

## ORIGINAL ARTICLE

# Novel role of Stat1 in the development of docetaxel resistance in prostate tumor cells

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A major obstacle for clinicians in the treatment of advanced prostate cancer is the inevitable progression to chemoresistance, especially to docetaxel. It is essential to understand the molecular events that lead to docetaxel resistance in order to identify means to prevent or interfere with chemoresistance. In initial attempts to detect these events, we analysed genomic differences between non-resistant and docetaxel-resistant prostate tumor cells and, of the genes modulated by docetaxel treatment, we observed Stat1 and clusterin gene expression heightened in the resistant phenotype. In this study, we provide biochemical and biological evidence that these two gene products are related. Stat1 and clusterin protein expression was induced upon docetaxel treatment of DU145 cells and highly overexpressed in the docetaxel-resistant DU145 cells (DU145-DR). The increase in total Stat1 corresponded to an increase in phosphorylated Stat1. Interestingly, there was no detectable difference between DU145 and DU145-DR cells expression of total Stat3 and phosphorylated Stat3. Treatment of DU145-DR cells with small interfering RNA targeted for Stat1 not only resulted in the knockdown of Stat1 expression, but it also caused the inhibition of clusterin expression. Thus, Stat1 appears to play a key role in the regulation of clusterin. Remarkably, inhibition of Stat1 or clusterin expression resulted in the re-sensitization of DU145-DR cells to docetaxel. These results offer the first evidence that Stat1, and its subsequent regulation of clusterin, are essential for docetaxel resistance in prostate cancer. Targeting this pathway could be a potential therapeutic means for intervention of docetaxel resistance.

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**Keywords:** stat1; clusterin; chemoresistance; prostate cancer; siRNA

## Introduction

Prostate cancer is the most frequently diagnosed non-cutaneous malignancy of males in the United States, ultimately affecting 35% of all American men (Nelson *et al.*, 2003; De Marzo *et al.*, 2004). The current standard therapies employed for localized prostate cancer include radiation, surgery or androgen ablation therapy. Although these therapies are relatively effective in the short term, they offer no significant benefit on long-term survival (Milbank *et al.*, 2002). Once these initial treatments are no longer effective, a major risk faced by prostate cancer patients is the development of metastases. Numerous chemotherapeutic regimens to treat advanced prostate cancer have not improved the overall survival of patients (Culine and Droz, 2000), but recent clinical trials using a combination of agents such as docetaxel and estramustine have shown a small survival benefit (Petrylak *et al.*, 2004; Tannock *et al.*, 2004). As a result of these important new studies, docetaxel is currently the drug of choice in prostate cancer chemotherapy. Unfortunately, clinical treatment with docetaxel often encounters a number of undesirable side effects, including drug resistance (Miller and Ojima, 2001; Geney *et al.*, 2002; Petrylak, 2005). Therefore, it has become essential to identify molecular events that may be associated with the development of docetaxel resistance.

Different tumors have various aberrations in signaling and growth stimulation pathways that drive cancer growth and induce chemoresistance. Although docetaxel resistance has been found to be dependent upon overexpression of P-glycoprotein in numerous tumor types, docetaxel resistance in prostate cancer has been shown to be independent of this protein (Makarovsky *et al.*, 2002). This information led us to search for other novel molecular targets in prostate cancer that could be the determinant for its docetaxel resistance phenotype. We successfully established a pair of docetaxel non-resistant and resistant cell lines to enable us to detect differences in gene expression between the two cell lines using cDNA microarray analysis. Of the genes modulated by docetaxel, we found two genes related to cell proliferation and apoptosis that were overexpressed in docetaxel-resistant prostate cancer cells, specifically, Stat 1 and clusterin.

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Clusterin, also referred to as testosterone-repressed prostate message-2 and sulfated glycoprotein-2, has a nearly ubiquitous tissue distribution and is apparently involved in a wide range of biological processes (Trogakos and Gonos, 2002). Clusterin expression has been associated with tissue remodeling, lipid transport, reproduction, complement regulation and apoptotic cell death (Ho *et al.*, 1998; Humphreys *et al.*, 1999; Miyake *et al.*, 2000b; O'Sullivan *et al.*, 2003). It has been reported that clusterin expression is associated with tumor formation and prostate cancer progression to androgen independence (Miyake *et al.*, 2000b). More importantly, it has been observed that prostate tumors that overexpress clusterin were more resistant to paclitaxel chemotherapy (Zellweger *et al.*, 2003), suggesting that clusterin may be an important protein either as a marker for resistance to chemotherapy or play a pivotal role in the development of drug resistance to chemotherapy (Miyake *et al.*, 2000a; Zellweger *et al.*, 2001).

Stat is comprised of a family of transcription factors that translocate from the cytoplasm to the nucleus of cells (Darnell, 1997). Its seven members, Stat1, 2, 3, 4, 5A, 5B and 6, influence different signal transduction pathways to influence normal physiological cell processes such as differentiation, proliferation, apoptosis and angiogenesis (Horvath, 2000). Stat1 has been implicated in modulating pro- and anti-apoptotic genes in stress-induced responses and is activated following cytokine signaling or by various stress-induced stimuli via phosphorylation on the C-terminal domain (Stephanou and Latchman, 2003). High levels of constitutively active Stat1 have been reported to be necessary to support cell survival in several different types of cancer (El-Hashemite *et al.*, 2004; Legrand *et al.*, 2004; Cochet *et al.*, 2006). Recently, it has been reported that chemotherapeutic agents, such as anthracyclines, potentiate Stat1 activation and enhance nuclear localization of phosphorylated Stat1 in cancer cells (Stephanou and Latchman, 2003; Thomas *et al.*, 2004).

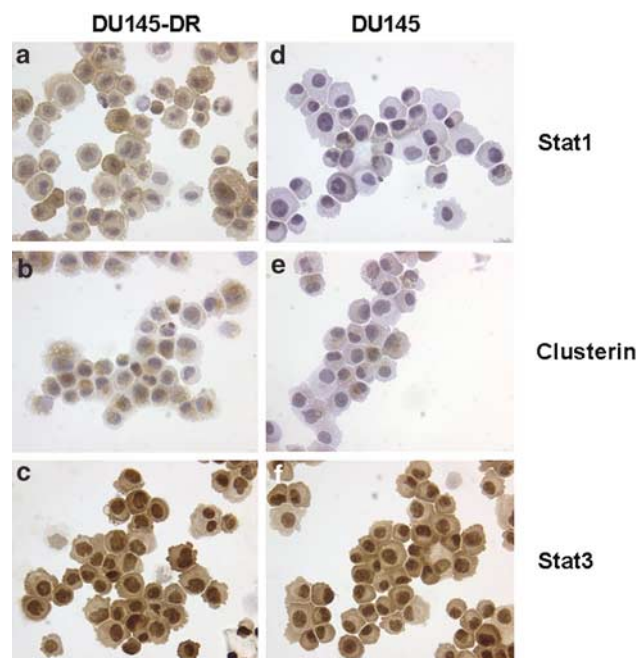
This current study was designed to investigate the role of Stat1 in docetaxel-induced drug resistance and its correlation with clusterin expression. Through our experiments, we have identified a previously unknown role of Stat1 in mediating the resistance to docetaxel. We demonstrated that Stat1, and not Stat3, was overexpressed in docetaxel-resistant prostate tumor cells and its induction of clusterin was involved in cell survival in the presence of docetaxel. We further provided evidence that blockage of Stat1 expression by small interfering RNA (siRNA) decreased clusterin expression and inhibited prostate cancer cell proliferation by re-sensitizing drug-resistant tumor cells to docetaxel. Together, these results indicated that Stat1 and clusterin are the key regulators of docetaxel resistance in prostate tumor cells.

## Results

### *Overexpression of Stat1 and clusterin in DU145-DR*

In an effort to understand the molecular basis for docetaxel resistance in prostate cancer cells, we

performed a microarray analysis on prostate cancer cells using the Affymetrix U133A array, which can detect 30 000 transcriptionally activated genes, and measured the differences in gene expression between resistant and non-resistant cells. Our microarray data showed that 559 genes had either an increase or decrease in expression in the resistant cell line. Table 1 shows a list of 30 genes that were overexpressed in the docetaxel-resistant cell line. Of those genes, we observed that two genes related to cell survival and proliferation were upregulated significantly in the resistant cell line, specifically, Stat1 and clusterin. To confirm our gene expression data, we examined for the presence of these proteins by immunohistochemistry staining of parental DU145 and docetaxel-resistant DU145-DR cells (Figure 1). DU145-DR stained positively for Stat1 (Figure 1a) and clusterin (Figure 1b) expression and at a substantially stronger intensity in comparison with DU145 cells (Figure 1d and e). The increase in clusterin expression in DU145-DR cells was especially noticeable in the cytoplasm of the cells (Figure 1b). The importance of this observation is that clusterin has been reported to have antiapoptotic characteristics only when it is located in the cytoplasm (Pucci *et al.*, 2004). As Stat3 has been linked with prostate cancer by other investigators, we also tested for Stat3 expression in both cell lines. In contrast to Stat1 expression, Stat3 expression was constant in both DU145 and DU145-DR cells (Figure 1c and f). To verify that the increased expression of Stat1 and clusterin, but not Stat3, correlated to



**Figure 1** Immunohistochemical detection of Stat1, clusterin and Stat3 in human prostate cancer cell lines, DU145 and DU145-DR. Bound antibodies were detected by peroxidase with diaminobenzidine as a substrate and cells were then counterstained with hematoxylin. Stat1 was visualized in (a, d), clusterin in (b, e) and Stat3 in (c, f). Magnification  $\times 100$ . This figure is a representative staining of greater than three experiments.

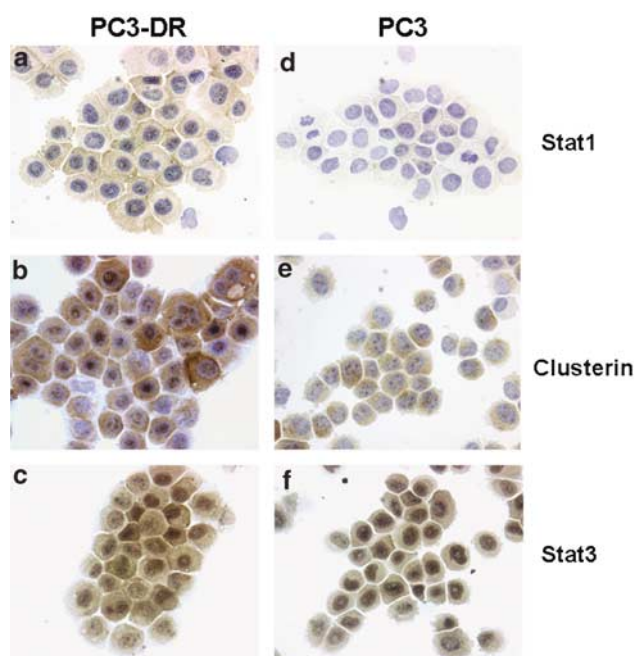
**Table 1** Overexpressed genes in docetaxel-resistant prostate tumor cells compared with normal prostate tumor cells

Unigene <sup>a</sup>	Description	Fold change
Hs.38084	Sulfotransferase family, cytosolic, 1C, member 1	16.6
Hs.171731	Solute carrier family 14, member 1	9.7
Hs.274376	Amylase, alpha 1A	8.7
Hs.9728	ALEX1 protein	7.6
Hs.44	Pleiotrophin	6.1
Hs.106070	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	5.8
Hs.79339	Lectin, galactoside-binding, soluble, 3 binding protein	5.5
Hs.123159	Sperm-associated antigen 4	5.4
Hs.279815	Cysteine sulfinic acid decarboxylase	4.9
Hs.155651	Forkhead box A2	4.5
Hs.155530	Interferon, gamma-inducible protein 16	4.4
Hs.288181	Cathepsin H	4.4
Hs.110637	Homeo box A10	4.3
Hs.172684	Vesicle-associated membrane protein 8	4.0
Hs.30524	Ring finger protein 24	3.9
Hs.39871	Myosin ID	3.8
Hs.38972	Tetraspan 1	3.6
Hs.78068	Carboxypeptidase Z	3.6
Hs.73885	HLA-G histocompatibility antigen, class I, G	3.4
Hs.75106	Clusterin	3.3
Hs.77961	Major histocompatibility complex, class I, B	3.1
Hs.75562	Discoidin domain receptor family, member 1	3.1
Hs.239189	Glutaminase	3.0
Hs.172210	MUF1 protein	2.8
Hs.18069	Legumain	2.3
Hs.21486	STAT1	1.8
Hs.301763	Formin binding protein 1	1.8
Hs.79069	Cyclin G2	1.7
Hs.111577	Integral membrane protein 2C	1.6
Hs.219614	F-box and leucine-rich repeat protein 11	1.5

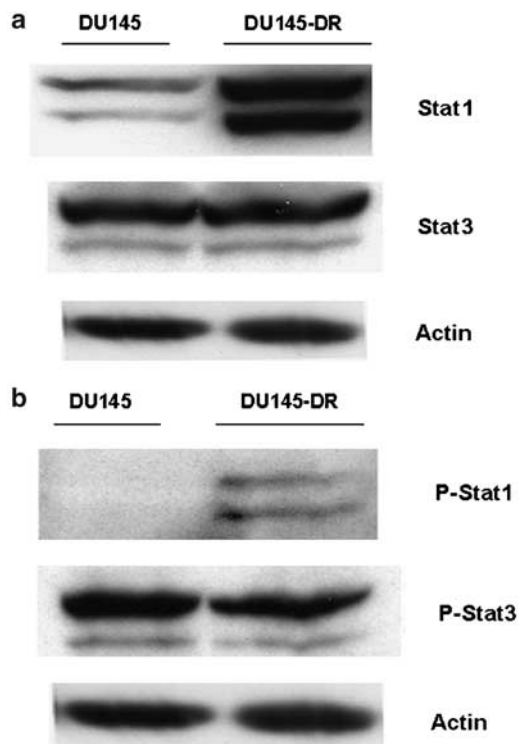
<sup>a</sup>UNIGENE represents the Unigene-*Homo sapiens* cluster number.

docetaxel resistance in prostate cancer and not only as a unique phenomenon in DU145-DR cells, we developed a second pair of docetaxel-sensitive/resistant prostate tumor cell lines, PC3 and PC3-DR. Immunostaining showed the same pattern as with DU145 and DU145-DR (Figure 2). PC3-DR cells expressed Stat1 (Figure 2a) and clusterin (Figure 2b) at a much greater extent than the normal PC3 cells (Figure 2d and e). Also, there was no significant difference in Stat3 expression between PC3-DR and PC3 cells (Figure 2c and f). Together, this information suggests that Stat1 and clusterin may be associated with docetaxel resistance in prostate cancer.

To verify that Stat1 was selectively upregulated in drug-resistant cells, we analysed protein levels by Western blotting. We found that Stat1 was expressed in low levels in DU145 and markedly overexpressed in DU145-DR (Figure 3a).  $\beta$ -Actin was used as a control to show equal loading. Not only was the total amount of Stat 1 increased in the DU145-DR cells, but also the amount of active phosphorylated Stat 1 was significantly increased (Figure 3b). In contrast, the constitutive expression of Stat3 and active phosphorylated Stat 3 was detected equally in both DU145 and DU145-DR (Figure 3a and b). These data demonstrated that Stat1 is overexpressed and activated in chemoresistant cells, whereas Stat3 is constitutively and equally expressed in both chemoresistant and non-resistant cells.



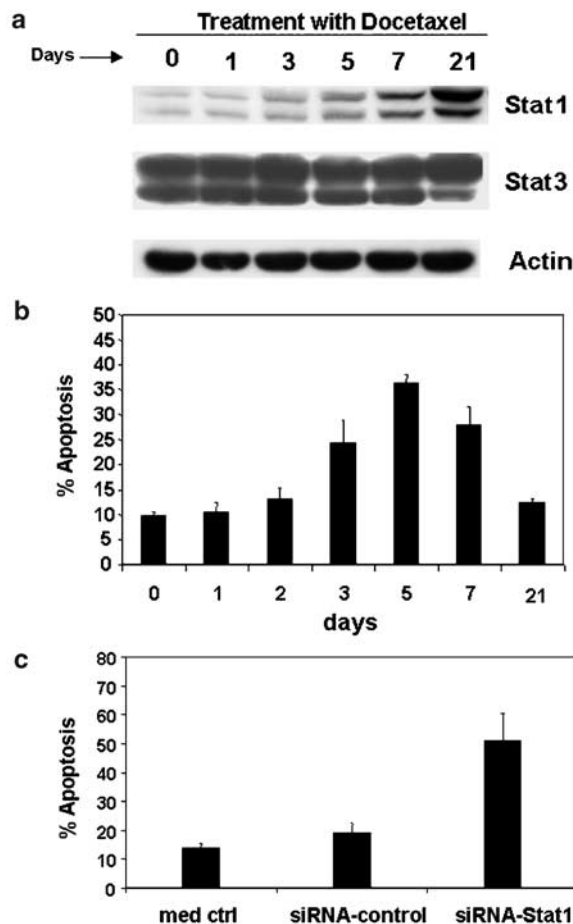
**Figure 2** Immunohistochemical detection of Stat1, clusterin and Stat3 in human prostate cancer cell lines, PC3 and PC3-DR. Bound antibodies were detected by peroxidase with diaminobenzidine as a substrate and cells were then counterstained with hematoxylin. Stat1 was visualized in (a, d), clusterin in (b, e) and Stat3 in (c, f). Magnification  $\times 100$ . This figure is a representative staining of greater than three experiments.



**Figure 3** Analysis of the activation state of Stat1 and Stat3 in DU145 and DU145-DR. (a) DU145 and DU145-DR lysates were immunoblotted with antibodies against Stat1, Stat3 and  $\beta$ -actin to detect total proteins. (b) The same lysates were immunoblotted with antibodies against phospho-Stat1 or phospho-Stat3. These data are representative of three separate experiments.

#### Induction of Stat1 expression with docetaxel

Based on our findings that Stat1 expression was elevated in DU145-DR cells, we further investigated whether docetaxel treatment was responsible for the induction of Stat1 expression in DU145-DR cells. As there is a report indicating that the chemotherapeutic drug doxorubicin is able to activate Stat1 activation and phosphorylation (Thomas *et al.*, 2004), we carried out a series of timed exposures to docetaxel for DU145. DU145 cells were incubated in 11 nM docetaxel-containing medium for 0, 1, 3, 5, 7 and 21 days and cell lysates were prepared at each time point. Western blot analysis indicated that untreated DU145 cells had a very low background level expression of Stat1 (Figure 4a). By day 3, docetaxel treatment had resulted in detectable Stat1 protein and it sequentially increased accordingly with the extension of docetaxel treatment. Significant Stat1 levels were seen by day 7 and by day 21 of drug exposure, Stat1 protein expression was maximal. The same lysates were probed for Stat3 expression in order to examine if docetaxel treatment selectively acted on Stat1 expression in DU145 cells (Figure 4a). Stat3 was constitutively expressed at a high level in both docetaxel-treated and untreated DU145 cells, and extending the time of docetaxel treatment had no effect on Stat3 expression. Equivalent  $\beta$ -actin expression in all groups indicated equal loading of proteins. Therefore, the results thus far



**Figure 4** Induction of Stat1 but not Stat3 expression by docetaxel in DU145 cells and effect of Stat1 inhibition on DU145 response to docetaxel. DU145 cells were cultured in the presence of 11 nM docetaxel for 0, 1, 3, 5, 7 or 21 days. (a) Lysates were made at each time point and analysed by Western blot analysis for expression of Stat1, Stat3 and  $\beta$ -actin. (b) Viability was assessed by Annexin V/PI (propidium iodide) staining for apoptotic cells on a FACScan. (c) Parental DU145 cells were pretreated with small interfering RNA-Stat1 and then cultured in 11 nM docetaxel medium for 48 h before assessment of apoptosis by Annexin V/PI staining. These data are representative of three separate experiments.

indicate that Stat1, and not Stat3, is upregulated by docetaxel treatment.

In order to examine if Stat1 expression correlated to cell survival, we analysed apoptotic capacity in parental DU145 cells upon initial exposure to docetaxel. Annexin V/PI (propidium iodide) staining indicated that apoptosis increased significantly from 0 to 5 days of docetaxel treatment and then the percentage of apoptotic cells began to lessen on the seventh day of treatment, indicating their early development of docetaxel resistance (Figure 4b). The percentage of apoptotic cells continued to decrease until the cells became completely resistant to docetaxel treatment by day 21. This time course of resistance to apoptosis correlated well to that of expression of Stat 1. In the first 5 days of initial exposure to docetaxel, when Stat1 was not present, DU145 cells readily underwent apoptosis. However,

when Stat1 was detectable at high levels at day 7 of treatment, apoptosis began to slow and survival was greatest at day 21 when these drug-treated cells had the highest expression of Stat1 (Figure 4a).

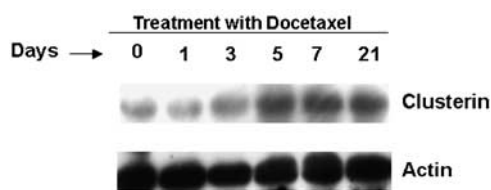
The correlation of Stat1 expression with resistance to apoptosis induced by docetaxel did not necessarily prove that Stat1 controlled cell survival mechanisms. For direct evidence of this pathway, we examined if inhibition of Stat1 expression in parental DU145 cells would sensitize them better to docetaxel-induced cell death. We thus pretreated parental DU145 tumor cells with siRNA targeted for Stat 1 and then exposed them to docetaxel. Pretreatment with siRNA-Stat1 significantly increased the percentage of apoptotic cells upon 2 days of docetaxel treatment, in comparison to either siRNA-control or media control (Figure 4c).

#### Induction of clusterin expression in DU145 with docetaxel

Because our initial gene profiling as well as immunohistochemical staining had indicated a significant difference in the level of expression for clusterin, we next speculated whether clusterin was also associated with docetaxel-induced resistance in DU145 cells. This approach is substantiated by a recent study linking clusterin and chemotherapy resistance (Miyake *et al.*, 2003). To probe this, we repeated the same experiment as in Figure 4a to test if prolonged docetaxel treatment could result in increased clusterin expression. Western blot analysis indicated that clusterin expression after docetaxel treatment mirrored the effect on Stat1 expression. Upon treatment with 11 nM of docetaxel for 0, 1, 3, 5, 7 and 21 days, clusterin protein expression increased sequentially with time of exposure (Figure 5). Like Stat1, it appears that an increasing level of clusterin occurs in response to docetaxel.

#### Dependence of clusterin expression on Stat1 in DU145-DR cells

It has been reported that clusterin expression is associated with tumor formation and prostate cancer progression to androgen independence (Miyake *et al.*, 2000b; Ouyang *et al.*, 2001). More importantly, it has been suggested that clusterin may play an antiapoptotic role in cancer cells (Bettuzzi *et al.*, 2000). Several lines of evidence suggest that overexpression of clusterin in prostate xenografts models induce chemoresistance. Both antisense oligonucleotides or small siRNA specific against clusterin induced spontaneous apoptosis and



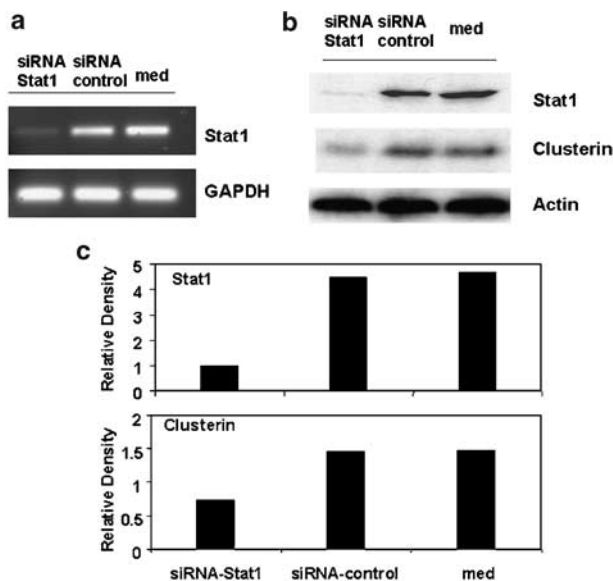
**Figure 5** Induction of clusterin expression by docetaxel in DU145 cells. DU145 cells were cultured in the presence of 11 nM docetaxel for 0, 1, 3, 5, 7 or 21 days. Lysates were made at each time point and analysed by Western blot analysis for expression of clusterin and  $\beta$ -actin.

increased the anticancer activity of chemotherapeutic agents (Miyake *et al.*, 2000a; Trougakos and Gonos, 2002). Although the above study provides strong evidence that clusterin may be involved in docetaxel-induced chemoresistance in DU145 cells, the importance of Stat1 in the progression to drug resistance in DU145 prostate cancer cells has not yet been defined. Also, it is unclear whether there is a significant correlation between Stat1 and clusterin. Therefore, we set out to define if clusterin protein expression was a result of Stat1 expression. Small interfering RNA against Stat1 was used to determine whether inhibition of Stat1 would affect clusterin expression. Either siRNA-STAT1 or mismatch siRNA (siRNA-control) was transfected into DU145-DR cells, and at 48 h, reverse transcriptase-polymerase chain reaction (RT-PCR) amplification was performed to ensure transfection efficiency. In addition to Stat1-specific primers, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were also included as a control. Stat1 siRNA almost completely knocked out Stat1 mRNA transcription, whereas GAPDH transcription remained unaffected (Figure 6a).

These cells were then analysed by Western blot analysis. The Stat1 siRNA-transfected cells demonstrated a marked decrease in Stat1 protein expression, whereas Stat1 expression after transfection with the siRNA-control as well as exposure to medium alone was unchanged (Figure 6b). More importantly, decreased Stat1 expression significantly inhibited the level of clusterin expression in siRNA-treated DU145-DR cells, whereas expression of clusterin was unaffected by siRNA-control-transfected DU145-DR cells, remaining at the same level as in untransfected cells (Figure 6b). Densitometric analysis confirmed that that siRNA-Stat1 had a specific knockdown effect on Stat1 and clusterin expression (Figure 6c). Small interfering RNA-Stat1 almost completely knocked down Stat1 expression while decreasing clusterin expression by 50%. Although clusterin expression is not completely blocked, it reflects the conditions seen in parental DU145 cells, where a basal level of clusterin expression still exists (Figure 1e) without Stat1 expression (Figure 1d). This basal level of clusterin (Figure 2e) is even more pronounced in parental PC3 cells, which do not express Stat1 (Figure 2d). From this information, it is clear that inhibition of Stat1 expression significantly downregulated the expression of clusterin.

#### Stat1 and clusterin small interfering RNA induce sensitivity of DU145-DR to docetaxel

Proliferative and apoptotic indices are two of the validation markers used to evaluate prostate cancer progression (Assikis *et al.*, 2004). To examine whether Stat1 siRNA would induce DU145-DR sensitivity to docetaxel and inhibit DU145-DR proliferation, DU145-DR cells were transfected with Stat1 siRNA and cell proliferation was quantified by [ $^3$ H] thymidine incorporation. We found that siRNA-Stat1-transfected drug-resistant cells became highly sensitive to docetaxel treatment (Figure 7a). Proliferation of siRNA-Stat1-transfected DU145-DR cells was significantly inhibited as

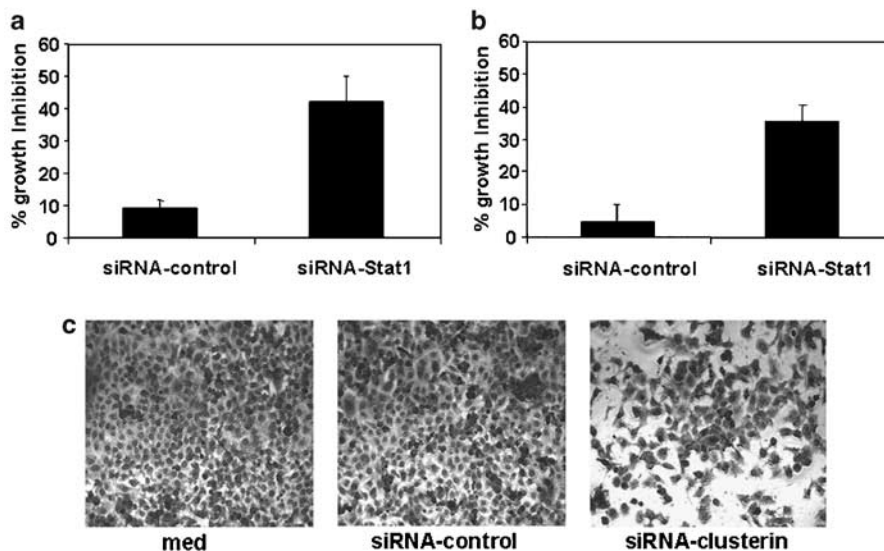


**Figure 6** Effective suppression of clusterin expression in DU145-DR cells after transfection with small interfering RNA (siRNA)-Stat1. **(a)** DU145-DR cells were transfected with plasmids containing either siRNA-Stat1 or siRNA-control for 72 h. Untransfected cells were used as a control (med). Stat1 mRNA levels were evaluated by reverse transcription-polymerase chain reaction amplification using primers for Stat1 and analysed by agarose gel electrophoresis. Glyceraldehyde-3-phosphate dehydrogenase mRNA expression was used as a control for equal gel loading. **(b)** Western blot analysis with specific antibodies was performed to detect the protein levels of Stat1, clusterin and  $\beta$ -actin. **(c)** Densitometric analysis using ImageQuant software (GE Healthcare, Piscataway, NJ, USA) was performed on the gel in **(b)** to estimate the level of reduction of Stat1 and clusterin in siRNA-Stat1 treated DU145-DR cells. These data are representative of three separate experiments.

compared to DU145-DR cells that had been transfected with siRNA-control. As we had already established that clusterin was under the influence of Stat1 and clusterin may act as a downstream mediator of Stat1, it is important to show that blocking clusterin would also induce DU145-DR sensitivity to docetaxel. Specific siRNA against clusterin was transfected into DU145-DR cells. Figure 7b demonstrated that transfection of siRNA-clusterin resulted in significant sensitivity to docetaxel, whereas transfection of siRNA-control resulted in a negligible amount of growth inhibition as compared to the medium control. Although greater than 90% of the siRNA-control-transfected cells remained viable and were able to incorporate [ $^3$ H] thymidine, the growth inhibition effects observed were specifically due to the specificity of siRNA targeting at clusterin expression. These results were further confirmed by visually examining the culture dishes by direct light microscopy of the remaining cells stained with Diff-Quik (Figure 7c). In contrast to the confluent layer of cells in untreated DU145-DR and control siRNA-transfected DU145-DR, the same cells transfected with clusterin siRNA for 72 h showed much less viable cells upon exposure to docetaxel. Thus, blockage of clusterin induces docetaxel sensitivity in DU145-DR cells. Overall, these data taken together indicate that Stat1 controls clusterin expression, which contributes to docetaxel resistance.

## Discussion

Because resistance of many tumors to established clinical treatment regimens still constitutes a major



**Figure 7** The effect of small interfering RNA (siRNA)-STAT1 and siRNA-clusterin on DU145-DR cells proliferation and sensitivity to docetaxel. **(a)** DU145-DR cells were either transfected with siRNA-STAT1 or siRNA-control for 5 h. **(b)** DU145-DR cells were either transfected with siRNA-clusterin or siRNA-control. The transfected cells were cultured in 11 nM docetaxel medium for 24 h, and then cultured for an additional 6 h in the presence of thymidine. Cells were harvested and counted on a  $\beta$ -scintillation counter. Percent growth inhibition was measured by comparing cpm of transfected cells to the cpm of untransfected cells. Error bars represent the standard deviation of three independent experiments. **(c)** siRNA-clusterin and siRNA-control pretreated DU145-DR cells or untransfected cells (med) were cultured in docetaxel-containing medium for 48 h and stained with Diff-Quik to visualize the cells.

concern in oncology, attempts to improve the survival of cancer patients depends largely on strategies to target tumor cell resistance (Peto, 2001). An important consideration at present in developing effective chemotherapeutic strategies for prostate cancer is the ability to identify their molecular pathways of resistance. It has been previously reported that taxane resistance can be contributed to multidrug resistance by overexpression of P-glycoprotein (P-gp). As docetaxel resistance in prostate cancer has been shown to be independent of P-gp overexpression (Makarovskiy *et al.*, 2002), our efforts focused on the search for a novel pathway of docetaxel-induced resistance in prostate cancer. Identification of molecules within this pathway could lead to potential new strategies to circumvent docetaxel resistance in prostate cancer patients.

We initially identified the two essential proteins for docetaxel resistance by studying the aberrant genomic expression of DU145-DR prostate cancer cells when compared to non-resistant DU145 cells. In the present study, we provide strong molecular, biochemical and biological evidence that Stat1 and clusterin play a critical role in DU145 cells progression to drug resistance. We observed that both Stat1 and clusterin were overexpressed in docetaxel-resistant DU145 cells when compared to non-resistant DU145 cells, whereas Stat3 was constitutively expressed at the same level in both resistant and non-resistant cell lines. Also, the increase in total Stat1 of DU145-DR cells led to a corresponding increase in active Stat1, whereas the amount of active Stat3 was uniform between both cell lines. Many studies have demonstrated that constitutive activation of Stat3 protein is associated with prostate cancer *in vivo* (Dhir *et al.*, 2002; Wang *et al.*, 2004), supporting existing knowledge that Stat3 plays a significant role for signaling in the growth and survival of various human malignancies such as prostate cancer. Stat3 activity is not only significantly increased in human primary prostate cancer tissues, but also increased in androgen-independent prostate cancer cells (DeMiguel *et al.*, 2002; Lee *et al.*, 2004). Disruption of Stat3 expression in human prostate cancer cells by either siRNA or antisense oligonucleotides suppresses cancer cell proliferation and induces apoptosis (Barton *et al.*, 2004; Lee *et al.*, 2004). However, our findings that Stat3 expression is unchanged upon acquisition of docetaxel resistance makes the argument that Stat3 is not the critical element in control of docetaxel resistance in prostate cancer. Our data show that initially, docetaxel induces a significant amount of apoptosis, but as Stat1 expression increases, the prostate tumor cells become less sensitive to docetaxel. Also, when DU145 tumor cells were pretreated with siRNA targeted for Stat1, docetaxel induced a much greater amount of apoptosis than DU145 cells that still expressed Stat1. Therefore, Stat1 may serve as a potential key target to prevent the development of docetaxel resistance in prostate cancer.

Another important finding from our study showed that expression of clusterin in resistant cells is dependent on Stat1. Inhibition of Stat1 expression by siRNA specific for Stat1 not only suppressed Stat1 expression,

but also resulted in decreased expression of clusterin. A key mechanism for most antitumor therapies such as chemotherapy, immunotherapy, cytokines or  $\gamma$ -irradiation is the induction of apoptosis. Often, resistance to any of those therapies is caused by proteins that inhibit some step in the apoptotic pathway (Hengartner, 2000; Lowe and Lin, 2000; Herr and Debatin, 2001; Debatin *et al.*, 2002; Johnstone *et al.*, 2002). Although clusterin has been shown to have both pro- and anti-apoptotic activities (Nickerson *et al.*, 1999; Miyake *et al.*, 2000c), the exact molecular pathway of clusterin remains elusive. Recently, it has been shown that the location of clusterin in the cytoplasm is a marker for advanced cancer that is resistant to apoptosis and has a greater metastatic potential (Pucci *et al.*, 2004). In our study, the increase in clusterin in our DU145-DR cells occurred in the cytoplasm, supporting the data that suggests that clusterin functions as an antiapoptotic protein when located in the cytoplasm. It is also significant that inhibition of Stat1 or clusterin protein expression is able to reverse docetaxel resistance and subsequently induce cell death. After DU145-DR cells were transfected with either siRNA targeted for Stat1 or clusterin, cell survival and proliferation were significantly decreased upon docetaxel treatment. Thus, clusterin appears to participate in evasion from apoptosis in prostate cancer, and thus clusterin may prove to be another valuable target as a therapeutic strategy to overcome docetaxel resistance that commonly occurs in these cancer patients.

Our findings constitute the first study to provide direct evidence that the pathway to docetaxel resistance in human prostate cancer cells involves Stat1 overexpression and activation and its subsequent regulation of clusterin. These findings are consistent with several recent studies showing that overexpression of clusterin in prostate xenograft models conferred chemoresistance, whereas antisense oligonucleotides or siRNA specific against clusterin induced spontaneous apoptosis and increased the anticancer activity of chemotherapeutic drugs (Miyake *et al.*, 2003; Trougakos *et al.*, 2004). Further research needs to be carried out in order to determine whether overexpression of Stat1 in xenograft prostate tumors results in docetaxel resistance.

Docetaxel is an effective chemotherapeutic agent offering one of the best response rates when used in the treatment of many other types of cancers including the lung, breast, and head and neck (Lacroix and Ligeza, 1998). However, as in prostate cancer, resistance to docetaxel treatment arises in all of the cancers mentioned above. Recently, high expression of clusterin has been associated with resistance to chemotherapy in lung adenocarcinoma (July *et al.*, 2004). Also, Stat1 expression and activation has been shown to play a key role for cell survival in lung cancer (Liu *et al.*, 2005). However, there has not been a study to date on whether Stat 1 expression affects clusterin expression in docetaxel-resistant lung cancer. Therefore, it will be important to investigate whether or not this novel Stat1/clusterin pathway that we have described here also influences docetaxel resistance in other cancers.

The exact molecular mechanism of how Stat1 and clusterin interact with each other is not fully understood and needs to be further defined. In our research, we have found several potential binding sites for Stat1 on the clusterin promoter. We believe that Stat1, as a transcription-activating factor, directly contributes to clusterin upregulation in docetaxel-resistant cells by binding to DNA and activating transcription of clusterin. Stat1 is known to have a regulatory role in androgen receptor expression and once it becomes phosphorylated and dimerized, it is translocated into the nucleus. There, it has been reported to bind the CBP p300 family of transactivator proteins that interact with a variety of DNA-binding factors and influence genes controlling cellular differentiation, cell cycle progression and transformation (Kominsky *et al.*, 2000). Whether Stat1 upregulation of clusterin involves this pathway needs to be determined.

In conclusion, we have identified Stat1 to critically control clusterin protein expression, which is associated with cell survival in DU145-DR cells that have developed docetaxel resistance. Thus, the targeting of Stat 1 could prove to be a promising new therapeutic strategy for the treatment of prostate cancer patients, either in combination with docetaxel to prevent development of docetaxel resistance, or to treat patients who are already resistant to docetaxel.

## Materials and methods

### *Cell culture and selection of docetaxel-resistant clones*

The human androgen-independent prostate carcinoma cell lines, DU145 and PC3, were obtained from American Type Culture Collection (Rockville, MD, USA) and were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) with 100 U/ml penicillin, 100 µg/ml streptomycin. Docetaxel-resistant clones of DU145 and PC3 were selected by culturing the cells in docetaxel in a dose-escalation manner. Initial culture was in 1 nM docetaxel. After the clones sensitive to 1 nM docetaxel were no longer present and the surviving DU145 and PC3 cells repopulated the flask and continued to divide through four passages, the concentration of docetaxel in the medium was increased to 5.5 nM and subsequently to 11 nM. The same selection methodology was followed with each increase in docetaxel concentration. Once DU145 and PC3 cells were freely dividing in 11 nM docetaxel mediums, they were considered as resistant and labeled DU145-DR and PC3-DR. All the cell cultures were maintained at 70% confluency and medium was changed every 48 h.

### *Immunohistochemistry*

Cytospins from DU145, DU145-DR, PC3, PC3-DR were fixed in a 3:1 solution of methanol and acetone for 1 h. Representative cytospins were then immunostained to analyse the expression of Stat1, clusterin and Stat3. Isotype controls were used as negative controls in the place of primary antibody in each experiment. The immunohistochemical staining was performed manually at room temperature, using the avidin-biotin-peroxidase complex method (Vectastatin Elite ABC kit; Vector Lab, Burlingame, CA, USA). Briefly, pretreatment for antigen retrieval with a pressure cooker involved heating

cytospins with a microwave oven in 250 ml of unmasking solution (Vector Lab) for 10 min at a high-power level, followed by 20 min of cooling. Endogenous peroxidase and nonspecific background staining were blocked by incubating slides with 50:50 solution of 3% hydrogen peroxide and methanol for 20 min. After washing with PBS for 5 min, slides were blocked with fetal calf serum for 20 min, followed by incubation with the primary monoclonal antibodies for Stat1, Stat3 (Cell Signaling, Beverly, MA, USA) and clusterin (Upstate Cell Signaling Solutions, Waltham, MA, USA) at a dilution of 1:100, for 2 h at room temperature. After rinsing with PBS for 5 min, slides were incubated with a biotinylated secondary antibody for 30 min and washed again. After washing with PBS for 5 min, slides were incubated with avidin-biotin complex for 30 min and washed again. The slides were developed with 3,3-diaminobenzidine (DAB Substrate kit for peroxidase, Vector Lab). All the slides were lightly counterstained with hematoxylin for 10 s before dehydration and mounting. Immunostaining was observed with a Leitz Orthoplan 2 microscope and images were captured by a CCD camera with the Smart Capture Program (Vysis, Downers Grove, IL, USA).

### *Western blotting analysis*

Protein expression of Stat1, Stat3 and clusterin for both DU145 and DU145-DR was determined by Western blot analysis by using specific antibodies. DU145 cells were exposed to docetaxel for 0, 1, 3, 5, 7 and 21 days, were harvested and solubilized by incubation at 4°C for 30 min in 1% NP-40, 10 mM Tris, 140 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide, 50 mM NaF, 1 mM ethylenediaminetetraacetic acid, 1 mM sodium orthovanadate, 0.25% Na Deoxycholate, 100 µl ALA, 100 µl cocktail I and 100 µl cocktail II. Cell lysates were centrifuged at 12 000 g for 15 min to remove nuclei and cell debris. The protein concentration of the soluble extracts was determined by using the Bio-Rad (Bradford) protein assay (Bio-Rad, Hercules, CA, USA). Separation of 50 µg of total protein was performed on 7.5% sodium dodecyl sulfate-polyacrylamide gels, and transferred to a nitrocellulose membrane before immunoblotting with primary monoclonal antibodies against Stat1 and Stat3 or rabbit anti-phosphoStat1 and anti-phosphoStat3 (Cell Signaling, Beverly, MA, USA). The specific proteins were detected by the enhanced chemiluminescence detection system (ECL, Amersham, Piscataway, NJ, USA). The equal loading of protein sample was verified with an  $\beta$ -actin-specific antibody (Sigma, St Louis, MO, USA).

### *Treatment of DU-145-DR cells with STAT1 and clusterin small interfering RNA*

Oligonucleotides representing siRNA against human Stat1 expression (siRNA-STAT1) and mismatch control oligonucleotides (siRNA-control) were purchased from SuperArray (Frederick, MD, USA). Kits included human siRNA-Stat1, siRNA-control for transfection and primers pair for RT-PCR. Also, a siRNA plasmid targeted for clusterin and a negative control plasmid (siRNA-control) was purchased from Upstate Cell Signaling Solutions. The sense strand for siRNA-clusterin is (5'-AACCTAGAAGAAGCCAAGAAG-3') and the anti-sense strand for clusterin is (5'-AATTCAAAAACCTA GAAGCCADU145-3'). Cells were seeded at  $5 \times 10^5$  cells in each of six-well plates with 2 ml of growth medium 18 h before transfection and were 70–80% confluent at the time of transfection. For inhibition of Stat1, 5 µl containing siRNA oligonucleotides (siRNA-STAT1 or siRNA-control) and 5 µl lipofectamine™ 2000 reagent (Invitrogen-Life Technologies

Inc., Carlsbad, CA, USA) were diluted in 250  $\mu$ l of Opti-MEM<sup>®</sup> I (Gibco, Carlsbad, CA, USA), separately. For inhibition of clusterin, 4  $\mu$ g of the clusterin siRNA plasmid and 10  $\mu$ l of lipofectamine was added to 285  $\mu$ l of Opti-MEM<sup>®</sup> I. The siRNA-STAT1, siRNA-clusterin or siRNA-control was preincubated with lipofectamine reagent in Opti-MEM<sup>®</sup> I for 20 min. Cells were transfected with lipofectamine + siRNA-STAT1, lipofectamine + siRNA-control, or left untransfected. The transfection was terminated after 5 h by aspirating the transfection medium and adding fresh RPMI 1640 containing 10% FBS and 11 nM docetaxel. The non-adherent cells were washed off and the remaining cells were incubated in medium containing 11 nM docetaxel at 37°C. Cells were harvested in TRIzol 48 h after treatment for RT-PCR. Cells were lysed 72 h after treatment for Western blot analysis to evaluate STAT1 and clusterin protein levels. Stat1 and clusterin expression in DU145-DR was compared to baseline expression of the same proteins in DU145.

#### Isolation of RNA and reverse transcriptase-polymerase chain reaction

Reverse transcriptase-polymerase chain reaction for Stat1 was assayed in DU145-DR cells. Briefly, total RNA was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). A total amount of 1  $\mu$ g of RNA was converted to cDNA by Omniscript reverse transcriptase in solution containing random hexanucleotide, deoxynucleoside triphosphate (dNTP), Rnase inhibitor and RT-buffer (Qiagen, Valencia, CA, USA). Aliquots of 1  $\mu$ l of DNA resulting from each RT reaction were then subjected to PCR. The temperature profiles of PCR were as follows: an initial denaturation step of 94°C for 5 min, followed by 25 cycles of 94°C for 15 s, 50°C for 15 s, 72°C for 30 s and a final elongation step of 72°C for 7 min. PCR was performed in reactions containing *Taq* DNA polymerase, dNTP PCR buffer and primers. The sense primer for Stat 1 was (5'-GATATAATTCACAAAATCAGAGAG-3') and the antisense primer for STAT1 was (5'-CTGATCCAGG CAGCGTTG-3'). The sense primer for the human GAPDH was (5'-CAAAAGGGTCATCATCTCTGC-3') and the antisense primers for the human GAPDH was (5'-GAGGGGC CATCCACAGTCTTC-3'). Reverse transcriptase-polymerase chain reaction products were analysed by agarose gel.

#### Induction of apoptosis by docetaxel

DU145 tumor cells were treated with 11 nM docetaxel from 0 to 21 days. DU145 cells were harvested at each time point, re-suspended with Annexin V binding buffer and then stained using the Annexin V Apoptosis Kit (BD Pharmagen, Franklin Lakes, NJ, USA). The stained samples were then analysed by flow cytometry using a FACScan. % Apoptosis includes both Annexin V+ and PI+ cells. Also, DU145 cells were

pretreated with either siRNA-Stat1 or siRNA-control. The transfection was terminated after 5 h by aspirating the transfection medium and adding fresh RPMI 1640 containing 10% FBS. After the cells were cultured in this medium for 48 h, 11 nM docetaxel containing medium was added. After an incubation of 48 h, the cells were harvested and stained with Annexin V/PI and analysed for apoptosis induction by flow cytometry.

#### [<sup>3</sup>H]Thymidine incorporation analysis

Relative growth rate of the siRNA-Stat1 and siRNA-clusterin-treated DU145-DR cells was assessed using the thymidine incorporation assay. Briefly, DU145-DR cells were plated in six-well plates at  $5 \times 10^5$  cells per well. After transfection, as described above, with specific siRNA-STAT1, siRNA-clusterin or siRNA-control, the cells were cultured in the presence of 11 nM docetaxel for 24 h and reseeded at a concentration of  $1.0 \times 10^4$  cells/well in a 96-well plate incubated for 18 h at 37°C. [<sup>3</sup>H]Thymidine was added to the 96-well plate at a final concentration of 1  $\mu$ Ci/ml and the cells were incubated for an additional 6 h. Cells were then processed through a harvester with distilled water onto glass fiber filters and counted on a  $\beta$ -scintillation counter. The mean  $\pm$  s.e. of the triplicate cultures was determined, and the percent growth inhibition was calculated as follows:

$$\begin{aligned} \% \text{ growth inhibition} &= (\text{cpm siRNA} - \text{control}) \\ &\quad - \text{cpm siRNA} - (\text{STAT1 or clusterin}) \\ &\quad / (\text{cpm siRNA} - \text{control}) \end{aligned}$$

A second plate of this experiment was stained with the Diff-Quik Stain Set (Harleco, Gibbstown, NJ, USA) to check for remaining viable cells. The cells were first fixed in methanol, and then stained with a buffered solution containing the dye, Eosin Y. Cells were then stained with a buffered solution containing the dyes, methylene blue and Azure A, and observed with a Leitz Orthoplan 2 microscope and images were captured by a CCD camera with the Smart Capture Program.

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