RESEARCH ARTICLE

Gelsolin Induces Promonocytic Leukemia Differentiation Accompanied by Upregulation of p21CIP1

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Abstract

Tumor suppressor genes have received much attention for their roles in the development of human malignancies. Gelsolin has been found to be down-regulated in several types of human cancers, including leukemias. It is, however, expressed in macrophages, which are the final differentiation derivatives for the monocytic myeloid lineage, implicating this protein in the differentiation process of such cells. In order to investigate the role of gelsolin in leukaemic cell differentiation, stable clones over-expressing ectopic gelsolin, and a control clone were established from U937 leukaemia cells. Unlike the control cells, both gelsolin-overexpressing clones displayed retarded growth, improved monocytic morphology, increased NADPH and NSE activities, and enhanced surface expression of the β-integrin receptor, CD11b, when compared with the parental U937 cells. Interestingly, RT-PCR and western blot analysis also revealed that gelsolin enhanced p21CIP1 mRNA and protein expression in the overexpressing clones. Moreover, transient transfection with siRNA silencing P21CIP1, but not the control siRNA, resulted in a reduction in monocytic differentiation, accompanied by an increase in proliferation. In conclusion, our work demonstrates that gelsolin, by itself, is capable of inducing monocytic differentiation in U937 leukaemia cells, most probably through p21CIP1 activation.

Keywords: Gelsolin - actin - promonocytic leukemia - differentiation

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Introduction

Acute myeloid leukaemia (AML) is characterized by blocked or impaired differentiation of haematopoietic stem cells, resulting in abnormal accumulation of immature precursors and suppression of growth and maturation of cells involved in normal haematopoiesis (Craig and Schiller, 2008). Classical treatments are usually toxic and non-specific, and are associated with difficulties (Cervio et al., 2012), such as economic burden (O'Donnell et al., 2012) and poor tolerance to myelo-suppressive chemotherapy, especially in elderly patients (Wiernik, 2011). Recent clinical trials demonstrate the efficacy of differentiation induction in the treatment of AML, either alone (Petrie, 2009), or in combination with intensive chemotherapy, thereby increasing the susceptibility of AML blasts to drug-induced apoptosis (Fredly, 2009).

The process of differentiation is usually accompanied by the activation of various molecules inside the cell, some of which are possibly actin-binding proteins. During dendritic cell maturation, for instance, the actin-regulatory protein, coffilin, is dephosphorylated (activated) and translocated to the cell membrane, suggesting a role for this protein in the rearrangement of the actin cytoskeleton (Verdijk et al., 2004). Also, in megakaryoblastic leukaemic cell lines, the expression of scinderin, another actin-regulatory protein, induces differentiation, maturation and apoptosis (Bhat et al., 2009). The actin cytoskeleton is an essential scaffold for integrating membrane and intracellular function, whose dynamics are regulated by a number of accessory proteins (Sato, 2012). It has been demonstrated that gelsolin, a ubiquitous protein expressed in almost all mammalian tissues (Kuzumaki and Maruta, 2002), also has an important role in actin reorganization (Furnish, 2001).

Gelsolin is a Ca^{2+} and polyphosphoinositide 4,5-bisphosphate (PIP_2)-regulated actin filament-severing and capping protein that is implicated in actin remodeling, both in growing and apoptotic cells (Patel, 2011). It was previously reported that the expression of gelsolin is frequently down-regulated in several types of human cancers, including stomach, bladder, colon and lung cancers (Tanaka et al., 2006). Whereas gelsolin is expressed in macrophages, which are the final differentiation derivatives for the monocytic myeloid lineage, the expression of this protein is strongly down-

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regulated in the U937, human myelomonocytic leukaemia, cell line. Interestingly, when this cell line is differentiated using tetradecanoyl-phorbolacetate (TPA) the mRNA and protein levels of gelsolin are elevated (Kwaitkowski, 1988). Additionally, gelsolin mRNA and protein levels are also enhanced during the differentiation of embryonal carcinoma cells (Dieffenbach, 1989).

Taken together these findings suggest the possible correlation of gelsolin levels with cytoskeletal reorganization and morphological changes during the process of differentiation in myeloid cell lines. In this study, we established gelsolin-overexpressing U937 clones, by means of retroviral infection and demonstrated that gelsolin, alone, is capable of inducing differentiation of the monocytic leukemia cell line U937.

Materials and Methods

Cell lines and construction of recombinant retrovirus vectors

U937 monocytic leukemia cells were purchased from the Health Science Research Resources Bank (Osaka, Japan) and cultured in 25 mm^3 flasks using RPMI medium (GIBCO, BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2/air incubator. The gelsolin retrovirus vector pLNChGSN was constructed from a pLNCX retroviral vector containing the Neo control gene (a kind gift from Dr. AD Miller, Fred Hutchinson Cancer Research Center) as previously described (Banno et al., 1999). Production of VSV-G pseudo-typed retroviruses was achieved by co-transfection of each retroviral vector and pMD.G, the plasmid encoding the VSV envelope protein VSV-G, into the human embryonal kidney packaging cell line 293gp/bsr (kind gifts from Dr. I. Verma, Salk Institute) as previously described (Ory, 1996). After 48 hours, virus-containing supernatants (VCM) of pLNCX and pLNChGSN were infected into NIH3T3 cells, cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 0.03% L-glutamine. Following selection with G418 (Sigma, St. Louis, MO) for one week, in the presence of polybrene (Sigma), the virus titer of pLNCX was $3x10^6$ CFU/ml and that of pLNChGSN was 10^6 CFU/ml.

Establishment of gelsolin-overexpressing U937 stable clones

To establish a gelsolin-overexpressing stable clone, U937 cells were centrifuged at 300 g for 3 min. The supernatant was replaced with VCM containing 10^6 CFU/ml of the virus and 6 microg/ml polybrene. The cells were then centrifuged at 2000 g for 1 hour at room temperature and incubated in VCM as previously described (Nam, 2011). Cells were treated with G418 (500 microg/ml) for 10 days and cloned by limiting dilution. Four clones were selected for primary analysis (UG3, UG4, UG6, UG11). Two clones showing the highest gelsolin expression (UG6 and UG11) were selected for further use. The same procedure was performed for U937 cells infected with the Neo control pLNCX retrovirus; one clone (UN5) was selected for further use.

Western blot and biological analysis

Western blot analysis: Whole cell lysates were prepared from parental U937 cells, the Neo clone (UN5), and gelsolin-overexpressing clones (UG3, UG4, UG6 and UG11) in SDS lysis buffer (25 mM Tris–HCl pH 6.8, 0.8% SDS, 4% glycerol, 2% 2-mercaptoethanol, 0.008% bromophenol blue) after washing the cells twice in PBS. For immunoblotting the protein extracts were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Cardiff, UK). Blocking was performed with 5% nonfat dry milk in TBST (50 mmol/L Tris-HCl (pH7.5), 150 mmol/l NaCl, 0.1% Tween-20) for 1 hour. The membranes were rinsed 3 times with TBST and incubated overnight at 4°C with a mouse monoclonal antibody against human gelsolin GS-2C4 (SIGMA) (1:5000), a mouse monoclonal anti-human p21CIP1 antibody (Cell Signaling Technology) and a mouse anti-human actin monoclonal antibody (1:5000) (Chemicon, Temecula, CA) in TBST. The next day, the membranes were washed several times with TBST and incubated with the secondary antibody: peroxidase conjugated affinity pure $F(ab')_2$ fragment goat anti-mouse IgG + IgM (H+L) (Jackson Immunoresearch laboratories Inc., West Grove, PA) in TBST for 1 hour at room temperature. Following another three washes with TBST, the membranes were incubated with ECL Western blotting detection reagents (Amersham Bioscience) for 1 min and the band intensity was analyzed by means of a Fuji film LAS-1000 Cool Scanner (Fuji Film, Kanagawa, Japan).

Growth curve: Parental U937 cells and stable clones (UN5, UG3, UG4, UG6, and UG11) were seeded at a density of 10^5 per well in 6 well-plates and incubated at 37°C in a 5% CO_2-incubator. Viable cells were counted every 24 hours for 5 days, based on trypan blue exclusion using a haemocytometer.

Apoptosis was investigated by Hoechst 33342 staining (Molecular Probes, Eugene, OR). In brief, 10^6 cells (U937, UN5, UG3, UG4, UG6, UG11) were cultured in 6 well-plates and incubated at 37°C in a 5% CO_2-incubator for 24, 48 and 72 hours. Hoechst 33342 (33 ng/ml) was added to the wells 5 min prior to microscopic observation. A minimum of 300 cells were counted for each cell line by means of a haemocytometer, under the UV light source of a Nikon biological microscope Eclipse TE300 (Nikon, Yokohama, Japan).

Morphological and flow cytometric analysis, and enzymatic staining

Giemsa staining: Cells, at a density of 10^6, were smeared onto glass slides, fixed with methanol for 1 min and air-dried. Slides were then stained with Giemsa solution (MERCK, Darmstadt, Germany) for 10 min and examined under a light microscope (Olympus BX50, Tokyo, Japan) to ascertain their morphology. At least three different fields were observed and more than 100 cells were counted per field.

Nitroblue tetrazolium (NBT) assay: Superoxide-generating activity of neutrophils was examined by the nitroblue tetrazolium (NBT) assay. NBT reduction

to formazan has been previously used as a marker for nicotinamide adenine dinucleotide phosphate (NADPH) oxidase(Jiang, 2008). Parental U937 cells and stable clones (UN5, UG6, and UG11), at a density of 10^6, were incubated with 0.2% NBT (Sigma), dissolved in PBS, for 30 min at 37°C in a 5% CO_2-incubator. The cells were smeared on glass slides, fixed with methanol for 1 min and dried in air. They were then counterstained with 0.15% safranin (Merck), dissolved in distilled water, for 1.5 min and observed under a light microscope using 40X, 60X and 100X magnification. At least three different fields were observed and more than 100 cells were counted per field. Cells that contained black-blue formazan precipitates were considered positive.

Non-specific Esterase staining: Parental U937 cells and stable clones (UN5, UG6, and UG11), at a density of 10^6, were smeared on glass slides, fixed for 1 min with methanol and air-dried. The slides were then stained for alpha-naphtyl esterase activity with 0.4% 1-naphtyl acetate (Sigma) in acetone mixed with pararosaniline HCl/sodium nitrite solution (Sigma) for 1 min. The slides were then rinsed in tap water, counter-stained with Mayer's Haematoxylin, rinsed again and air-dried (Kim and Feldman, 2002). Cells were then viewed under a light microscope using 40X, 60X and 100X magnification. At least three different fields were observed and more than 100 cells, containing red-brown granules, were counted per field.

Flow cytometric analysis: The surface marker antigen (β-integrin receptor) CD11b was monitored in U937 parental cells and UN5, UG6, and UG11 clones by flow cytometry (Yamamoto et al., 2009). Cells, at a density of 10^6, were washed 3 times in PBS containing 0.1% bovine serum albumin (BSA) (Wako, Osaka, Japan) and 0.05% sodium azide (FACS buffer). The cells were re-suspended in 90 microl FACS buffer and incubated with 10 microl fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-human CD11b (Sigma), or mouse IgG1/FITC as a negative control (Dako, Golstrup, Denmark), at 4°C for one hour in the dark. Cells were again washed three times in FACS buffer, passed through a mesh to remove debris and re-suspended at a concentration of 10^6/ml in FACS buffer. Fluorescence intensity was detected by means of a BECTON DICKINSON FACS Calibur (San Jose, CA).

PCR, RNAi, statistics

RT-PCR analysis: In an effort to delineate the molecules affected by gelsolin over-expression, flanking primers were designed for the gelsolin, PU1, and p21CIP1 genes, including GAPDH as an internal control. The forward and reverse PCR primers used were gelsolin: 5'-TAT GAA TTC GAC ACC GCC AAG GAG GAT-3' and 5'-TAT CTC GAG TTA GGA AAG GTA GGA CAA GCC-3' and PU1: 5'-CCA TCA GAA GAC CTG GTG C-3' and 5'-GAT GCT GTC CTT CAT CTC G-3'; respectively. For p21CIP1 and GAPDH previously reported primers (Matsuzaki et al., 2003; Zeng and Davis, 2003) were used. Total RNA was isolated from parental U937 cells, the UN5 control clone and gelsolin-overexpressing clones (UG6 and UG11) using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Two micrograms of RNA were reverse-transcribed using SuperScript II and random primers (Invitrogen). Two dilutions for cDNA were prepared (10X and 100X) and PCR reactions were performed using a Gene Amp PCR 9700 (PE Applied Biosystem, Foster City, CA). The PCR reaction cycle consisted of an initial denaturation step at 94°C for 5 min, followed by 33 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min (for gelsolin and PU1), 30 cycles at 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec (for p21CIP1) or as previously described (for GAPDH) (Matsuzaki et al., 2003), and then followed by a final 7 min extension at 72°C. PCR reactions were run in 2% agarose gel electrophoresis and band images were detected as previously described (Tanaka et al., 2006).

RNA interference: To elucidate the role of p21CIP1 in the differentiation process induced by gelsolin overexpression, UG6 and UG11 clones were transfected by means of a siRNA system for p21CIP1 (Cell Signalling Technology, Beverly, MA). Fluorescein conjugated-control siRNA (SignalSilence: Cell Signaling) was used as a negative control. Experiments were performed using Nucleofector technology (Amaxa Biosystems, Gaithersburg, MD), and the electroporation was performed according to the manufacturer's instructions. Briefly, for each reaction, 10^6 UG6 or UG11 cells were mixed with a total of 100 nM of each siRNA for knockdown of expression in 100 μl of V solution (Amaxa). Following electroporation, samples were transferred into 12-well plates and incubated at 37°C in 5% CO_2 condition. Cell counting, Giemsa staining, and Western blot analyses for p21CIP1, were performed using an anti-p21CIP1 mouse monoclonal antibody (Cell signaling technology) at 24, 48, and 72 hour intervals, as mentioned earlier in this section.

Statistical analyses

Statistical analyses for data were done using two-tailed, unpaired Student's t-tests. Differences were considered to be statistically significant at $P<0.01$.

Results

Gelsolin overexpression induces changes in morphology and growth patterns

Gelsolin was not detected in both parental U937 cells and the Neo control clone UN5, while gelsolin-overexpressing U937 clones UG3, UG4, UG6 and UG11, displayed expression of gelsolin (90 kDa) (Figure 1A). The level of actin, as an internal control, remained unchanged in all cell lines. UN5 cells exhibited exponential growth and continued to proliferate in a fashion similar to the parental U937 cells. Growth pattern of the lowest gelsolin expressing clone, UG3 was the same as parental cells and UN5. Overexpression of gelsolin in both UG6 and UG11 clones, however, resulted in clear inhibition of cellular proliferation between days 3 and 6. Growth rate of UG4 which the expression of gelsolin was lower than UG6 and UG11 was in the middle of high gelsolin expressing clones UG6, UG11 and low expressing UG3 clone, parental cells and UN5 (Figure 1B)

Time course studies using Hoechst staining

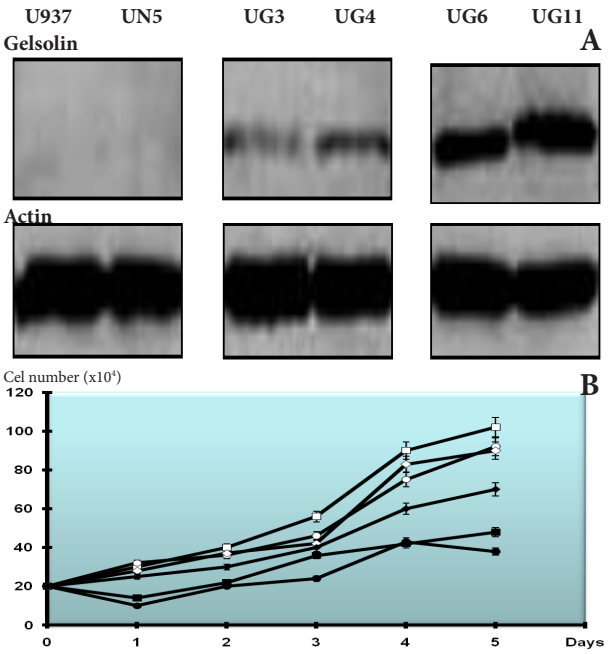

Figure 1. Isolation and Growth Suppression. A) Western blot analysis in parental U937 cells, the Neo control clone UN5, and gelsolin-overexpressed stable clones UG3, UG4, UG6 and UG11. Actin was used as an internal control. B) Growth curve of parental U937 (❑), UN5 (○), UG3 (▽), UG4 (◆), UG6 (■): and UG11 (●).

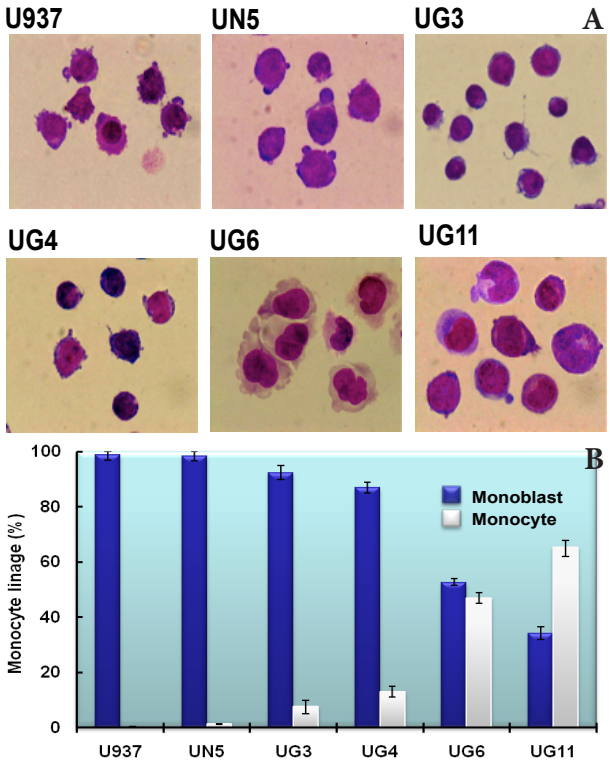

Figure 2. Induction of Monocytic Differentiation in Gelsolin-overexpressing U937 Clones. A) Giemsa staining of parental U937 cells, and UN5, UG6 and UG11 clones. Gelsolin-overexpressing UG6 and UG11 cells show greater monocytic morphology than UG3, UG4, the parental U937 and UN5 control cells (100x). B)The percentage of monocytic differentiation in the parental U937 cells and UN5 control cells, UG3, UG4, UG6 and UG11 clones.The error bars indicate standard deviations from three different microscopic fields that were randomly chosen and contained more than 100 cells per field having monoblast or monocyte characteristics.

demonstrated no significant change in apoptosis between parental, UN5 and UG6, UG11 cells (data not shown).

To determine whether the gelsolin-overexpressing cells gave rise to terminally differentiated monocytes/macrophages, they were examined for morphological changes by Giemsa staining. The parental U937 cells and UN5 cells displayed a morphology identical to that of monoblasts (99% and 98%, respectively) (Figure 2A, 2B). These cells had small amounts of cytoplasm with round and hyperchromic nuclei containing prominent blast-like nucleoli. In contrast, UG6 and UG11 cells were larger, and uniformly differentiated into monocytes with increased cell size, a greater cytoplasmic to nuclear ratio, a vacuolated cytoplasm, cytoplasmic granularity, kidney shape or lobular nuclei with finely dispersed chromatin, and inconspicuous nucleoli (Figure 2A). This monocytic morphology was observed in the range of 50-70% in the gelsolin overexpressors, UG6 and UG11. Meanwhile this value was between 8-13% in the case of lower expressing clones UG3 and UG4 (Figure 2B). These findings confirm that gelsolin overexpression induces growth retardation and morphological differentiation in U937 cells probably based on concentration.

Induction of monocytic differentiation markers in gelsolin-overexpressing U937 clones

Functional differentiation of monoblasts into monocytes was histologically quantified by the cell's ability to produce a superoxide, generated via NADPH oxidase, and to reduce the tetrazolium salt NBT to insoluble formazan, which forms a black-blue precipitate in these cells (Jiang et al., 2008). As shown in Figure 3, NBT-reducing ability was enhanced in UG6 (70%) and UG11 (68%) cells, when compared with UN5 (2%) and parental (2%) cells (Figure 3B).

Since the NBT-reducing ability has also been used to observe differentiation into the granulocyte lineage, the measurement of non-specific esterases activity was used to distinguish differentiation into the monocyte lineage. NSE is a heterogeneous and ubiquitous group of carboxyl esterases which are detected cytochemically with alpha-naphthyl ester substrates and diazotized couplers (Miranda, 2002). In monocytes NSE can react with alpha-naphthyl ester substrates that lead to the appearance of red-brown granules (Miranda et al., 2002). As shown in Figure 4A and 4B, 55-45% of UG6 and UG11 cells were positive for NSE staining, when compared with UN5 (5%) and parental U937 (3%) cells.

The expression of CD11b (a beta-integrin receptor) is enhanced in myelomonocytic progenitors following differentiation to more mature cell types (Yamamoto et al., 2009). Flow cytometric analysis revealed that the mean percentages of CD11b-positive cells in parental U937 and UN5 cells were 12%~10%, whereas those in UG6 and UG11 cells increased to 40%~36% (Figure 5A).

All these data demonstrate that gelsolin plays an important role in the monocytic differentiation of U937 cells, also in terms of functional activity.

Gelsolin overexpression upregulates p21CIP1

Availability of PU1 is crucial for normal myeloid

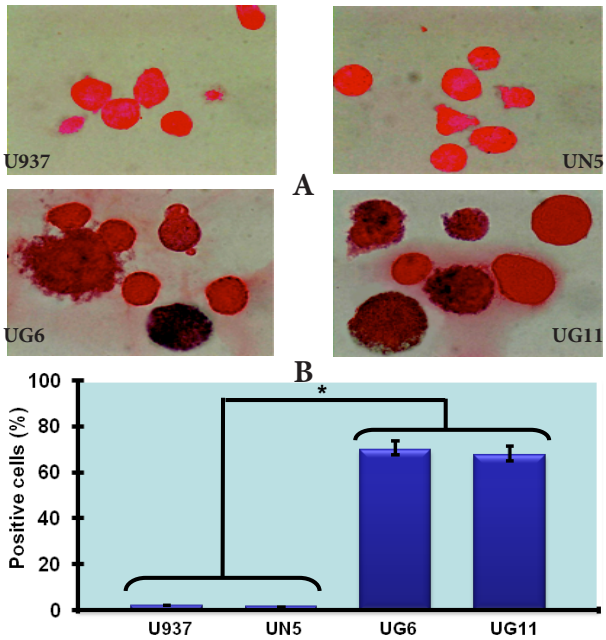

Figure 3. Nitroblue Tetrazolium (NBT)-reducing activity in Gelsolin-overexpressing U937 Clones. A) Safranin counterstaining in parental U937 cells, control UN5 cells and gelsolin-overexpressing UG6 and UG11 cells. Parental U937 cells and UN5 cells contained few NBT positive cells whereas gelsolin-overexpressing UG6 and UG11 cells displayed a lot of NBT-positive cells (100X). B) Cells that contained dark precipitation were counted as positive for NBT-reducing activity. The error bars indicate standard deviations from three different microscopic fields that were randomly chosen and contained more than 100 cells per field (*P<0.01).

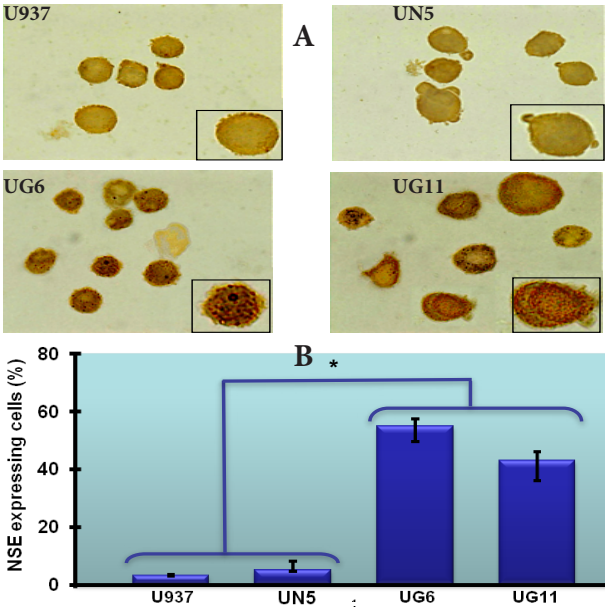

Figure 4. Nonspecific Esterase Activity in Gelsolin-overexpressing U937 Clones. A) 1-naphtyl acetate staining with haematoxylin counterstaining in parental U937 cells, Neo control UN5 cells, and gelsolin-overexpressing UG6 and UG11 cells. Low esterase activity was observed in parental U937 cells and UN5 cells whereas high esterase activity was observed in the gelsolin-overexpressed UG6 and UG11 cells (100X). Right bottom corner insert shows higher magnification (200X). B) Cells that contained red-brown granules were counted as positive for NSE staining. The error bars indicate standard deviations from three different microscopic fields that were randomly chosen and contained more than 100 cells per field (* P<0.01).

development and some of the molecular events in developing neutrophils and macrophages have been shown to be critically dependent on PU1 (Mak, 2011). Therefore, the expression of the transcription factor PU1 was examined. UG6 and UG11 cells showed similar expression levels of PU1, when compared with parental U937 cells and UN5 cells. Since p21CIP1 is required for initiation of the early steps of vitamin D3 (VD3) - induced monocytic differentiation of U937 cells (Koschmieder et al., 2007; Moosavi and Yazdanparast, 2008), we hypothesized that this gene could be potentially involved in the gelsolin-induced differentiation of these cells. UG6 (13-fold) and UG11 (25-fold) cells showed up-regulation of p21CIP1 mRNA levels, while its expression in parental and UN5 cells was very low (Figure 5B). The protein levels of p21CIP1 were also elevated in the gelsolin-overexpressing clones (Figure 5C). These data imply that the differentiation resulting from gelsolin overexpression in U937 cells is associated with the activation of p21CIP1.

P21CIP1 silencing reverses the differentiation and growth suppression induced by gelsolin

To understand the role of the p21CIP1 gene in gelsolin overexpression-induced monocytic differentiation, gene knockdown experiments were performed using siRNAs. Western blot analysis for UG6 and UG11 cells, transfected

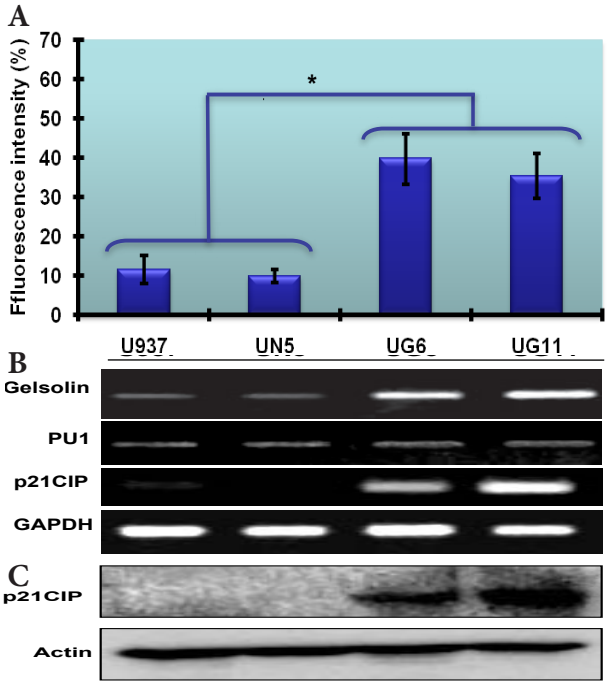

Figure 5. CD11b Expression and RT-PCR Analysis. A) Flow cytometry, using a FITC-conjugated monocyte/macrophage terminal differentiation cell surface marker CD11b antibody in parental U937 cells, Neo control UN5 cells, and gelsolin-overexpressing UG6 and UG11 cells. The error bars indicate standard deviations from triplicate experiments (*P<0.01). B) RT-PCR analysis of gelsolin, PU1 and p21CIP1 mRNAs in parental U937 cells, control UN5 cells, and gelsolin-overexpressing UG6 and UG11 cells. The level of p21CIP1 mRNA increased 13-25 fold in gelsolin-overexpressing clones while the level of PU1 mRNA remained unchanged in all cells. GAPDH mRNA was used as an internal control. C) Western blot analysis for p21CIP expression also shows up-regulation of p21CIP1 at the protein level.

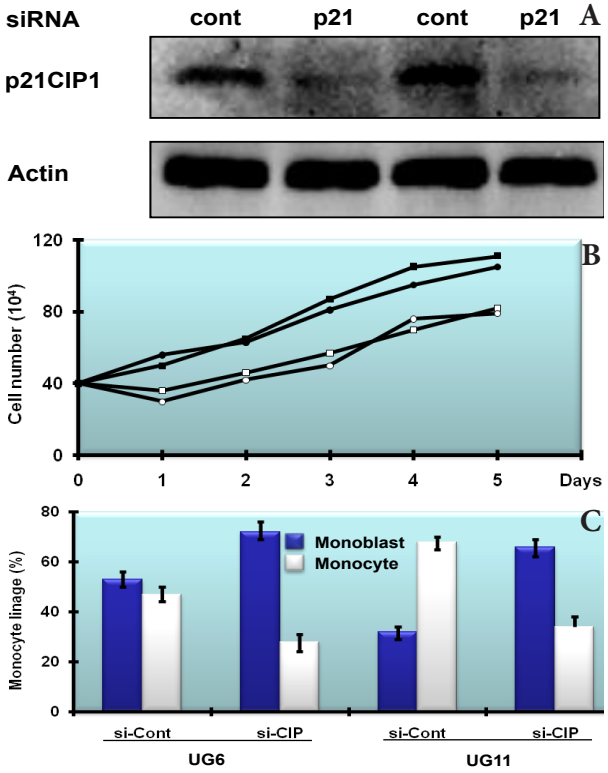

Figure 6. A) Western Blot Analysis for U937 Cells Transfected with siRNA for p21CIP1. Control basal p21CIP1 mRNA levels before transfection and proliferation of p21 siRNA-transfected UG6 and UG11 cells, compared to control siRNA transfected cells (siRNA proliferation: 72h after transfection). **B) Growth Curve for U937 Gelsolin-overexpressing Clones Transfected with siRNA for p21CIP1.** Control time points were selected according to the maximal effect of siRNA on proliferation (The optimal time point for siRNA was 48h). UG6 Si-Cont (❑), UN11 Si-Cont (❍):, UG6 Si-p21CIP1 (■): and UG11 Si-p21CIP1 (●).The error bars indicate standard deviation. **C) The Percentage of Monocytic Differentiation in U937 Gelsolin – overexpressing Clones Transfected with siRNA for p21CIP1 Compared to the Same Clones Transfected with Control siRNA.** Time points were selected according to the maximal effect of siRNA on proliferation which was determined in separate experiments. (The optimal time points were 48h). Error bars; Standard deviation from three different microscopic fields that were randomly chosen and contained more than 100 cells per field that had characteristics of monoblasts or monocytes.

with siRNA for p21CIP1, demonstrated 90.6% and 95.2% knockdown respectively, after 48 hours, which was the best point for silencing (Figure 6A). The growth ratio of the p21CIP1-silenced UG6 and UG11 cells demonstrated a slight increase, when compared with that of the control siRNA-transfected cells (Figure 6B). Morphological analysis, 48 hours after nucleofection, revealed that the monocyte ratio of the p21CIP1-silenced UG6 and UG11 cells was reduced to 28% and 34%, respectively, while control siRNA-transfected UG6 and UG11 cells showed the same monocytic ratio (47% and 68%) as that of untreated UG6 and UG11 clones (50% and 70%) (Figure 6C). All these results strongly suggest that p21CIP1 plays an important role in the growth inhibition and monocytic morphological differentiation observed in gelsolin overexpression-induced monocytic differentiation of U937cells.

Discussion

Tumor suppressor genes have received much attention for their roles in the development of human malignancies. Their gene products, however, function as negative growth regulators and are highly expressed in many tissues during embryonic development, suggesting that they might also act as critical proteins in differentiation and development (Lai, 2012).

Previously, it was reported that the expression of gelsolin is frequently silenced in various cancers, both in cell lines as well as in human tissue tumors (Tanaka et al., 2006). Since overexpression of wild-type gelsolin inhibited the growth of colon, bladder and lung cancer cell lines *in vitro* and *in vivo*, it was proposed that gelsolin can function as a tumor suppressor (Sazawa et al., 2002; Sagawa et al., 2003). It has been shown that one mechanism accounting for the inhibition of cell proliferation and tumorigenicity was related to gelsolin suppressing the activation of protein kinase Cs (PKCs) involved in phospholipid signaling pathways (Sagawa et al., 2003). Furthermore, it has also been demonstrated that transfectants of a highly metastatic murine melanoma cell line, B16-BL6, expressing ectopic gelsolin, exhibited retardation of cell spreading, reduced chemotactic migration to fibronectin and suppressed lung colonization in a spontaneous metastasis assay (Fujita et al., 2001).

There is evidence implicating other tumor suppressors, such as, the retinoblastoma gene (Goodrich, 2006), p53 (Spike and Wahl, 2011), DCC gene (Zhao, 2012), and Differentiation-Related-Gene-1 (Drg-1) (Dong et al., 2005) in cellular differentiation. Results of the molecular control of changes in the normal developmental program in myeloid leukaemia have shown that genetic abnormalities which give rise to malignancy can be bypassed, and their effects nullified, by inducing differentiation, which stops cells from multiplying (Lai et al., 2012). Specific haematopoietic transcription factors are crucial for differentiation to particular lineages during normal differentiation, and are controlled by specific patterns of expression and protein interactions (Petrie, 2009). PU1, an Ets family member, is one of the master transcription factors identified to regulate development of both granulocytes and monocytes/macrophages (Shima and Kitabayashi, 2011). However, in our gelsolin-induced monocytic differentiation, the mRNA expression of PU1 was unchanged.

Myeloid maturation of CD34+ precursor cells is associated with a marked increase in the tumor-suppressor p21CIP1 expression, both at RNA and protein levels (Ullmannová, 2003). In our study, p21CIP1 was also upregulated in gelsolin-overexpressing U937 cells, however the level of the tumor suppressor p53 (activated form) remained unchanged (data not shown). These data suggest that expressions of gelsolin and p21CIP1 are functionally associated with the regulation of cell differentiation. To confirm whether the up-regulation of the p21CIP1 gene was associated with the progression

of monocytic differentiation of the U937 cell line, we investigated the effect of a siRNA, which inhibits the expression of p21CIP1. SiRNA caused reduction in p21CIP1 but a control siRNA did not. The knockdown of p21CIP1, however, showed reduced growth rate and a morphological monocytic differentiation phenotype. Therefore, taken together, our results strongly suggest that p21CIP1 acts as an effector of cytoplasmic gelsolin and plays an important role in gelsolin-induced monocytic differentiation of U937 cells.

A myeloid leukaemia suppressor gene encoding sequence-specific single-stranded DNA binding protein 2 (SSBP2) which may function by direct interaction with cytoskeletal elements, and thus altering signal transduction, was reported to be a potential novel regulator of haematopoietic growth and differentiation, whose loss confers a block in differentiation advantage to myeloid leukaemic cells (Liang, 2005). Our work confirms that the cytoskeletal tumor suppressor, gelsolin, can induce monocytic myeloid differentiation in addition to growth retardation in the human monocytic cell line U937, thus providing further evidence that several tumor suppressor genes have differentiation-inducing function.

Studies with myeloid leukaemic cells have shown that some leukaemic cells can be induced to differentiate by cytokines that control normal haematopoiesis, and that myeloid leukaemic cells resistant to normal cytokines can be induced to differentiate by compounds that use alternative differentiation pathways (Humeniuk, 2009). The maturation arrest of acute promyelocytic leukaemia (APL), for instance, can be reversed with all-trans retinoic acid (Germain, 2006). More recent trials involving differentiation induction demonstrate that this can be applied even in combination with intensive chemotherapy to increase the susceptibility of AML blasts to drug-induced apoptosis (Fredly et al., 2009). Besides the standard therapy for AML, the use of site-specific ligands, receptors and cytokines, disruption of dominant fusion leukaemogenic proteins, chromatin remodeling and the combination of the above with cytotoxic chemotherapy have been shown to be synergistic in inducing myeloid differentiation and apoptosis in several AML cell lines and in patients with APL (Koeffler, 2010). In this report, we demonstrate that an actin regulatory-protein, gelsolin, by itself, has induced growth retardation and monocytic differentiation in U937 cells. Recent evidence suggests that several members of the gelsolin family might possess unexpected nuclear functions, including roles in regulating transcription (Li, 2012). For future studies, an *in vivo* system is necessary to develop a model for gene therapy. This could provide promising new strategies for the use of gelsolin as a differentiation-inducing agent in the therapy of promonocytic leukemia cells.

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