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Loss of signal transducer and activator of transcription 1 is associated with prostate cancer recurrence.

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Abbreviations:

STAT: signal transducers and activators of transcription factors

JAK : janus kinase

SOCS: suppressors of cytokine signalling

ER: oestrogen receptor

PR: progesterone receptor

TMA: tissue micro array

PSA: prostate specific antigen

TURP: transurethral resection of the prostate

TRUS transrectal ultra sound

IHC: immunohistochemistry

WST: water soluble tetrazolium salts

AR: androgen receptor

IQR: interquartile range

siRNA: small interfering ribonucleic acid

IFN: interferon

Key words: androgen receptor, signal transduction, prognostic biomarker

Abbreviated title: STAT1 expression in prostate cancer.

Abstract

STAT1 loss has previously been implicated in cell line studies to modify prostate cancer cell growth and survival, however the clinical significance of this has not been previously established. This study investigated if STAT1 loss was associated with patient outcome measures and the phenotypic consequence of STAT1 silencing. STAT1 expression was assessed in two patient cohorts with localised (n=78) and advanced prostate cancer at initial diagnosis (n=39) by immunohistochemistry (IHC). Impact of STAT1 silencing on prostate cancer cells lines was assessed using Cell Death detection ELISA, TLDA gene signature apoptosis arrays, WST-1 assay, xCELLigence system, clonogenic assay and wound healing assay. In the localised patient cohort, low expression of STAT1 was associated with shorter time to disease recurrence (3.8 vs 7.3 years, $p=0.02$) and disease specific survival (6.6 vs 9.3 years, $p=0.05$). In the advanced patient cohort, low expression was associated with shorter time to disease recurrence (2.0 vs 3.9 years, $p=0.001$). When STAT1 was silenced in PC3 cells (AR negative) and LNCaP cells (AR positive) silencing did not influence levels of apoptosis in either cell line and had little effect on cell viability in the LNCaP cells. In contrast, STAT1 silencing in the PC3 cells resulted in a pronounced increase in cell viability (WST-1 assay: mock silenced vs STAT1 silenced, $p<0.001$), clonogenicity (clonogenic assay: mock silenced vs STAT1 silenced, $p<0.001$) and migration (wound healing: mock silenced vs STAT1 silenced, $p<0.001$). In conclusion, loss of STAT1 may promote prostate cancer recurrence in AR negative patients via increasing cell viability.

Introduction

Prostate cancer is a major cause of death in the developed world and is the most common cancer amongst men in the UK. Treatment options vary depending on grade and staging of patients. For patients with localised prostate cancer, active surveillance, radical prostatectomy or radiotherapy may be offered, but within this patient group a sub cohort will develop a subsequent recurrence and require further intervention [1]. For patients diagnosed with advanced disease, hormone therapy is normally the first choice of treatment with the majority of these patients eventually recurring with castrate resistant disease [2]. Therefore a major challenge facing today's urologists is predicting which patients are most likely to recur and what therapeutic strategy to employ when they do. In order to identify biomarkers and novel therapeutic options for these patients, further understanding of the pathways associated with promoting prostate cancer recurrence is required.

Deregulation of the signal transducers and activators of transcription factors (STAT) along with the negative feedback regulators of the Janus-activated kinase (JAK), such as members of the SOCS family have been implicated in prostate cancer cell growth and survival [3, 4]. STAT members, a group of seven cytoplasmic proteins, act as transcription factors to elicit their effects via control of transcriptional expression of multiple genes. Aberrant activation of some STAT members, in particular STAT3 and STAT5, have been found in a large number of human tumours, acting as pro-survival signals for tumour cells via tight regulation of cell cycle progression, cellular transformation, and prevention of apoptosis [5, 6].

Contrary to the function of other family members' in cancer development, STAT1 is believed to act as a tumour suppressor by playing an important role in growth progression and apoptosis. In early studies of the role of STAT1 in cancer, it was observed that STAT1^{-/-} mice formed significantly more carcinogen-induced

sarcomas than wild type mice [7, 8]. Recently, it was demonstrated in a subset of patients with ER \pm /PR $^+$ breast cancer, that STAT1 expression is lost or significantly reduced in the neoplastic cells compared to normal breast epithelium [9]. Moreover, female mice lacking longitudinal expression of STAT1, spontaneously developed mammary gland cancers of the luminal subtype [9]. Numerous *in vitro* studies have also suggested that STAT1 may function as a tumour suppressor by regulating the expression of caspases such as caspase 1, 2, 3, and 7 [10, 11] upregulating p27^{Kip1} expression [12] or interacting with p53 or BRCA1 [13, 14]. Paradoxically, STAT1 accumulation and hyper-activation has also been observed in multiple types of cancers, offering a survival advantage to these tumours. Elevated levels of STAT1 in squamous cell carcinoma cell lines have been linked to acquisition of resistance to radiation or chemotherapy treatment [15]. Furthermore, STAT1 along with clusterin protein expression was induced by docetaxel treatment in prostate cancer cells, DU145 and overexpressed in a docetaxel-resistant cell line (DU145-DR)[16]. Therefore, STAT1 expression might be a double-edged sword that functions to either suppress or promote cancer development depending on the tissue or cellular context.

The current study was designed to investigate if STAT1 expression was associated with recurrence or patient survival in prostate cancer specimens at initial diagnosis.

Materials and Methods

Patient cohort. Cohort 1 contained 78 patients with localised prostate cancer at initial diagnosis from Greater Glasgow and Clyde NHS Trust, all of whom were diagnosed between 1993 and 2002. Ethical approval was obtained from the West of Scotland Research Ethics Committee (05/S0704/94). Each patient in cohort 1 was required to have hormone naïve prostate tissue samples available to provide

sufficient material for construction of a tissue micro array (TMA). These patients were identified retrospectively and samples were retrieved from archived stored specimens. Protein expression in cancer tissue was assessed using TMAs. TMAs were constructed using 0.6mm² cancer tissue cores taken from representative areas of tumour from each patient, as identified by a pathologist. All TMA blocks were constructed in triplicate, to account for intra-tumour disease heterogeneity. 5µm thick sections were used in all experiments.

Cohort 2 contained 39 patients with advanced prostate cancer at initial diagnosis from Greater Glasgow NHS Trust, all of who were diagnosed between 1984 and 2000. Ethical approval was obtained from West of Scotland Research Ethics Committee (98UR004). Each patient was diagnosed with advanced prostate cancer and had tissue available for analysis. Each patient was observed to respond to maximum androgen blockade (androgen deprivation therapy and LHRH agonists) and response was defined as a fall in PSA levels of at least 50%. Tissue was obtained from patients by TRUS-guided biopsy or TURP, TMA was constructed (as described above) from the TURP specimens but full sections were used for the TRUS specimens. These patients were identified retrospectively and samples were retrieved from archived stored specimens. All tumours had patient identification removed, and the clinical information database was anonymised.

Immunohistochemistry (IHC). Tissue sections were dewaxed in xylene and rehydrated in a series of graded alcohol. Antigen retrieval was performed under pressure for 5 min in Tris-EDTA buffer (pH 8.0). Endogenous peroxidase was blocked in 3% hydrogen peroxide for 10 min and non specific binding blocked using 5% horse serum in tris buffered saline. Incubation with primary STAT1 antibody was carried out in a humidified chamber overnight at 4°C (1:200, Cell Signaling 9175, UK). Tissue was then incubated in Envision solution (DAKO UK) for 30 min and

developed by application of 3,3'-diaminobenzidine as a chromogen (DAKO UK). Sections were counterstained with hematoxylin, dehydrated through graded alcohol and xylene, and mounted in DPX. Prostate tissue was included as positive control and an isotype-matched antibody was used on prostate samples to provide a negative control. Specificity of the antibody was confirmed by a single band of 87 kDa on western blotting in cell lysates of PC3 cells treated with mock siRNA or siRNA targeting STAT1 (Dharmacon, ThermoScientific, UK; Figure 1A) and positive IHC staining was blocked by a peptide specific for the STAT1 antigen (Cell Signaling, UK; Figure 1B). AR expression was already available for these cohorts from previous studies [17, 18]

Scoring method. Protein expression levels were assessed blindly by two independent observers using a weighted histoscore method also known as the H-score at magnification $\times 400$. Each cellular location (membrane, cytoplasm, and nuclei) was scored separately. The weighted histoscore method assesses the staining intensity and the percentage of cells stained with that intensity within the full sample. It is calculated by $(1 \times \% \text{ cells staining weakly positive}) + (2 \times \% \text{ cells staining moderately positive}) + (3 \times \% \text{ cells staining strongly positive})$. This provides a semi-quantitative classification of staining intensity, with the maximum score 300 (if 100% of cells stain strongly positive) and minimum score 0 (if 100% of cells are negative) [19].

Cell Culture and Reagents. Prostate cancer cells; LNCaP and PC3 were obtained from American Type Culture Collection (ATCC, LGC Standards, UK).

Transient transfection. LNCaP and PC3 cells were plated in 6-well plates at concentration of 2×10^5 cells/well. At 70% confluent they were transfected with 80nM siRNA STAT1 (Dharmacon, ThermoScientific, UK) or 80nM non-targeting of human genome control siRNA (Dharmacon, ThermoScientific, UK) or sterile 1x siRNA buffer

(control; Dharmacon, ThermoScientific, UK) using Lipofectamine RNAiMAX (Invitrogen, UK) as per manufacturers instructions. Best knockdown was observed at 72h post transfection as assessed by western blotting (Figure 1C).

Western Blot analysis. Changes observed at the protein level in prostate cancer cell lines were assessed by western blotting as previously described [20]. The PVDF membranes were probed with primary antibodies STAT1 (1:3000; Cell Signaling 9175, UK) and STAT3 (1:5000; Cell Signaling 9132, UK). Following visualisation with ECL plus (Amersham, UK) membranes were stripped and re-blotting with β -tubulin (Abcam 21058, UK) to ensure equal protein loading.

Cell Death Detection ELISA Plus. 24h after transient transfection, LNCaP and PC3 cells were trypsinised and plated at 3×10^4 cells/mL in quadruplicates for untreated cells, cells transfected with control siRNA, and cells transfected with siRNA STAT1 in 96-well plates. Cell Death Detection ELISA Plus kits (Roche Applied Science, UK) were used to measure apoptosis at the 72 hour time point by quantifying histone-DNA complexes generated after inhibitor treatment, as per the manufacturer's protocol. HRP cleavage of ABTS substrate was measured by absorbance at 405nm.

Taqman low density arrays. 72 hours following transfection, RNA was extracted using Trizol (Invitrogen, UK) as per manufacturer's instructions. Preparation of cDNA and RT-PCR were performed using TaqMan RT-PCR methodology and reagents (Perkin-Elmer Applied Biosystems).

384 TLDA gene signature apoptosis arrays containing 93 genes related to apoptosis and 3 candidate endogenous controls were employed to determine if STAT1 silencing induced change in expression of apoptotic genes. Each sample from 3 independent silencing experiments on PC3 cells was run in duplicate. All results were analysed simultaneously by RQ Manager Software (ABI, UK) and the threshold

cycle (Ct) values of the genes of interest were calibrated against 18S Ct (• Ct). Results are presented in fold change in the target gene relative to 18S endogenous control using the $2^{-\Delta\Delta CT}$ method.

Cell Viability. 24 hours after transient transfection, LNCaP and PC3 cells were trypsinised and plated at 3×10^4 cells/mL in quadruplicates for untreated cells, cells transfected with control siRNA, and cells transfected with siRNA STAT1 in 96-well plates. Proliferation of the cells was assessed 72 hours following transfection by WST-1 assay as previously described by Tatarov et al. [20]. In brief, cell viability was measured by mitochondrial dehydrogenase induced cleavage of water soluble tetrazolium salt, via incubation with WST-1 for 2 hours at 37°C with 5% CO₂ in air (Roche Applied Science, UK). Absorbance was then measured at 450nm.

Furthermore, cells were also plated at 3×10^4 cells/mL in octuplicates in E-Plate VIEW96 (Roche, UK) and their viability and proliferation was measured in real-time using the xCELLigence system (Roche,UK) as per manufacturer's instructions over 144 hours post-transfection.

Clonogenics assay. 24 hours after transient transfection in 6-well plates, PC3 cells were trypsinised and plated at 400 cells/plate in triplicate plates for untreated cells, cells transfected with control siRNA, and cells transfected with siRNA STAT1 in 60mm Nunclon dishes. Following a 10 day incubation in the dishes, PC3 cells were washed with ice-cold PBS and fixed with 3mL of methanol for 10 min. Methanol was aspirated off and dishes were left to dry for 20 min. Colonies were stained with Crystal Violet solution for 10 min followed by a washing step with water. Colonies were counted in each dish and presented as surviving fraction (mean siRNA STAT1 transfected colonies divided by mean control).

Cell migration assay. Wound-healing assay was carried out to investigate the ability of PC3 cells to migrate into a denuded area after being transfected with siRNA for STAT1. Wounds were made using a fine pipette tip through the cell monolayer (three wounds per each well) of PC3 cells 48 hours post transfection with siRNA STAT1 in 6-well plates. The medium was then replaced with standard medium prior to assessing the wound closure using a Zeiss Axiovert S100 microscope at $\times 20$ magnification. The assay was done over 20 hours with images taken from four fields per each well at 0, 4, 10, 16 and 20 hours. Closure of wound was assessed by measurement of the distance between the edges of the wound at each time point using ImageJ software.

Statistical Analysis. Statistical analysis was done using SPSS statistical package (version 19.0) and GraphPad Prism4. Survival analysis including time to disease recurrence and disease specific survival was conducted using the Kaplan-Meier method and curves were compared with the log-rank test. Multi variate analysis was performed using the Cox Regression model. *In vitro* experiments were analysed using one-way Anova and are expressed as mean \pm s.e.m or % control mean (mean of treatment divided by mean of control $\times 100$).

Results

STAT1 expression is associated with recurrence. To determine the expression levels of STAT1 in earlier and more advanced stages of prostate cancer, two different patient cohorts were utilised. For the expression of STAT1 in earlier stages of the disease, TMAs of paraffin embedded samples from 78 patients with localised disease were utilised (cohort 1). While to determine the expression of STAT1 in

patients with advanced prostate cancer a cohort of 39 patients was utilised (cohort 2).

Patient characteristics for cohort 1 can be seen in Table 1. Briefly, at diagnosis the median age was 71, Gleason grade was 7 and PSA level was 19 ng/mL. Data for disease recurrence was available for 63 patients of which 40 relapsed, median disease recurrence time was 2.99 years. At last follow-up 18 patients developed metastases, 17 patients were still alive, 42 had died of their disease and 19 died of inter-current disease. Median follow up was 10.5 years. Ki67 proliferation index and AR expression were already available for this cohort and had median histoscore of 3 and 67.67 units, respectively.

STAT1 expression was assessed in all specimens available in both stromal and tumour cells (Figure 2A). In the tumour cells median membrane STAT1 histoscore was 50 units (IQR: 10.8-86.7), median cytoplasmic STAT1 histoscore was 85 units (IQR: 50-127.5), median nuclear STAT1 histoscore was 35 units (IQR: 6.7-65.0). In the stromal cells only nuclear expression was observed with a median expression of 3 units (IQR: 0-15). Specimens were divided in to those with low expression (< lower quartile) or high expression (> lower quartile) and Kaplan-Meier curves constructed to assess if expression was associated with time to disease recurrence (recurrence) or disease specific survival. Stromal STAT1 expression, tumour cytoplasmic STAT1 expression and tumour nuclear STAT1 expression were not associated with time to disease recurrence or disease specific survival. However, membrane STAT1 expression in the tumour cells was significantly associated with time to disease recurrence ($p= 0.02$; Figure 2B). Those patients with high membrane STAT1 expression had a mean time to disease recurrence of 7.3 years (IQR: 5.5-9.1) compared to 3.8 years (IQR: 2.2-5.3) for those with low expression. Membrane STAT1 expression in the tumour cells was also significantly associated with disease

specific survival ($p=0.05$) (Figure 2C). Those patients with high membrane STAT1 expression had a mean time to death (disease specific survival) of 9.3 years (IQR: 7.8-10.9) compared to 6.6 years (IQR: 4.6-8.6) for those with low expression. In addition, membrane STAT1 expression was inversely associated with recurrence ($p=0.019$) and ki67 proliferation index as assessed by chi square test ($p=0.004$).

STAT1 expression was also assessed in the second patient cohort with advanced disease at initial diagnosis (table 1). Briefly, at diagnosis the median age was 70, Gleason grade was 8 and PSA level was 41 ng ml^{-1} . All patients had disease recurrence; median disease recurrence time was 2.64 years. At diagnosis 25 patients had locally advanced disease and 14 patients had metastatic disease, during follow-up a further 6 patients developed metastases. No patients were alive at last follow up, 29 died of their disease and 10 died of inter-current disease. Ki67 proliferation index and AR expression was already available for this cohort, and had a median histoscore of 1.5 and 100 units, respectively.

STAT1 expression was observed in the membrane, cytoplasm and nuclear compartments of the tumour cells and cytoplasmic and nuclear compartments of stromal cells. Median membrane tumour cell STAT1 histoscore was 16 units (IQR: 0-42), median cytoplasmic tumour cell STAT1 histoscore was 50 units (IQR: 15-94) and median nuclear tumour cell STAT1 histoscore was 8 units (IQR: 0-43). In the stromal compartment, median cytoplasmic stromal cell STAT1 histoscore was 0 units (IQR: 0-0.33), and median nuclear stromal cell STAT1 histoscore was 0.33 units (IQR: 0-3.58).

STAT1 expression in the stromal cells, membrane of tumour cells and nucleus of tumour cells were not associated with clinical outcome measures or survival. Cytoplasmic STAT1 expression when subdivided into high and low groups at the lower quartile (LQ), was significantly associated with time to disease recurrence ($p=$

0.001; Figure 2D). Those patients with high cytoplasmic expression had a mean time to disease recurrence of 3.9 years (IQR: 3.0-4.7) compared to 2 years (IQR: 1.6-2.3) for those with low expression. When these patients were stratified by AR expression, this association was lost in the group of patients expressing high levels of AR in their tumour ($p>0.05$) but was maintained in the group with low AR tumour expression ($p=0.002$). In addition, membrane and cytoplasmic tumour cell STAT1 expression was inversely associated with Gleason Grade ($p=0.02$ and $p=0.05$ respectively) and ki67 proliferation index ($p=0.024$) as assessed by chi square test.

STAT1 expression loss was not associated with levels of apoptosis in prostate cancer cell lines. Having observed an association between loss of STAT1 expression and shorter time to disease recurrence and decreased specific survival, the phenotypic consequence of STAT1 loss in prostate cancer cells was investigated. A transient STAT1 silencing model was employed in order to assess the effect of STAT1 loss in AR negative PC3 cells as well as AR positive LNCaP cells (Figure 1C).

The effect of silencing STAT1 upon cellular apoptosis in both PC3 cells and LNCaP cells was investigated using the cell death ELISA plus kit. Apoptosis was measured in PC3 and LNCaP control cells (untreated cells), siRNA control cells (cells silenced with scrambled siRNA) and cells silenced for STAT1 expression at 72 hours. No difference in apoptotic rate of the cells was observed between any of the treatment groups of the PC3 cells (Figure 3A) or the LNCaP cells (Figure 3B), suggesting that STAT1 loss does not influence apoptotic rate in prostate cancer cell lines.

We further explored the influence of STAT1 loss on apoptosis by studying changes of expression in response to STAT1 loss in a panel of genes known to be involved in the cellular apoptotic pathways. Change in gene expression was assessed 72 hours following silencing in a panel of 96 genes. No genes were observed to have a fold

change in expression of 2 or more in response to STAT1 loss. This supports our observation that STAT1 loss was not associated with induction of apoptosis in PC3 cells (Figure 3C).

Loss of STAT1 expression is associated with cell viability in prostate cancer cell lines. The effect of silencing STAT1 on cell viability was measured by WST-1 assay. Cell viability was measured in PC3 and LNCaP control cells (untreated cells), siRNA control cells (cells silenced with scrambled siRNA) and cells silenced for STAT1 at 72 hours. Silencing of STAT1 resulted in a significant increase in cell viability in PC3 cells (Control: 98.1 ± 2.3 %, siControl: 105.6 ± 4.7 %, siSTAT1: 179.4 ± 9.5 %) ($p < 0.001$) (Figure 4A). However no increase in viability was observed in LNCaP cells (Control: 100 ± 4.4 %, siControl: 94.9 ± 3.4 %, siSTAT1: 108.8 ± 3.4 %) ($P > 0.05$) (Figure 4B).

Results on cell viability were confirmed by monitoring the proliferation of prostate cancer cells following silencing of STAT1 over a 144 hour period (6 days) using the xCELLigence system. Following silencing of STAT1, PC3 cells exhibited an increase in proliferation over mock silenced cells (Control) and scrambled siRNA (siControl) from 64 hours post-transfection and this increased proliferation was continuously observed throughout the period that the cells were monitored (Figure 4C). LNCaP cells silenced for STAT1 also showed an increase in proliferation around 72 hours post-transfection (Figure 4D), but this did not continue for the full 144 hour period.

As we observed that loss of STAT1 influenced cell viability and proliferation of PC3 cells, we then proceeded to assess the impact of STAT1 loss on the capability of PC3 cells to form clones. The number of clones formed in the silenced cells with siRNA STAT1 (1.4 ± 0.04) was significantly higher than those measured in the control (1.0 ± 0.05) and siRNA control cells (0.9 ± 0.05 , $p < 0.001$; Figure 5 A and B). Furthermore, the effect of STAT1 loss on migration of PC3 cells was measured using

a wound healing assay. Wound closure was observed at a faster rate in cells silenced with siRNA STAT1 cells in comparison with control cells (Figure 6A and B). More specifically 6h following generation of the wound, closure of the wound was more apparent in the PC3 cells silenced with siRNA STAT1 (wound closure $19.8\% \pm 7.6\%$) in comparison to control cells (wound closure $7.5\% \pm 4.5\%$, $p < 0.001$; Figure 6A and 6B), this was also observed at 10 and 14 hours, with the difference in wound closure being most pronounced at 14 hours (wound closure of control $30.1\% \pm 1.8\%$, vs wound closure of siSTAT1: $76.9\% \pm 2.7\%$, $p < 0.001$).

Discussion

Numerous transcription factors, growth factors and protein kinases have been associated with the progression of prostate cancer. However, the inability to completely control and thus eradicate the disease reveals that other unknown oncogenic signalling pathways may be involved in regulation of this disease. STAT1 signalling has previously been reported to be associated with cell viability in prostate cancer [21, 22] and we further investigated this in patient specimens. We observed that loss of STAT1 expression in patients with either localised or advanced prostate cancer at initial diagnosis was associated with shorter time to disease recurrence as well as shorter disease specific survival for patients with localised disease. Recently, EZH2 was demonstrated to up-regulate the STAT1 tumour suppressor action in DU145 and PC3 cells and therefore may be employed as a novel therapeutic agent in a subset of patients with low STAT1 levels [23]. In addition, Chan et al. reported that loss of STAT1 expression was linked with breast cancer development and progression, as STAT1^{-/-} mice are highly susceptible to mammary tumour formation [9]. Conversely, in haematopoietic tumours such as leukaemia, high levels of STAT1 accelerated the expression of tumours independently of the IFN signaling pathway

[24], moreover in human soft tissue sarcoma specimens high expression of STAT1 was associated with reduced disease specific survival [25]. This contradictory data reveals that STAT1 may have different roles in different types of tumours either as a promoter or suppressor of the progression of the disease.

Interestingly, the localisation of STAT3 associated with disease recurrence was different in localised and advanced tumours. In localised tumours, membrane STAT3 was associated with shorter time to disease recurrence, suggesting that classical pathways may be important at this stage. However, in advanced prostate cancer, cytoplasmic STAT1 was associated with shorter time to disease recurrence, suggesting a differential role in this disease stage. Ng et al. have shown that cytoplasmic STAT3 can inhibit the microtubule destabilising protein, stathmin, to allow stabilisation of microtubules and promote cell migration (ref). Also, Teng et al. have also shown that cytoplasmic STAT3 interacts with β PIX to modulate RAC1 mediated cell migration and metastasis, suggesting a possible role for STAT3 in prostate cancer metastasis. It is possible that a similar relationship between classical and alternative pathways for STAT1.

The key role of androgens and AR, not just in early development but also in the progression of prostate cancer is very well characterised. In order to evaluate a potential regulation of AR on STAT1 expression in clinical setting, we further stratified localised and advanced prostate cancer patient cohorts based on expression of AR. When localised tumours were stratified into high and low AR-expression groups, associations between membrane STAT1 expression and time to disease recurrence was significant for both stratified groups (results not shown). But, in patients diagnosed with advanced disease, the association was lost in those that were AR positive but remained in those with no or low AR expression. This suggest that in advanced prostate cancer loss of STAT1 expression maybe a poor prognostic factor,

and this may be specific to a subgroup of patients with low nuclear AR expression. Furthermore, a study using the AR positive cell line LNCaP and the AR overexpressing cell line LNCaP-ARhi, demonstrated that *STAT1* expression was not regulated in response to androgens or AR [26]. These facts combined suggest that STAT1 functions independently of the AR and it may be possible to use loss of STAT1 to identify patients with aggressive prostate cancer. Unfortunately, the number of patients with advanced prostate cancer in our cohort was relatively small, and this precluded any meaningful analysis of survival in this group; however, this data is interesting and further investigation in a larger cohort would be highly informative.

In addition, to our observations that STAT1 expression inversely correlates with ki67 proliferation index in the tissue specimens, we also in our cell line experiments observed that STAT1 plays a key role in regulating proliferation and cell viability in prostate cancer AR negative cells. Induction of STAT1 loss via a siRNA approach, demonstrated that loss of STAT1 did not influence proliferation or cell viability in LNCaP AR positive cells but did in PC3 AR negative cells. Indeed, Wee et al. recently reported in prostate cancer cells that Myc induced regulation of the STAT1 pathway acted to induce tumour suppressor signalling but a similar effect was not observed when PI3K was employed to regulate the STAT1 pathway, again demonstrating the complexity of this pathway in prostate cancer [23]. Both the cell lines used in the current study were derived from patients with advanced disease, and these results are in agreement with the observations we made in the clinical studies where, STAT1 was associated with recurrence in patients with advanced prostate cancer and AR negative tumours but was not associated with recurrence in patients with advanced prostate cancer and AR positive tumours. In addition, it would be useful to study the effect of STAT1 loss in combination with AR expression in early prostate cancer cell lines. In AR negative cells, we observed that the

clonogenic capacity of PC3 cells increase with STAT1 loss and also wounds healed significantly quicker. These results again support our hypothesis that STAT1 loss may promote prostate cancer recurrence in a subset of patients. It is difficult to convey using the wound healing assay alone, if the results observed were due to an increase in the motility and migration of the cells or was simply due to an increase in proliferation rate. It would be interesting in future studies to probe this in more detail and establish if STAT1 loss only impacts on cell proliferation or if it can also increase the metastatic potential of the tumours by increasing cell migration and invasion.

Additional evidence to support a role of STAT1 in progression of prostate cancer is provided by Roca et al. who reported that STAT1 was an important factor in the epithelial to mesenchymal transition, which is important for both cancer progression and development of metastases [27]. Wee et al., who reported that Myc can regulated STAT1 signalling, observed that this effect was observed in both DU145 and PC3 cells, which are both AR negative but not in LNCaP and 22RV1 cells which are both AR positive [23]. They hypothesize this due to Myc sensitivity in these cells, however an alternative explanation could be that STAT1 was not regulated in the LNCaP and 22RV1 due to AR status and not Myc status [23], or conversely the observations made in the current study in the contrasting role of STAT1 loss in PC3 and LNCaP cells could be due to Myc sensitivity and not AR status.

In conclusion, the current study begins to build a body of evidence supporting the use of STAT1 loss as a prognostic marker for prostate cancer patients at diagnosis as well as in a subgroup of patients with low AR expression. Furthermore STAT1 loss was associated with increased proliferation in the tissue specimens and increase proliferation and cell viability of prostate cancer AR negative cells in our cell line experiments, suggesting up-regulation of STAT1 expression may serve as a novel therapeutic approach for prostate cancer. However further investigations in larger

patient cohorts, utilising over expression of STAT1 in cell lines to compliment the siRNA results and *in vivo* mechanistic studies are required to confirm the role of STAT1 in prostate cancer development and progression.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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Table 1. Clinicopathological characteristics of patients with localised prostate cancer (cohort 1) and advanced prostate cancer (cohort 2).

Clinicopathological characteristics	Localised disease	Advanced disease
	Cohort 1 (M (IQR))	Cohort 2 (M (IQR))
Age (years)	71 (67-76)	70 (63-73)
Gleason Grade	7 (6-8)	8 (6-9)
PSA (ng/ml)	19 (6-78)	41 (14-43)
Disease Recurrence (years)	2.99 (1.81-3.98)	2.6 (1.92-4.89)
Follow Up (years)	10.5 (9.7-13.5)	
Ki67 proliferation index	1 (0-3)	1.5 (0-5.5)
AR expression	67.7 (42.5-81.67)	100 (60-120)
STAT1 expression		
Membrane - tumour	50 (10.8-86.7)	16 (0-42)
Membrane - stroma	0	0
Cytoplasmic - tumour	85 (50-127.5)	50 (15-94)
Cytoplasmic - stroma	0	0 (0-33)
Nuclear - tumour	35 (6.7-65)	8 (0-43)
Nuclear - stroma	3 (0-15)	0.33 (0-3.58)

M=Median, IQR=intra-quartile range

Titles and legends for figures

Figure 1. STAT1 antibody specificity and STAT1 silencing at 72 hours (A) STAT1 antibody on western blot detects a single band of 87 kDa in PC3 cells expressing STAT1 but not in PC3 silenced for STAT1. (B) demonstrates (i) that there is no non specific staining in the negative control, (ii) that STAT1 is expressed in prostate cancer tissue and (iii) that this can be blocked when the tissue is pre incubated with a peptide specific to the STAT1 antigen. (C) STAT1 antibody on western blot detects a single band of 87 kDa in both LNCaP and PC3 cells expressing STAT1 but not in LNCaP and PC3 cells silenced for STAT1. This western blot confirms that silencing for STAT1 is maintained at 72 hours and that silencing is specific to STAT1 as no effect is seen when lysates are probed with STAT3 antibody.

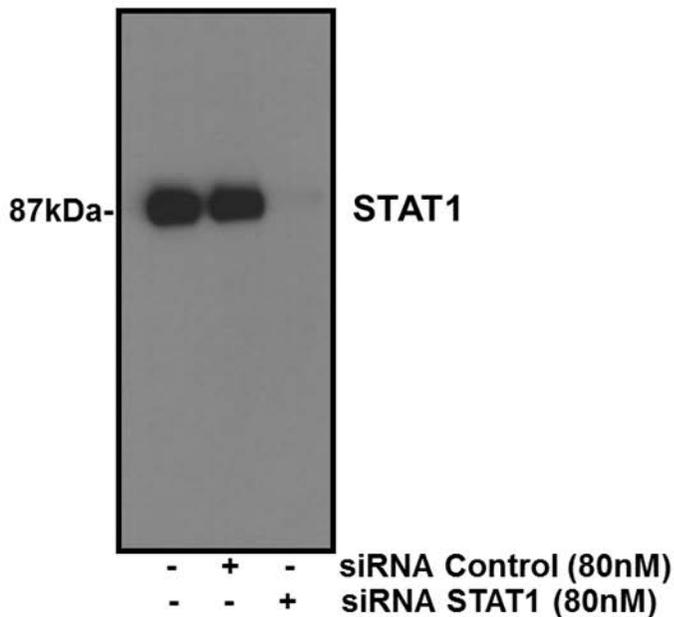
Figure 2. Loss of STAT1 is associated with poor prognosis in prostate cancer patients. (A) Representative tissue of prostate cancer specimens with low expression of STAT1 and high expression of STAT1. (B) Kaplan-Meier plot depicting that low membrane expression of STAT1 in prostate cancer patients with localised disease at diagnosis leads to significantly shorter time to disease recurrence (light grey line) compared to patients showing moderate to high expression of STAT1 (black line). (C) Kaplan-Meier plot depicting that low membrane expression of STAT1 in prostate cancer patients with localised disease at diagnosis leads to shorter disease specific survival time (light grey) compared to patients showing moderate to high expression of STAT1 (black line). (D) Kaplan-Meier plot depicting that low cytoplasmic expression of STAT1 in patients with advanced prostate cancer at initial diagnosis leads to significantly shorter time to disease recurrence (light grey line) compared to patients showing moderate to high expression of STAT1 (black line).

Figure 3. STAT1 silencing does not influence apoptosis in prostate cancer cell lines. (A) Silencing STAT1 had no significant effect on levels of apoptosis in PC3 cells. (B) Silencing STAT1 had no significant effect on levels of apoptosis in LNCaP cells. (C) Silencing STAT1 does not significantly influence gene expression of apoptotic genes using the TLDA gene signature apoptosis array.

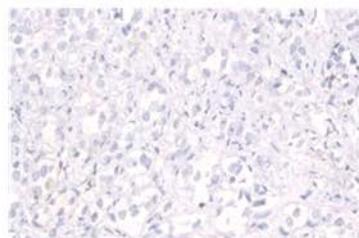
Figure 4. STAT1 silencing increases AR negative prostate cancer cell line viability. (A) Silencing STAT1 in PC3 cells resulted in a significant increase in cell viability as assessed by WST-1 assay. (B) Silencing STAT1 in LNCaP cells did not result in a significant change in cell viability as assessed by WST-1 assay. (C) Silencing STAT1 in PC3 cells resulted in an increase in cell proliferation as assessed by xCELLigence over 144 hour period. (D) Silencing STAT1 in LNCaP cells did not result in a change in cell proliferation as assessed by xCELLigence over 144 hour period. Graphical representation of results as mean \pm sem of n=3 independent experiments, * p <0.05, *** p <0.0001.

Figure 5. STAT1 silencing increases AR negative prostate cancer clonogenic capacity. (A) Photographic representation demonstrating that STAT1 silencing increases the clonogenic capability of PC3 cells. (B) Graphical representation demonstrating that STAT1 silencing increases the clonogenic capability of PC3 cells. Graphical representation of results as mean \pm sem of n=3 independent experiments, * p <0.05, *** p <0.0001.

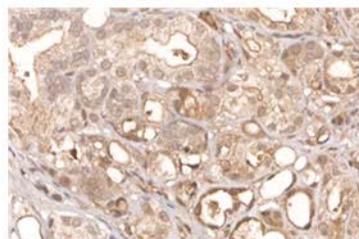
Figure 6. Silencing STAT1 in PC3 cells lead to an increase in wound closure monitored at 0, 6, 10, 14 and 20h post wounding of the cells. (A) Photographic representation demonstrating that STAT1 silencing increases the wound closure of PC3 cells. (B) Graphical representation demonstrating that STAT1 silencing increases the wound closure of PC3 cells. Graphical representation of results as mean \pm sem of n=3 independent experiments, * p <0.05, *** p <0.0001.

A.**B.**

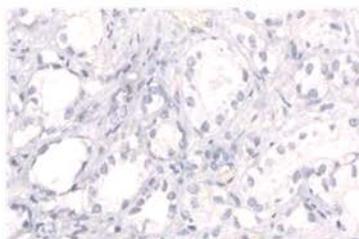
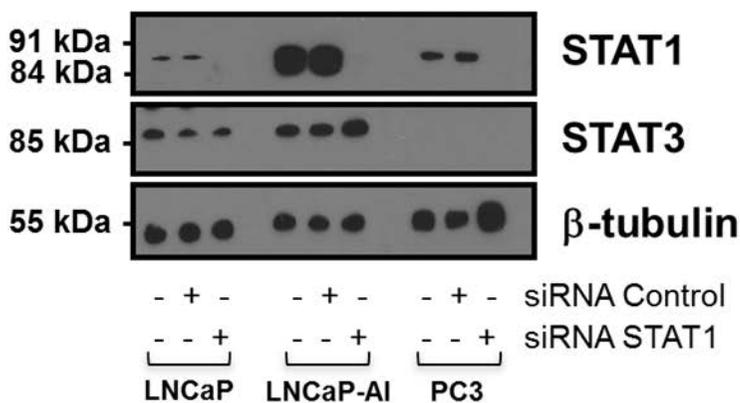
i. -ve Control

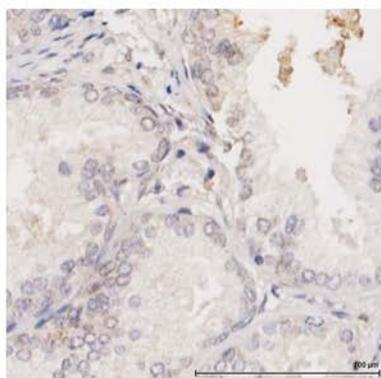


ii. STAT1 Ab

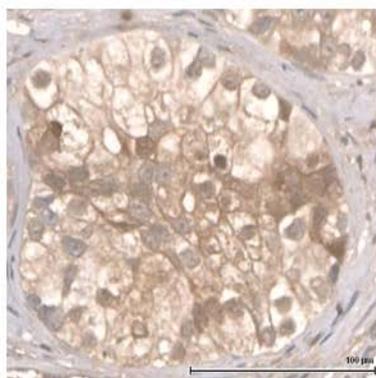
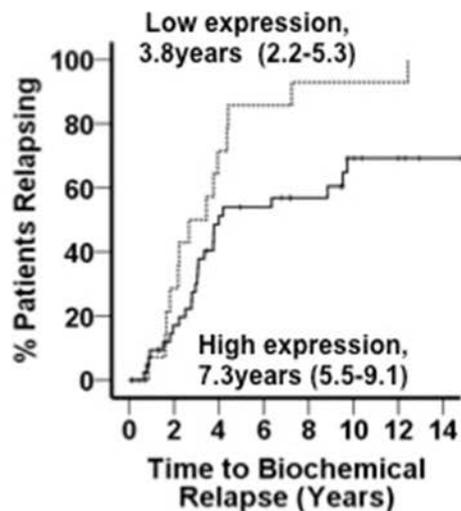
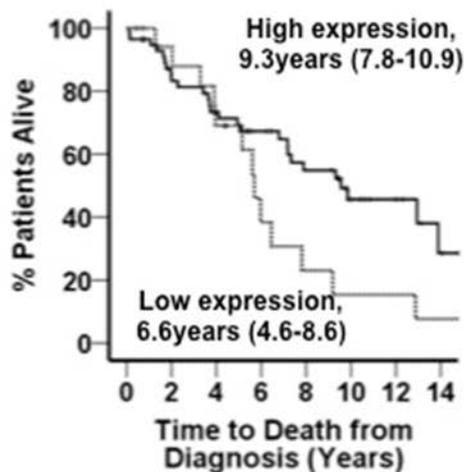
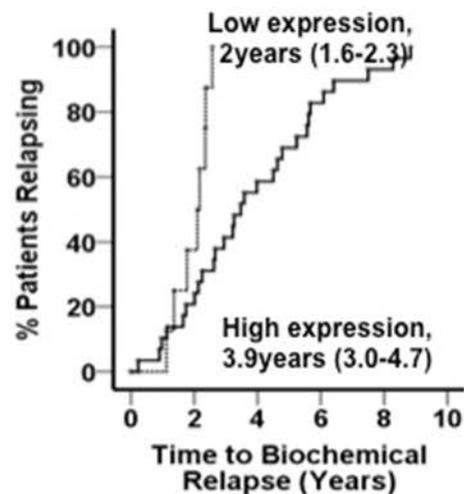


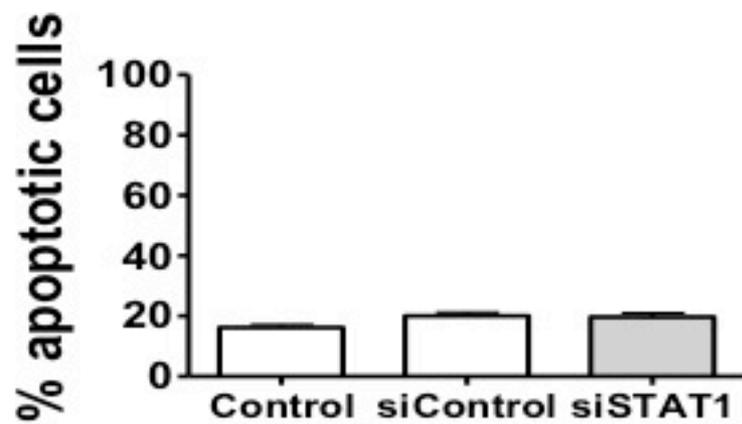
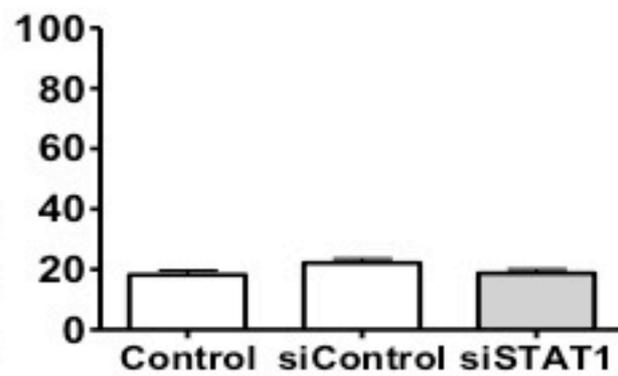
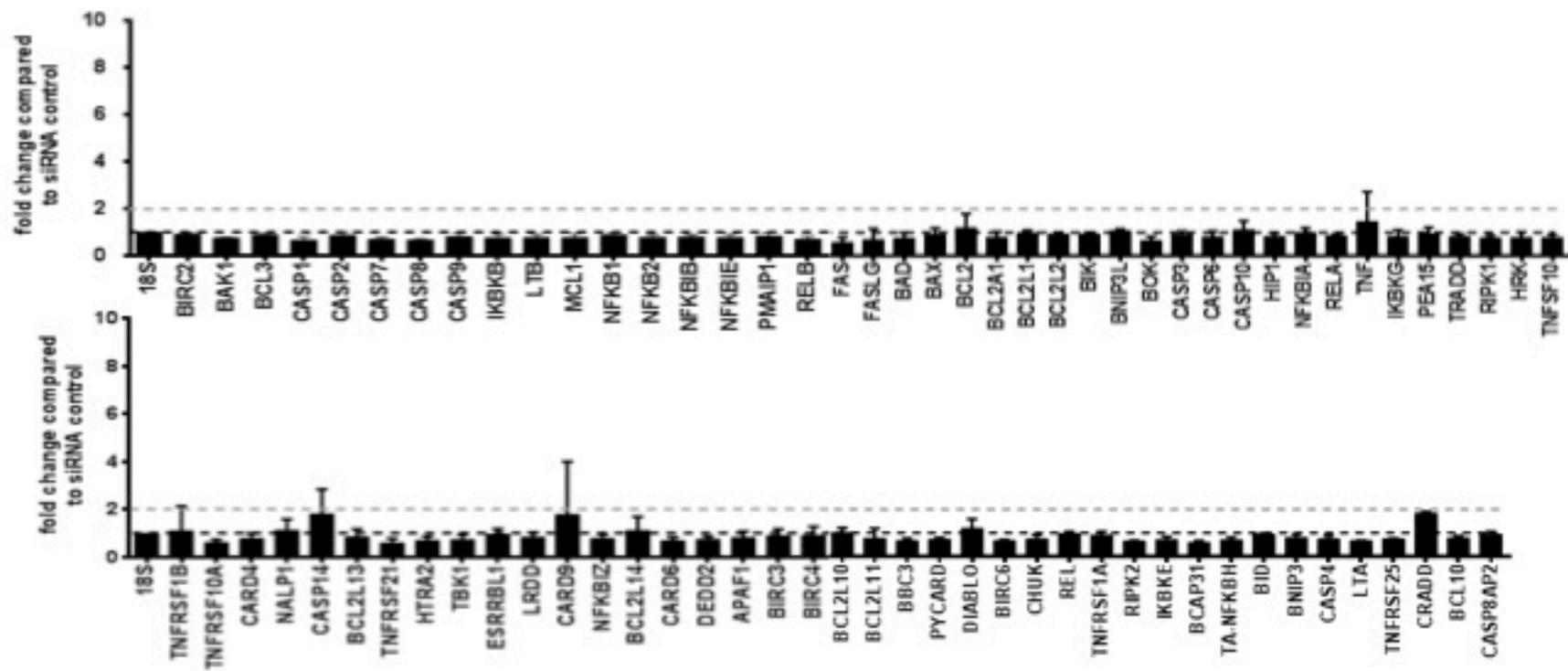
iii. STAT1 Ab + Peptide

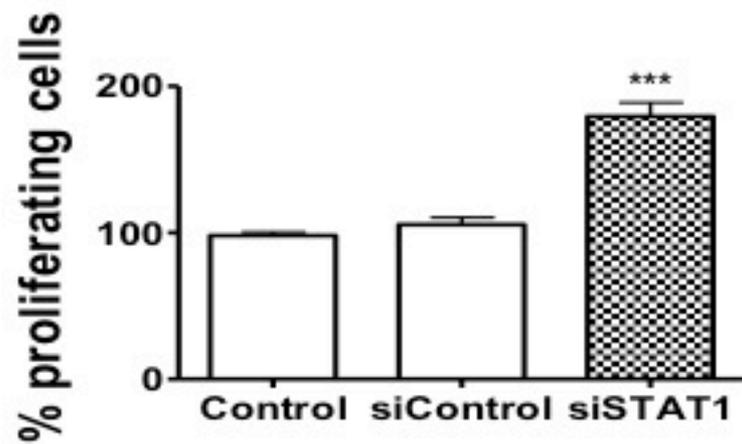
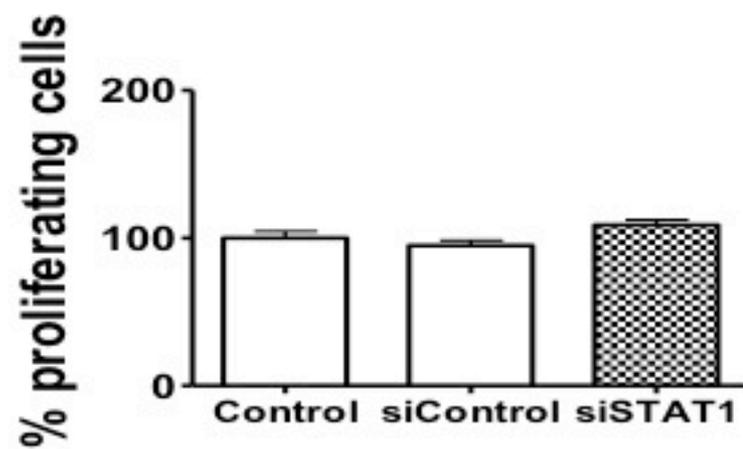
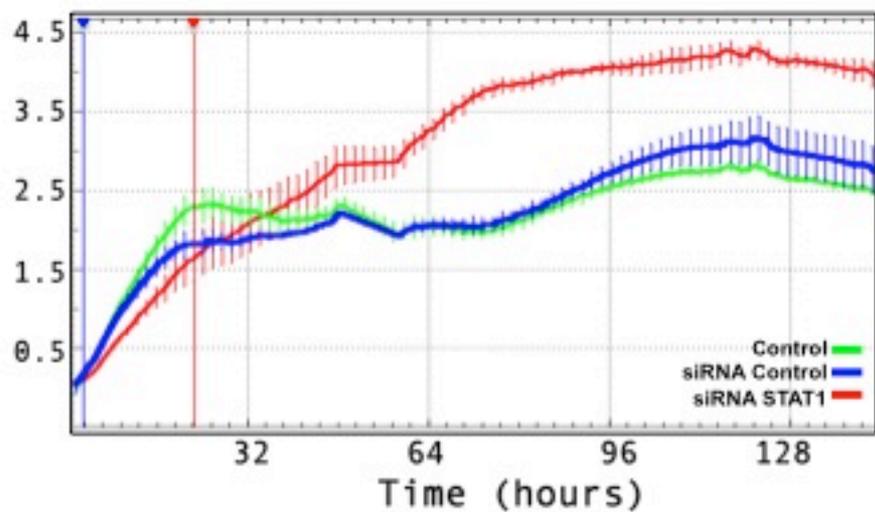
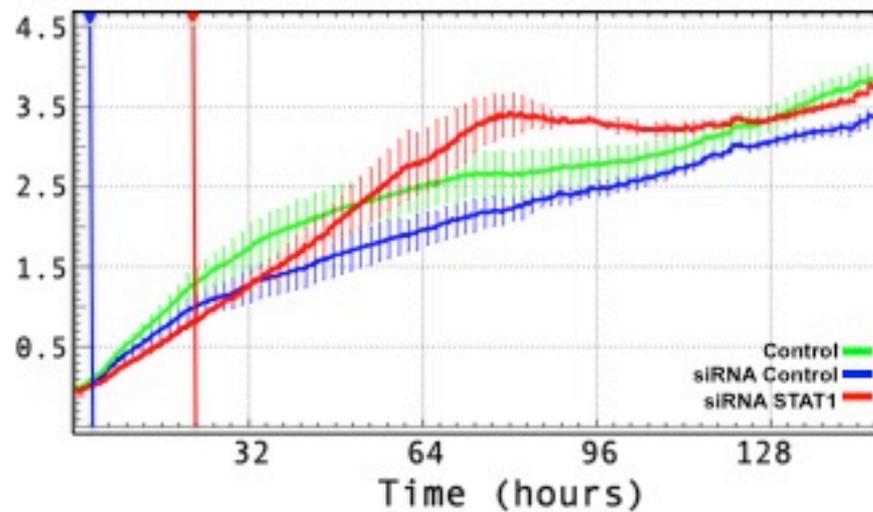
**C.**

A Low expression of STAT1

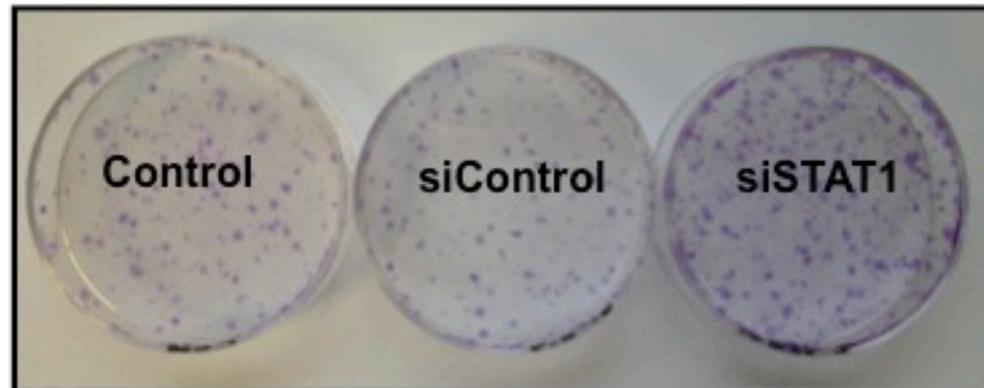
High expression of STAT1

**B****C****D**

A.**B.****C.**

A.**B.****C.****D.**

A.



B.

