Lack of reproducible growth inhibition by *Schlafen1* and *Schlafen2 in vitro*

Liang Zhao [a], Brent Neumann [a], Kathleen Murphy [a,1], John Silke [b], Thomas J. Gonda [a,*]

[a] *University of Queensland Diamantina Institute for Cancer, Immunology and Metabolic Medicine, Brisbane, Australia*
[b] *Department of Biochemistry, La Trobe University, Melbourne, Australia*

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ABSTRACT

The *Schlafen* gene family has been implicated in lymphoid and myeloid maturation and differentiation as well as inflammation. However, little is known about the functions of this gene family except that anti-proliferative activities, particularly for *Schlafen1*, the prototype member of the family, have been reported. This was shown mainly by ectopic expression of *Schlafen1* in murine fibroblasts resulting in growth inhibition and a G1 cell cycle arrest apparently via repression of *Cyclin D1* expression. However, we have been unable to reproduce these findings. *Schlafen1* and *Schlafen2* failed to inhibit cell proliferation, cause G1 cell cycle arrest, or affect *Cyclin D1* level in murine fibroblasts. This was regardless of whether overexpression was constitutive, induced or from transient transfections. Moreover, in our hands, *Schlafen1* and *-2* do not appear to regulate the activity of *Cyclin D1* promoter. Importantly, we also showed that *Schlafen1* and *-2* do not play anti-proliferative roles in more physiologically-relevant myeloid cell lines. We therefore suggest that *Schlafen1* and *Schlafen2* might not have obligatory anti-proliferative activities, at least *in vitro*, and that efforts to explore their functions should be directed to other aspects, such as haemopoietic development and immune response.

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Introduction

The up-regulation of *Schlafen* (*Slfn*) gene family members has been found in a variety of contexts, such as lymphocyte maturation and differentiation [1,2], inflammation [3–8], macrophage activation [3,9,10] and myeloid differentiation [3,11]. However, the roles of *Slfn* genes in these processes have only been poorly characterized to date.

Slfn1 and -2 proteins are the shortest forms within the family. *Slfn1*, the prototype member of the family, was identified in a screen for genes up-regulated during thymocyte maturation [1]. Expression in mouse fibroblasts was reported to repress cell growth and arrest cells at G1 phase [1,3,12] via down-regulation of *Cyclin D1* at transcriptional level [12]. *Slfn2*, the family member bearing the most similarity to *Slfn1*, was suggested to have a much stronger growth inhibitory activity than *Slfn1* [1].

The present study argues against the notion of anti-proliferative activities of *Slfn1* and *-2*. We show, by using a variety of approaches and cell lines, that expression of *Slfn1* and *-2* fails to suppress cell proliferation and induce G1 cell cycle arrest. We also present data arguing that *Schlafen1* and *-2* are unlikely to inhibit *Cyclin D1* at transcriptional level. Importantly, we show that *Slfn1* and *-2* do not confer growth inhibition in myeloid cells, where both genes are up-regulated during terminal myeloid differentiation.

Materials and methods

Cell culture

Recombinant murine Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and Interleukin-3 (IL-3) were produced from insect cell lines provided by Dr. Anna Brown and A/Prof. Richard D'Andrea (Hanson Institute, Adelaide, Australia). The murine myelomonocytic FDC-P1 cell line [13] was maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS) and 20 FDU/ml of GM-CSF. The murine myeloid progenitor FDB1 cell line [14] was maintained in IMDM supplemented with 10% FBS and 13 FDU/ml of IL-3. The estrogen-inducible Myb-transformed cell line, ER-MYB [15], was maintained in IMDM supplemented with 10% FBS, 80 FDU/ml of GM-CSF and 1 μM of β-estradiol. NIH/3T3 and 293T cell line were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS.

To induce differentiation of FDB1 or ER-MYB cells, cells were washed with PBS thrice and cultured in IMDM supplemented with 10% FBS, 46 FDU/ml of GM-CSF (FDB1) or 80 FDU/ml of GM-CSF and 100 nM of β-estradiol antagonist ICI-182780 (ER-MYB).

RT-Quantitative PCR (RT-qPCR)

Total RNA was extracted using RNeasy Mini kit (Qiagen), treated with DNase I (Fermentas) and reverse transcribed with SuperScript III (Invitrogen). qPCRs were performed with iQ SYBR Green Super-mix (Bio-Rad) on a RotorGene 3000 (Corbett Research, Sydney,

Australia). Primers used were: Slfn1 forward CCAGACCAGCACCTG-CAC, reverse AAGAGGTTGGAGGGGGCTCAT; Slfn2 forward GCTTTAA-TGCAGCAAGGAACAAAGA, reverse TGGGCTTTGGCACTTGGAA; and HPRT forward GCAGTACAGCCCCAAAATGG, reverse AACAAAGTCTG-GCCTGTATCCAA.

Plasmid construction

The *Slfn* coding regions were PCR modified to encode epitope-tagged (FLAG or HA, fused to C- or N-terminus) Slfn proteins, and were then cloned into lentiviral or retroviral expression vectors, i.e. pF 5xUAS MCS [16] and pMYs-IRES-GFP [17].

Tamoxifen-inducible lentiviral overexpression of Slfn1 and 2

To produce recombinant lentivirus, 293T cells were transfected with pF 5xUAS lentiviral vector, pCMV δR8.2, Rev plasmid and VSV-G plasmid using Lipofectamine 2000 (Invitrogen). Lentiviral supernatants were harvested 48 h after transfection and concentrated using a Vivaspin-20 column (Sartorius).

NIH/3T3 cells were infected by incubating cells with supernatants for 6 h. FDB1 cells were infected with lentiviral supernatants using the spinoculation method. Briefly, cells were seeded into 6-well plate and spun with supernatants for 60 min. Cells were first infected with Gal4-ER^{T2}-VP16 virus [16] and subjected to hygromycin selection. Hygromycin resistant cell lines (dubbed NIH/3T3/GEV and FDB1/GEV, respectively) were subsequently infected with pF 5xUAS lentiviruses for overexpressing *Slfn1* and *2*, and selected for puromycin resistance. Expression of *Slfns* was induced with 4-hydroxy tamoxifen (4-OHT) in resulted cell lines (100 nM for NIH/3T3/GEV cells and 1 μM for FDB1/GEV cells).

Cell proliferation assay

Cell proliferation was assessed using CellTiter 96 AQueous reagent (Promega) as per manufacturer's instructions. Each experiment was performed in triplicate and repeated at least twice.

Cell cycle profile analysis

Cells were harvested and fixed in 75% ethanol at 4 °C overnight, then stained with 50 μg/ml propidium iodide in the presence of 100 μg/ml RNase A in the dark for 30 min. The DNA content was measured using FACSCanto (Becton Dickinson) and data were analyzed using ModFit software. Each experiment was performed in triplicate and repeated at least twice.

Western blot analysis

Cell lysates were prepared in 1× SDS sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% w/v bromophenol blue), separated by SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were incubated with primary antibodies at 4 °C overnight followed by rinses and addition of HRP-conjugated secondary antibodies (Sigma). Immunoreactive proteins were visualized using ECL Western substrates (Pierce). The following primary antibodies were used: anti-HA antibody (Roche, 3F10), anti-Cyclin D1 antibody (Cell Signalling, # 2926), anti-FLAG M2 (Sigma, F3165) and anti-β-Tubulin (Sigma, T0198).

Reporter assays

NIH/3T3 cells were transiently transfected with *Cyclin D1* promoter reporter plasmid (provided by Prof. Richard Pestell [18]), *Slfn1*, *Slfn2* or *E1A 12S* expression plasmid (provided by Prof. Antony Braithwaite [19]), pCMX-β-Gal plasmid (provided by Dr. Dennis H. Dowhan [20]).

Similarly, NIH/3T3/GEV cells inducible for *Slfn1* or *Slfn2* were transfected with *Cyclin D1* promoter plasmid and pCMX-β-Gal in the presence or absence of 100 nM of 4-OHT. Cells were harvested 48 h after transfection and assayed for luciferase and β-Galactosidase activities on a luminometer (Berthold) by using luciferase assay kit (Promega) and Accelerator II reagent (Tropix) respectively. Relative luminescence unit (RLU) was calculated by normalizing luciferase activity to β-Galactosidase activity. All transfections were done in triplicate and repeated at least twice.

Retroviral expression of Slfn1 and Slfn2 in FDC-P1 cells

293T cells were co-transfected with pMYs-IRES-GFP retroviral constructs and packaging vector pEQ$_{Eco}$ using Lipofectamine 2000 (Invitrogen). Retroviral supernatants were harvested 48 h after transfection and used to infect FDC-P1 cells using spinoculation protocol described above. Cells were sorted using a MoFlo station for GFP expression 48 h after infection.

Immunofluorescence microscopy

Cells were fixed in 10% formalin and incubated with the following primary antibodies for 1 h: anti-FLAG M2 (Sigma, 1:100), anti-Slfn2 (Santa Cruz, 1:25), and anti-Cyclin D1 (Cell Signalling, 1:100); followed by incubation in the dark with Cy5, FITC or Texas Red conjugated secondary antibodies. Finally, after staining with 4,6-diamidino-2-phenylindole for 10 min, coverslips were mounted. Fluorescent signal was examined and imaged using a Carl Zeiss Axioskop 2 plus microscope with an AxioCam 2 camera.

Results

Both Slfn1 and Slfn2 are up-regulated upon myeloid differentiation

Slfn2 first came to our attention from a screen for genes differentially expressed during the differentiation of ER-MYB myeloid progenitor cells [21]. Both *Slfn1* and *Slfn2* are up-regulated strongly during this process (Fig. 1A, 3F and Ref. [22]). This was also observed in differentiation of another myeloid progenitor cell line FDB1 (Fig. 1B).

Induction of Slfn1 or Slfn2 in NIH/3T3 cells fails to confer growth inhibition or G1 cell cycle arrest

Ectopically expressed *Slfn1* was reported to inhibit proliferation of murine fibroblasts [1,3] by inhibiting the G1 to S phase transition via repression of *Cyclin D1* expression [12]. *Slfn2* was also proposed to disrupt cell growth, as attempts to establish murine fibroblast cell lines constitutively or conditionally expressing *Slfn2* constantly failed [1].

However, we failed to reproduce these results in our NIH/3T3 murine fibroblast cells. We employed a tightly-regulated two-component tamoxifen lentiviral system [16] (depicted in Fig. 2A) to inducibly overexpress *Slfn1* and *Slfn2*. In the presence of tamoxifen, Slfn1 or -2, encoded by the pF 5xUAS vector, is expressed from the 5xUAS promoter that is bound and activated by Gal4-ER^{T2}-VP16 (GEV16) fusion protein, encoded by the pFU GEV16 vector.

The proliferation of synchronized NIH/3T3/GEV cells upon induction of *Slfn1* and *-2* was assessed. Cells were pre-treated with or without 4-OHT for 24 h, synchronized to G0 phase by serum starvation for 24 h, then re-stimulated with serum in the presence or absence of 4-OHT. As shown in Fig. 2B, no significant difference in growth rate over 7 days upon induction for *Slfn1* or *2* was observed, except that all cells including controls treated with 4-OHT showed decreased proliferation.

Cell cycle profiles were also analyzed at 0, 24 and 48 h after serum re-stimulation. No significant change in the percentage of cells at G0/G1 phase was observed at either 24 or 48 h after the serum re-stimulation
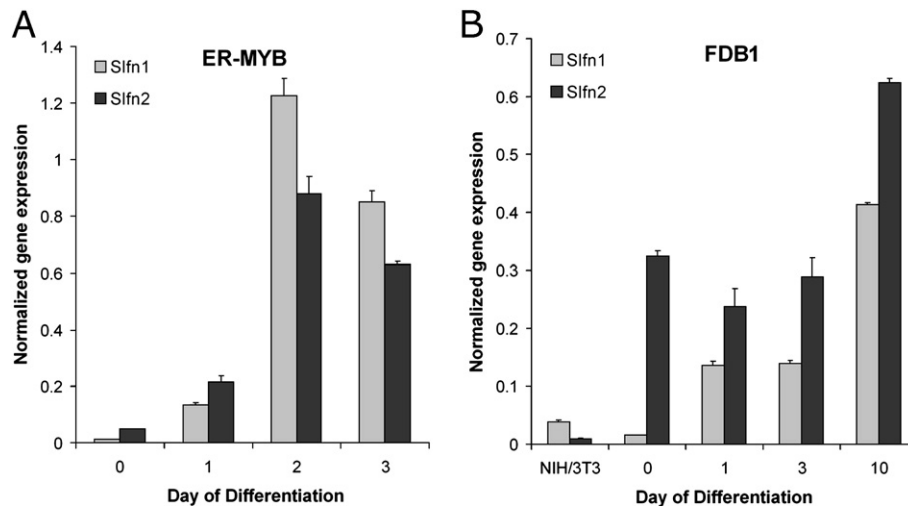
Fig. 1. Both *Slfn1* and *Slfn2* are up-regulated upon terminal myeloid differentiation. ER-MYB (A) or FDB1 (B) cells were induced to differentiate and harvested at indicated time points. Total RNA was extracted and RT-qPCR analysis was performed. The expression level of *Slfn1* or *2* was normalized to hypoxanthine–guanine phosphoribosyltransferase (HPRT). Panel (B) also shows that *Slfn1* and *-2* are expressed at very low levels in NIH/3T3 cells. Error bars represent S.E.

(Fig. 2C). Induction for Slfn1 and -2 was confirmed using Western blotting (Fig. 2D).

As inhibition of *Cyclin D1* expression was proposed [12] as the mechanism of G1 cell cycle arrest caused by *Slfn1*, Cyclin D1 levels were also examined in these cells. No significant change in Cyclin D1 level was observed following induction for Slfn1 or -2 (Fig. 2D), although there was a slight decrease in Cyclin D1 levels in all cells treated with 4-OHT, which might underlie the observed repression of cell proliferation by 4-OHT.

Slfn1 and Slfn2 do not inhibit Cyclin D1 promoter activity

Brady et al. [12] reported that *Slfn1* inhibits *Cyclin D1* promoter activity in a luciferase reporter assay. Since no significant decrease in Cyclin D1 levels was observed in NIH/3T3 cells expressing *Slfn1* or *-2* (Fig. 2D), we examined whether *Slfn1* or *Slfn2* could inhibit the *Cyclin D1* promoter activity in our hands.

Two *Cyclin D1* promoter constructs were tested, in which a 1.7 or 3.3 kb *Cyclin D1* promoter drives expression of firefly luciferase. NIH/3T3 cells were co-transfected with a reporter construct and a *Slfn1*, *Slfn2* or adenovirus *E1A 12S* expression construct. *E1A 12S* served as a positive control as it is known to inhibit *Cyclin D1* promoter activity [18]. As shown in Fig. 2E, both *Slfn1* and *Slfn2* failed to inhibit the activity of *Cyclin D1* promoter significantly, whereas the positive control *E1A 12S* strongly inhibited the activity ($P<0.01$) of *Cyclin D1* promoter.

Reporter assays were also conducted in NIH/3T3 cells inducible for *Slfn1* or *-2*. Consistently, no significant inhibition of *Cyclin D1* promoter activity was observed upon induction for *Slfn1* or *-2* (Fig. 2F).

Overexpression of Slfn1 or Slfn2 in FDC-P1 and FDB1 myeloid cells fails to repress cell proliferation

As neither *Slfn1* nor *-2* is naturally expressed in fibroblasts (Fig. 1B), we reasoned that it would be more important to investigate whether they would have anti-proliferative activities in physiologically more relevant cells. We therefore examined myeloid cells in which *Slfn* genes are up-regulated upon differentiation (Fig. 1 and Ref. [3,11]).

FDC-P1 is a growth factor dependent murine myelomonocytic cell line that is deficient in differentiation [13]. We first attempted to constitutively express *Slfn1* and *-2* in this cell line, reasoning that this would probably induce repression of FDC-P1 cell proliferation if *Slfn1* and *Slfn2* have anti-proliferative activities in myeloid cells. However, overexpression of *Slfn1* or *-2* failed to repress the proliferation of FDC-

P1 cells (Fig. 3A). Expression of *Slfn1* and *-2* was confirmed by immunofluorescence microscopy (Fig. 3B).

We next examined bi-potent myeloid FDB1 cells [14] in which *Slfn1* and *-2* are up-regulated upon differentiation (Fig. 1B). These cells proliferate continuously when stimulated with IL-3, but undergo granulocyte-macrophage differentiation and cease proliferation after several days in response to GM-CSF. Hence it would be a better cell model than FDC-P1 to study potential myeloid differentiation-related effects of *Slfn* genes [23].

The tamoxifen-inducible system (see above and Fig. 2A) was employed to overexpress *Slfns* in FDB1 cells. Slfn2 was strongly induced in these cells kept either in IL-3 or in GM-CSF (Fig. 3E). However, the induction of Slfn1 was not detectable using Western blotting (data not shown), possibly due to the sub-optimal sequence around its start codon. Similar to FDC-P1, FDB1 cells failed to show significant growth inhibition upon induction of *Slfn2* either when maintained in a proliferating state (in IL-3) or when induced to differentiate (in GM-CSF), as shown in Figs. 3C and D.

Slfn2 and Cyclin D1 are co-expressed in the cytoplasm of differentiated ER-MYB cells

Slfn2 is highly expressed in differentiated ER-MYB cells (Fig. 1A and Ref. [22]) which are primarily macrophages. We therefore examined Cyclin D1 levels in these macrophages, reasoning that if *Slfn2* inhibits *Cyclin D1*, low levels of Cyclin D1 would be expected. To the contrary, both Slfn2 and Cyclin D1 are up-regulated and co-expressed in the cytoplasm of differentiated ER-MYB cells (Fig. 3F).

Discussion

Our data do not support obligatory anti-proliferative activities of *Slfn1* and *Slfn2* in murine fibroblasts or in myeloid cells. We failed to reproduce the reported growth inhibition and G1 arrest effects of *Slfn1* and *-2* in NIH/3T3 cells [1,3,12]. Moreover, we did not observe significant changes in *Cyclin D1* level in response to overexpression of *Slfn1* or *Slfn2*, although *Slfn1* was previously reported to directly inhibit transcription of *Cyclin D1* [12]; this was proposed as a mechanism by which *Slfn1* repressed cell growth and caused a G1 arrest. In fact, we found that neither *Slfn1* nor *Slfn2* repressed *Cyclin D1* promoter activity in reporter assays. Consistent with this, transient transfections of NIH/3T3 cells with *Slfn1* or *Slfn2* expression plasmids with or without epitope tags also showed no G1 cell cycle arrest and no significant change of Cyclin D1 level (data not shown).
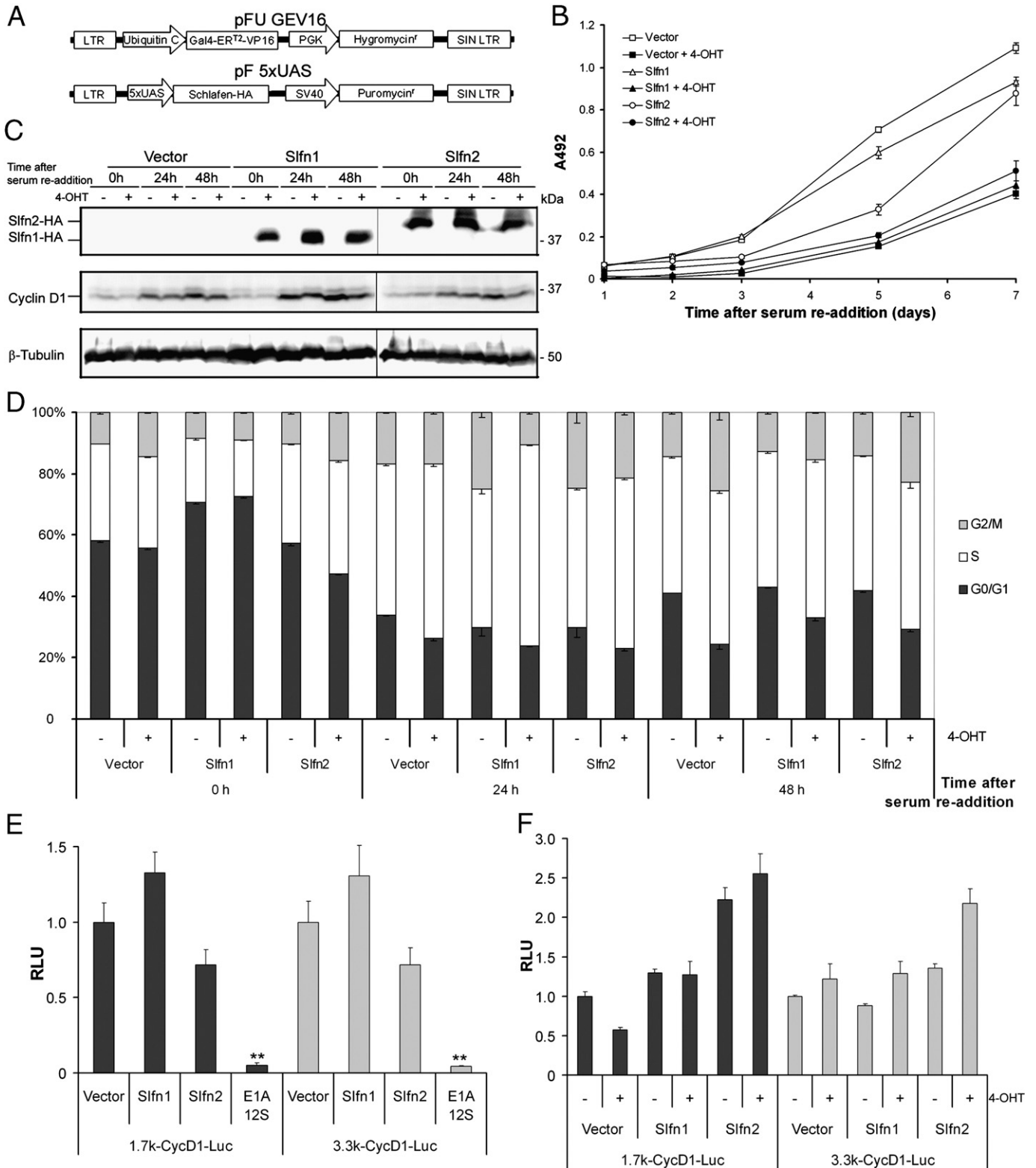
Fig. 2. Induction for *Slfn1* or *2* in NIH/3T3 cells does not confer growth inhibition or G1 cell cycle arrest. (A) Schematic representation of the tamoxifen-inducible expression system [16] for overexpressing *Slfn* cDNAs. (B) Induction of *Slfn1* or *Slfn2* in synchronized NIH/3T3 cells does not cause significant growth inhibition over 7 days. Cells were treated with or without 4-OHT for 24 h, serum starved for another 24 h, then released from G0 phase by serum re-stimulation with or without 4-OHT. (C) Induction for *Slfn1* or *Slfn2* in synchronized NIH/3T3 cells does not arrest cells at G1 phase. Cells were treated as in (B) and cell cycle profiles were measured by propidium iodide staining at each time point. (D) Western blot analysis showing the induction of *Slfn1* or *-2* in synchronized cells. No significant change of *Cyclin D1* levels in *Slfn1* or *-2* expressing cells was observed. The blot was also probed for β-Tubulin as a loading control. (E and F) Neither *Slfn1* nor *Slfn2* inhibits the activity of *Cyclin D1* promoter. NIH/3T3 cells (E) were co-transfected with the −1.7k-CycD1-Luc or −3.3k-CycD1-Luc reporters in conjunction with *Slfn1*, *Slfn2* or *E1A 12S* expression constructs. NIH/3T3 cells inducible for *Slfn1* or *-2* (F) were co-transfected with *Cyclin D1* promoter reporters in the presence or absence of 4-OHT. A β-*Galactosidase* (β-*Gal*) expression plasmid was included in all transfections as a control for transfection efficiency. Luciferase activity was assayed 48 h after transfection and normalized to β-Gal activity. Data are presented as means ± S.E. (**) denotes significance (*P* < 0.01, Student's *t*-test) when compared to control cells transfected with empty vector.
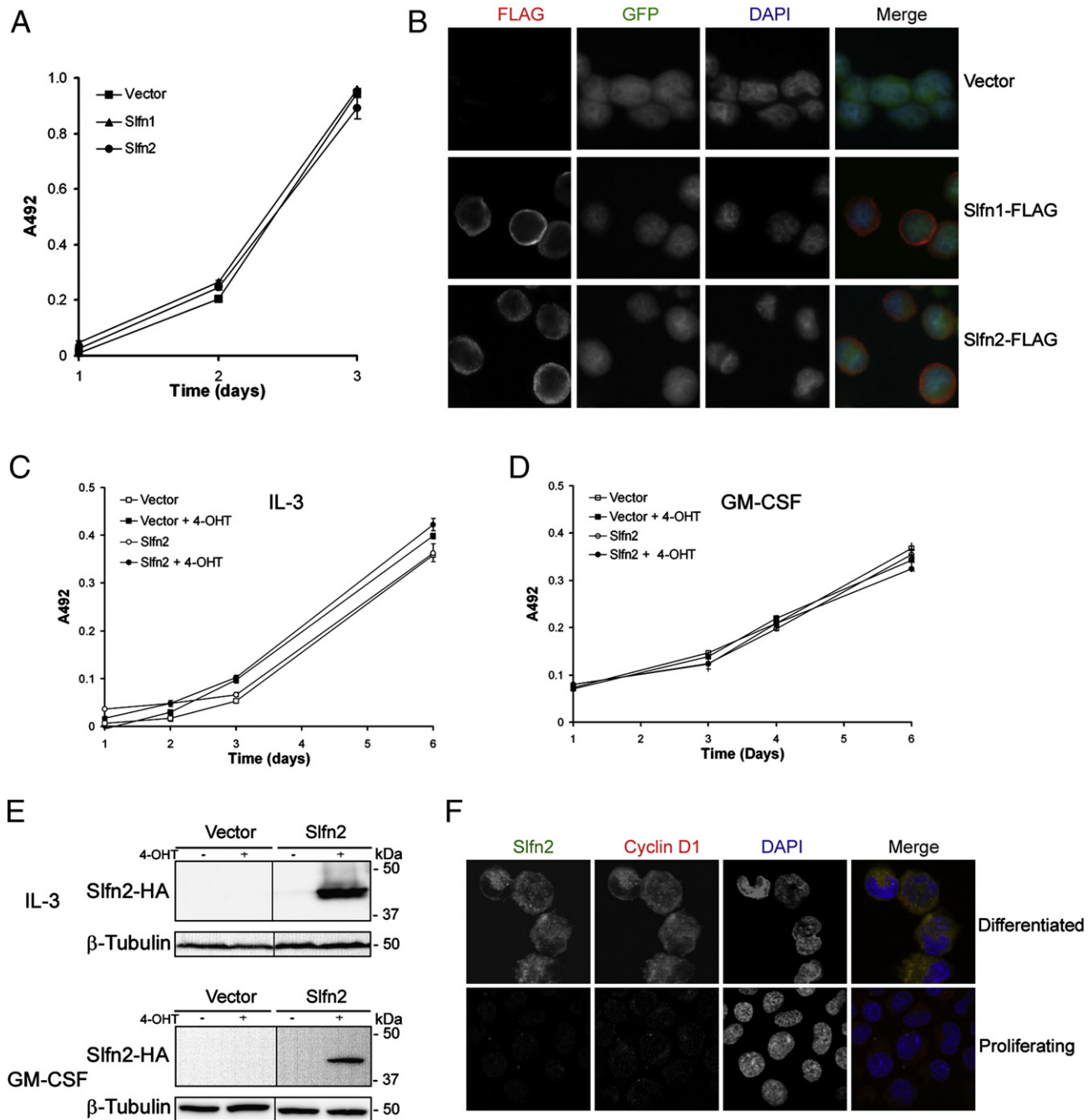
Fig. 3. Expression of either *Slfn1* or *Slfn2* does not affect proliferation of myeloid cells. (A) Overexpression of *Slfn1* and *-2* in FDC-P1 cells does not repress cell proliferation. (B) Immunofluorescence microscopy showing the cytoplasmic expression of Slfn1 and *-2* in FDC-P1 cells. (C and D) Induction of Slfn2 in FDB1 cells does not cause inhibition of cell proliferation when cells are maintained in either proliferative or differentiation-inducing conditions. Data are presented as means±S.E. (E) Western blot analysis showing induction of Slfn2 in FDB1 cells growing in each condition. The membranes were also probed for β-Tubulin as a loading control. (F) Immunofluorescence microscopy showing the cytoplasmic co-expression of Slfn2 and Cyclin D1 in differentiated, but not undifferentiated, proliferating ER-MYB cells.

At the moment, we cannot explain the discrepancies between our data and previous reports. It might for example, reflect the variability between NIH/3T3 cell lines used in the different laboratories. However we did note that in two of the published studies [1,12], the anti-proliferative activity of *Slfn1* was shown in one particular clonal cell line, 18–9, which was only one out of ten individual clones. Moreover, there have already been some discrepancies about the reported anti-proliferative activities of *Slfn1*: Schwarz et al. [1] reported that *Slfn1* expressing (18–9) cells do not undergo apoptosis and the growth inhibition is gradually relieved after 4 days, whereas Geserick et al. [3] suggested that *Slfn1* expressing cells died eventually of apoptosis. We also note that there is no direct evidence supporting the anti-

proliferative activity of *Slfn2* except that attempts trying to establish stable *Slfn2* expressing clones or lines failed [1].

Recently, the *Slfn* gene family has been associated with terminal myeloid differentiation [3,11]. In agreement with this, we also found that in two myeloid progenitor cell lines, ER-MYB and FDB1, *Slfn1* and *-2* are expressed at relatively low levels when these cells are in an undifferentiated, proliferative state, whereas both genes are up-regulated significantly during terminal myeloid differentiation.

Given the minimal levels of *Slfn1* and *-2* in NIH/3T3 cells, we believe that it is important to explore the functions of *Slfn1* and *Slfn2* in physiologically-relevant cell types. These include myeloid cells where *Slfn1* and *-2* are normally up-regulated during terminal differentiation.

In agreement with our results in NIH/3T3 cells, we found that *Slfn1* and *Slfn2* do not cause any growth inhibition when overexpressed in FDC-P1 or FDB1 myeloid cells. In fact, as Slfn1 and Slfn2 proteins are predominantly localized in the cytoplasm in various cell types (Ref. [22] and Figs. 2B and F), they may not normally play such a role of transcriptional repression of *Cyclin D1*. Moreover, we have found that Slfn2 and Cyclin D1 are co-expressed in the cytoplasm of differentiated ER-MYB cells, further supporting our argument.

In addition to myeloid cells, *Slfn* genes are expressed in the lymphoid compartment, and decreased proliferation and increased apoptosis were observed in thymocytes from *Slfn1* transgenic mice [1]. Thus, lymphoid cells may be an appropriate system for investigating possible anti-proliferative or pro-apoptotic functions of *Slfn* genes.

In conclusion, we have presented data showing that neither *Slfn1* nor *Slfn2* has obligatory anti-proliferative activities in murine myeloid cells or fibroblasts; furthermore, we do not find that these genes suppress *Cyclin D1* expression in either cell type. Although we still do not fully understand the functions of *Slfn1* and *Slfn2*, our findings should stimulate efforts to explore their involvement in haemopoietic cell development and immune response by looking beyond direct regulation of proliferation.

Acknowledgments

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