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Innovation in the Neo-Adjuvant Chemotherapy and its Implication in the Recurrence of Tumor: A scope Review

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ABSTRACT

Tumor Neo-Adjuvant Therapy (NAT) manages restorative mediation that can expand the in adequacy of the primary treatment of the tumor. A definitive objective of NAT is to dispose of a lingering store of tumor-causing cells that isn't assessable by the primary treatment (PT). This methodology when utilized as compound medications is called Neo-Adjuvant Chemotherapy (NACT). Mortality because of explicit tumor recurrence is as high as 13.5% following 3 years of PT. The high occurrence of drug resistance has been proposed a sone of the potential foundations for the recurrence process. The correlative association between drug resistance cells (DRC), quiescent cells (G_0) (stage of the cell cycle) with the recurrence process has a in recognition in the last decade. Not many investigations have been focused to target DRC (G_0) engaged in the recurrence. Likewise, none of the accessible NACT is a counted for to focus these particular cell type (G_0) with in the tumor which emphasize the importance of the current review. Moreover, there is a need for developing a genetic functional reporter assay construct specific to the G_0/G_1 transition of the cell cycle. The design of the quiescent specific genetic assay calls for the characterization of the genomic organization of regulatory elements associated with the gene involved in the G_0/G_1 transition. PubMed and Ovid Medline databases were scanned for full content articles distributed between 2008 and 2020 with detailed examination related to tumor recurrence, DRC, G_0cells, and accessible NACT in the field of oncology. In this scoping review,19 novel potential NACT agents along with its mode of action have been proposed that could be further examined by researchers in both *Insilico* and In vivo/In vitro set-up. Additionally, using multiple computational analysis software, the first time *Insilico* synthesis soft transcriptional/translational reporter specific to G_0/G_1 transition (human GOS-2-GFP) has been predicted here as a proof of concept. The 5'UTR pro-motor region, potential enhancer region, transcription binding site, promotor motif, micro RNA Regulatory Elements (MREs) have been characterized for G_0/G_1 transition human gene GOS2. For functional analysis, g RNA/Cas9 -GOS-2 *Insilico* mutational construct has been designed against quiescent specific molecular marker of GOS-2 gene. These *Insilico* findings could be considered as a molecular foundation for G_0/G_1 or quiescent biology that can have tremendous implication in treating recurrence of specific tumor that develops from DRC quiescent cells or $G1/G_0$ transition cells.

Keywords: Checkpoint Abrogation, Advanced Cancer, DNA Repair

INTRODUCTION

The tumor is heterogeneous that consists of multiple cell types [1]. The primary cause of tumor which includes environmental conditions, genetic factors manifested by stress, infection, or local injury [2,3]. The complexity and heterogeneity of the tumor a rise that is contributed by variation in the type, number and frequency of the somatic mutation [4]. Multiple types of tumor therapy have been the focus of tumor researchers [5-9] and the limitations of these approaches have been reported for the recurrence of the tumor after the primary treatment (PT) [10]. For convenience, the literature reviewed here is restricted to then on-blood solid tumor. The diverse nature of the tumor tissue with its heterogeneous cell types responds differently to the treatment process. The responsive groups have been assigned as drug-sensitive cells (DSCs)while on-responsive cells are

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defined as drug-resistant cells (DRCs) [11] or phenomenon identified as drug re-sensitivity (DR) [12].

90% tumor-related death has been reported due to the drug resistance and unproductiveness of the treatment [13-16]. Mechanism associated with DRCs include pharmacokinetics failure, ATP-Efflux pump, clonal expansion of resistance cells, and cell cycle dynamics (G_0 transition to G_1) [1-5] **(Figure 1 and Table S1)**. The DR in the solid tumor cells based on the cell cycle dynamics (G_0) and its role in the recurrence of the tumor is the focus of the present review.

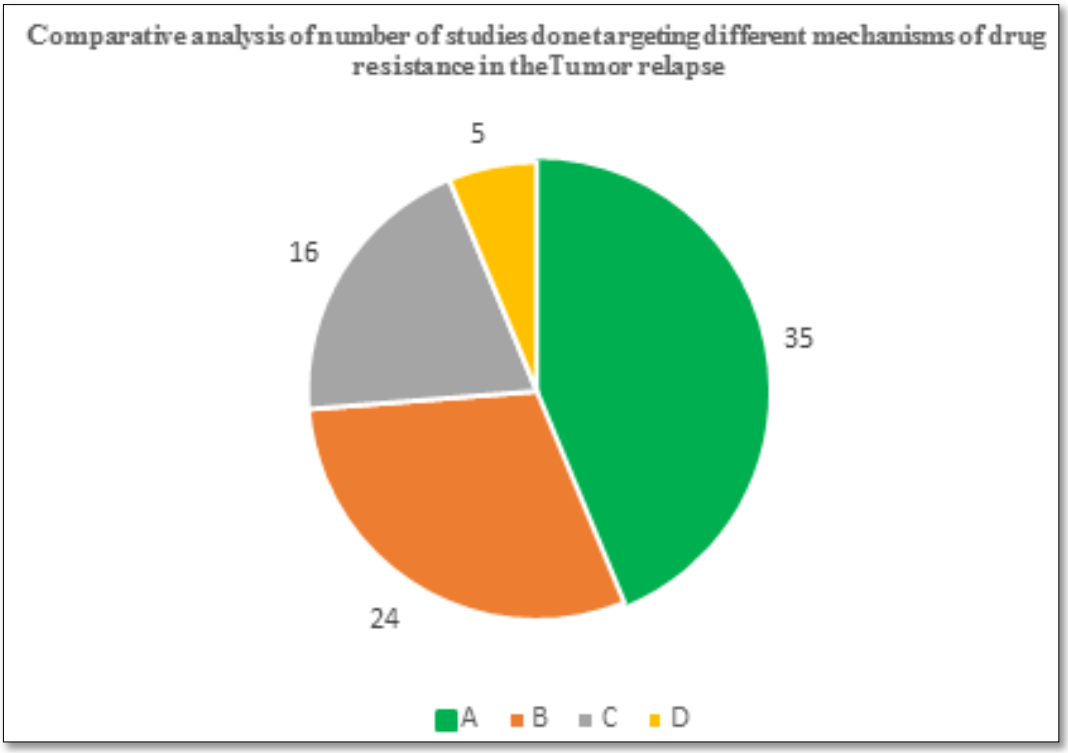


Figure 1. Comparing total number of publications in the field of tumor relapse and drug resistance targeting multiple mechanism that includes Quiescent cell biology (green), ATP-Efflux pump (brown), Clonal expansion (grey) and Pharmacokinetics failure (yellow). Key words used for primary screening of the literature includes Tumor recurrence, quiescent biology, ATP-Efflux pump, Pharmacokinetics, Pharmacodynamics etc. The raw data was collected from NCBI-PubMed, Ensemble etc. and data can be visualized in the supplementary table **(Table S1)**.

A-Quiescent Biology, B- ATP-Efflux Pump, C-Failure of Pharmacokinetics, D-Clonal expansion of drug resistance cells

The cell cycle consists of multiple mini steps of G_1, S, G_2 & M that is involved in the cell division [6] and DNA replication. An additional state that exits outside the cell cycle (quiescence-G_0) that are neither dividing or nor synthesizing DNA but remains metabolically active and considered as a distinct phase of the cell cycle [7]. The G_0 cells have been categorized as DRCs and are responsible for the recurrence of the tumor [4,8] among other possible mechanisms mentioned above. It has been suggested that once the treatment for the cancer therapy is over, the drug resistant G_0(DRC) undergoes the transition to the G1 and/or in the process contributes to the recurrence of the tumor [9]. In the field of tumor therapy, there is a need for identifying agents that can sensitize the DRC(G_0) to the drug-sensitive proliferative state and in the process eradicate the reservoir for the generation of cells contributing to the recurrence process during the post-PT [6]. In this context, it is important to mention that for the effective management of the tumor therapy, the process has been classified into two steps-the PT consists of drugs that induce apoptosis [6], inhibits protein and/or DNA synthesis/replication and thus affecting host cells translation/transcription in the multiple levels [7-9]. The mechanism mainly targeted by these drugs are microtubule dynamics affecting cell division along [10,11] with the use of alkylating/methylating agents [12,13] **(Table 1)**.

Table S1. Multiple key words were used as shown for primary screening of the literature associated with Drug resistance in the tumor tissue. Representative studies done on the mechanisms associated with the drug resistance and recurrence of the tumor are shown in the table. Average was calculated and plotted as shown in **Figure 1**.

Set	Key words used for screening number of publications in PubMed	Number of Publications	
A	Drug resistance tumor recurrence	7070	
B	Drug resistance tumor recurrence Quiescent	27	
C	Drug resistance tumor recurrence ATP efflux Pump	15	
D	Drug resistance tumor recurrence Clonal expansion	13	
E	Drug resistance tumor recurrence Pharmaceutical failure	5	
A'	Drug resistance tumor relapse	10182	
B'	Drug resistance tumor relapse quiescent	44	
C'	Drug resistance tumor relapse quiescent ATP Efflux Pump	34	
D'	Drug resistance tumor relapse Clonal expansion	19	
E'	Drug resistance tumor relapse Pharmaceutical failure	5	
			Average
A'	Drug resistance tumor relapse	10182	
Average C/C'(C'')			
	15	34	24.5
Average D/D'(D'')	13	19	16
Average E/E'(E'')	5	5	5
B''=A		35	
C''= B		24	
D''=C		16	
E''=D		5	

The focus of the present. Review is Neo-Adjuvant Therapy (NAT) [14-16,29] especially chemotherapeutic (C) part of the NAT that is focused on the relapse of the tumor tissue (NACT). The most common form of NAT (non-chemotherapy) used is surgery and irradiation that is still relevant in the management of solid tumor therapy that may be pre-operative or post-operative [30-33]. The NAT aims to eliminate the residual tumor cells that are not accessible by the primary treatment (PT). Moreover, it should prevent there-establishment of the tumor or the relapse after the PT course is stopped. There is a requirement for improving the existing NAT to be used against solid tumors specially chemo-NACT as very few of them are focused on the G_0DRCs [6,7]. The process of identifying, screening and investigating potential NACT agents should fulfill one critical criterion - the DRCs(G_0) should be sensitized to undergo cell cycle entry viaG_0-G_1 transition before PT. In preparation for predicting and proposing potentially novel NACT agents against tumor recurrence, it is very crucial to understand the cell biology of G_0 cells and mechanisms that can be manipulated for the artificial transition of (G_0) to the (G_1 phase). The genome and cell cycle dynamics of *Saccharomyces cerevisiae* is relatively well conserved with that of the mammalian cell cycle [7,8]. Using literature review on cross-species comparison of cell cycle dynamics across multiple species, the following concerns were raised that are as follows-

- Is there any reporter assay available that can track the duration of the G_0 phase and monitor the effect of

Table 1. List of PT agents used during anti-tumor treatment for non-brain types with their potential mode of action.

Tumor Type	Adjuvant	Clinical trial	Mode of action
Melanoma	Interferon α-2b	CT [17]	JAK-STAT
	Dacarbazine	CT [18]	Alkylate DNA
	Temozolomide	CT [19]	Methylate DNA
Colorectal	Fluorouracil	CT [20]	TS* inhibitor
Pancreatic	Gemcitabine	CT [21]	DNA inhibition
Bladder	Platinum based	NR [22]	Alkylate- like DNA
Breast	Cyclophosphamide	CT [23]	RNA/DNA crosslink
	Methotrexate	CT [24]	Inhibits DHFR**
	Fluorouracil	CT [25]	TS* inhibitor
	Doxorubicin***	CT [26]	Inhibits Topoisomerase II
Ovarian	Cisplatin	CT [27]	Inhibit cell division
Cervical	Platinum based	CT [28]	Antineoplastic drug
Testicular	Not well defined	N/A	N/A

: TS-Thymidylate Synthase; **: DHFR-Dihydrofolate reductase; *: Affects the relaxing of supercoiling of enzyme and thus inhibit transcription. CT; clinical Trial; NR; not reported*

drugs/Inhibitor on the G_0 dynamics?

- What is the genomic information (promotor analysis, motifs, enhancers, microRNA etc.) one should have to make an appropriate G_0-specific reporter construct?
- Can we use already available drugs (pro-proliferative factors) that have the potential to induce G1 entry?
- It is of importance to note that among all the known mechanism associated non-responsive of the tumor to PT, the review focusses on the drug resistance originating because of quiescent cells.

Materials & Methods

Literature review

PubMed, Uniport, Ensemble, UCSC databases was used to identify publication related to Tumor recurrence, drug resistance and quiescence biology.

In silico analysis-Sequence analysis

Blast, BlastP, Smart Blast, EMBOSS Needle, Blat was used to align the RNA and Protein sequence. Promotor analysis to identify Transcription factor binding site-Augustus version [8] for promotor analysis and PROMO from TRANSFECTS. Micro RNA Regulatory Element identifier Target Scan Human was used for the identification of MREs along with sequence-specific comparison of micro RNA target site in the 3'UTR of GOS-2 gene.

Gene Enhance locator

The potential enhances relementin 5'UTR was examined during UCSC genome browser and Eukaryotic Promotor Database.

Conserved domain analysis

The software like CCD in NCBI, InterPro in EMBL-EBI was used for identifying con- served domains in the protein's primary structure.

Restriction digestion mapping

GenScript, Restriction mapper 3 software were used for mapping the restriction enzymes sites in both promotor region of the GOS-2 gene as well as coding region of the GFP **(Table S2)**.

Insilico Primer designing

Primer 3 and Primer-Blastin NCBI was used for designing gene specific primers from different regions of the genome **(Table 4)**.

Generation of restriction site in the primers specific for GOS-2 (5'UTR/CDS) for GOS-2 transcriptional assay

The primers are designed following the standard guidelines of 5'UTR-sitting sequence (4 nucleotide), restriction site (6 nucleotide) and GOS2 specific sequence. Kp1 and EcoR1 restriction digestion sites were added in the respective 5'UTR/3'UTR of the GOS2 specific primer. The generation of restriction sites with PCR were then digested with both the restriction enzymes and subsequently ligated to the vector (pPD95.75). For Kp1 (438bp) RE, GOS2-F-5'-AGGGT-GGTACC-GAGAGGAGGAGAACGCTGAG - 3' and for EcoR1 (1012bp), GOS2-R-5'-GGCGGC-GAATTC-CTCTCGGAGGCGGGAATG - 3' was used using Primer 3 software.

Repeat masker analysis

In order to avoid repeats within the PCR amplified regions of the template, Repeat-Masker software was used.

Table S2. The sequence collected from NCBI-GENE identified (NM_015714.4) was analyzed in the RestrictionMapper 3 software for mapping the restriction enzyme sites present in the first 170bp 5'UTR of the gene.

Name	Sequence	Site Length	Overhang *	Frequency	Cut Positions
PvuII	CAGCTG	6	blunt	1	36
AvaII	GGWCC	5	five_prime	1	69
BbvCI	CCTCAGC	7	five_prime	1	57
Bpu10I	CCTNAGC	6	five_prime	1	57
BsmAI	GTCTC	5	five_prime	1	19
FauI	CCCGC	5	five_prime	1	153
HgaI	GACGC	5	five_prime	1	87
PleI	GAGTC	5	five_prime	1	20
BfiI	ACTGGG	6	three_prime	1	31
BseMII	CTCAG	5	three_prime	1	48
BsrI	ACTGG	5	three_prime	1	26
EciI	GGCGGA	6	three_prime	1	24
NmeAIII	GCCGAG	6	three_prime	1	65
SduI	GDGCHC	6	three_prime	1	160
TauI	GCSGC	5	three_prime	1	9

**=The 5'UTR, 3'UTR mentioned are within the context of initial upstream 5'UTR region of the coding sequence*

In silico PCR

Using In silico PCR facility in UCSC genome browser, the primer designed were used to perform Insilico PCR.

Tissue culture

Mammalian breast cell culture line was maintained according to the standard procedure namely Hela and MDAMB231celllines [9].

Transduction of viral construct (FUCCI) in the cell lines

FUCCI plasmid with its G_1 specific Cdt1-RFP fused protein along with M-G_2 specific Geminin-GFP protein is introduced in the cell lines during the proliferative phase of the cell cycle. The technology is called as FUCCI (Fluorescence ubiquitination cell cycle indicator (FUCCI), a translational reporter assay where genes are do unregulated by the ubiquitination process [10,11]. The expression of Cdt1-RFP showing red fluorescence indicates cells in G_1 phase of the cell cycle and expression of geminin-GFP showing green fluorescence indicates G_2 phase of the cell cycle [12]. It's an advanced biomedical technique to monitor the individual stages of the cell cycle under live condition using fluorescent microscopy.

Premo FUCCI Cell Cycle Sensor BamMam 2.0

The two viral vectors consist of Premo gemin in-GFP and Premo Cdt-1-RFP were introduced separately into the Hela and MDAMB231 cell lines as previously described [13]. The two constructs were mixed prior to adding the solution in the cell lines. 40ul of Premo Geminin-GFP is added to the 2 ml of culture medium (DMEM) in 12 well tissue culture plate. Cell culture medium was replaced with 2 ml of transduction solution and cells were incubated for 2 h at 37ºC. Additional enhancer solution provided in the kit (1X BacMam) was mixed with fresh medium and finally added to the cells for further incubation at 37ºC. Fresh medium was given to the transduced cells followed by 16 h incubation at 37ºC.

Imaging with Spinning Disc fluorescent microscopy

The transduced cells under the influence tryps in gets detached from the cell culture plate and after 20 h transferred to 35 mm glass bottom tissue spinning Disk 2.6-Image Processing Software-Image J and Photo shop and movies were made using Xen-blue/black software. Time lapse images were captured after regular time point with the automatic setting parameters which attached to a colored camera. The imaging continued for 72 h post-transduction.

FACS analysis

In order to study the successful transduction efficiency of the Fluorescent probes in the cell culture, FACS analysis was performed as described earlier [14]. The minimum of 50% transduction efficiency was used as a threshold in FACS analysis that was further processed for the visualization under microscopy **(Figure S1)**.

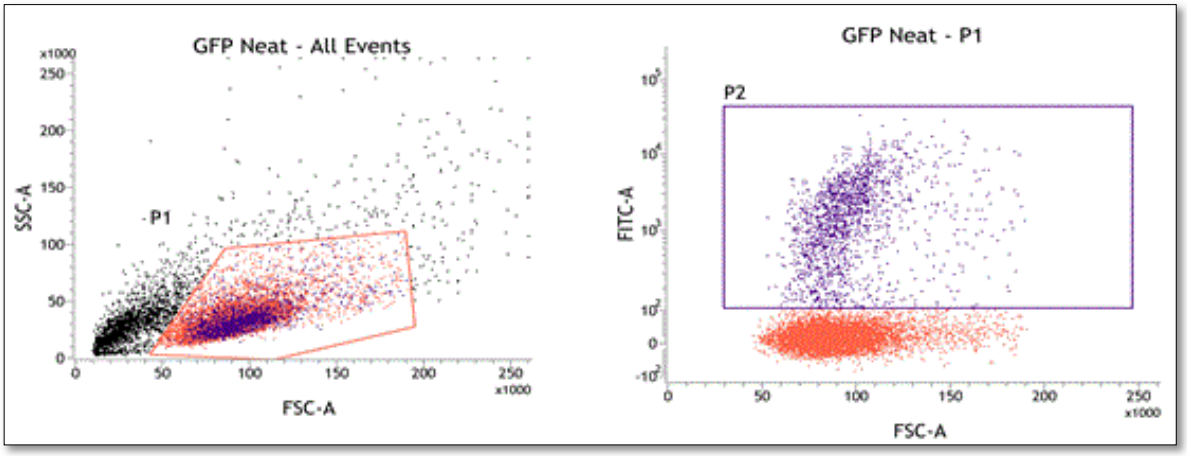


Figure S1. Dot-blot using FACS analysis- The transduction of the viral constructs into the mammalian cell lines could be represented in the Dot blot as shown above. The left-hand panel represents distribution of the cells in the based on the size, the most optimal being with the assigned box whereas the uneven or double lets cells are eliminated from the analysis (shown in black color). The right-handpanelrepresentstheGFPlabelledcellsindarkblueassignedinsidethebox.Theanalysiswas carried using FITC-A filter in the FACS instruments.

RESULTS

Cell cycle detection visualization and associated functional drug assay using molecular markers fused to are porter gene has been described in the literature [15,16]. The identification of different stages of cell cycle is done using markers in G_1 (Cdt-1-RFP-red) and G_2 phase (Geminin-GFP-green) of the cell cycle [4,12,15]. However, there is a gap in the information regarding visualization and identification of the quiescent cells (G_0) for downstream functional analysis. Neither any transcriptional/translational reporter assay associated with the quiescent specific cells have been documented nor any gene editing tool targeting quiescent cells have been described. In order to select appropriate gene reporter system for understanding quiescent cell biology, it is of critical importance to understand and study the regulatory elements associated with the 5'UTR and 3'UTR of the gene related to G_0/G_1 transition process.

In silico synthesis of G_0 specific transcriptional GOS 2-GFP fusion reporter

Using PubMed, Ensemble and UCSC genome browser, a total of 6 genes have been screened that shows high level expression in the G_0 phase of the cell cycle and its down regulation is accompanied by transition to the G_1 phase **(Table 2)**. G_0/G_1 switch - 2 (GOS-2) first identified in chicken, a protein coding gene with multiple function in the apoptotic pathway and lipid metabolism [29,30]. In silico sequence analysis shows human GOS-2 gene is 876bp long with 5'UTR-170bp, CDS-312bp, 3' UTR-394bp along with positional sequence information of the initiation codon **(Figures 2 and 3)**. The human GOS-2 translated protein is 103aa long and using Smart BLAST analysis, it shows 49.51% identity with the chicken sequence **(Figure 3c)**. Using conserved domain identifier (CDD) in NCBI, the gene has been clustered to the superfamily of $G_0\_G_1\_switch\_2$ genes **(Figure 3d).**

In Silico synthesis of quiescent cell specific transcriptional reporter of Human GOS-2gene (hGOS-2-GFP). The genomic organization of the human GOS-2 genem RNA (NM_015714.4) fused to the coding region of the GFP. The fusion protein is a transcriptional reporter and hence only promotor region of the h GOS-2geneis considered for the analysis. The sequence was identified using UCSC genome browser that interactly visualize genomic data. The m RNA positional information was gathered by RNA fold software to identify the promotor, coding sequence and downstream 3' prime region of the gene as shown above. The typical promotor consists of proximal, distal and core regions having binding sites for RNA polymerase (RNA Pol II},

General Transcription Factors (GTFs) and tissue-specific Transcription Factors (TFs). The 5'UTR (untranslated region) of Human GOS-2sequence is shown on the left panel with its ATG initiation codon (Bold). The coding region of the GFP (C.Elegans) (as a part of vector pPD95_75 (Addgene)is used for the analysis. The sequence analysis of the GFP was

Table 2. List of the genes that has been proposed to be involved in the transition/regulation of the quiescent (G_0) to (G_1) phase in the different cell types.

Gene	Proliferation	Type of cell	Potential Adjuvant
p27Kip1 [34]	Indirectly	Fibroblast	Yes
Neuroligin-3 [35]	Yes (PI3K/AKT)	NSC/OSC	Yes
GOS2(chicken) [36]	G0/G1 switch	Blood/Lung	Yes
egr-1 [37]	G0/G1 switch	Blood	Yes
Cyr61 [38]	G0/G1 switch	Fibroblast	Yes
ATF2 [39]	G0/G1 switch	cell culture	Yes

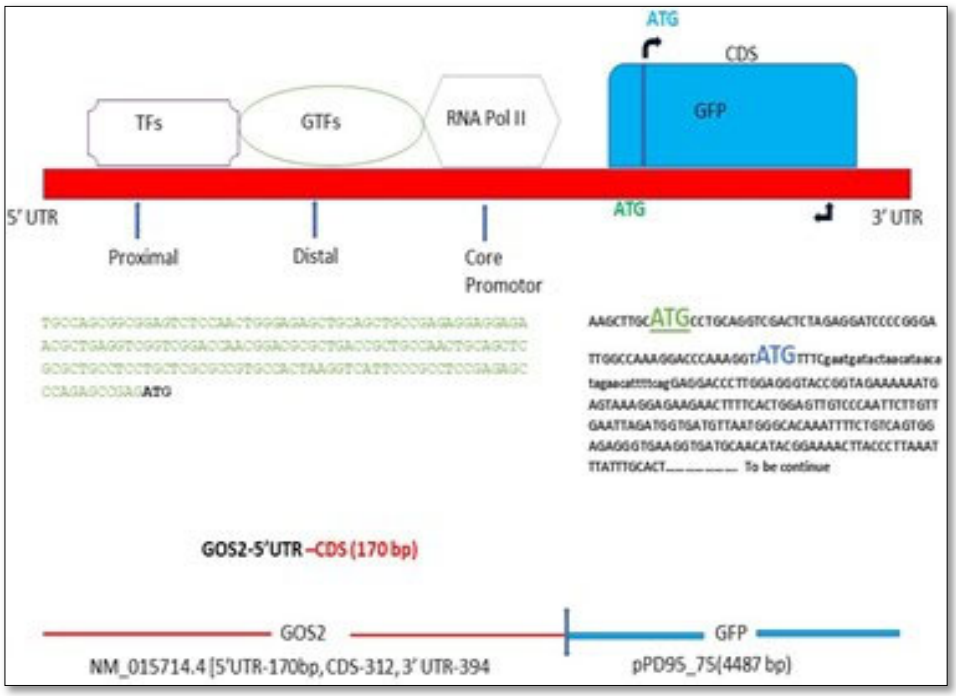


Figure 2. Transcriptional reporter constructs specific to quiescent cell (human GOS-2 gene).

identified from the publicly available web portal. ppD95_75 (AndrewFire-Addgeneplasmid#1494: http://n21.net/addgene:1494:RRID:Addgene_1494]. Analysis of protein sequence using CCD and Blast P shows pfam gene is the only other member of this superfamily **(Figure 3d)** that are known to be involved in apoptosis by preventing the complex formation of BAX-BCL2 protein [29]. As a proof of concept to design a reporter assay specifying, marking and visualizing G_0 phase pf the cell cycle, GOS-2 gene sequence was selected based on its role in the transition process from G_0 to G_1 **(Table 4)**. For the *In silico* synthesis of the transcriptional reporter (GOS2-GFP), promotor region of the human GOS2 (NM_015714.4) was used along with the coding region of the GFP (pPD95_75) [31].

(a) NM_015714.4 – 876 bp G0/G1 switch protein 2 [Homo sapiens] [5'UTR-170bp, CDS-312bp, 3' UTR-394bp

TGCCAGCGGCGGAGTCTCCAACTGGGAGAGCTGCA
GCTGCCGAGACCAACGGACGCGCTGACCGCTGCC
AACTCAGCTGCTG
CCTCCTGCTCGCGCCGTGCCACTAAGGTCATTCC
CGCCTCCGAGAGCCCAGAGCCGAG**ATG**GAAACG
GTCCAGGAATCC
CCCTGGCCAAGGAGATGATGGCCCAGAAGCGCA
AGGGGAAGATGGTGAAGCTGTACGTCGTGGTGC
TCGGGGAGAC

TGTGTGCAGCCCCTTCACGGCCGCCAGACGTCTG
CAGCCGTGGCGGAGCTGCAGGCCGCCCTGGAGC
GACAGGAGAAGC
AAGCCCTGCAGGAGAAAGGCAAGCAGCAGGACA
CGGTCCTCGGCGGCCGGGCCCTGTCCAACCGGCAG
CACGCCTGGAA
CTGTGGGAGACCAGCGGAGTGGGAGGGAGACGC
AGTAGACAGAGACAGACCGAGAGAGGAATGGAG
AGACAGGGG
CGCGCGCACAGGAGCCGACTCCGCTGGGAGAGTGC
AGGAGCACGTGCTGTTTTTTATTTGGACTTAACTT
CAGACCGC

TGACATCTAGAACTGACCTACCACATCCACCAAA
GGAGTTTGGGATTGAGTTTTGCTGCTGTGCAGCA
CTGCATTGTC
ATGACATTTCCAACACTGTGTGAATTATCTAA**AT**
GCGTCATTTTGCACTAGGGAGGAAGGATAA**ATG**
CTTTTT**ATG**TTA
TTATTAATTATTACA**ATG**ACCA

(b) NP_056529.1 – 103aa G0/G1 switch protein 2 [Homo sapiens]

METVQELIPLAKEMMAQKRKGKMVKLYVLGSVL
ALFGVVLG
LMETVCSPFTAARRLRDQEA

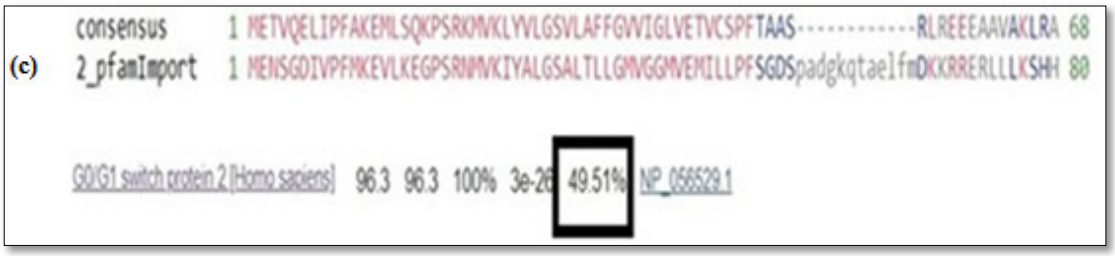


Figure 3. Complete mRNA sequence of human GOS-2gene (NM_015714.4) with its sequence positional information of 5UTR, CDS and 3'UTR region Different color codes were used for identifying 3 important regions of the transcript. The corresponding protein coding region is highlighted above for the human GOS-2 protein (NP_056529.1) (A). The protein is 103 amino acid long (B). Using conserved domain data base (CDD) in NCBI, the superfamily was identified as G_0/G_1 switch 2 family of genes with conserved pfam sites as shown above in the (C). The chicken protein GOS-2 shows 49.51% identity with its human counterpart (D). The identity of the protein region was analyzed using Smart Blast, a tool used for identifying known homologous sequences (Smart basic local alignment search tool).

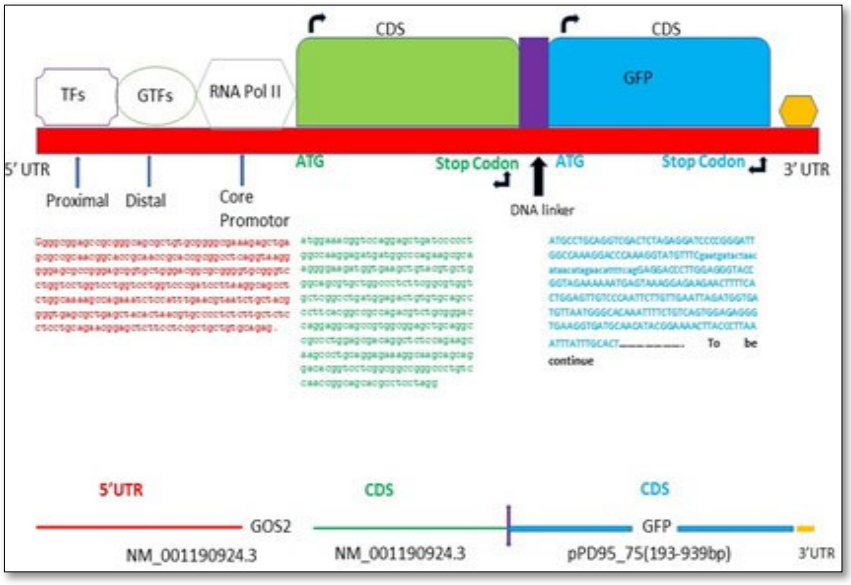


Figure 4. Translational reporter constructs specific to the quiescent G_0 cells- The 5'UTR and Coding region was extracted using human $_{G0}$S-2 (NM_001190924.3) gene using NCBI and EPD. The promotor region considered for the constructs runs-170 bp upstream from the transcription initiation site. The whole extent of the coding sequence was considered from the GOS-2 gene sequence. The DNA linker of Bam HI was used to join the fused transcript of GOS-2 coding region and GFP coding region located in the vectorpPD95.75 (4487bp).

In silicosynthesis of quiescent specific (G_0) translational reporter fused with the GEP

The translational reporter system gives much better resolution in terms of expression profiling and subcellular localization with additional features of 3'UTR associated post- translational modification [32]. Multiple ways translational reporter system could be synthesized and in the present review, the proposed construct consists of 5'UTR/promotor of gene of interest (GOS-2) followed by the coding sequence of GOS-2 and coding sequence of the reporter (GFP) with 3'UTR extension **(Figures 4 and S2)**. For the generation of the translational reporter construct, 170 bp upstream of the TSS are considered for the *In silico* analysis. The DNA linker used for the fusion of the CDS of GOS-2 gene and GFP gene present in the vector (pPD95.75) that was done by using Bam HI DNA linker **(Figure S2)**.

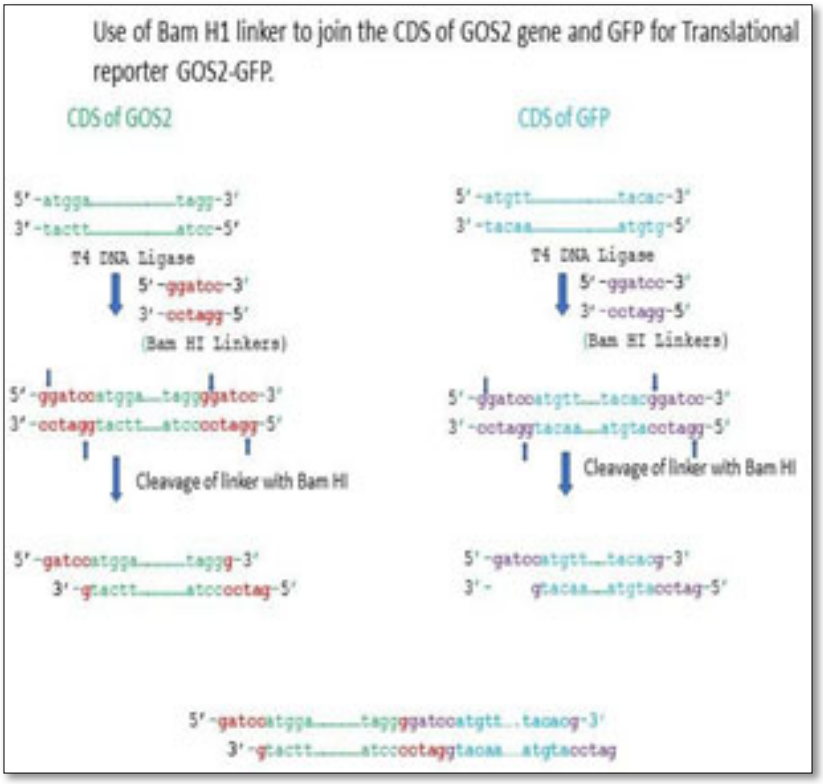


Figure S2. For generation of Translational Reporter constructs**,** BamH1linker was used for joining the coding sequence of GOS-2 gene and coding sequence of the GFP present in the vector.

In silico synthesis of gene editing tool of gRNA/C as 9 constructs targeting G_0 specific quiescent specific GOS-2 molecular marker

Targeted gene editing using gRNA/Cas 9 has been extensively used in the field of genome manipulation [33]. It is a genome editing tool using guide RNA (gRNA)and scaffold RNA (scRNA)to identify target sequence of 20 nt long to generate double stranded cut by Caspase 9 enzyme [40]. The activation of the DNA repair mechanism contributes to insertion or deletion of nucleotides in the target sequence finally resulting in the frame shift mutation [40]. In the present scope review, Ex on 1 and Ex on 2 was used a sagRNA target construct that runs from 263-282 bp and 329-348 bp of GOS2gene (NM_001190924.3) respectively. The PAM (protospacer adjacent motif) sequence for both the g RNA/Cas9 system are TGG (283-285bpposition) &AGG (348-351bpposition) immediate downstream of gRNA sequence belonging to Ex on 1 and Ex on 2. The gRNA/Cas9 makes a double stranded cut of 3 nucleotide upstream of the PAM sequence in 279 and 346 position respectively. The DNA repair mechanism would try to amend the double stranded cut and, in the process, generating frameshift mutation that finally affects the function of the gene **(Figures 5 and 6)**. The plasmid pSpCas (BB)-2A-GFP(PX458) is digested by 2XBbsl to generate overhangs followed by the synthesis of the sense and antisense g RNA oligos with 5' & 3' 2XBbsl overhangs that complement the vector overhang **(Figures 6a and 6b)**. Typical DNA ligation with T_4 DNA polymerase and subsequent transformation and cloning into the bacterial cell **(Figures 6c and 6d)**. Extraction and purification of the plasmid for downstream functional analysis along with microscopy and phenotypic analysis are among multiple approaches that could be exploited to in order to study quiescent biology and G_0/G_1transition.

Promotor analysis of G_0-speciftc Human geneG0S2 Identification of several motifs in the upstream -1000 to 100 bprelative toTSS.

Eukaryotic Promotor data base was used for identifying upstream 5'UTR region of the quiescent G_0-specific GOS2 gene in Human (NM_015714.4). Sequence retrieval tool was used to extract the promotor sequence-2000bp upstream relative to TSS **(Figure S3)**. Promotor motifs were identified using search motif tool in the library from-1000 bp to100 bp of the TSS. The two TATA-box were identified at -664bp and-29 bp position relative to TSS with an initiator at-402 position. GC-boxin-47position followed by the presence of another Gcbox with in the first cod on at position 8 downstream of TSS. Moreover, two CCAAT-Box at position -587 bp and -547bp is present relative to the TSS.

Computational prediction of transcription factors binding site associated with the promotor region of G_0- specific gene (GOS2) using PROMO virtual laboratory

Using PROMO virtual laboratory, prediction of the potential transcription binding sites was analyzed encompassing-3000bp to 0bprelative to TSS with dis similarity margin of less or equal to15%. 81 different types of transcription factors binding site has been identified with human quiescent specific GOS-2 upstream promotor sequence (NM_015714) relative to the TSS **(Table 3)**. Among the predicted transcription factor binding site identified in the promotor region of the quiescent cell specific geneGOS-2gene in the present analysis, some are general transcription factors or while others are involved in the specific transcriptional program like HOXD9/HOXD10 **(Table 3)**. On the detail analysis of transcription factor binding site, it was observed that there is lot variation in the number of potential binding sites associated with some transcription factors like C/EBP-$\beta$and RAR-α1 are 113 and 1 respectively **(Table 3).**

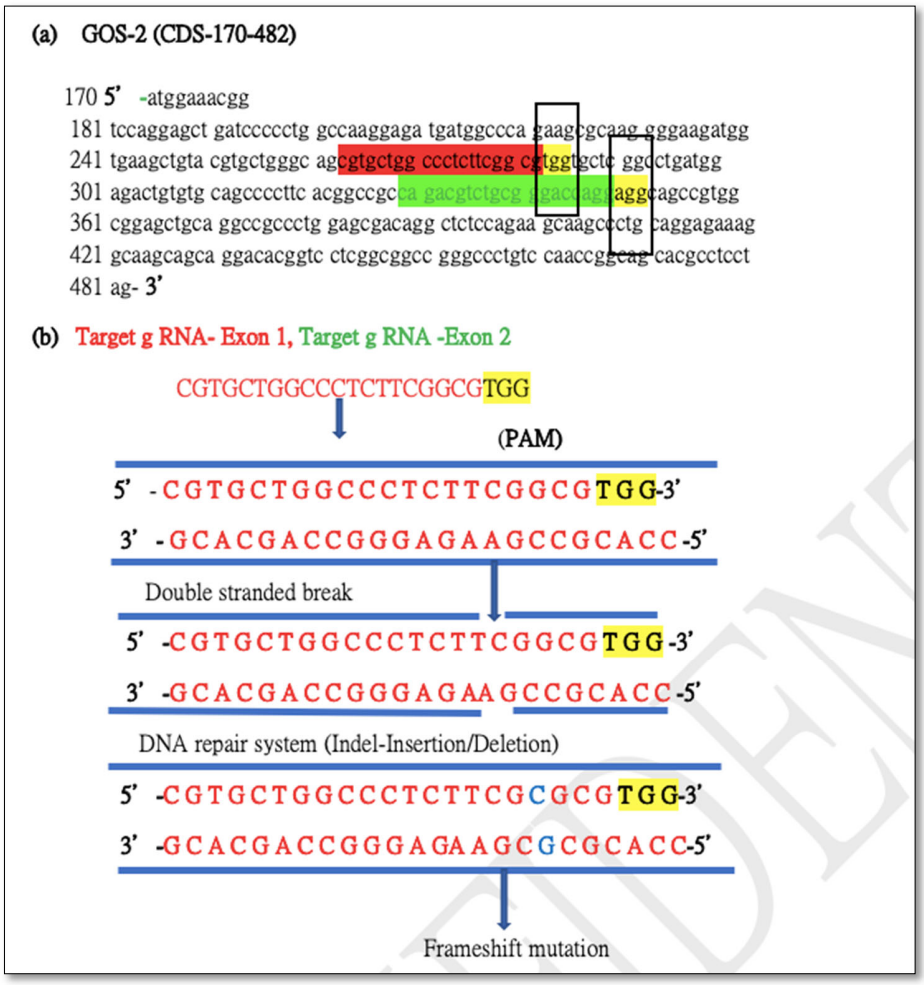


Figure 5. *In silico* synthesis and designing of g RNA/Cas9 construct targeting Exon 1/2 of quiescent specific GOS-2 gene. The double stranded cut is made at 3 nucleotides upstream of PAM- (Protospacer Adjacent Motif) sequence finally resulting in frame shift mutation.

Table 3a. List of transcription factors and number of sites for each transcription factor in the 5'UTR region of the quiescent specific gene (GOS-2).

Genes	NTFBS*	Genes	NTFBS*
C/EBPβ	113	USF1	4
GR	29	MAZ	5
HNF-1B	10	AhR: Arnt	4
STAT1β	14	TFIID	35
STAT5A	5	C/EBPα	26
PPAR-α: RXR-α	4	NFI/CTF	30
GR-β	116	RAR-β	13
RelA	3	GATA-3	1
CTF	2	p53	23
LEF-1	13	COUP-TF1	3
YY1	20	NF-Y	4
GATA-1	15	POU2F1	3
MEF-2A	6	c-Fos	2
ER-α	11	PR B	26
ETF	1	HNF-1C	15
NF-AT1	2	NF-AT2	12
Sp1	1	XBP-1	39
NF-KappaB	2	RAR-β: RXR-α	4
NF-kappaB1	1	NF-AT1	17
HNF-4α	1	SRY	13
GR-α	145	POU2F2 (Oct-2.1)	2

**NTFBS-Number of transcription factor binding site*

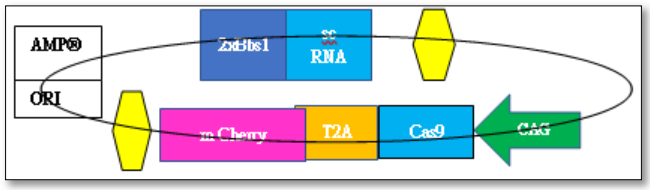
Table 3b. List of transcription factors and number of sites for each transcription factor in the 5' UTR region of the quiescent specific gene (GOS-2).

Genes	NTFBS*	Genes	NTFBS*
ER-α	11	RAR-α1	1
TFII-I	78	PU.1	1
HNF-1A	7	PR A	26
TBP	2	FOXP3	62
GATA-1	15	STAT4	49
GATA-3	1	ATF3	6
GATA-2	3	c-Jun	29
c-Ets-1	49	RBP-Jkappa	5

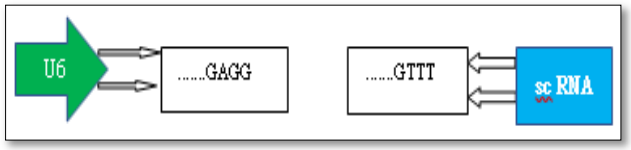
c-Myc	2	TCF-4E	13
EBF	8	VDR	2
GCF	6	IRF-2	1
AP-2αA	26	TCF-4	3
HOXD9	15	HNF-3α	27
HOXD10	15	c-Myb	24
PEA3	2	c-Ets-2	17
RXR-α	33	ENKTF-1	27
Elk-1	20	NF-1	26
Pax-5	23	Ik-1	2
AR	7	AP-1	9
Sp1	1	PXR-1: RXR-α	2
NF-ATF2	12	USF2	1
C/EBPα	26	TBP	2
PU1	1	NF1	26
E2F1	9	T3R β 1	7
IRF-1	17	NF-AT1	2

The plasmid pSpCas (BB)-2A-GFP(PX458) is digested by 2XBbsl to generate overhangs followed by the synthesis of the sense and antisense g RNA oligos with 5' & 3' 2XBbsl overhangs that complement the vector overhang **(Figures 6a and 6b)**. Typical DNA ligation with T_4 DNA polymerase and subsequent transformation and cloning into the bacterial cell **(Figures 6c and 6d)**. Extraction and purification of the plasmid for downstream functional analysis along with microscopy and phenotypic analysis are among multiple approaches that could be exploited to in order to study quiescent biology and G_0/G_1 transition.

(a) Schematic representation of the expression plasmid for cloning of g RNA/Cas 9 cassette



(b) Bbs1 restriction enzyme for 1-hour 37°C



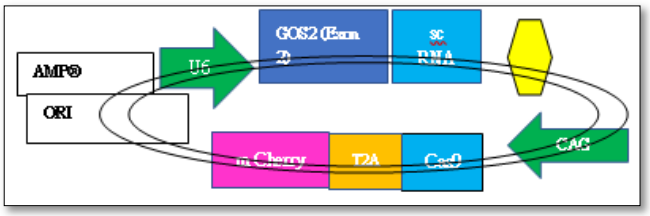
(c) Generation of sticky end with 2x Bbs1 enzyme

[Sticky end g RNA sequence (Exon2, GOS-2]
Sense Oligo: 5'-CACCCAGACGTCTGCGGGACCAGG-3'
Antisense Oligo:3' GTCTGCAGACGCCCTGGTCC-CAAA-5'
[g RNA reverse complementSticky end]

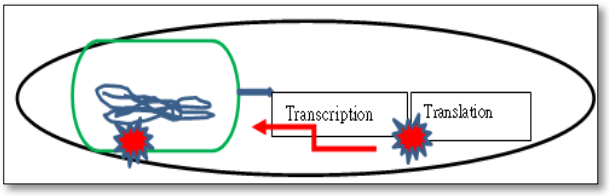
(d) Mix oligo to form duplex-95^{0}C

5'-CACC C A G A C G T C T G C G GG A C C A G G G T C T G C A G A C G C CC T G G T C CCAAA-5'

(e)



(f) Transformation of expression plasmid into *E.Coli*



(g) Ready for all sorts of functional analysis to check for mutation, subcellular location, gene expression profiling, phenotypic affect, toxicity assay etc.

Figure 6a. The expression vector (pSpCas9(BB)-2A-GFP-(PX458) consists of Cas9 enzyme in frame with m Cherry fused by T2A linker driven by CAG promotor and expression of g RNA (GOS-2-Exon 2)/sc RNA driven by U6 promotor. Schematic representation of the expression factor (a), restriction digestion site of the vector (b), generation of sense/antisense oligos (c), mixing of oligos (d)

Figure 6b. (e) Subcloning of GOS2-Exon-2 g RNA oligo duplex sequence (f), transformation in bacterial cell, (g) Cloning, extraction of plasmid followed by functional analysis.

***In silico* identification of potential enhancer region in the quiescent specific GOS-2 gene**

The combination H3K4me1 and H3K27AC positive regions in the regulatory sites has been used extensively for identifying enhancers [41]. Using UCSC genome browser and EPD, covering -3000bp relative to the TSS, 5 regions in the genome of the G_O-specific GOS-2 gene has been identified using the characteristic feature of H3K4me1 and H3K27AC positive region in the regulatory sequence of the gene of the interest **(Figure 7)**. Five regions have been highlighted that could have the potential for finding the enhancer element associated with quiescent specific GOS-2 gene. This information would be a good tool to understand the regulation of gene expression during G_0/G_1 transition process.

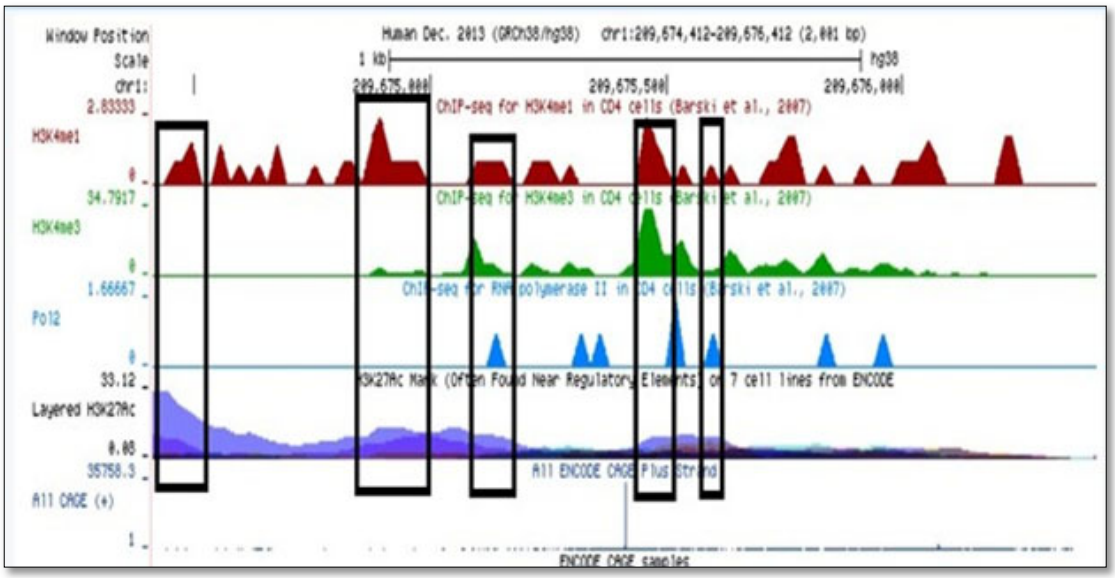


Figure 7. Using Eukaryotic Promotor Database and UCSC genome browser, it was possible to predict the potential enhancer region present on the-3000bp5' UTR region of the quiescent specific GOS-2 gene. Presence of peaks associated with H3K4 me1 and H3K27 AC could be used as a potential indicator for the presence of the enhancer region in the gene. The topline H3K4 me 1 data (dark brown) and bottom-line of H3K27AC (dark blue) with the associated peak area could be a one of the criteria for identification of enhancer region. At least 5 regions in the genome of GOS-2gene could be seen highlighted for the presence of potential enhancer region.

Target prediction of microRNA Regulatory Elements (MREs) in the 3'UTR region of the G_0-specific GOS-2 gene

In order to find potential microRNA regulatory elements, present in the 3'UTR region of the GOS-2 gene, the NCBI and Ensemble reference sequence used for the 3'UTR analysis are NM_01574.4 and ENST00000367029.4. The sequence position of 482-876bp in NM_01574.4 is equivalent to 1-394bp analyzed for the identification of potential mi RNA in the Target Scan Human software. All the miRNA sites recognized in the 3'UTR are having 7mer-m8 sites **(Figures 8a and 8b)**.

Pro-proliferating Drugs that has the potential to be used as G_0/G_1 transition agent NACT agents or against brain cancer

As the recurrence of the solid tumor is most prevalent in the case of brain tumor as compared to the other types of tissue relapse [44-46], the known pro-proliferating agents that are

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type
Position 222-228 of G0S2 3' UTR hsa-miR-424-5p	5' ...UUUGGGAUUGAGUUUUGCUGCUG... 3' AAGUUUUGUACUUAACGACGAC	7mer-m8
Position 222-228 of G0S2 3' UTR hsa-miR-16-5p	5' ...UUUGGGAUUGAGUUUUGCUGCUG... 3' GCGGUUAUAAAUGC--ACGACGAU	7mer-m8
Position 222-228 of G0S2 3' UTR hsa-miR-497-5p	5' ...UUUGGGAUUGAGUUUUGCUGCUG... 3' UGUUUGGUGUCACACGACGAC	7mer-m8
Position 222-228 of G0S2 3' UTR hsa-miR-15b-5p	5' ...UUUGGGAUUGAGUUUUGCUGCUG... 3' ACAUUUGGUACUACACGACGAU	7mer-m8
Position 222-228 of G0S2 3' UTR hsa-miR-195-5p	5' ...UUUGGGAUUGAGUUUUGCUGCUG... 3' CGGUUAUAAAGAC--ACGACGAU	7mer-m8
Position 222-228 of G0S2 3' UTR hsa-miR-15a-5p	5' ...UUUGGGAUUGAGUUUUGCUGCUG... 3' GUGUUUGGUAAUACACGACGAU	7mer-m8
Position 222-228 of G0S2 3' UTR hsa-miR-6838-5p	5' ...UUUGGGAUUGAGUUUUGCUGCUG... 3' UCCUCAGAACGGUG-ACGACGAA	7mer-m8

Figure 8a. The 3'UTR region of quiescent GOS-2 specific sequence gathered from ensemble (ENST00000367029.4) encompassing 394 bp (482-876) downstream of the coding sequence was analyzed for potential miRNA regulatory target sequence in Target Scan Human, a prediction of microRNA targets software analysis tool [21]. The sequence of 222-228bp shown in the figure has the potential miRNA target sequence recognized by different types of microRNA.

Figure 8b. Species -specific comparison of the potential miRNA target sequence present in the 3'UTR region of the quiescent specific GOS-2 gene the ensemble ID is ENST00000367029.4 and the predicted target sequence run from 222-228bp [42,43].

generated in a spatiotemporal restricted manner and secreted in brain would have the maximum chance of inducing transition in the neuronal quiescent cells to enter the cell cycle and in the process transitioning DRCs to DSCs **(Table 4)**.

The generation of neurotransmitters are site specific as some of them have function in subventricular zone while others having role in the ventral forebrain involved in the process of cell proliferation **(Table 5)**. It indicates the use of these potential NACT agents against specific tumor has to be

Table 4. List of neurotransmitters that have the potential to be used as NACT agents against brain tumor. All of the neurotransmitters proposed to activate the process of proliferation except GABA that inhibitor of its receptor has the potential to be used as adjuvant in case of the specific tumor.

Neurotransmitter	Cell cycle	Tissue/Species	Potential adjuvant drug
Acetylcholine	Proliferation [47]	Neuron/Rat	Yes
Dopamine	Proliferation [48]	Neuron/Rat	Yes
Norepinephrine	Proliferation [49]	SVZ/DG	Yes
Serotonin	Proliferation [50]	SM	Yes
GABA	Proliferation [51]	SVZ/DG	Yes (γ-GABAA inhibitor)
Glutamate	Proliferation [52]	Forebrain	Yes

**All the potential adjuvants might act differentially in acting as pro-proliferation (G0-G_1 transition) factor depending on the origin of the tumor. Some adjuvant would be effective against SVZ/dentate gyrus/striatal neurons*

taken into consideration depending on the site of their origin for their effectiveness.

Identification of the cellular mechanism predicted to be used as a target mechanism for designing NACT drugs against drug resistant cells.

The transition of cell fate is associated with not only molecular dynamics but morphological deviations during the process [53]. The subcellular re-organization of G_0 cells associated with its transition to G_1 phase have been investigated in detail including presence of G_0 unique morphological features like mRNA granules of P-bodies, proteasome storage granules (PSG) etc. [53,54]. These molecular events unique to G_0 phase that coincide with activation or repression of certain genes could be examined as a potential NACT drug development strategy **(Table 5)**.

Materials and Methods

Literature review: PubMed, Uniport, Ensemble, UCSC databases was used to identify publication related to Tumor recurrence, drug resistance and quiescence biology.

***In silico* analysis-Sequence analysis**

Blast, BlastP, Smart Blast, EMBOSS Needle, Blat was used to align the RNA and Protein sequence.

Promotor analysis to identify Transcription factor binding site

Augustus version [8] for promotor analysis and PROMO from TRANSFECTS.

Micro RNA Regulatory Element identifier

Target Scan Human was used for the identification of MREs along with sequence-specific comparison of micro RNA target site in the 3'UTR of GOS-2 gene.

Enhancer locator

The potential enhances relementin 5'UTR was examined using UCSC genome browser and Eukaryotic Promotor Database.

Conserved domain analysis

The software like CCD in NCBI, InterPro in EMBL-EBI was used for identifying con- served domains in the protein's primary structure.

Restriction digestion mapping

GenScript, Restriction mapper 3 software were used for mapping the restriction enzymes sites in both promotor region of the GOS-2 gene as well as coding region of the GFP **(Table S2)**.

***Insilico* Primer designing**

Primer 3 and Primer-Blastin NCBI was used for designing gene specific primers from different regions of the genome **(Table 4).**

Generation of restriction site in the primers specific for GOS-2 (5'UTR/CDS) for GOS-2 transcriptional assay

The primers are designed following the standard guidelines of 5'UTR-sitting sequence (4 nucleotide), restriction site (6 nucleotide) and GOS2 specific sequence. Kp1 and EcoR1 restriction digestion sites were added in the respective 5'UTR/3'UTR of the GOS2 specific primer. The generation of restriction sites with PCR were then digested with both the restriction enzymes and subsequently ligated to the vector (pPD95.75). For Kp1 (438bp) RE, GOS2-F-5'-AGGGT-GGTACC-GAGAGGAGGAGAACGCTGAG - 3' and for EcoR1(1012bp), GOS2-R-5'-GGCGGC-GAATTC-CTCTCGGAGGCGGGAATG-3' was used using Primer 3 software.

Table 5. Morphological unique features of the quiescent (G_O) cells along with the list of proteins involved in the morphological characteristic of G_0-G_1 transition. These proteins are potential NACT drugs or NACT drug target mechanism that can be investigated further for its effectiveness and feasibility.

Drug Target	Mechanism unique to G_0	Function	Inhibitor/activator	Mode of action
CPEB1 [55]	mRNA granules [55]	Translational repression	Pin 1 [56]	Dephosphorylated Pin 1 induce ubiquitination
ELF4E	mRNA granules [57]	5'Initiation complex	Maskin [58]	Disassembly of initiation complex
Ccr4-Not	m RNA granules [59]	Deadelyation and RNA decay [60]	Neomycin [60]	Deadenylase activity is inhibited
Spg5 [61]	Proteasome storage granules (PSG)	Proteasome accessory protein	Evolocumab (PCSK9) NCT04101643-Clinical trial [62]	Bortezomib? degradation of enzyme
NaTB acetylation complex [63]	PSG	Proteasome -modifying enzyme	miRNA (Has-mir-215-5p)	3'mRNA degradation
Dihydrofolate reductase	Cytoplasmic clustering of enzyme involved in purines /pyrimidine synthesis	Synthesis of purines/pyrimidines	Methotrexate [64]	Inhibitor of folic acid synthesis
Ste5	Absence of Actin cables to induce cytokinesis	Endocytosis, cytokinesis	Activator- -Formin [65]	via actin cables of Rho 1 &Myo 2 [66]

Repeat masker analysis

In order to avoid repeats within the PCR amplified regions of the template, Repeat- Masker software was used.

***In silico* PCR**

Using *In silico* PCR facility in UCSC genome browser, the primer designed were used to perform *In silico* PCR.

Tissue culture

Mammalian breast cell culture line was maintained according to the standard procedure namely Hela and MDAMB 231 cell lines [9].

Transduction of Viral construct (FUCCI) in the cell lines

FUCCI plasmid with its G_1 specific Cdt1-RFP fused protein along with M-G_2 specific Geminin-GFP protein is introduced in the cell lines during the proliferative phase of the cell cycle. The technology is called as FUCCI (Fluorescence ubiquitination cell cycle indicator (FUCCI), a translational reporter assay where genes are down regulated by the ubiquitination process [10,11]. The expression of Cdt1-RFP showing red fluorescence indicates cells in G_1 phase of the cell cycle and expression of geminin-GFP showing green fluorescence indicates G_2phase of the cell cycle [12]. It's an advanced biomedical technique to monitor the individual stages of the cell cycle under live condition using fluorescent microscopy.

Premo FUCCI Cell Cycle Sensor BamMam 2.0

The two viral vectors consist of Premo geminin-GFP and Premo Cdt-1-RFP were introduced separately into the Hela and MDAMB 231 cell lines as previously described [13] **(Figure 9)**. The two constructs were mixed prior to adding the solution in the cell lines. 40 ul of Premo Geminin-GFP is added to the 2 ml of culture medium (DMEM) in 12 well tissue culture plate. Cell culture medium was replaced with

2ml of transduction solution and cells were incubated for 2 h at 37ºC. Additional enhancer solution provided in the kit (1X BacMam) was mixed with fresh medium and finally added to the cells for further incubation at 37ºC. Fresh medium was given to the transduced cells followed by 16 h incubation at 37ºC.

Imaging with Spinning Disc fluorescent microscopy

The transduced cells under the influence trypsin gets detached from the cell culture plate and after 20 h transferred to 35 mm glass bottom tissue culture plate for imaging using spinning Disk 2.6-Image Processing software-Image J and Photo shop and movies were made using Xen-blue/black software. Time lapse images were captured after regular time point with the automatic setting parameters which attached to a colored camera. The imaging continued for 72 h post-transduction.

FACS analysis

In order to study the successful transduction efficiency of the Fluorescent probes in the cell culture, FACS analysis was performed as described earlier [14]. The minimum of 50% transduction efficiency was used as a threshold in FACS analysis that was further processed for the visualization under microscopy.

Discussion

Synthesis of *In silico*G_0specific transcriptional, translational reporter construct, gene editing using g RNA/Cas 9 construct targeting G_0-specific human gene GOS-2

The most common biomedical approach used for the identification and quantification of cell cycle stages is FACS (Fluorescent Activated Cell Sorting) [67]. The duration of the individual stages of the cell cycle (G_1/S/G_2/M), their visualization and their temporal dynamics during transition dynamics could be achieved in a limited manner using FACS approach in the study of cell cycle biology. In order to overcome this limitation in the field of cell biology, FUCCI (Fluorescent Ubiquitination Cell Cycle Indicator) reporter system has been introduced to investigate dynamics of individual stages of the cell cycle where RFP fused to Cdt1 gene labels G_1 phase cells(red) while GFP fused to geminin label cells in G_2 phase (green). The intermediate stage of S phase cell shows yellow color while the M stage and quiescent cell (G_0) remain colorless [68] **(Figures 7 and 8)**.

There is absence of information regarding way to identify quiescent cell or G_0 cell not even with FUCCI system. This review is first of its kind to address the question of approaching FUCCI like system for identification of quiescent cells which is proposed to be used in conjunction with the existing FUCCI system. The gene GOS-2 (G_O/G_1 switch gene 2) has been selected to be used for G_0 specific assay based on its stage specific expression in the quiescent

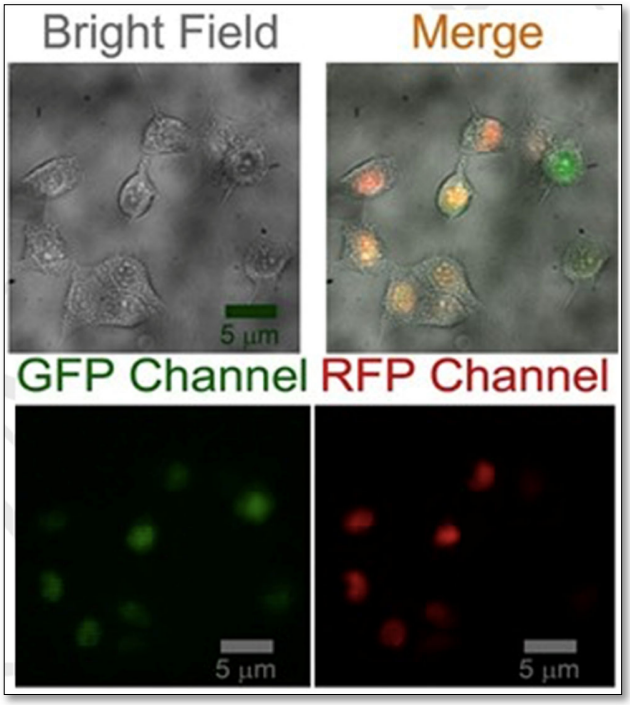


Figure 9. Snap shots of Hela cell live cell imaging is detected under the Florescent microscope (10X magnification). GFP fused to Geminin gene are shown to be express strongly specially only the cells in the G_2 phase cells. (A) Weak green signal indicates S phase cells. RFP fused to Cdt1gene shows red color represents the cells in the G_1 phase pf cell cycle. (B) The transition of G_1 cells to S phase shown in the bottom panel where downregulation of Cdt1 gene is accompanied by upregulation of Geminin gene imparting a yellow color (D)The outline of the cell is shown with the bright field microscopy. 10X magnification with scale bar of 5 μm.

phase of the cell cycle. As a proof of concept, an *Insilico* transcriptional G_0 specific reporter system and *In silico* translational G_0-specific reporter system has been introduced in this review **(Figures 2 and 4)**. As there are limitation with *In silico* transcriptional assay, a more innovative *In silico* translational assay has been proposed as the later approach has the potential to consider the post-transcriptional modification associated with protein trafficking which is a critical feature in studying quiescent biology. Additionally, for investigating loss or gain of function associated with quiescent biology and G_0-G_1 transition, a g RNA/Cas9/GOS2 cloning system has been proposed, designed and developed that can target quiescent specific GOS-2 gene finally resulting in the frameshift mutation **(Figures 5 and 6)**. In order to avoid the degeneracy of the genetic code, two exons were targeted inside the open reading frame (ORF) **(Figures 5 and 6).** Other G_0 specific molecular markers should be examined further for the

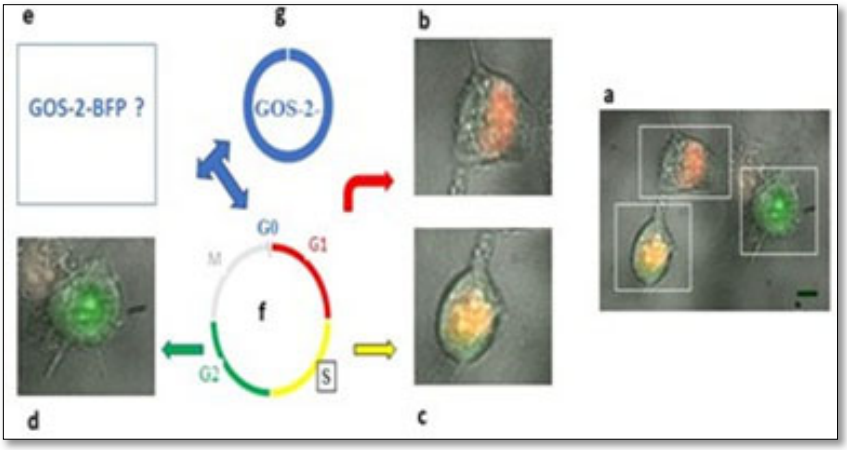


Figure 10. Overview of FUCCI technology developed by Sakaue-Sawano [12] for identification of G_1/S/G_2stage of the cell cycle **(Figures 8a-8f)**. For Mitotic (M) phase, visual morphological attachment is possible under time lapse imaging in Hela cell line. The FUCCI system lacks any gene specific reporter for quiescent phase which is predicted here via Insilico analysis (e.g., maybe G_0 specific gene GOS-2 can be fused to Blue Fluorescent Protein (BFP) or any other color detecting system other red, green and yellow **(Figures 8e and 8g)**. The GOS-2-BFP could be used along with conventional FUCCI system. Experiment was performed by SS and visualized under fluorescent microscopy in the department of Chemistry and department of BSBE, IIT-Bombay. The magnification is10X. The Scale bar is 5 μm.

synthesis of both *Insilico* and *In vivo/In vitro* translational reporter assay.

Identification of potential transcription factor binding site, promotor motif identifier and locating enhancer and micro RNA regulatory element in 5' & 3' UTR region of G_0-specfic GOS-2 genome

Using multiple software like EPD and PROMO, it was possible to identify potential functional promotor motifs, transcription factor binding site in the 5'UTR region quiescent specific GOS-2 gene **(Figure S3 and Table 3)**. The presence of multiple promotor motifs like CCAAT-box, Initiator, TATA box (RNA polymerase binding site) and CCAAT-box were identified in EPD within -1000 to -100 bp relative to TSS **(Figure S3)**. The analysis of promotor binding sites within -3000bp of the TSS might indicate the role of GOS-2 in multiple signaling transduction pathways. Presence of Transcription Factor Binding Site (TFBS) for factors like HOXD9/HOXD10 in GOS-2 promotor might indicate the role of TFBS in limb bud development as well as in Wilm's Tumor. A lot of genes involved in T/B cell differentiation, Immunological response, cytokine development has been identified in the present analysis which includes c-Ets-1/2, Foxp2, RELA, SPI, LEF1, VDR, C/EBP- β, XBP, RBPJ, PAX-5, c-MyC, PU-1 etc. Few of the genes screened in this preliminary analysis present in the promotor region of GOS-2 has been implicated in Alzheimer's disease and colorectal tumor like ENKTF1, Retinoic acid receptor-RXR α, ETS like 1 protein ELK-1 **(Table 3)**. The above preliminary promotor analysis of quiescent cell specific molecular marker (GOS-2) gene could be used as a template for further investigation for the role of GOS-2 gene and subsequently its role in G_0/G_1 transition and its pathological correlation with multiple diseases like Pitts-Hopkins syndrome, Osteosarcoma endometrial carcinoma, Multiple sclerosis etc. (Data not shown but could be send if interested). The enhancer plays an important role in the regulation of gene expression and as the present gene under review GOS-2 is involved in G_0/G_1 transition, it is very crucial to understand and locate the enhancer region within the promotor region of the gene. Using UCSC genome browser and EPD software, identification of potential location for the presence of the enhancer was examined. Typically, in Chip-Seq analysis, presence of peaks associated with H3K4me1 and H3K27ac is considered as good indicator for the presence of the enhancers. For GOS-2 gene, 5 potential regions have been located within the genome that could be further studied *In silico* and *In vitro* for its role in the spatio-temporal regulation of GOS-2 expression and its association with G_0/G_1 transition. The process of transition of different phases of the cell cycle including G_0/G_1 conversion is associated with alteration of gene expression profile involving activation/repression of stage specific transcription factors. The 3 'UTR are a potential site for the presence of micro RNA regulatory elements (MREs) that are involved in the repression of the transcript. Using Target Scan Human, the predicted micro target site was identified in the 3'UTR of GOS-2 gene (222bp-228bp) **(Figure 8a)**. Cross-species comparison of the MREs was done and the sequence conservation is highlighted across multiple species (**Figure 8b)**. This observation is important and miRNA target region has to be considered at the time of designing the translation reporter construct specially with GOS-2 gene where 3'UTR is a critical part of the GOS-2-Translational reporter construct. The present approach for the generation of GOS-2

translational reporter assay has its own limitation as the potential protein coding region to be ubiquinited for the degradation process during G_0/G_1 transition has not be considered which needs further detailed sequences analysis both in transcript level and in protein level.

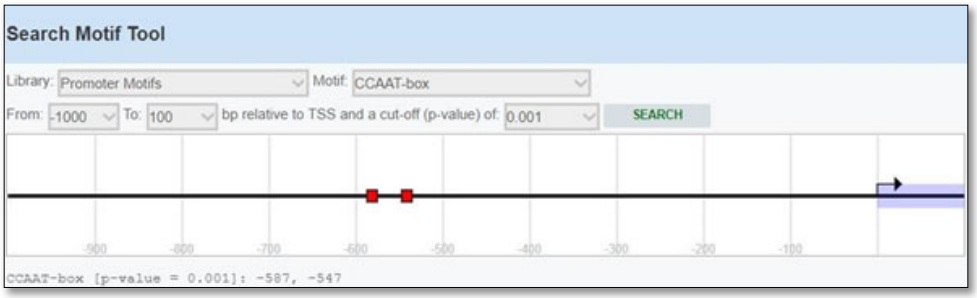


Figure S3a. Identification of promotor motifs in the 5'UTR region of GOS-2 gene in the range -1000 bp to 100 bp relative to the TSS-CCAAT-box.

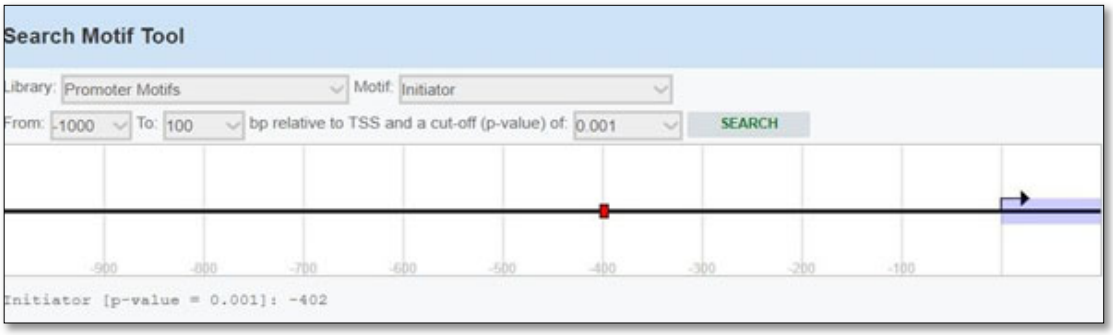


Figure 3b. Initiator.

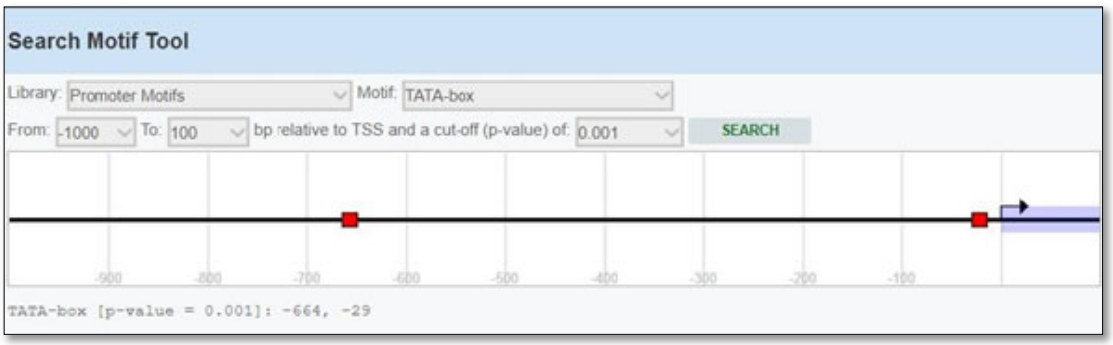


Figure S3c. TATA-box

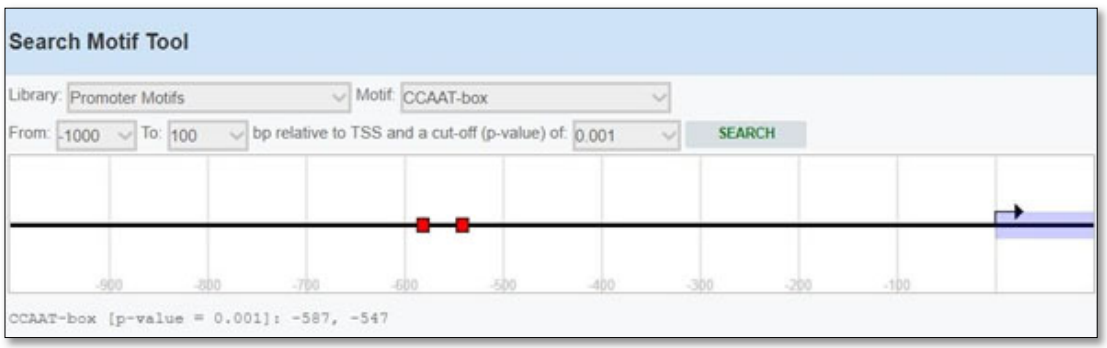


Figure S3d. CCAAT-box.

Limitation of presently available NATC agents against tumor

The NACT approach mainly comprised of surgical intervention, chemotherapy along with use of chemoradiotherapy [42,43]. As the present review focuses only on the management of the NACT agents dealing with the DRCs, discussion of other approaches is beyond the scope of this review. A list of widely used neoadjuvant, adjuvants and antimitotic have been described **(Table 4)**. The use of these drugs considerably reduces the extent of the recurrence of the tumor [18,20]. The most widely used mechanism used by these drugs include acting as alkylating agent, methylating agent etc. **(Table 4)** hampering DNA replication, DNA transcription and protein translation. Similarly, in the case of different types of brain tumor, the drug target identical mechanism but few of them are more cell type specific targeting G_1 phase cells (e.g. vatalanib) or metaphase (vincristine) **(Table 3)**.

Limitation of the NATC drug development strategy against tumor relapse proposed and predicted in this review

The genes and mechanism proposed here to be used as drugs and drug target for to be investigated as potential Neo-adjuvant chemotherapeutic (NACT) agent against recurrence of the tumor has its own limitations as described below-The analysis is cross-species (G_o specific gene expression and their potential role might differ from species to species and it has to be examined properly), tissue specific modality (e.g., neurotransmitter might act as pro-proliferative factor in tumors arising in macroscopic tissue specific manner), differential response to the potential NACT drugs (G_o cells might respond differentially to the potential drugs proposed that is open for further investigation). Translational reporter assay is more informative than transcriptional reporter assay and translation reporter assay needs further detailed sequence analysis to examine the implication of ubiquitous degradation pathway. Use of G_0 specific genes other than GOS-2 has to analyzed to be for G_0 specific reporter assay. Comparison of whole genome transcriptome analysis between cells in the $G_1/S/G_2/M$ and G_0 cells under the regulation of growth factors would help us better understand the up-regulation/down regulation of genes associated with the G_0/G_1 transition.

CONCLUSION

A slight decrease in the rate of the tumor recurrence process aided by potential novel NACT agents can have drastic effect in the life span of recurring tumor patient.19 potential novel NACT drugs and drug target mechanism identified in this review is open for further investigation in *In silico*, *In vitro* and *In Vivo* setup. First time *In silico* synthesis of transcriptional and translational reporter construct associated with G_0/G_1 transition has been described that can be used along with already existing FUCCI reporter system for better understanding of G_0/G_1 transition process. The identification of transcriptional binding site and list of transcription factors of G_0/G_1 transition human GOS-2 gene along with identification of its potential enhancer, promoter motif, micro RNA regulatory elements and *In silico* design of g RNA/Cas 9 -GOS-2 quiescent specific mutant construct can trigger molecular studies associated with G_0/G_1 transition that might improve the therapeutic approach against tumor recurrence [69-78].

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