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Insights on ornithine decarboxylase silencing as a potential strategy for targeting retinoblastoma

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ABSTRACT

Ornithine Decarboxylase (ODC) is a key enzyme involved in polyamine synthesis and is reported to be up regulated in several cancers. However, the effect of ODC gene silencing in retinoblastoma is to be understood for utilization in therapeutic applications. Hence, in this study, a novel siRNA (small interference RNA) targeting ODC was designed and validated in Human Y79 retinoblastoma cells for its effects on intracellular polyamine levels, Matrix Metalloproteinase 2 & 9 activity and Cell cycle. The designed siRNA showed efficient silencing of ODC mRNA expression and protein levels in Y79 cells. It also showed significant reduction of intracellular polyamine levels and altered levels of oncogenic LIN28b expression. By this study, a regulatory loop is proposed, wherein, ODC silencing in Y79 cells to result in decreased polyamine levels, thereby, leading to altered protein levels of Lin28b, MMP-2 and MMP-9, which falls in line with earlier studies in neuroblastoma. Thus, by this study, we propose ODC silencing as a prospective strategy for targeting retinoblastoma.

1. Introduction

Pyridoxal-phosphate (PLP) dependent enzymes play an important role in the biosynthesis of amino acids [1]. These enzymes shown to be up regulated in different types of diseases like Parkinson disease, epilepsy, African sleeping sickness and cancer [2]. Ornithine decarboxylase (ODC) is a PLP dependent type IV enzyme, which participates in polyamine synthesis. ODC converts L- ornithine to putrescine, which is the first stage in polyamine synthesis pathway [3,4]. Polyamines are found both in eukaryotic and prokaryotic species. It is known to play an essential role in cell proliferation, differentiation and apoptosis in eukaryotic species [5,6]. Elevated levels of polyamines are reported to accelerate tumor metastasis. Inhibition of polyamines synthesis is also shown to be beneficial as evidenced in multiple clinical trial studies on cancer [7–11]. Hence, ODC serves as a potential target for different types of cancers [12–18] .

Hypusination of Eukaryotic translation initiation factor 2 (eIF5A2), (Hypusinated by spermidine), [19–21] is evidenced to up regulate the levels of Oncogenic LIN28b [22]. Up regulation of LIN28b in cancer cells leads to quenching of premature Let-7 tumour suppressor mi-RNAs. Further, it leads to altered levels of matured Let-7 mi-RNAs, thereby resulting in enhanced cancer cell proliferation [22]. An earlier study in retinoblastoma also reported the down regulation of Let-7 miRNA clusters across tissue, serum and cellular levels [23]. Over expression of LIN28b is also shown to increase the levels of c-myc in neuroblastma cells [24].

Recent *in vitro* studies report polyamines to regulate the LIN28/Let-7 pathway in colorectal cancer cells [25]. Similarly, it was also reported in animal model of neuroblastoma [26]. Matrix Metalloproteinases (MMP) play an important role in cancer cell proliferation and migration [27]. An earlier study inferred a close relatedness of decreased polyamine levels with down regulation of MMP-2 and MMP- 9, as observed in case of Difluoromethylornithine (DFMO) a known inhibitor of ODC treated Non-melanoma skin cancers (NMSCs) [28]. All these studies infer ODC to be a potential hub protein for targeting cancer, as it is the key enzyme involved in the synthesis of polyamines. Retinoblastoma is a highly vascularized and solid tumour of eye caused due to mutation in *rb1* gene [23]. The potential of ODC silencing in retinoblastoma needs to be understood for utilization in therapeutic applications. Hence, in this study, ODC gene silencing in human retinoblastoma Y79 cells was

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Table 1
showing the shortlisted siRNA's targeting ODC and Primers of ODC and 18s Rrna.

S. NO	Name	Sequence
1.	siRNA1(si 1)	AntisensesiRNA 5-3:'5AGCGUUGGACAAAUACUUUCCGUCA-3'
2.	siRNA2(si 2)	AntisensesiRNA 5-3:5'-GCAAGUUUAGCUUGAAUUAAGGGAT-3'
3	ODC	Forward primer: 5-ATATCGATGTTGTTGGTGTC-3 Reverse primer:5-AAGCAGATACATGCTGAAAC-3
4	18s	Forward primer:5-GTGGAGCGATTTGTCTGGTT-3 Reverse primer:5-GGACATCTAAGGGCATCACAGA-3

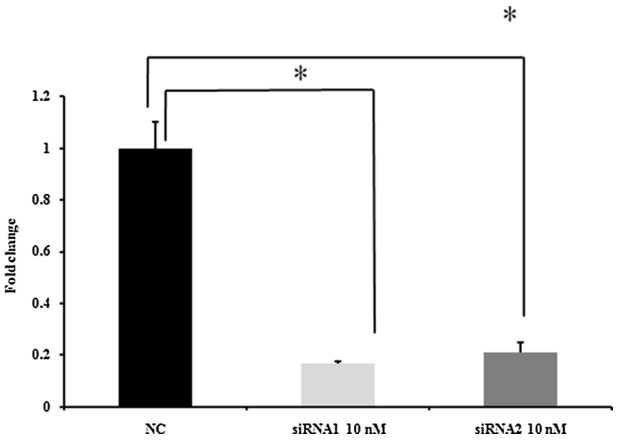

Fig. 1. Silencing of ODC in Y79 cell lines using two different siRNA1 (2a), siRNA2 (2b) and Negative Control siRNA (NC) at 48 h . (Student's *t* test was used for statistical analysis: NS-Non significant, *p < 0.05, **p < 0.01, ***p < 0.001, significance expressed as comparison with Negative Control siRNA (NC)).

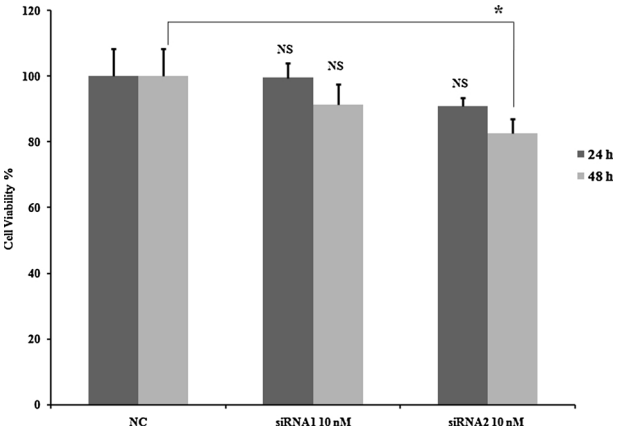

Fig. 2. Showing the cell viability observed in siRNA1, siRNA2 and Negative Control siRNA (NC) treated cells for 24 h & 48 h at 10 nM concentration. (Student's t test was used for statistical analysis: NS-Non significant,*p < 0.05 expressed as comparison with Negative Control siRNA (NC)).

performed and its impact on intracellular polyamine levels, regulation of LIN28b, MMP-2, MMP-9 and cell cycle were studied, towards providing insights on therapeutic applications.

2. Materials and methods

2.1. Cell culture

Human retinoblastoma Y79 cell line was procured from National Centre for Cell Science (NCCS, Pune, India). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and cultured at 37 °C in 5% CO2 atmosphere.

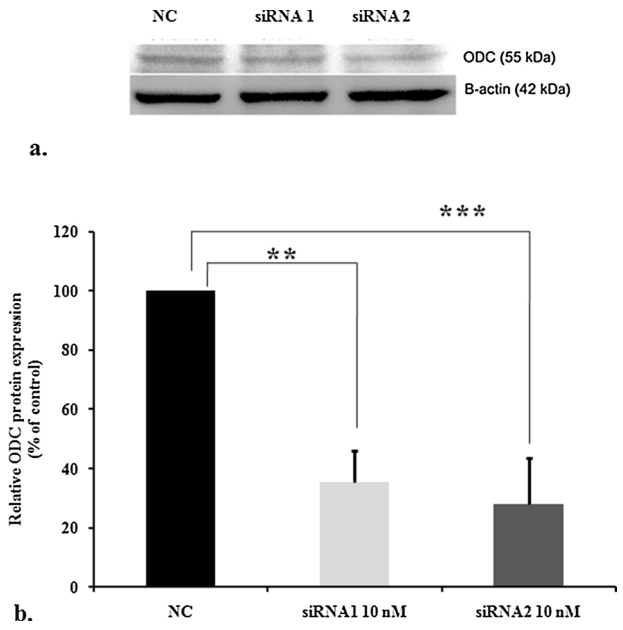

Fig. 3. Showing the impact of ODC silencing on protein level expression in Y79. (3a) Western blot analysis of ODC post silencing in Y79 cells (beta-actin expression levels were to normalize ODC expression level). (3b) Densitometry analysis of western blot showing the relative band intensity of ODC expression of siRNA transfected cells compared to NC siRNA transfected cells at 48 h. (Student's t test was used for statistical analysis: *p < 0.05, **p < 0.01, ***p < 0.001 when compared with Negative Control siRNA (NC)).

2.2. Design of ODC siRNA

The double stranded siRNA's targeting ODC expression were designed using Integrated DNA Technologies (IDT) web tool. Among the different siRNAs predicted, the top two ranking were procured from IDT (Table 1). A scrambled siRNA that does not target human genes was used as negative control (NC) [29]. The top ranking siRNA-1, siRNA-2, and scrambled siRNA (NC) in varying concentrations were used for silencing experiment. Each concentration (5 Nm &10 nM) was incubated for 15 min with Lipofectamine RNAiMAX transfection reagent (Cat # 13778030) and was further used to transfect the Y79 cells.

2.3. Real time PCR

For the real time PCR experiments, Y79 cells transfected with different concentration of siRNA (5 Nm & 10 nM) were harvested after 24 h & 48 h. Total RNA from these cells were extracted using TRIzol reagent (Sigma) by following manufacturer's protocol. Further, the RNA levels were quantified using NanoDrop ND-1000 spectrophotometer. Following which, 1 µg of total RNA was used to synthesize the cDNA (iScript cDNA synthesis kit, Biorad, USA). Subsequently, 1 µl of cDNA (50 ng) was used to study the ODC mRNA expression levels. Quantitative real-time PCRs were performed using ROCHE with SYBR Green chemistry (Roche). Primers for qRT-PCR were commercially procured (Sigma) and its sequences are given in Table 1. Real-time PCR cycle is set as follows: denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s and extension at 72 °C for 25 s. At 10 nM of concentration, both the siRNA's showed significant silencing. Hence, 10 nM was fixed as optimal concentration for ODC silencing studies. Each sample was run in triplicates and Ct was determined for the target transcripts. Relative expression levels of the target genes were normalized to 18s rRNA.