Down-regulation but not phosphorylation of stathmin is associated with induction of HL60 cell growth arrest and differentiation by physiological agents

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Abstract Stathmin is a cytosolic phosphoprotein that has an important but, as yet, undefined role in cell proliferation and differentiation. Induction of growth arrest and differentiation of HL60 cells to monocytes by phorbol 12-myristate 13-acetate is associated with rapid phosphorylation of the protein. Stathmin phosphorylation was not seen when HL60 cells were induced to differentiate to monocytes, by 1α,25-dihydroxyvitamin D3, and to neutrophils, by *all-trans* retinoic acid and granulocyte colony stimulating factor. In all the above instances, stathmin expression was down-regulated. Thus, increased stathmin phosphorylation is not required for cell growth arrest or differentiation or down-regulation of stathmin expression.

Key words: Stathmin; Cell proliferation; Growth arrest; Protein phosphorylation

1. Introduction

Stathmin is a phylogenetically conserved, abundant cytosolic protein which is present within cells in non-phosphorylated and variously phosphorylated forms [1]. This protein has also been termed p19 or metablastin [2], prosolin [3], 19K [4], Lap18 [5] and Op18 [6]. Both the extent to which the protein is phosphorylated and the level of expression of stathmin have been associated with cell proliferation [3,7–10], development [11–14] and differentiation [2,15,16]. In vitro analysis has shown that stathmin is phosphorylated on serine residues by MAP kinase [17], cAMP-dependent protein kinase [12,18] and $p34^{cdc2}$ kinase [18]. It has also been shown that the extent to which stathmin is phosphorylated is related to the progression of cells through the cell cycle, with the protein becoming maximally phosphorylated during G2/M [19].

Phosphorylation of stathmin has been described in association with a wide variety of cellular events [2,4,20–26]. In particular, induction of growth arrest upon treatment of cells with phorbol 12-myristate 13-acetate (PMA) is accompanied by rapid and extensive phosphorylation of stathmin in HL60 cells and other myeloid cell lines [3,27,28], A431 epidermal carcinoma cells [29] and proliferating peripheral blood lymphocytes [7]. In cell lines that subsequently undergo differentiation following PMA treatment, for example HL60 and K562 cells, there is also a decline in the level of stathmin expression [27,28,30]. Findings such as these have been used to suggest that stathmin phosphorylation and subsequent down-regulation are important in the processes of cell growth arrest and/or differentiation.

HL60 cells can be induced to differentiate to monocytes by 1α,25-dihydroxyvitamin D3 (vitD3) [30] and to neutrophils by *all-trans* retinoic acid (ATRA) together with recombinant human granulocyte colony stimulating factor (hrG-CSF) [31]. In the present report we describe the changes in the extent of stathmin phosphorylation and expression following induction of growth arrest and differentiation of HL60 cells by these physiological agents. The rapid and transient hyperphosphorylation of stathmin that occurs when PMA is used to elicit growth arrest and differentiation of HL60 cells was not observed when HL60 cells were induced to differentiate by physiological agents. As cell growth arrest occurred the proportion of stathmin that was phosphorylated decreased and the level of stathmin declined.

2. Materials and methods

2.1. Induction and quantitation of HL60 cell differentiation

The maintenance of the promyeloid cell line HL60 [32] and induction of HL60 monocyte differentiation by treatment with PMA have been described previously [3]. Treatment of HL60 cells with 10 nM ATRA (Sigma, Poole, UK), in combination with 30 ng/ml hrG-CSF (Neuphogen; Amgen, Cambridge, UK), to induce neutrophil differentiation, and 100 nM vitD3 to induce monocyte differentiation have also been described previously [30,31]. HL60 cell differentiation was assessed by measuring the proportion of cells able to phagocytose complement coated yeast cells [33] and the proportion expressing the monocyte specific enzyme α-napthyl acetate esterase [34]. The proportions of HL60 cells within compartments of the cell cycle were determined by flow cytometric analyses of cells that had been stained with propidium iodide.

2.2. Immunohistochemistry

The proportions of cells expressing a proliferation-associated protein, the Ki-67 antigen [35], and stathmin following vitD3 or ATRA/hrG-CSF treatment were determined by double immunostaining of cytocentrifuged cell preparations, as described previously [36]. The results are a mean (± S.E.M.) of data from three experiments.

2.3. Gel electrophoresis and Western blotting

Levels of stathmin expression and the extent of phosphorylation in control and treated cells were determined by 1- and 2-dimensional gel electrophoresis followed by immunostaining of Western blots with an

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; ATRA, *all-trans* retinoic acid; hrG-CSF, recombinant human granulocyte colony stimulating factor; vitD3, 1α,25-dihydroxyvitamin D3.

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antiserum to stathmin and [^{125}I]protein A, as described previously [28]. Autoradiography films (Hyperfilm-MP X-ray film; Amersham Int. plc, Amersham) were preflashed, to allow quantitative densitometric analysis of the autoradiographs, and exposed at −70°C with intensifying screens. Autoradiographs were scanned using a laser densitometer (Ultroscan XL, Pharmacia LKB, Milton Keynes, UK) and analysis of gel scans was undertaken using Gelscan XL laser densitometer programme software (Pharmacia LKB).

3. Results

3.1. Changes in stathmin expression during HL60 monocyte differentiation

Treatment of HL60 cells with 100 nM vitD3 induced monocyte differentiation as shown by an increase in the number of phagocytic cells from <0.5% in untreated cells to 77 ± 1% at day 5. The number of cells expressing the monocyte-specific enzyme α-naphthyl acetate esterase increased from 1 ± 0.5% to 81 ± 6%. Cell cycle analyses of vitD3 treated HL60 cells revealed a time-dependent accumulation of cells into the G_0/G_1 phase of the cell cycle (Fig. 1, left panel). In control cells and for 2 days after addition of vitD3, 52 ± 8% of HL60 cells were in G_0/G_1, 43 ± 8% in S and 6 ± 1% in G_2/M. After 5 days of exposure to vitD3, 90 ± 2% of HL60 cells were in G_0/G_1 indicating that the majority of the cells in the culture had arrested their growth.

PMA-induced monocyte differentiation of HL60 cells results in loss of stathmin and Ki-67 proteins, as detected by immunocytochemistry [28]. Treatment of HL60 cells with vitD3 also resulted in a decrease in the proportion of cells that stained positively for stathmin and the Ki-67 antigen (Fig. 1, left panel). On the double immunostains performed, there was a close relationship between strong cytoplasmic immunopositivity for stathmin and nuclear immunopositivity for the Ki-67 antigen. After 3 days of treatment with vitD3, some cells were weakly positive for stathmin but negative for the Ki-67 antigen, with the numbers of these cells decreasing with time, as described for PMA-treated HL60 cells [28]. 93–96% of HL60 cells in control cultures stained strongly for stathmin and the Ki-67 antigen. In vitD3-treated cultures, the proportion of cells expressing both proteins remained high (86%–92%) for 2 days after addition of vitD3. Thereafter, the proportion of cells expressing stathmin and Ki-67 antigen declined in a time dependent manner up to day 5, when 22 ± 6% of cells were stained.

Down-regulation of stathmin protein was quantitated by densitometric analyses of immunostained Western blots of cell extracts (Fig. 1, left panel). In exponentially growing HL60 cultures there was a variation in the level of expression of stathmin of 6% (n = 3). Stathmin expression remained constant for up to 2 days after exposure to vitD3, thereafter there was a time-dependent reduction in the total level of stathmin. After 3 days treatment with 100 nM vitD3, HL60 cells expressed 63 ± 16% of the level of stathmin in untreated cells and after 5 days exposure to vitD3 only 5 ± 2% of the initial level of stathmin was detectable.

3.2. Changes in stathmin expression during HL60 neutrophil differentiation

HL60 cells exposed to 10 nM ATRA in combination with 30 ng/ml hrG-CSF grew exponentially for 2 days after which time cell growth arrest was observed. The proportion of phagocytic cells increased from <0.5% to 67 ± 3% at day 5. Cell cycle analyses of HL60 cells exposed to ATRA/hrG-CSF revealed an accumulation of cells in G_0/G_1 phase of the cell cycle (Fig. 1, right panel). In control cells and up to 2 days after addition of ATRA/hrG-CSF, 48 ± 2% of HL60 cells were in G_0/G_1, 44 ± 2% in S and 4 ± 0.5% in G_2/M. After 5 days of exposure to ATRA/hrG-CSF, 85 ± 3% of HL60 cells were in G_0/G_1. The patterns of down-regulation of expression of stathmin and of Ki-67 antigen in HL60 cells that had been treated with ATRA/hrG-CSF were similar to those observed in vitD3 treated HL60 cells (Fig. 1, right panel). A high proportion of cells expressing both proteins (90%–92%) was observed for 2 days after addition of ATRA/hrG-CSF. Thereafter, the proportion of cells expressing stathmin and Ki-67 antigen declined until after 5 days exposure to ATRA/hrG-CSF 20 ± 8% of cells were positive.

Quantitation of these changes in the levels of expression of stathmin was undertaken by Western blotting. As shown in Fig. 1 (right panel), ATRA/hrG-CSF treatment resulted in down-regulation of stathmin levels in HL60 cells. After 5 days exposure to ATRA/hrG-CSF, 24 ± 5% of the initial level of stathmin was detectable.

3.3. Stathmin phosphorylation during HL60 monocyte and neutrophil differentiation

Stathmin is present within HL60 cells as a non-phosphorylated (n) and two phosphorylated forms (p1 and p2). In exponentially growing HL60 cultures a low level of phosphorylation of stathmin was always detected (see Fig. 2). Stathmin was present as 82 ± 7.% n and 18 ± 7% p1 (n = 21). The p2 form was not detected. As reported previously, time course analyses of PMA-treated HL60 cells revealed a rapid increase in the phosphorylation status of stathmin [3,27] (see Fig. 3). After 1 h of exposure to 10 nM PMA, stathmin was present as 20 ± 7% n, 70 ± 10% p1 and 10 ± 4% p2 (n = 5) (see Fig. 2). This maximal level of phosphorylation of stathmin was maintained for up to 6 h after exposure to PMA. At 4 h, stathmin was present as 31 ± 3% n, 68 ± 4% p1 and 2 ± 1% p2 (n = 5). Thereafter, a decline in the phosphorylation status of stathmin was observed. After 24 h of exposure to 10 nM PMA, the level of phosphorylation was similar to that observed in exponentially growing cells (88 ± 7% n and 12 ± 7% p1). Treatment of HL60 cells for 36, 48 and 72 h with PMA resulted in a time-dependent dephosphorylation of stathmin. At 72 h, stathmin was present as 98 ± 2% n and 2 ± 2% p1 (n = 5), which is a level of phosphorylation lower than that detected in exponentially growing HL60 cells.

In contrast to the dramatic changes observed after PMA-treatment of HL60 cells, induction of HL60 differentiation with

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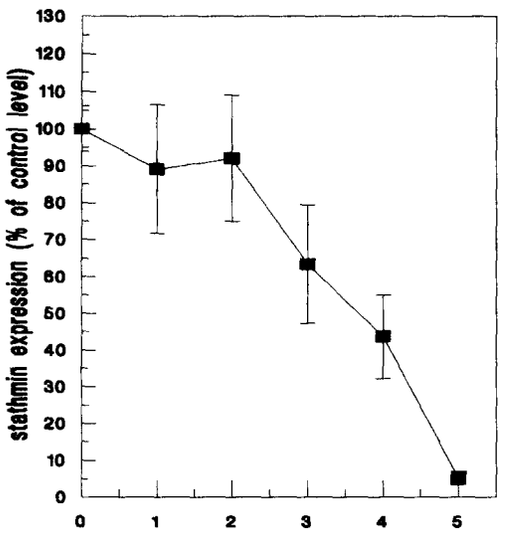
Fig. 1. Changes in stathmin expression during HL60 neutrophil and monocyte differentiation. HL60 cells were induced to differentiate to monocytes, by vitD3 (left panels), and to neutrophils, by ATRA/hrG-CSF (right panels). Relative levels of stathmin were measured by immunostaining of Western blots of cell extracts (top panels). The middle panels show the proportions of cells stained for stathmin and the Ki-67 antigen. Proportions of cells in the G_1 (■), S (□) and G_2/M (▲) phases of the cell cycle are shown (bottom panels).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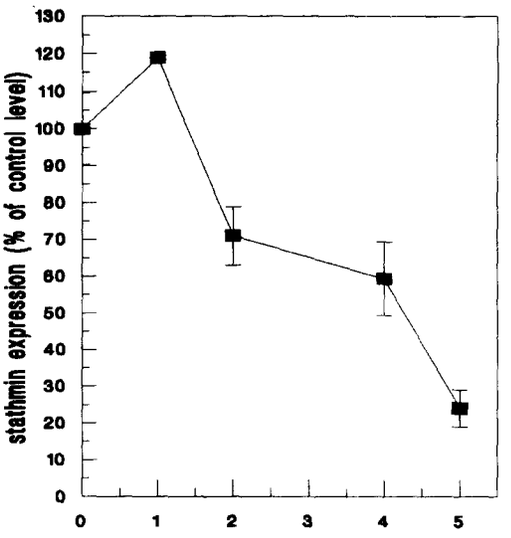
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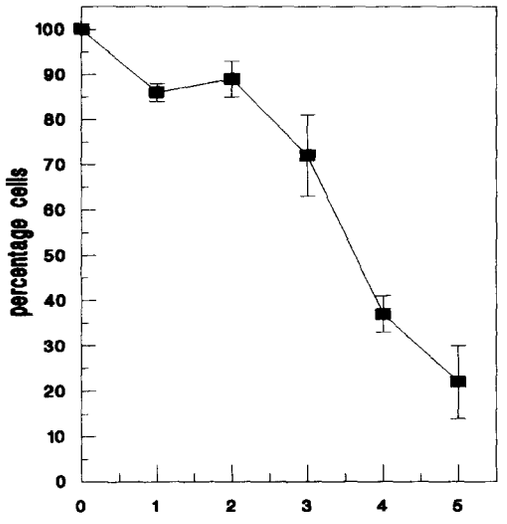
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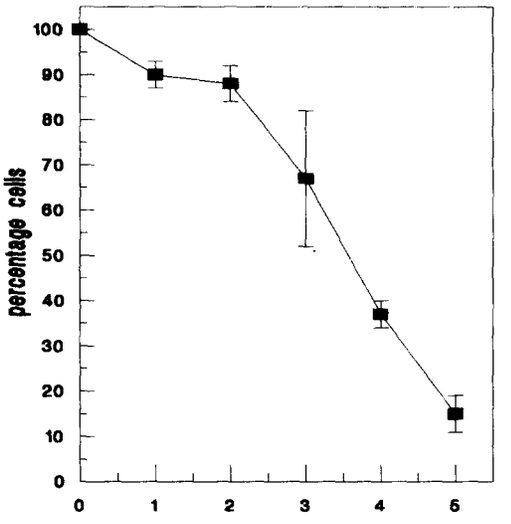
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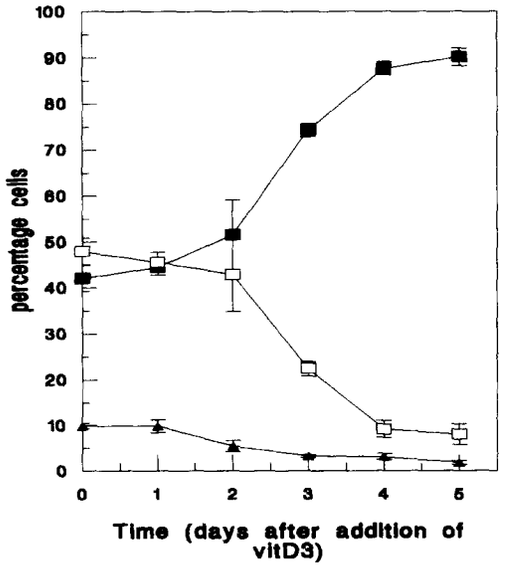
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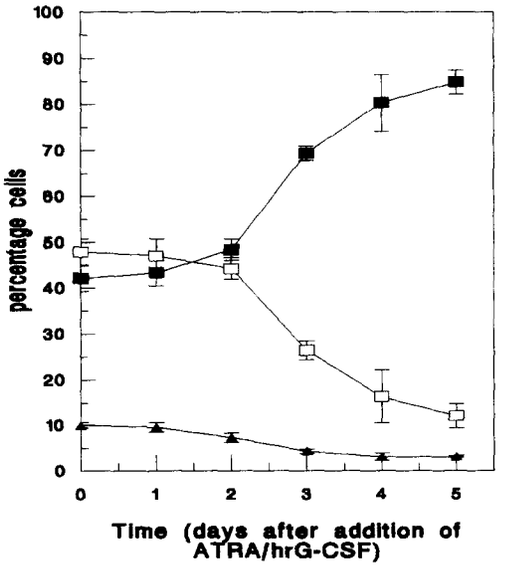
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Time (days after addition of ATRA/hrG-CSF)

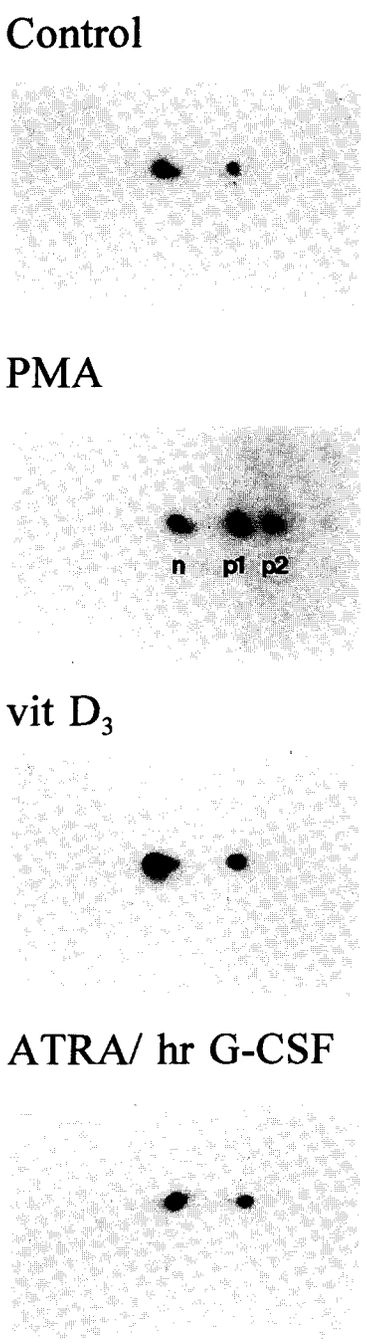

Fig. 2. Treatment of HL60 cells with PMA, but not vitD3 and ATRA/ hrG-CSF, results in rapid stathmin phosphorylation. Autoradiographs of immunostained Western blots of HL60 cell extracts after treatment of cells for 4 hours with either PMA, ATRA/hrG-CSF or vitD3 are shown. n = non-phosphorylated form; p1 = major phosphophorylated form; and p2 = minor phosphorylated form.

vitD3 and ATRA/hrG-CSF did not result in any rapid and significant changes in the level of stathmin phosphorylation during the first 24 h after treatment (Fig. 3). Beyond 24 h, there was a time-dependent dephosphorylation of stathmin. After 5 days of exposure to vitD3 stathmin was present as 99 ± 1% n

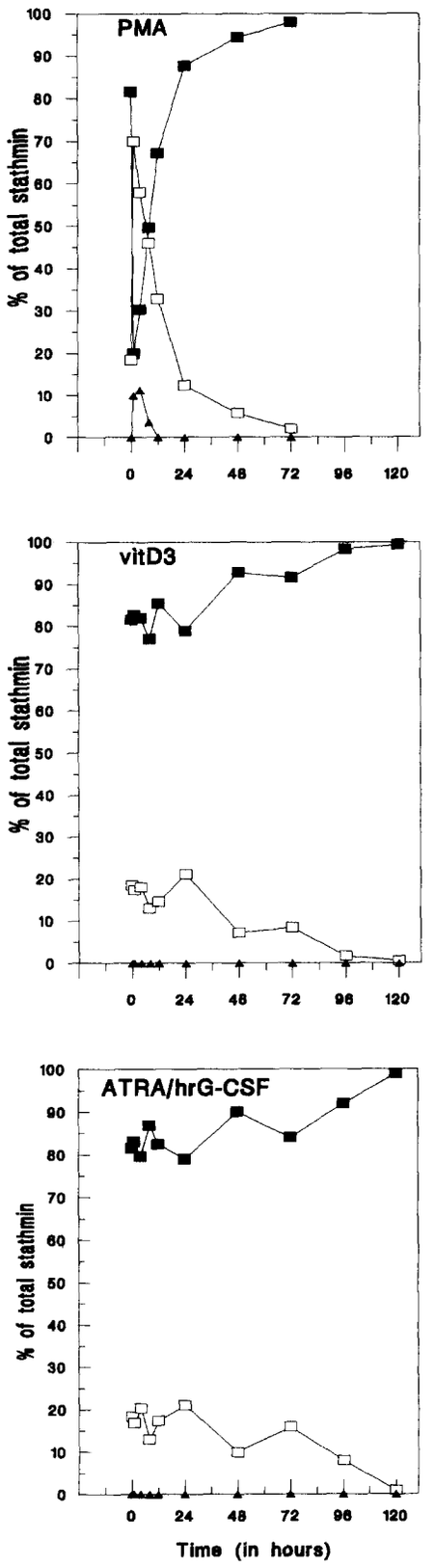

Fig. 3. Changes in stathmin phosphorylation during HL60 neutrophil and monocyte differentiation. Immunostaining of Western blots of 2-dimensional gels of cell extracts was undertaken to determine the proportions of stathmin present in a non-phosphorylated (■), and the two phosphorylated forms p1 (□) and p2 (▲). HL60 cells were induced to differentiate to monocytes, by PMA and by vitD3, and to neutrophils, by ATRA/hrG-CSF.

and 0.6 ± 1% pl (n = 5) and after 5 days of treatment with ATRA/hrG-CSF stathmin was present as 99 ± 1% n and 0.5 ± 0.5% pl (n = 5). This dephosphorylation occurred over the time period when the majority of cells were exiting from the cell cycle and when down regulation of stathmin protein and Ki-67 antigen were detected (Fig. 1).

4. Discussion

In most cell lineages stathmin expression in vivo is confined to cells that are, or have recently been, proliferating [36]. In this and in previous studies of cells in vitro, stathmin expression falls in association with induction of cell growth arrest [8,27,28,30,37].

Rapid and transient phosphorylation of stathmin has been described in cell types, including myeloid, lymphoid and epithelial cells, that growth arrest and differentiate following treatment with PMA [3,7,27–29]. Hence, it has been suggested that hyperphosphorylation of the protein is an important event in these cellular processes. However, we have described a high level of stathmin phosphorylation following PMA treatment of plasmacytoma cell lines and these cells did not undergo any subsequent change in their proliferative activity [28]. In addition, PMA-induced hyperphosphorylation of stathmin has been related to a number of other cellular events, such as peptide hormone secretion [1]. It is therefore possible that this widely reported phosphorylation is simply a non-specific effect of PMA and has no direct functional significance. In this study it was observed that extensive phosphorylation of stathmin did not occur when physiological agents, as opposed to PMA, were used to elicit growth arrest and differentiation in HL60 cells. Thus, stathmin phosphorylation is not necessary for these cellular changes to occur. This study showed that there was a gradual decrease in the proportion of phosphorylated stathmin protein which coincided with the period during which cell growth arrest was occurring. This is consistent with a reduction in the proportion of cells in the G_2/M phases of the cell cycle when stathmin phosphorylation is at its highest level [19].

It is concluded that the level of stathmin expression is linked to the proliferative status of cells. However, phosphorylation of the protein is not a consistent finding in relation to cell growth arrest and differentiation and so is not essential for these cell processes to occur.

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