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Nicotinamide (vitamin B3) increases the polyploidisation and proplatelet formation of cultured primary human megakaryocytes

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Summary

Megakaryocytic (Mk) cell maturation involves polyploidisation, and the number of platelets produced increases with Mk DNA content. Ploidy levels in cultured human MK cells are much lower than those observed *in vivo*. This study demonstrated that adding the water-soluble vitamin nicotinamide (NIC) to mobilised peripheral blood $CD34^+$ cells cultured with thrombopoietin (Tpo) more than doubled the percentage of high-ploidy (≥8N) MK cells. This was observed regardless of donor-dependent differences in Mk differentiation. Furthermore, MK cells in cultures with NIC were larger, had more highly lobated nuclei, reached a maximum DNA content of 64N (vs. 16N with Tpo alone), and exhibited more frequent and more elaborate cytoplasmic extensions. NIC also increased the ploidy of cultured primary murine MK cells and a cell line model (CHRF-288) of Mk differentiation. However, NIC did not alter Mk commitment, apoptosis, or the time at which endomitosis was initiated. Despite the dramatic phenotypic differences observed with NIC addition, gene expression microarray analysis revealed similar overall transcriptional patterns in primary human Mk cultures with or without NIC, indicating that NIC did not disrupt the normal Mk transcriptional program. Elucidating the mechanisms by which NIC increases Mk maturation could lead to advances in the treatment of Mk and platelet disorders.

Keywords: megakaryocyte, ploidy, nicotinamide, proplatelet.

As the immediate precursors to platelets, megakaryocytic (Mk) cells are central to blood coagulation and haemostasis. Mk differentiation from haematopoietic stem cells (HSCs) involves the development of polyploid cells via endomitosis – a modified cell cycle in which several rounds of DNA replication occur without cytokinesis (Vitrat *et al*, 1998; Zimmet & Ravid, 2000). The increase in DNA content is associated with the development of multilobated nuclei and increases in cytoplasmic volume and cell surface area. This is followed by the extension of proplatelets (long branched cytoplasmic protrusions) from which platelets are released (Italiano *et al*, 1999; Patel *et al*, 2005). Mk maturation is further characterised by the induction of apoptosis (Zauli *et al*, 1997; Ryu *et al*, 2001), which is thought to be correlated to and occur concurrently with platelet formation (Li & Kuter, 2001). Thrombopoietin (Tpo) is the primary regulator of megakaryopoiesis, inducing both recruitment of progenitor cells into the Mk lineage and polyploidisation (Banu *et al*, 1995; Debili *et al*, 1995; Kaushansky, 1995).

Megakaryocytic cell commitment and the molecular and cellular mechanisms through which MK cells differentiate and mature remain poorly characterised, due in large part to the rarity of MK cells *in vivo*. Elucidating these mechanisms is key to understanding megakaryopoiesis under physiological and pathophysiological conditions and could provide the basis for clinical advances for the treatment of Mk and platelet disorders, such as essential thrombocytopenia, refractory thrombocytopenia in myelodysplastic syndromes, megakaryoblastic leukaemia and thrombocythaemia. Furthermore, *ex vivo* culture of HSCs under conditions that

promote Mk commitment, expansion and differentiation would enable the production of immature and mature Mk cells for transplantation therapies and may also allow for the production of platelets *in vitro* for use in platelet transfusions (Maurer *et al*, 2000).

In vitro models have proven useful for studying the molecular events governing megakaryopoiesis. However, it is not yet possible to achieve ploidy levels in cultured human cells that are as high as those observed *in vivo*. Human bone marrow Mk cells reach a maximum DNA content of 128N, with a modal ploidy of 16N (Tomer, 2004). In contrast, cultured human Mk cells typically reach a maximum DNA content of 16N (Guerriero *et al*, 2001; Lannutti *et al*, 2005; Ma *et al*, 2005; Raslova *et al*, 2006), with only a few reports of cultures containing a low frequency of cells with a ploidy of 32N or 64N (Mattia *et al*, 2002; De Bruyn *et al*, 2005). Culture conditions that yield high Mk ploidy are desirable because there is a direct correlation between Mk DNA content and platelet production (Mattia *et al*, 2002). Some progress has been made to increase Mk ploidy using genetic modification or chemical modulation of signalling pathways. However, most studies have been conducted using Mk cell line models and murine Mk cells, with only a limited number of studies performed using primary human Mk cells (Guerriero *et al*, 2001, 2006; Lannutti *et al*, 2005; Ma *et al*, 2005).

We have used gene expression microarrays to characterise the changes in gene transcription that accompany Mk differentiation (C. Chen & P. G. Fuhrken, unpublished observations). These studies revealed the upregulation of several members of the silent information regulator 2 (Sir2) family of histone/protein deacetylases with Mk differentiation. Nicotinamide (NIC), also known as water-soluble vitamin B3, is a known Sir2 inhibitor. The present study characterised the effects of NIC on primary human Mk maturation *in vitro*. NIC was demonstrated to greatly enhance Mk endomitosis and proplatelet formation, irrespective of donor-dependent variations in Mk maturation. In doing so, we also provided a novel experimental model to explore the complex process of Mk differentiation in which cells achieve a maximal ploidy of 64N, which is close to that reached *in vivo*.

Materials and methods

Human Mk culture

Unless otherwise noted, all reagents were obtained from Sigma-Aldrich (St Louis, MO, USA). Cultures were initiated with previously frozen human mobilised peripheral blood (mPB) $CD34^+$ cells (AllCells, Berkeley, CA, USA). Cells were cultured in T-flasks and maintained at a density of 100 000–200 000 cells/ml with a constant liquid depth of 0·33 cm. All cultures were performed using X-VIVO 20 (BioWhittaker, Walkersville, MD, USA) supplemented with 100 ng/ml Tpo (generously provided by Genentech, South San Francisco, CA, USA). Beginning at various time points (days 0, 3, 5, 7), cells were continuously treated with NIC. Every other day, half-media exchanges were performed using fresh medium containing 100 ng/ml Tpo and either 3 or 6·25 mmol/l NIC. Cells receiving no NIC were used as a control. Cells were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 and 95% air.

Murine Mk culture

Bone marrow cells were isolated from normal male CD-1 mice (Charles River, Wilmington, MA, USA) with approval from the Northwestern University Animal Care and Use Committee. Mice were sacrificed using CO_2 and cervical dislocation and the femurs were dissected. Cells from the bone marrow were collected by flushing the bones with Hank's Balanced Salt Solution (HBSS) containing 1% penicillin/streptomycin using a syringe and a 21-gauge needle, until the bones appeared white. Bone marrow cells were washed with phosphate-buffered saline (PBS) and resuspended in ACK buffer (0·15 mol/l NH_4Cl, 1·0 mmol/l $KHCO_3$, 0·1 mmol/l Na_2EDTA, pH 7·2–7·4) for red cell lysis. Cells were plated in 6-well plates at a concentration of 1×10^6 cells/ml in Dubelcco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 1% penicillin/streptomycin and 100 ng/ml human Tpo (Genentech). Cultured murine Mk cells mature more rapidly than human Mk cells; therefore, NIC was added on day 1 after seeding and DNA content was assessed using flow cytometry on day 4.

Human megakaryoblastic cell line culture

The human megakaryoblastic CHRF-288-11 (CHRF) cell line (Witte *et al*, 1986; Fugman *et al*, 1990) was generously provided by Dr R. Smith (NIH, Bethesda, MD, USA). CHRF cells were cultured in Iscove's modified Dubelcco's medium (IMDM) supplemented with 10% FBS (HyClone). Cells were treated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) to induce megakaryocytic differentiation, as characterised by morphological changes including adherence, increased cell size and the formation of long cytoplasmic extensions. NIC was added from the time of PMA addition.

Mk culture with Sir2/PARP inhibitors

Synthetic inhibitors of Sir2 or poly (ADP-ribose) polymerase (PARP) were dissolved in dimethyl sulfoxide (DMSO) and added to human Mk cultures beginning on day 5. Half-media exchanges were performed every other day for inhibitor replacement. Sir2 inhibitors were tested in the following concentration ranges: sirtinol (Calbiochem, San Diego, CA, USA) (1–50 μmol/l) and splitomicin (1–100 μmol/l). PARP inhibitors were tested in the following concentration ranges: 3-aminobenzamide and benzamide (0·1–6·25 mmol/l), 5-iodo-6-amino-1,2 benzopyrone (INH_2BP, Calbiochem)

(1–100 μmol/l). Cells treated with an equal volume of DMSO were included as a control.

Mk characterisation

Flow cytometric detection of CD41 expression. Cells were washed with PBS containing 2 mmol/l EDTA (to reduce platelet adhesion) and 0·5% bovine serum albumin (BSA) [PEB], incubated with phycoerythrin (PE)-labelled anti-CD41 antibody (Becton Dickinson, San Jose, CA, USA) for 30 min at room temperature, and then analysed on a Becton Dickinson LSRII flow cytometer using FACSDiva software (Becton Dickinson). 7-amino-actinomycin D (7-AAD; 2 μg/ml) was added to samples shortly prior to acquisition to exclude dead cells.

Flow cytometric analysis for Mk apoptosis. Simultaneous staining for CD41, Annexin V and 7-AAD was used to detect viable Mk cells undergoing apoptosis ($CD41^+$, Annexin V^+, $7AAD^-$). Briefly, cells were washed with PEB and incubated with PE-conjugated anti-CD41 antibody (Becton Dickinson). After washing, cells were resuspended in Annexin V binding buffer (10 mmol/l HEPES, 140 mmol/l NaCl, 2·5 mmol/l $CaCl_2$, pH 7·4) containing fluorescein isothiocyanate (FITC)-conjugated Annexin V and 7-AAD and analysed by flow cytometry. The ratio of the number of apoptotic Mk cells to the total number of $7AAD^-$ Mk cells was used to calculate the percentage of apoptotic Mk cells.

Flow cytometric analysis for Mk ploidy. Cells were labelled with FITC-conjugated anti-CD41 antibody (Becton Dickinson) and fixed with 0·5% paraformaldehyde (15 min at room temperature), followed by permeabilization with cold 70% methanol (1 h at 4°C). Cells were treated with RNAse followed by 50 μg/ml propidium iodide to stain DNA, and analysed by flow cytometry. The percentage of high-ploidy Mk cells was determined from the ratio of the number of $CD41^+$ Mk cells with 8N or higher DNA ploidy to the total number of $CD41^+$ Mk cells. The geometric mean (GM) ploidy was determined as described by Iancu-Rubin *et al* (2005).

Morphological analysis. Cells collected at different days of culture were cytocentrifuged (Shandon Cytospin3; Thermo Electron, Waltham, MA, USA) onto glass slides, stained with Wright–Giemsa (Quik Stain II; Camco, Ft Lauderdale, FL, USA) and observed by light microscopy. To obtain images of proplatelet-forming Mk cells, cells were fixed *in situ* with 2% paraformaldehyde prior to cytocentrifugation. Cells were observed with a Leica DM IL inverted contrasting microscope (Heidenheim, Germany) fitted with a SPOT Insight 2MP Firewire Colour Mosaic camera (Diagnostic Instruments, Sterling Heights, MI, USA). Images were captured using SPOT software (Diagnostic Instruments). Cells displaying one or more cytoplasmic extensions were counted in five fields of view at 400× magnification.

PARP activity assay

The Universal Colorimetric PARP Assay Kit (Trevigen, Gaithersburg, MD, USA) was used according to the manufacturer's instructions to measure the incorporation of biotinylated poly (ADP-ribose) onto histone-coated plates by PARP that was present in cell lysates. The incorporated biotin was detected using horseradish peroxidase (HRP)-conjugated streptavidin and a colorimetric HRP substrate. Cell extracts were prepared on days 5 (2 h after NIC treatment), 7 and 9, from cells cultured with Tpo only and with Tpo + 6·25 mmol/l NIC.

Microarray analysis of gene expression

Primary human Mk cultures were performed as described above; 6·25 mmol/l NIC was supplemented to cultures beginning on day 5. Cells were sampled on days 5 (before NIC addition), 6, 8 and 10, from both the Tpo only and Tpo + NIC conditions and frozen for later analysis.

Detailed protocols for microarray sample preparation, hybridisation and data analysis are provided in Appendix SI. Briefly, RNA was isolated, linearly amplified and hybridised to Human 1A(v2) 60-mer oligonucleotide microarrays following the manufacturer's protocols (Agilent Technologies, Wilmington, DE, USA). Hybridisations were performed in a reference design with each biological sample, labelled with cyanine 3 (Cy3), co-hybridised with amplified Universal Reference RNA (Stratagene, LaJolla, CA, USA) labelled with Cy5. All hybridisations were performed in duplicate or triplicate. Raw and normalised data were deposited in the Gene Expression Omnibus (GSE4974; http://www.ncbi.nlm.nih.gov/geo/).

Quantitative (Q)-RT-PCR

Quantitative (Q) reverse transcription (RT) polymerase chain reaction (PCR) was performed using the High-Capacity cDNA Archive Kit and Assays-on-Demand Taqman Kit with the accompanying protocols (Applied Biosystems, Foster City, CA, USA). The following primer codes were used: c-Myb, Hs00193527_m1; GUS-β, Hs99999908_m1; RPLP0, Hs99999902_m1; 18s, Hs99999901_s1. PCR reactions were scaled-down to 25 μl and performed on a Bio-Rad iCycler (BioRad, Hercules, CA, USA). A standard curve using serial dilutions of the reference RNA (Stratagene) was used to verify linearity, and samples were diluted to ensure they remained within the linear range for the assay. The amount of mRNA for each sample was normalised using the average of three housekeeping genes (glucuronidase-β, large ribosomal protein P0, and 18s ribosomal RNA), as recommended by Applied Biosystems.

Statistical analysis

Statistical comparisons between cultures with Tpo only and those with Tpo + NIC were performed using a paired Student's *t*-test. *P*-values <0·05 were considered significant.

Results

NIC increases Mk ploidy and proplatelet formation in a dose-dependent manner

We first examined the effects of different amounts of NIC on primary human Mk maturation in the presence of Tpo (100 ng/ml). Replicate cultures with mPB $CD34^+$ cells from the same donor were performed in which 3, 6·25, 12·5 or 25 mmol/l NIC was added beginning on day 5. NIC inhibited cell growth in a dose-dependent manner (Fig S1); 12·5 mmol/l NIC completely inhibited growth and 25 mmol/l was cytotoxic. Therefore, NIC doses ≥ 12·5 mmol/l were not analysed further. Cell growth with 3 mmol/l NIC was similar to that with Tpo only and 6·25 mmol/l NIC had an intermediate effect. Morphological analysis of cells cultured with 3 mmol/l or 6·25 mmol/l NIC revealed the presence of more highly lobated nuclei and a dose-dependent increase in cell size (Fig 1A). DNA histograms show that NIC addition also yielded much greater DNA content (Fig 1B). Mk cells reached a maximum ploidy of 64N for 6·25 mmol/l NIC and 32N for 3 mmol/l NIC compared with 16N for cells cultured with Tpo only. NIC also increased the percentage of high-ploidy (≥8N) Mk cells. On day 13, the percentages of high-ploidy Mk cells for the Tpo only, Tpo + 3 mmol/l NIC, and Tpo + 6·25 mmol/l NIC conditions were 23·6 ± 0·3, 48·1 ± 2·7 and 62·2 ± 0·1 respectively. Addition of NIC also increased the number of proplatelet-bearing Mk cells, as well as the size and complexity of their cytoplasmic extensions (Fig 1C).

Consistency of NIC-enhanced Mk ploidy and proplatelet formation across donors

All further mPB $CD34^+$ cell cultures were carried out using 6·25 mmol/l NIC. A total of 10 primary Mk cultures were initiated using cells from seven different donors. The effects of NIC on Mk ploidy were consistent across all cell samples tested (Fig 2A), despite significant donor-to-donor variability in Mk maturation. The percentages of high-ploidy Mk cells on day 11 were 13·5–34·6% and 25·0–53·4% for the Tpo only and Tpo + NIC cultures respectively. However, in all cases there was a 1·5- to 2·5-fold increase in high-ploidy Mk cells with 6·25 mmol/l NIC (day 11, data not shown). NIC consistently shifted the distribution of Mk ploidy towards the higher classes (Fig 2B). The percentages of 16N, 32N and 64N Mk cells (days 11/12) were increased on average from 6·7, 1·0 and 0 respectively, for Tpo only to 14·8, 6·7 and 1·7 for Tpo + NIC cultures. Similarly, the GM ploidy for $CD41^+$ cells (days 11/12) was increased by 31–81% with NIC (Fig 2C). Proplatelet-formation was also donor-dependent, with approximately half of the Tpo only cultures producing only a few proplatelet-forming (PPF) cells by day 13 and the other half producing a significant number by day 9 (Fig 2D). With the exception of one donor sample, for which no PPF cells were detected, NIC increased the number of PPF Mk cells by approximately fivefold (Figs 1C and 2D).

NIC increases polyploidisation of primary murine Mk cells and a megakaryoblastic cell line

Nicotinamide also increased polyploidisation of both primary murine Mk cells and a cell line model of Mk differentiation. Murine Mk cells derived from cultures of bone marrow mononuclear cells showed an increase in cell size and polyploidisation when treated with 6·25 mmol/l NIC. By day 4, Mk cells reached a maximal DNA content of 64N with NIC vs. 32N for cultures with Tpo only (Fig 3A); 12·5 mmol/l NIC yielded a slightly greater increase in Mk ploidy compared with that for 6·25 mmol/l NIC (data not shown). Similar increases in Mk ploidy were obtained in a second experiment when $Sca\text{-}1^+$-selected murine bone marrow cells were treated with Tpo plus 6·25 mmol/l NIC (data not shown). NIC also enhanced polyploidisation of the megakaryoblastic CHRF cell line, which undergoes polyploidisation upon stimulation with PMA. Addition of NIC with PMA increased the percentage of high-ploidy CHRF cells (Fig 3B). By day 9, cultures treated with PMA + 12·5 mmol/l NIC contained 47% high-ploidy cells vs. 29% for cells with PMA only. A dose of 6·25 mmol/l NIC increased Mk ploidy to a lesser extent and doses greater than or equal to 25 mmol/l NIC were cytotoxic (data not shown). NIC had no effect on ploidy in the absence of PMA, and the maximum DNA content (32N) reached by CHRF cells with PMA was not affected by NIC.

NIC has no effect on primary Mk commitment or apoptosis

The kinetics of CD41 expression, total-cell expansion and apoptosis also varied for cells from different donors. By day 11, cultures with Tpo only exhibited 3·7- to 7·6-fold total-cell expansion, whereas those with Tpo + 6·25 mmol/l NIC exhibited only 2·4- to 4-fold cell expansion (Fig 4A). While it inhibited cell growth, NIC had no effect on Mk commitment, as indicated by the percentage of $CD41^+$ Mk cells in cultures of primary human (Fig 4B) or murine Mk cells (data not shown). Of the eight primary human Mk cultures in which CD41 expression was characterised, the percentage of $CD41^+$ cells on day 11 ranged from 68–88% for both the Tpo only and Tpo + NIC conditions. Mk polyploidisation and apoptosis are typically correlated events. However, the increase in DNA content observed for cells treated with 6·25 mmol/l NIC was not accompanied by any significant change in the kinetics of apoptosis (Fig 4C).

NIC effects on ploidy and cell growth extend across a wide range of Mk maturation states

In order to examine whether the NIC-mediated increase in Mk ploidy varied with the stage of Mk maturation at which NIC is added, cultures initiated with mPB $CD34^+$ cells were continuously treated with 6·25 mmol/l NIC beginning on days 0 and 3, as well as on day 5 as described above. NIC addition at day 0 or

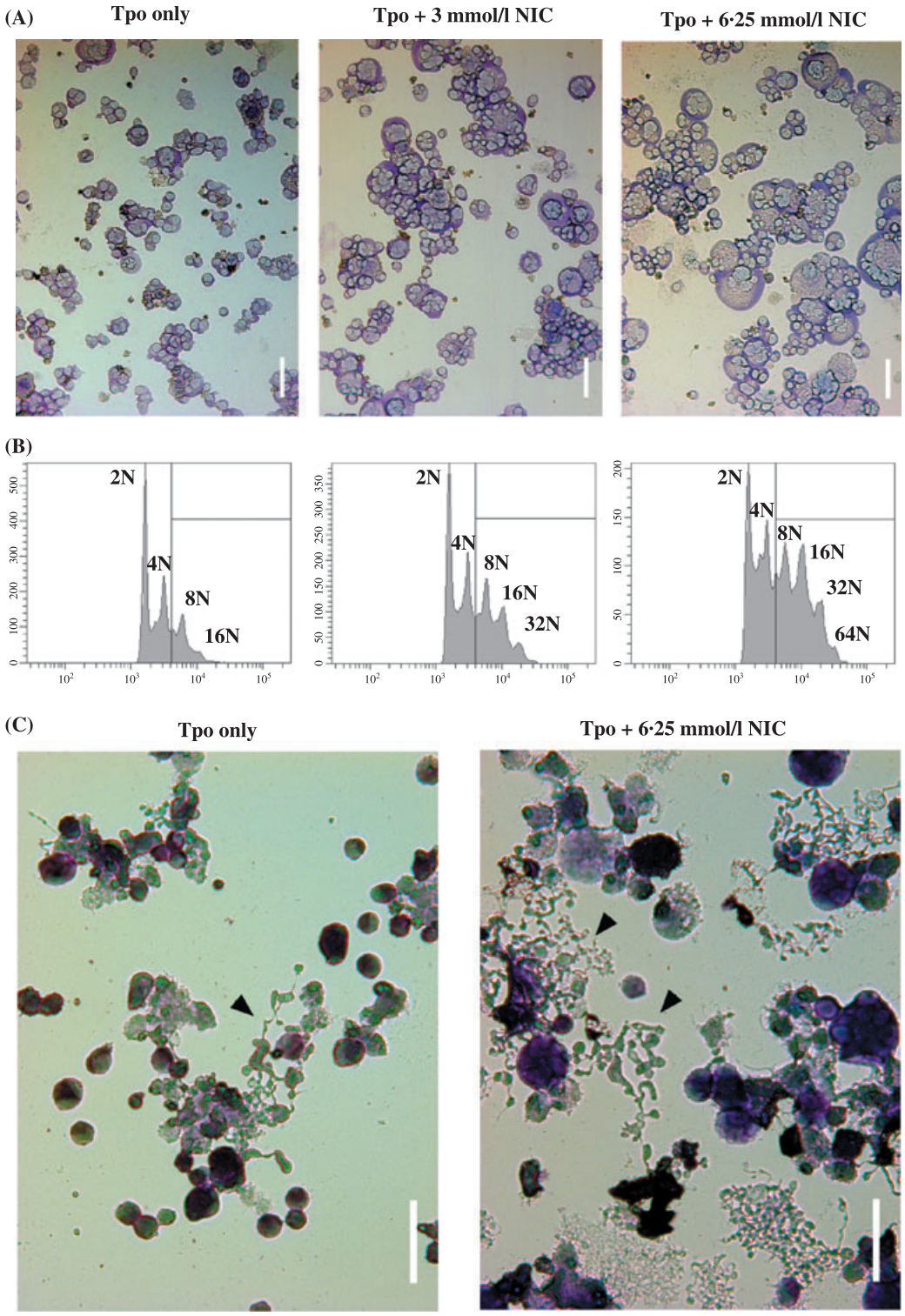

Fig 1. Nicotinamide (NIC) increases primary human megakaryocytic (Mk) cell size, DNA content and proplatelet formation. (A) Morphology of Mk cells examined by staining cytocentrifuged cells with Wright-Giemsa. mPB $CD34^+$ cells were cultured with 100 ng/ml thrombopoietin (Tpo), Tpo + 3 mmol/l NIC or Tpo + 6·25 mmol/l NIC. Images are shown for cells on day 11. A dramatic dose-dependent increase in cell size, along with more highly lobated nuclei, is observed with NIC treatment (magnification 200×; scale bar 50 μm). (B) DNA content was evaluated by propidium iodide staining of permeabilized Mk cells. DNA histograms are shown for $CD41^+$ Mk cells cultured with Tpo only (left), Tpo + 3 mmol/l NIC (middle) or Tpo + 6·25 mmol/l NIC (right). Gate shows high-ploidy $CD41^+$ Mk cells with DNA content greater than or equal to 8N. (C) Proplatelet-bearing Mk cells (arrowheads) from cultures with Tpo only or Tpo + 6·25 mmol/l NIC were examined by *in situ* fixation followed by cytocentrifugation and staining with Wright-Giemsa (day 11; magnification 400×; scale bar 50 μm).

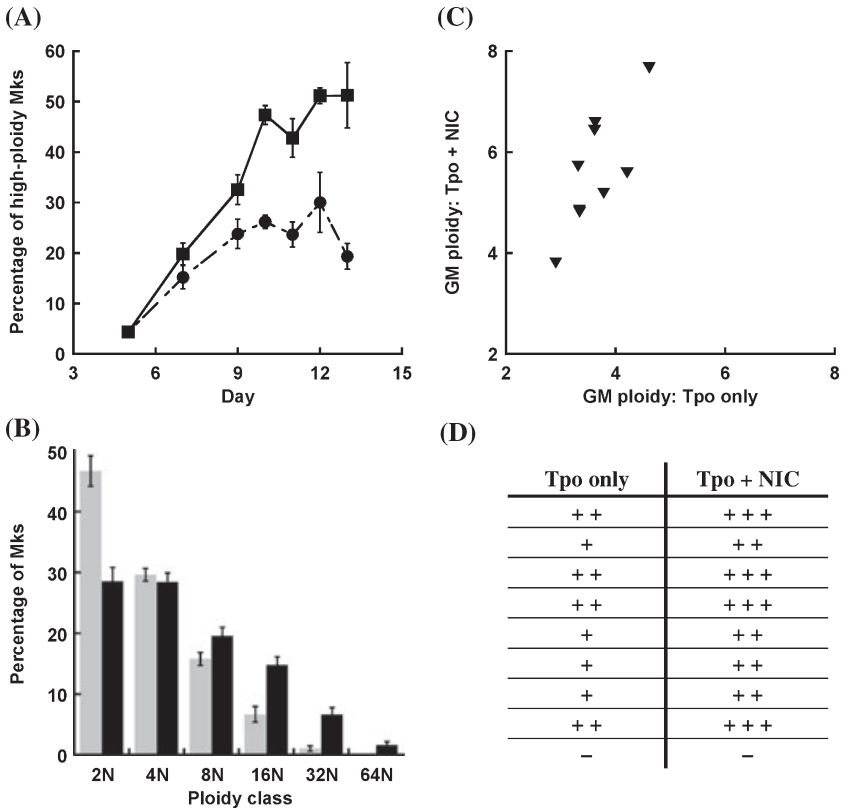

Fig 2. Nicotinamide (NIC) consistently increases the DNA content and proplatelet formation of primary human megakaryocytic (Mk) cells. (A) Percentage of high-ploidy (≥8N) $CD41^+$ Mk cells derived from mPB $CD34^+$ cells cultured with thrombopoietin (Tpo) only (circles) and Tpo + 6·25 mmol/l NIC (squares). Each data point represents the mean ± SEM of two to seven separate experiments. $P < 0{\cdot}02$ for all time points except for day 12 ($P = 0{\cdot}13$; $n = 2$) (B) Ploidy distribution of primary human Mk cells. Data represents the mean ± SEM of nine separate experiments in which Mk ploidy was analysed on either day 11 or 12 for cultures with Tpo only (grey) and Tpo + 6·25 mmol/l NIC (black). $P < 0{\cdot}05$ for all ploidy classes except for 4N (C) Relationship between the geometric mean (GM) ploidy of $CD41^+$ Mk cells on day 11 or 12 in cultures with Tpo only *versus* that with Tpo + 6·25 mmol/l NIC for different donor samples. (D) Numbers of proplatelet-forming (PPF) Mk cells in cultures of mPB $CD34^+$ cells with Tpo only and Tpo + 6·25 mmol/l NIC for different donor samples. Mk cells bearing one or more cytoplasmic extension were counted in five fields of view on day 11. Cultures were classified according to the number of PPF Mk cells: (–) 0, (+) 1–10, (++) 10–50, (+++) >50. Counting was performed for four cultures and the remaining cultures were classified by estimation.

day 3 led to a similar increase in the percentage of high-ploidy Mk cells as that observed with addition at day 5 (Fig 5A). Further, for NIC addition beginning at day 0 or day 3, the percentage of $CD41^+$ cells was also similar to those for NIC addition beginning at day 5 and in Tpo only cultures (Fig 5B). However, total-cell fold-expansion was substantially reduced by addition of NIC on day 0 (~30% less than for NIC addition on day 5; Fig 5C and D). The effect of day 3 NIC addition on cell growth was culture-dependent. For donor cells that matured slowly, reaching only 37% $CD41^+$ cells by day 7, addition of NIC at day 3 inhibited growth to a similar extent as for cells receiving NIC on day 0 (Fig 5C). However, for donor cells that matured more typically and reached 57% $CD41^+$ cells by day 7, adding NIC at day 3 had less of an inhibitory effect, such that the cells expanded in a similar manner to those with NIC addition at day 5 (Fig 5D). The effects on Mk ploidy for NIC addition on day 7, the point at which large increases in ploidy are first observed, also varied with the kinetics of cell maturation. For donor cells that matured typically with 15% high-ploidy Mk cells by day 7, NIC addition on day 7 decreased cell growth (Fig 5D) and increased Mk ploidy (data not shown) in a similar manner to that for cells treated on day 5. In contrast, for donor cells that matured more rapidly, such that the percentage of high-ploidy Mk cells had reached 30% by day 7, there was no effect of NIC addition at day 7 and the ploidy was similar to that of cells cultured with Tpo only (data not shown). Overall, these results suggest that NIC has a similar inhibitory effect on the growth of Mk cells and other myeloid cells during the proliferative phase of culture, with no effect on Mk commitment, such that earlier NIC addition more extensively decreases cell production with no change in the fraction of $CD41^+$ cells. Furthermore, NIC addition produces a similar increase in the ploidy of committed Mk cells until late in Mk maturation.

Pharmacological inhibition of Sir2 or PARP does not enhance polyploidisation

In order to investigate the mechanism by which NIC increases Mk ploidy and proplatelet formation, we examined two known

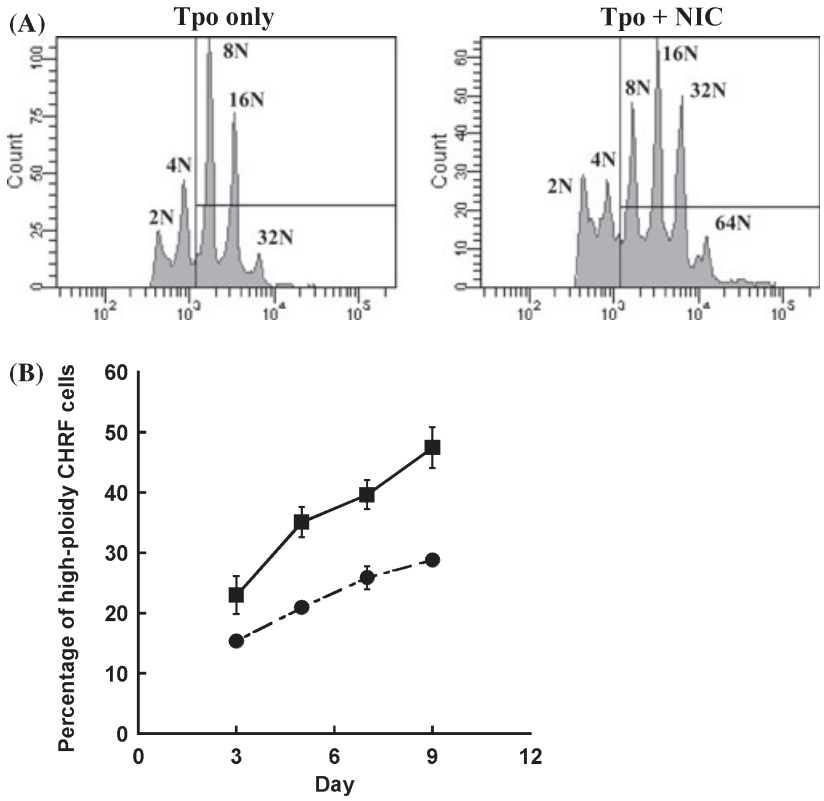

Fig 3. Nicotinamide (NIC) enhances polyploidisation of primary murine megakaryocytic (Mk) cells and a megakaryoblastic cell line. (A) DNA histograms of primary murine $CD41^+$ Mk cells. Murine bone marrow mononuclear cells were cultured with 100 ng/ml human thrombopoietin (Tpo). A total of 6·25 mmol/l NIC was added on day 1 after seeding, and $CD41^+$ Mk ploidy was evaluated using flow cytometry on day 4. (B) Percentage of high-ploidy CHRF cells cultured with phorbol 12-myristate 13-acetate (PMA) only (circles) and PMA + 12·5 mmol/l NIC (squares). NIC was added at the time of PMA stimulation. Each data point is the mean ± SEM of three to five separate experiments. $P < 0{\cdot}02$ for all time points.

roles of NIC – inhibition of Sir2 and PARP activity (Bitterman *et al*, 2002; Jagtap & Szabo, 2005). As indicated above, gene expression analysis by our group revealed upregulated expression of SIRT1, SIRT2 and SIRT7 with Mk differentiation in cultures treated with a cytokine cocktail (C. Chen & P. G. Fuhrken, unpublished observations). Therefore, we examined the effects of two Sir2 inhibitors – sirtinol and splitomicin, which are known to inhibit SIRT1 and SIRT2 activity (Bedalov *et al*, 2001; Grozinger *et al*, 2001). Doses ranged from a concentration that was cytotoxic, to one that had no effect on cell growth or viability (Fig S2A and B), but no increase in Mk polyploidisation was found in mPB $CD34^+$ cell cultures at any of the concentrations tested (Table SI). NIC is also known to exhibit weak inhibition of PARP activity. Therefore, mPB $CD34^+$ cells were also cultured with several pharmacological inhibitors of PARP. Benzamide and 3-aminobenzamide are similar in structure to NIC and, as with NIC, both are active in the millimolar range (Shino *et al*, 2003; Szabo, 2003; Scott *et al*, 2004). In contrast, 5-iodo-6-amino-1,2-benzopyrone (INH_2BP) is more potent and is used at micromolar concentrations (Endres *et al*, 1998; Du *et al*, 2003). As for the Sir2 inhibitors, a wide range of concentrations was tested (Fig S2C–E), but none of the PARP inhibitors increased Mk polyploidisation (Table SI). Furthermore, the measured PARP activity was very low, even for cells cultured without NIC (Fig S3). This was consistent with downregulation of PARP gene expression by approximately twofold during Mk maturation in cultures with or without NIC (data not shown).

Global transcriptional analysis shows similar gene expression in Mk cultures with or without NIC

Gene expression microarrays were used to identify differences in gene transcription associated with NIC-mediated increases in endomitosis and proplatelet formation; 6·25 mmol/l NIC was added to mPB $CD34^+$ cell cultures on day 5 and cells were sampled for microarray analysis on days 5 (before NIC addition), 6, 8 and 10, from both the Tpo only and Tpo + NIC conditions. Two replicate biological experiments (MKI and MKII) were conducted. Surprisingly, although very dramatic phenotypic differences were observed with NIC addition for both experiments, the overall transcriptional patterns were very similar to those in the cultures with Tpo only. As expected, previously identified Mk-associated genes (Balduini *et al*, 2006) were strongly upregulated with Mk differentiation under both culture conditions in both experiments, indicating that NIC did not cause large disruptions in the normal transcriptional program of Mk cells (Fig S4A). Mk differentiation, as indicated

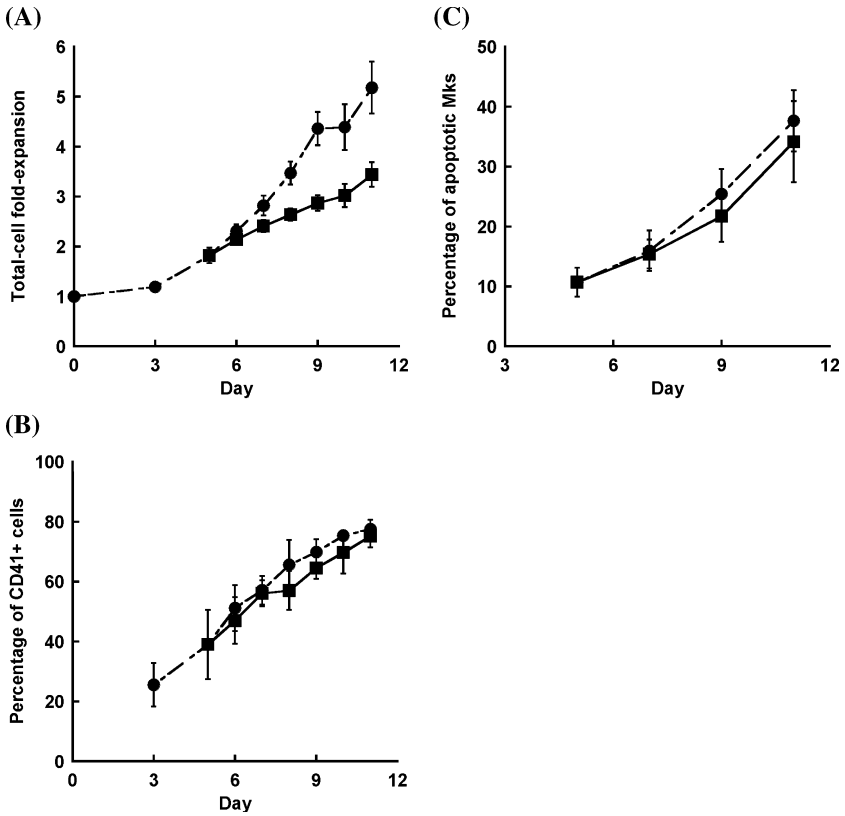

Fig 4. Nicotinamide (NIC) inhibits cell growth but has no effect on primary megakaryocytic (Mk) cell commitment or apoptosis. mPB $CD34^+$ cells were cultured with 100 ng/ml thrombopoietin (circles); 6·25 mmol/l NIC was added to cultures beginning on day 5 (squares). (A) Cells were counted at different days of culture and are reported as the total-cell fold-expansion. Each data point represents the mean ± SEM of 5–10 separate experiments. $P < 0{\cdot}01$ for all time points except for day 6 ($P < 0{\cdot}05$) (B) Analysis of CD41 expression during Mk differentiation. The number of $CD41^+$ Mk cells as a percentage of viable cells was evaluated by flow cytometry. Each data point represents the mean ± SEM of two to six experiments. (C) Kinetics of $CD41^+$ Mk apoptosis as measured by AnnexinV staining. Each data point represents the mean ± SEM of four separate experiments.

by CD41 expression and polyploidisation, occurred more slowly in MKII, and this was reflected by a delay in the upregulation of many Mk-associated genes. Out of more than 18 000 genes probed by the microarray, only 59 genes were found to be differentially regulated by NIC (two-way ANOVA, $P < 0{\cdot}001$) in both cultures (Fig 6). Among these, the transcription factor MYB (also known as c-Myb), which was downregulated with Mk differentiation in both the Tpo only and Tpo + NIC cultures (Fig S4B), was downregulated to a greater extent with NIC. Greater downregulation of c-Myb expression with NIC was confirmed by Q-RT-PCR (Fig S5). Mice with *N*-ethyl-*N*-nitrosourea-induced mutations in c-Myb exhibited excessive megakaryopoiesis characterised by increased numbers of bone marrow Mk cells and high peripheral blood platelet counts (Metcalf *et al*, 2005). Forced expression of c-Myb resulted in a block of terminal Mk differentiation, suggesting that downregulation of c-Myb was necessary for Mk maturation (Sakamoto *et al*, 2006). Lower expression of c-Myb in NIC-treated Mk cells may be an indication of greater Mk maturity. Among the genes expressed higher in the presence of NIC, protein disulfide isomerase family A, member 5 (PDIA5, PDIR), was found to be upregulated with Mk maturation (Fig S4B). PDIA5 is a member of the family of oxidoreductases (Hayano & Kikuchi, 1995) that facilitate protein folding by catalysing the formation and reduction of disulphide bonds in the endoplasmic reticulum (Jessop *et al*, 2004). Greater upregulation of PDIA5 may be related to the observation that cells cultured in the presence of NIC contain approximately twofold more protein by day 9 than do cells cultured with Tpo only (data not shown).

Discussion

It has proven difficult to obtain high-ploidy human Mk cells *in vitro*. Cultured primary human Mk cells typically reach a maximum DNA content of only 16N (Guerriero *et al*, 2001; Lannutti *et al*, 2005; Ma *et al*, 2005; Raslova *et al*, 2006), with only a few reports of cultures containing Mk cells with a maximum DNA content of 32N (De Bruyn *et al*, 2005) or 64N (Mattia *et al*, 2002). Also, cultures with Tpo only typically contain 15–25% high-ploidy (≥8N) Mk cells (De Bruyn *et al*, 2005; Ma *et al*, 2005). Consistent with past results, we obtained a maximum Mk DNA content of 16N in seven cultures and 32N in two cultures with Tpo only. The maximum percentage of high-ploidy Mk cells in these cultures ranged from 13·5–34·6%, which is similar to what is typically observed.

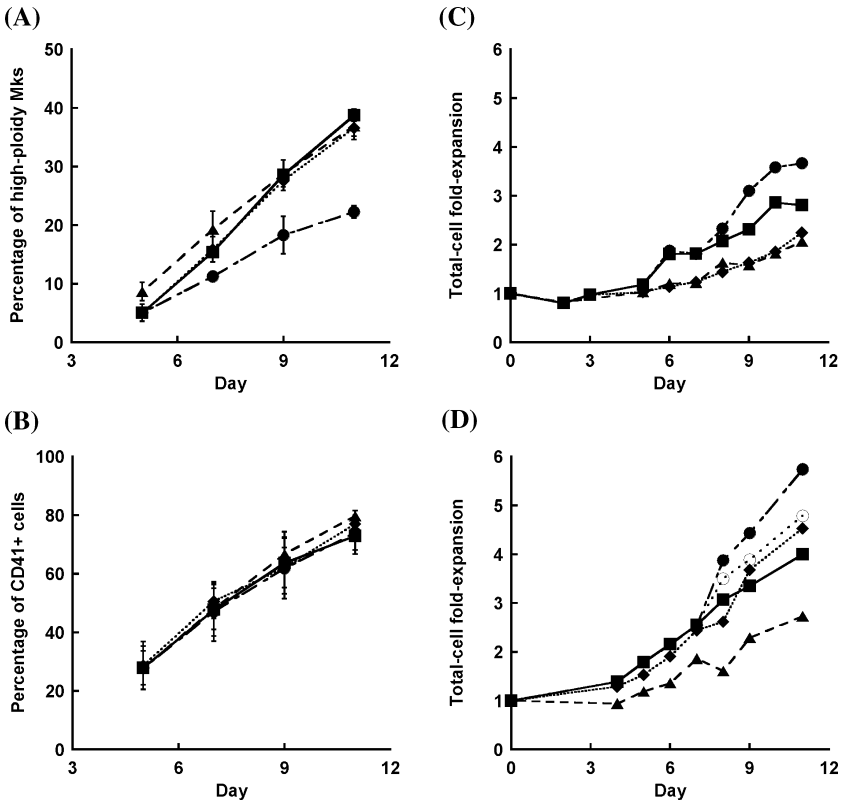

Fig 5. Effects of nicotinamide (NIC) on ploidy and cell growth extend across a wide range of megakaryocytic (Mk) maturation states. mPB $CD34^+$ cells were cultured with 100 ng/ml thrombopoietin. Beginning on days 0 (triangles), 3 (diamonds), 5 (squares) and 7 (open circles), 6·25 mmol/l NIC was added to the cultures. Cells receiving no NIC (closed circles) were included for comparison. DNA content and CD41 expression were assessed by flow cytometry. (A) The percentage of high-ploidy $CD41^+$ Mk cells and (B) the percentage of $CD41^+$ Mk cells are shown for NIC addition on days 0, 3 and 5. Each data point represents the mean ± SEM of two separate experiments. Total-cell fold-expansion is shown for both (C) a slow-maturing culture and (D) one in which cells differentiated more typically, as determined by CD41 expression and Mk ploidy.

Some progress has been made to increase Mk ploidy based on advances in the understanding of Mk development. Although most of these studies have been performed using primary murine Mk cells or Mk cell lines, four recent studies with primary human cells have also demonstrated enhanced Mk maturation. Treatment with the Src kinase inhibitor SU6656 increased Mk cell size and the number of high-ploidy Mk cells in cultures of human bone-marrow-derived $CD34^+$ cells and in cultures of bone marrow mononuclear cells from patients with myelodysplastic syndromes (Lannutti *et al*, 2005). In another study, addition of the chemokine stromal-cell-derived factor-1α (SDF-1α) to cells cultured with Tpo increased proplatelet frequency twofold and approximately doubled the number of Mk cells with both four and eight nuclear lobes compared to those with Tpo only (Guerriero *et al*, 2001). A similar increase in the number of Mk cells with four and eight nuclear lobes was found using the MEK inhibitor PD98059 (Guerriero *et al*, 2006). However, even with SU6656, SDF-1α or PD98059, the highest ploidy class reported was 16N. In another study, treatment of Mk cells with an anti-CD226 monoclonal antibody (expressed on Mk cells and platelets and hypothesised to play a role in Mk development as an adhesion molecule) in combination with lymphocyte-function-associated antigen-1 (LFA-1) increased the percentage of high-ploidy Mk cells from 15·6% to 35·9% and increased the maximum Mk ploidy to 32N (Ma *et al*, 2005).

The present study demonstrated that Mk polyploidisation and proplatelet formation were greatly increased when the water-soluble vitamin NIC was added to mPB $CD34^+$ cell cultures in the presence of Tpo, irrespective of donor-dependent differences in Mk differentiation. NIC-treated primary human Mk cells reached a DNA content of 64N. Also, cultures with NIC exhibited ∼50% high-ploidy Mk cells. Together, the Mk ploidy distribution and percentage of high-ploidy Mk cells obtained with NIC were greater than what has been previously reported for cultured primary human Mk cells. The increase in ploidy with NIC more than offset the decrease in total Mk production, such that NIC addition at day 5 also led to an increase in the total Mk DNA content. Mk cells in NIC-supplemented cultures also exhibited more elaborate cytoplasmic extensions and a greater frequency of proplatelet formation. NIC also increased the DNA content of both primary murine Mk cells and a megakaryoblastic cell line (CHRF-288-11). However, NIC did not induce polyploidisation of CHRF cells in the absence of PMA, and did not alter Mk commitment or the time at which polyploidisation was

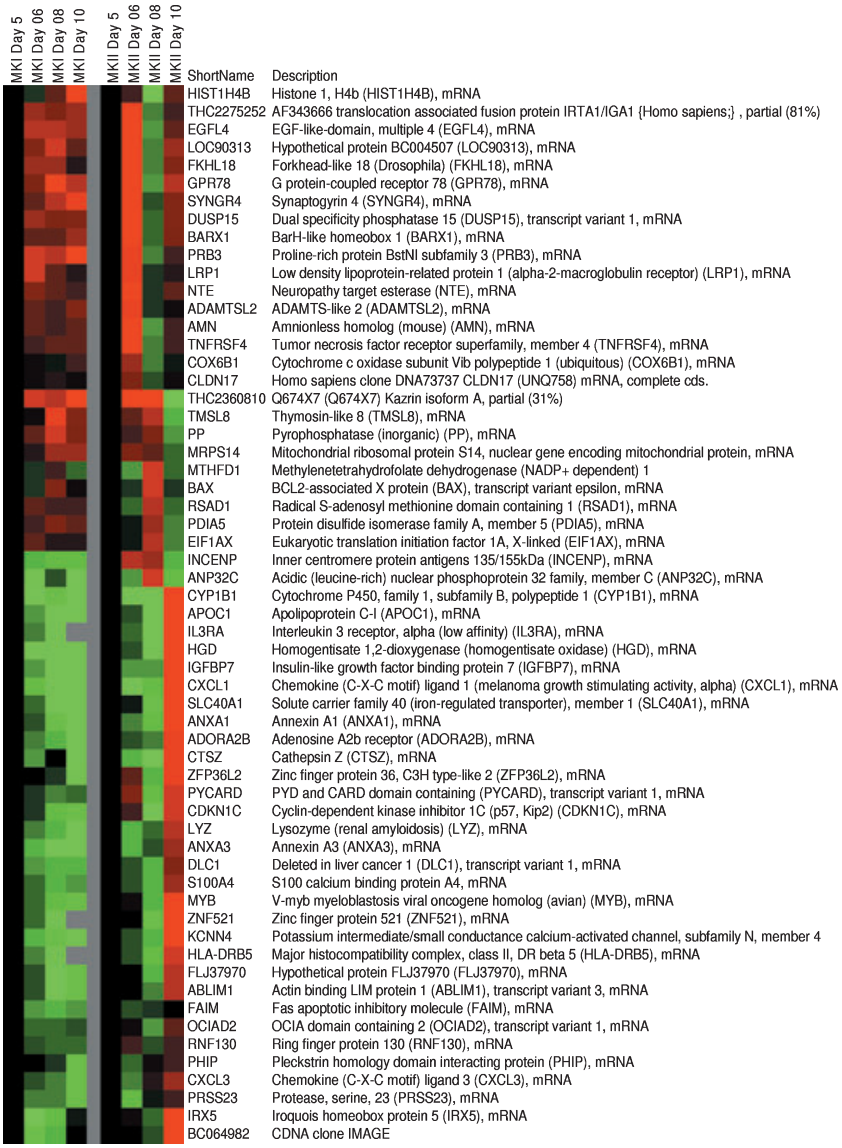

Fig 6. Nicotinamide (NIC)-associated differences in gene expression. Profiles of NIC effects on gene expression for two biological replicate experiments, MKI (left) and MKII (right). Time course data for 59 genes that were found to be differentially regulated by NIC in both experiments (two-way ANOVA ($P < 0{\cdot}001$)) is shown, progressing from left to right for cells analysed on days 5 (prior to NIC addition), 6, 8 and 10. Genes are hierarchically clustered and the colour denotes the ratio of expression level in Tpo + NIC cultures compared to those with Tpo only (saturated red = twofold upregulation, saturated green = twofold downregulation, grey = no data).

initiated in primary human Mk cultures, even when NIC was added on day 0 (Fig 5A). These results suggest that NIC does not induce endomitosis *per se*, but rather enhances the process once it has been initiated.

The mechanisms that underlie the ability of NIC to enhance Mk maturation have yet to be established. Surprisingly, very similar global transcriptional profiles were observed with or without NIC, despite the dramatically different phenotypes. NIC has a variety of known roles, including serving as a precursor to nicotinamide adenine dinucleotide (NAD^+) (Micheli *et al*, 1990; Evans *et al*, 2002; McLure *et al*, 2004), activating the phosphoinositide-3 kinase /Akt signaling pathway (Chong *et al*, 2004, 2005) and inhibiting the activity of both PARP (Jagtap & Szabo, 2005) and the Sir2 family of histone/protein deacetylases (sirtuins) (Bitterman *et al*, 2002). PARP catalyses the NAD^+-dependent addition of poly (ADP-ribose) onto nuclear proteins, including histones and plays a role in DNA replication (D'Amours *et al*, 1999). PARP-1 has been shown to interact with many cell-cycle regulators such as p21 (Frouin *et al*, 2003), proliferating cell nuclear antigen (PCNA) (Frouin *et al*, 2003) and p53 (Kumari *et al*, 1998). However, several different pharmacological inhibitors of PARP, including INH_2BP, which is a more potent PARP inhibitor than NIC, did not increase Mk ploidy. Sirtuins catalyse a unique NAD^+-dependent reaction and are required for a wide variety of biological processes, including transcrip-

tional silencing, regulation of apoptosis and lifespan regulation (Blander & Guarente, 2004; Grubisha *et al*, 2005). NIC inhibits the ability of SIRT1 to deacetylate p53 (Bitterman *et al*, 2002) and also inhibits the activity of SIRT2, which is an α-tubulin deacetylase that has been shown to play a role in cell-cycle regulation (Dryden *et al*, 2003). While we initially examined NIC because it is a sirtuin inhibitor, two additional chemical inhibitors of SIRT1 and SIRT2 failed to increase Mk ploidy. Therefore, we conclude that NIC does not enhance Mk maturation by inhibiting SIRT1 or SIRT2 activity. However, we are not able to rule out effects on SIRT7 because this molecule has not been well characterised. Recently, it has been shown that SIRT7 localises to the nucleus (Michishita *et al*, 2005; Ford *et al*, 2006) and is necessary for cell survival (Ford *et al*, 2006). However, it is not yet known whether nicotinamide, sirtinol and/or splitomicin inhibit SIRT7 activity.

Our results suggest several potential applications for NIC both *in vitro* and *in vivo*. We provide a novel culture system that can be used to investigate the complex process of Mk differentiation. Importantly, NIC increases the rate of endomitosis without affecting apoptosis, such that Mk cells reach ploidy levels closer to those observed *in vivo*. As there is a direct correlation between Mk DNA content and the number of platelets produced (Mattia *et al*, 2002), these results suggest a possible therapeutic benefit of NIC in providing Mk cells for transplantation therapies and/or platelets for use in transfusions. NIC may be especially useful for increasing the ploidy of umbilical-cord-blood-derived Mk cells, which typically show less extensive polyploidisation compared with Mk cells from other haematopoietic cell sources (van den Oudenrijn *et al*, 2000). Furthermore, NIC has been administered safely at pharmacological doses for a variety of therapeutic applications, dating back to the late 1950s for treatment of psychiatric conditions such as schizophrenia. More recently, NIC has undergone clinical testing for use as a preventative agent for type I diabetes (Elliott *et al*, 1996; Lampeter *et al*, 1998; Gale *et al*, 2004). Doses up to 3 g/d have been reported to be generally well tolerated with few side effects (Knip *et al*, 2000). *In vivo* administration of NIC may have therapeutic potential for the management of Mk and platelet disorders, such as essential thrombocytopenia, refractory thrombocytopenia in myelodysplastic syndromes and megakaryoblastic leukaemia.

In summary, although the molecular and cellular mechanisms through which Mk cells differentiate and mature remain poorly characterised, we have shown that Mk maturation *in vitro* can be dramatically enhanced by adding the water-soluble vitamin NIC. Elucidating the mechanisms by which NIC increases Mk ploidy could provide the basis for clinical advances for the treatment of Mk and platelet disorders.

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References

Balduini, A., d'Apolito, M., Arcelli, D., Conti, V., Pecci, A., Pietra, D., Danova, M., Benvenuto, F., Perotti, C., Zelante, L., Volinia, S., Balduini, C.L. & Savoia, A. (2006) Cord blood *in vitro* expanded CD41 cells: identification of novel components of megakaryocytopoiesis. *Journal of Thrombosis and Haemostasis*, **4,** 848–860.

Banu, N., Wang, J.F., Deng, B., Groopman, J.E. & Avraham, H. (1995) Modulation of megakaryocytopoiesis by thrombopoietin: the c-Mpl ligand. *Blood*, **86,** 1331–1338.

Bedalov, A., Gatbonton, T., Irvine, W.P., Gottschling, D.E. & Simon, J.A. (2001) Identification of a small molecule inhibitor of Sir2p. *Proceedings of the National Academy of Sciences of the United States of America*, **98,** 15113–15118.

Bitterman, K.J., Anderson, R.M., Cohen, H.Y., Latorre-Esteves, M. & Sinclair, D.A. (2002) Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. *Journal of Biological Chemistry*, **277,** 45099–45107.

Blander, G. & Guarente, L. (2004) The Sir2 family of protein deacetylases. *Annual Review of Biochemistry*, **73,** 417–435.

Chong, Z.Z., Lin, S.H. & Maiese, K. (2004) The NAD+ precursor nicotinamide governs neuronal survival during oxidative stress through protein kinase B coupled to FOXO3a and mitochondrial membrane potential. *Journal of Cerebral Blood Flow and Metabolism*, **24,** 728–743.

Chong, Z.Z., Lin, S.H., Li, F. & Maiese, K. (2005) The sirtuin inhibitor nicotinamide enhances neuronal cell survival during acute anoxic injury through AKT, BAD, PARP, and mitochondrial associated "anti-apoptotic" pathways. *Current Neurovascular Research*, **2,** 271–285.

D'Amours, D., Desnoyers, S., D'Silva, I. & Poirier, G.G. (1999) Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. *Biochemical Journal*, **342** (Pt 2), 249–268.

De Bruyn, C., Delforge, A., Martiat, P. & Bron, D. (2005) *Ex vivo* expansion of megakaryocyte progenitor cells: cord blood versus mobilized peripheral blood. *Stem Cells and Development*, **14,** 415–424.

Debili, N., Wendling, F., Cosman, D., Titeux, M., Florindo, C., Dusanter-Fourt, I., Schooley, K., Methia, N., Charon, M., Nador, R., Bettaieb, A. & Vainchenker, W. (1995) The Mpl receptor is expressed in the megakaryocytic lineage from late progenitors to platelets. *Blood*, **85,** 391–401.

Dryden, S.C., Nahhas, F.A., Nowak, J.E., Goustin, A.S. & Tainsky, M.A. (2003) Role for human SIRT2 NAD-dependent deacetylase activity in control of mitotic exit in the cell cycle. *Molecular and Cellular Biology*, **23,** 3173–3185.

Du, L., Zhang, X., Han, Y.Y., Burke, N.A., Kochanek, P.M., Watkins, S.C., Graham, S.H., Carcillo, J.A., Szabo, C. & Clark, R.S. (2003) Intra-mitochondrial poly(ADP-ribosylation) contributes to NAD+ depletion and cell death induced by oxidative stress. *Journal of Biological Chemistry*, **278,** 18426–18433.

Elliott, R.B., Pilcher, C.C., Fergusson, D.M. & Stewart, A.W. (1996) A population based strategy to prevent insulin-dependent diabetes

using nicotinamide. *Journal of Pediatric Endocrinology & Metabolism*, **9,** 501–509.

Endres, M., Scott, G.S., Salzman, A.L., Kun, E., Moskowitz, M.A. & Szabo, C. (1998) Protective effects of 5-iodo-6-amino-1,2-benzopyrone, an inhibitor of poly(ADP-ribose) synthetase against peroxynitrite-induced glial damage and stroke development. *European Journal of Pharmacology*, **351,** 377–382.

Evans, J., Wang, T.C., Heyes, M.P. & Markey, S.P. (2002) LC/MS analysis of NAD biosynthesis using stable isotope pyridine precursors. *Analytical Biochemistry*, **306,** 197–203.

Ford, E., Voit, R., Liszt, G., Magin, C., Grummt, I. & Guarente, L. (2006) Mammalian Sir2 homolog SIRT7 is an activator of RNA polymerase I transcription. *Genes & Development*, **20,** 1075–1080.

Frouin, I., Maga, G., Denegri, M., Riva, F., Savio, M., Spadari, S., Prosperi, E. & Scovassi, A.I. (2003) Human proliferating cell nuclear antigen, poly(ADP-ribose) polymerase-1, and p21waf1/cip1. A dynamic exchange of partners. *Journal of Biological Chemistry*, **278,** 39265–39268.

Fugman, D.A., Witte, D.P., Jones, C.L., Aronow, B.J. & Lieberman, M.A. (1990) In vitro establishment and characterization of a human megakaryoblastic cell line. *Blood*, **75,** 1252–1261.

Gale, E.A., Bingley, P.J., Emmett, C.L. & Collier, T. (2004) European Nicotinamide Diabetes Intervention Trial (ENDIT): a randomised controlled trial of intervention before the onset of type 1 diabetes. *Lancet*, **363,** 925–931.

Grozinger, C.M., Chao, E.D., Blackwell, H.E., Moazed, D. & Schreiber, S.L. (2001) Identification of a class of small molecule inhibitors of the sirtuin family of NAD-dependent deacetylases by phenotypic screening. *Journal of Biological Chemistry*, **276,** 38837–38843.

Grubisha, O., Smith, B.C. & Denu, J.M. (2005) Small molecule regulation of Sir2 protein deacetylases. *FEBS Journal*, **272,** 4607–4616.

Guerriero, R., Mattia, G., Testa, U., Chelucci, C., Macioce, G., Casella, I., Samoggia, P., Peschle, C. & Hassan, H.J. (2001) Stromal cell-derived factor 1alpha increases polyploidization of megakaryocytes generated by human hematopoietic progenitor cells. *Blood*, **97,** 2587–2595.

Guerriero, R., Parolini, I., Testa, U., Samoggia, P., Petrucci, E., Sargiacomo, M., Chelucci, C., Gabbianelli, M. & Peschle, C. (2006) Inhibition of TPO-induced MEK or mTOR activity induces opposite effects on the ploidy of human differentiating megakaryocytes. *Journal of Cell Science*, **119,** 744–752.

Hayano, T. & Kikuchi, M. (1995) Molecular cloning of the cDNA encoding a novel protein disulfide isomerase-related protein (PDIR). *FEBS Letters*, **372,** 210–214.

Iancu-Rubin, C., Nasrallah, C.A. & Atweh, G.F. (2005) Stathmin prevents the transition from a normal to an endomitotic cell cycle during megakaryocytic differentiation. *Cell Cycle*, **4,** 1774–1782.

Italiano, Jr, J.E., Lecine, P., Shivdasani, R.A. & Hartwig, J.H. (1999) Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *Journal of Cell Biology*, **147,** 1299–1312.

Jagtap, P. & Szabo, C. (2005) Poly(ADP-ribose) polymerase and the therapeutic effects of its inhibitors. *Nature Reviews. Drug Discovery*, **4,** 421–440.

Jessop, C.E., Chakravarthi, S., Watkins, R.H. & Bulleid, N.J. (2004) Oxidative protein folding in the mammalian endoplasmic reticulum. *Biochemical Society Transactions*, **32,** 655–658.

Kaushansky, K. (1995) Thrombopoietin: the primary regulator of platelet production. *Blood*, **86,** 419–431.

Knip, M., Douek, I.F., Moore, W.P., Gillmor, H.A., McLean, A.E., Bingley, P.J. & Gale, E.A. (2000) Safety of high-dose nicotinamide: a review. *Diabetologia*, **43,** 1337–1345.

Kumari, S.R., Mendoza-Alvarez, H. & Alvarez-Gonzalez, R. (1998) Functional interactions of p53 with poly(ADP-ribose) polymerase (PARP) during apoptosis following DNA damage: covalent poly(ADP-ribosyl)ation of p53 by exogenous PARP and noncovalent binding of p53 to the M(r) 85,000 proteolytic fragment. *Cancer Research*, **58,** 5075–5078.

Lampeter, E.F., Klinghammer, A., Scherbaum, W.A., Heinze, E., Haastert, B., Giani, G. & Kolb, H. (1998) The Deutsche Nicotinamide Intervention Study: an attempt to prevent type 1 diabetes. DENIS Group. *Diabetes*, **47,** 980–984.

Lannutti, B.J., Blake, N., Gandhi, M.J., Reems, J.A. & Drachman, J.G. (2005) Induction of polyploidization in leukemic cell lines and primary bone marrow by Src kinase inhibitor SU6656. *Blood*, **105,** 3875–3878.

Li, J. & Kuter, D.J. (2001) The end is just the beginning: megakaryocyte apoptosis and platelet release. *International Journal of Hematology*, **74,** 365–374.

Ma, D., Sun, Y., Lin, D., Wang, H., Dai, B., Zhang, X., Ouyang, W., Jian, J., Jia, W., Xu, X. & Jin, B. (2005) CD226 is expressed on the megakaryocytic lineage from hematopoietic stem cells/progenitor cells and involved in its polyploidization. *European Journal of Haematology*, **74,** 228–240.

Mattia, G., Vulcano, F., Milazzo, L., Barca, A., Macioce, G., Giampaolo, A. & Hassan, H.J. (2002) Different ploidy levels of megakaryocytes generated from peripheral or cord blood CD34+ cells are correlated with different levels of platelet release. *Blood*, **99,** 888–897.

Maurer, A.M., Liu, Y., Caen, J.P. & Han, Z.C. (2000) Ex vivo expansion of megakaryocytic cells. *International Journal of Hematology*, **71,** 203–210.

McLure, K.G., Takagi, M. & Kastan, M.B. (2004) NAD+ modulates p53 DNA binding specificity and function. *Molecular and Cellular Biology*, **24,** 9958–9967.

Metcalf, D., Carpinelli, M.R., Hyland, C., Mifsud, S., Dirago, L., Nicola, N.A., Hilton, D.J. & Alexander, W.S. (2005) Anomalous megakaryocytopoiesis in mice with mutations in the c-Myb gene. *Blood*, **105,** 3480–3487.

Micheli, V., Simmonds, H.A., Sestini, S. & Ricci, C. (1990) Importance of nicotinamide as an NAD precursor in the human erythrocyte. *Archives of Biochemistry and Biophysics*, **283,** 40–45.

Michishita, E., Park, J.Y., Burneskis, J.M., Barrett, J.C. & Horikawa, I. (2005) Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. *Molecular Biology of the Cell*, **16,** 4623–4635.

van den Oudenrijn, S., von dem Borne, A.E. & de Haas, M. (2000) Differences in megakaryocyte expansion potential between CD34(+) stem cells derived from cord blood, peripheral blood, and bone marrow from adults and children. *Experimental Hematology*, **28,** 1054–1061.

Patel, S.R., Richardson, J.L., Schulze, H., Kahle, E., Galjart, N., Drabek, K., Shivdasani, R.A., Hartwig, J.H. & Italiano, Jr, J.E. (2005) Differential roles of microtubule assembly and sliding in proplatelet formation by megakaryocytes. *Blood*, **106,** 4076–4085.

Raslova, H., Baccini, V., Loussaief, L., Comba, B., Larghero, J., Debili, N. & Vainchenker, W. (2006) Mammalian target of rapamycin (mTOR) regulates both proliferation of megakaryocyte progenitors and late stages of megakaryocyte differentiation. *Blood*, **107,** 2303–2310.

Ryu, K.H., Chun, S., Carbonierre, S., Im, S.A., Kim, H.L., Shin, M.H., Shin, H.Y., Ahn, H.S., Woo, S.Y., Seoh, J.Y. & Fraser, J.K. (2001) Apoptosis and megakaryocytic differentiation during *ex vivo* expansion of human cord blood CD34+ cells using thrombopoietin. *British Journal of Haematology*, **113,** 470–478.

Sakamoto, H., Dai, G., Tsujino, K., Hashimoto, K., Huang, X., Fujimoto, T., Mucenski, M., Frampton, J. & Ogawa, M. (2006) Proper levels of c-Myb are discretely defined at distinct steps of hematopoietic cell development. *Blood*, **108,** 876–903.

Scott, G.S., Szabo, C. & Hooper, D.C. (2004) Poly(ADP-ribose) polymerase activity contributes to peroxynitrite-induced spinal cord neuronal cell death *in vitro*. *Journal of Neurotrauma*, **21,** 1255–1263.

Shino, Y., Itoh, Y., Kubota, T., Yano, T., Sendo, T. & Oishi, R. (2003) Role of poly(ADP-ribose) polymerase in cisplatin-induced injury in LLC-PK1 cells. *Free Radical Biology & Medicine*, **35,** 966–977.

Szabo, C. (2003) Nicotinamide: a jack of all trades (but master of none?). *Intensive Care Medicine*, **29,** 863–866.

Tomer, A. (2004) Human marrow megakaryocyte differentiation: multiparameter correlative analysis identifies von Willebrand factor as a sensitive and distinctive marker for early (2N and 4N) megakaryocytes. *Blood*, **104,** 2722–2727.

Vitrat, N., Cohen-Solal, K., Pique, C., Le Couedic, J.P., Norol, F., Larsen, A.K., Katz, A., Vainchenker, W. & Debili, N. (1998) Endomitosis of human megakaryocytes are due to abortive mitosis. *Blood*, **91,** 3711–3723.

Witte, D.P., Harris, R.E., Jenski, L.J. & Lampkin, B.C. (1986) Megakaryoblastic leukemia in an infant. Establishment of a megakaryocytic tumor cell line in athymic nude mice. *Cancer*, **58,** 238–244.

Zauli, G., Vitale, M., Falcieri, E., Gibellini, D., Bassini, A., Celeghini, C., Columbaro, M. & Capitani, S. (1997) *In vitro* senescence and apoptotic cell death of human megakaryocytes. *Blood*, **90,** 2234–2243.

Zimmet, J. & Ravid, K. (2000) Polyploidy: occurrence in nature, mechanisms, and significance for the megakaryocyte-platelet system. *Experimental Hematology*, **28,** 3–16.

Supplementary material

The following supplementary material is available for this article online:

Appendix SI. Supplemental methods

Table SI. No increase in Mk ploidy with Sir2 or PARP inhibitors.

Fig S1. Dose-dependent growth inhibition by NIC.

Fig S2. Effects of Sir2 and PARP inhibitors on cell growth.

Fig S3. PARP activity in primary Mk cultures is low and is not affected by NIC.

Fig S4. Gene expression profiles of Mk-associated and NIC-regulated genes.

Fig S5. Q-RT-PCR analysis of c-Myb expression in primary human Mk cells from experiment MKI.

This material is available as part of the online article from http://www.blackwell-synergy.com