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The effects of plasma gelsolin on human erythroblast maturation for erythrocyte production

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ABSTRACT

Gelsolin is an actin binding protein present in blood plasma and in cytoplasm of cells including macrophages. Gelsolin has important functions in cell cycle regulation, apoptotic regulation, and morphogenesis. Even though bone marrow macrophages and serum factors are critical for regulating erythropoiesis, the role of gelsolin on human erythroblasts has not been studied. Here, we investigated the effects of human recombinant plasma gelsolin (pGSN) on human immature erythroblasts. CD34+ cells isolated from cord blood were differentiated into erythroid cells in serum-free medium. When pGSN was applied to the culture medium, it accelerated basophilic and polychromatic erythroblast maturation and increased the enucleation rate with highly expressed erythropoiesis-related mRNAs. Also, pGSN was effective in reducing dysplastic changes caused by vincristine, suggesting its role in cell cycle progression at G2/M checkpoints. Also, pGSN activated caspase-3 during maturation stages in which caspase-3 functions as a non-apoptotic maturational signal or a pro-apoptotic signal depending on maturation stages. Our results suggest that pGSN has a pivotal role in maturation of erythroblasts and this factor might be one of the way how bone marrow macrophages and previously unknown serum factors work to control erythropoiesis. pGSN might be used as additive for *in vitro* production of erythrocytes.

1. Introduction

Erythroblasts proliferate, differentiate, and finally enucleate within specialized niches called erythroblastic islands in which erythroblasts cluster around macrophages (Chasis and Mohandas, 2008). Even though it is very clear that macrophages are critical for erythropoiesis, the factors through which they act are not fully understood. Gelsolin (GSN) is a multifunctional actin binding protein involved in cell motility, cell morphogenesis, and remodeling of the cytoskeleton, controlled by Ca^{2+} and phospholipid (Yin, 1987; Yin and Stossel, 1979). GSN was first found in the cytosol of macrophages (Yin and Stossel, 1979), and three isoforms have been identified till now: an intracellular type (cytoplasmic type, cGSN), an extracellular type (secreted or plasma type) that is 23 amino acids longer than the cytoplasmic isoform, and a third isoform (GSN-3) that is 11 amino acids longer than the cytoplasmic isoform. Intracellular GSN is present in the cytoplasm and mitochondria, while extracellular GSN is found in the blood plasma (Chaponnier et al., 1986; Wen et al., 1996; Yin et al., 1984; Zapun et al., 2000; Li et al., 2012). GSN is involved in cell cycle control and enhances the G2/M checkpoint function of many cancer cells (Sakai et al., 1999; Deng et al., 2015). It may also inhibit apoptosis by preventing the loss of mitochondrial membrane potential (Koya et al., 2000). However, depending on the cell types and experimental conditions, GSN showed pro-apoptosis or anti-apoptosis (Nishio and Matsumori, 2009). Moreover, GSN might have a different function between species (Leifeld et al., 2006).

Little is known about the role of GSN in human erythroblasts. Cantu et al. reported that in GSN knock- out mice, terminal maturation and enucleation of erythroblasts failed due to an altered balance between actin polymerization and depolymerization (Cantu et al., 2012). However, the observed defective erythropoiesis may have been due to gelsolin-defective abnormal macrophages or altered gelsolin levels in the bloodstream, not just due to the gelsolin-defective erythroblasts. Moreover, that study was performed in mice not in human cells. Hence it could not establish the effects of GSN itself on human erythroblasts.

Erythroblasts committed from hematopoietic stem cells (HSCs) undergo a multi-step process of differentiation from proerythroblasts to

Abbreviations: GSN, gelsolin; pGSN, human recombinant plasma gelsolin; cGSN, cytoplasmic gelsolin; HSCs, hematopoietic stem cells; GATA-1, GATA-binding factor 1; VLA-4, very late antigen-4; ICAM-4, intracellular adhesion molecule-4; DLC-1, deleted in liver cancer-1; RhoA, ras homolog gene family member A; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Cdk, cyclin-dependent kinase.

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basophilic erythroblasts, polychromatic erythroblasts, orthochromatic erythroblasts, enucleated reticulocytes and finally mature red blood cells (RBCs) (Gabet et al., 2011; Testa, 2004). Cell morphology, cellular characteristics, and apoptotic mechanisms all differ depending on the degree of maturation of erythroid cells (Testa, 2004). These processes might be related to gelsolin especially plasma isoforms that are secreted by adjacent macrophages and other erythroblasts.

In the present work, we examined the effects of human plasma gelsolin on the various developmental stages of human immature erythroblasts by adding human recombinant plasma gelsolin protein (pGSN) to erythroblast differentiation medium. Our results indicate that pGSN facilitates erythroblast maturation, acts as a G2/M checkpoint, and corrects the myelodysplasia induced by serum-free culture conditions or by a G2/M blocker.

2. Materials and methods

2.1. Isolation of human CD34+ cells

Umbilical cord blood was collected after obtaining a written consent from healthy pregnant women. The study was approved by the IRB of Hanyang University Guri Hospital in Korea. Mononuclear cells (MNCs) of human umbilical cord blood were separated on Ficoll-Paque gradients and CD34+ cells were isolated using an Easysep Human CD34 Positive Selection Kit (Stemcell Technologies, Vancouver, Canada). The erythroid cell purity was evaluated by the expression of the glycophorin A (GPA) by flow cytometry and was higher than 90% after culture day 8.

2.2. Cell culture

CD34+ cells were cultured in serum-free medium (Stemline II, Sigma, Aldrich, St Louis, MO) supplemented with 150 μg/ml holotransferrin (Sigma), 90 ng/ml ferric nitrate, 30.8 μM vitamin C, 160 μM 1-thioglycerol, 50 μg/ml insulin (Sigma), 4 μM L-glutamine, 2 μg/ml cholesterol, 0.05% pluronic F-68, 0.5 μl/ml lipid mixture. Growth factors supplemented in the media were erythropoietin (6 U/ml, R&D systems, Minneapolis, MN), stem cell factor (100 ng/ml, R&D systems), and interleukin-3 (10 ng/ml; Sigma) for 0–7 days of culture, erythropoietin (3 U/ml) and stem cell factor (50 ng/ml) for 8–13 days, and erythropoietin (2 U/ml) for 14–18 days. The medium was changed every 2 days.

2.3. pGSN treatment

As Koya et al. have reported that recombinant gelsolin inhibited mitochondrial membrane potential loss at 0.4 μM (Koya et al., 2000), and the median level of GSN levels in human plasma is about 200 μg/ml (2.3 μM) (Jagadish et al., 2012), erythroblasts were treated with pGSN at concentrations of 0, 0.4, 1.0, and 2.0 μM. Around at culture day 8 or 13, the cultured cells were evaluated for their maturation states by cell morphology after Wright-Giemsa staining. If >70% of cells were at basophilic (experiment 1) or polychromatic erythroblasts (experiment 2), pGSN was added to culture media for 24–48 h.

For the toxicity test, cells were cultured for 24 h in the presence of pGSN dissolving solution (50% glycerol in Tris buffer).

2.4. Morphological analysis

Cells were stained with Wright-Giemsa (Sigma) after sedimentation using a cytospin (Hanil Science Industrial, Korea). Cell size, maturation, and myelodysplasia were evaluated by a microscope (Nikon Eclipse E400) in a blinded manner by two experts.

2.5. Flow cytometry

Cells were harvested and washed with phosphate buffered saline (PBS) containing 1% fetal bovine serum (FBS). Cells were labeled with CD235a (GPA)-PE, CD235a-FITC (Life Technologies, CA, USA), CD71-PE/Cy7 (BioLegend, San Diego, CA, USA), CD71-PE (BioLegend), CD49-FITC (BD Pharmingen, San Diego, CA), CD44-FITC (BioLegend) antibodies at 4 °C, for 20 min. Cells were then fixed with 1.3% formaldehyde for 10 min and permeabilized with 0.2% TX-100 at room temperature (RT) for 5 min. Cell were washed 2 times with 1% FBS and labeled with CD233-PE (Miltenyi Biotec, Auburn, CA) for 20 min. To confirm purity, cells were double-labeled for anti-CD13-APC (BD Biosciences) and anti-CD11b-FITC (BD Biosciences). In order to measure the activation of caspase-3, we used a CaspGLOW Fluorescein Active Caspase-3 Staining Kit (BioVision, California, USA). Labeled cells were analyzed by flow cytometry (Accuri™ C6, BD Biosciences).

2.6. qRT-PCR

Total RNA was purified from cells with Trizol Reagent (Ambion, Austin, TX) and measured using a Nanodrop (BioSpecNano Spectrophotometer, Shimadzu, Japan). To synthesize cDNA, we used SuperScript III Reverse Transcriptase (Invitrogen). mRNA expression levels were measured by qRT-PCR in duplicate. GAPDH mRNA was used for normalization. The primer sequences (forward/reverse) used were:

GATA-1, 5′-CCAAGCTTCGTGGAACTCTC-3′/5′-CCTGCCCGTTTACTGACAAT-3′

VLA-4, 5′-AGGATGGTGTAAGCGATGGC-3′/5′-TGCTGAAGAATTGGCTGAAGTGGTGG-3′

ICAM-4, 5′-CCGGACTAAGCGGGCGCAAA-3′/5′-AGCCACGAACTCCGGGCTCA-3′

DLC-1, 5′-AGTGCGTGCAACAAGCGGGT-3′/5′-TCCGGGTAGCTCTCGCGGTT-3′

RhoA, 5′-CTCATAGTCTTCAGCAAGGACCAGTT-3′/5′-ATCATTCCGAAGATCCTTCTTATT-3′

GAPDH, 5′-GAAGGTGAAGGTCGGAGT-3′/5′-GACAAGCTTCCCGTTCTCAG-3′

2.7. Cell cycle analysis

Cells were harvested and washed with PBS containing 1% FBS. Cells were fixed with 70% ethanol at −20 °C for 4 h. Thereafter, cells were washed twice with PBS containing 1% FBS and stained with propidium iodide (PI) and analyzed by flow cytometry (FACS Canto II, BD Biosciences).

2.8. PKH26 assay

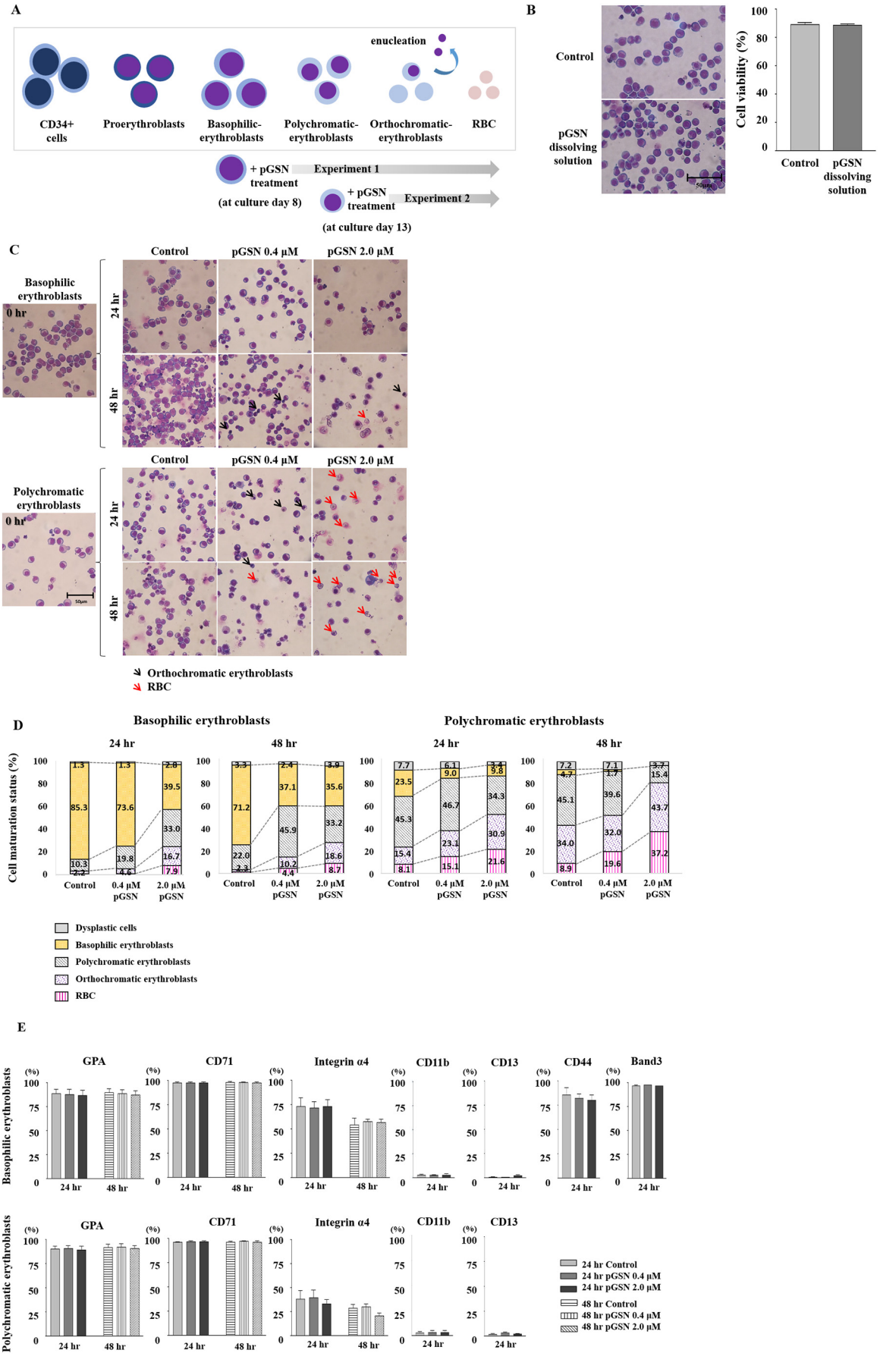
Cells were washed twice with serum-free media, and the supernatant was removed. Cells were labeled with PKH26 (PKH26 red fluorescent cell linker kit, Sigma) at RT for 1 min and staining was stopped by addition of FBS. Subsequently, the cells were washed twice with medium and mean fluorescence intensity (MFI) was measured by flow cytometry (Accuri™ C6, BD Biosciences).

2.9. Cell apoptosis analysis

Cultured cells were harvested and washed with PBS containing 1% FBS. Cells were labeled with Annexin V-FITC (BD Biosciences) and PI (Sigma) and analyzed by flow cytometry (Accuri™ C6, BD Biosciences).

2.10. Confocal microscope analysis

Cells were harvested and washed with PBS containing 1% FBS. Cells were fixed with 3.7% paraformaldehyde for 10 min and then


A
enucleation
CD34+ cells
Proerythroblasts
Basophilic-erythroblasts
Polychromatic-erythroblasts
Orthochromatic-erythroblasts
RBC
+ pGSN treatment
(at culture day 8)
Experiment 1
+ pGSN treatment
(at culture day 13)
Experiment 2
B
Control
pGSN dissolving solution
Cell viability (%)
Control
pGSN dissolving solution
C
Basophilic erythroblasts
Polychromatic erythroblasts
0 hr
24 hr
48 hr
Control
pGSN 0.4 μM
pGSN 2.0 μM
Orthochromatic erythroblasts
RBC
D
Basophilic erythroblasts
Polychromatic erythroblasts
Cell maturation status (%)
Control
0.4 μM pGSN
2.0 μM pGSN
Dysplastic cells
Basophilic erythroblasts
Polychromatic erythroblasts
Orthochromatic erythroblasts
RBC
E
GPA
CD71
Integrin α4
CD11b
CD13
CD44
Band3
Basophilic erythroblasts
Polychromatic erythroblasts
24 hr Control
24 hr pGSN 0.4 μM
24 hr pGSN 2.0 μM
48 hr Control
48 hr pGSN 0.4 μM
48 hr pGSN 2.0 μM

permeabilized with 0.2% TX-100 at RT for 5 min. Cells were washed with PBS. Next, cells were labeled with His-Tag (D3I1O) XP® Rabbit mAb (Alexa Fluor® 647 conjugate; Cell Signaling, Beverly, MA) at RT for 1 h and washed twice with PBS. Cells were labeled with DAPI and washed with PBS. Cells were analyzed by confocal microscope (TCS SP5, Leica).

2.11. Treatment with LPA and LPA inhibitors

LPA (Sigma) and Ki16425 (Cayman Chemical, Ann Arbor, MI), an antagonist of $LPAR_1$ and $LPAR_3$, was diluted in dimethyl sulfoxide (DMSO). pGSN 2 μM, LPA (5 μM), and Ki16425 (20 μM) added to culture media for 24 h according to the previous report (Chiang et al., 2011). Cultured cells were evaluated for their maturation status by cell morphology and viability.

2.12. ELISA

Basophilic erythroblasts (at culture day 10) and polychromatophilic erythroblasts (at day 14) were cultured at a density of 5×10^5/ml for 24 h in serum-free media. The cell supernatants were collected and assayed for secreted gelsolin concentration by enzyme-linked immunosorbent assay (ELISA). Ninety-six-well plates were coated with supernatants in triplicate for overnight at 4 °C. Then, the plates were washed 3 times with 0.05% tween-20 in PBS. The plates were blocked with 10% FBS in PBS for 2 h at RT. Then, the plates were washed and incubated with anti-GSN antibody (1:500 dilution; Abnova, Taipei, Taiwan) in 10% FBS in PBS. After 2 h, the plates were washed 3 times using wash buffer and incubated with secondary antibody (1:2000; Jackson Immunoresearch, West Grove, PA) for 1 h. After washing, the plates were incubated with TMB (Tetramethyl benzidine) ELISA substrate (Biolegend, San Diego, CA) in 30 min in the dark and then stop solution was added (GenDEPOT, Barker, TX). The absorbance was read at 490 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). For each assay, pGSN concentrates of a known amount were used to generate a standard curve.

2.13. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed by one-way ANOVA or *t*-test using GraphPad Prism version 3 (GraphPad, San Diego, CA). A *p*-value of <0.05 was considered significantly different.

3. Results

3.1. pGSN accelerates maturation of immature erythroblasts

The cord blood CD34+ cells were cultured until day 10 and day 14 when the basophilic and polychromatic erythroblasts become a main portion. The expansion-fold from day 0 was 2341.6 (mean;n = 10) at day 10 and 10,540.1 at day 14. To confirm the effect of the pGSN on immature erythroid maturation, immature erythroblasts at two maturation stages of basophilic erythroblasts and polychromatic erythroblasts were treated with pGSN for 24–48 h (Fig. 1A). At each maturation phase before pGSN treatment, the purity of basophilic erythroblasts and polychromatic erythroblasts were >84% and 70% of total cultured cells, respectively. When cells were cultured for 24 h in the presence of pGSN dissolving solution used for 2.0 μM pGSN in order to determine the possible toxicity, cell viability (88.3%) was similar to that of the control (88.7%), and cell morphology was unaffected (Fig. 1B).

When basophilic or polychromatic erythroblasts were treated with pGSN for 24–48 h, both cell and nuclear sizes were consistently reduced (Fig. 1C) and terminal erythropoiesis was accelerated with higher percentages of orthochromatic erythroblasts and enucleated retculocytes in a dose-dependent pattern.

When cells at a basophilic erythroblast stage were treated pGSN, the mean proportion of polychromatic erythroblasts was increased almost twice in the 0.4 μM pGSN condition and three times in 2.0 μM pGSN compared to that of controls at 24 h. At 48 h of pGSN treatment, the proportion of reticulocytes was 2.3% in the 0.4 μM pGSN condition but was increased to 8.7% in the 2.0 μM pGSN condition. When the cells at the polychromatic erythroblast stage were treated pGSN, the mean proportion of orthochromatic erythroblasts was increased almost twice in 2.0 μM pGSN condition and the proportion of reticulocytes were increased twice in the 0.4 μM pGSN condition and four times in the 2.0 μM pGSN condition compared to the control at 48 h (Fig. 1D).

The maturation status was evaluated by cell surface markers. Erythroid specific marker, GPA, was expressed >86.9% in basophilic erythroblasts and >88.9% in polychromatic erythroblasts (Fig. 1E). The surface expression of CD71 on basophilic and polychromatic erythroblasts was, however, minimally affected by pGSN treatment with expression >95.8% in the mature condition, because CD71 expression does not change substantially until the fully mature erythrocyte stage and the cells during our culture period were just recently enucleated reticulocytes (Chen et al., 2009; Hu et al., 2013). Also, band 3 was already fully expressed at basophilic erythroblast stage as 96.5%, 97.2%, 96.3% at 24 h of 0, 0.4 μM, and 2.0 μM pGSN treatment (Fig. 1E). Therefore, the maturation change could not be assessed by Band 3 (n = 3).

With the treatment of pGSN for 24 h, basophilic erythroblasts showed decreased expression of CD44 that declines continuously during erythroblast maturation (control, 85.3%; pGSN 0.4 μM, 81.6%; pGSN 2.0 μM, 79.8%). As the integrin α4 expression normally decreases in the very late stage of erythropoiesis (Hu et al., 2013), the decrease of integrin α4 expression was not much different during the basophilic erythroblast stage even with pGSN treatment, but was more decreased during the polychromatic erythroblast stage (control, 38.0%; pGSN 2 μM, 32.6% at 24 h, n = 3; control, 28.5%; pGSN 2 μM, 20.5% at 48 h, n = 3).

The purity of the erythroid cells among the total cultured cells were >96% when measured by non-erythroid cell lineage markers of CD11b (monocytes) and CD13 (myeloid cells).

Altogether, GPA, CD71, CD44, and Band3 were inadequate to assess the maturation status during our culture period, which were relevant data to the previous report (Hu et al., 2013). However, as integrin α4 expression decreases during the very late maturation stage, its expression decreased during the polychromatic erythroblast stage after pGSN treatment in a pGSN concentration-dependent pattern, suggesting the cells were more maturated in the presence of pGSN.

Fig. 1. Effects of pGSN on maturation of immature erythroblasts. (A) Experimental design. Basophilic erythroblasts or polychromatic erythroblasts were differentiated from cord blood CD34+ cells and treated with pGSN. (B) As a vehicle control, basophilic erythroblasts were treated with the pGSN dissolving solution for 24 h (n = 3). (C) Erythroblasts were treated with pGSN for 24–48 h as basophilic erythroblasts and polychromatic erythroblasts, respectively. Cell morphology was evaluated by Wright-Giemsa staining. The number of mature erythroblasts increased with concentration of pGSN. Black arrows, orthochromatic erythroblasts; red arrows, enucleated red cells (scale bar, 50 μm). Representative images are shown (n = 4). (D) The mean percentage of mature erythroblasts increased with pGSN dose of 4 independent experiments. The proportion of RBCs was increased especially at the mature stage of polychromatic erythroblasts (>200 cells scored in 4 independent fields by two experts, n = 4). (E) Erythroid cell surface markers were measured by flow cytometry for the maturation stage using antibodies against GPA, CD71, CD44, integrin α4 and Band 3 at 24–48 h of pGSN treatment in the basophilic erythroblasts and polychromatic erythroblast stage, respectively (n = 3). As other cell lineage markers, CD11b and CD13 were assessed for basophilic erythroblasts (n = 3) and polychromatic erythroblasts (n = 5).

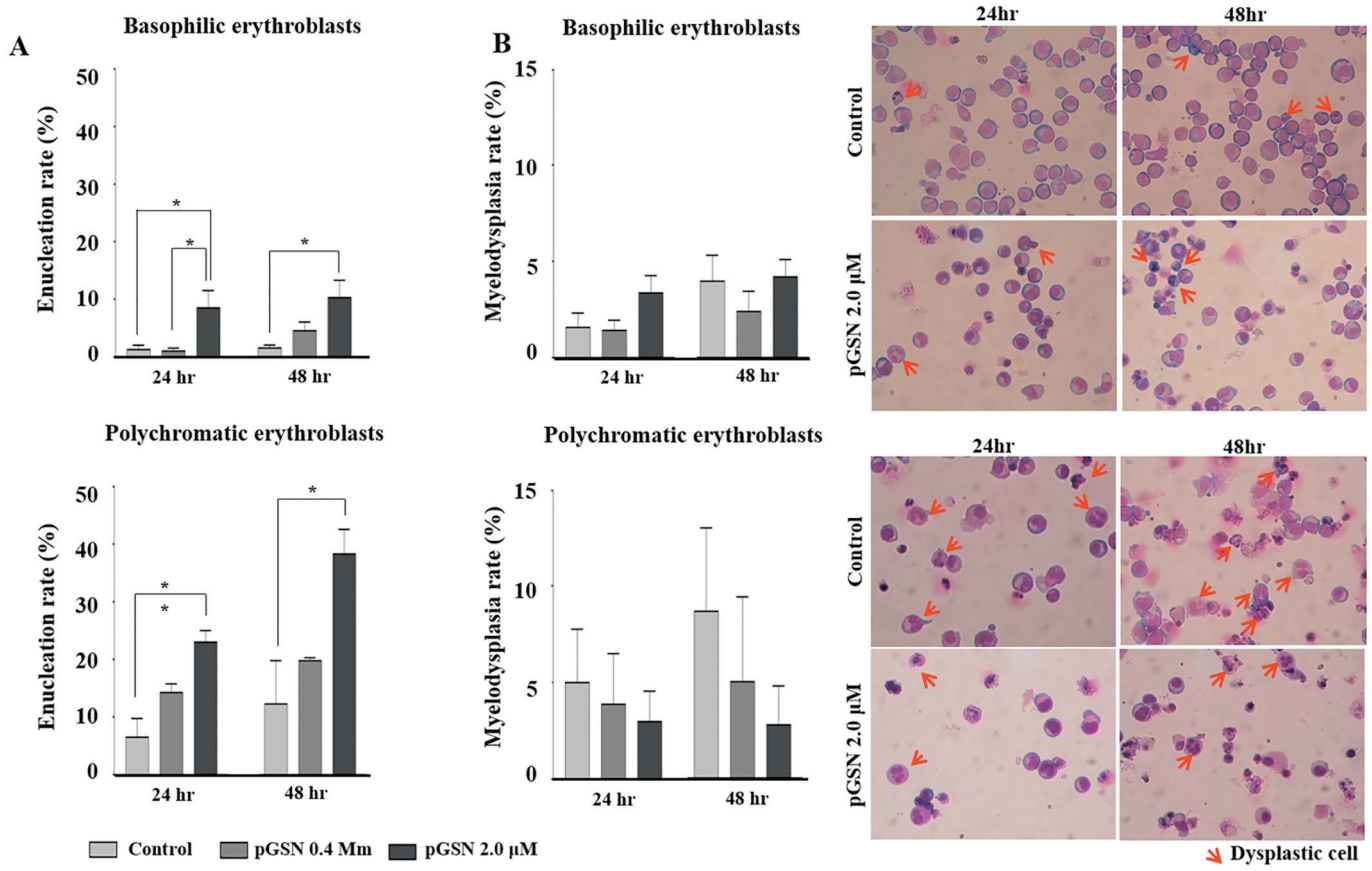


Fig. 2. Effect of pGSN on enucleation of erythroblasts and dysplastic cells. Basophilic and polychromatic erythroblasts were treated with pGSN for 24–48 h (>200 cells scored in 4 independent fields by two experts, n = 4). (A) The enucleation rate of erythroblasts was increased and (B) the dysplasia rate of the maturation phase of polychromatic erythroblasts was decreased (n = 3). (*$p < 0.05$, ** $p < 0.01$).

3.2. pGSN facilitates enucleation and reduces dysplasia of erythroblasts

Enucleated erythrocytes were significantly increased by treatment with 2.0 μM pGSN for 24 h, and the effect was more evident in polychromatic erythroblasts ($p < 0.01$) than in the basophilic stage ($p < 0.05$) (Fig. 2A). While myelodysplasia, as shown by abnormal cytokinesis and fragmented nuclei, is commonly found in serum-free *in vitro* cultures, treatment with pGSN in polychromatic erythroblasts decreased the ratio of dysplastic cells in a dose-dependent manner (Fig. 2B). However, the effect was not significant on dysplastic cells in a basophilic erythroblast state, probably due to the lower frequency of dysplastic cells at this early stage.

3.3. pGSN elevates mRNA expression levels related to terminal erythropoiesis

The effects of pGSN treatment on maturation status were assessed at the mRNA level. When basophilic erythroblasts were treated with pGSN, transcripts of GATA-1, which is one of the most important transcription factors regulating erythropoiesis, increased 5.5-fold at 24 h (Fig. 3A). Previous reports have shown that RhoA, ICAM-4, and DLC-1 mRNA levels increase during erythroblast maturation and enucleation, ICAM-4 interacts with DLC-1 and regulates cell morphology and enucleation (Choi et al., 2013). When basophilic erythroblasts were treated with pGSN, mRNA levels of ICAM-4 and DLC-1 were significantly increased at 48 h ($p < 0.05$) (Fig. 3A). During the polychromatic erythroblastic phase, GATA-1 mRNA expression was also increased with a two-fold increase that is a lower increase than that in basophilic erythroblasts. However, DLC-1 mRNA that increases along with terminal maturation and enucleation showed a significant increase during polychromatic phases, suggesting that pGSN activates downstream signals related to enucleation (Fig. 3B).

RhoA which is a small GTPases of the Rho family that regulates cell morphology, motility, and cell cycle were also increased about 1.7-fold at 24 h and 4.2-fold at 48 h in response to pGSN treatment. On the contrary, VLA-4 which is an integrin α4β1 for ICAM-4 was unaffected. These results demonstrate that pGSN increased the expression of terminal erythropoiesis-related genes in erythroblasts.

3.4. pGSN causes faster erythroid cell maturation via the reduced number of cell division at each maturation stage

Erythroblasts naturally undergo several cell divisions at each maturation stage. As our cell population is heterogeneous and contains enucleated reticulocyte, the cell proliferation-fold only cannot measure the cell division number. To clarify whether appearance of more mature cells by the treatment of pGSN is due to shortening of the cell-cycle time or the decrease in the number of cell divisions at each maturation stage, cells were labeled with the PKH26 and cultured with various concentration of pGSN. Then, the diluted fluorescent intensity by cell division was analyzed by measuring mean fluorescence intensity (MFI) by flow cytometry (Fig. 4A). The histograms of PKH26 fluorescence indicated that the cell division frequency was exactly the same for the control and pGSN treated samples for 24–48 h (Fig. 4B). This result suggests that pGSN does not shorten the time of cell cycle, but reduces the average number of cell divisions at each stage of the immature erythroblast and so leads to faster maturation than the control.

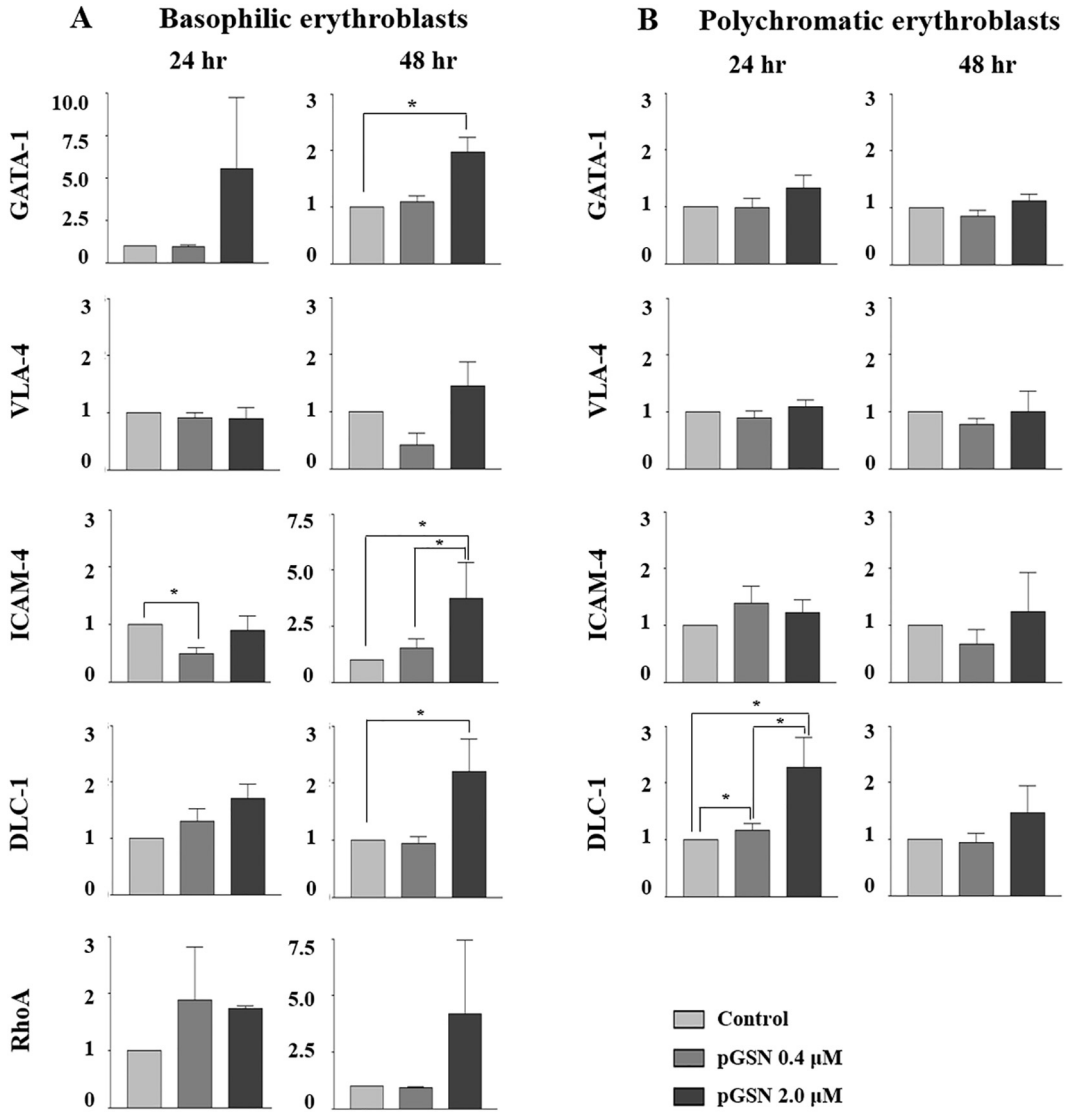


Fig. 3. Effect of pGSN on erythropoiesis-related mRNA levels. (A) Basophilic erythroblasts (n = 3) and (B) polychromatic erythroblasts (n = 4) were treated with pGSN for 24–48 h. Total mRNA expression levels of GATA-1, DLC-1, ICAM-4, VLA-4, and RhoA were analyzed by qRT-PCR. The mRNA expression levels were normalized to that of GAPDH. (*$p < 0.05$).

3.5. pGSN affects the erythroblast cell cycle and reverses G2/M blocking induced by vincristine

To further evaluate whether the effects of pGSN on dysplasia were related to the cell cycle, we analyzed the cell cycle of the erythroblasts. when basophilic erythroblast were treated with pGSN for 24–48 h, G1 was increased and G2/M decreased in a dose dependent pattern, resulting in a significant decrease of G2/G1 ratio at 2.0 μM pGSN ($p < 0.05$) (Fig. 4C). As this change of cell cycle proportion could be due to either the faster maturation effect by pGSN or the direct effect of pGSN on the cell cycle, further experiments using the cell cycle blocker were performed.

To see whether pGSN is able to correct erythroid dysplasia reversibly induced by a G2/M blocker, basophilic erythroblasts were treated with vincristine at the indicated concentrations for 24 h. To determine the appropriate concentration of vincristine to apply before pGSN treatment, cell viability and dysplasia were assessed at the indicated concentrations. A vincristine concentration of 5 ng/ml was used subsequently because it showed higher viability than, and a comparable degree of erythroid dysplasia to higher concentrations of vincristine (Fig. 5A and B). After treatment with vincristine, G0/G1 and S phases relatively decreased and G2/M was significantly increased ($p < 0.01$) (Fig. 5C and D), confirming the vincristine-induced G2/M phase arrest. As expected, cells treated with both pGSN and vincristine contained increased proportions of cells in G0/G1 and S phases and decreased proportion in G2/M phase in a dose-dependent pattern with significant change at 2.0 μM pGSN ($p < 0.05$) (Fig. 5C and E). This result suggests that pGSN enhances the G2/M checkpoint function in dysplastic immature erythroblasts and reverses the G2/M blocking effect of vincristine.

3.6. pGSN activates caspase-3 and apoptosis during erythroid differentiation

Caspase-3 is a key apoptotic signal but normally stimulated during early and late erythroid cells for erythroid cell maturation (Zermati et al., 2001). GSN is cleaved by caspase-3 in cytoplasm and its fragments can be either an anti-apoptotic or a pro-apoptotic signal depending on cell types and conditions (Carlile et al., 2004). To evaluate whether human plasma GSN is related to apoptosis or non-apoptotic maturation effector, we observed whether gelsolin induces caspase-3 along with apoptotic markers at each maturation stage.

In control conditions, proerythroblasts, basophilic erythroblasts, and polychromatic erythroblasts showed small peaks with caspase-3

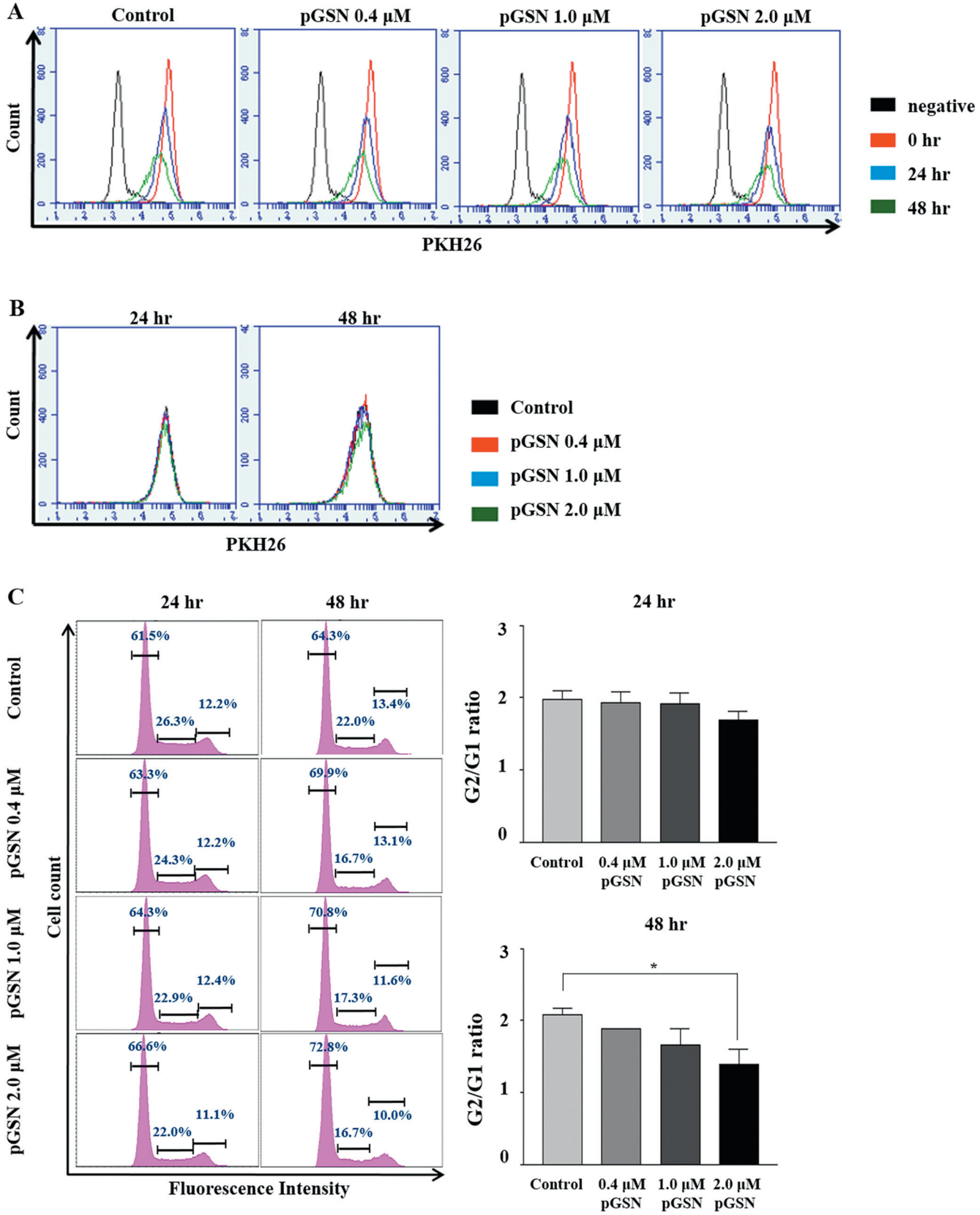


Fig. 4. pGSN does not increase the frequency of cell divisions but causes cytokinesis. (A) Basophilic erythroblasts were stained with PKH26 and cultured in the presence of pGSN. Mean fluorescence intensity (MFI) was analyzed by flow cytometry to evaluate cell division frequency. (B) The PKH26-labeled treated and control cells divided at the same frequency at each concentration of pGSN (n = 3). (C) The cell cycles of pGSN-treated erythroblasts were analyzed by flow cytometry after PI staining (n = 4). pGSN increased the proportion of G0/G1 phase cells and decreased the proportion of S/M phase cells in a dose-dependent pattern, represented by G2/G1 ratio. (*$p < 0.05$ *vs.* control group).

activity, which are known as non-apoptotic maturational signals in these stages of maturation (Zermati et al., 2001; Carlile et al., 2004). With pGSN treatment at each phase, basophilic and polychromatic erythroblasts showed significantly increased levels of caspase-3 ($p < 0.05$) (Fig. 6A), but levels of caspase-3 at a proerythroblast stage did not change significantly. Therefore, pGSN activates caspase-3 depending on maturation stages.

To determine whether the increased caspase-3 activity is due to apoptosis or non-apoptotic maturation effects, cells were labeled with Annexin V and propidium iodide (PI) and analyzed by flow cytometry. pGSN treated basophilic and polychromatic erythroblasts showed significantly increased levels of both early and late apoptotic cells ($n = 5$) (Fig. 6B).

These results suggest that pGSN significantly increased apoptosis *via* caspase-3 activation during basophilic and polychromatic erythroblast stages. These results suggest that pGSN functions as pro-apoptotic signals that might remove dysplastic cells that are caused by *in vitro* culture conditions that lacks pGSN sources such as macrophages and serum.

3.7. pGSN enters erythroblasts

To further evaluate whether extracellular pGSN could enter the human erythroid cells, the basophilic erythroblasts and K562 cells were treated with 2.0 μM His-tagged pGSN for 24 h and then stained with antibodies against the His-tag. The result by confocal microscopy showed that His-tagged pGSN entered the human erythroid cells (Fig. 7A) as well as K562 cells (Fig. 7B), but not in control cells.

4. Discussion

In the present study we made recombinant human plasma-type gelsolin and examined its effect on human erythroblasts. We found that pGSN accelerated erythroid cell maturation and enucleation, and induced caspase-3 expression which is critical for the maturation of erythroblasts. It also corrected transient myelodysplasia *in vitro* especially in polychromatic erythroblasts, suggesting that it controls cell cycle and terminal maturation.

Erythroblasts committed from HSCs undergo a series of differentiation steps from proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts, orthochromatic erythroblasts, reticulocytes, and erythrocytes (Gabet et al., 2011; Testa, 2004). The erythroid cells have different characteristics at each stage of maturation (Testa, 2004). For example, caspase-3 expression is elevated in basophilic erythroblasts and orthochromatic erythroblasts and functions as a non-apoptotic maturation signal (Carlile et al., 2004). Even though *in vivo* models such as knock out mice would be more accurate for analyzing *in vivo* functions of a certain protein, evaluating the sole effects of plasma gelsolin in erythroid cells is not possible without hampering functions of cytoplasmic gelsolin as the two types of gelsolin are originated from one gene. Moreover, gelsolin is also present in many other cells and can be secreted from them including macrophages which are critically important in controlling erythropoiesis. Our results using recombinant gelsolin protein showed their effects not just in a RNA level but with clear cellular changes such as enhanced maturation and enucleation.

Cell cycle analysis using PKH26 showed that pGSN did not shorten the time of cell division and seemed to reduce the number of divisions to accelerate maturation. It increased the ratio of mature orthochromatic erythroblasts and enucleated reticulocytes when analyzed by morphology and maturation markers expression. In mice it is well established that basophilic erythroblasts undergo 2 mitoses to generate, sequentially, 2 polychromatic erythroblasts, and 4 orthochromatic erythroblasts (Liu et al., 2013). However, in human erythroblasts it is suggested that the erythroblasts undergo an additional mitoses (Stohlman Jr. et al., 1968) and Hu J et al. showed that there are 3 mitoses at the early basophilic erythroblasts, 2 at late basophilic, and 1 at the polychromatic erythroblastic phase (Hu et al., 2013). Also, in our data, the cell expansion-folds from the culture day 10, when the almost all cells are basophilic erythroblasts, were >60-fold until reticulocytes, suggesting that cells were divided about 6 times. Since the poly- or orthochromatic erythroblasts came out earlier with pGSN, we assessed whether pGSN induced a decreased number of cell division (lower than 6 times) or reduced cell cycle time but probably with the same mitosis number. If pGSN can accelerate the maturation speed by the shortened cell division time, PKH experiments would have shown the different peaks in Fig. 4B. However, the number of cell cycle at the same time is exactly the same but with more mature cells between the control and pGSN conditions. Therefore, we suggest that the erythroblast did not proliferate up to the maximal number of the cell division but differentiated to the late basophilic erythroblasts or polychromatic erythroblasts, enabling faster erythropoiesis in the presence of pGSN.

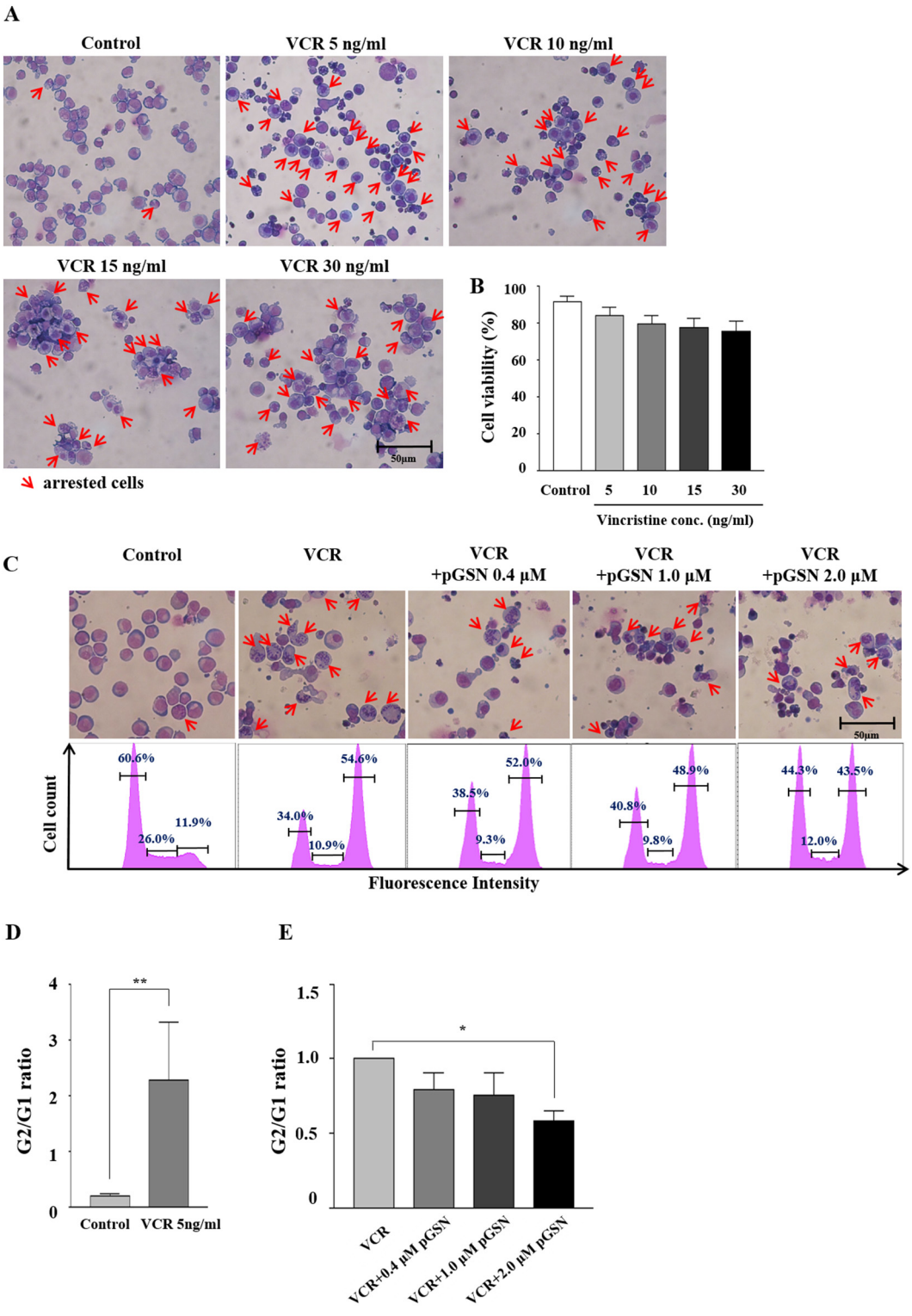
We also evaluated the effects of pGSN in the mRNA level related to erythropoiesis. pGSN increased GATA-1 mRNA levels, especially in more immature erythroblasts. Gelsolin regulates severing and capping of actin filaments in cells (Sun et al., 1999) and actin filaments are one of the major components of the cytoskeleton, which is critical in erythroblast enucleation (Konstantinidis et al., 2012). However, how gelsolin affects these processes is not fully understood in erythroblasts. Therefore, we assessed levels of transcripts of DLC-1 which increases around the terminal maturation and enucleation period, and of RhoA which regulates actin cytoskeleton remodeling. An ICAM-4 ligand interacts with VLA-4 and DLC-1 during cell differentiation and increases during erythroblast maturation (Choi et al., 2013).

We found that all of these mRNA increased in response to pGSN during immature erythroblast maturation. Expression of GATA-1 and ICAM-4 were elevated in a basophilic phase and DLC-1 mRNA increased in both the basophilic and polychromatic phases, suggesting a close relationship with pGSN signaling during erythropoiesis and enucleation. However, the detailed signaling by pGSN requires further study.

Gnanapragasam MN et al. showed that the cell cycle pattern changes very minimal until polychromatic phase, but at the orthochromatic stage, the S phase proportion was markedly shortened followed by delayed G1 accumulation, which are necessary for cell cycle exit and enucleation (Gnanapragasam et al., 2016). If the cell population had more mature erythroid cells, they would show shortened S and prolonged G1 phases compared to the control. So, in our experiments of Fig. 4C, we could not say that the effect of gelsolin directly affect the cell cycle but could be just the consequence of faster maturation by pGSN. However, these results also support that pGSN enhanced maturation of erythroid cells.

As gelsolin is known to be associated with the G2/M checkpoint (Sakai et al., 1999), we tested whether pGSN could reverse the abnormal cytokinesis in myelodysplasia. As vincristine is a vinca alkaloid, widely used anti-neoplastic agent and induces myelodysplasia by cell cycle arrest (Tsutsui et al., 1986; Tu et al., 2013), we generated dysplastic erythroblasts by treatment with vincristine. In response to treatment with vincristine and pGSN, cells in a G1 phase increased and those in G2/M phase decreased in proportion. Thus, pGSN appears to reverse the G2/M arrest induced by vincristine. Also, pGSN reduced the abnormal cytokinesis, which is frequently observed in serum-free cell culture conditions. The decreased dysplasia and erythroid differentiation/maturation

Fig. 5. Effects of pGSN on cell cycle progression and reversal of G2/M blockage induced by vincristine in immature erythroblasts. (A) Basophilic erythroblasts were treated with 5, 10, 15, and 30 ng/ml of vincristine (VCR) for 24 h ($n = 3$). (B) Then, the viability of the erythroblasts was measured by trypan blue staining ($n = 3$). (C) Basophilic erythroblasts were treated with 5 ng/ml of vincristine and with the indicated levels of pGSN for 24 h. Then, cell cycles were analyzed by flow cytometry after PI staining. Representative images of cytospun cells stained with Wright-Giemsa, and cell cycle analysis ($n = 3$). (D) Graphs showing that G2/M phase was arrested by vincristine treatment, represented by G2/G1 ratio ($n = 3$). ($^{**}p < 0.01$ *vs.* control group). (E) The G2/M phase arrest by vincristine in basophilic erythroblasts was reversed by pGSN treatment in a dose-dependent pattern, represented by G2/G1 ratio normalized to the vincristine condition ($n = 3$, $^{*}p < 0.05$).


A
Control
VCR 5 ng/ml
VCR 10 ng/ml
VCR 15 ng/ml
VCR 30 ng/ml
50μm
arrested cells
B
Cell viability (%)
100
80
60
40
20
0
Control
5
10
15
30
Vincristine conc. (ng/ml)
C
Control
VCR
VCR +pGSN 0.4 μM
VCR +pGSN 1.0 μM
VCR +pGSN 2.0 μM
50μm
Cell count
60.6%
11.9%
26.0%
54.6%
34.0%
10.9%
52.0%
38.5%
9.3%
48.9%
40.8%
9.8%
44.3%
43.5%
12.0%
Fluorescence Intensity
D
G2/G1 ratio
**
4
3
2
1
0
Control
VCR 5ng/ml
E
G2/G1 ratio
*
1.5
1.0
0.5
0
VCR
VCR+0.4 μM pGSN
VCR+1.0 μM pGSN
VCR+2.0 μM pGSN

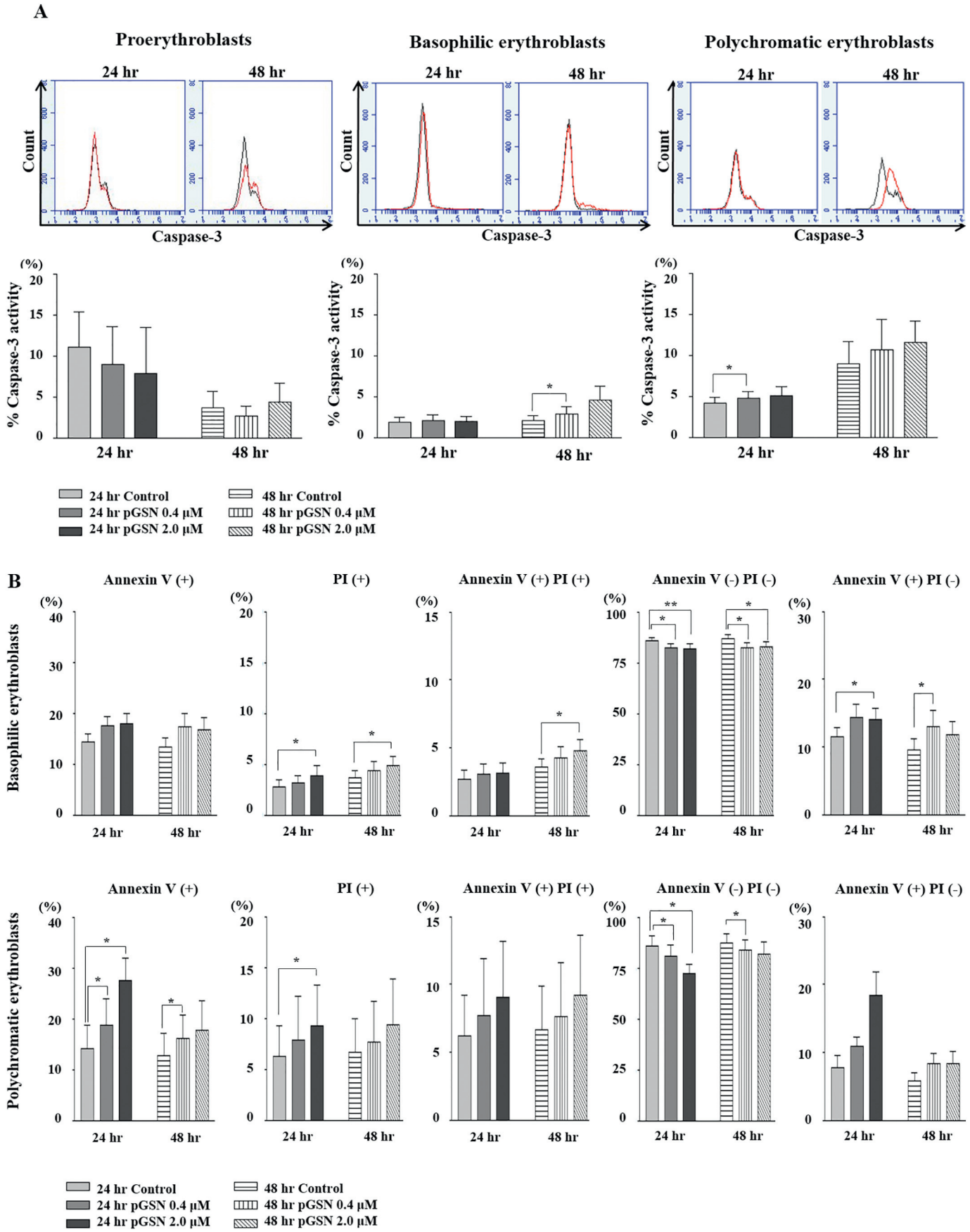


Fig. 6. pGSN effects on caspase-3 activation with increased apoptosis at mature erythroblasts (A) Erythroblasts at each maturation stage were treated with 2.0 μM pGSN for 24–48 h. Then, caspase-3 activities were analyzed by flow cytometry. pGSN induces early and late apoptosis in both polychromatic- and orthochromatic erythroblasts but not in proerythroblasts. Black line: control; Red line: pGSN 2.0 μM. (B) Cells were stained with Annexin V and PI after treatment of pGSN and then analyzed by flow cytometry. ($n = 5$, $^{*}p < 0.05$, $^{**}p < 0.01$).

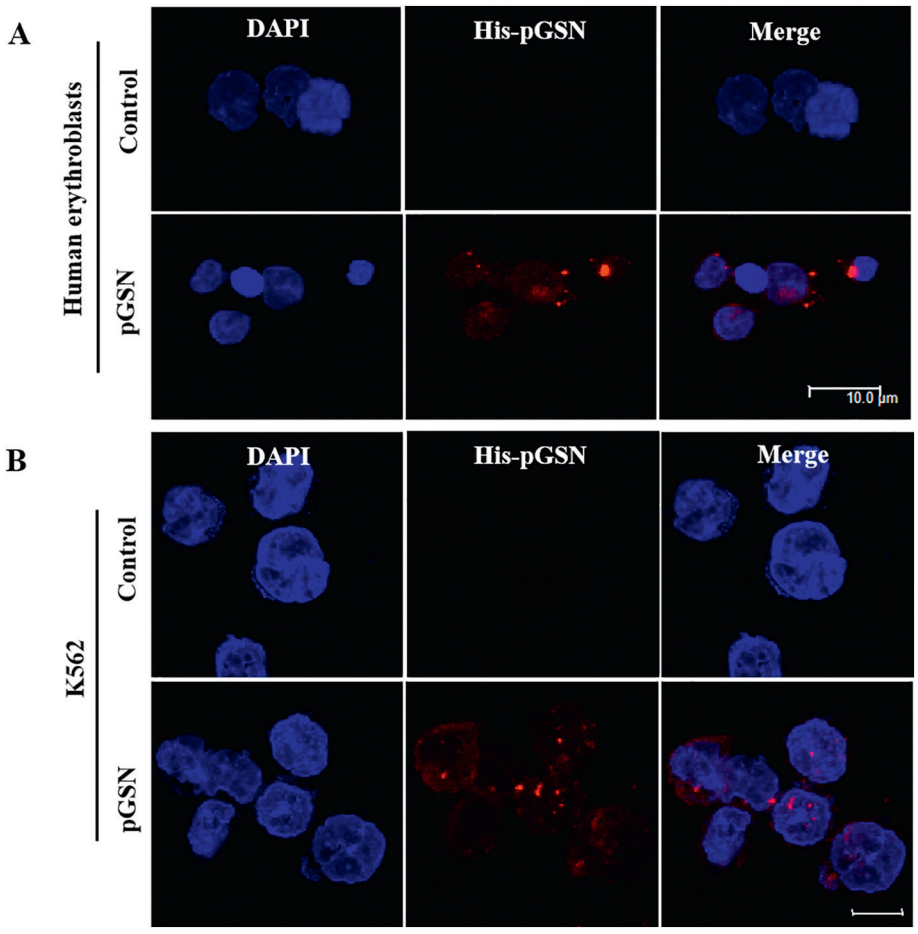


Fig. 7. Confocal images of human erythroblasts with His-tagged pGSN. (A) Basophilic erythroblasts and (B) K562 cells were treated with or without 2.0 μM His-tagged pGSN for 24 h. Cells were stained with DAPI and anti-His and analyzed by confocal microscopy. (Blue; DAPI, Red: His-Tag).

but with the increased ratio of apoptosis by pGSN might be the result that pGSN blocked the transit of dysplastic cells from the G2M phase. In our experiments, pGSN accelerated caspase-3 expression at both basophilic and polychromatic erythroid stages and increased apoptotic signals. Levels of caspase-3 were slightly increased in proerythroblasts but significantly increased in more mature erythroblasts, suggesting its different functions depending on the cell maturation status.

The mechanism of pGSN on erythropoiesis could be intracellular or extracellular effect. In the Fig. 7, we, for the first time, demonstrated that pGSN can pass the cell membrane and enter inside the cells. Next, for the possible extracellular mechanism, the previous report demonstrated that lysophosphatidic acid (LPA) bind to the gelsolin (Mintzer et al., 2006) and LPA-bound pGSN deliver LPA to LPA receptors on cell membrane and then pGSN augment LPA stimulation of nuclear signals and protein synthesis in rat cardiac myocytes (RCMs) that express Edg-2 and -4 receptors (or LPA receptors) (Goetzl et al., 2000). LPA, a growth factor-like phospholipid, regulates numerous physiological functions, including cell proliferation and differentiation. Chiang C. et al. have demonstrated that LPA enhances erythropoiesis by activating the LPA receptor subtype-3 (LPAR3) under erythropoietin induction (Chiang et al., 2011). Lin K. et al. also demonstrated that LPAR3 agonist enhances erythropoiesis in erythroblasts derived from cord blood derived-CD34+ cells (Lin et al., 2016).

So, we hypothesized that extracellular pGSN affects cell maturation or viability *via* the delivery of LPA to LPAR3. Therefore, experiments using pGSN with or without LPA or LPA antagonist (Ki16425) were performed to assess whether pGSN effects may have a relationship with LPA activity *via* its receptors on erythroid cells. In our experiments (Supple Fig. 2), pGSN addition enhanced erythropoiesis as in our other results. However, the sole supplement of LPA, LPAR3 antagonist, or both did not alter the viability or maturation status compared to the control. However, together with pGSN, LPAR3 antagonist caused more maturation, while LPA hampered erythropoiesis, unlikely to the previous report. Therefore, the hypothesis that pGSN could enhance erythropoiesis extracellularly with LPA through LPAR3 activation doesn't seem to be relevant to our results. We suggest that LPA that bind to pGSN inhibited for pGSN to enter the cells therefore diminish the intracellular amount of pGSN. However, to elucidate the precise mechanisms, extensive experiments are required further.

This further suggests that pGSN can promote the natural process of erythroid cell maturation in terms of cytokinesis and dysplasia, and could help in manufacturing erythrocytes *in vitro* in the absence of macrophages and serum. Our findings also provide a clue that pGSN might be used as a therapeutic reagent for patients with erythroid dysplasia who have a low plasma gelsolin level. Further *in vivo* studies are required to establish which sources of pGSN among macrophages, plasma, or platelets are critical for erythropoiesis in bone marrow.

5. Conclusion

We demonstrate for the first time that pGSN promotes maturation and the cell cycle in human basophilic and polychromatic erythroblasts by correcting abnormal cytokinesis, accelerating erythropoiesis, and increasing terminal erythropoiesis/enucleation-related signaling as well

as enucleated red cells. Furthermore, pGSN could be used as a reagent to produce RBCs *in vitro* in serum- and macrophage-free culture conditions and as a possible therapeutics to treat erythroid dysplasia.

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