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PMA Induces SnoN Proteolysis and CD61 Expression through an Autocrine Mechanism

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Abstract

Phorbol-12-myristate-13-acetate, also called PMA, is a small molecule that activates protein kinase C and functions to differentiate hematologic lineage cells. However, the mechanism of PMA-induced cellular differentiation is not fully understood. We found that PMA triggers global enhancement of protein ubiquitination in K562, a myelogenous leukemia cell line and one of the enhanced-ubiquitination targets is SnoN, an inhibitor of the Smad signaling pathway. Our data indicated that PMA stimulated the production of Activin A, a cytokine of the TGF- β family. Activin A then activated the phosphorylation of both Smad2 and Smad3. In consequence, SnoN is ubiquitinated by the APC^{Cdh1} ubiquitin ligase with the help of phosphorylated Smad2. Furthermore, we found that SnoN proteolysis is important for the expression of CD61, a marker of megakaryocyte. These results indicate that protein ubiquitination promotes megakaryopoiesis via degrading SnoN, an inhibitor of CD61 expression, strengthens the roles of ubiquitination in cellular differentiation.

Keywords

Ubiquitin; SnoN; Cdh1; Smad2; Activin A; differentiation

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1. Introduction

Ubiquitin is an 8 kDa protein that is covalently conjugated to other proteins via an isopeptide linkage between its C-terminal glycine and a primary amino group on its substrates, which is usually from a lysine side chain [1–2]. This process, known as ubiquitination, is mediated by the sequential actions of three enzymes: a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin ligase (E3) that is directly involved in substrate recognition [1–2]. One of the major consequences of ubiquitination is proteolysis via the 26S proteasome, the primary pathway that regulates the levels of many short-lived proteins in eukaryotes. The ubiquitin and proteasome system is an important machine that regulates virtually all biological processes, such as cell cycle, DNA damage repair and immune responses etc [1–3].

While comprehensive information about the function of the ubiquitin and proteasome system in cell cycle control, DNA damage control, immune response, and proliferation have accumulated [3–7], much less is known about the roles of the ubiquitin and proteasome system in cellular differentiation. Cellular differentiation is the process by which stem cells or lineage-specific progenitor cells become more specialized cell types and is essential for development of multicellular organisms. One of many differentiation processes in mammals is megakaryopoiesis by which undifferentiated bipotent progenitor cells undergo terminal differentiation into highly differentiated, polyploid megakaryocytes [8–9]. Megakaryopoiesis is mainly regulated by lineage-specific transcription factors, such as Fli-1 and GATA2 [9]. The role of the ubiquitin and proteasome pathway in megakaryopoiesis is only starting to unfold [10–11].

Phorbol-12-myristate-13-acetate, also known as PMA, is a small molecule that activates protein kinase C [12–13]. PMA has been shown to induce megakaryopoiesis-like differentiation in certain leukemia cell lines [12–13], but the mechanism is unclear. Here we report that PMA induces expression of CD41 and CD61, which form a heterodimer, a bona fide marker of megakaryocytes [8]. We observed that two lineage-specific transcription factors, Fli-1 and GATA2 are also up-regulated by PMA. Moreover, we found that PMA induced the ubiquitination and degradation of SnoN, an inhibitor of the Smad signaling pathway. The ubiquitination of SnoN was mediated by APC^{Cdh1} and Smad2 that is phosphorylated upon PMA treatment. Furthermore, we found that PMA induced SnoN proteolysis via the induction of Activin A, a cytokine of the TGF β family, which suggests that PMA activated an autocrine mechanism to control SnoN turnover and megakaryopoiesis.

2. Materials and methods

2.1. Plasmids and small hairpin RNA (shRNA)

Open reading frames of human SnoN and Cdh1 were amplified by PCR with Phusion high fidelity DNA polymerase (New England Biolab) from HeLa cDNAs that were prepared using Superscript III cDNA synthesis kit (Invitrogen). Purified PCR products were cloned into a Gateway entry vector, pENTR/D-Topo (Invitrogen). After verification of their

sequences, the entry clones were shuttled to Gateway recipient vectors using LR clonase (Invitrogen).

Lentiviral shRNA vectors of human ACVR2A, Alk4, Cdh1, Smad2 and Smad3 were designed according to a published method [14].

2.2. Antibodies and other reagents

The following antibodies were employed in this study: Activin A (R&D SYSTEMS), Cdt2 (Bethyl Laboratories), Cul1, DDB1 and Skp2 (Invitrogen), Cdh1 (BD Biosciences), Cyclin B1, Fli-1 and Ski (Santa Cruz), SnoN (Abcam), CD41, CD61, GATA1, GATA2, Id2, Smad2, Smad3, Phospho-Smad2 (Ser465/467) and Phospho-Smad3 (Ser423/425) (Cell Signaling). The K48 and the K63 linkage-specific polyubiquitin antibodies were reported previously [15].

Phorbol-12-myristate-13-acetate (PMA), SB-431542 and A83-01 were purchase from Calbiochem, Tocris Bioscience and Reagents Direct, respectively.

2.3. Cell culture, cell line and lentivirus infection

K562 cells were maintained in RPMI1640 media supplemented with 10% fetal bovine serum and 5% CO₂ at 37 °C. The procedures of lentivirus production and infection were reported previously [16]. K562-His-Bio-Ubiquitin and Biotin-tagged SnoN cells were established by infection of lentivirus.

2.4. Cell lysis, and western blot

For western blot, cells were harvested and lysed in SDS lysis buffer by heating at 100 °C for 10 minutes. The lysate was then cleared by ultracentrifugation at room temperature. 10 µg lysate was separated in SDS-Polyacrylamide gels (SDS-PAGE) and then transferred to PVDF membrane for detection with antibodies.

2.5. Pulldown Experiment

Biotinylated proteins were purified according to previous report [17].

2.6. Flow cytometry Analysis

1×10⁶ DMSO or PMA treated K562 cells were washed twice with PBS containing 2%FBS (PBS-2%FBS), resuspended in 80 µl PBS-2%FBS, and incubated with the fluorescent-conjugated antibodies for 30 minutes on ice. Cells were then washed twice with PBS-2%FBS, resuspended in 0.5 ml PBS-2%FBS and were subjected to FACS analysis. The antibodies used in this experiment included: Alexa-647-conjugated anti-human CD61 (Biolegend Cat#336407); FITC-conjugated anti-human CD41 (Biolegend, Cat# 303703); FITC-conjugated mouse IgG1 κ isotype control (Biolegend Cat#400110) and Alexa-647-conjugated mouse IgG1 κ isotype control (Biolegend Cat#400130). Data obtained from the experiment were analyzed with Kaluza.

2.7. Quantitative RT-PCR, ELISA, and luciferase assay

Total RNAs isolated using Trizol (Invitrogen) were employed to make cDNAs using Superscript III cDNA synthesis kit (Invitrogen). Quantitative real-time PCR (qPCR) was performed using Roche LightCycler 480 SYBR Green I Master mix on Roche LightCycler 480 machine.

The concentration of Activin A secreted to the cultured medium of K562 cells was determined by ELISA using a kit from R&D Systems (Cat# DAC00B). Briefly, K562 cells (1×10^6 /ml) were treated with DMSO or PMA at 20nM for the indicated time. The medium was collected and the concentration of Activin A was assayed according to the manual of the kit. Each sample was measured at triplicates to obtain standard deviation.

Luciferase assays were done using Dual-Luciferase Reporter Assay System (Promega).

3. Results

3.1. PMA induces the expression of CD41 and CD61 in K562 cells

PMA is an activator of protein kinase C and can trigger megakaryocyte differentiation in multiple cell lines [12–13]. One example is K562, a myelogenous leukemia cell line that maintains a differential potential for both erythrocytes and megakaryocytes. Upon hemin treatment, K562 can be differentiated into erythrocytes [18]. In contrast, PMA induces K562 to differentiate into megakaryocyte cells [12–13].

To recapitulate the capability of PMA to induce differentiation of megakaryocyte cells, we examined the expression of both CD61 and CD41 proteins, which form a heterodimer and are *bona fide* markers of megakaryocytes [8]. K562 cells express no detectable CD61 or CD41 (Figure 1A). Using a western blot approach, we found that PMA induced CD61 expression in 24 hours and CD41 in 48 hours after PMA treatment (Figure 1A). Flow cytometry analysis with both CD61 and CD41 antibodies showed that ~12.93% K562 cells expressed both markers at 48 hours after PMA stimulation. At 96 hours post PMA treatment, ~56.85% of K562 cells became doubly positive for both signals (Figure 1B). The induction of both CD61 and CD41 is likely regulated at transcriptional level, because mRNAs of both markers were increased upon PMA induction (Figure 1C, Supplemental Figure 1A). Interestingly, however, CD61 expression was induced much earlier than CD41. In fact, both mRNA and protein of CD61 were detectable as early as ~6 and ~8 hours after PMA treatment, respectively (Figure 1C, Supplemental Figure 1B), whereas CD41 expression was not detectable until 48 hours after PMA application (Figure 1A, Supplemental Figure 1A).

Cellular differentiation is often driven by lineage-specific transcription factors [9]. Therefore, we examined expression of both Fli-1 and GATA2, two important transcription factors of megakaryocytes and found that expressions of both genes were enhanced by PMA (Figure 1A). Together, these data confirm that PMA can promote K562 to differentiate into megakaryocyte cells.

3.2. Overall ubiquitination is enhanced during PMA-induced megakaryopoiesis

To explore the potential roles of the ubiquitin signaling pathway in PMA-induced K562 differentiation, we first expressed a biotin-tagged version of ubiquitin in K562 cells (K562-Bio-Ub) (Supplemental Figure 2). As reported previously [17, 19–20], the expression of biotinylated ubiquitin allows us immunoprecipitate ubiquitinated proteins under denaturing conditions. Mammalian cells contain only a few endogenous biotinylated proteins [21]. Therefore, more specific results can be achieved. We treated K562-Bio-Ub cells with PMA to trigger differentiation. Cells were collected at four and eight hours post PMA treatment. Ubiquitinated proteins were collected using streptavidin resin. Purified ubiquitinated proteins were separated in a SDS-PAGE gel and detected by western blot with the anti-Ubiquitin antibody FK2, which specifically binds to conjugated ubiquitin. Some ubiquitinated proteins were collected from cells treated with DMSO as a control (Figure 2A). However, much more ubiquitinated proteins were purified after PMA treatment (Figure 2A). These data suggest that the whole ubiquitination machinery was much more active after PMA stimulation.

3.3. Cdh1 is upregulated during PMA-induced megakaryopoiesis

Differentiated cells must exit cell cycle at first. Therefore we sought to analyze the expression of several E3s that are involved in cell cycle control. We observed that expression of most E3s was either relatively constant, as observed for DDB1 (Figure 2B) or was decreased, as in the case of Cdt2, Skp2, Cul1, and CDC20 (Figure 2B). Cdh1 expression, however, was enhanced (Figure 2B). Cdh1 is an activator of anaphase promoting complex/Cyclosome (APC/C), which suggests that the APC/C^{Cdh1} ubiquitin ligase was more active upon PMA treatment. The fact that Cdc20, the other activator of APC/C ubiquitin ligase, was down-regulated (Figure 2B) implied that the APC/C^{Cdh1} ubiquitin ligase was specifically activated. This finding suggests that the ubiquitin substrates of APC/C^{Cdh1} should become more unstable during PMA-induced differentiation.

3.4. Degradation of SnoN is mediated via the ubiquitin and proteasome pathway

APC/C^{Cdh1} is the ubiquitin ligase that is mainly involved in cell cycle regulation [6–7]. Recent studies, however, indicated that APC/C^{Cdh1} is also important for cellular differentiation [22–25]. APC/C^{Cdh1} regulates the ubiquitination and degradation of several differentiation inhibitors, such as SnoN, an inhibitor of Smad signaling pathway [26–28]. Therefore, we surveyed the expression of SnoN, a substrate of APC/C^{Cdh1} [29]. We found that SnoN was down-regulated (Figure 3A). Both MG132 and Bortezomib, two inhibitors of the 26S proteasome, could restore the SnoN expression (Figure 3B and 3C), indicating that SnoN was degraded by the 26S proteasome. Ski, a homolog of SnoN, and Cyclin B1, another substrate of APC/C^{Cdh1} were also down-regulated (Figure 3B and 3C). However, neither MG132 nor Bortezomib could restore their expression, suggesting that Ski and Cyclin B1 were suppressed via a pre-proteolysis mechanism. Interestingly, Id2, another reported substrate of APC/C^{Cdh1} was up-regulated upon PMA stimulation for 24 hours (Figure 3B), whereas its expression could be enhanced by MG132 regardless of PMA treatment (Figure 3B), implying that the enhancement of Id2 expression was a pre-proteolysis event as well.

To further confirm that SnoN is ubiquitinated upon PMA treatment, we purified ubiquitinated proteins from K562-Bio-Ub cells treated with PMA using streptavidin beads. Ubiquitinated proteins were resolved on a SDS-PAGE gel as reported [17]. Anti-SnoN antibody was then employed to examine whether SnoN was ubiquitinated. As shown in Figure 3D, the ubiquitination of SnoN was undetectable prior to PMA application, but was robustly induced 8 hours after PMA stimulation, although the overall expression of SnoN was reduced upon PMA treatment.

To characterize the linkage of the polyubiquitin chain attached to SnoN upon PMA treatment, we expressed Biotin-tagged SnoN in K562 cells (K562-Bio-SnoN) and treated K562-Bio-SnoN cells with PMA for 24 hours (Figure 3E). MG132 was added to accumulate more ubiquitinated SnoN two hours before sample collection. We then purified Bio-SnoN using streptavidin resin under a denaturing condition. Ubiquitinated and unmodified SnoN proteins were resolved on a SDS-PAGE gel. We found that purified Bio-SnoN proteins reacted with anti-K48 polyubiquitin-specific antibody (Figure 3F), but not with anti-K63 polyubiquitin-specific antibody (data not shown). Residual ubiquitinated SnoN was identified before PMA treatment (Figure 3F, upper panel), perhaps due to the over-expression of Bio-SnoN. However, stronger signal was detected upon PMA treatment (Figure 3F, upper panel), although less SnoN proteins were purified (Figure 3F, low panel). These data strongly support that K48 polyubiquitin chain was synthesized on SnoN upon PMA treatment.

Together, these data indicate that PMA induces SnoN turnover via the ubiquitin and proteasome pathway.

3.5. SnoN is an inhibitor of CD61 expression

To evaluate the physiological function of SnoN destruction in PMA-induced differentiation, we over-expressed exogenous SnoN in K562 cells using lentivirus infection. Ectopic expression of either untagged or Flag/HA-tagged SnoN reduced CD61 induction (Figure 4A, compare Lanes 2, 4 and 6), implying that SnoN is an inhibitor of K562 differentiation. The expression of exogenous SnoN was decreased after PMA treatment (Figure 4A, Compare Lanes 3–6), further supporting that PMA-induced SnoN reduction is a post-transcriptional event.

SnoN contains a D-box motif which is a common degron sequence among the substrates of the APC/C^{Cdh1} ubiquitin ligase [27–28]. We mutated two critical residues (arginine-164 and leucine-167) of the D-box motif of SnoN into alanines (Supplemental Figure 3) and ectopically expressed the D-box mutant of SnoN in K562 cells using lentivirus infection. We found that the D-box mutant inhibited CD61 induction (Figure 4B, compare Lanes 2, 4 and 6). Interestingly, the D-box mutant was more resistant than wild type SnoN to PMA-induced proteolysis (Compare Figure 4A and 4B), indicating that the D-box motif is important for PMA-triggered SnoN turnover. Therefore, it is highly possible that the APC/C^{Cdh1} protein complex is the ubiquitin ligase to induce SnoN proteolysis.

3.6. Cdh1 is required for SnoN degradation

At least three ubiquitin ligases, including APC/C^{Cdh1}, Smurf2 and Arkadia, have been reported to ubiquitinate SnoN [27–28, 30–32]. The observations that Cdh1 expression was enhanced by PMA (Figure 2B) and that the D-box mutant of SnoN was less liable for proteolysis (Figure 4A & 4B) suggested that APC/C^{Cdh1} was the prime ubiquitin ligase for SnoN during PMA-induced differentiation. To prove this hypothesis, we silenced Cdh1 in K562 cells using a lentivirus vector that expressed a shRNA targeting human Cdh1 (Figure 4C). We found that both SnoN degradation and CD61 induction were blocked (Figure 4C). These results confirm our hypothesis that Cdh1 is in charge of SnoN ubiquitination and degradation during PMA-induced megakaryopoiesis.

3.7. Smad2 and Smad3 are activated upon PMA treatment

Our results indicate that Cdh1 controlled SnoN turnover during PMA-induced megakaryopoiesis. To further support our hypothesis, we over-expressed Cdh1 in K562 cells using lentivirus infection method (Figure 4D). However, we found that over-expressing Cdh1 alone could not repress SnoN expression (Figure 4D), although expression of Cyclin B1, (Figure 4D) was repressed. This finding indicates that additional cofactors might be required for Cdh1-mediated SnoN proteolysis. In the TGF β signaling pathway, phosphorylation of Smad2 and/or Smad3 is required for APC/C^{Cdh1}-mediated SnoN destruction [22, 27–28]. Therefore, we analyzed the phosphorylation status of both Smad2 and Smad3 upon PMA treatment and observed both Smad proteins were phosphorylated upon PMA treatment (Supplemental Figure 4). Interestingly, the induced phosphorylation of both Smads by PMA was maintained over the course of PMA treatment (Supplemental Figure 4). Moreover, the phosphorylation of Smad3 was stimulated as early as 2 hours after PMA application, whereas that of Smad2 was triggered around 4–8 hours (Figure 5A). Considering that the ubiquitination and degradation of SnoN appeared around 6–8 hours after PMA treatment (Figure 3D & Figure 5A), we believe that Smad2 was the major cofactor to assist Cdh1 to ubiquitinate SnoN.

Both Smad2 and Smad3 are transcription factors that are activated by ligands of the TGF β subfamily, including TGF β , Activin and Nodal etc. To investigate whether the TGF β signaling pathway was involved in SnoN degradation and PMA-induced megakaryopoiesis, we employed SB-431542, an inhibitor of type I TGF β subfamily receptor kinases, including Alk4, Alk5 and Alk7 [33]. As expected, SB-431542 inhibited the phosphorylation of Smad2 and Smad3, SnoN degradation, as well as induction of both CD41 and CD61, but not Fli-1 (Figure 5B). Application of A83-01 [34], another specific inhibitor of type I TGF β subfamily receptor kinases produced similar results (Figure 5B). These data suggest that TGF β subfamily receptor kinases play important roles in promoting megakaryopoiesis through the ubiquitination and proteolysis of SnoN, whereas the induction of Fli-1 is independent of signals triggered by TGF β subfamily receptors.

3.8. Smad2 is required for SnoN turnover

Given the fact that the phosphorylation of Smad2 appears at the similar time to SnoN proteolysis after PMA treatment, Smad2 phosphorylation might be required for SnoN turnover. To confirm our hypothesis, we silenced Smad2 in K562 cells using lentivirus

expressing shRNAs (Figure 5C). We found that Smad2 depletion efficiently blocked PMA-stimulated SnoN degradation and CD61 induction (Figure 5C). Therefore, Smad2 is required for SnoN degradation and CD61 induction. Considering that overexpressing Cdh1 could not trigger SnoN proteolysis (Figure 4D), we believe that the activated Smad2 signaling pathway plays important roles in SnoN turnover and CD61 induction.

To further prove that the role of Smad2 in PMA-induced SnoN proteolysis, we constructed a SnoN A3 mutant (T89A/L90A/Q92A) that is defective in Smad2 binding [26]. We expressed the SnoN A3 mutant in K562 cells using a lentivirus infection approach and found that this SnoN mutant was resistant to PMA-induced SnoN degradation (Figure 5D). Consistent with this observation, we found that the SnoN A3 mutant could attenuate the induction of CD61 (Figure 5D). Together, these data support the idea that Smad2 functions as an adaptor to assist APC/C^{Cdh1} to ubiquitinate SnoN and PMA-stimulated SnoN proteolysis is important for CD61 expression.

3.9. Expression of Activin A and its receptors is enhanced upon PMA treatment

Both Smad2 and Smad3 are phosphorylated when TGF β receptors are activated by their ligands, such as TGF β , Activin or Nodal. To determine which TGF β receptors were involved in PMA-induced phosphorylation of Smad2 and Smad3, as well as SnoN proteolysis, next, we examined the expression of Alk4 and Alk5 after PMA stimulation. Semi-quantitative RT-PCR results indicated that Alk4 was up-regulated, while Alk5 is slightly down-regulated (Supplemental Figure 5A). RT-PCR based on two pairs of primers produced similar induction pattern of Alk4 (Supplemental Figure 5A, Lanes 3–6), suggesting that up-regulated Alk4 might be responsible for the activation of Smad2 and Smad3. The induction of Alk4 was further confirmed by real time quantitative-PCR (qPCR) (Figure 6A). Alk4 is a type I receptor of activin. Upon ligand binding to type II receptor of activin, type II receptor phosphorylates a type I receptor, such as Alk4, which consequentially phosphorylates Smad2 and Smad3. Among two type II receptors that associate with Alk4, ACVR2B was not expressed in K562 cells (data not shown), whereas ACVR2A mRNA was slightly upregulated by PMA at later time points (Figure 6B), implying that ACVR2A is responsible for the activation of Smad2 and Smad3 signaling events.

The main ligands that activate Alk4 and ACVR2A receptors are Activins. Among them, Activin B transcript was not detectable in K562 cells, whereas Activin A mRNA was detected by semi-quantitative RT-PCR (Supplemental Figure 5B). Upon PMA stimulation, Activin A was highly induced, while Inhibin A, an inhibitor of Activins, was slightly up-regulated (Supplemental Figure 5B). The induction of Activin A was further validated by qPCR (Figure 6C), indicating that Activin A is the main ligand to trigger the TGF β signaling pathway during PMA-induced megakaryopoiesis. To explore the potential role of transcription in up-regulation of Activin A upon PMA treatment, we constructed a firefly luciferase reporter under the control of an Activin A promoter. Dual luciferase reporter assay showed that firefly luciferase activities were enhanced by ~7.51 and ~24.14 folds after 8 and 24 hours of PMA treatments, respectively (Supplemental Figure 6). All together, these data suggest that PMA turned on the Activin A-ACVR2A-Alk4 signaling cascade that leads

to Smad2 phosphorylation and SnoN degradation. The induced Activin A expression is likely regulated at transcriptional level.

Activin A is a secretable protein. Based on our data, we expect to detect Activin A in the culture media after PMA treatment. Using an ELISA approach with an anti-Activin A antibody, we detected ~10.28 pg/ml and ~640.01 pg/ml of Activin A in the culture media after 4 hours and 8 hours of PMA treatment, respectively, but not in the media after 8 hours of DMSO treatment (Figure 6D). We found ~23.916 ng/ml of Activin A in the media after 24 hours of PMA application (Figure 6D). These data indicate that Activin A was synthesized and secreted into the culture media upon PMA treatment.

3.10. Activin A and its receptors are required for SnoN degradation and CD61 expression

To validate the roles of ACVR2A and Alk4 in PMA-induced Smad2 phosphorylation, SnoN turnover and CD61 induction, we silenced their expression using shRNA technology (Figure 7A). We found that PMA-stimulated Smad2 phosphorylation, SnoN proteolysis and CD61 induction were attenuated when either ACVR2A or Alk4 was knocked down in K562 cells (Figure 7B). Together, these data support that ACVR2A and Alk4 play important roles in PMA-induced megakaryopoiesis.

To confirm the function of Activin A in PMA-induced Smad2 phosphorylation, SnoN turnover and CD61 induction, we first treated K562 cells with an anti-Activin A antibody together with PMA. We found that PMA-induced Smad2 phosphorylation, SnoN turnover and CD61 induction were attenuated by anti-Activin A antibody (Figure 7C). These data further validate the important role of Activin A in PMA-induced megakaryopoiesis. However, Smad3 phosphorylation was slightly reduced at high doses of Activin A antibody, but not at low doses (Figure 7C), indicating that PMA might initiate an alternative pathway to phosphorylate Smad3. Moreover, Fli-1 induction was insensitive to Activin A antibody treatment (Figure 7C), implying that PMA used an alternative pathway to induce Fli-1 expression as well. To prove our observation, we treated K562 cells with 10 ng/ml of Activin A and found that Activin A can induce phosphorylation of both Smad2 and Smad3, and SnoN degradation as early as 30 minutes (Figure 7D, left panel). These data indicate that Activin A alone can efficiently activate its receptor kinases which then phosphorylate both Smad2 and Smad3, and promote SnoN turnover. However, we were not able to detect any CD61 proteins by western blot (data not shown), suggesting that Activin A alone is insufficient to induce K562 differentiation. Similar data were obtained when we treated K562 cells with different amounts of recombinant Activin A for 8 hours (Figure 7D, right panel), further validating our initial observations.

To explore the possibility that different differentiation factors were secreted into the culture media after PMA treatment, we first stimulated K562 cells with either DMSO or PMA for 3 hours. We then washed PMA away with PBS and incubated K562 cells in the culture media without PMA or DMSO for another 5 hours. Conditioned media were collected and used to treat K562 cells for 24 hours. We found the PMA-treated conditional media can stimulate expressions of CD61 and Fli-1, and SnoN degradation (Figure 7E). These data suggest that beside Activin A, other differentiation factors were also secreted into the culture media after PMA treatment and they are important for PMA-induced megakaryopoiesis.

4. DISCUSSION

4.1. Overall ubiquitination is associated with megakaryopoiesis

Protein ubiquitination is an indispensable process that regulates many biological activities [2], although very limited information is available for the roles of ubiquitination in megakaryopoiesis [10–11]. The ubiquitin and proteasome pathway has been shown to degrade cMPL, a specific receptor for TPO, a specific ligand that is required for megakaryopoiesis. In this scenario, the ubiquitination of cMPL by its ubiquitin ligase, c-Cbl, likely plays a negative feedback role in megakaryopoiesis [10]. However, it was unclear whether ubiquitination could promote megakaryocyte differentiation until this study. Our data reveal some unprecedented observation, that is, the overall ubiquitination of proteins can be highly enhanced during PMA-induced megakaryopoiesis (Figure 2A). Our results also indicate that the enhanced ubiquitination did not apply to every ubiquitin substrates, because the ubiquitination of SnoN was increased, whereas that of GATA2 was decreased (data not shown). In concert with the differential regulation among ubiquitin substrates, ubiquitin ligases, i.e. specific factors in the ubiquitin signaling pathway, were also differentially regulated, as we observed that several cell cycle-related ubiquitin ligases were down-regulated, whereas APC/C^{Cdh1} was up-regulated (Figure 2B). APC/C^{Cdh1} ubiquitin ligase has been shown to mediate cellular differentiation of several tissue types [22–23, 25]. Whether APC/C^{Cdh1} is the sole ubiquitin ligase in charge of PMA-induced megakaryopoiesis remains to be determined. However, our preliminary results suggest that this may not be the case. We found that the monoubiquitination of Histone H2A at lysine-119 was rapidly increased after PMA treatment (data not shown). The monoubiquitination of Histone H2A at lysine-119 has been linked to transcriptional repression [35]. Clearly, there is no direct connection between APC/C^{Cdh1} and H2A monoubiquitination. Therefore, it is possible that the activity of the H2A ubiquitin ligase is enhanced or the signaling event leading to H2A monoubiquitination is more active upon PMA treatment. Considering cellular differentiation is a process during which cells switch from one type to another, it is conceivable that many proteins, such as cell cycle regulators, must be degraded in order for cells to exit cell division. Therefore, it will be very interesting to determine whether the enhancement of the overall ubiquitination is a universal phenomenon of cellular differentiation. Obviously, enhanced ubiquitination during PMA-induced megakaryopoiesis should not be limited to H2A and SnoN. Therefore, it will be equally important to determine which proteins are ubiquitinated during PMA-induced megakaryopoiesis and what is their biological significance.

4.2. Cdh1-mediated SnoN ubiquitination and turnover is required for CD61 induction

SnoN is a component of the Smad signaling pathway and plays important roles in development [26, 36]. As an inhibitor of the TGF β signaling pathway, SnoN binds to Smad4 to repress TGF β target genes in the absence of ligand and is involved in the negative feedback regulation of TGF β signaling pathway [37]. TGF β family ligands induce the phosphorylation and nuclear translocation of Smad2/3. In consequence, SnoN is ubiquitinated by the APC/C^{Cdh1} ubiquitin ligase with Smad2 or Smad3 as cofactors [22, 27–28], and degraded via the 26S proteasome. TGF β -induced SnoN proteolysis is important for the transactivation of TGF β targeting genes.

Our data suggest that PMA triggers phosphorylation of both Smad2 and Smad3 (Figure 5 and Supplemental Figure 4). However, it appears that Smad2 is essential for PMA-induced SnoN turnover and CD61 induction (Figure 5). Our data also indicate that SnoN is an inhibitor of CD61 expression and its proteolysis is important for CD61 induction (Figure 4A, 4B and 5D).

4.3. The Activin A-dependent pathway is induced by PMA through an autocrine mechanism

Megakaryopoiesis is an important process for hematopoietic progenitor cells to differentiate into megakaryocytes that produce platelets [8]. Current studies focus on the functions of megakaryocyte-specific transcription factors, such as Fli-1, GATA2 and RUNX1 [9], as well as the signaling pathway through thrombopoietin (TPO) and its receptor, cMPL [8, 38]. However, either TPO- or Mpl-knockout mice still remain 15~20% production of platelets and megakaryocytes [39–40], indicating that alternative signaling pathways exist to induce megakaryopoiesis. Therefore, our data might lead to identify additional pathways linked to megakaryopoiesis.

We found that PMA can induce Activin A expression (Figure 6C and 6D). This observation should not be restricted to K562 cells, because Activin A was up-regulated in CHRF-288-11, another megakaryocytic cell line, upon PMA treatment [41]. Activin A was required for PMA-induced SnoN turnover and CD61 induction (Figure 7), indicating that like TGF β , Activin A can trigger SnoN proteolysis via the ubiquitin and proteasome pathway. The induction of Activin A was a very quick and persistent process. This may explain why SnoN degradation was maintained at least four days after PMA treatment (Figure 3A), although TGF β -induced SnoN turnover is often a transient event [30]. Our data also show that PMA can up-regulate the expression of both Alk4 and ACVR2A (Figure 6), two receptors of Activin A. Consequently, the Smad2 signaling pathway was activated through phosphorylation. The phosphorylated Smad2 was then translocated into the nucleus where it triggered SnoN ubiquitination and degradation by the APC/C^{Cdh1} ubiquitin ligase and the 26S proteasome. Furthermore, we found that the SnoN degradation via the ubiquitin and proteasome pathway is important for CD61 induction. In summary, PMA induces SnoN proteolysis and CD61 expression through an autocrine mechanism (Figure 8).

Beside Activin A, our data also suggest that additional differentiation factors are secreted into the culture media and should play important roles in PMA-induced megakaryopoiesis (Figure 7).

Megakaryopoiesis is an important differentiation process to produce megakaryocytes and platelets which prevents bleeding [8]. Many studies focus on dissecting the roles of TPO, c-MPL, and the signaling pathway via TPO and c-MPL in megakaryopoiesis. However, neither TPO nor c-MPL is essential for megakaryopoiesis and platelets products [39–40], indicating that alternative pathway must exist for control of megakaryocyte differentiation. We found that SnoN is an inhibitor of CD61 expression, and the Smad2- and Cdh1-regulated SnoN ubiquitination and degradation is important for PMA-induced CD61 induction. Moreover, we found that PMA promotes production of Activin A which stimulates Smad2 phosphorylation and SnoN turnover. Therefore, the Activin A-Smad2/

Cdh1-SnoN signaling cascade provides new insight for megakaryopoiesis and could lead to the discovery of alternative pathways in control of megakaryopoiesis. Given the fact that Activin A, together with BMP4 can induce the formation of erythrocytes from non-mesodermal structures [42], it will be interesting to determine whether Activin A-stimulated SnoN degradation is important for erythrocyte differentiation. Our data also reveal a positive role of the ubiquitin and proteasome pathway in megakaryopoiesis. Certainly, the interplay between the ubiquitin signaling pathway and megakaryopoiesis will be a new research area that is worth for further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

PMA induces megakaryopoiesis of K562 cells. Cells were treated with PMA and collected at different time points.

(A). Analyze expressions of CD41, CD61, GATA2 and Fli-1 using western blots.

(B). Analyze CD41 and CD61 expression using Flow cytometry.

(C). Analyze CD61 expression using qPCR.

Figure 2.

The ubiquitination machinery is altered upon PMA treatment. K562-His-Bio-Ub or K562 cells were treated with PMA and collected at different time points.

(A). Overall ubiquitination was enhanced by PMA. K562-His-Bio-Ub cells were collected after 4 and 8 hours of PMA treatment. Ubiquitinated proteins were purified using Streptavidin beads under denatured conditions and subjected to SDS-PAGE electrophoresis. α-Ubiquitin (FK2) antibody was employed to detect ubiquitinated proteins.

(B). Different Ubiquitin-related genes were regulated by PMA. K562 cells were collected at different days post PMA treatment and subjected to SDS-PAGE electrophoresis. Antibodies against various ubiquitin ligases were used for western blots.

Figure 3.

SnoN is degraded via the ubiquitin and proteasome pathway upon PMA treatment. Western blots were employed to detect protein expression.

(A). SnoN was down-regulated by PMA.

(B). MG132 inhibited SnoN degradation. K562 cells were treated with PMA for 24 hours. MG132 at 20 μ M was added to block protein degradation.

(C). Bortezomib blocked SnoN turnover. K562 cells were treated with PMA for 24 hours. Bortezomib at 20 μ M was added to block protein degradation.

(D). SnoN was ubiquitinated. K562-Bio-Ub cells were collected after 8 hours of PMA treatment. Ubiquitinated proteins were purified using Streptavidin beads and subjected to SDS-PAGE electrophoresis. α -SnoN antibody was employed to detect ubiquitinated SnoN. (E). Exogenous Bio-SnoN responded to PMA treatment. K562 and K562-Bio-SnoN cells were treated with PMA for 24 hours. MG132 at 40 μ M was added to block SnoN turnover 2 hours before sample collection. Bio-SnoN was detected using Streptavidin-HRP. (F). K48 polyubiquitin chain was identified on SnoN. Collected cells from Figure 3E were lysed in a denaturing buffer and Bio-SnoN proteins were purified using Streptavidin beads. After resolving on a SDS-PAGE gel, ubiquitinated Bio-SnoN proteins were detected using ant-K48 polyubiquitin chain-specific antibody (upper panel). An α -SnoN antibody was employed to detect Bio-SnoN (low panel).

Figure 4.

Cdh1 is important for PMA-induced proteolysis of SnoN whose over-expression could attenuate CD61 induction. Both untagged and Flag/HA-tagged SnoN were expressed in K562 cells. PMA was used to trigger SnoN turnover and CD61 expression. Cells were collected at 7 or 24 hours post PMA treatment. ShRNA targeting Cdh1 was expressed using lentivirus system. Western blots were employed to detect protein expressions.

(A). Exogenous SnoN inhibits CD61 induction.

(B). The D-box mutant of SnoN was more stable and could block CD61 induction.

(C). Silencing Cdh1 inhibited SnoN turnover and CD61 induction. Endogenous Cdh1 was silenced using lentivirus expressing shRNA in K562 cells. Cells were collected at 7 hours (*) post PMA treatment to detect SnoN, Cdh1 and Vinculin. Cells were also collected at 24 hours (**) post PMA treatment for better results of CD61 induction using western blots.

(D). Over-expression of Cdh1 could reduce Cyclin B1, but not SnoN expression. Exogenous Cdh1 was over-expressed in K562 cells using lentivirus approach. Infected cells were subjected to western blots.

Figure 5.

Activated Smad2 signaling is required for SnoN turnover and CD61 induction.

(A). Both Smad2 and Smad3 were phosphorylated upon PMA treatment. K562 cells were treated with PMA for different times. Western blots were employed to detect expression of Smad2, Smad3, Phospho-Smad2 (Ser465/467) and Phospho-Smad3 (Ser423/425).

(B). Small molecule inhibitors of TGF β type I receptors blocked SnoN proteolysis and CD61 expression. SB413542 (10 μ M) or A83-01 (1 μ M) was added at the same time as PMA was done in K562 cells. Cells were collected 24 hours later and subjected to western blots.

(C). Knocking down Smad2 inhibited SnoN turnover and CD61 expression. Endogenous Smad2 was silenced using lentivirus expressing shRNA in K562 cells. Cells were collected at 8 hours post PMA treatment and subjected to SDS-PAGE electrophoresis and western blots.

(D). The SnoN A3 mutant inhibited CD61 induction. Threonine-89, Leucine-90 and Glutamine-92 of SnoN were mutated to Alanines as a SnoN A3 mutant (T89A/L90A/Q92A). The untagged A3 mutant was expressed in K562 cells using lentivirus infection. Expression of SnoN and CD61 were detected using western blot.

Figure 6.

The Activin A-ACVR2A-Alk4 signaling axis is activated upon PMA treatment. K562 cells were collected at different time points after PMA treatment for cDNA preparation using reverse transcription.

(A). Expression of Alk4 was examined using qPCR.

(B). Expression of ACVR2A was examined using qPCR.

(C). Expression of Activin A was examined using qPCR.

(D). Activin A proteins were secreted into the culture media after PMA treatment. Culture media were collected at different time points after PMA treatment. Activin A proteins were detected using ELISA.

Figure 7. Activin A and its receptors play important roles in PMA-induced megakaryopoiesis. (A). ACVR2A or Alk4 was silenced in K562 cells using shRNA. qPCR was used to show knockdown efficiency. (B). Silencing either ACVR2A or Alk4 could attenuate PMA-stimulated Smad2 phosphorylation, SnoN degradation and CD61 induction. Control and knockdown cells were treated with PMA for 8 hours and cells were collected for western blots.

(C). Activin A antibody could attenuate PMA-induced megakaryopoiesis. Different amounts of Activin A antibodies were used to treat K562 cells at the same time as PMA. 24 hours later, cells were collected for western blots.

(D). Recombinant Activin A proteins triggered Smad signaling pathway and SnoN turnover. Left Panel: Time course of Activin A treatment. Recombinant Activin A proteins (10 ng/ml) were employed to treat K562 cells. Cells were collected at different time points for western blots.

Right Panel: Dosage effect of Activin A. Different amounts of recombinant Activin A proteins were employed to treat K562 cells for 8 hours. Cells were collected for western blots.

(E). Conditional media could trigger the similar effect to PMA treatment. K562 cells were treated with either DMSO or PMA for 3 hours. PMA was then washed away with PBS and cultured for another 5 hours without PMA or DMSO to produce conditional media.

Conditioned media were collected and used to treat K562 cells for 24 hours. Cells were collected for western blots.

Figure 8.

Model of PMA-induced SnoN turnover and CD61 expression. PMA stimulates expression of Activin A which activates Smad2 signaling pathway via activating its receptors, such as Alk4. Phosphorylated Smad2 functions as a cofactor for the APC^{Cdh1} ubiquitin ligase to ubiquitinate SnoN. SnoN functions as an inhibitor of CD61 expression and its proteolysis via the ubiquitin and proteasome pathway is important for PMA-induced CD61 induction.