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Functional Characterization of hNUDC As a Novel Accumulator That Specifically Acts on In Vitro Megakaryocytopoiesis and In Vivo Platelet Production

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Abstract Human NUDC (hNUDC) has been previously described as a human homolog of a fungal nuclear migration protein. It is a multifunctional interactive protein that forms an association with the microtubule motor complex in a variety of cells. Our recent studies demonstrated that hNUDC could bind specifically to the thrombopoietin receptor (Mpl) and suggest a potential role for hNUDC in megakaryocytopoiesis and thrombopoiesis. The present study is designed to define its biological activity. We demonstrate that the recombinant hNUDC significantly increases megakaryocyte maturation in serum-free liquid-cultured human $CD34^+$ cells and stimulates colony formation in serum-free semi-solid cultures. Flow cytometry analyses also confirm the stimulatory effect of hNUDC on megakaryocyte polyploidization and in vitro platelet production. In vivo experiments further demonstrate that the administration of hNUDC substantially enhance the number of circulating platelets in normal mice. J. Cell. Biochem. 98: 429–439, 2006.

Key words: hNUDC; TPO; Mpl; megakaryocyte; platelet

Megakaryocyte maturation is a unique developmental process that requires both mitotic cell division and endomitotic nuclear replication from the hematopoietic progenitor cells. Megakaryocytes enter a maturation stage by switching from a normal mitotic cell cycle to an endomitotic cell cycle, which is believed to be a form of abortive mitosis that is characterized by inability to complete the late stages of mitosis and cytokinesis [Nagata et al., 1997; Vitrat et al., 1998]. At the end of polyploidization, the megakaryocyte increases synthesis of specific platelet proteins and the size of its cytoplasm, leading to enhanced production of platelets [Italiano et al., 1999].

The production of platelets from cells of the megakaryocyte lineage is regulated by a series of cytokines including interleukin-1 (IL-1), interleukin-3 (IL-3), interleukin-6 (IL-6), interleukin-11 (IL-11), stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and thrombopoietin (TPO) [Kaushansky et al., 1986; Ishibashi et al., 1989; Paul et al., 1990; Burstein et al., 1992; Tanaka et al., 1992; Bartley et al., 1994; Brandt et al., 1994; Lok et al., 1994; Wendling et al., 1994; de Sauvage et al., 1994]. So far, TPO is the most extensively studied member of cytokine family. The binding of TPO to its receptor, thrombopoietin receptor (Mpl), transduces a cascade of signals in megakaryocyte cells, leading to megakaryocyte colony-formation units (CFU-MK) in vitro and elevated platelet levels in vivo [Alexander et al., 1995; Kaushansky and Drachman, 2002]. However, the signaling events that induce the final stages of proplatelet production and platelet release remain ambiguous. Recent studies have found that the last steps of platelet formation do not entirely depend on TPO [Nagahisa et al., 1996; de Sauvage et al., 1996;

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Bunting et al., 1997], as $TPO^{-/-}$ mice were not more vulnerable to hemorrhage than normal mice, and retained approximately 15% of the circulating platelets and 15% of marrow and spleen megakaryocytes [de Sauvage et al., 1996; Bunting et al., 1997]. This suggests the possibility of other factors being involved.

We have recently used the extracellular portion of Mpl as the bait protein in a yeast two-hybrid system to isolate a full-length human cDNA identical in sequence to *hnudC* [Pan et al., 2005]. GST Pull-down, coimmunoprecipitation and colocalization of hNUDC and Mpl in human megakaryocyte cells confirmed this interaction [Pan et al., 2005].

Based on the similarity of its C-terminus to that of fungal NUDC from *Aspergillus nidulans*, hNUDC was defined as a nuclear migration protein [Matsumoto and Ledbetter, 1999]. In humans, hNUDC is expressed in most tissues and very highly in hematopoietic precursor cells where hNUDC was implicated in the promotion of hematopoietic cell growth [Miller et al., 1999]. In cultured HeLa cells and *C. elegans* embryos, disruption of the homologs of NUDC using gene silencing and overexpression techniques resulted in multinucleate cells [Aumais et al., 2003]. Because of the abundance of hNUDC in hemopoietic cells and because megakaryocyte maturation requires endomitotic nuclear replication, we were led to investigate the possible physiological role of hNUDC in megakaryocyte maturation, polyploidization and platelet production.

MATERIALS AND METHODS

Materials

A monoclonal antibody against hNUDC was prepared in our laboratory by standard techniques from mice immunized with bacterial expressed recombinant hNUDC [Pan et al., 2005]. *Pichia* Expression Kit was obtained from Invitrogen (Carlsbad, CA). BD TALON Metal affinity Resins was obtained from BD Biosciences (Clontech, Palo Alto, CA) R-phycoerythrin (R-PE)- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies against rabbit or mouse IgM + IgG, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), FITC-conjugated anti-human CD41, allophycocyanin (APC)-conjugated anti-human CD34, PE-conjugated-anti-CD41, and APC-conjugated-anti-CD62P monoclonal antibodies were purchased from BioLegend (San Diego, CA). MegaCult kit was obtained from Stem Cell Technologies (Vancouver, BC, Canada). Recombinant human TPO was purchased from Sigma Chemicals Co. (St. Louis, MO).

Construction of *P. pastoris* Expression Vector pPICZαA -hNUDC-His

The full-length cDNA encoding hNUDC was originally isolated as a 1.3-kb fragment in a pB42AD vector from a fetal liver cDNA library [Pan et al., 2005]. A cDNA fragment encoding C-terminal 6× His-tagged hNUDC was amplified by two-step PCR using the pB42AD-hNUDC as a template with the following primers: 5′-CCGCTCGAGAAAAGA***ATG***GGCGGAGAGC-AGGAG-3′ (forward primer), 5′-ATGGTGAT-GGTGATGGTTGAATTTAGCCTT-3′ (reverse primer I), and (5′-GCTCTAGA***CTA***GTGATGG-TGATGGTGATGGTTGAA-3′ (reverse primer II). The forward primer contains an ATG codon (bold and italic), followed by a Kex2 signal cleavage site (double underlined) and *Xho*I site (underlined) at the 5′ end. Reverse primer I contains 5× His codons (double underlined) at the 3′ end. Reverse primer II contains 6× His codons (double underlined), followed by a stop codon (bold and italic) and an *Xba*I site (underlined) at the 3′ end. Forward and reverse primer I were used to generate a 1,023-bp fragment. This fragment was used in a second PCR reaction with forward primer and reverse primer II, which overlapped reverse primers I by 21 nucleotides. Following digestions with *Xho*I and *Xba*I, the resulting fragment was cloned into vector pPICZαA, in frame and downstream of the α-factor signal sequence to give pPICZαA-hNUDC-His.

Expression and Purification of Recombinant hNUDC-His

The *BstX*I-linearized pPICZαA-hNUDC-His or an empty vector was transformed into *P. Pastoris* KM71 by electroporation. Transformants were selected on plates with YPDS medium (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, and 2% agar) and Zeocin (100 μg/ml) for 3 days at 30°C. For the verification of gene integration into the KM71 genome, genomic DNA from transformants was isolated and examined by PCR. To test for the most productive transformants, colonies were grown in 10 ml of BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0,

1.34% yeast nitrogen base, 0.00004% biotin, and 1% glycerol) at 30°C for 24 h. Cells were collected by centrifugation and gently resuspended in 10 ml of liquid BMMY medium (same as BMGY medium but containing 0.5% methanol instead of 1% glycerol). Secretion of hNUDC-His was monitored by SDS–PAGE at day 0, 1, 2, 3, and 4 after induction with methanol. The transformant with the highest protein yield was used for large-scale protein expression.

Large-scale expression was achieved using 500 ml culture in a 2-liter flask. Cells grown in BMGY at 30°C to a density of $OD_{600} = 2$ to 6 were harvested, resuspended in 100 ml of BMMY and incubated with shaking at 30°C. The culture was continued for a total of 3 days and methanol was added to the medium daily to yield a final concentration of 0.5%. The culture supernatant was concentrated using a Minitan Ultrafiltration System (Millipore, Bedford, MA), dialyzed against PBS, pH 8.0, and loaded on a pre-packed cobalt-based immobilized metal affinity chromatography (Co^{2+}IMAC) column (Clontech, Palo Alto, CA). The column was washed with three column volumes of 300 mM NaCl, 20 mM sodium phosphate, pH 8.0. The protein was eluted with the same buffer containing 150 mM imidazole.

Cell Source and $CD34^+$ Cell Purification

Umbilical cord blood was obtained from Guangdong Hematopoietic Stem Cell Therapy Technology Center. Mononuclear cells were isolated using Ficoll-Hypaque (density, 1.077; Amersham Biosciences, NJ) density gradient centrifugation. $CD34^+$ cells were then isolated using the Dynal CD34 Progenitor Cell Selection System (Dynal, Oslo, Norway). The purity of $CD34^+$ cells was 84–98%.

Megakaryocyte Colony Forming (CFU-MK) Assay

$CD34^+$ cells (5×10^3 cells/ml) were plated in triplicate 35-mm tissue culture dishes with 0.95% methylcellulose in serum-free media containing penicillin/streptomycin/glutamine supplemented with 0.05–0.5 μg/ml hNUDC-His or 0.05 μg/ml recombinant TPO. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 12 days, multipotential (granulocyte, erythroid, macrophage, megakaryocyte, and GEMM) progenitor cell-derived colonies were scored by microscopy analysis.

For the definitive detection of CFU-MKs, these colony-forming cells were evaluated by immunohistochemical staining in a collagen-based serum-free medium using the MegaCult kit according to manufacturer instructions. Briefly, harvested $CD34^+$ cells were plated in MegaCult serum free medium supplemented with hNUDC-His or recombinant TPO, and incubated at 37°C in a humidified atmosphere of 5% CO_2. After 12 days, results of colonies were identified using a monoclonal anti-CD41 by anti-alkaline phosphatase technique according to the manufacturer's instructions.

Flow Cytometric Analysis of Human Megakaryocyte Cells

Purified $CD34^+$ cells were cultured in serum-free liquid media (Stem SpanTM SFEM, StemCell Technologies, Inc., Vancouver, BC) containing penicillin/streptomycin/glutamine supplemented with 0.05–0.5 μg/ml hNUDC-His or 0.05 μg/ml TPO. Cells were removed on day 12 and stained with FITC-conjugated anti-human CD41 and APC-conjugated anti-human CD34 antibodies. Control staining was performed with isotype-matched control antibodies. After staining, the cells were washed in cold PBS, fixed and permeabilized using 1% paraformaldehyde in PBS, and analyzed on a FACsort flow cytometer (Becton Dickinson, Mountain View, CA) with the Cell Quest software package. The DNA content of $CD41^+$ cells was estimated using propidium iodide (PI).

In Vitro Generation of Megakaryocyte Cells From $CD34^+$ Cells and Detection of Proplatelet-Bearing Megakaryocytes

Purified $CD34^+$ cells were stimulated to form megakaryocytes in serum-free liquid media by the addition of 0.05 μg/ml TPO. After 9 days, megakaryocytes were purified using the MiniMACS selection system (Miltenyi Biotec) and washed twice in serum-free media. The megakaryocytes were cultured in 24-well plates at a concentration of 1×10^3 cells/well in serum-free media containing 0.05–0.5 μg/ml hNUDC-His or 0.05 μg/ml TPO for observation of proplatelet formation after incubation for 72 h. Proplatelet-bearing megakaryocytes were defined as cells exhibiting one or more cytoplasmic processes with areas of constriction.

Detection of In Vitro Platelet Production

Nine-days-TPO-expanded megakaryocytes (1×10^6) were cultured in serum-free media containing hNUDC-His or TPO for a further 72 h. The culture medium was gently collected and centrifuged at 150*g* for 20 min to remove the nucleated large cells. The supernatant was fixed with 1% paraformaldehyde for 1 h and centrifuged at 1,000*g* for 10 min. Platelet pellets were washed with PBS, suspended in 100 μl PBS/BSA, and incubated with a PE-conjugated-anti-CD41 monoclonal antibody for 45 min. In parallel, activated platelets were stained with PE-anti-CD41 and APC-conjugated -anti-CD62P antibodies following 1 U/ml thrombin stimulation according to the method described by Choi et al. [1995]. Both non-activated and activated platelets were enumerated by flow cytometer (Becton Dickinson) as particles with the same scatter properties as blood platelets.

Platelet Counts in Mice

Pathogen-free BALB/c mice 8 to 10 weeks old were subcutaneously injected with hNUDC-His for 7 days with varying dosages of 2.5, 10, and 20 μg/kg, respectively. TPO (2.5 μg/kg) was used as a positive comparison. PBS containing 100 μg/ml of BSA was used as a negative control. Venous blood (20 μl) was collected from a small lateral cut in a tail vein on days 0, 4, 7, 10, 13, 16, and 19. Platelets were counted using of an F-820 Sysmex electronic blood cell analyzer (Sysmex Corp Ltd., Japan).

RESULTS

Overexpression and Purification of hNUDC-His

Recombinant hNUDC-His was expressed under the control of the yeast α-factor promoter. This system allows removal of the α-factor signal peptide by the yeast Kex2 enzyme as the fusion protein enters the secretory pathway. A time-course analysis was performed with the culture supernatants being taken after 0, 1, 2, 3, and 4 days to examine protein production in *P. pastoris* KM71 by SDS–PAGE (Fig. 1A). A band corresponding to approximately 45 kDa was observed after 1 day and became more pronounced as time progressed (Fig. 1A). hNUDC-His was not observed in the supernatant of yeast cells before induction or in the induced cells transformed with the pPICZαA vector only (Fig. 1A). hNUDC-His was purified by affinity purification from culture supernatant harvested on day 3, resulting in a yield of ~20 mg/L of culture (Fig. 1B). The purified hNUDC-His protein was confirmed by Western blot using an anti-hNUDC monoclonal antibody (Fig. 1C).

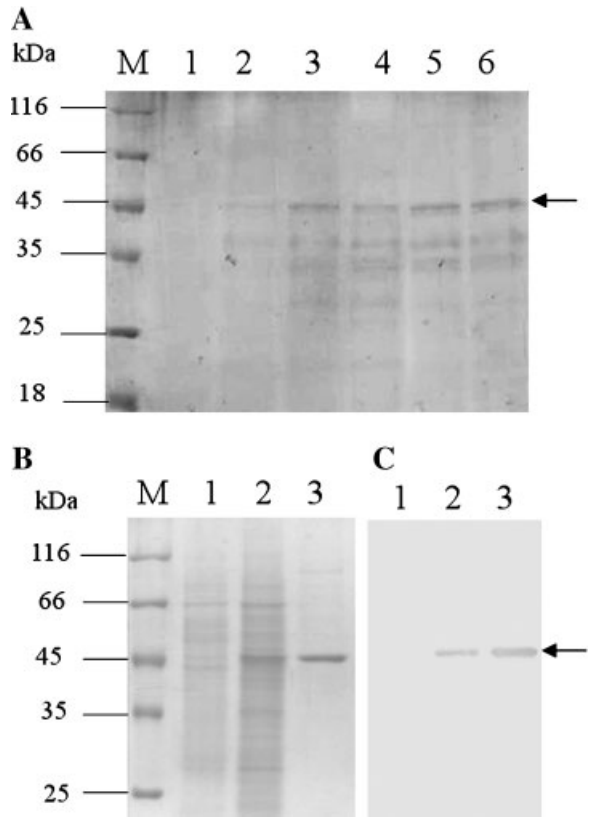

Fig. 1. SDS–PAGE gel and Western blot analysis of hNUDC-His expressed in *P. pastoris*. **A**: Samples from culture supernatants after induction for 0–4 days were run on a 12% SDS–PAGE gel and stained with Coomassie Brilliant Blue. M, molecular mass protein markers; (**Lane 1**) culture supernatant from a yeast clone transformed with empty pPICZαA; (**Lane 2**) culture supernatant from a yeast clone transformed with pPICαA-hNUDC-His before methanol induction (Day 0); (**Lanes 3–6**) culture supernatants from a yeast clone transformed with pPICZαA-hNUDC-His after induction for 1, 2, 3, and 4 days with 0.5% methanol. **B**: hNUDC-His was purified by immobilized metal affinity chromatography from the yeast culture supernatant. M, molecular mass protein markers; (Lane 1) culture supernatant from a yeast clone transformed with pPICZαA-hNUDC-His before methanol induction; (Lane 2) culture supernatants from a yeast clone transformed with pPICZA-hNUDC-His after induction for 3 days with 0.5% methanol. Lane 3: hNUDC-His eluted from Co^{2+} affinity chromatography. **C**: Immunoblotting of hNUDC-His with an anti-hNUDC monoclonal antibody. The arrows indicate the band corresponding to hNUDC-His.

The Effect of hNUDC-His on Human CFU-MK

Purified $CD34^+$ cells were plated in methylcellulose cultures for colony formation. The

proportions of CFU-MKs, estimated from colonies of 2–20, 21–50, and >50 cells per colony were increased when cells were exposed to hNUDC-His (0.05 or 0.1 μg/ml) or TPO (0.05 μg/ml) (Fig. 2A). A dose of 0.5 μg/ml of hNUDC-His slightly reduced the number of CFU-MKs (Fig. 2A). The majority of the increased number of CFU-MKs in hNUDC-His treated cells were 3–20 cells/colony, whereas the number of CFU-MKs with greater than 50 cells per colony were increased in the presence of TPO. CFU-MKs were further demonstrated by culture of $CD34^+$ cells in the presence of hNUDC-His or TPO in a collagen-based media designed to support CFU-MK growth. The CFU-MKs were specifically identified by immunostaining with an antibody against CD41, specific for megakaryocytes and platelets. The colonies formed in a collagen-based media had the same highly characteristic morphologies of colonies with that in methylcellulose (Fig. 2B and C). There were no other progenitor cell-derived colonies were found methylcellulose media based on a microscopy observation (Fig. 2C).

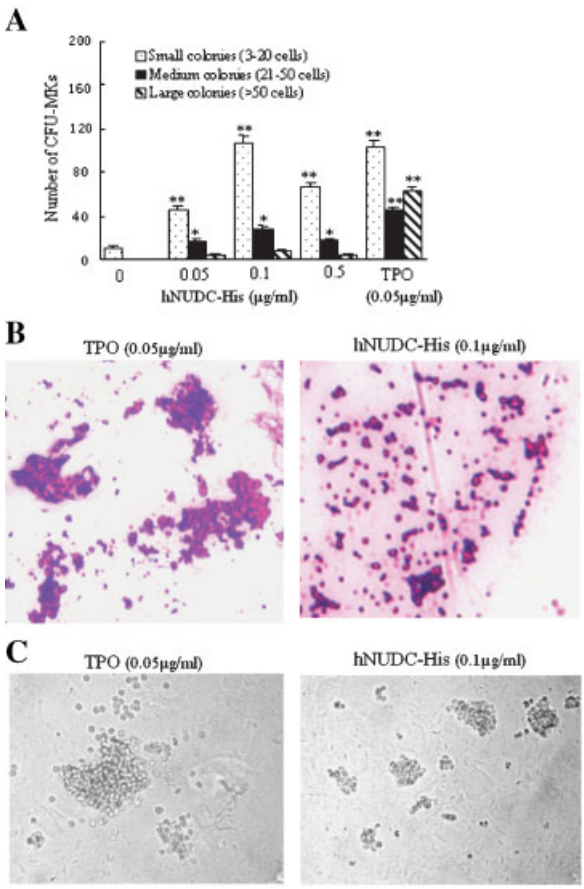

Fig. 2. The effect of hNUDC-His on CFU-MK. **A**: The number of CFU-MKs in cultured $CD34^+$ cells after incubation in the absence or the presence of hNUDC-His or TPO. Colonies classified according to their size were evaluated by inverted light microscopy. Experiments were performed in triplicate in four assays. Results are expressed as the mean number ± SE of megakaryocyte colonies. Statistically significant compared with PBS (*, $P < 0.05$; **, $P < 0.01$). **B**: CFU-MKs were grown on collagen matrix as described in ''Materials and methods.'' Cells were fixed and immunohistochemically examined using a monoclonal anti-CD41. Red spots represent individual megakaryocyte colonies. **C**: Morphologic aspects CFU-MKs in cultured $CD34^+$ cells after incubation in the absence or the presence of hNUDC-His or TPO. Colonies were evaluated by inverted light microscopy (Original magnification 100×).

The Effect of hNUDC-His on Human Megakaryocyte Differentiation

In the presence of hNUDC-His or TPO, purified $CD34^+$ cells differentiated into cells expressing the human megakaryocyte specific membrane marker $CD41^+$. The proportions of $CD41^+$ cells were 28.2 ± 2.5%, 59.9 ± 6.2%, and 51.7 ± 3.8% at doses of 0.05, 0.1, or 0.5 μg/ml hNUDC-His, respectively (Fig. 3A). TPO (0.05 μg/ml) produced $CD41^+$ at a higher percentage of 78.0 ± 13%. In the absence of either protein the proportion of $CD41^+$ was 5.43 ± 1.0% (Fig. 3A). Cells were also analyzed by flow cytometry to determine polyploidy classes for the $CD41^+$ cells (Fig. 3B). The mean proportions of megakaryocytes greater than or equal to 8N increased by 18.4% at a dosage of 0.05 μg/ml hNUDC-His (2N = 61.8 ± 3.8%; 4N=19.8±2.3%; 8N = 10.2 ± 1.2%; 16N = 5.4 ± 1.5%, and 32N = 2.8 ± 1.5%), 26.6% at a dosage of 0.1 μg/ml hNUDC-His (2N = 55.6± 2.5%; 4N = 17.6 ±1.1%; 8N = 10.6 ± 1.0%; 16N=10.3 - ± 1.0%, and 32N = 5.9 ± 0.8%), and 25% at a dosage of 0.5 μg/ml hNUDC-His (2N = 58.9 ± 2.3%; 4N = 15.6 ± 1.1%; 8N = 10.1 ± 1.0%; 16N = 9.5 ± 1.0%, and 32N = 5.9 ± 0.5%) (Fig. 3B). The number of cells with a ploidy of 8N, 16N, or 32N was lower in TPO-treated cells (2N = 75.36 ± 4.3%; 4N = 14.2 ± 2.3%; 8N =7.6 ± 1.9%; 16N = 2.7 ± 0.5%, and 32N= 0.14 ± 0.5%) than in those from hNUDC-His-treated cells (Fig. 3B). Cell ploidy was also confirmed by morphologic analysis using May-Grüwald-Giemsa staining (Fig. 3C). In TPO-treated cultures, the majority of cells showed two to four nuclear lobes, whereas in hNUDC-His-treated cultures, large populations of cells showed multiple nuclei enveloped by a thin layer of cytoplasm and with the cells

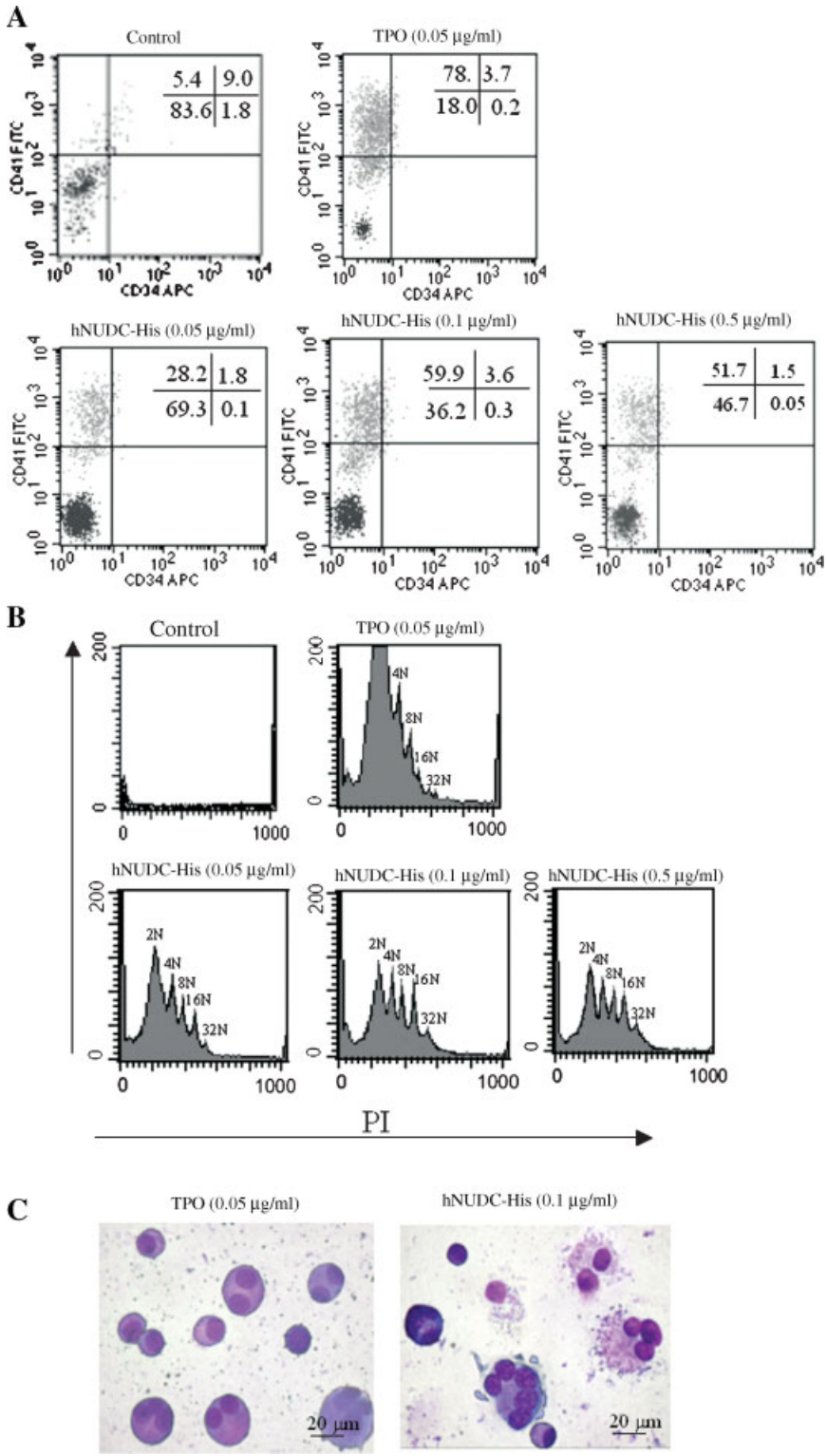

Fig. 3. The effect of hNUDC-His on expression of CD41$^+$ cells and their DNA content. **A**: Cytometric analysis of the expression of the CD41 marker on expanded megakaryocytes in cultured CD34$^+$ cells after incubation with hNUDC-His or TPO. CD41$^+$ cell numbers were estimated with FITC-conjugated anti-human CD41a monoclonal antibody. **B**: The DNA content of CD41$^+$ cells was examined by propidium iodide (PI) staining. The figure is representative of five independent experiments. **C**: Morphologic aspects of expanded megakaryocytes were observed by light microscopy after May-Grüwald staining.

entering the proplatelet formation stages (Fig. 3C).

The Effect of hNUDC-His on Megakaryocyte-Bearing Proplatelets

Enriched megakaryocytes generated from $CD34^+$ cells in the presence of TPO were cultured in serum-free liquid medium in the presence of either hNUDC-His or TPO and megakarocyte-bearing proplatelets were scored at 72 h (day 12). With no added either hNUDC-His or TPO proplatelets were formed at a low level. TPO only increased 1.9-fold the number of proplatelets compared to that of untreated cells (Fig. 4A). In contrast, hNUDC-His increased 2.3-, 4-, and 3.6-fold the number of proplatlets at doses of 0.05, 0.1, or 0.5 μg/ml hNUDC-His, respectively (Fig. 4A). Figure 4B showed the morphological changes after 72 h of the purified megakaryocytes undergoing hNUDC-His, TPO, or buffer treatments respectively. hNUDC-His-treated cells were characterized by significantly higher proportions of proplatelets containing platelet-size particles as well as long cytoplasmic extensions (Fig. 4B). TPO-treated cells were smaller and had fewer cytoplasmic extensions compared with hNUDC-His-treated cells (Fig. 4C). Control cells had the most round morphology with only few cytoplasmic extensions (Fig. 4D).

The Effect of hNUDC-His on In Vitro Platelet Production

Above results suggest that hNUDC-His supports terminal differentiation of megakaryocytes. To further characterize whether hNUDC-His supports platelet formation from proplatelet formed cultures, flow cytometry was used to determine platelet particles from these culture supernatants. In this experiment,

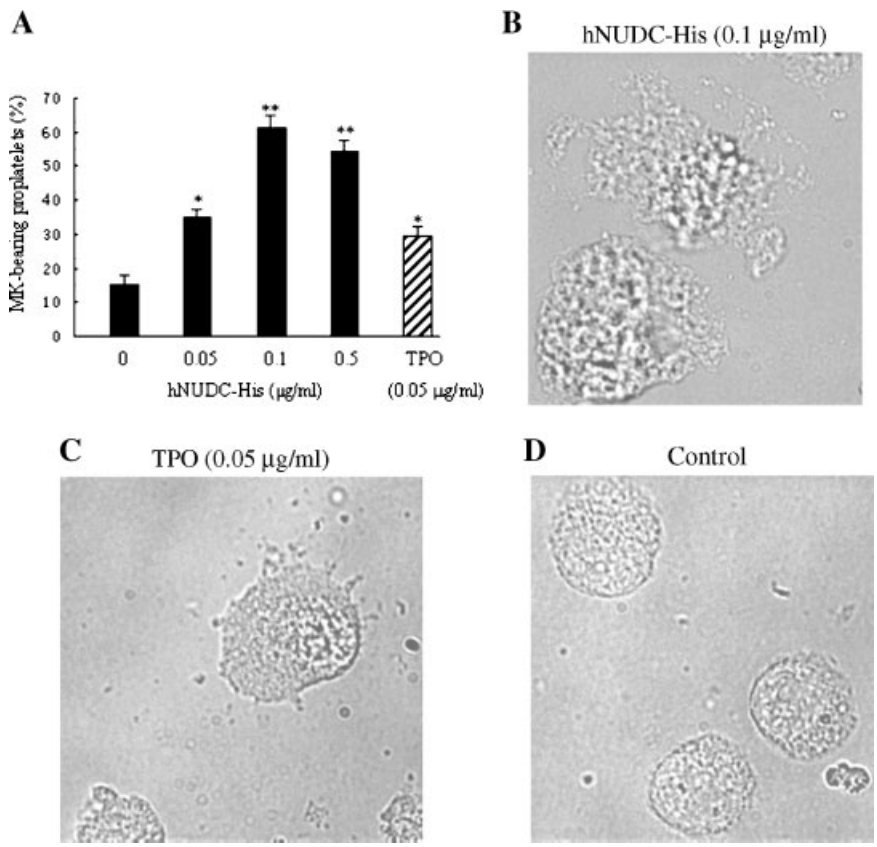

Fig. 4. The effect of hNUDC-His on proplatelet formation. $CD34^+$ cells were cultured in the presence of TPO for 9 days. Purified megakaryocyte cells were washed three times with PBS buffer and subsequently cultured with hNUDC-His or TPO for 72 h. **A**: The numbers of proplatelets were determined using an inverted microscope as megakaryocytes showing one or more cytoplasmic expansions with constriction areas. Experiments were performed in triplicate in four assays. Statistically significant compared with PBS (*, $P < 0.05$; **, $P < 0.01$). **B–D**: Morphology of cultured megakaryocyte cells at day 12 in the presence of hNUDC-His or TPO and control illustrates proplatelet-bearing megakaryocytes as shown in an inverted microscope (original magnification 400×).

hNUDC-His or TPO was added to TPO-derived megakaryocyte cells and cells were grown for an additional 72 h. As shown in Figure 5A, stimulation with increasing doses of hNUDC-His (0.05–0.5 μg/ml) led to increasing percentages of $CD41^+$ platelets from 76 ± 6 to 83 ± 9. The CD41 expression of TPO-stimulated platelets was markedly lower ($48.5 \pm 2\%$) than that of hNUDC-His-stimulated platelets. Incubation of megakaryocyte with buffer had no detectable

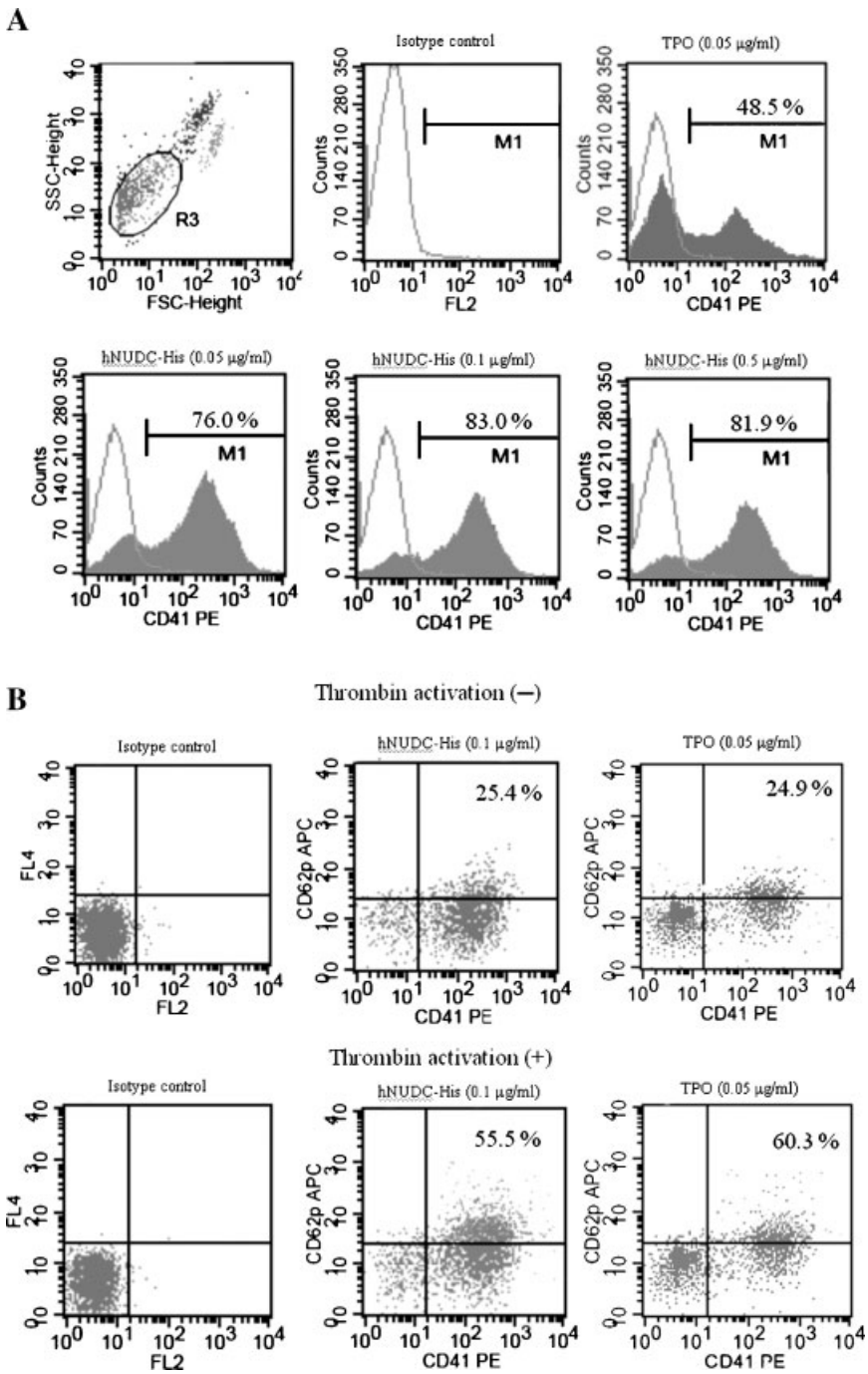

Fig. 5. Effect of hNUDC-His on platelet production in cultured megakaryocyte cells. **A**: Culture-produced platelets in the presence of hNUDC-His or TPO were defined as $CD41^+$ elements with the same scatter properties as blood platelets (R3). **B**: Culture-produced platelets in the presence of hNUDC-His or TPO were double-color staining with anti-CD62P in combination with anti-CD41a before (–) and after (+) 1.0 U/ml thrombin activation. These data are representative of four experiments.

platelet production (data not shown). An additional experiment was performed for the determination of CD62P expression on $CD41^+$ platelets upon activation with thrombin. In both hNUDC-His (0.1 μg/ml) or TPO (0.05 μg/ml) stimulated platelets, $CD62^+$ platelets increased from $25.4 \pm 0.9\%$ to $55.5 \pm 5\%$ and $24.9 \pm 2\%$ to $60.3 \pm 5\%$, respectively, after addition of thrombin (Fig. 5B). A notable percentage of $CD62P^+$ expression on hNUDC-His- or TPO-stimulated $CD41^+$ platelets without thrombin treatment may be attributed by accidental activation occurring during isolation procedure.

The Effect of hNUDC-His on Platelet Production in Normal Mice

To confirm that recombinant hNUDC-His is capable of stimulating the production of platelet in vivo, we detected platelet numbers in normal mice after administration of hNUDC-His. As shown in Figure 6, hNUDC-His significantly increased platelet counts in a dose-dependant manner between 2.5 and 20 μg/kg. Peak platelet counts were achieved on days 7 to 10, with statistically significant increases relative to control. On day 7, platelet counts were increased 41.6, 51.1, and 61.3% for the doses of 2.5, 10, and 20 μg/kg hNUDC-His, respectively ($n = 10$, $P < 0.01$). On day 10, platelet counts were increased 47, 61.4, and 81.2% for the doses of 2.5, 10, and 20 μg/kg hNUDC-His, respectively ($n = 10$, $P < 0.01$). At each time point, the platelet counts observed in TPO-treated mice were much higher than those receiving hNUDC-His (Fig. 6). Platelet numbers gradually declined on day 13 and returned to preinjection levels on day 19 (Fig. 6).

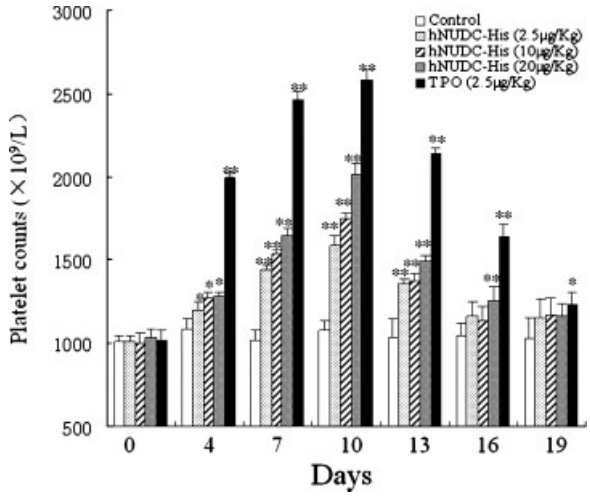

Fig. 6. The effect of hNUDC-His on platelet counts in normal mice. Mice received hNUDC-His or TPO at the indicated dosage once a day for 7 consecutive days. Error bar represent the standard deviation of the mean value. Each dose resulted in statistically significant increases in platelet counts relative to control. *, $P < 0.05$; **, $P < 0.01$.

DISCUSSION

Previous studies by our research group demonstrated that hNUDC binds specifically to Mpl [Pan et al., 2005]. The domain of hNUDC that bound to Mpl spanned a portion of 100 to 238 amino acids [Pan et al., 2005]. Immunofluorescence staining of human megakaryocyte cells indicated that punctuated expression of hNUDC colocalized with Mpl in the plasma membrane of immature megakaryocytes as well as in cytoplasmic extensions of maturing megakaryocytes [Pan et al., 2005]. Another important aspect that hNUDC was prominently associated with microtubules in the proplatelet extensions of megakaryocytes [Pan et al., 2005].

In our present study, we showed that recombinant hNUDC-His fusion proteins produced by *P. pastoris* were biologically active as assessed by both in vitro and in vivo bioassays. hNUDC-His significantly stimulated colony formation and increased megakaryocyte differentiation in serum-free cultured $CD34^+$ cells. Perhaps the most surprising finding of this study is that hNUDC-His accelerated the process of megakaryocytic polyploidization followed by proplatelet formation and platelet release. By testing the dose response characteristics of the yeast-expressed hNUDC-His on $CD34^+$, it was noted that a concentration of 0.1 μ/ml was the most effective dose to promote human megakaryocytes proliferation, differentiation, and maturation. Higher doses did not significantly modify these results and only a slight decrease was observed when 0.5 μg/ml was added in each experiment.

Comparing the in vitro actions of hNUDC-His and TPO, we concluded that hNUDC promoted proliferation less efficiently than TPO but enhanced megakaryocyte polyploidization, proplatelet formation, and in vitro platelet production more potently than TPO. We therefore postulate that TPO is more involved with the early stages of megakaryocytic differentiation, whereas hNUDC acts to a greater degree on the later stages of differentiation. Our results are consistent with previous observations that TPO induces low levels of megakaryocyte

polyploidization and proplatelet formation in vitro [Nagahisa et al., 1996; de Sauvage et al., 1996]. However, our in vivo data indicate that the administration of hNUDC to normal mice enhances blood platelet numbers to a lesser degree when compared with TPO-administered mice. It is clear that, in vivo TPO is the most active factor in regulating platelet formation. However, the separate tasks of the two ligands and the interactions between TPO and hNUDC within in vivo systems remain unclear.

hNUDC has been found to exert hematopoietic activities affecting proliferation or functions in a wide variety of cell types [Miller et al., 1999; Zhang et al., 2002]. It has also been shown to be involved in cellular mitosis, cytokinesis, and neuronal migration [Morris et al., 1998; Aumais et al., 2003]. The region of hNUDC that is homologous to fungal NUDC was shown to form an intracellular association with Lis1 and the dynein motor complex and colocalized with dynein/dynactin at the microtuble-organizing center (MTOC) in neuron and fibroblasts [Aumais et al., 2001]. Cytoplasmic dynein has been shown to be involved in microtubule (−)-end-directed platelet movement [Rothwell and Calvert, 1997]. Because hNUDC binds Mpl as well as dynein, the regulation of hNUDC may also be crucial not only in terms of its cytokine action but also with regard to other roles. The mechanisms of the final stage of platelet formation are still elusive but the emerging evidence indicates that the process is shaped by a series of events of microtubule reorganization [Tajika et al., 1996], including the formation of the molecular motors kinesin and dynein [Rothwell and Calvert, 1997]. Furthermore, megakaryocytes migrate into the bone marrow stromal niche [Avecilla et al., 2004] apparently before the final stages of megakaryocyte maturation and platelet release [Avecilla et al., 2004]. An intriguing possibility emerges that hNUDC may play a key role in associations with dynein/Mpl in the secretory pathway, and in delivery of platelets to specialized sites through the associate with microtubules.

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