Plasma-membrane-anchored growth factor pro-amphiregulin binds A-type lamin and regulates global transcription

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Summary
Amphiregulin (AR), a member of the EGF family, is synthesized as a type I transmembrane protein precursor (proAR) and expressed on the cell surface. Shedding of proAR yields a transmembrane-cytoplasmic fragment (AR-CTF), as well as a soluble AR. Here we demonstrate that the proAR-shedding stimuli trigger endocytosis of both AR-CTF and un-shed proAR. ProAR translocates from the plasma membrane to the inner nuclear membrane, whereas AR-CTF is translocated to the lysosome via retrograde membrane trafficking. Nuclear envelope localization of proAR involves truncation of the C-terminus, which subsequently activates the ER-retrieval signal. The truncated form of proAR interacts with A-type lamin and is retained at the inner nuclear membrane. Heterochromatin formation is then induced and global transcription is transiently suppressed. This study gives new insight into epigenetic chromatin organization in mammalian cells: a plasma-membrane-anchored growth factor is targeted to the inner nuclear membrane where it participates in dynamic chromatin organization and control of transcription.

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Key words: Amphiregulin, Heterochromatin, Inner nuclear membrane, Lamin, RNA polymerase II transcription

Introduction
Over the 25 years, research on the epidermal growth factor (EGF) family and EGF receptors has provided numerous key insights into development, homeostasis and disease. The EGF family comprises 13 members, all of which are synthesized as type I transmembrane protein precursors and are subsequently expressed on the plasma membrane (Higashiyama et al., 2008). These transmembrane forms serve not only as juxtacler factors (Anklesaria et al., 1990; Higashiyama et al., 1995; Singh and Harris, 2005), but also as intermediaries of EGF-receptor (EGFR) transactivation (Blobel, 2005; Higashiyama et al., 2008), which can play a major role in signaling by G-protein-coupled receptors (GPCRs), cytokine receptors and receptor tyrosine kinases (Rozengurt, 2007). Transactivation of EGF-Rs is mediated, in many cases, by soluble forms of EGF-family ligands, which are cleaved from their membrane-anchored forms (proforms) in a process termed ‘ectodomain shedding’ (Higashiyama et al., 2008).

The precursor of amphiregulin (proAR), which is a member of the EGF family, shares overall structural homology with other members of the EGF family. ProAR shedding from the cell surface involves a disintegrin and metalloprotease 17 (ADAM17) (also known as tumor necrosis factor-α converting enzyme or TACE) (Sahin et al., 2004; Hinkle et al., 2004). Ectodomain shedding of proAR is induced by stimuli, such as 12-0-tetradecanoylphorbol-13-acetate (TPA). Activation of proAR shedding produces a plasma-membrane-anchored remnant C-terminal fragment (AR-CTF) and leaves a significant amount of un-shed proAR on the cell surface (Tokumaru et al., 2000). The fate of AR-CTF and proAR post-activation of the shedding process remains unclear. Recently we reported that ectodomain shedding of proHB-EGF, another member of the EGF family, evokes two independent signaling pathways: EGFR signaling induced by the shed extracellular domain and signaling driven by a remnant peptide comprised of the transmembrane and cytoplasmic domains (Nanba et al., 2003). Cytoplasmic domain signaling is mediated by interaction with at least two transcriptional repressors, known as promyelocytic leukemia zinc finger (PLZF) and B-cell lymphoma 6 (Bcl6), reversing PLZF- and Bcl6-mediated gene repression (Nanba et al., 2003; Kinugasa et al., 2007). We have also demonstrated translocation of proHB-EGF from the cell surface to the inner nuclear membrane (INM), where binding to the transcriptional repressors probably occurs (Hieda et al., 2008).

The nuclear envelope consists of the INM and an outer nuclear membrane (ONM), which are joined at the nuclear pore membrane. The ONM is structurally continuous with the peripheral ER. The nuclear lamina is a scaffold-like network of protein filaments underneath the INM. The scaffold consists primarily of type V intermediate filament proteins: lamin A/C, which is encoded by a single gene (LMNA) and expressed only in differentiated cells; and lamin B, which is encoded by two human genes (LMNB1 and LMNB2) and found in nearly all somatic cells (Vlcek et al., 2001; Gruenbaum et al., 2005). Lamins support a broad range of functions through interaction with
INM translocation of proAR

Various proteins, including INM proteins, chromatin, components of the RNA-polymerase-II-dependent transcription complex, and DNA replication complexes, are translocated to various diseases termed laminopathies. To date, there are about 50 known mutations in LMNA that cause laminopathies. These diseases affect striated muscle (Emery-Dreifuss muscular dystrophy or EDMD), cardiac muscle (dilated cardiomyopathy CMD1A), limb-girdle muscle and neurons (Charcot-Marie-Tooth disorder AR-CMT2B1), adipose tissue (familial partial lipodystrophy FPLD), and adipocytes and bone (mandibuloacral dysplasia MAD) (Mounkes et al., 2003).

The results of the present study demonstrate that (1) AR-CTF and un-shed proAR are internalized in response to ectodomain-shedding stimuli and then translocated to lysosomes and ER or nuclear envelope, respectively; (2) un-shed proAR is subsequently translocated to the INM where it is retained; and (3) the INM-targeted un-shed proAR induces heterochromatin formation and suppression of global transcription.

**Fig. 1.** ProAR translocates from the plasma membrane to the inner nuclear membrane. (A) Schematic representation of human AREG gene products. The AR coding region is translated as a precursor form (pre-proAR) with five structural domains consisting of predicted 252 amino acids (Plowman et al., 1990). The signal sequence is trimmed off, and the resulting protein is expressed on the plasma membrane as proAR whose N-terminal sequence is not yet determined (*). In response to various stimuli, proAR is shed at the juxtamembrane domain, resulting in the production of AR and AR-CTF. Anti-AR-N and anti-AR-C pAbs specifically recognize the extracellular and the cytoplasmic domains of proAR, respectively. The single line is the epitope region of anti-AR-N pAb, and double line is that of anti-AR-C pAb. (B) HeLa cells were transfected with a full-length AREG plasmid, then incubated with or without TPA. Cells were immunostained with anti-AR-N or anti-AR-C pAbs. (C) HeLa cells transiently expressing AREG were incubated with 20 μg/ml cycloheximide for 60 minutes. The cells were then treated with TPA and immunostained with anti-AR-N pAb. (D) HeLa cells transiently expressing AREG were permeabilized with digitonin and then fixed. The cells were re-permeabilized with (left panels) or without (right panels) Triton X-100, and immunostained with anti-AR-C (green) or anti-lamin B (red) pAbs. The lower panels are high magnification images of the boxed regions labelled 1 and 2. Scale bars: 5 μm. (E) Ultra-thin sections were stained with anti-V5 mAb and 15 nm gold-conjugated secondary antibodies. Right panel, without TPA; middle panel, with TPA; left panel, high magnification image of boxed region in middle panel. PM, plasma membrane; NE, nuclear envelope. Scale bars: 200 nm.
Results

Un-shed proAR and AR-CTF are targeted to the INM and lysosome, respectively.

Amphiregulin (AR; official symbol AREG) is synthesized as a type I transmembrane protein (pre-proAR) and is expressed on the plasma membrane as a 25-50 kDa precursor (proAR). ProAR contains an extracellular EGF-like domain, a transmembrane segment and a short cytoplasmic tail (Fig. 1A). ProAR is cleaved at the juxtamembrane domain via metalloprotease activation, yielding a soluble AR and a C-terminal fragment that contains transmembrane and cytoplasmic segments (AR-CTF). HeLa cells transiently transfected with AREG were immunostained with two polyclonal antibodies, anti-AR-N and anti-AR-C, which recognize the extracellular and cytoplasmic domains of proAR, respectively. ProAR-positive staining was visualized at the plasma membrane and the perinuclear organelles under steady-state conditions (Fig. 1B, upper panels). The perinuclear signal colocalized with the Golgi complex marker protein, GM130 (data not shown). In the presence of a shedding stimulus (TPA), the anti-AR-N and anti-AR-C signals were both observed in the reticular meshwork, evidently in the ER and nuclear envelope (Fig. 1B, lower). This result suggests translocation of un-shed proAR from the plasma membrane to the ER or nuclear envelope. To verify that the proAR in the reticular meshwork was not newly synthesized protein, de novo synthesis was blocked with cycloheximide. In the presence of cycloheximide, the proAR signal in the Golgi disappeared; however, proAR staining was evident at the plasma membrane and shifted to the ER and nuclear envelope after TPA stimulation (Fig. 1C). Thus, proAR on the plasma membrane translocates to the ER and nuclear envelope.

If proAR is targeted to the INM, cytoplasmic domains face the nucleoplasm and can physically interact with proteins localized inside the nucleus. Thus, it is crucial to determine whether nuclear-envelope-targeted proAR localizes to the ONM or to the INM. We took advantage of the fact that low concentrations of digitonin selectively permeabilize the plasma membrane, but leave the nuclear membrane intact (Adam et al., 1990). Digitonin treatment allowed detection of anti-AR-C signal at the plasma membrane and Golgi without TPA treatment and at the ER after TPA treatment. Lamin B signal was not detected, whether cells were treated with TPA or not. Lamins form a network of filaments underlying the INM and require Triton X-100 permeabilization for antibody detection. After Triton X-100 treatment and digitonin permeabilization, an AR-C signal was detected at the nuclear envelope.

Fig. 2. ProAR is targeted to the nuclear envelope. (A) Schematic presentation of AR deletion mutants. All constructs (AR-V5, AR-ΔC1-V5, AR-ΔC2-V5, AR-ΔC3-V5 and AR-ΔC4-V5) were V5-tagged at the C-terminus. (B) Three monoclonal antibodies mAb2, mAb5 and mAb9 against the cytoplasmic domain of proAR were established. The core epitope region for each antibody is underlined. (C) Total lysates of HeLa cells expressing AR-V5 or AR-ΔC-V5 mutants were analyzed by immunoblotting with the three mAbs. An anti-V5 mAb was used as a positive control. (D) Lysates of HeLa cells expressing wild-type AREG were immunoblotted with anti-AR-C mAbs. (E and F) HeLa cells transiently expressing wild-type AREG were incubated with or without TPA for 30 minutes and immunostained with the anti-AR-C mAbs (E) or mAb2 and anti-LAMP-1 mAbs (F). (G) HeLa cells transiently expressing wild-type AREG were treated with 50 nM Bafilomycin (Baf) for 60 minutes and incubated with TPA in the presence of Baf. The cells were then immunostained with mAb2 and anti-LAMP-1 mAb. Scale bars: 5 μm.
envelope in response to TPA treatment and a lamin B signal was evident, which was independent of TPA treatment (Fig. 1D). The detection of AR-C signal after Triton X-100 and digitonin treatment indicated that the cytoplasmic domain of proAR was exposed into the nucleoplasm after TPA stimulation (Fig. 1D, lower panel). These results suggest that the un-shed proAR could be targeted to the INM after TPA treatment. Next, we detected proAR at the nucleoplasmic face of the nuclear envelope in TPA-treated cells using immunoelectron microscopy (Fig. 1E). We conclude that un-shed proAR can localize at the INM upon shedding stimuli.

To identify the functional domain(s) involved in intracellular localization, we prepared monoclonal antibodies (mAbs) against the cytoplasmic domain of proAR, and obtained three clones (termed mAb2, mAb5 and mAb9) that recognized distinct epitopes (Fig. 2B). To identify the epitope region, we used the C-terminal deletion mutants of AR (Fig. 2A). The ability of mAb2, mAb9 and mAb5 to bind proAR was abrogated by deleting the C-terminal 5, 15 and 20 amino acids of proAR, respectively (Fig. 2C). In western blot analysis, mAb5 efficiently recognized proAR, but not the ~10 kDa AR-CTF (Fig. 2D). The mAb9 bound both proAR and AR-CTF, whereas mAb2 preferentially bound AR-CTF (Fig. 2D). Because AR-CTF encompasses the mAb5 epitope region, these results suggest that the mAb5 epitope region might be modified in AR-CTF. Immunofluorescent microscopy revealed that all mAbs stained the plasma membrane and the Golgi complex in the absence of TPA, although the signal from mAb2 was weaker than that from the other antibodies (Fig. 2E, upper panel). After TPA treatment, mAb5 and mAb9 detected proAR localized in the ER and nuclear envelope (Fig. 2E, lower panel), which was also detected with anti-AR-N pAbs (data not shown). However, mAb2 did not detect proAR in the ER and nuclear envelope (Fig. 2E, lower panel), suggesting that the mAb2 epitope might be masked or removed in proAR localized in this region. Interestingly, organelles detected by mAb2 were partially recognized by a mAb that was specific for LAMP-1, which is a lysosome marker protein (Fig. 2F), but were not recognized by anti-AR-N pAb (data not shown). In the presence of bafilomycin (Baf), which inhibits the lysosomal proton pump, thereby suppressing membrane trafficking through lysosomes, the signal for mAb2 significantly colocalized with the LAMP-1 signal (Fig. 2G).

These results imply that un-shed proAR targeted to the ER and nuclear envelope might be modified at the C-terminus (i.e. in the mAb2 epitope region), whereas AR-CTF accumulates in lysosomes. We speculate that masking of the epitope recognized by mAb5 in AR-CTF might signal lysosome targeting. Thus, we focused hereafter on the localization and function of un-shed proAR.

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**Fig. 3.** ProAR interacts with A-type lamin. (A) HeLa cells transiently expressing wild-type AREG were incubated with or without TPA. Cells were stained with anti-AR-C pAb and anti-lamin A/C mAb. Arrowheads indicate AREG-expressing cells. Scale bars: 5 μm. (B) Lysates of HeLa cells transiently transfected with or without wild-type AREG and treated with TPA for 60 minutes, were subjected to immunoblotting using anti-lamin A/C mAb (left) or anti-lamin B pAb (right). The left lanes show standard proteins. (C) HeLa cells transiently expressing wild-type AREG were incubated with or without TPA for 60 minutes. The cell lysates were subjected to immunoblotting with an anti-AR-C pAb (lane 1 and lane 2). The cell lysates were immunoprecipitated with an anti-lamin A/C mAb or normal mouse IgG, followed by western blotting using anti-AR-C pAb and anti-lamin A/C mAb. Arrows indicate the molecular mass of lamin A/C-interacted (upper) and AR-CTF (lower). (D) Schematic representation of GST-fused lamin A deletion mutants. (E) The cell lysates from cells transiently expressing wild-type AREG were incubated with GST or GST-lamin A derivatives, which were pulled down by glutathione-Sepharose beads. Bound proteins were analyzed by western blotting using the anti-AR-C pAb (upper panel). GST fusion proteins were analyzed by SDS-PAGE and stained with CBB (lower panel).
Un-shed proAR interacts with A-type lamin

Some proteins are tethered to the INM because of interactions with INM-resident proteins (Holmer and Worman, 2001). The intensity of anti-lamin A/C mAb staining decreased in AR-expressing cells after TPA treatment (Fig. 3A), even though lamin protein levels remained unchanged (Fig. 3B). This observation suggests a physical interaction between AR and A-type lamin. Immunoprecipitation was used to examine this possibility. Un-shed proAR, but not AR-CTF, co-precipitated with an anti-lamin A/C mAb; TPA treatment markedly enhanced binding between AR and lamin A/C (Fig. 3C). Precipitated un-shed proAR migrated slightly faster than proAR, suggesting that it was smaller than proAR (Fig. 3C). Neither normal mouse IgG nor anti-lamin B pAb immunoprecipitated un-shed proAR (Fig. 3C and supplementary material Fig. S1A). GST-tagged deletion mutants of lamin A pulled down proAR from the total cell lysate. This result demonstrates that a sequence within the lamin A region spanning residues 247-355 (shared with lamin C) is essential for the interaction with AR (Fig. 3D,E).

Next, siRNA duplexes targeting lamin A/C were used to examine the lamin A/C requirement for INM localization of un-shed proAR. Transfection with siRNA duplexes resulted in a marked and specific reduction in levels of lamin A/C protein (Fig. 4A). Lamin A/C downregulation abrogated localization of un-shed proAR in the nuclear envelope, but did not affect accumulation of proAR in the perinuclear structure in the presence of TPA (Fig. 4B). Control siRNA or knockdown of lamin B did not affect nuclear envelope targeting of proAR (Fig. 4B and supplementary material Fig. S1B). Thus, it was concluded that lamin A/C is essential for localization of proAR to the nuclear envelope.

Un-shed proAR that interacts with A-type lamin is truncated at the C-terminus

Because the proAR that interacted with lamin A/C migrated slightly faster than proAR (Fig. 3C) and mAb2 did not recognize the proAR targeted to the ER and nuclear envelope in immunofluorescence studies (Fig. 2E), we hypothesized that the epitope recognized by mAb2 might be removed from INM-localized proAR. This hypothesis was tested by immunoprecipitation studies. As shown in Fig. 5A, mAb2 was unable to detect proAR that had been co-

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**Fig. 4.** A-type lamin is essential for targeting of proAR to the nuclear envelope. (A) HeLa cells were transfected with 20 nM or 50 nM of lamin A/C-targeting duplex siRNA (si-lamin A/C) or control siRNA (si-control). After 72 hours, the cells lysates were analyzed by western blotting with anti-lamin A/C mAb or anti-lamin B pAb. (B) HeLa cells were transfected twice: first with 20 nM of control siRNA (upper) or lamin A/C-siRNA (lower); and, after 48 hours, with wild-type AREG plasmid. After 24 hours, cells were stimulated with or without TPA, and then fixed. Cells were stained with anti-AR-C pAb and anti-lamin A/C mAb. The lower panels (1 and 2) are high magnification images of the boxed areas above. Scale bars: 5 μm.

**Fig. 5.** Un-shed proAR that interacts with A-type lamin is truncated at the C-terminus. (A) HeLa cells transiently expressing wild type AREG were incubated with or without TPA. Cell lysates were immunoprecipitated with anti-lamin A/C mAb and analyzed by western blotting using the mAb5 and mAb2. (B) HeLa cells transiently expressing AR-V5 were incubated with or without TPA. Cell lysates were immunoprecipitated with anti-lamin A/C mAb. Cell lysates (upper) and precipitated proteins (lower) were analyzed with indicated mAbs. (C) Cell lysates of HeLa cells expressing AR-V5, AR-ΔC1-V5 (lane 1), AR-ΔC2-V5 (lane 2) or AR-ΔC3-V5 (lane 3) were immunoprecipitated with anti-lamin A/C mAb. Cell lysates and precipitated proteins were analyzed with β-actin, anti-V5 mAb or mAb5.
precipitated with lamin A/C. This result implies that proAR that interacts with lamin A/C lacks the portion of the C-terminus containing the epitope recognized by mAb2. We examined this possibility in greater detail using C-terminal-V5-tagged proAR (Fig. 2A). Anti-AR-N and anti-AR-C pAbs recognized the co-precipitated proAR, but an anti-V5 mAb did not (Fig. 5B). These results demonstrate that the proAR that interacts with lamin A/C did not contain the V5 tag. Furthermore, two deletion mutants, AR-ΔC1-V5 and AR-ΔC2-V5 (Fig. 2A) were co-precipitated with an anti-lamin A/C mAb. Thus, the C-terminal 10 amino acids are not required for lamin A/C binding (Fig. 5C). All proAR proteins co-immunoprecipitated with anti-lamin A/C mAb were the same size (Fig. 5C, bottom panel) and were not detected using an anti-V5 mAb (Fig. 5C, second panel from the bottom). Taken together, these results show that proAR interacting with lamin A/C is truncated at the C-terminus. Mass spectrometric analysis of proAR co-immunoprecipitated with anti-lamin A/C mAb was performed to determine the cleavage site. However, the lysine- and arginine-rich sequence in the cytoplasmic domain made it difficult to identify the enzymatic digest products.

Lys239 and Lys240 in the cytoplasmic domain of proAR are essential for targeting to the nuclear envelope

Most ER-resident type I transmembrane proteins have the ER-retention signal, K-K-x-x (x is any amino acid), at the C-terminus (Nilsson et al., 1989; Jackson et al., 1990). Interestingly, the K-K-x-x motif exists in the cytoplasmic domain of proAR, although it is not exposed at the distal terminus (Fig. 6A). When Ala residues were substituted for Lys239 and Lys240 (AR-m2) in proAR, targeting to the nuclear envelope did not occur, even after TPA treatment. Instead, the proAR-m2 accumulated in the perinuclear region colocalized with TGN46, a trans-Golgi marker (Fig. 6B). These results indicate that Lys239 and/or Lys240 are essential for ER targeting. To analyze the mechanism of proAR targeting to the ER, a V5 epitope tag was inserted into proAR just downstream from the transmembrane domains (AR-V5-C). The V5-tag insertion had no effect on localization of proAR to the plasma membrane and Golgi under steady state conditions. Likewise, insertion of the V5 tag did not affect targeting to the nuclear envelope in response to TPA stimulation (Fig. 6C,D). Notably, deletion of 10 residues at the C-terminus (AR-V5-ΔC10) to expose 239KKLR242-induced localization of proAR to the ER and nuclear envelope, even in the absence of TPA treatment (Fig. 6D). However anti-lamin A/C mAb staining was only slightly reduced in AR-V5-ΔC10 transfectants (compare Fig. 6E and Fig. 3A) and the interaction with lamin A/C was very weak (Fig. 6F). We then created an 11-residue deletion mutant (AR-V5-ΔC11), which exposed 238RKKL241 at the C-terminus, and observed ER and nuclear envelope localization in the steady state. Anti-lamin A/C mAb staining was markedly reduced and a significant interaction with lamin A/C was observed (Fig. 6E,F). In addition, a 12-residue deletion mutant of...
proAR (AR-V5-ΔC12), in which 237ERKK240 was exposed at the C-terminus, did not localize to the ER and nuclear envelope (data not shown). These results indicate that 239KKLR242 and 238RKKL241 in the cytoplasmic domain of proAR function as ER-retention signals when exposed at the distal C-terminus, but the activity of AR-V5-ΔC11 seems to be equivalent to that of the wild type, unlike AR-V5-ΔC10. Thus, the exact cleavage site remains to be identified. Although it is plausible that lamin-A/C-interacting proAR might lack 11 amino acids at the C-terminus, resulting in ER and nuclear envelope targeting.

Nuclear-envelope-targeted proAR masks a lamin A/C epitope in dense heterochromatin
The intensity of lamin A/C mAb staining was decreased in cells expressing nuclear-envelope-targeted proAR, even though A-type lamin protein levels remained unchanged (Fig. 3A,B) and anti-lamin A/C pAb stained these cells (data not shown). Epitope masking of lamin A/C in immunofluorescence studies as a result of interactions with chromatin and other proteins is a well known phenomenon (Hozák et al., 1995; Dyer et al., 1997; Kumaran et al., 2002; Markiewicz et al., 2005). To explore epitope masking due to lamin interactions with chromatin or other proteins, AR-expressing cells were treated with DNase I, NaCl and/or Triton X-100 before fixation (Markiewicz et al., 2005). Triton X-100 and/or NaCl extraction had no affect on lamin A/C staining. When cells were treated with Triton X-100, NaCl and DNase I, the lamin A/C signal was apparently recovered (Fig. 7A). We tried to extract DNA using NaCl and DNase I; however, the efficiency of chromosomal DNA extraction, judged by Hoechst 33342 staining, was too low (data not shown). These results suggest that epitope masking occurs because of physical associations between lamin and chromatin. Intriguingly, the epitope for the anti-lamin A/C mAb is located in a region spanning amino residues 356-571 (data not shown), which corresponds to the chromatin-binding domain (Zastrow et al., 2004).

Nuclear-envelope-targeted proAR induces diverse heterochromatin assembly
To verify the physiological role of nuclear-envelope-targeted AR, we focused on the proAR-lamin-A/C interaction. We observed that nuclear envelope targeting of proAR induced heterochromatinization, as evidenced by strong staining with Hoechst 33342 (Fig. 7B). Overexpression of other INM proteins (e.g. HB-EGF and lap2β) did not induce this heterochromatinization (supplementary material Fig. S3). Higher-order assembly of chromatin is thought to be largely determined by post-translational methylation of histone tails at H3 Lys9 (H3K9), which is essential for localization of HP1. As expected, it could be observed that Hoechst 33342 staining was accompanied by an increase in trimethylation of H3K9 (H3K9me3) in cells with nuclear-envelope-targeted proAR. Moreover, the heterochromatin protein, HP1β colocalized with Hoechst 33342 staining in these cells (Fig. 7B). Western blot analysis confirmed the relative increase of H3K9me3 in cells transiently expressing proAR after TPA treatment and cells expressing AR-Δ11 without TPA treatment (Fig. 7C).

Nuclear-envelope-localizing proAR suppresses global transcription
Heterochromatin can propagate, and thereby influence, gene expression in a region-specific
and sequence-independent manner. When heterochromatin spreads across domains, it generally causes epigenetic repression of nearby sequences. Thus, the diverse heterochromatin formation in AR-expressing cells prompted us to examine global transcriptional activity. Synthesis of RNA transcripts was monitored using incorporation of bromo-uridine (Br-U) (Iborra et al., 1998). This analogue does not affect activity of the major polymerizing enzyme RNA polymerase II. Newly synthesized Br-U-labeled RNA (Br-RNA), with the exception of nucleolar RNA, can be detected with anti-BrdU mAb after formaldehyde fixation (Koberna et al., 1999). In the present study, newly synthesized RNA was dramatically reduced in cells with nuclear-envelope-targeted proAR, even though Br-RNA intensity in untransfected cells was not affected by TPA treatment (Fig. 8A) and overexpressed HB-EGF or lap2β (supplementary material Fig. S2B,C). To exclude the possibility that reduced transcriptional activity was due to cell death, cell proliferation and Br-U incorporation were monitored for 24 hours. Quantitative analysis showed that global transcription was dramatically suppressed after exposure to a shedding stimulus by approximately 80% for up to 4 hours, after which time transcription was recovered within 24 hours. Statistical analysis showed that there are significant differences between the presence and absence of shedding stimuli. Interestingly, ectopic expression of AR-V5-ΔC11, which is localized to the nuclear envelope in the absence of shedding stimuli suppressed global transcription without TPA treatment (Fig. 8C). Moreover the global transcription in the AR-V5-C-expressing cells in which lamin A/C was knocked down was not affected (supplementary material Fig. S3). These results indicate that proAR-lamin-A/C interaction induces heterochromatinization and global transcriptional repression. The total pool of hyperphosphorylated and hypophosphorylated largest RNA polymerase II subunit was unchanged in western blot analysis (data not shown). Heterochromatin assembly is commonly associated with large-scale chromatin condensation and reorganization of the nuclear domain. Both these conditions reduce the accessibility of transcription machinery to heterochromatic loci. Thus, it was concluded that nuclear-envelope-targeting of proAR suppressed the global transcription. This might be due to reduced accessibility rather than regulation of transcriptional machinery.

**Discussion**

A model of proAR targeting from the plasma membrane to the inner nuclear membrane

The efficiency of proAR-ectodomain shedding is moderate (Tokumaru et al., 2000), resulting in the production of AR, AR-CTF and un-shed proAR. Herein, we provide evidence that proAR is translocated from the plasma membrane to the INM, where it is tethered by interaction with A-type lamin. Based on our results, we propose a model for this retrograde transport pathway, as shown in Fig. 9. First, proAR is primarily localized at the plasma membrane. Shedding stimuli induce internalization of both AR-CTF and un-shed proAR. Internalized AR-CTF and un-shed proAR are then targeted to different destinations, the lysosome and INM, respectively, suggesting different functions. The molecular mechanisms of the differential intracellular sorting remain unknown, but two types of modifications in the cytoplasmic region may be key events: epitope masking and truncation of the C-terminus. Masking of the epitope recognized by mAb5 in AR-CTF was suggested by the observation that this antibody was barely able to detect AR-CTF, whereas mAb2 showed a strong signal. We speculate that this masking modification might act as a lysosomal-sorting signal. AR-CTF is likely to have a full-length cytoplasmic tail given the fact that it can bind to mAb2, even though...
INM-targeted proAR is truncated at the C-terminus. Deletion mutants of proAR suggest that the R/K-K-x-x sequence might function as an ER-retrieval signal. Disruption of this ER-retrieval signal sequence in wild-type proAR (AR-m2) abrogated the INM targeting typically observed in response to shedding stimuli. Thus, proAR utilizes this ER retrieval signal to localize to the ER. Truncation of the cytoplasmic tail might be crucial in determination of the intracellular fate of AR-CTF compared with un-shed proAR. AR-V5-ΔC11 is localized at the nuclear envelope without shedding stimuli and decreased lamin A/C staining. AR-V5-ΔC11 is co-precipitated with anti-lamin-A/C antibody. AR-V5-ΔC10 is localized at the nuclear envelope in immunofluorescence studies; however, its effect on the lamin A/C staining and transcriptional repression is not relevant to cells expressing the wild-type proAR. Therefore we assume that removal of the 11 amino acids at the C-terminus of proAR is necessary to achieve nuclear targeting of proAR. This is the first report to describe exposure and activation of the internal ER-retrieval signal in a mammalian protein; it has been described for some protein toxins (e.g. Pseudomonas exotoxin) (Sandvig and van Deurs, 2002). Accumulation of the AR-m2 mutant at the TGN suggests that proAR may translocate by way of the TGN, where truncation of the C-terminus might occur.

After translocation to the ER, proAR can diffuse laterally between the peripheral ER, ONM and INM via the nuclear pore complex, because here the cytoplasmic domain is small enough to pass through the aqueous channel. Alternatively, proAR can be translocated from the ONM to the INM via active transport, which requires protein interactions. Some integral INM proteins possess a basic-sequence motif that resembles a ‘classical’ nuclear localization signal (NLS) and binds to karyopherin-α (King et al., 2006). However, the cytoplasmic domain of proAR did not show any detectable NLS activity for soluble reporter proteins (data not shown).

Possible mechanism and roles for proAR-induced heterochromatin formation and transient suppression of global transcription

We observed that INM targeting of proAR triggers heterochromatin assembly and negatively regulates transcription by RNA polymerase II. Chromosomal DNA contains a high density of repetitive DNA elements, including constitutive heterochromatin. However, heterochromatin is also found at developmentally regulated loci, where the chromatin state can change in response to cellular signals and gene activity. This type of heterochromatin is known as facultative heterochromatin. The basic mechanisms that underlie proAR-induced heterochromatin formation are largely unclear. However, lamin-proAR interaction is likely to enhance the lamin-chromatin interaction because of the masking of an antigenic epitope in the chromatin-binding domain of lamin A/C. The ProAR-interacting domain in lamin A/C is shared with other lamin-A/C-binding proteins, such as the Kruppel/TFII-related zinc finger protein MOK2 (Dereuillet et al., 2002), retinoblastoma protein (Rb) (Ozaki et al., 1994) and LAP2α (Markiewicz et al., 2005), suggesting that this might have an important role in their function. Rb and LAP2α are well known regulators of cell cycle and gene transcription in the nucleoplasm. Thus, chromatin organization might be dynamically regulated via these soluble lamin-binding proteins throughout the nucleoplasm. Furthermore, the ability of heterochromatin to spread in cis and to be coordinately regulated with other heterochromatin regions in trans may play a role in diverse heterochromatin assembly. Interestingly, the disease of premature aging, Hutchinson-Gilford Progeria Syndrome (HGPS), is caused by a 50-residue deletion mutation at the C-terminus of lamin A (LAΔ50). Nuclei in LAΔ50 mutant cells display a loss of heterochromatin, indicating that the C-terminal region of lamin A/C plays a role in chromatin organization. ProAR-dependent heterochromatin formation might be mediated via regulation of lamin function. Heterochromatinization is nearly synonymous with epigenetic gene silencing; however, there are some reports that suggest heterochromatin formation is required for activation of gene expression (Weiler and Wakimoto, 1995; Lu et al., 2000; Yasuhara and Wakimoto, 2006).

AR has been characterized as a growth factor involved in pathophysiological cell proliferation and differentiation. Thus, proAR translocation into the INM and interaction with lamin A/C, resulting in the induction of heterochromatinization and global transcriptional suppression, might participate in dynamic epigenetic reprogramming of gene expression during cell proliferation and differentiation.
Materials and Methods

**Plasmids**

Human AREG cDNA was subcloned into the EcoRI-Xhol sites of the pME18s vector (full-length AREG) or V5-tagged-pME18s vector (AR-V5). AR-V5-C was generated by insertion of a sequence encoding the V5-tag using PCR with appropriate oligonucleotide DNA primers producing the coding sequence. Mutants of truncated forms of AREG were generated using PCR and inserted into the EcoRI-Xhol sites of pME18s (AR-AC-V5) or pME18s-V5 (AR-AC1-V5-AR-AC4-V5). The 10- or 11-residue deletion mutant of AREG or AR-V5-C were generated using PCR and inserted into the EcoRI-Xhol sites of pME18s (AR-AC11, AR-V5-AC10, and AR-V5-AC11). Mutants with Ala substitutions at Lys239 and Lys240 of wild-type AR were generated using PCR-based site directed mutagenesis. All the cDNA constructs described previously (Ozaki et al., 1994) were generated using a CEFQ 8000 DNA Analysis System (Beckman Coulter), pGEX-lamin A derivatives were gifts from Toshinori Ozaki (Ozaki et al., 1994). The plasmids pME18S-HB-EGF-V5-C and pGEG-C1-hlap2IIR were described previously (Hieda et al., 2008).

**Antibodies**

Affinity-purified rabbit pAbs against synthetic peptides corresponding to the cytoplasmic region of proAR (residues 233-250), i.e. AR-C pAb was obtained from Immuno Biological Laboratories (IBL). Goat pAb against the extracellular region of the cytoplasmic region of AR, anti-AR-N pAb was purchased from R&D Systems. The mouse anti-v5 mAb Was from Invitrogen. Mouse anti-lamin A/C mAb (636), the mouse anti-LAMP-1 mAb (HAA3) and goat anti-lamin B pAb (M-20) were from Santa Cruz Biotechnology. Mouse anti-β-actin mAb (AC-15) was from Sigma. Sheep anti-human TGN46 pAb was from Serotec. Mouse anti-GM130 mAb was from BD Transduction Laboratories. HRP-conjugated secondary Abs were from Promega. FITC- or Cy5-conjugated secondary antibodies were from Jackson Immuno Research Laboratories. Anti-HIP1β rat mAb (MACS), anti-histone H3 dimethyl K9 mAb (AB2), anti-histone H3 trimethyl K9 pAb were from Abcam. Rabbit anti-GFP pAb was purchased from Medical & Biological Laboratories (MBL).

**Preparation of monoclonal antibodies**

Monoclonal antibodies were generated using the rat lymph node method (Sado et al., 1995). Briefly, Wky/Ncrj rats (Charles River Japan) were immunized with an emulsion containing a KLH-conjugated synthetic peptide, which corresponds to that of the cytoplasmic region of AR, and Freund’s complete adjuvant. Cells from the lymph nodes were fused with mouse myeloma Sp2/0 cells. The hybridomas were screened using ELISA and three clones (No.2, No.5 and No.9) were established. The isotype subclass of mAb2, mAb5 and mAb9 were determined to be IgM, IgG2b and IgG1, respectively, using a rat monoclonal antibody ID/SP Kit (Zymed Laboratories).

**Cell culture, transfection and TPA treatment**

HeLa cells were grown in DMEM (Nikken Biomedical Laboratory) supplemented with 10% fetal bovine serum (HyClone). Transfections were performed using Lipofectamine2000 (Invitrogen). Obtained from B-Bridge International. RNA dimers were transfected into HeLa cells using Lipofectamine2000 (Invitrogen). The isotype subclass of mAb2, mAb5 and mAb9 were determined to be IgM, IgG2b and IgG1, respectively, using a rat monoclonal antibody ID/SP Kit (Zymed Laboratories).

**Digitonin permeabilization**

For digitonin permeabilization, the cells were incubated with transport buffer (20 mM HEPES pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EDTA and 2 mM DTT) containing 33 μg/ml digitonin on ice for 5 minutes. After permeabilization, cells were washed and fixed with 4% formaldehyde in transport buffer.

**Preparation of cell lysate**

Cells were lysed in ice-cold lysis buffer, PBS (-) containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail (Roche). Cell lysates were incubated for 30 minutes at 4°C and centrifuged at 9100 g for 10 minutes. The resulting supernatants were used as total cell lysates.

**Immunoprecipitation**

Total cell lysates were incubated with anti-lamin A/C mAb for 2 hours at 4°C. Protein-G-Sepharose beads (Amersham Biosciences) were then added to the mixture. After incubation for 1 hour, the suspension was centrifuged, and the collected protein-G-Sepharose beads were washed three times with lysis buffer. The bound proteins were analyzed by western blotting.

**GST pull-down assays**

GST and GST-lamin mutants were produced in and purified from E. coli, as described previously (Ozaki et al., 1994). Total cell lysates were mixed with 2 μg recombinant GST or GST-lamin mutants and glutathione-Sepharose beads for 2 hours at 4°C. The bound proteins were analyzed by SDSPAGE, followed by western blotting.

**Immunofluorescence microscopy**

Cells were fixed with 4% formaldehyde for 20 minutes and permeabilized with 0.2% Triton X-100 for 10 minutes at room temperature. Cells were blocked with 2% BSA and incubated with primary antibodies overnight at 4°C. Cells were viewed with an epifluorescence microscope (IX70, Olympus).

**siRNA knockdown**

A specific RNA dimer that targets the coding sequence of human A-type lamins at nucleotides 608-626 was obtained from Dharmacon. Specific RNA dimers corresponding to B-type lamins, lamin B1 and lamin B2 (Tasi et al., 2006) were obtained from B-Bridge International. RNA dimers were transfected into HeLa cells using Lipofectamine2000 (Invitrogen).

**Br-U incorporation and its quantification**

Cells were grown in 5 mM Br-U for 30 minutes and fixed with 4% paraformaldehyde. Br-RNA was immunolabeled using a rat anti-BrdU mAb (Abcam). The fluorescent intensity in the nucleus was measured in 200 cells with Image-pro plus (MediaCybernetics). The analysis was performed in triplicate and results were considered significant at a level of P<0.05.

**Nuclear extraction**

HeLa cells transiently expressing proAR were extracted using a protocol described previously (Markiewicz et al., 2005) with some modifications. Cells were rinsed twice with TM buffer (20 mM Tris-HCl pH 7.5, 3 mM MgCl2) and then incubated for 10 minutes on ice in TM buffer containing 0.4% Triton X-100, 0.5 mM CuCl2 and protease inhibitors. After washing with TM buffer, cells were incubated with DNase I (20 U/ml) for 20 minutes at 37°C, or/and incubated with 2 μM NaCl for 5 minutes on ice.

**immunogold electron microscopy**

The cells were fixed with 2% paraformaldehyde/0.25% glutaraldehyde, dehydrated and embedded in LR White resin. Ultrathin sections were blocked with 1% BSA and incubated with anti-mouse IgG (15 nm; BBInternational, UK). After incubation, the grids were counterstained with 2% uranyl acetate and lead citrate and examined with a JEOL JEM1230 transmission electron microscope.

**pGEX-lamin A derivatives**

A derivatives were a gift from Toshinori Ozaki (Chiba Cancer Research Institute). Mouse myeloma cells (SP2/0) were a gift from Yoshikazu Sado (Shigei Medical Research Institute). We thank Hidehiko Iwabuki for technical help with plasmid construction and Higashiyama’s laboratory members for useful discussion. This study was supported by Grants-in Aid for Scientific Research No.19570182 to MH, and No. 17014068 and No. 17390081 to S.H. from the Ministry of Education, Culture, Sports, Science and Technology and from Japan Society for the Promotion of Science, and Precursory Research for Embryonic Science and Technology (Information and Cell Function), JST, Japan.

**References**


Figure S2.

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Figure S3.

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*: si-lamin A/C (-)/Br-U incorporation (+)
*: si-lamin A/C (+)/Br-U incorporation (+)
*: si-lamin A/C (-)/Br-U incorporation (-)