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An in vitro model for Pelger-Huët anomaly
Stable knockdown of lamin B receptor in HL-60 cells

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Key words: lamin B receptor, nuclear structure, Pelger-Huët anomaly, neutrophil, macrophage

Abbreviations: LBR, lamin B receptor; RA, retinoic acid; ECL, enhanced chemiluminescence; GFP, HL-60/S4 cell subline which has a stable green fluorescent protein expressing gene; S4, HL-60/S4 cell subline; sh1, HL-60/S4 cell subline with a stable LBR knockdown; ELCS, envelope-limited chromatin sheets; TPA, phorbol ester; PHA, Pelger-Huët anomaly; NE, nuclear envelope; ER, endoplasmic reticulum

The principal human blood granulocyte (neutrophil) possesses a lobulated and deformable nucleus, important to facilitate rapid egress from blood vessels as these cells migrate to sites of bacterial or fungal infection. This unusual nuclear shape is a product of elevated levels of an integral membrane protein of the nuclear envelope lamin B receptor (LBR) and of decreased amounts of lamin A/C. In humans, a genetic deficiency of LBR produces Pelger-Huët anomaly, resulting in blood neutrophils that exhibit hypolobulated nuclei with redistributed heterochromatin. Structural changes in nuclear architecture occur during granulopoiesis within bone marrow. The exact mechanisms of this nuclear shape change and of heterochromatin redistribution remain largely unknown. As a tool to facilitate analysis of these mechanisms, a stable LBR knockdown subline of HL-60 cells was established. During in vitro granulopoiesis induced with retinoic acid, the LBR knockdown cells retain an ovoid shaped nucleus with reduced levels of lamin A/C; while, the parent cells develop highly lobulated nuclei. In contrast, macrophage forms induced in LBR knockdown cells by in vitro treatment with phorbol ester were indistinguishable from the parent cells, judged by both nuclear shape and attached cell morphology. The capability of differentiation of LBR knockdown HL-60 cells should facilitate a detailed analysis of the molecular relationship between LBR levels, granulocyte nuclear shape and heterochromatin distribution.

Introduction

Lamin B receptor (LBR), encoded by a single gene, is an integral membrane protein of the nuclear envelope (NE), interacting with lamin B and heterochromatin and functioning to regulate interphase nuclear shape and heterochromatin distribution within myeloid cells.[1,2] Genetic deficiency of LBR in humans results in Pelger-Huët anomaly (PHA, MIM 169400),[3] characterized by neutrophil nuclear hypolobulation and redistribution of peripheral heterochromatin towards the center of the nucleus. A more severe manifestation of human LBR deficiency is observed in the lethal fetal HEM/Greenberg skeletal dysplasia, in which cholesterol metabolism is profoundly affected.[4] A homologous LBR deficiency in mouse results in Ichthyosis (*ic*), changing the normally ring-shaped neutrophil nucleus to ovoid shape with central clustering of the pericentric heterochromatin and associated with an increased death of homozygous mutant fetuses.[5] Considerable knowledge about the nuclear changes in heterozygous (+/*ic*) and homozygous (*ic*/*ic*) Ichthyosis was achieved by the creation of immortalized bone marrow cells from the mouse mutants, which can be differentiated to granulocyte form in vitro by the addition of retinoic acid (RA).[6,7] As yet, no similar immortalized human PHA bone marrow cells have been created to allow the study of nuclear changes during RA induced granulocyte differentiation in vitro. However, the established human acute myelocytic cell line (HL-60)[8] (and a more recent subline HL-60/S4,[9]) can be differentiated with RA in vitro from ovoid to lobulated nuclear shape, exhibiting an increase in cellular levels of LBR and a decrease in lamin A/C and vimentin content.[10,11] Consequently, the authors of this paper conceived that an LBR knockdown within HL-60 cells might mimic human PHA, furnishing a convenient model cell line for the study of myeloid differentiation in the absence of LBR and for elucidating mechanisms underlying the acquisition of aberrant nuclear architecture. We describe creation and characterization of a stable LBR knockdown subline derived

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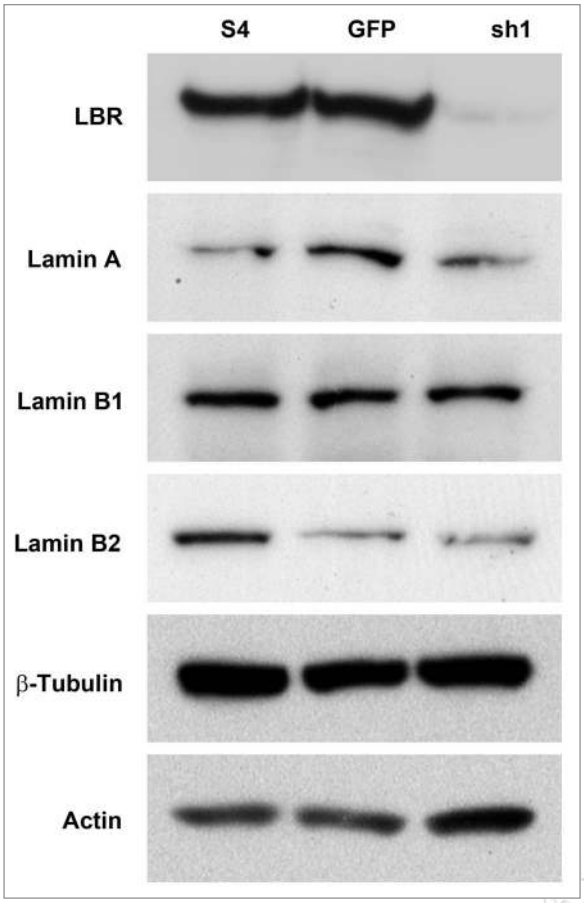

Figure 1. Immunoblotting of the three undifferentiated HL-60 cell sublines with anti-LBR, lamins, β-tubulin and actin. Cell sublines: S4, parent; GFP, infected with GFP expressing vector; sh1, short hairpin knockdown of LBR.

from HL-60/S4 cells infected with a lentiviral construct encoding a short-hairpin RNA targeting human LBR.

Results

Formation of a subline of HL-60/S4 with a stable LBR knockdown. Employing lentiviral vectors, we have established two stable cell sublines of HL-60/S4: one encoding a short hairpin RNA targeting human LBR; the other, expressing GFP. Both of these sublines exhibited vigorous growth and healthy microscopic appearance. The LBR knockdown subline is denoted "sh1"; the GFP expressing subline, denoted "GFP"; the parent subline, denoted "S4". Total cell extracts of the undifferentiated S4, GFP and sh1 sublines were run on a 4–20% gradient SDS-PAGE and analyzed by immunoblotting with antibodies against LBR, lamins A, B1 and B2, β-tubulin and actin (**Fig. 1**). The ECL (enhanced chemiluminescence) images clearly show a massive reduction of LBR in the sh1 cells, compared to either the S4 or GFP cells (which displayed similar levels of LBR). There were no comparable changes in the levels of the lamins, β-tubulin or actin in these three undifferentiated cell sublines.

A comparison of calculated doubling times for the undifferentiated S4 and sh1 sublines (based upon cell titers during exponential growth) indicated that these two sublines grew at comparable rates: S4 doubled in ~16 hrs; sh1, in ~17 hrs. Cell cycle analysis was also performed on these two undifferentiated sublines in their exponential growth phases, following ethanol fixation, RNAse digestion and propidium iodide staining. Analysis of S4 revealed: G_1 ~36%; S ~50%: G_2 ~12%. In contrast, sh1 cells appeared to spend somewhat more time in S phase, exhibiting: G_1 ~23%; S ~65%; G_2 ~10%. Despite these small differences in doubling time and cell cycle parameters, these cell sublines grow robustly with minimal indication of cell death during the exponential phase.

Granulocyte differentiation with retinoic acid. When HL-60/S4 cells are exposed to 1 μM RA for 4 days, at least ~80% of the cells exhibit nuclear lobulation and formation of nuclear envelope-limited chromatin sheets (ELCS).[10,12] In parallel to this nuclear change from ovoid to lobulated shape, nuclear envelope and cytoskeletal protein changes have been documented, employing both immunofluorescence and immunoblotting methods:[11,13,14] the protein expression level of LBR increases; the low levels of lamin A/C decrease further; vimentin undergoes a massive reduction; β-actin increases slightly. Many other proteins exhibited little or no change in amount or cellular location; e.g., lamin B2; lamin B1 (which may decrease slightly); LAP2β; emerin; HP1α; SUN2; α-tubulin. We sought to determine how the knockdown of LBR in the sh1 subline might influence these previously described phenotypic effects of RA induced granulocyte differentiation.

An immunofluorescent comparison of S4 and sh1 cells, with and without RA treatment and stained for LBR is presented in **Figure 2**. All image data in this figure were collected with identical microscope conditions on a Zeiss 710 confocal microscope, allowing semi-quantitative comparison of antibody staining intensities. Only selected images (**Fig. 2** bottom row, sh1* cells) were subjected to image processing (see below). Undifferentiated S4 nuclei are mostly ovoid or indented with clear LBR localization in the NE. After 4 days of RA treatment, the differentiated nuclei exhibit lobulation and indentation, with bright anti-LBR staining of ELCS, as previously described.[10-12,14,15] Overall, the differentiated NE reveals a stronger staining by anti-LBR, than that seen within undifferentiated S4 cells, as previously described[14,15] and in agreement with immunoblot data (see below). By contrast, the sh1 cells present negligible staining with anti-LBR (although a faint increase is seen after RA treatment). The bottom row (**Fig. 2**) presents the same sh1 images as in the middle row, processed with Photoshop to facilitate visualization of nuclear shape. DAPI staining of DNA demonstrates that undifferentiated and RA treated sh1 cells primarily possess ovoid shaped nuclei. This apparent lack of nuclear shape change within sh1 cells, following 4 days of RA treatment, was confirmed by thin section electron microscopy (**Fig. 3**). These images convincingly demonstrate that the vast majority of S4 cells display nuclear lobulation and ELCS following 4 days of RA treatment, as shown previously;[10] but the sh1 cell nuclei remain primarily ovoid in shape.

An extensive immunoblotting comparison of S4 and sh1 cells, with and without RA treatment, is presented in **Fig. 4** and summarized in **Table 1**. The major conclusions are as follows: (1) LBR is severely reduced in the sh1 cells, exhibiting a trace amount in undifferentiated cells which increases after RA treatment; (2) Lamin A/C, which is normally quite low in undifferentiated S4 cells (and may be slightly reduced in sh1 cells), is reduced further after RA in S4 and sh1 cells; (3) Lamin B1 is relatively unchanged comparing S4 and sh1 (±RA); (4) Lamin B2 is strong and unchanged comparing S4 and sh1 (±RA); (5) Vimentin is weak in undifferentiated S4 and sh1 cells (perhaps slightly stronger in sh1 cells), virtually disappearing after RA treatment in both sublines; (6) SUN1 is very weak in undifferentiated cells (perhaps slightly stronger in sh1 cells), virtually disappearing like vimentin after RA; (7) SUN2 appears to display a slight increase in both S4 and sh1 after RA; (8) Emerin, α-tubulin, HP1α, HP1β and HP1γ are present and essentially unchanged, comparing S4 and sh1 ± RA. These conclusions are largely consistent with previous immunoblot comparisons of S4 cells (±RA).[11,14]

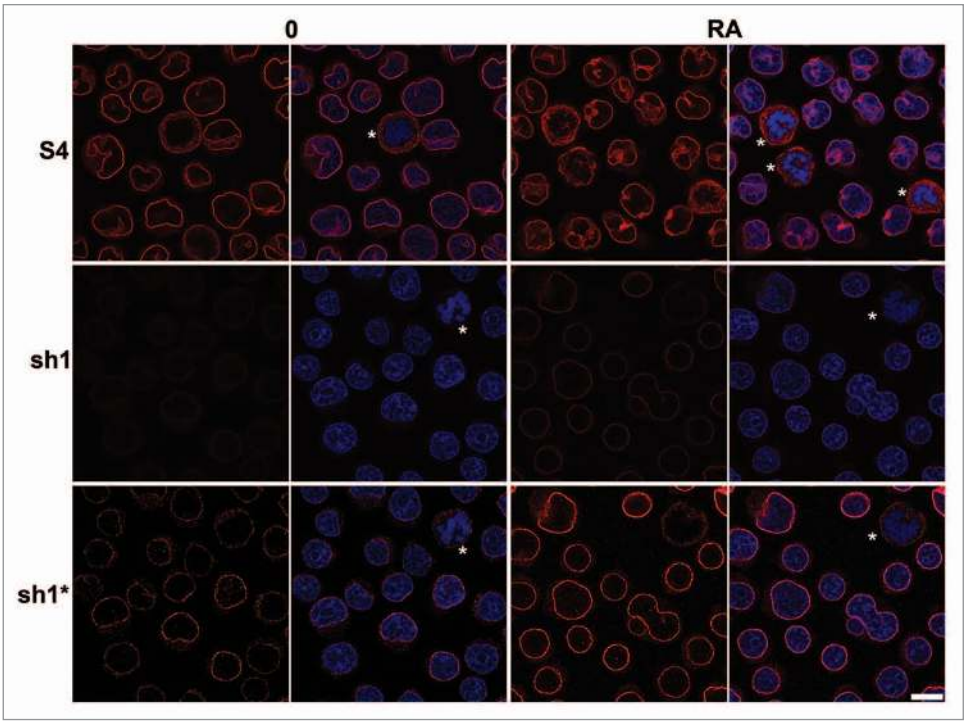

Figure 2. Confocal immunofluorescent staining of undifferentiated and RA treated S4 and sh1 cells with anti-LBR. All data was collected with identical microscope settings. Cell states: 0, undifferentiated cells, leftmost two columns; RA, differentiated for four days, rightmost two columns. Top row: S4, parent HL-60/S4 cell subline. Middle row: sh1, HL-60/S4 cell subline with a stable LBR knockdown. Bottom row: sh1*, same images as the middle row after image processing. Magnification bar: 10 μm.

Macrophage differentiation with phorbol ester. When HL-60 cells, which normally grow as suspension cultures, are treated with the phorbol ester TPA, they quickly attach, cease to divide and differentiate into macrophage cells.[10,11,14,16,17] The nuclei in HL-60/S4 cells remain ovoid in the macrophage state and there is a striking increase in lamin A/C, vimentin, plectin and nesprin 1 giant proteins.[11,14] We inquired whether LBR knockdown in sh1 cells results in obvious perturbations of TPA induced macrophage differentiation. **Figure 5** presents the results of Wright-Giemsa staining of S4 and sh1 cells, undifferentiated or treated with TPA for 3 days. The undifferentiated cells were cytocentrifuged onto coverslips, flattening the cells prior to staining; the TPA treated cells grew attached to coverslips and were, consequently, less flattened prior to staining. Even so, it is clear that the S4 and sh1 cells look very much alike, before or after TPA treatment. The TPA treated cells display a characteristic mixture of solitary attached cells with long cytoplasmic extensions interspersed with clusters of cells. **Figure 6** presents an immunostaining experiment with S4 and sh1 cells treated with TPA (4 days), methanol and acetone fixed

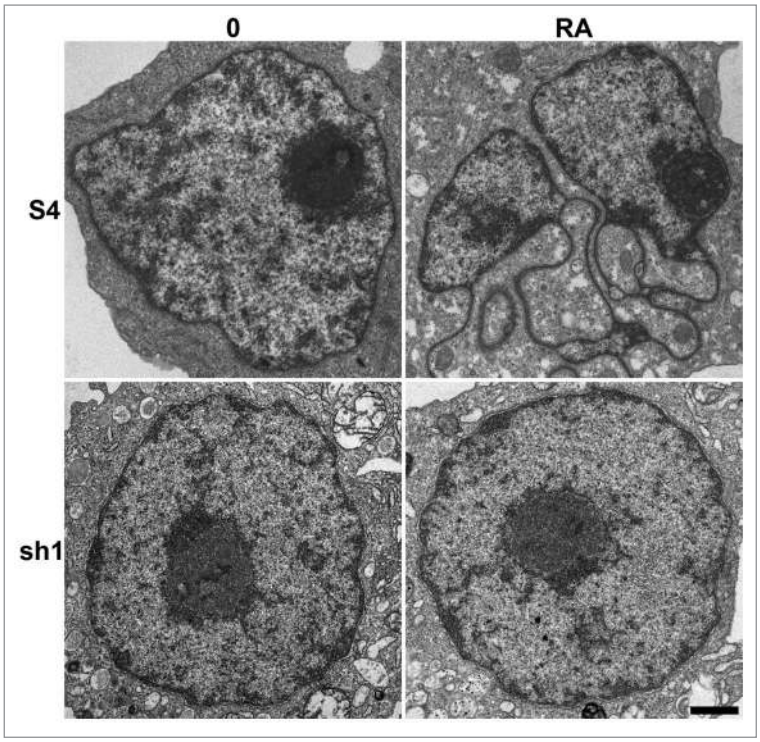

Figure 3. Thin section electron microscopy of undifferentiated and RA treated S4 and sh1 cells. Note the appearance of a lobulated nucleus and chromatin sheets (ELCS) in the RA treated S4 cells and their absence in the RA treated sh1 cells. Magnification bar: 1 μm.

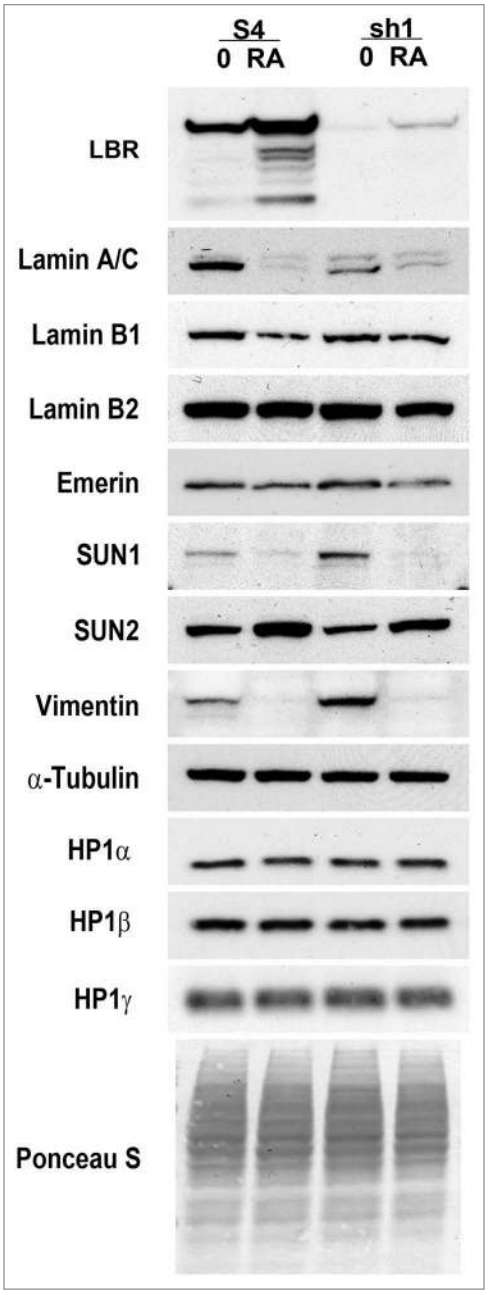

Figure 4. Immunoblotting of total cell extracts from undifferentiated and RA treated S4 and sh1 cells. Cell states: 0, undifferentiated cells; RA, differentiated for four days. The Ponceau S stained membrane demonstrates that the protein loads were well matched. LBR exhibits a small amount of degradation.

and reacted with anti-LBR and anti-lamin B2. It is clear that there is much more LBR in the S4 cells, than the sh1 cells, with localization in both the cytoplasm and the NE. The nuclei are ovoid in shape in both cell sublines, with comparable NE staining by anti-lamin B2. Immunoblotting experiments were also performed upon extracts of S4 and sh1 (±) TPA (**Fig. 7**). Comparable to previous experiments on S4 cells,[14] these experiments demonstrate that both S4 and sh1 cells reveal an increased synthesis of lamin A and plectin following treatment with TPA; the levels of SUN2 and β-actin remain largely unchanged in the same experiment. In summary, we showed that the near absence of LBR in sh1 cells has only a minimal effect on the differentiation of HL-60/S4 cells to a macrophage form following exposure to TPA.

Discussion

The present study demonstrates that LBR knockdown in the sh1 subline of HL-60/S4 cells results in one obvious phenotypic consequence: maintenance of an ovoid nuclear shape in sh1 cells induced to granulocytic differentiation with RA. This phenotype is in sharp contrast to S4 cells (and GFP cells) where RA treatment consistently leads to nuclear lobulation and the formation of ELCS.[1,2,10,12] The absence of nuclear lobulation in granulocytic forms of sh1 cells is in agreement with the observation of ovoid nuclei within granulocytes of human PHA[3] and of mouse Ichthyosis,[5] both genetic conditions resulting in significantly reduced levels of LBR. This LBR dosage effect is nicely demonstrated in a recent study,[18] where the average number of human granulocyte nuclear lobes correlates with the level of cellular LBR, encompassing 0 to 3 copies of the LBR gene. Aside from this one significant morphologic characteristic, the changes (or lack of changes) in the levels of various NE and cytoskeletal proteins, previously documented within granulocytic S4 cells,[11,14] appear unperturbed by the absence of LBR in sh1 cells (**Fig. 4 and Table 1**). Likewise, the morphological and immunochemical changes observed when S4 cells are differentiated into macrophages by treatment with TPA, remain largely unaltered by the absence of LBR in sh1 cells (**Figs. 5–7**).

The mechanism relating LBR content to granulocyte nuclear lobulation remains obscure. The primary hypothesis involves a structural interpretation of LBR[2] residing within the inner nuclear membrane and interacting with peripheral heterochromatin and the lamina, which is more deformable in granulocytes due to reduced levels of lamin A/C. It is also speculated that LBR, which possesses sterol reductase activity, might be involved with nuclear membrane growth. Furthermore, LBR is one of the first NE proteins to bind to chromosomes during post-mitotic nuclear reformation, which may be an important time during the cell cycle for determining interphase nuclear architecture. The influence of LBR dosage on nuclear shape, although most dramatic in granulocytic cells, has been detected in other cell types. Lymphoblastoid cells from normal individuals frequently display irregular or indented nuclear shape; whereas those from individuals with PHA show a more ovoid nuclear shape.[3] In addition, U2OS cells that overexpress LBR frequently exhibit irregular and indented nuclear shape, not seen in wildtype or LBR knockdown cells.[19]

It is anticipated that the development of a stable LBR knockdown HL-60 subline, capable of multiple paths of differentiation in vitro, will contribute to a deeper understanding of the structural and functional role of LBR within myeloid cells. These cells should permit one to ask whether LBR plays a pivotal role in establishing or stabilizing certain routes of differentiation in pluripotent hematopoietic cells.

Materials and Methods

Cell culture. HL-60/S4 was cultivated in RPMI 1640 medium plus 10% heated fetal calf serum and pen/strep as described earlier.[11] RA and TPA stocks were added to cells as described earlier.[11] The stable viral infected GFP and sh1 sublines were maintained in the same RPMI 1640 medium plus 1 μg/ml puromycin. During RA or TPA differentiation of the GFP and sh1 sublines, puromycin was not present within the media.

Measuring cell growth parameters. Growth curves for the S4 and sh1 cells were obtained using a Vi-CellXR Cell Viability Analyzer (Beckman Coulter, Brea CA). Cells were initiated at 10^5 cells/ml and viable cells measured on a daily basis (4 days). Viability was routinely >95%. Cell cycle measurements were made at the Flow Cytometry Core Facility (German Cancer Research Center), with data acquisition on a FACSCalibur (BD Biosciences, San Jose CA). Cells were fixed overnight with 70% ethanol (-20°C), washed in PBS, digested with RNase (50 μg/ml, 30 min, 37°C) and stained with Propidium Iodide (50 μg/ml, 10 min) before analysis.

LBR silencing. Lentiviral vectors were produced by co-transfection of HEK293T cells with the psPAX2, pMD2.G and pLKO.1 constructs (MISSION TRC-Hs 1.0).[22] Transfections were carried out using TransIT (Mirus Bio Corporation, Madison, WI). Virus was harvested 72 hours after transfection. Infections of HL60/S4 cells were carried out in the presence of 8 μg/ml of polybrene. Virus-containing supernatant was removed after 24 hours. shRNA construct MISSION NM_002296.2-548s1c1 was used for LBR silencing and pLKO.1-puro CMV GFP was used as a control (both Sigma-Aldrich, St. Louis MO). Initial selection with puromycin was performed in 12 well plates, which included untreated and "mock" (polybrene only) samples.

Immunoblotting and immunostaining. The numerous antibodies employed in this study have been described earlier.[7,11,14,15] These antibodies include: (1) guinea pig anti-LBR, emerin and plectin; (2) rabbit anti-SUN1 and SUN2; (3) mouse monoclonal anti-lamins A/C, B1, B2 and anti-vimentin, α-tubulin, β-actin, HP1α, HP1β and HP1γ. In addition, for **Figure 1**, the following antibodies were employed: anti-actin, pan-isoforms (20-33; A5060, Sigma Aldrich, St. Louis MO) and β-tubulin (ab6046, Abcam, Cambridge MA). Total cell extracts were prepared in 1 x Laemmli sample buffer as described previously.[11,14] Immunoblotting was performed by SDS-PAGE in 4–20% BioRad gradient gels, followed by transfer to Millipore Immobilon-P membranes and an ECL reaction captured with X-ray film.[11,14] During immunostaining, undifferentiated or RA treated cells were allowed to settle onto fresh poly-lysine-coated slides in a moist chamber (30 min, RT), fixed with 4% paraformaldehyde/PBS or with -20°C methanol/acetone, washed, permeabilized (0.1% Triton X-100/PBS) and blocked with 5% normal goat serum/PBS, using conditions previously published.[15] For some imaging experiments (**Fig. 6**), data were collected on a DeltaVision Core microscope (Applied Precision Inc., Issaquah WA) using either a 40x, 60x or a 100x objective. Images were deconvolved using the built in SoftWoRx software. For others (**Fig. 2**), images were obtained on a Zeiss 710 confocal microscope using a 63x objective. Image collection parameters were kept constant allowing a semi-quantitative

Table 1. Relative changes in the levels* of various proteins during RA treatment of S4 and sh1 cells

	S4		sh1	
Protein	**0**	**RA**	**0**	**RA**
LBR	+	++	~0	+/-
Lamin A/C	+	~0	+	+/-
Lamin B1	+	+	+	+
Lamin B2	+	+	+	+
Emerin	+	+	+	+
SUN1	+/-	~0	+/-	~0
SUN2	+	+	+	+
Vimentin	+	~0	+	~0
α-Tubulin	+	+	+	+
HP1α	+	+	+	+
HP1β	+	+	+	+
HP1γ	+	+	+	+

*The relative protein levels were evaluated from immunoblots. Each antibody reaction was evaluated under the same ECL exposure conditions on a single blot. Notation: ++, very strong; +, medium to strong; +/-, trace reaction; ~0, negligible reaction.

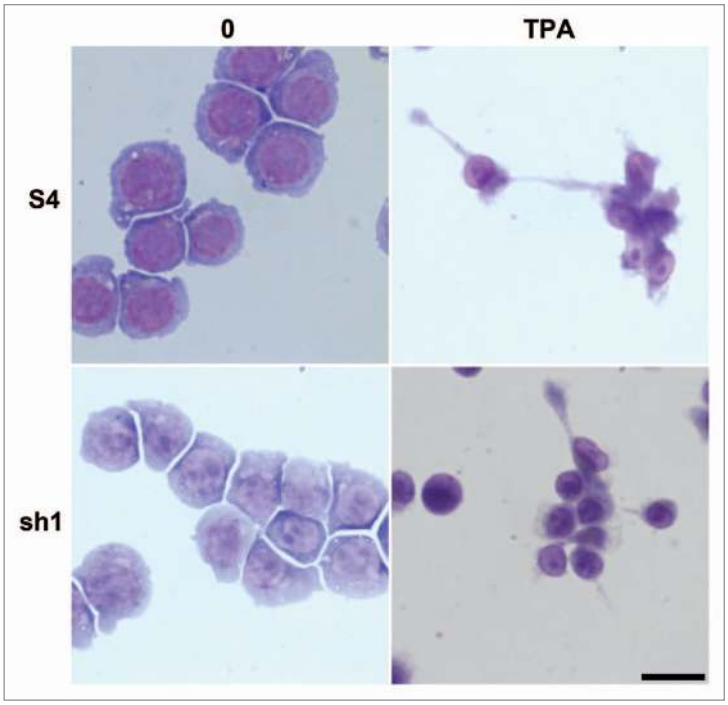

Figure 5. Wright-Giemsa stained preparations of S4 and sh1 cells following differentiation to macrophage form. Cell states: 0, undifferentiated; TPA, macrophage differentiation by TPA treatment for three days. Sublines: S4, top row; sh1, bottom row. Magnification bar: 10 μm.

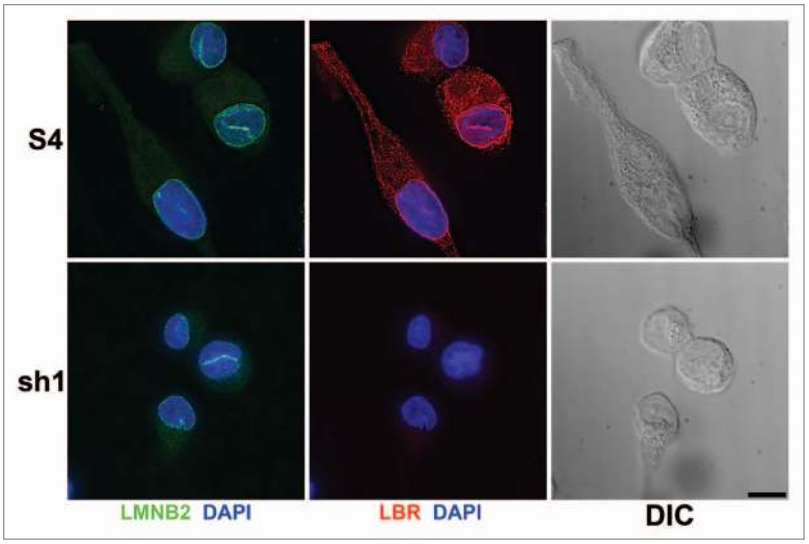

Figure 6. Deconvolved immunostaining images of S4 and sh1 cells after differentiation to macrophage with TPA. The left column of images is a merge of staining with anti-lamin B2 (green) and DAPI (blue); middle column, merge of anti-LBR (red) and DAPI (blue). The right column (DIC) includes differential interference contrast images of the same fields, more clearly indicating the cytoplasm and cell membranes with protrusions. Magnification bar: 10 μm.

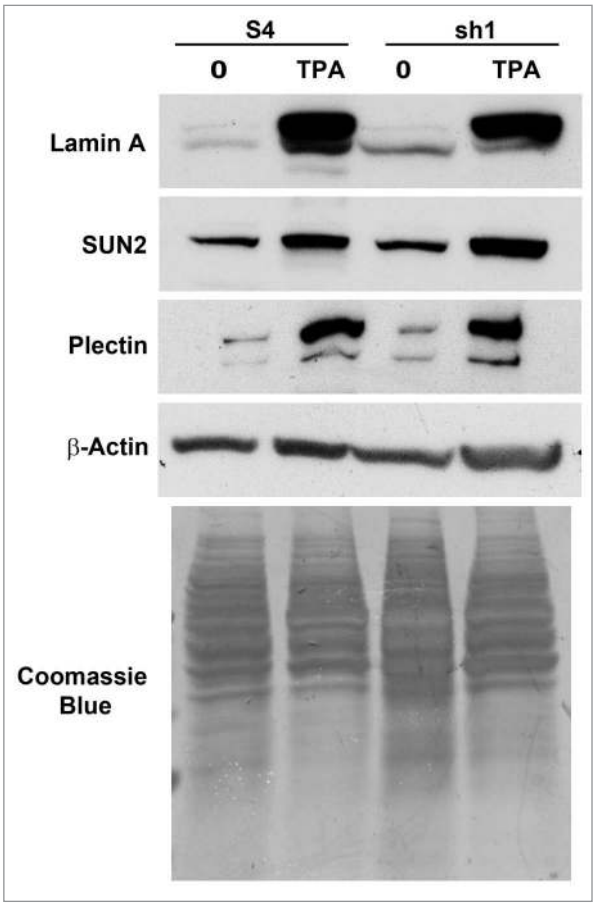

Figure 7. Immunoblotting of total cell extracts from undifferentiated and TPA treated S4 and sh1 cells. Cell states: 0, undifferentiated cells; TPA, macrophage differentiation for four days. The Coomassie Blue stained membrane demonstrates that the protein loads were well matched.

comparison of the levels of anti-LBR staining in the different samples. The data was collected by Dr. Sven Poppelreuther at the Carl Zeiss MicroImaging Application Center, Im Neuenheimer Feld 581, 69120 Heidelberg. Except for **Figure 2** (bottom row), no image processing was applied to the confocal data; Photoshop was employed to permit clearer visualization of nuclear shape (bottom row).

Electron microscopy. The suspension cells HL-60/S4 and HL-60/sh1, undifferentiated or treated with RA for 4 days, were centrifuged into a pellet, fixed with glutaraldehyde and OsO_4, washed, dehydrated, embedded in epon, thin sectioned and stained, as described in previous publications.[10,23] Images were collected on a Philips 400 electron microscope at the German Cancer Research Center (Heidelberg).

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