

# Off-target response to decoy oligodeoxynucleotide treatment

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## Abstract

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**Background:** The transcription factor PAX2 regulates key developmental processes, including mediation of resistance to apoptosis. Inappropriate PAX2 expression has been implicated in facilitating tumour survival, and we have previously shown that siRNA-mediated blockade of PAX2 signalling at the transcript level in EJ bladder carcinoma cells promotes cell death. In this study, we attempted to disrupt PAX2 transcriptional activity in EJ cells by using a decoy oligodeoxynucleotide (ODN).

**Results:** We could not show an interaction between PAX2 and our PAX2 decoy ODN, and in both PAX2-positive EJ and PAX2-negative HEK293 control cells, decoy and control ODN transfection resulted in a marked retardation of cell growth, irrespective of sequence, but not in COS7 and NZM4 melanoma cells.

**Conclusions:** Our data indicate that decoy ODN transfection had off-target effects that inhibited cell growth in a cell line-dependent manner, and we suggest caution is required to determine the specificity of decoy ODN sequences before considering their application as a potential therapeutic agent.

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## Background

The paired box (PAX) family of transcription factors figure prominently in key developmental processes that promote proliferation, migration, and cell lineage specification, with several PAX genes implicated in orchestrating cell survival through mediation of apoptotic resistance. Evidence of a survival function for PAX2 in developing cells/tissues has been provided by studies on human patients with PAX2 mutations that cause renal-coloboma syndrome, a condition associated with small kidneys, ocular defects and hearing loss - symptoms which arise due to elevated apoptosis [1]. Pax2 mutant mice also display reduced kidney size as a result of increased apoptosis [2], a phenotype rescued by the caspase inhibitor Z-VAD-fmk [3]. In mouse kidney cells, Pax2 expression increases in response to elevated sodium chloride concentration, which is thought to protect against apoptosis caused by osmotic stress in the kidney [4].

The pro-survival role of PAX2 during development is recapitulated in several tumourigenic situations (reviewed in [5]), and blocking PAX2 activity at the transcript level has proved successful in antagonising this characteristic. Inhibition of PAX2 using antisense oligonucleotide strategies in Kaposi sarcoma cells disrupted cell migration, reduced invasive potential, and triggered cell death [6]. RNAi-mediated knockdown of PAX2 in ovarian and bladder cancer cell lines inhibited growth and promoted apoptosis [7], whereas over-expression of PAX2 conferred apoptotic resistance in PAX2-negative HEK293 cells [8]. The nature of the mechanism via which PAX2 promotes tumour survival is not fully understood, although PAX2 has recently been reported to act as a putative antagonist of the tumour suppressor PTEN [9], while the closely related PAX8 has been suggested to transcriptionally activate the pro-survival gene BCL2 [10], and repress TP53 [11].

Double-stranded ODN delivered into the cell may act as a decoy by binding to a specific transcription factor, and block transcriptional activity by competing with binding sites in genomic DNA, thus preventing expression of target genes. Decoy ODN incorporating regions corresponding to transcription factor binding sites have been used to interfere with the transcriptional activity of relatively few DNA-binding transcription factors, most prominently nuclear factor-kappa B (NF- $\kappa$ B) and E2F. Transfection of E2F decoy ODN inhibited smooth muscle proliferation and formation of vascular lesions in rat models of carotid injury [12], and have been reported to block cell cycle progression and proliferation [13–15]. NF- $\kappa$ B decoy ODN have been reported to reduce inflammation in animal models of a variety of diseases [14, 16–18], to inhibit intimal hyperplasia following vascular injury [19, 20], to suppress NF- $\kappa$ B-dependent activation of the human immunodeficiency virus enhancer [21], and show promise in ameliorating injury during allograft rejection [22, 23]. Other transcription factors targeted with this approach include IRF-1 [24], AP-1 [25, 26],

Sp1 [27], Stat6 [28,29], Stat1 [30], Stat3 (reviewed in [31]), and Ets [32,33].

We have previously shown that siRNA-mediated knockdown of PAX2 transcripts causes decreased proliferation and increased apoptosis in the EJ bladder carcinoma cell line [7]. To further elucidate the mechanism via which PAX2 mediates acquisition of malignant characteristics, we sought to inhibit PAX2 activity at the protein level. We designed a decoy ODN incorporating a consensus PAX2 DNA binding sequence, and characterised the response of transfecting decoy ODN in four cell lines including the EJ bladder carcinoma cell line.

## Results

### Decoy and control ODN treatment inhibited proliferation of EJ cells

Our initial experiments compared the confluency of cell populations 48 hours post-transfection with PAX2 decoy ODN. Visual examination revealed that PAX2 decoy ODN transfection caused a marked decrease in cell number compared to untreated controls (Fig. 1A). Unexpectedly, both mismatch and polyA negative control ODN transfections also resulted in marked reduction in cell number, similar to that of PAX2 decoy ODN transfection (Fig. 1A), whereas treatment with transfection reagent alone achieved the same degree of confluency as untreated controls (data not shown), suggesting that reduced cell number was not caused by transfection reagent cytotoxicity. To quantify these observations, we compared growth rates in treated cells by using an MTT-based cell proliferation assay 24, 48, and 72 hours post-transfection. Cells treated with decoy, mismatch or polyA ODN all displayed a retarded growth rate compared to controls (Fig. 1B), suggesting that ODN treatment had a negative impact on EJ cell growth, irrespective of ODN sequence. Assays of apoptotic activity indicated that there was no increase in cell death in ODN-treated cells compared to controls (data not shown), suggesting that the effects on cell growth were as a result of inhibited proliferation. Transfection of single ODN strands into EJ cells had no effect on growth rate, while co-transfection of unannealed complimentary ODN strands demonstrated a mild retardation of growth, presumably due to spontaneous annealing events before or after delivery into the cell (data not shown).

### Decoy and control ODN-mediated growth inhibition was cell-line specific

Next, we performed decoy ODN transfection in three additional cell lines - COS7 (monkey kidney), HEK293 (human embryonic kidney), and NZM4 (human malignant melanoma). PAX2 decoy ODN transfection had no effect on PAX2-negative COS7 cells (Fig. 2A). Mismatch ODN caused a small increase (24 hours) then decrease (48 hours) in proliferation of COS7 cells relative to transfection reagent control,

but although statistically significant, we do not consider this biologically so (Fig. 2A). Transfection of the PAX2 decoy ODN into HEK293 cells, which do not express detectable PAX2 protein (Torban et al., 2000), significantly impaired cell growth, but not in PAX2-negative NZM4 cells (Fig. 2B). These results suggest that the effect of ODN transfection was cell line-specific, and impaired growth of PAX2-negative HEK293 cells indicated that the PAX2 decoy ODN was not interacting with PAX2.

### **PAX2 decoy ODN did not obviously interact with PAX2**

To determine whether there was any interaction between PAX2 and the PAX2 decoy ODN, we transfected EJ cells with biotinylated decoy and mismatch ODN, and incubated cell lysates isolated 24 and 48 hours post-transfection with streptavidin beads prior to PAX2 protein profiling by Western blot. We found that there was no difference in PAX2 signal irrespective of streptavidin incubation (Fig. 3A). Densitometry analysis by using ImageJ software showed that PAX2 band intensity normalised to  $\beta$ -actin loading control was not decreased following streptavidin incubation compared to non-biotinylated ODN (data not shown). We attempted to elute any decoy ODN-PAX2 complex attached to streptavidin by boiling the beads, with the resultant supernatant analysed by using Western blot (Fig. 3B). No PAX2 signal was detected following this process, suggesting that either limited or no interaction took place between PAX2 and the decoy or mismatch ODN.

### **PAX2 decoy ODN did not activate interferon response genes**

Given it appeared unlikely that the observed phenotype in EJ and HEK293 cells was caused by PAX2-specific decoy ODN activity, we then investigated whether the inhibition of cell growth observed in this study could be explained by an ODN-induced antiviral interferon response. The relative expression of *OAS1*, *MX1*, *IFITM1* and *IL6*, four genes with roles in the mediation of the interferon (IFN) response, was assessed by using quantitative real-time RT-PCR (qPCR). Transfection reagent alone caused a small but significant increase in *IFITM1* and *IL6* expression compared to media-only (Fig 4). *IFITM1* expression increased slightly, but significantly, in response to decoy ODN transfection compared to transfection reagent-only controls, whereas *IL6*, *MX1*, and *OAS1* did not (Fig. 4). These data suggest that growth inhibition was probably not caused by an interferon response.

## Discussion

We present a cautionary note that has implications for the application of decoy ODN intervention as a therapeutic strategy. Whilst other studies have indicated the efficacy of decoy ODN against certain transcription factors, our results show that cell growth in two of four cell lines studied was retarded following transfection of decoy ODN, and in EJ cells this was regardless of ODN sequence, suggesting a cell line-specific response not just to decoy ODN but to double-stranded ODN in general. Determining the nature of this variable response is important as non-specific activity restricts the extent to which the decoy ODN approach could be deployed therapeutically.

In our experiments we did not detect an interaction between PAX2 and either the PAX2 decoy ODN, or the mismatch control ODN. Although the biotin-streptavidin assay has been successfully used by others to show decoy ODN binding to a target protein [34], it is possible that, in our hands, immunoblotting was not sensitive enough to detect changes in PAX2 protein level caused by interaction with the biotinylated decoy ODN. However, whether our PAX2 and mismatch decoy ODN bound to PAX2 or not is overshadowed by the observation that HEK293 cells, which do not contain detectable levels of endogenous PAX2, also showed inhibited growth when transfected with our PAX2 decoy ODN. It is possible that the decoy ODN was targeting other endogenous PAX genes; HEK293 cells contain low levels of PAX5 transcript, and no detectable PAX8 (AJ, unpublished observations), whereas EJ cells express PAX8 and low levels of PAX5 [7]. This possibility, and the mechanism of the ODN-mediated growth inhibition reported here await further investigation.

Expression of IFN target genes post-transfection were relatively unchanged in our study compared to other IFN-response studies that report up to 100-fold increase for *IFITM1* [35], up to 1000-fold for *MX1* [36], and 100- to 1000-fold for *OAS1* [37], although this may reflect defective interferon signalling in our cell types, which is reported to be a feature of many tumour cells [37]. That expression of *OAS1*, a classic interferon target gene, did not increase in our study is further evidence that a decoy ODN-induced IFN response did not occur in our cell types. Further, IFN induction pathways are probably suppressed in HEK293 cells due to constitutive expression of the adenoviral *E1A* gene, yet HEK293 cells showed strong inhibition of proliferation in response to PAX2 decoy ODN transfection, suggesting that the ODN-mediated growth inhibition was caused by mechanisms other than IFN induction.

Our results are in agreement with other reports of sequence-independent non-specific effects of decoy ODN transfection [38,39], and show some similarities to the off-target effects more recently described for antisense and siRNA intervention strategies (reviewed in [40]). In agreement with our observations

regarding cell type, Reynolds et al. [41] provide evidence that siRNA-induced IFN responsiveness is both cell-type and duplex-length specific. In addition, Anderson et al. [42] recently showed that the phenotypic effects of the antisense BCL2 ODN G3139 were BCL2-independent, and caused by the off-target induction of a stress response rather than specific down-regulation of BCL2. Gene expression profiling data suggests that siRNA commonly have off-target effects resulting in a measurable phenotype [43], and we can see no reason why the same should not be so for decoy ODN, especially considering the often degenerate DNA binding sites recognised by transcription factors. The major refinements so far described for the decoy ODN technique have focussed on improving the robustness of the decoy to withstand nuclease attack through various modifications, such as phosphorothioate additions and circular dumbbell design (reviewed in [44]), but there is little published data that we are aware of that has specifically investigated the mechanism or frequency of decoy ODN-mediated off-target effects.

## Conclusion

Given our PAX2 decoy ODN caused growth inhibition in PAX2-positive EJ cells and PAX2-negative HEK293 cells, but not in PAX2-negative COS7 and NZM4 cells, we recommend that the PAX2 decoy ODN sequences presented in this study not be used, or at least used with caution, by others wishing to target PAX2 transcriptional activity by using decoy ODN. We suggest that future work should be directed towards identifying and abrogating the potential for non-specific off-target effects induced by decoy ODN transfection.

## Methods

### Decoy ODN design

PAX2 decoy ODN incorporated the published PAX2 paired domain recognition site [45]. The likelihood of secondary structure formation was assessed using Sigma Genosys software online, and successive nucleotide substitutions were introduced into the starting sequence to identify ODN in which secondary structure was predicted to be weak or absent. These were interrogated using the web-based ConSite tool to display the consensus transcription factor binding sites contained within [46]. In this way, a 30-mer decoy ODN sequence containing a PAX2 consensus binding site (5'-AGTCACGG-3') was obtained, flanked by buffer regions to protect against nuclease attack. ConSite was similarly applied to screen decoy sequences with missense mutations to produce a mismatch control ODN containing no detectable PAX2 consensus DNA binding sites. A 30-mer double-stranded polyA ODN was included in the study as a second negative

control ODN. Pax 2 Decoy ODN (PAX2 consensus binding site emphasised), 5'-GGCGACAGAATGAGTCACGGGACCAGTGCG-3'; mismatch ODN (differences to decoy sequence underlined), 5'-GGCGACAGAATTCGTCAACGGACCAGTGCG-3'

### **Cell culture and ODN transfections**

EJ, COS7 and HEK293 cells were cultured in DMEM (GIBCO) supplemented with 10% foetal calf serum (GIBCO) and 2mM L-glutamine (GIBCO). NZM4 cells were cultured in alpha-MEM (GIBCO), supplemented with insulin-transferrin-selenium (ITS; Roche), 1% penicillin/streptomycin, and 10% FCS. Confluent populations of EJ cells were passaged at a dilution of 1:10, COS7 and HEK293 at 1:5, and NZM4 at 1:4, and seeded into 24 well plates. Twenty four hours later, media was aspirated and cells washed twice with PBS. For transfections, annealed ODN were pre-combined with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions in serum-free OPTI-MEM (GIBCO; EJ, COS7, HEK293) or MEM-alpha (GIBCO) supplemented with ITS (NZM4). For each transfection, ODN were at a final concentration of 100 nM in 500  $\mu$ L media with 1  $\mu$ L Lipofectamine 2000 (DNA:Lipofectamine 2000 ratio, 1:1). Five hours post-transfection, cells were washed twice with PBS, and normal growth media added. For proliferation experiments using 96-well plates, reagents were scaled accordingly. All ODN were synthesised and HPLC purified by Sigma Genosys. Complimentary ODN were annealed in Annealing Buffer (Tris-HCl, pH 8.0, 10 mM; NaCl, 20 mM; EDTA, 1 mM) by heating at 90°C for 1 minute, then allowed to cool to room temperature.

### **MTT proliferation assay**

Cells were passaged as above, and seeded into 96-well plates (100  $\mu$ L final volume), and transfected with decoy ODN, mismatch ODN, polyA ODN, or Lipofectamine 2000 only. Proliferation was measured 24, 48 and 72 hours post-transfection by using an MTT Proliferation Assay (Roche) according to the manufacturer's instructions. Proliferation data was combined from three separate experiments.

### **Western blotting and streptavidin pull-down**

Whole cell extracts were prepared from EJ cells 24 and 48 hours post-treatment with standard or biotinylated decoy or mismatch ODN, using RIPA buffer (150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS) containing Complete protease inhibitors (Roche). Protein was quantified by using a BCA assay (Pierce), and 10  $\mu$ g protein for each sample was applied either directly to 10%

SDS-polyacrylamide gels or pre-incubated with streptavidin beads. Streptavidin pull-down assays were performed as described [34]. Briefly, after a 1 hour incubation at room temperature, the lysate-bead mixture was centrifuged, and the supernatant applied to a 10% polyacrylamide gel as above. To investigate whether detectable PAX2 protein was retained on the streptavidin beads, the streptavidin beads were then boiled for 5 minutes to induce release of bound biotinylated ODN-protein complex, and supernatant loaded onto a 10% polyacrylamide gel, as above. Proteins were transferred using a semi-dry transfer unit (Amersham) to PVDF membranes (Millipore) and blocked in 5% milk powder/0.1% Tween-20 for 60 minutes at room temperature, prior to overnight incubation at room temperature with a primary anti-PAX2 antibody (Zymed; 1:500 dilution). Antibody was detected with peroxidase-conjugated goat anti-rabbit secondary antibody (Zymed), using an enhanced chemiluminescence kit (ECL, Amersham), and recorded by exposure to ECL Hyperfilm (Amersham).

### Quantitative real-time RT-PCR

RNA was isolated from EJ cells 24 hours after treatment with media only, Lipofectamine 2000 only, or decoy ODN by using an RNeasy kit (Qiagen) according to the manufacturer's instructions. Random hexamer-primed cDNA was generated from 1  $\mu$ g Total RNA by using Superscript III (Invitrogen) as per the manufacturer's instructions. qPCR was performed by using Platinum SYBR green qPCR SuperMix UDG (Invitrogen) according to the manufacturer's instructions, with a final primer concentration of 150  $\mu$ M. Triplicate qPCR reactions were run on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). *GAPDH*, *B2M*, *PPIB*, and *18S* were screened to identify the two most stable reference genes in our treated and control samples as determined by analysis with geNorm software [47], with both *B2M* (geNorm M = 0.152) and *GAPDH* (geNorm M = 0.153) subsequently used as reference genes to normalise the expression of *OAS1*, *MX1*, *IFITM1* and *IL6* in EJ cells treated with decoy ODN or transfection reagent-only relative to media-only controls. qBase software [48] was used to analyse the qPCR data. Primer sequences (all intron-spanning): *GAPDH* forward 5'-TGCACCACCAACTGCTTAGC-3', *GAPDH* reverse 5'-GGCATGGACTGTGGTCATGA-3' (RTprimerDB assay ID 3; [49]); *B2M* forward 5'-AGATGAGTATGCCTGCCGTGT-3', *B2M* reverse 5'-TTTCGCTCTGGTCCGTCTTG-3'; *IFITM1* forward 5'-TCCCTGTTCAACACCCTCTTCT-3, *IFITM1* reverse 5'-GTCACGTCCCAACCATCTT-3'; *IL6* forward 5'-CCACACAGACAGCCACTCAC-3', *IL6* reverse 5'-AGGTTGTTTTCTGCCAGTGC-3' [50]; *MX1* forward 5'-CAGCACCTGATGGCCTATCA-3', *MX1* reverse 5'-ACGTCTGGAGCATGAAGAACTG-3' (RTPrimerDB assay ID 3231); *OAS1* forward 5'-



AGGTGGTAAAGGGTGGCTCC-3', *OAS1* reverse 5'- ACAACCAGGTCAGCGTCAGAT-3' [51].

## Abbreviations

IFN, interferon; ITS, insulin-transferrin-selenium; ODN, oligodeoxynucleotide; qPCR, quantitative real-time RT-PCR.

## Competing interests

The authors declare that they have no competing interests.

## Authors contributions

ME was responsible for the conception of this study. ER, AJ, and ME contributed to the experimental design, and writing of the manuscript. ER designed the decoy sequences, performed the experiments, and provided initial interpretation of the data, and drafts of the manuscript. AJ carried out final data analysis and preparation of the figures, and compiled the final draft of this manuscript. All authors read and approved the final manuscript .

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## Figures

### Figure 1 - Decoy ODN inhibits cell growth in EJ cells

A, EJ cells transfected with PAX2 decoy, mismatch, or polyA ODN show decreased density compared to media-only controls 48 hours post-transfection. Images are representative of multiple different experiments. Magnifications x 100. B, Proliferation of EJ cells is inhibited by PAX2 decoy, mismatch, and polyA ODN. Error bars represent the standard deviation from the mean of three replicate experiments. Significant differences between means was determined by using two-way ANOVA; \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ .

### Figure 2 - ODN-mediated growth inhibition is cell line-specific

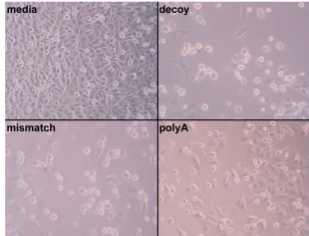
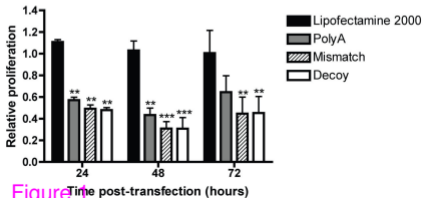
Proliferation of COS7 (A), HEK293 and NZM4 cells (B) relative to media-only controls 24, 48, and 72 hours post-transfection with PAX2 decoy, mismatch or, polyA ODN . Error bars represent the standard deviation from the mean of three replicate experiments. Significant differences between means was determined by using two-way ANOVA; \*\*  $p \leq 0.01$ , \*\*\* $p \leq 0.001$ .

### **Figure 3 - PAX2 decoy and mismatch ODN do not bind PAX2**

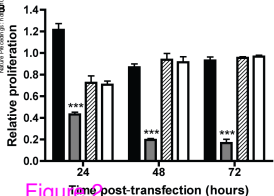
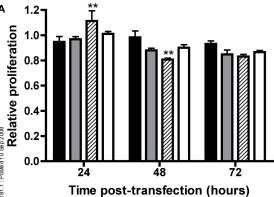
Cell lysates were prepared from EJ cells 24 and 48 hours post-transfection with decoy ODN, or biotinylated equivalents. A, lysates for each sample were applied directly to 10% SDS-polyacrylamide gel either with (+) or without (-) pre-incubation with streptavidin beads. B, Following incubation with cell lysates, streptavidin beads were boiled for 5 minutes to induce release of bound biotinylated ODN-protein complex. D, PAX2 decoy ODN; BD, biotinylated PAX2 decoy ODN; BM, biotinylated mismatch ODN.

### **Figure 4 - Growth inhibition is not likely to be caused by an interferon response**

The relative expression of four different genes with roles in the mediation of the interferon response was assessed by qPCR 24 hours post-treatment. Quantities of the respective genes are normalised to the geometric means of both *GAPDH* and *B2M* reference genes, and expressed relative to the media-only control. Error bars represent the standard deviation from the mean of three replicate reactions. Significant differences between means was determined by using two-way ANOVA; \*  $p \leq 0.05$ , \*\*\*  $p \leq 0.001$ .

**A****B**  
Nature Precedings | doi:10.1038/npre.2008.2261.1.1 | Posted 10 Sep 2008**Figure 1**

**A**



**Figure 2**

**A**

Untreated

24 hr

48 hr

24 hr

48 hr

D

BD

BM

D

BD

BM

PAX2

- streptavidin

PAX2

+ streptavidin

NeuroPrecedings : hdl:10.1016/j.pre.2008.2.281.1 : Posted 10 Sep 2008

**B**

Untreated

D

BD

BM

24 hr

24 hr

48 hr

24 hr

48 hr

24 hr

48 hr

PAX2

Figure 3



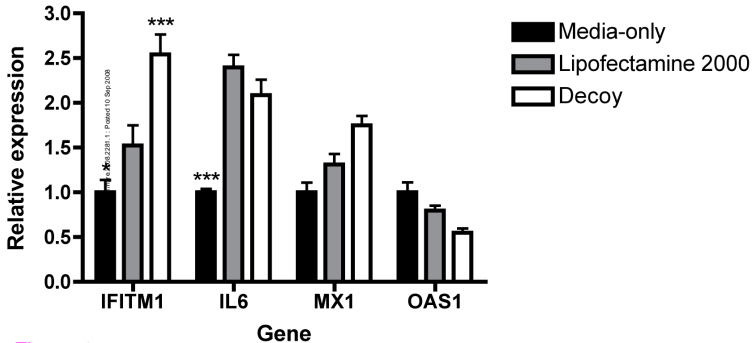


Figure 4