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CD226 is involved in megakaryocyte activation and early-stage differentiation

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ABSTRACT

This study was conducted to investigate the effect of CD226 on the differentiation, activation, and polyploidization of megakaryocytes (MKs) and explore the potential mechanism. Dami (megakaryocyte line) cell maturation was induced by phorbol 12-myristate 13-acetate. *CD226* was silenced by infection with a *CD226*-specific shRNA lentiviral vector. The mRNA level of *CD226* was detected by qRT-PCR. The expressions of Dami cells surface CD226, MK specific markers CD41 and CD62P, and DNA ploidy in Dami cells and *CD226* knockdown (KD) cells were evaluated by flow cytometry. The effect of CD226 on the expression of megakaryocyte-associated transcription factors was measured by western blot and confocal analysis. Transfection with CD226 shRNA lentivirus dramatically decreased the level of CD226 and expression of CD62 P in Dami cells. Silencing of *CD226* caused morphological changes and differentiation retardation in low-ploidy MK. Furthermore, *CD226* knockout (KO) mice exhibited increased 2N–4N low-ploidy MK and decreased ≥8N polyploidy. Interestingly, silencing of *CD226* in megakaryocytic cells down-regulated the expression of early stage transcription factors includes GATA-binding factor 1 (GATA-1) and friend leukemia integration 1 (FLI-1), but not late-stage nuclear factor, erythroid 2 (NF-E2). CD226 is involved in MKs activation and polyploidy cell cycle control.

1. Background

Megakaryocytes (MKs) are the precursors of platelets. These cells undergo proliferation, differentiation, and maturation followed by multiple rounds of endomitosis and polyploidy, finally fragmenting into platelets (Eliades et al., 2011). Dysfunction of MK proliferation, polyploidization, and maturation may lead to severe platelet disorders, which are related to various diseases such as thrombocytopenic purpura, anemia, and cardiovascular diseases. Thrombocytopenia has harmful effects in patients of HIV, dengue, and liver cirrhosis as well as during chemotherapy (Gutti et al., 2018).

CD226 is a member of the Ig superfamily and contains two Ig-like domains in its extracellular region. It was initially named T lineage-specific activation antigen and was found to modulate the differentiation of cytotoxic T lymphocytes (Burns et al., 1985). Subsequently, Burns and colleagues found that CD226 is also expressed on the platelet membrane with approximately 1 200 copies/platelet, and thus the antigen was re-named platelet and T cell activation antigen 1 (Scott et al., 1989). Later, Shibuya et al. proposed the designation of DNAX accessory molecule 1 and described CD226 as an important adhesion molecule that triggers cytotoxic T lymphocytes and natural killer (NK) cells (Shibuya et al., 1998).

CD226 is expressed on most monocytes, NK cells, T cells, a subset of B cells (Shibuya et al., 1998). CD226 interacts with other cells bearing its ligands Necl-5 (poliovirus receptor, CD155) and nectin-2 (PRR2, CD112) (Bottino et al., 2003; Tahara-Hanaoka et al., 2004). CD226 was demonstrated play roles in transendothelial migration in monocytes, tumor immunosurveillance receptor in NK cells and cytotoxic T lymphocytes, polarization of T cells into the Th1 type, and viral infections via inflammatory monocytes and NK cells (Fuchs and Colonna, 2006; Iguchi-Manaka et al., 2008; Xiong et al., 2015; Lenac Rovis et al., 2016; Gaud et al., 2018). Therefore, CD226 is involved in cancers, inflammation, viral infections immune tolerance, and autoimmune diseases (Morisaki et al., 2012; Stein et al., 2017; Liu et al., 2018; Zhang et al., 2016; Zhang et al., 2018). Downstream signaling transduction of CD226 depends on its association with important integrin molecule

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leukocyte function-associated antigen 1 (CD11a/CD18) to induce tyrosine phosphorylation of CD226 and Fyn protein tyrosine kinase activation (Shibuya et al., 1999; Xu and Jin, 2010).

Additionally, CD226 is expressed on MKs/platelets. This molecule is involved in platelet activation, aggregation independent of granule secretion, and adhesion of platelets and MK cells to vascular endothelial cells (Scott et al., 1989). Ma et al. demonstrated that CD226 is expressed on the megakaryocytic lineage and hematopoietic stem/progenitor cells. A CD226 monoclonal antibody combined with a leukocyte function-associated antigen 1 monoclonal antibody increased the ploidy of the MK derived from adult-derived $CD34^+$ cells (Ma et al., 2005). However, the mechanism of CD226 in the differentiation and activation of MK remains unknown.

Here, the effects of CD226 on MK maturation and development in the megakaryocytic Dami cell line and *CD226* gene knockout (KO) mice were tested. Both *CD226*-silenced Dami cells and *CD226* KO mice exhibited blockage in 2N–4N low-ploidy MK compared to their counterpart controls. Additionally, a deficiency of CD226 reduced the expression of CD62P and megakaryocyte-associated transcription factors, thereby inhibiting MKs activation and maturation.

2. Materials and methods

2.1. Reagents and antibodies

PMA and the protease inhibitor panel were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium, fetal bovine serum, antibiotics, and Trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA, USA). SYBR Green Real-time PCR Master Mix and the reverse transcription kit were purchased from Takara Bio, Inc. (Shiga, Japan). The rat anti-mouse CD41-APC antibody and rat anti-mouse CD62p-PE antibody were purchased from eBioscience (San Diego, CA, USA). The mouse anti-human CD226 antibody (clone no. C9) was prepared and used as we previously reported (Xu et al., 2009). Rabbit anti-human FLI-1 antibody, rabbit anti-human GATA-1 antibody, and mouse anti-human β-actin antibody were purchased from BOSTER (Hubei, china). Mouse anti-human NF-E2 antibody and horseradish peroxidase-labeled goat anti-rabbit/goat anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

2.2. Cell culture

Dami (human megakaryocytic) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C with 5% CO_2 and saturated humidity. In this experiment, the cells were exposed to 100 nM PMA to induce MKs.

2.3. Mice

Male *CD226* KO mice (C57BL/6 background, 8–10 weeks) were kindly provided by Prof. Marco Colonna. Genotyping was performed following a standard protocol and three primers were used: 1. TGTTC AAATCTAACCCAGAG; 2. GCAGAAGCCAGTACAGTTTGAAGTT; 3. TGTGACTTGGGAGCTCTGCAGC. C57BL/6 mice were purchased from the animal center of the Fourth Military Medical University. Genotypes were determined by PCR analysis of DNA from the mouse tails. Animal protocols were approved by the Animal Care and Use Committee of the Fourth Military Medical University.

2.4. Transduction of lentivirus

For *CD226* gene silencing, lentivirus encoding CD226 shRNA or scramble control shRNA was synthesized by Genechem (Shanghai, China) and used as we previously reported [30]. The CD226 shRNA lentiviral vector contains a GFP reporter. Dami cells were seeded and infected with lentivirus with different MOIs. At 48 h after viral incubation, the cells were assessed for *CD226* gene expression.

2.5. qRT-PCR

Total RNA was extracted from the cells using TRIZOL Reagent (Invitrogen Life

Technologies, Carlsbad, CA, USA). First-strand cDNA was synthesized using the PrimeScript® RT reagent (Takara). Human CD226, GATA-1, FLI-1, NF-E2, BDNF, cyclin D1, and cyclin D3 mRNA expression was analyzed using the SYBR 2× Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). *GAPDH* was used as the housekeeping gene. The primers are listed in Table 1 (supplementary data).

2.6. Ploidy analysis

After Dami cells were treated with 100 nM PMA for 24 h or mouse bone marrow isolation, 2×10^6 cells were harvested and washed once with cold PBS containing 2.5% fetal bovine serum and 0.5% NaN_3. Next, the cells were stained with CD41-APC and fixed in 70% cold alcohol for more than 2 h. After washing, the cells were stained with 0.5 mL propidium iodide (PI) at 50 μg/mL in PBS containing 0.1% Triton-X 100 and 100 U/mL RNase (Biyuntian, Shanghai, China). Cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) and DNA ploidy was analyzed using FlowJo 7.6.1 software (Ashland, OR, USA).

2.7. Flow cytometric analysis

Approximately 2×10^6 cells were collected into 1.5-mL tubes and washed once with a stream of prechilled washing buffer. Following incubation with Fc-blocker (BD Biosciences), the cells were incubated with a specific antibody or isotype antibody in the dark at 4 °C for 30 min and washed twice. Samples were analyzed using a FACS Calibur flow cytometer. Data were processed using FlowJo 7.6.1 software.

2.8. Western blot

Cells were lysed in RIPA lysis buffer (supplemented with 1 μM PMSF). Protein concentrations were measured using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of proteins were resolved by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat dry milk and 0.1% Tween-20 in PBS, the membranes were incubated with specific antibodies for CD226, GATA-1, FLI-1, NF-E2, and β-actin at 4°C overnight. After the blots were incubated in corresponding horseradish peroxidase-conjugated secondary antibodies, immunoreactive proteins were detected by enhanced chemiluminescence.

2.9. Confocal laser scanning microscopy images analysis

The sections were blocked with 10% normal goat serum and incubated overnight at 4 °C with anti-GATA-1, FLI-1, and NF-E2 primary antibodies. After three washes, binding of the primary antibodies was detected using Cy3 goat anti-rabbit or anti-mouse secondary antibodies. The nuclei of all cells were stained with DAPI nuclear dye. Images were captured with a Zeiss LSM 800 confocal microscope (Oberkochen, Germany) and data were analyzed by ZEN software.

2.10. Statistical analysis

The difference between two groups was analyzed by unpaired Student's *t*-test (GraphPad Prism 5, GraphPad software, La Jolla, CA,

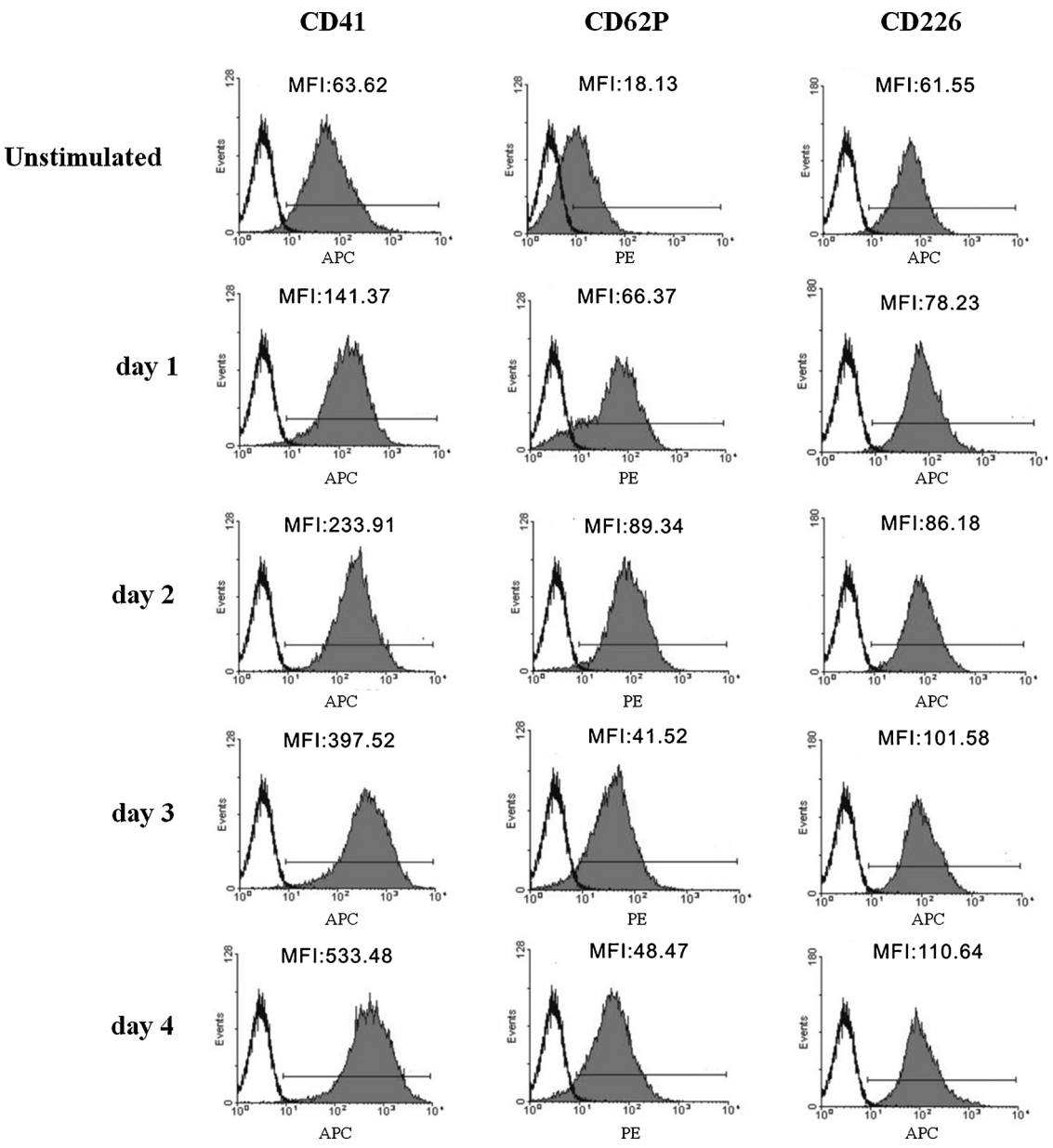

Fig. 1. CD41, CD62P, and CD226 expression following PMA stimulation detected by flow cytometry. Dami cells treated with 100 nM PMA for 1, 2, 3, and 4 days in culture. Unstimulated group showing the expression levels without any treatment at day 0. MFI: mean fluorescence intensity.

USA). A P value < 0.05 was considered statistically significant.

3. Results

3.1. Expression of CD226 is stable during MK development

Dami is a commonly used model cell line for MK development upon phorbol 12-myristate 13-acetate (PMA) stimulation and its characteristics have been widely described [20]. CD41 is a specific megakaryocytic marker. CD62 P is also known as P-selectin, platelet activation-dependent granule membrane protein and is expressed on activated platelets, MKs and endothelial cells. As expected, CD41 expression was constantly elevated in the MKs in a time-dependent manner upon stimulation with PMA from day 0 (unstimulated) to day 4. The expression of CD62 P was increased after stimulation and reached a peak at day 2. However, further stimulation did not increase CD62 P expression and after day 2, its expression was decreased.

Next, we evaluated whether cell surface CD226 expression was altered upon treatment with PMA. We found that CD226 was expressed at high levels in Dami cells and was stable during MK development upon stimulation with PMA (Fig. 1). Subsequent experiments were performed to determine if CD226 functions in MK activation and development.

3.2. Silencing CD226 significantly inhibits PMA-induced activation of MK

To test the function of CD226 in MK differentiation, we used lentiviral shRNA to knock down *CD226* in Dami cells as they differentiated *in vitro*. By evaluating the GFP fluorescence level, we demonstrated that lentivirus encoding CD226 shRNA (shCD226) infected Dami cells successfully. The qPCR assay showed that compared to the control vector at a multiplicities of infection (MOI) of 200, the efficiency of CD226 shRNA lentivirus silencing at an MOI of 200 was greater than 80%. According to western blotting and flow cytometry analysis, the protein level of CD226 and cell surface CD226 were both decreased significantly (Fig. 2). These results demonstrate that CD226 was

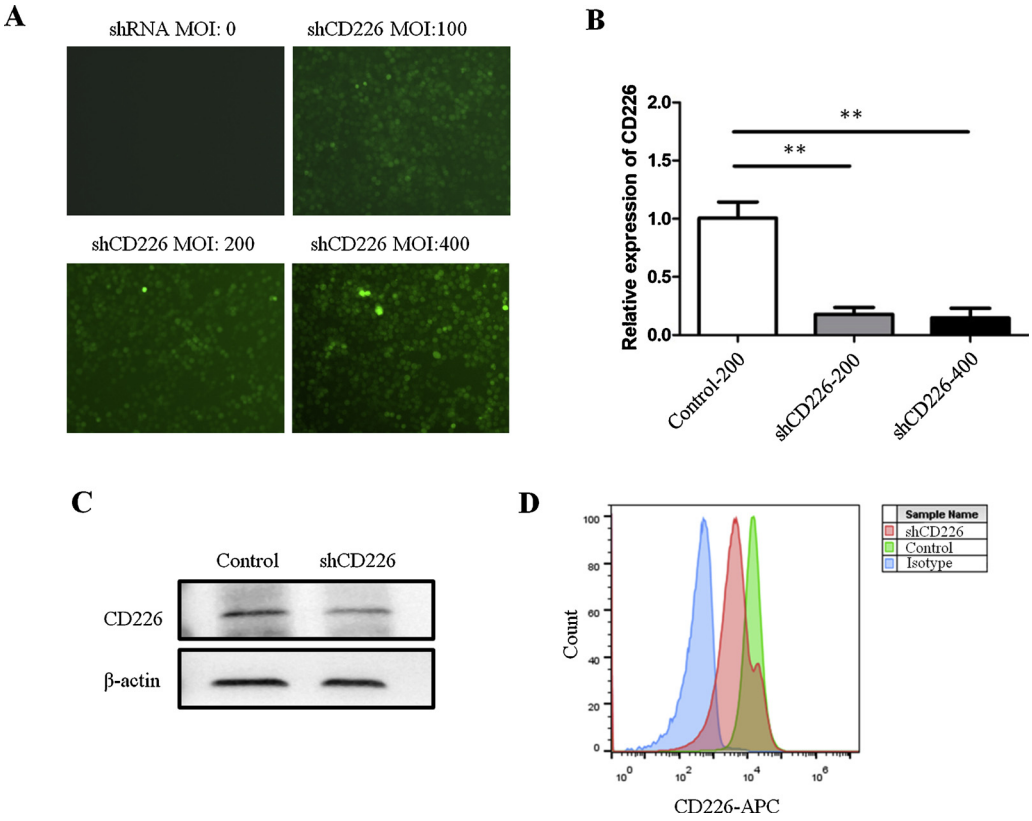

Fig. 2. Efficiency of human *CD226* gene silencing in Dami cells. (A) Cells were infected with lentivirus encoding CD226 shRNA (shCD226) at different MOIs, and transfection efficiency was evaluated by GFP fluorescence expression. (B) Measurement of CD226 levels (fold-change) by qPCR; Dami cells were infected with control shRNA (MOI 200) or CD226 shRNA (MOI 200 and 400) for 48 h. $^{**}P < 0.01$. (C) Western blot analysis of lysates from Dami cells infected with control or CD226 shRNA lentiviral vector, and probed with anti-CD226 and anti-β-actin antibodies. (D) Control or CD226 shRNA lentiviral vector with flow cytometric histogram of cell surface CD226 expression infected for 48 h.

successfully silenced in Dami cells using the CD226 shRNA lentiviral vector.

Functional studies using lentivirus-mediated CD226 inhibition led to dramatically down-regulated CD62 P levels in Dami cells stimulated with PMA from days 1 to 3. However, CD41 expression was gradually elevated during PMA stimulation, and silencing of *CD226* in Dami cells did not significantly alter CD41 expression (Fig. 3).

3.3. Silencing CD226 causes development retardation in low-ploidy of MK

At 48 h after lentivirus infection, the morphology of Dami cells was observed under a light microscope. Examination of randomly selected fields revealed a proportion of cells with large diameters of > 20 μm, with some cells > 50 μm A previous study demonstrated that the diameter of Dami cells is > 20 μm during differentiation into MK [21]. However, in the CD226 shRNA lentivirus-treated group, the Dami cells remained small at < 20 μm, indicating retardation of cell differentiation (Fig. 4A).

DNA staining with PI was conducted to distinguish between typical low ploidy (2N–4N) and high ploidy (≥8N) based on the DNA density in Dami cells. Ploidy values in unstimulated control Dami cells were predominantly diploid-tetraploid (2N–4N, 98%), with a small proportion of polyploidy (≥8N, 2%). As expected, Dami cells displayed increased polyploidization in response to PMA stimulation, reaching 8 N or higher in 25% of the cell population (2N–4N, 75%). Interestingly, silencing of *CD226* blocked the formation of tetraploid DNA, and CD226 had no effects on PMA-induced polyploidization in MK cells. Based on these findings, we predicted that CD226 is involved in the differentiation of MK cells. Silencing of *CD226* lead to the 2N–4N low ploidy and blocked polyploidy formation.

To determine whether CD226 participates in MK development, MK in the bone marrow of *CD226* KO mice were analyzed by flow cytometry. MKs were selected based on CD41-APC staining and analyzed based on PI staining. In agreement with our data in Dami cells, *CD226*-deficient MKs were less polyploid than their wild-type (WT) counterparts (Fig. 4D). Taken together, these results confirm that the absence of CD226 leads to defects in the polyploidization of MK.

3.4. Silencing CD226 decreases early-stage transcription factors GATA-1 and FLI-1expression in MK

Next, we evaluated the expression of transcription factors involved in MK maturation. The relative expression of specific mRNAs was detected by qRT-PCR. GATA-1 and FLI-1 are the main transcription factors involved in the early stage of MK differentiation, and both *GATA-1* and *FLI-1* mRNAs were decreased significantly after CD226 shRNA lentiviral vector infection for 48 h. Expression of NF-E2, the main transcription factor involved in the late stage of MK differentiation and platelet formation, was evaluated. We found that the *NF-E2* expression level was not changed in CD226 shRNA lentivirus-infected Dami cells compared to in control shRNA-infected cells.

Furthermore, brain-derived neurotrophic factor (BDNF), which is also expressed on MK and may interact with the CD226 receptor, was also evaluated. The expression of BDNF was not changed in CD226 shRNA lentivirus-infected Dami cells. Additionally, has been reported that D-type Cyclins may participate in MK cells differentiation. Our results showed that cyclin D1 expression remained relatively unchanged. However, cyclin D3 was significantly increased in *CD226*-silenced Dami cells.

The western blotting results of these transcription factors are shown in Fig. 5G. In agreement with the data obtained by qRT-PCR, the protein levels of GATA-1 and FLI-1 were decreased significantly after CD226 shRNA lentivirus treatment, while the expression of NF-E2 did not significantly change.

Confocal analysis revealed that compared to GATA-1 and FLI-1, NF-E2 expression was relatively lower in Dami cells. The cells were successfully infected with CD226 shRNA lentivirus based on GFP fluorescence analysis. The results indicated GATA-1 was mainly localized in the nuclei, while FLI-1 and NF-E2 were distributed in both the nuclei and cytoplasm. Inhibition of CD226 decreased GATA-1 and FLI-1

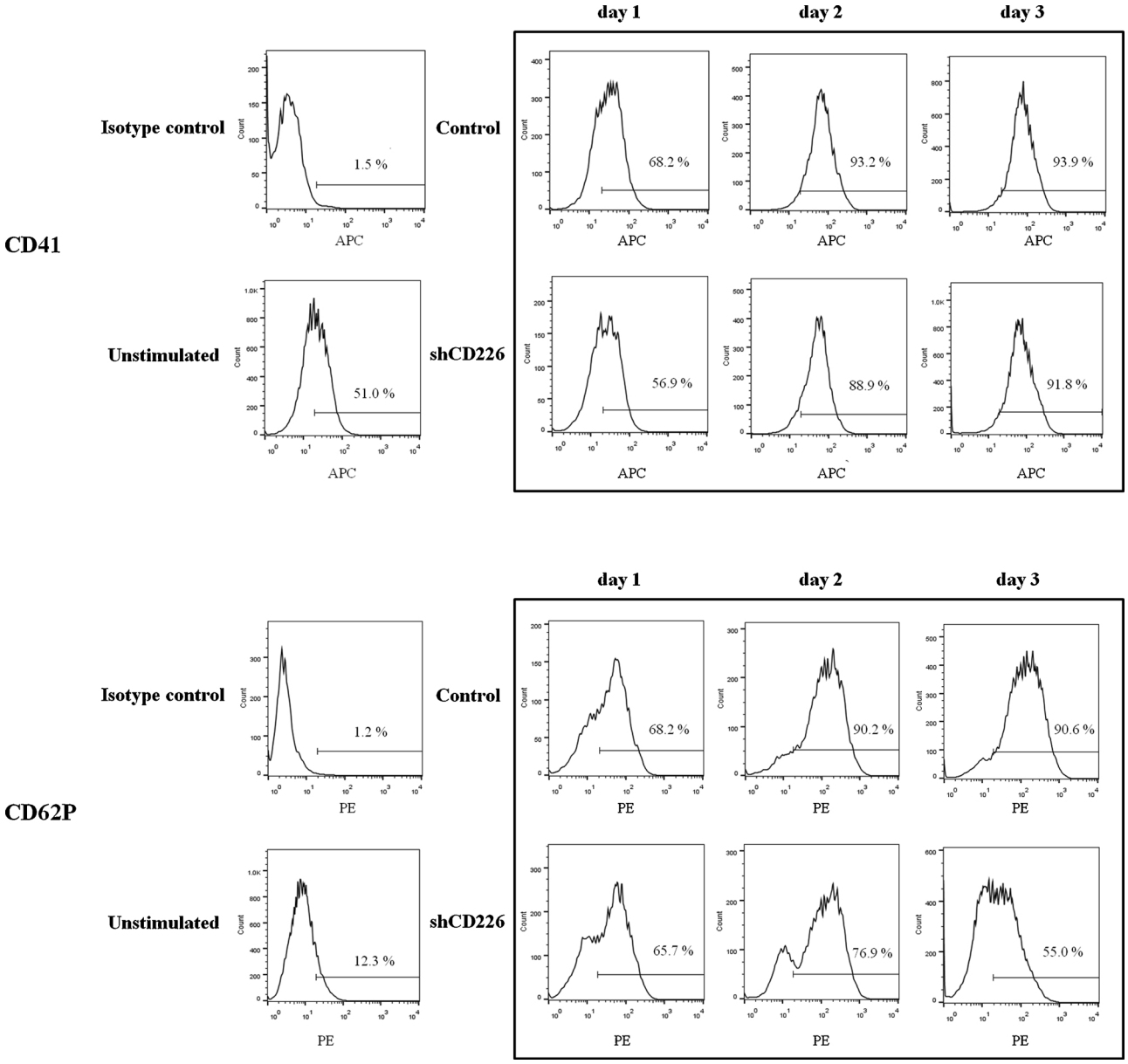

Fig. 3. Inhibition of CD226 decreases surface expression of CD62P. Flow cytometric analysis of control and CD226 shRNA-infected cells labeled with anti-CD41, anti-CD62P, and an isotype matched IgG. Data shown represent three independent experiments.

expression compared to that in the control group. However, consistent with the results obtained by qPCR and western blot assays, NF-E2 remained unaffected after *CD226* gene silencing (Fig. 6). These results suggest that CD226 plays an important role in the early stage of MK differentiation.

4. Discussion

Under physiological conditions, the polyploidization of MK determines the quantity and quality of platelet production. Despite the importance of MKs and platelets in health and a variety of diseases, the mechanisms regulating their formation are not well-understood. The results of the present study suggest that CD226 plays a role in MK development and activation.

We found a progressive increase in CD41 expression and stable high-level expression of CD226 on the PMA-stimulated Dami cells membranes. Additionally, CD62P exhibited an expression peak at day 2 after PMA stimulation. After *CD226* expression was knocked down by CD226 shRNA lentivirus in Dami cells, the MK and platelet activation marker CD62P was significantly inhibited. These data suggest that CD226 is involved in MK activation.

MK differentiation is regulated by thrombopoietin and cytokines such as interleukin 3 (IL-3), IL-6, and IL-11 (Yang et al., 2000). Megakaryocytopoiesis also involves specific transcription factors, including GATA-1, FLI-1, and NF-E2. GATA-1 and FLI-1 are involved in MK differentiation and endomitosis (Shivdasani et al., 1997; Hart et al., 2000). Additionally, GATA-1 controls MK growth and polyploidization, which are independent of MK terminal differentiation (Muntean et al., 2007). NF-E2 is the main transcription factor involved in the final step of platelet assembly and release known as proplatelet formation (Shivdasani et al., 1995). In this study, we found that CD226 down-regulation decreased the level of MK maturation, which was associated with increased low ploidy (2N–4N) both in Dami cells and gene knockout mice. This may be because silencing of *CD226* decreased the expression of the MK-related early-stage transcription factors GATA-1 and FLI-1.

Furthermore, cyclin D1 and D3, but not D2, were reportedly expressed on primary murine MK (Wang et al., 1995). D-type Cyclins play

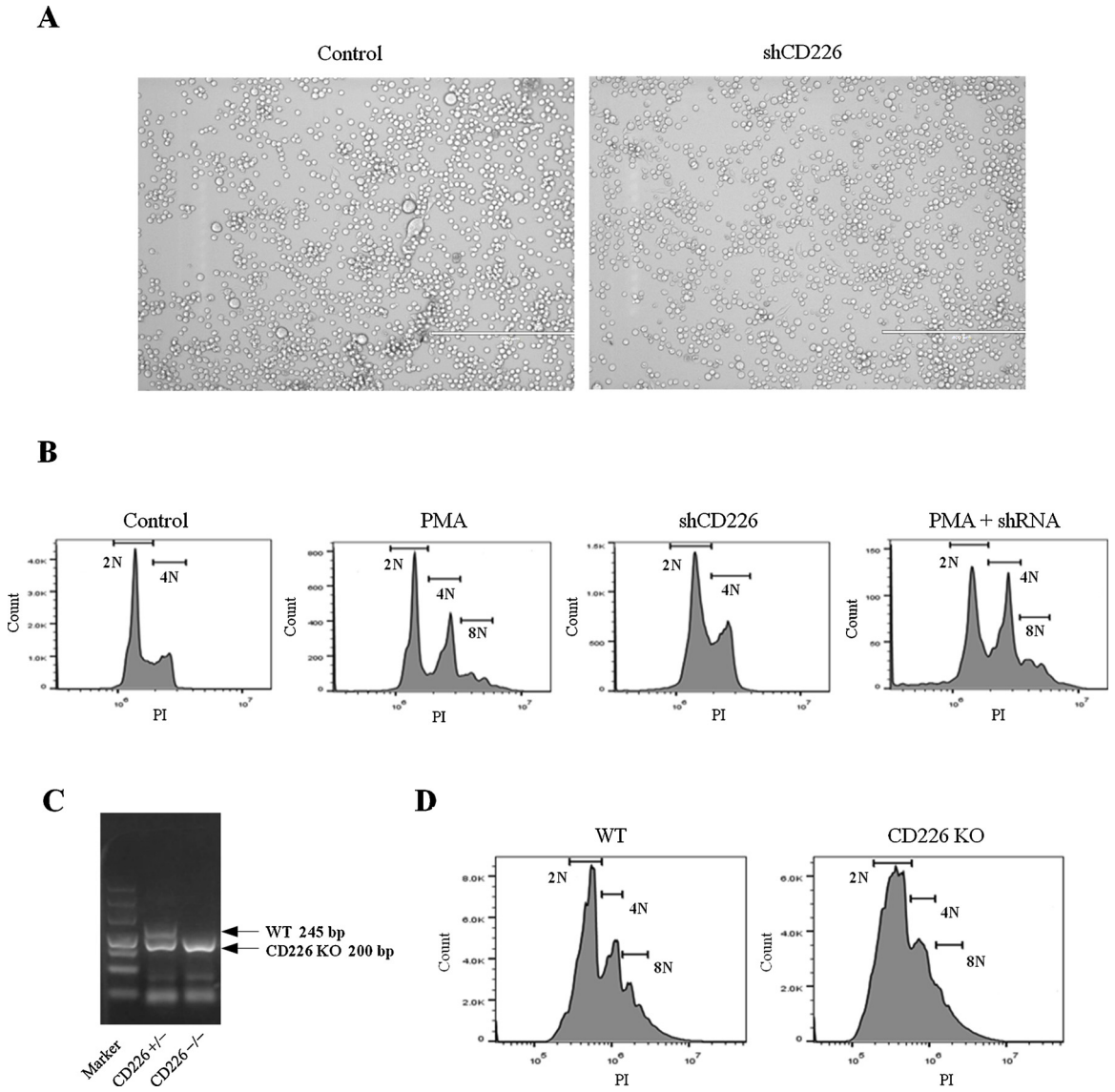

Fig. 4. Deficiency of CD226 blocks ploidy in low-ploidy MK. (A) Morphological changes induced in Dami cells treated with control lentivirus and CD226 shRNA lentivirus after 48 h. Cells were observed by optical microscopy and representative images are shown. Scale bar = 400 μm. (B) DNA ploidy analysis of Dami cells by PI staining and flow cytometry. Control cells and CD226 shRNA infected were treated with or without PMA. Representative histograms of PI staining are shown. (C) PCR genotyping of *CD226* KO mice. The 245-bp band represents the WT gene amplified product and 200-bp band represents the *CD226* gene KO amplification product. $CD226^{-/-}$, homozygote; $CD226^{+/-}$, heterozygote. (D) DNA ploidy analysis of MK from bone marrow of WT and *CD226* KO mice by flow cytometry. Cells and stained with APC conjugated anti-mouse CD41 antibody and PI. MKs were gated based on CD41 staining, and representative histograms of PI staining are shown. Data shown represent three independent experiments.

key roles in endomitosis, and thus are essential for megakaryocytopoiesis. Particularly, cyclin D3 is present at high levels in MK, and transgenic mice with cyclin D3 in the platelet lineage display a striking increase in endomitosis. However, overexpression of cyclin D3 leads to poor development of demarcation membranes and platelet fragmentation (Zimmet et al., 1997). In this study, we observed that although transcription factors related to MK differentiation, GATA-1 and FLI-1, were both decreased, DNA in the tetraploid form was modestly elevated in Dami cells. Few MK reached a high ploidy class of ≥8N or greater. The increase in tetraploid DNA may partially relate to cyclin D3 elevation after silencing of *CD226*.

5. Conclusion

Taken together, a deficiency of CD226 may decrease MK CD62P expression, key transcription factors levels, and high ploidy formation, thereby inhibiting MK activation and maturation. Our findings suggest the thrombopoietic potential of CD226 in MK differentiation.

Conflicts of interest

The authors declare no commercial or financial conflicts of interest.

Funding sources

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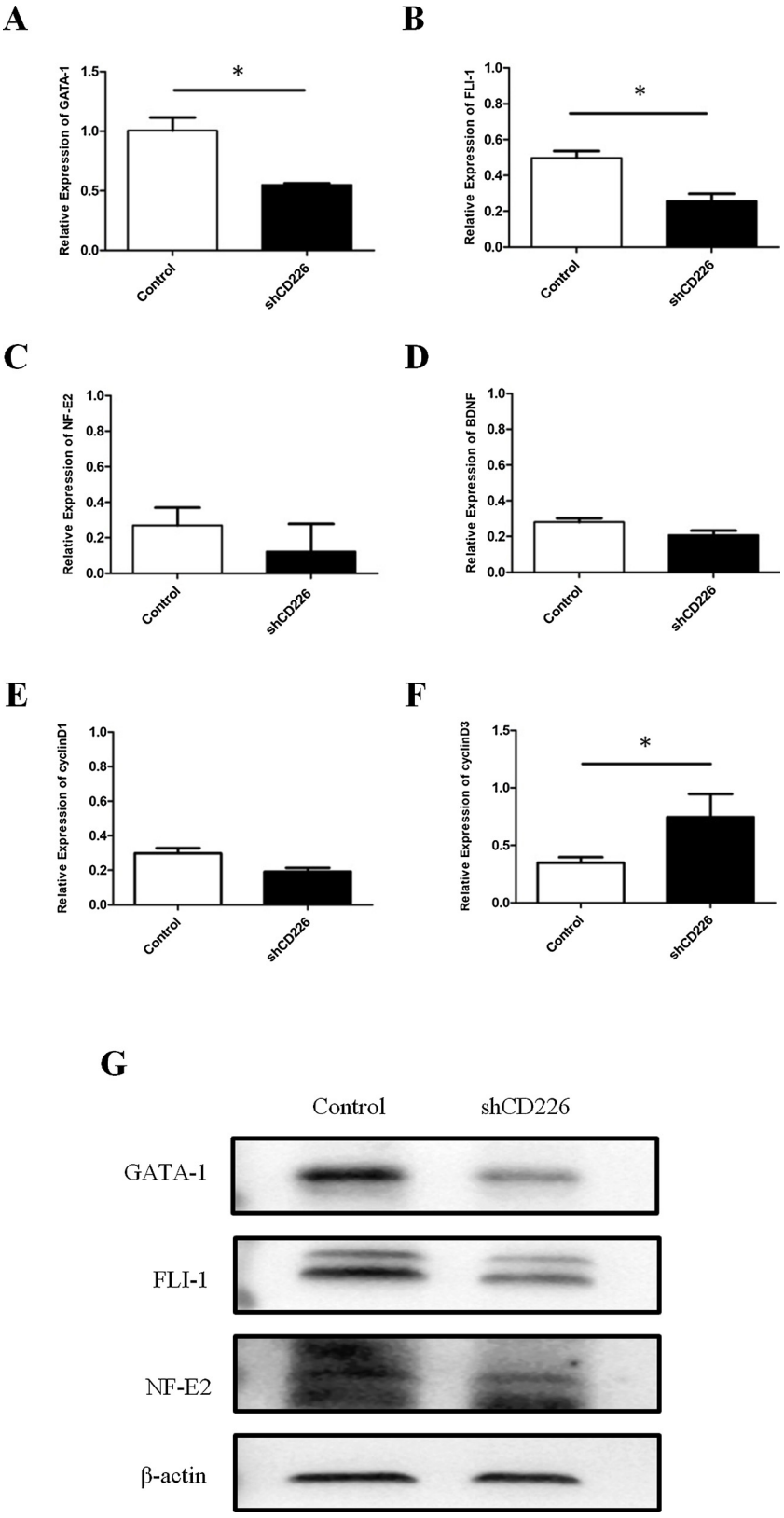

Fig. 5. qRT-PCR analysis of MK differentiation markers. (A) *GATA-1*; (B) *FLI-1*; (C) *NF-E2*; (D) *BDNF*; (E) *cyclin D1*; (F) *cyclin D3*. Data were normalized to *GAPDH* mRNA. The data represent the means ± SEM of three independent experiments. * $P < 0.05$. (G) Western blot analysis of key transcription factors in MK differentiation. Dami cells were treated with control lentivirus and CD226 shRNA lentivirus. Blots were probed with the indicated antibodies; β-actin was used as a loading control. Images are representative western blot results of three independent experiments.

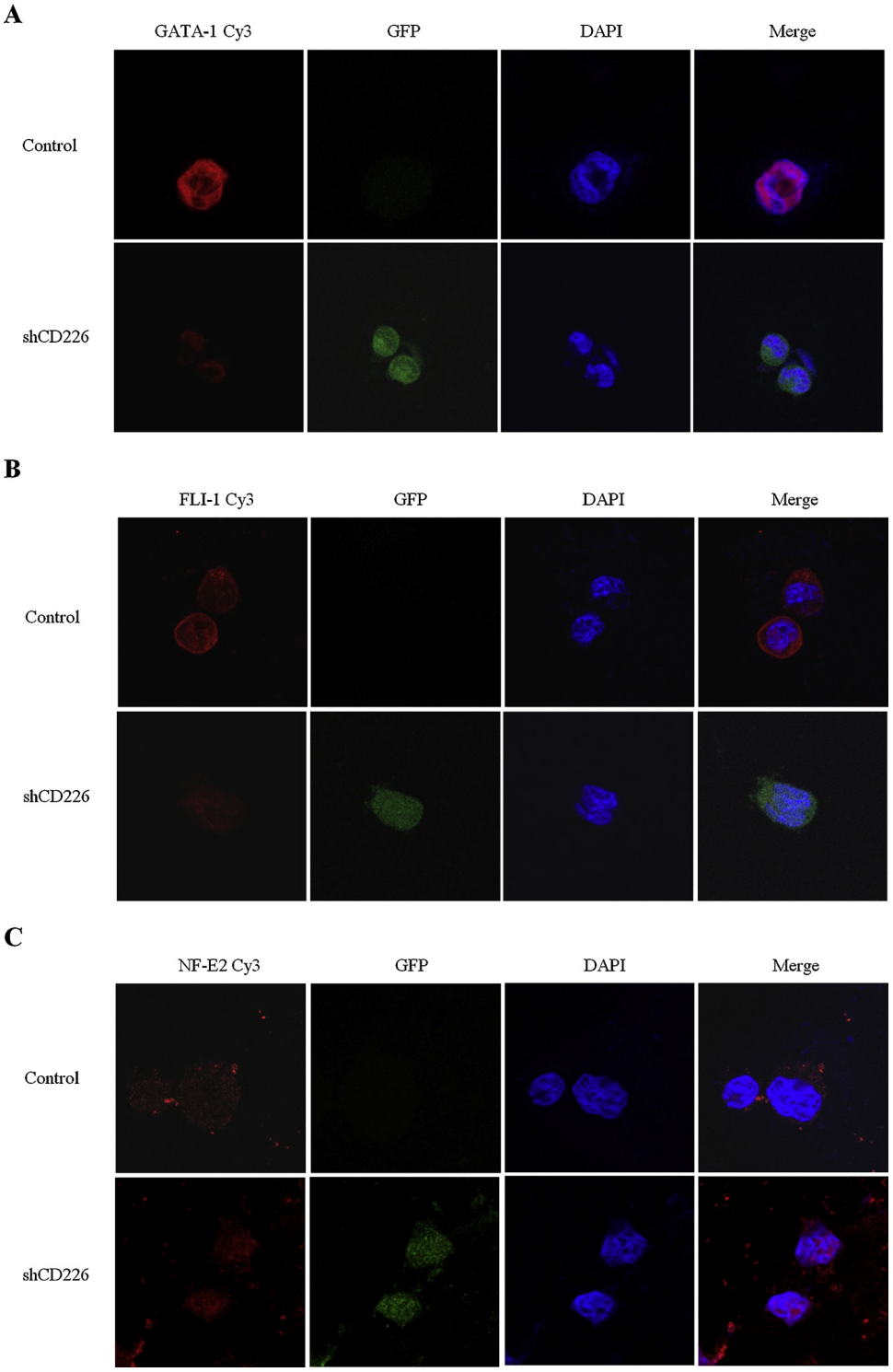

Fig. 6. Effect of CD226 inhibition on transcription factor expression by confocal analysis. Immunostaining was performed using Cy3 goat anti-rabbit or goat anti-mouse secondary antibody (Red) to test for antibody specificity. Anti-human GATA-1 (A), FLI-1 (B), and NF-E2 (C) antibodies were used to detect transcription factor expression and distribution in Dami cells infected with control lentivirus and CD226 shRNA lentivirus (with GFP reporter). Nuclei were counterstained with DAPI (Blue). Data shown represent three independent experiments (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Pathology and Immunology, Washington University School of Medicine).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.01.013.

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