PML structures suggests that they may be tethered at each end. Based on these collective findings, we suggest that novel PML structures may be involved in the dramatic architectural remodeling of nuclei that occurs during the brief transition when changes in nuclear programming begin to sweep across the genome. For example, these findings raise the possibility that the unique PML structures have a structural role involving interaction with lamin, potentially linked to changing nuclear shape. We were initially surprised to see that, during early stages of differentiation, there were large groups of cells that synchronously underwent shape changes, sometimes dramatic (Fig. 1B, 3 and 4E). In general, these cells appeared healthy, did not stain for apoptosis markers, and apparently represented small "clones" of cells with similar cell shape changes; these could be directly adjacent to a group of cells with no shape change (indicating it was not caused by culture conditions). Changes in nuclear shape in cells of early embryos (in vivo) have been reported and recently discussed (Brandt et al., 2006; Pilot et al., 2006), thus this aspect of cultured hES cells may well reflect natural features of embryonic cells, although this has not received substantial attention in ES cell literature. These shape changes in otherwise healthy cells may reflect either transient formation of irregular contours that arise as nuclear size and structure are being remodeled (but will resolve to a more regular oval or round shape), or more permanent formation of the unusual multi-lobed structure that characterizes cells such as granulocytes (neutrophils) (reviewed in Melcer et al., 2006). While speculative, it is interesting to consider that the cell type most affected by PML mutation in acute promyelocytic leukemia is the granulocyte, a cell with a remarkably complex, multi-lobed shape (reviewed in Hoffmann et al., 2007; Olins et al., 2008). In APL, the promyelocytic cells with regular oval nuclei fail to differentiate into multi-lobed granulocytes (reviewed in Strudwick et al., 2002).

Clearly, the nuclear structure of human ES cells is markedly different from that of somatic or more differentiated cells, as shown here not only for PML bodies but other ubiquitous compartments of somatic nuclear structure, such as SC35 domains. These differences coupled with the changes to nuclear size, shape, and lamina-associated proteins further highlight the remarkably rich opportunity ES cells present to understand how various aspects of nuclear structure relate to the state of pluripotency and self-renewal of hESC, and the epigenetic programming of genome at the onset of cell-type commitment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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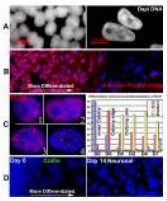

Figure 1. Differentiating hESC exhibit sweeping epigenetic and morphological changes

(A&B left) Small (5um) tightly packed round cells are characteristic of undifferentiated hESC. They stain brightly for pluripotency markers such as Alkaline Phosphatase (AP) **(B, Left**, red**) (A+B Right)** Differentiating cells peripheral to the colonies exhibit diminished expression of pluripotency markers (AP) and changes in nuclear size and shape. **(C & Graph)** SC35 (red) domain formation occurs as cells differentiate; either peripheral to the colonies in D0 cultures or with directed differentiation. **(C1)** Undifferentiated hESC show diffuse SC35. **(C2-C3)** SC35 domain formation starts as cells begin to differentiate. **(C4)** Mature SC35 domains, typical of somatic cells, are not abundant until D7 of differentiation. **(D)** Cajal bodies are absent in undifferentiated cells (left), are found in some differentiating peripheral cells in D0 cultures, and most differentiated cells (D14 Neuronal).

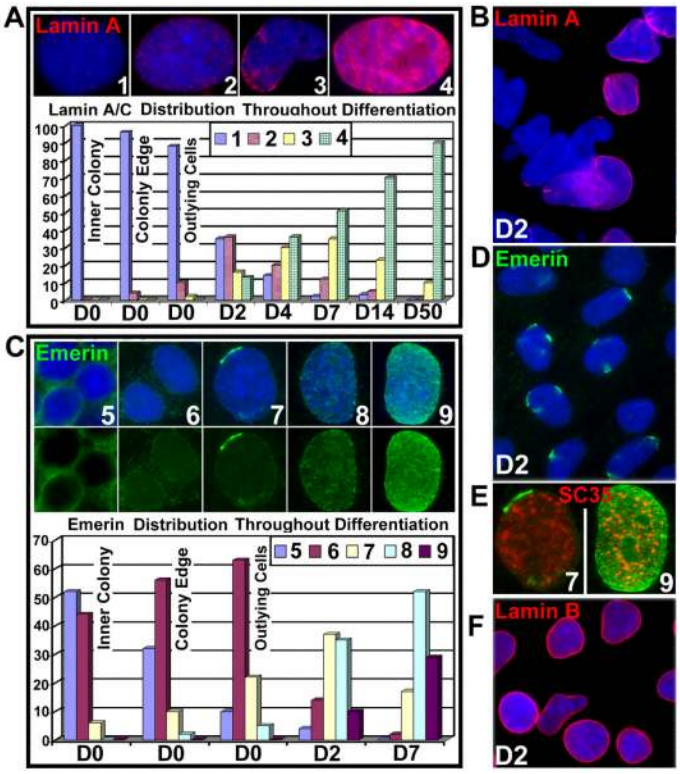

Figure 2. Maturation of the nuclear envelope (NE) occurs during hESC differentiation

(A) Lamin A (red) is not expressed in undifferentiated hESC (A1). It initiates expression in patches during early differentiation (A3), and is abundantly expressed throughout the cell by D4-7 (A4). **(B)** Initiation of lamin A often coincides with nuclear shape change. **(C)** Emerin (green) appears cytoplasmic in undifferentiated hESC (C5) but then declines (C6). Emerin localization to the NE also starts in patches concurrent with localization of Lamin A (C7) and is seen abundantly expressed by D4-7 (C8-9). **(D)** A field of "Patchy" localized Emerin in differentiating hESC. **(E)** Patchy emerin is usually seen in cells exhibiting undefined SC35 domains (cell from C7), while abundant emerin expression is usually seen in cells with clearly defined SC35 domains (cell from C9). **(F)** Lamin B is always present in undifferentiated 0 day hESC.

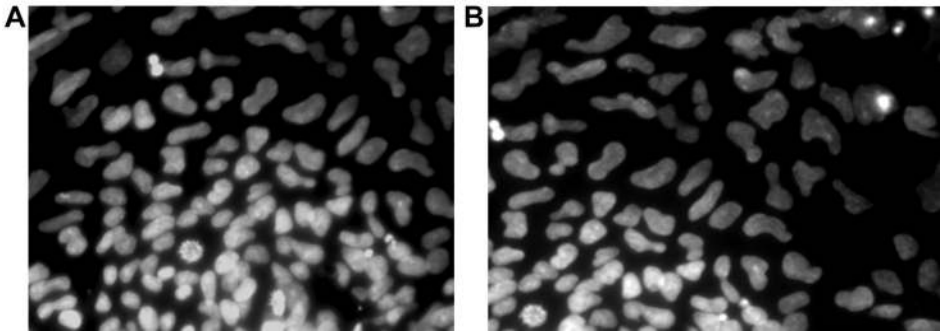

Figure 3. Distinct transformations in nuclear size and shape occur during a brief window of early differentiation

DNA staining reveals dramatic nuclear size and shape changes, in which occur often in synchronous waves of differentiating hES cells. **(A)**Undifferentiated cells in close proximity to each other within colonies (bottom) have smaller more uniform nuclei. Nuclear shape changes are prevalent in less densely packed areas of "differentiated" cells at the outer edge of the colony (top) **(B)** Moving away from the origin of the colony, nuclear size and shape change increases significantly in cells peripheral to the colony.

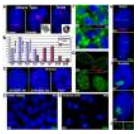

Figure 4. hES cells exhibit unusual PML structures

(A) Large unique linear (left) and rosette (middle) PML structures (red) are found in hESC cultures. Torus structures (right) are also seen, similar in shape to somatic PML bodies, but approximately 2-3 fold larger. **(B)** Unique PML structures are less abundant within undifferentiated colonies, and increase in abundance toward the colony's edge and in differentiating cells outside the colony (**see also E**). The frequency decreases quickly upon differentiation, while the large torus structures peak later in differentiation. **(C-D)** Undifferentiated hESC (H9) also contain more somatic-like PML (C red, D green) structures, however they are much fewer in number per cell, and their numbers increase with differentiation (Tig-1 are somatic fibroblasts). **(F)** Unique PML structures are occasionally found in cells that still stain brightly for pluripotency markers (AP, green). **(G)** They are usually found in hES cells (top) lacking heterchromatin (HP1, green), which is found in cells containing more somatic-type PML bodies (bottom). Multiple examples of linear PML structures are shown **(H)**, Linear Structures appear taut and can range in size from 2-10um. **(I)** PML Rosette structures can range in size from 2-8um in diameter and resemble "looping" threads or a honey-comb. **(J)** Both Linear and Rosette Structures appear to dissolve forming multiple small round PML structures.

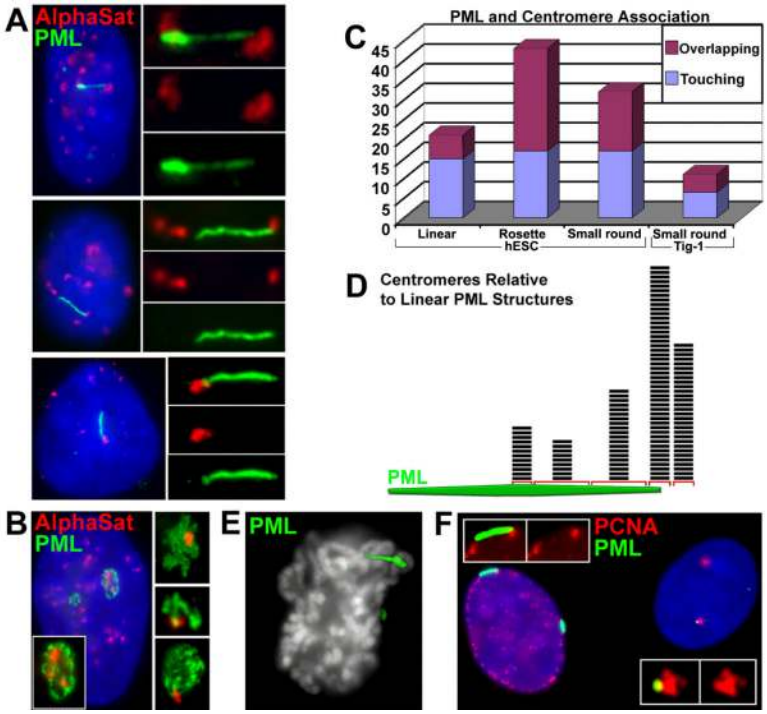

Figure 5. Unique PML structures exhibit a relationship to heterochromatin

(A) Linear PML Structures (green) often exhibit a relationship to centromeres (alpha satellite, red), at one or both ends of the structure, and appear "taut". **(B)** Rosette PML structures also commonly encircle one or more centromeres. **(C)** The small round "somatic-like" PML structures in hESC also associate with centromeres, which is 2.5 fold higher than normal PML bodies in somatic cells. **(D)** Centromere interaction along the linear PML structure mapped predominantly to the ends suggesting a non random association. **(E)** Some linear PML structures maintained an association to chromatin (dapi, white) during mitosis. **(F)** PML structures associate with PCNA foci (red) in late S-phase cells, supporting an association to late replicating heterochromatin.

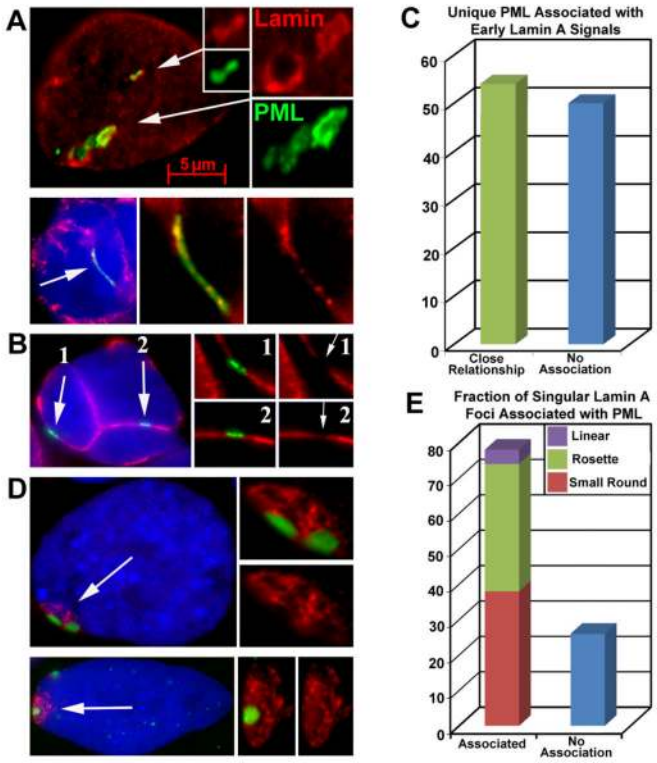

Figure 6. Interaction of Unique PML structures with the nuclear lamina

(A) Unique PML structures (green) frequently show a striking relationship with the nuclear lamina (red), either at the nuclear periphery (**B** 1st arrow) or within laminar folds as the cells undergo shape change (arrow **A** or 2nd arrow **B**). **(B)** PML structures often fill gaps in the lamina signal (see arrows in separated channels at right). **(C)** Approximately 52% of differentiated cells (D2) with Unique PML structures showed an interaction with Lamin A. **(D)** Lamin A expression begins focally at the NE of differentiatining cells. Unique PML structures are frequently found within these large foci. **(E)** Most hES cells containing a singular Lamin foci (75%) showed a close relationship with Unique PML structures.