Overexpression of nuclear distribution protein (hNUDC) causes pro-apoptosis and differentiation in Dami megakaryocytes

Y. Xiao*, Y. Zheng*, P. Tan*, P. Xu* and Q. Zhang*

*Key Laboratory of Gene Engineering of Education Ministry, School of Life Sciences, Zhongshan University, Guangzhou, 510275, China

Received 3 March 2013; revision accepted 1 May 2013

Abstract

Objectives: Overexpression of hNUDC, a member of the nuclear distribution protein family, reduces cell population growth in prostate cancer cell lines, concurrent with induced morphological change and enhanced polyploidization. These phenomena are also closely associated with terminal phases of megakaryocyte maturation.

Materials and methods: In Dami cells, MTT and trypan blue assays were used to investigate cell viability and proliferation effects of hNUDC, and flow cytometry was used to analyse cell cycle and DNA content. Real-time RT-PCR was employed to detect mRNA expression. Activations of caspase-3, ERK, Akt and Stat-5 were determined by immunoblotting. May-Grünwald–Giemsa staining was performed to reveal cell morphology.

Results and conclusion: Functional studies using adenovirus-mediated hNUDC overexpression led to inhibition of megakaryocyte proliferation *via* cell cycle arrest in G2/M transition phase. This process could have been mediated by upregulation of p21 and downregulation of its downstream targets, including cyclin B1, cyclin B2 and c-myc. Enhanced apoptosis in turn ensued, characterized by increased caspase-3 activation, upregulation of pro-apoptotic Bax and downregulation of anti-apoptotic Bcl-2. Furthermore, hNUDC overexpression elevated the level of megakaryocyte maturation, associated with increased polyploidy, cell morphological changes and increased expression of cell surface differentiation markers, including CD10, CD44, CD41 and CD61. Our results further suggest that

the ERK signalling pathway was involved in hNUDC overexpression-induced apoptosis. Taken together, this study provides experimental evidence for overexpression of hNUDC in Dami cells and suggests that activation of apoptotic machinery may be involved in megakaryocytic differentiation.

Introduction

The human homologue of fungal nuclear distribution protein (hNUDC) is an important protein active in cell proliferation, cytokinesis and mitotic spindle formation, in a variety of cell types and tissues (1–3). Alteration of hNUDC levels by either small interfering RNA-mediated gene silencing or adenovirus-mediated overexpression has resulted in inhibition of proliferation and creation of multinucleate cells, in cultured HeLa and *Caenorhabditis elegans* (4). A further noteworthy study found that hNUDC overexpression significantly inhibited tumourigenicity of prostate cancer cells, leading to failure of cytokineses, aberrant cell cycle progression and morphological changes, as demonstrated in LNCaP, DU145 and PC-3 cell lines (5). These phenotype characteristics are common occurrences during megakaryocyte development (6). Recently, hNUDC has been found to act as a secondary ligand for thrombopoietin receptor (Mpl) involved in regulating proliferation and differentiation of different types of megakaryocytes (7–10). Moreover, intracellular co-localization of hNUDC and Mpl from endoplasmic reticulum to cell membrane, has raised the possibility that binding of these proteins is involved in regulation of hNUDC release (11,12), thus linking biological effects of hNUDC to Mpl.

Induction of megakaryocyte terminal differentiation occurs on initiation of the cells' senescence, preceding release of mature platelets (13,14); yet in-depth study regarding exact mechanisms underlying megakaryocyte apoptosis and terminal differentiation has not up to now been performed. Here, we have chosen the Dami cell line,

Correspondence: P. Xu and Q. Zhang, School of Life Sciences, Zhongshan University, Guangzhou 510275, China. Tel.: +86 20 84038085; Fax: +86 20 84036551; E-mails: xupeilin.gz@gmail.com and lsszq@mail.sysu.edu.cn

derived from an acute megakaryoblastic leukaemia cell system, with well-known biological background (15,16), to examine effects of hNUDC overexpression on megakaryocyte apoptosis and differentiation. Although evidence suggests that exogenous hNUDC, acting through Mpl, imposes an extracellular effect on Dami megakaryocyte formation (10), endogenous expression of hNUDC in the cell line remained to be explored. To establish a physiological model for hNUDC overexpression, we generated an adenoviral vector carrying an hNUDC cDNA insert to transfect the Dami cells. Using this system, we examined effects of hNUDC overexpression on megakaryocyte proliferation, cell cycle, apoptosis and differentiation *in vitro*. Moreover, we examined involvement of signalling molecules and changes in transcription factors that occur during megakaryocyte apoptosis and differentiation.

Materials and methods

Reagents

Polyclonal rabbit antibody against hNUDC was prepared as previously described (7). Anti-P-STAT5, anti-P-AKT, anti-P-ERK1/2 and anti-T-ERK1/2 were purchased from Millipore Co. (Bedford, MA, USA). Anti-T-STAT5, anti-T-AKT, anti- β -actin and anti-cleaved caspase-3 were purchased from Cell Signaling Technology (Beverly, MA, USA). Phycoerythrin (PE)-conjugated anti-CD41 monoclonal antibody was purchased from Beckman Coulter (Fullerton, CA, USA) and Alexa Fluor 594 goat anti-rabbit IgG antibody was supplied by Molecular Probes (Carlsbad, CA, USA). Real-time PCR reagents for LightCycler 480 SYBR Green I Master were purchased from Roche Applied Science (Indianapolis, IN, USA) and RT-PCR primers were purchased from Invitrogen Technologies (Shanghai, China). LipofectamineTM 2000 reagent kit and propidium iodide (PI) staining solution were purchased from Invitrogen (Carlsbad, CA, USA). 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Transfection with recombinant adenoviruses

Recombinant adenovirus Ad-hNUDC was constructed as previously described (12). Dami cells purchased from ATCC were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS and 1% penicillin/streptomycin/glutamate at 37 °C in 5% CO₂ atmosphere. Confluent Dami cells were transfected with the optimal dose of Ad-hNUDC or Ad-GFP for 24 h until number of GFP-expressing cells had reached more than 90%. After transfection, cells were starved in

serum-free medium for 12 h; medium was then replaced with serum-free medium containing 1% Nutridoma (this time point was designated as day 0).

Cell viability assay

Twenty-four hours after transfection of cells with Ad-GFP or Ad-NUDC, they were harvested and reseeded into 96-well plates at 4000 cells per 200 μ l serum-free medium per well, in triplicate. Cells were then cultured for 0, 24, 48 or 72 h at 37 °C in a humidified incubator. MTT reagent (5 mg/ml in phosphate-buffered saline) was added to each well and incubated for 2 h. Plates of cultured cells were centrifuged at 500 *g* for 5 min at 4 °C and MTT solution was removed by aspiration. Solubilization/stop solution (100 μ l) was added, and plates were incubated overnight at 37 °C. Absorbance was recorded by microplate reader at 540 nm wavelength. Effect on cell population growth inhibition was quantified as percentage of viable cells, where vehicle-treated cells were taken to be 100% viable.

Trypan blue assay was employed to determine number of living cells in cultures. In this test, dead cells permit entry of dye and appear blue by microscopy, whereas living cells exclude the dye and appear translucent. Number of living cells was determined using a hemacytometer and results are expressed as mean cell number $\pm$ SD of triplicate cultures.

Protein extraction and immunoblotting

Cells were homogenized in lysis buffer (20 mmol/L Tris-HCl, pH 7.5, 1% NP-40, 5 mmol/L sodium pyrophosphate, 5 mmol/L EDTA, pH 8.0, 1 mmol/L sodium vanadate, 150 mmol/L NaCl, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 μ mol/L PMSF). After centrifugation at 10 000 *g* for 10 min at 4 °C, equal concentrations of proteins were resolved by 10% or 12% SDS-PAGE, and transferred to polyvinylidene fluoride membranes (Hybond ECL; Amersham Biosciences Inc., Piscataway, NJ, USA). After blocking with 5% non-fat dry milk and 0.1% Tween-20 in PBS, membranes were incubated with specific antibodies at dilutions specified by the manufacturer. Blots were then incubated in corresponding HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive proteins were detected using enhanced chemiluminescence western blotting detection; blots were analysed in three independent experiments.

Immunofluorescence staining

Dami cells were grown on coverslips in complete medium, fixed in 4% formaldehyde at room temperature for

15 min and permeabilized at room temperature with 0.1% Triton X-100. Subsequently coverslips were incubated in anti-cleaved-caspase-3 antibody (1:250 dilution) in PBS containing 2% BSA, for 16 h at 4 °C, followed by incubation in secondary Alexa Fluor 594 goat anti-mouse antibody (1:500; Molecular Probes, Eugene, OR, USA) for 1 h. DNA was then labelled with DAPI for 5 min in the dark, followed by three washes in PBS. Samples on slides were mounted in ProLong Antifade (Molecular Probes) and visualized using fluorescence microscopy.

RNA isolation and real-time RT-PCR

Total RNA was isolated using TRIzol RNA isolation kit according to the manufacturer's protocol (Invitrogen). First strand cDNA was synthesized using QuantiTect cDNA synthesis kit (Qiagen, Valencia, CA, USA); specific primers were designed with Primer Express software (Applied Biosystems, Foster City, CA, USA) and are shown in Table 1. Gene expression levels were analysed by real-time RT-PCR with LightCycler 480 SYBR Green I Master (Roche). After initial denaturation at 95 °C for 10 min, cDNA was amplified by 45 cycles of 10 s at 95 °C, 20 s at 60 °C and 20 s at 72 °C. Relative RNA levels were determined by analysing changes in SYBR green fluorescence during PCR using the $\Delta\Delta C_t$ method. To confirm amplification of specific transcripts, melting curve profiles were produced at the end of each

reaction. All results were obtained from at least three independent experiments. mRNA levels from all genes were normalized using β -actin as internal control.

Flow cytometry

For cell cycle and ploidy assessments, 5×10^5 Dami cells were washed and resuspended in PBS, then fixed in 70% ice-cold ethanol overnight at 4 °C. They were then incubated in 500 μ l PI staining solution, containing 20 μ g/ml PI, 100 U/ml DNase-free RNase, and 0.1% v/v Triton X-100, for 15 min at 37 °C in the dark. All samples were analysed with FACSCalibur, using CellQuest software (Becton-Dickinson, Mountain View, CA, USA).

Cell morphology assays

The Dami cells were infected and starved in serum-free medium overnight, then cultured for 72 h. They were then washed in PBS and transferred to slides, then fixed by methanol prior to being stained with May-Grünwald-Giemsa solution for 15 min at room temperature. Stained samples were examined by phase contrast microscopy.

Statistical analyses

To evaluate changes in expression profiles of proteins of interest, one-way ANOVA and Tukey's *post hoc* tests

Table 1. Primers for RT-PCR analysis

Genes	Forward primers	Reverse primers
<i>GATA-1</i>	ATCAGCACTGGCCTACTACAGAG	GAGAGAAGAAAGGACTGGGAAAG
<i>GATA-2</i>	GCACCTGTTGTGCAAATTGT	GCCCCCTTCTTGCTCTTCTT
<i>c-myb</i>	GAAGGTCGAACAGGAAGGTTATCT	GTAACGCTACAGGGTATGGAACA
<i>Scl-1</i>	CCCTATGTTACCAACCAACAAC	AAGGCCCGTTACATTCT
<i>FLI-1</i>	ACTTGGCCAAATGGACGGGACTAT	CCCGTAGTCAGGACTCCCCG
<i>NF-E2</i>	CCACTTCCTCCACCACCTTA	TCGGATTCTGGGTCTTCTTG
<i>FOG-1</i>	GTTCCCTCCGCAGTACGTGT	GTTGACGTTGCTGAAGGTGA
<i>HoxB4</i>	TCTTGGAGCTGGAGAAGGAA	GTTGGGCAACTGTGGTCTT
<i>p21</i>	TGCCCAAGCTCTACCTTCC	ACAGGTCCACATGGTCTTCC
<i>c-myc</i>	TTCTAAGAGAAATGTCTTGAGCAAC	TCAAGACTCAGCCAAGGTTGTG
<i>cyclin B1</i>	AAGAGCTTTAAACTTTGGTCTGGG	CTTTGTAAAGTCCTTGATTTACCATG
<i>cyclin B2</i>	AAAGTTGGCTCCAAAGGGTCTT	GAAACTGGCTGAACCTGTAAAAAT
<i>Bax</i>	TTTCTCACGGCAACTTCAAC	GGAGGAAGTCCAATGTCCAG
<i>Bad</i>	GTTCCAGATCCCAGAGTTTG	CCTCCATGATGGCTGCTG
<i>Bcl-xL</i>	ACATCCCAGCTCCACATCAC	CGATCCGACTCACCAATACC
<i>Bcl-2</i>	AGGATTGTGGCCTTCTTTGAGTT	GCCGGTTCAAGTACTCAGTCAT
<i>CD 10</i>	GGTCATAGGACACGAAATCA	AGATCACCAAACCCGGCACT
<i>CD44</i>	GATCCACCCCAATTCCATCTGTGC	AACCGCGAGAATCAAAGCCAAG
<i>CD41</i>	CTCCTTTGACTCCAGCAACC	CTGTTCTGCTCCCTCTCACC
<i>CD61</i>	TATAGCATTGGACGGAAGGC	GACCTCATTGTTGAGGCAGG
β -actin	GATCATGTTTGAGACCTTC	GGCATACCCCTCGTAGATG

were used. Differences between the two groups were analysed using Student's *t*-test for independent samples. All data are expressed as mean $\pm$ SD; $P < 0.05$ considered to represent statistical significance. Minitab 15.0 software (Minitab Inc., State College, PA, USA) was used for statistical analyses.

Results

Inhibition of cell viability by adenoviral gene transfer of hNUDC into Dami cells

First we determined overexpression of hNUDC protein in Ad-hNUDC-transfected cells, compared to cells transfected with empty vector (Ad-GFP). Expression level of 45-kDa form of hNUDC was significantly higher 24 h post-transfection and plateaued by 48–72 h, compared to controls (Fig. 1a). Effect of hNUDC overexpression on cell population growth was determined by MTT assay and trypan blue staining 0–72 h after transfection. As expected, proliferation was significantly higher in Ad-GFP-transfected cells at 48 and 72 h (Fig. 1b,c); in contrast, cell viability was suppressed in Ad-hNUDC-transfected cells under the same conditions (Fig. 1b, c). The effect of hNUDC overexpression on specific phases of cell cycle was examined by flow cytometry. Compared to controls, percentage of cells transfected with Ad-hNUDC for 24 h were approximately 21% higher in G2/M phase ($P < 0.05$), 20% lower in G1 ($P < 0.05$) and 29% higher in S phase ($P < 0.05$) (Fig. 1d). These results indicate that hNUDC overexpression arrested cells in G2/M phase of the cell cycle.

To elucidate molecular mechanisms of inhibition of Dami cell proliferation, we performed real-time RT-PCR to analyse level of mRNA expression of cyclin B1, cyclin B2, c-myc and p21 – crucial regulators of cell cycle progression. Quantitative RT-PCR experiments revealed that cyclin B1 and cyclin B2 mRNAs reduced nearly 75% and 78% ($P < 0.05$) respectively; similarly, expression of c-myc was reduced by 51%. In contrast, p21 transcription was increased by 76% (Fig. 1e).

Ad-hNUDC transfection induced cleavage of caspase-3 by changing expression Bcl-2 family members

To determine whether the inhibitory effect of hNUDC overexpression on Dami cell population expansion was related to activation of apoptotic pathways, we performed western blot analysis to determine cleavage and activation of caspase-3. As shown in Fig. 2a, cleavage of 32 kDa pro-caspase-3 into 17- and 19-kDa subunits of activated caspase-3 was evident in hNUDC-transfected cells, but not in Ad-GFP transfected cells. Activated

caspase-3 initiates caspase-activated DNase, which triggers chromatin condensation, margination of chromatin to periphery of the nuclear envelope, and DNA fragmentation (17). Therefore, we evaluated changes in translocation of caspase-3 with anti-cleaved caspase-3 monoclonal antibody, and alteration of chromatin morphology using DAPI staining. As shown in Fig. 2b, activation of caspase-3 in Ad-hNUDC-transfected cells led to the appearance of nuclei with apoptotic features, not seen in Ad-GFP-transfected cells. As alteration in Bcl-2 family proteins are known to initiate caspase-3 signalling, we next determined the effect of Ad-hNUDC transfection on expression of anti-apoptotic proteins Bcl-xL and Bcl-2, as well as pro-apoptotic proteins Bax and Bad. Real-time RT-PCR results indicated that compared to control cells, hNUDC overexpression in Ad-hNUDC transfected cells increased expression of Bax up to 5.8-fold ($P < 0.05$), reduced Bcl-2 expression by 2.5-fold ($P < 0.01$), and did not affect expression of Bad or Bcl-xL (Fig. 2c).

Effects of hNUDC overexpression on megakaryocytic differentiation

In order to determine whether growth inhibition induced by hNUDC overexpression would be associated with megakaryocytic differentiation, we examined cells for morphological modifications, surface marker expressions and DNA content. May-Grünwald-Giemsa staining revealed that our Dami cells transfected with Ad-hNUDC for 72 h displayed over all cell enlargement, shrinkage of cytoplasm, membrane blebbing, and condensation of nuclei (Fig. 3a). In contrast, Ad-GFP-transfected cells were markedly smaller with predominantly amorphous nuclei (Fig. 3a). Flow cytometry indicated that percentages of high-ploidy were $22 \pm 3.7\%$ at 2N, $30 \pm 4.4\%$ at 4N, $15 \pm 1\%$ at 8N, $11 \pm 1.8\%$ at 16N, in Ad-hNUDC transfected cells (Fig. 3b), whereas Ad-GFP transfected cells were predominantly 2N ($48.62 \pm 4.8\%$) and 4N ($23.65 \pm 2.7\%$), with only 5.03 ± 1.5 8N and 2.9 ± 1 16N respectively (Fig. 3b). In addition, megakaryocytic lineage-specific differentiation markers CD10, CD44, CD41 and CD61 (18–20) were assessed by real-time RT-PCR. Expression of these markers was notably increased on Ad-hNUDC-transfected cells, compared to Ad-GFP-transfected cells (Fig. 3c).

Overexpression of hNUDC altered coordinate expression of transcription factors

Megakaryocyte differentiation is governed by hierarchical and sequential activation of specific transcription factors. We next analysed mRNA levels of transcription factors known to promote differentiation of megakaryo-

Figure 1. Effect of hNUDC overexpression on Dami cell population growth. (a) Overexpressed hNUDC levels were compared to native expressed hNUDC. Dami cells were transfected with either Ad-hNUDC or Ad-GFP. Western blotting using anti-hNUDC antibody to detect protein levels at 12, 24, 48 and 72 h after transfection. Determination of protein levels of β -actin was used as a control, to ensure equal protein loading. The histogram represents relative levels of controls for hNUDC normalized to β -actin. Representative results of three independent experiments are shown. (b) hNUDC overexpression inhibiting cell population growth was determined at indicated times using MTT. (c) Number of live cells was determined by trypan blue staining at different time points. Results represent mean $\pm$ SD from experiments performed in triplicate. (d) hNUDC overexpression induced cell cycle arrest. Cell cycle distribution was determined by propidium iodide staining and FACS after 24 h transfection. Percentage of cells in G1, S-phase and G2 from one of three datasets is presented. (e) Alteration in expression of cell cycle modulated genes. Total RNA was extracted from Ad-hNUDC and Ad-GFP after 24 h transfection. mRNA levels were quantified by real-time RT-PCR. Overexpression of hNUDC-induced cell cycle arrest associated with downregulation of cyclin B1, cyclin B2, c-myc and upregulation of p21 in Ad-hNUDC-transfected, compared to Ad-GFP-transfected control cells. All measurements are shown relative to expression levels of β -actin. Experiments were repeated three times and representative results are shown. ** $P < 0.01$; *** $P < 0.001$.

cytes, including Scl-1, HoxB4, GATA-1, GATA-2, NF-E2, FOG-1, Fli-1 and c-myb. mRNA expression levels of these transcription factors was quantified in Ad-hNUDC-transfected cells and compared to those of Ad-GFP-transfected ones, using real-time RT-PCR. Notably, essential regulators of megakaryocyte differentiation, including NF-E2, GATA-2 and FOG-1, were highly expressed in Ad-hNUDC-transfected cells, revealing 9.5-fold, 4.4-fold and 11.7-fold increases, respectively, compared to Ad-GFP-infected control cells (Fig. 4). GATA-1 had 30.1% increase in expression, compared to control cells (Fig. 4). HoxB4 and c-myb mRNA levels were downregulated in Ad-hNUDC-transfected cells (Fig. 4).

No significant reduction in expression of Scl-1 and Fli-1 was observed in Ad-hNUDC-transfected cells (Fig. 4).

Overexpression of hNUDC activated ERK1/2 in megakaryocytic differentiation

Among kinases known to play an important role in coordinating a variety of cell processes including growth, differentiation, and in some cases, apoptosis, the three best characterized mammalian proteins are ERK1/2, PI3K/AKT and JAK/STAT (21,22). To investigate whether hNUDC overexpression would affect activation of ERK, AKT and STAT-5, western blot assays, using

Figure 2. Effect of hNUDC overexpression on Dami cell apoptosis. (a) Expression of cleaved caspase-3. Total extracts were prepared from cells incubated for 24 h and analysed by immunoblotting, using anti-cleaved caspase-3 antibody. Blots were also probed against β -actin antibody to verify the protein loading. (b) Effect of hNUDC overexpression on cleaved caspase-3 translocation. Dami cells were labelled for indirect fluorescence by means of anti-cleaved caspase-3 antibody. Nuclei are visualized with DAPI (blue). Images show examples of nuclear localization of caspase-3 in Ad-hNUDC-transfected cells, not found in control cells. (c) Expression of apoptosis-related genes in Dami cells. Relative to expression levels of selected genes of the *Bcl-2* family, including *Bax*, *Bad*, *Bcl-xL* and *Bcl-2*, in Ad-hNUDC-transfected cells, compared to Ad-GFP-transfected cells, quantified by real-time RT-PCR analysis. All measurements are shown relative to expression levels of β -actin. Values based on three independent experiments with triplicate measurements for each. ** $P < 0.01$; *** $P < 0.001$.

antibodies against phosphorylated forms of the kinases, were performed on extracts from 12 h-transfected cells, 0–48 h after starvation. Our data showed that Ad-hNUDC-transfected cells displayed increased phosphorylation of ERK1/2 beginning at the 6 h time point and maintained over the following 14 h period (Fig. 5). In Ad-GFP-transfected control cells, phosphorylation of

Figure 3. Influence of hNUDC overexpression on differentiation. (a) Cell morphology of Dami cells transfected with Ad-hNUDC or Ad-GFP for 3 days. Cyto-centrifuged preparations stained with May-Grünwald–Giemsa reagent ($\times 40$). Cells appear multinucleate or with polilobulated nuclei, evident in Ad-hNUDC infected categories, but not in Ad-GFP-infected cells. (b) Flow cytometric analysis of DNA content. Cells transfected with Ad-hNUDC or Ad-GFP for 3 days were stained with propidium iodide and subjected to cell cycle analysis. Percentage of polyploid cells is shown. (c) Real-time RT-PCR analysis of cell surface markers for differentiation. Relative expression levels of CD10, CD44, CD41 and CD61 in Ad-hNUDC-transfected cells, compared to Ad-GFP-transfected cells, quantified by real-time RT-PCR. All measurements shown relative to expression levels of β -actin. Values based on three independent experiments with triplicate measurements for each. *** $P < 0.001$.

ERK1/2 increased slightly between 24 and 36 h, but still remained lower than that observed in Ad-hNUDC-transfected cells (Fig. 5). Activation of STAT-5 in the cells was constitutive (Fig. 5). Ad-hNUDC-transfection did not alter phosphorylation level of AKT. These results

Figure 4. Profile of mRNA expressions of differentiation-related transcription factors in hNUDC-overexpressing cells. Total RNA was isolated from Ad-hNUDC- or Ad-GFP-transfected cells. Relative expression levels of indicated transcription factors in Ad-hNUDC-transfected cells were compared to Ad-GFP-transfected cells. All measurements shown relative to expression levels of β -actin. Values based on three independent experiments with triplicate measurements for each. ** $P < 0.01$; *** $P < 0.001$.

indicate that overexpression of hNUDC activated the ERK1/2 pathway, but not AKT or JAK pathways, in our Dami cells.

Effect of PD98058 on ERK1/2 activation and expression of apoptosis and differentiation markers in Ad-hNUDC transfected cells

To gain better understanding of the role of the MAPK pathway in apoptosis and differentiation in response to hNUDC overexpression in Dami cells, we pre-treated them with an ERK-specific inhibitor PD98059, or DMSO, prior to transfection of Ad-hNUDC. ERK1/2 phosphorylation was then measured at different time points (0, 6, 12, 18, 24 and 30 h). Apoptosis was assessed by western blot analysis of cleaved caspase-3 and real-time RT-PCR quantification of changes in expression levels of antiapoptotic proteins Bcl-2 and Bcl-xL, pro-apoptotic proteins Bax and Bad. Cell differentiation was assessed by changes in transcript levels of cell surface markers, CD10, CD44, CD41 and CD61. As seen in Fig. 6a, increase in phosphorylated ERK1/2 expression in hNUDC-overexpressing cells was abrogated on application of PD98059. In addition, enhanced expression of active caspase-3 was inhibited by PD98058 (Fig. 6b). Furthermore, expressions of Bax, Bad and Bcl-xL in Ad-hNUDC-transfected cells treated with PD98058 were reduced by 82%, 14% and 86% respectively (Fig. 6c). However, expression of Bcl-2 was increased 2.64-fold, compared to Ad-hNUDC-transfected cells (Fig. 6c). Pre-treatment with PD98059 did not significantly affect mRNA levels of CD10, CD44,

CD41 and CD61 compared to their mRNA levels in hNUDC overexpressing cells (Fig. 6d). Our results indicate that MAPK pathways could be necessary for hNUDC overexpression-induced apoptosis but were not involved in cell differentiation.

Discussion

Overexpression of hNUDC in a number of cancer cell lines (such as LNCaP, DU145 and PC-3), has been shown to cause failure of cytokinesis, aberrant cell cycle progression and formation of multinucleate cells (5). These phenomena have also been recognized in the central mechanism of platelet production from megakaryocytes (13–15). Results from our study support the hypothesis that elevated hNUDC expression in Dami cells raises their apoptotic threshold in a manner similar to that observed in some prostate cancer cells lines.

Our study has shown that overexpression of hNUDC in Dami cells significantly attenuated level of cell proliferation through G2/M phase arrest. This observation is in line with earlier publications showing that hNUDC overexpression causes arrest of cell cycle progression at G2/M transition in prostate cancer cells (5). As a consequence, cells arrested in G2/M attain nuclear enlargement and polyploidization, possibly through failure to complete both mitosis and cytokinesis (5). Molecular analysis has found that the cell cycle is stopped at the G2/M checkpoint *via* upregulation of p21 and downregulation of cyclin B1, cyclin B2 and c-myc. Activity of cyclin B1 and cyclin B2 was significantly observed in G2/M and plays an essential role in the G2/M transition process (23). Downregulation of c-myc is an important event that has been connected to terminal differentiation and growth arrest of several cell types (24,25) and p21 is a critical event in c-myc-dependent cell-cycle progression (26,27). Although it is not clear whether p21 suppresses transcriptional activation of cyclin B1, cyclin B2 and c-myc, it is clear that this specific regulation may be necessary in G2/M transition in megakaryocytes (28). Indeed, previous reports have indicated that p21 is implicated in megakaryocyte polyploidization; overexpression of p21 in UT-7 cells of megakaryocyte phenotype leads to nucleus polylobulation (29). In addition, Zhang *et al.* also reported that endomitosis was closely associated with reduced levels of cyclin B protein in a murine megakaryocytic cell line MegT, generated by targeted expression of temperature-sensitive simian virus 40 large T antigen, to megakaryocytes of transgenic mice (30). Moreover, *in vitro* studies have demonstrated that c-myc is required for proliferation of both megakaryocyte and erythrocyte progenitors (31). In accordance with these results and our observations, we conclude

Figure 5. Effects of overexpression of hNUDC on signalling pathways. Extracts from Ad-hNUDC-transfected cells were subjected to western blot analysis using indicated antibodies for indicated times. hNUDC overexpression up-regulated p-ERK1/2 in a time-dependent manner, while P-STAT-5 and P-AKT expressions were not influenced by hNUDC overexpression. The histogram represents relative levels to controls for P-ERK, P-STAT-5 and P-AKT normalized to total ERK, STAT-5 and AKT. Representative results of three independent experiments are shown.

Figure 6. MEK1/2 inhibitors blocked hNUDC overexpression-induced ERK activation. (a) Inhibition of ERK phosphorylation by PD98059 as determined by western blotting. DMSO (control) or PD98059 (30 μ M) was added to Dami cells prior infection with Ad-hNUDC. P-ERK1/2 and T-ERK1/2 protein levels were determined using anti-P-ERK1/2 or anti-T-ERK1/2 antibodies at indicated time points. (b) Influence of inhibition of the ERK pathway on apoptosis, analysed by caspase-3 assay. Whole cell lysates from indicated cells were blotted for caspase-3 for western blot analysis; protein loading was verified analysing the same blot using an anti- β -actin antibody. (c) Inhibition of the ERK pathway by specific inhibitors and influence of such inhibition on Bax, Bad, Bcl-xL and Bcl-2 expression. mRNA levels were quantified by real-time RT-PCR. (d) Inhibition of the ERK pathway by specific inhibitors and influence of such inhibition on cell surface marked expression. Values based on three independent experiments, each performed in triplicate compared to DMSO-treated Ad-hNUDC-transfected cells after normalization to β -actin. * P < 0.05; *** P < 0.001.

that cell programming during polyploidization in megakaryocytes is associated with reduced levels of cyclin B1, cyclin B2 and c-myc, but with increased p21.

Our data also show that hNUDC overexpression exerts its pro-apoptotic function by caspase-3 activation in a megakaryocytic model. A further interesting finding

is that caspase-3 selectively translocates into nuclei. Nuclear localization of caspase-3 has previously been reported by De Botton *et al.* in human megakaryocytes derived from CD34 cells (32). Abundance of caspase-3 in nuclei suggests that caspase-3-mediated apoptosis is primarily routed through mitochondrial pathways (32). Bcl-2 family proteins are involved in regulation of programmed cell death, as the relative ratio between pro- (Bax and Bad) and anti-apoptotic proteins (Bcl-2 and Bcl-xL) dictates fate of the cells (33). Our results have shown that overexpression of hNUDC in Dami cells increased levels of Bax, but downregulated Bcl-2. Activation of Bax can cause changes in mitochondrial membrane potential and trigger caspase activation, whereas Bcl-2 proteins play important roles in governing the intrinsic mitochondria death pathway. Thus, hNUDC overexpression facilitates pro-apoptotic protein Bax, but suppresses anti-apoptotic protein Bcl-2 as seen in this study, could tip the balance towards megakaryocyte apoptosis and reduce cell survival. Of note, Bad and Bcl-xL were statistically unchanged in hNUDC overexpression culture; thus, Bad and Bcl-xL may not be involved in control of apoptosis of Dami cells.

Overexpression of hNUDC also appeared to induce differentiation of Dami cells. The differentiation phenotype induced by hNUDC overexpression in Dami cells is usually referred to as 'megakaryocytic' based on expression of markers including CD41, CD61, CD10 and CD44, as they progresses from progenitor cells to megakaryocytes then to platelets (18–20). We observed elevated mRNA levels of CD10, CD44, CD41 and CD61. These results suggest that hNUDC overexpression is involved in regulating cell population growth and directly contributes to cell differentiation. We also observed that hNUDC overexpression induced cell morphological changes and enlargement in cell size. Further evidence to link hNUDC overexpression and excessive differentiation of megakaryocytes is seen by higher ploidy of DNA content.

A series of transcription factors is known to act in coordination during megakaryocyte differentiation, but individual functions in hNUC-induced megakaryocytic apoptosis and differentiation remain to be determined. Our data indicate that overexpression of hNUDC induces significant changes in levels of mRNAs for NF-E2, GATA-1, GATA-2, FOG-1, c-myb and HoxB4, with no effects on Scl-1 and FLI-1. Among those transcription factors, NF-E2, GATA-1, GATA-2 and FOG-1 play important roles in megakaryocytic development (34–37), while Scl-1, Flt-1, c-myb and HoxB4 may have roles at early stages (38–40). Most of these changes are congruent with predictions raised in previous literature regarding megakaryocyte differentiation.

We further provide evidence that significant increase in ERK1/2 activity was induced in Ad-hNUDC-infected cells. Interestingly, hNUDC overexpression did not alter activation of AKT and STAT-5. In addition, treatment with MEK inhibitor PD98059 resulted in reduced activation of ERK1/2. When we blocked ERK signalling in Ad-hNUDC-transfected cells, expression levels of caspase-3 were significantly reduced, suggesting that ERK signalling is required for Ad-hNUDC-mediated pro-apoptosis in Dami cells. Inhibition of ERK also caused dramatic increase in Bcl-2 expression and we also noted concomitant reduction in levels of Bax, Bad and Bcl-xL expressions. These observations support the notion that activation of Bcl-2 family members may participate in megakaryocyte apoptosis by regulation of the MAPK/ERK pathway. Surprisingly, blocking the ERK1/2 pathway had no profound effect on CD10, CD44, CD41 and CD61 expression in hNUDC overexpression cells, suggesting that ERK1/2 signalling is dispensable in differentiation triggered by overexpression of hNUDC, in Dami cells.

In conclusion, our findings suggest that hNUDC overexpression induced multiple cell events simultaneously, including pro-apoptosis, cell cycle arrest and megakaryocyte differentiation. Pro-apoptosis was likely to have been induced in a caspase-dependent pathway with upregulation of Bax and concurrent downregulation of Bcl-2. Signal transduction studies demonstrated that the ERK pathway was required for apoptosis of megakaryocytes, but did not effect their differentiation.

References

- 1 Zhang MY, Huang NN, Clawson GA, Osmani SA, Pan W, Xin P *et al.* (2002) Involvement of the fungal nuclear migration gene nudC human homolog in cell proliferation and mitotic spindle formation. *Exp. Cell Res.* **273**, 73–84.
- 2 Zhou T, Zimmerman W, Liu X, Erikson RL (2006) A mammalian NudC-like protein essential for dynein stability and cell viability. *Proc. Natl. Acad. Sci. USA* **103**, 9039–9044.
- 3 Aumais JP, Tunstead JR, McNeil RS, Schaar BT, McConnell SK, Lin SH *et al.* (2001) NudC associates with Lis1 and the dynein motor at the leading pole of neurons. *J. Neurosci.* **21**, RC187.
- 4 Aumais JP, Williams SN, Luo W, Nishino M, Caldwell KA, Caldwell GA *et al.* (2003) Role for NudC, a dynein-associated nuclear movement protein, in mitosis and cytokinesis. *J. Cell Sci.* **116**, 1991–2003.
- 5 Lin SH, Nishino M, Luo W, Aumais JP, Galfione M, Kuang J *et al.* (2004) Inhibition of prostate tumor growth by overexpression of NudC, a microtubule motor-associated protein. *Oncogene* **23**, 2499–2506.
- 6 Li J, Kuter DJ (2001) The end is just the beginning: megakaryocyte apoptosis and platelet release. *Int. J. Hematol.* **74**, 365–374.
- 7 Pan RM, Yang Y, Wei MX, Yu XB, Ge YC, Xu P (2005) A microtubule associated protein, (hNUDC), binds to the extracellular domain of thrombopoietin receptor (Mpl). *J. Cell. Biochem.* **96**, 741–750.

- 8 Wei MX, Yang Y, Ge YC, Xu P (2006) Functional characterization of hNUDC as a novel accumulator that specifically acts on in vitro megakaryocytopoiesis and in vivo platelet production. *J. Cell. Biochem.* **98**, 429–439.
- 9 Tang YS, Zhang YP, Xu P (2008) hNUDC promotes the cell proliferation and differentiation in a leukemic cell line via activation of the thrombopoietin receptor (Mpl). *Leukemia* **22**, 1018–1025.
- 10 Pang SF, Li XK, Zhang Q, Yang F, Xu P (2009) Interference RNA (RNAi)-based silencing of endogenous thrombopoietin receptor (Mpl) in Dami cells resulted in decreased hNUDC-mediated megakaryocyte proliferation and differentiation. *Exp. Cell Res.* **125**, 1352–1357.
- 11 Zhang YP, Tang YS, Chen XC, Xu P (2007) Regulation of cell differentiation by hNUDC via a Mpl-dependent mechanism in NIH 3T3 cells. *Exp. Cell Res.* **313**, 3210–3221.
- 12 Zheng YB, Xiao YY, Tan P, Zhang Q, Xu P (2012) Live-cell visualization of intracellular interaction between a nuclear migration protein (hNUDC) and the thrombopoietin receptor (Mpl). *PLoS One* **7**, e51849.
- 13 Zauli G, Vitale M, Falcieri E, Gibellini D, Bassini A, Celeghini C *et al.* (1997) In vitro senescence and apoptotic cell death of human megakaryocytes. *Blood* **90**, 2234–2243.
- 14 Li J, Xia Y, Bertino AM, Coburn JP, Kuter DJ (2000) The mechanism of apoptosis in human platelets during storage. *Transfusion* **40**, 1320–1329.
- 15 Greenberg SM, Rosenthal DS, Greeley TA, Tantravahi R, Handin RI (1988) Characterization of a new megakaryocytic cell line: the Dami cell. *Blood* **72**, 1968–1977.
- 16 Lev PR, Goette NP, Glembofsky AC, Laguens RP, Cabeza Meckert PM, Salim JP *et al.* (2011) Production of functional platelet-like particles by the megakaryoblastic DAMI cell line provides a model for platelet biogenesis. *Platelets* **22**, 26–36.
- 17 Boatright KM, Salvesen GS (2003) Mechanisms of caspase activation. *Curr. Opin. Cell Biol.* **15**, 725–731.
- 18 Corbel C, Vaigot P, Salaün J (2005) (alpha) IIb Integrin, a novel marker for hemopoietic progenitor cells. *Int. J. Dev. Biol.* **49**, 279–284.
- 19 Belhacène N, Maulon L, Guérin S, Ricci JE, Mari B, Colin Y *et al.* (1998) Differential expression of the Kell blood group and CD10 antigens: two related membrane metalloproteinases during differentiation of K562 cells by phorbol ester and hemin. *FASEB J.* **12**, 531–539.
- 20 Koshiishi I, Shizari M, Underhill CB (1994) CD44 can mediate the adhesion of platelets to hyaluronan. *Blood* **84**, 390–396.
- 21 Steelman LS, Pohnert SC, Shelton JG, Franklin RA, Bertrand FE, McCubrey JA *et al.* (2004) JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. *Leukemia* **18**, 189–218.
- 22 Dreesen O, Brivanlou AH (2007) Signaling pathways in cancer and embryonic stem cells. *Stem Cell Rev.* **3**, 7–17.
- 23 Manni I, Mazzaro G, Gurtner A, Mantovani R, Haugwitz U, Krause K *et al.* (2001) NF-Y mediates the transcriptional inhibition of the cyclin B1, cyclin B2, and cdc25C promoters upon induced G2 arrest. *J. Biol. Chem.* **276**, 5570–5576.
- 24 Gartel AL, Shchors K (2003) Mechanisms of c-myc-mediated transcriptional repression of growth arrest genes. *Exp. Cell Res.* **283**, 17–21.
- 25 Henriksson M, Luscher B (1996) Proteins of the Myc network: essential regulators of cell growth and differentiation. *Adv. Cancer Res.* **68**, 109–182.
- 26 Gartel AL, Tyner AL (2002) The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. *Mol. Cancer Ther.* **1**, 639–649.
- 27 Claassen GC, Hann SR (2000) A role for transcriptional repression of p21CIP1 by c-Myc in overcoming transforming growth factor β -induced cell-cycle arrest. *Proc. Natl. Acad. Sci. USA* **97**, 9498–9503.
- 28 Baccini V, Roy L, Vitrat N, Chagraoui H, Sabri S, Le Couedic JP *et al.* (2001) Role of p21(Cip1/Waf1) in cell-cycle exit of endomitotic megakaryocytes. *Blood* **98**, 3274–3282.
- 29 Kikuchi J, Furukawa Y, Iwase S, Terui Y, Nakamura M, Kitagawa S *et al.* (1997) Polyploidization and functional maturation are two distinct processes during megakaryocytic differentiation: involvement of cyclin-dependent kinase inhibitor p21 in polyploidization. *Blood* **89**, 3980–3990.
- 30 Zhang Y, Wang Z, Ravid K (1996) The cell cycle in polyploid megakaryocytes is associated with reduced activity of cyclin B1-dependent cdc2 kinase. *J. Biol. Chem.* **271**, 4266–4272.
- 31 Brewer G (2000) Regulation of c-myc mRNA decay in vitro by a phorbol ester-inducible, ribosome-associated component in differentiating megakaryoblasts. *J. Biol. Chem.* **275**, 33336–33345.
- 32 De Botton S, Sabri S, Daugas E, Zermati Y, Guidotti JE, Hermine O *et al.* (2002) Platelet formation is the consequence of caspase activation within megakaryocytes. *Blood* **100**, 1310–1317.
- 33 Haskard DO, Mason JC (2007) A protein kinase Cepsilon-anti-apoptotic kinase signaling complex protects human vascular endothelial cells against apoptosis through induction of Bcl-2. *J. Biol. Chem.* **282**, 32288–32289.
- 34 Lecine P, Blank V, Shivdasani R (1998) Characterization of the hematopoietic transcription factor NF-E2 in primary murine megakaryocytes. *J. Biol. Chem.* **273**, 7572–7578.
- 35 Levin J, Peng JP, Baker GR, Villeval JL, Lecine P, Burstein SA *et al.* (1999) Pathophysiology of thrombocytopenia and anemia in mice lacking transcription factor NF-E2. *Blood* **94**, 3037–3047.
- 36 Chang AN, Cantor AB, Fujiwara Y, Lodish MB, Droho S, Crispino JD *et al.* (2002) GATA-factor dependence of the multitype zinc-finger protein FOG-1 for its essential role in megakaryopoiesis. *Proc. Natl. Acad. Sci. USA* **99**, 9237–9242.
- 37 Tsai FY, Keller G, Kuo FC, Weiss M, Chen J, Rosenblatt M *et al.* (1994) An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371**, 221–226.
- 38 Emambokus N, Vegiopoulos A, Harman B, Jenkinson E, Anderson G, Frampton J (2003) Progression through key stages of haemopoiesis is dependent on distinct threshold levels of c-Myb. *EMBO J.* **22**, 4478–4488.
- 39 Zhong Y, Sullenbarger B, Lasky LC (2010) Effect of increased HoxB4 on human megakaryocytic development. *Biochem. Biophys. Res. Commun.* **398**, 377–382.
- 40 Shivdasani R (2001) Molecular and transcriptional regulation of megakaryocyte differentiation. *Stem Cells* **19**, 397–407.