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Transcriptome analysis identifies *TODL* as a novel lncRNA associated with proliferation, differentiation, and tumorigenesis in liposarcoma through FOXM1

Deepika Kanojia [a,*], Anuradha Kirtonia [b], Namburi Sai Valli Srujana [a], Sree Priyanka Jeevanandan [a], Pavithra Shyamsunder [a], Shruthi Sanjitha Sampath [a], Pushkar Dakle [a], Anand Mayakonda [a,c], Harvinder Kaur [a], Jiang Yanyi [a], H.Phillip Koeffler [a,d], Manoj Garg [b,*,1]

[a] Cancer Science Institute of Singapore, National University of Singapore, 117599, Singapore
[b] Amity Institute of Molecular Medicine & Stem Cell Research (AIMMSCR), Amity University, Sector-125, Noida, Uttar Pradesh 201313, India
[c] Division of Cancer Epigenomics, German Cancer Research Center (DKFZ), Heidelberg 69120, Germany
[d] Division of Hematology/Oncology, Cedars-Sinai Medical Center, University of California, School of Medicine, Los Angeles, CA 90048, USA

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ABSTRACT

Liposarcoma, the most common soft tissue sarcoma, is a group of fat cell mesenchymal tumors with different histological subtypes. The dysregulation of long non-coding RNAs (lncRNAs) has been observed in human cancers including a few studies in sarcoma. However, the global transcriptome analysis and potential role of lncRNAs remain unexplored in liposarcoma. The present investigation uncovers the transcriptomic profile of liposarcoma by RNA sequencing to gain insight into the global transcriptional changes in liposarcoma. Our RNA sequencing analysis has identified that many oncogenic lncRNAs are differentially expressed in different subtypes of liposarcoma including *MALAT1*, *PVT1*, *SNHG15*, *LINC00152*, and *MIR210HG*. Importantly, we identified a highly overexpressed, unannotated, and novel lncRNA in dedifferentiated liposarcomas. We have named it *TODL*, transcript overexpressed in dedifferentiated liposarcoma. *TODL* lncRNA displayed significantly higher expression in dedifferentiated liposarcoma cell lines and patient samples. Interestingly, functional studies revealed that *TODL* lncRNA has an oncogenic function in liposarcoma cells by regulating proliferation, cell cycle, apoptosis, differentiation, and tumorigenesis in the murine model. Silencing of *TODL* lncRNA highlighted the enrichment of several key oncogenic signaling pathways including cell cycle, transcriptional misregulation, FOXM1 network, p53 signaling, PLK1 signaling, FoxO, and signaling Aurora signaling pathways. RNA pull-down assay revealed the binding of *TODL* lncRNA with FOXM1, an oncogenic transcription factor, and the key regulator of the cell cycle. Silencing of *TODL* lncRNA also induces adipogenesis in dedifferentiated liposarcomas. Altogether, our finding indicates that *TODL* could be utilized as a novel, specific diagnostic biomarker, and a pharmacological target for therapeutic development in controlling aggressive and metastatic dedifferentiated liposarcomas.

1. Introduction

Soft tissue sarcomas are rare mesenchymal tumors of connective tissue that include more than 50 heterogeneous subtypes [1,2]. Liposarcoma, a tumor of adipocytes, is the most frequent histological subtype of adult soft tissue sarcoma accounting for 15–20% of all sarcomas

Abbreviations: TODL, Transcript overexpressed in dedifferentiated liposarcoma; LncRNA, Long non-coding RNA; FOXM1, Forkhead box protein M1; FISH, Fluorescence in situ hybridization; GFP, Green fluorescent protein; HAT, Human adipose tissue; Nt, Non-target; OE, Overexpression; ESC, Epithelial stem cells; RLS, Retroperitoneal liposarcoma; EMT, Epithelial-mesenchymal transition; shRNA, Short hairpin RNA.

* Corresponding authors.
E-mail addresses: dkanojia23@outlook.com (D. Kanojia), mgarg@amity.edu, nuscsimg@gmail.com (M. Garg).
[1] ORCID: 0000-0002-3492-2957.

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[3,4]. Liposarcoma is further sub-classified into four molecularly and histologically distinct subtypes: well-differentiated, dedifferentiated, myxoid, and pleomorphic liposarcoma [5–8]. Management of liposarcoma includes multimodality treatment with surgical resection as a major prerequisite along with chemo and radiation therapy [4,5].

Well-differentiated and dedifferentiated liposarcomas are the most common subtypes representing more than 40% of all liposarcomas [1]. These two subtypes differ in histological and tumor behavior. Well-differentiated liposarcomas are low-grade tumors with mature fat cells. However, dedifferentiated liposarcomas are high-grade, more aggressive, and non-adipogenic metastatic tumors [9]. The majority of dedifferentiated liposarcomas arise de novo but some well-differentiated tumors progress to a high-grade dedifferentiated form of the liposarcoma [9]. The molecular basis for the progression of low-grade to high-grade liposarcomas has not been extensively explored. Various studies have indicated many therapeutic interventions that might treat the disease, but none has been translated into clinical applications [1]. Until now, only Trabectedin has been approved by the Food and Drug Administration to treat patients with a myxoid subtype of liposarcoma [5]. Genomic sequencing studies have indicated various important mutations in protein-coding genes that could be used for targeted therapy [8,10–13]. Also, RNAi and drug studies highlighted ponatinib, a multi-targeted tyrosine kinase inhibitor for potential therapy against liposarcomas [14].

Genomic complexity at the transcriptomic level for many organisms has increased exponentially. Less than 2% of the whole genome codes for proteins and ~98% of transcription occurs from non-coding RNAs that could be crucial for driving cancer phenotypes [15]. Long non-coding RNAs (lncRNAs) are non-coding transcripts containing > 200 nucleotides in length, frequently transcribed by RNA polymerase II, spliced, and polyadenylated that cannot be translated into proteins [16–19]. LncRNAs have emerged as an important category of ncRNAs consisting of very large numbers and regulating diverse functions [20]. LncRNAs have a genome-wide and tissue-specific expression making these molecules promising biomarkers and therapeutic targets for human malignancies [16,19,21].

Previous global genomic and proteomic studies identified various tumor-specific targets for liposarcoma, but lncRNAs and their role in liposarcoma tumor progression are not studied in depth. In the present study, we identified and characterized the lncRNAs landscape in liposarcomas. We noted a novel unannotated lncRNA, which is highly and specifically expressed only in dedifferentiated liposarcomas. Therefore, we termed this novel lncRNA as *TODL*; transcript overexpressed in dedifferentiated liposarcoma. Further, our functional investigations strongly suggested that *TODL* is an oncogenic lncRNA that promotes cell cycle progression, aberrant proliferation, adipogenesis *in vitro,* and tumor growth in the xenograft model through its interaction with FOXM1. Knockdown of *TODL* lncRNA revealed significant enrichment of major signaling pathways such as FOXM1 network, transcriptional misregulation, FoxO signaling, cell cycle, Aurora kinase pathway, PLK1 signaling, and p53 cascade. Collectively, the results of this study indicated that *TODL* could be explored as a novel diagnostic biomarker and therapeutic target for controlling high-grade dedifferentiated liposarcomas.

2. Materials and methods

2.1. Cell culture

Ten human liposarcoma cells were used in this study. T778 and T1000 (recurrent well-differentiated liposarcoma) were provided by Dr. Pedeutour; LPS141 and LP6 (dedifferentiated liposarcoma) were provided by Dr. Christopher DM Fletcher [22]; GOT-3 [23] (recurrence of a myxoid variant of a well-differentiated liposarcoma) and MLS-402 (myxoid liposarcoma) [24] were generous gifts from Dr. Åman and LiSa-2 (metastatic poorly differentiated pleomorphic liposarcoma) was kindly provided by Dr. Moller [25]. Adipose-derived mesenchymal stem cell (ASC52telo) was purchased from ATCC and maintained in a stem cell medium according to culture instructions. Human adipose tissue (HAT) total RNA was purchased from ClonTech. All the liposarcoma cells were maintained in RPMI media supplemented with fetal bovine serum in a humidified incubator at 37^0 C with 5% CO2 [8]. All cells tested negative for mycoplasma and authenticated by short tandem repeat analysis.

2.2. RNA preparation and real-time quantitative PCR (qPCR)

Total RNA was extracted using the RNeasy kit (Qiagen) followed by on-column DNaseI treatment. RNA from cytoplasmic and nuclear fractions was isolated using the PARIS kit (Ambion). cDNA was prepared by Transcriptor First Strand cDNA Synthesis Kit (Roche) for real-time qPCR. Kapa SYBR fast qPCR Master Mix (KAPA Biosystems) was used for performing real-time qPCR assays. All the primer sequences are given in Supplementary Table ST1.

2.3. RNA sequencing and analysis

Total RNA samples were subjected to strand-specific RNA-seq libraries that were constructed by TruSeq Library Prep kit (Illumina) according to the manufacturer's instructions and sequencing was performed using an Illumina HiSeq2000 platform (paired-end reads of 100 bases). RNA sequencing analysis for lncRNA identification was performed as described [26]. Briefly, reads were mapped to Hg19 using Tophat v2.0.11 [27]. The aligned reads were de novo assembled using Cufflinks v 2.2.1 [28] with minimum read coverage of ≥ 1 read per base, size filter of > 200 bp, and exon filter of ≥ 2 exons. BEDTools were applied to intersect de novo transcripts and to filter out incompletely assembled mRNAs and other unannotated isoforms. Sixpack tool from EMBOSS [29] and HMMER3 [30] was used to list out the longest ORF for each transcript. Next, we filtered out any transcript under evolution pressure and based on a coding potential threshold using PhyloCSF [31].

2.4. RNA- fluorescence in situ hybridization (FISH)

Stellaris RNA-FISH probe was designed to bind to *TODL* lncRNA using the online tool of Biosearch Technologies and the probe conjugated with fluorescent Quasar 570 molecule was purchased. RNA-FISH was performed according to the manufacturer's instructions. Briefly, cells were grown on chamber slides, fixed using formalin, and permeabilized with alcohol. The hybridization reaction of fluorescent probes was done at 37^0 C for 16 h in the incubator. After washing with stringent buffers, cells were mounted with DAPI containing mounting media, and images were captured using a confocal microscope.

2.5. RNA interference

Dicer-Substrate Short Interfering RNAs (DsiRNAs) were designed to target *TODL* lncRNA and were purchased from Integrated DNA Technologies (IA, USA) along with control non-targeting DsiRNAs (Nt siRNA). Cells were transfected with 5 nM each of *TODL* siRNA1, *TODL* siRNA2, and Nt siRNA using Lipofectamine RNAi Max (ThermoFisher Scientific) according to the manufacturer's instructions [14]. Stable knockdown of *TODL* lncRNA was generated using TODL-specific short hairpin RNA (shRNA) and non-targeting shRNA (Nt shRNA) in a lentiviral pLKO.1 puro vector system. Sequences of siRNAs and shRNAs used in the study are provided in Supplementary Table ST2.

2.6. Overexpression system

TODL transcript was amplified and cloned in a pLKO.1-puro-CMV-TurboGFP vector removing GFP from the vector. The original vector was used as a negative control. A stable overexpression system was

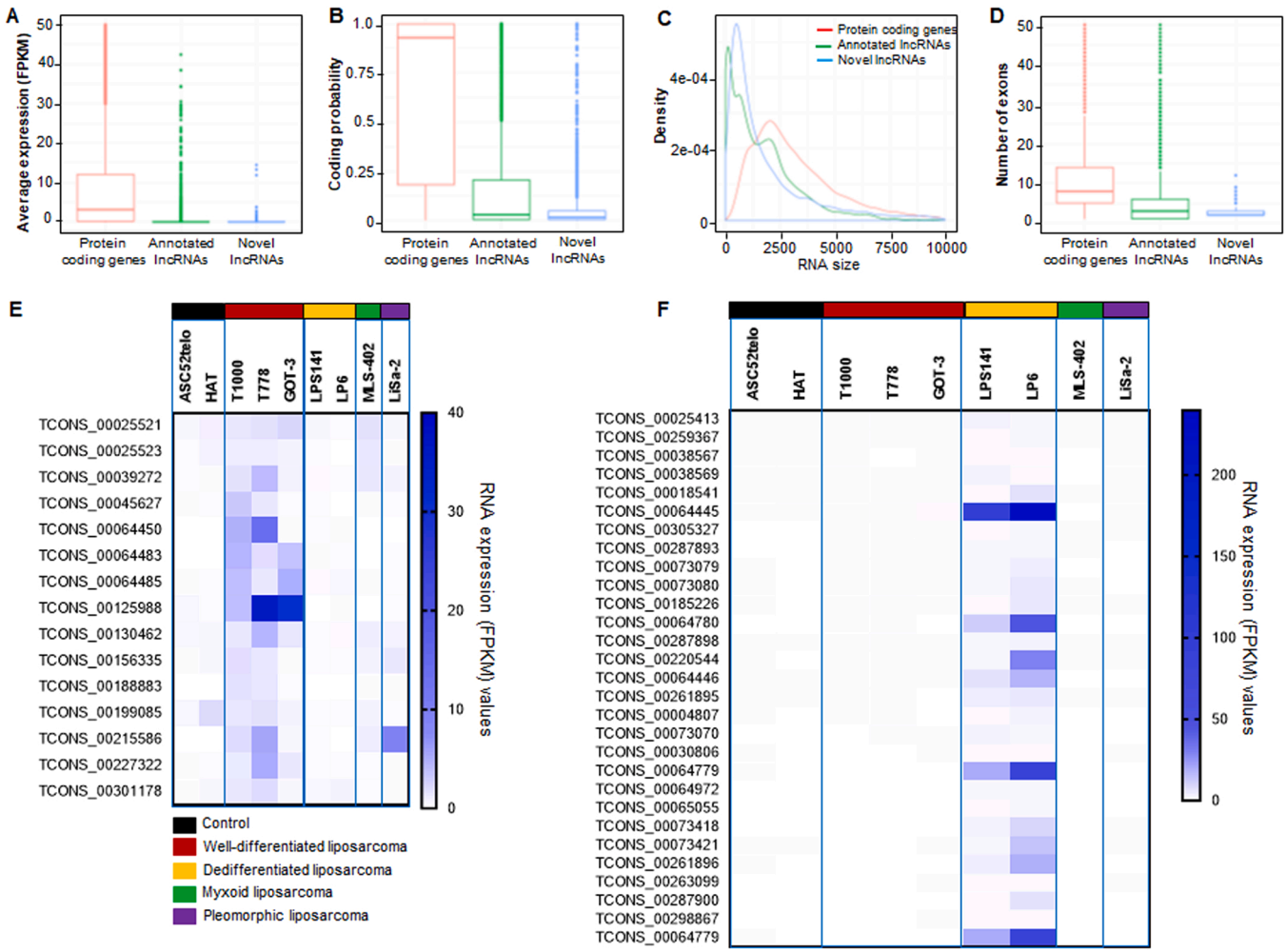

Fig. 1. RNA sequencing analysis in liposarcoma cells of different subtypes. (A) Average expression values in FPKM (Fragments Per Kilobase of transcript per Million mapped reads) of protein-coding genes, known annotated lncRNA transcripts, and novel unannotated lncRNAs in LPS cell lines. (B) The coding potential of all categories of transcripts is described in terms of coding probability. (C) Comparison of transcript size distributions of mRNA genes, known and novel lncRNAs were analyzed from RNA seq data. (D) Distribution of the number of exons identified per transcripts in protein-coding genes, known and novel lncRNAs. The heatmap displays RNA expression values of novel unannotated lncRNAs identified in well-differentiated liposarcoma (E), and dedifferentiated liposarcoma (**F**) cells compared to controls; ASC52telo and HAT. The intensity of the blue color represents FPKM values obtained from RNA-seq.

created by transduction and selection with puromycin.

2.7. Cell growth assay

Cells were seeded in 96-well plates at 3000 to 4000 cells per well and cultured for 3–5 days. At the end of incubation, substrate MTT (Sigma-Aldrich) was added to each well, incubated for 2–4 h, and MTT STOP solution was added to stop the reaction. Absorbance was measured at 570 nm on a Tecan microplate reader as described earlier [32].

2.8. Colony formation assay

Cells were seeded at a density of 1500 to 2000 cells per well in a 6-well plate and allowed to grow for 10–15 days with twice a week change of the medium. Once colonies were visible, the medium was removed, cells were washed with PBS, and colonies were fixed with methanol and stained with 0.01% crystal violet in 25% methanol. After staining for 1 h, cells were washed with water, and images were acquired using a bright field microscope.

2.9. Xenograft animal studies

All the animal experiments were performed according to the ethical regulations of the Institutional Animal Care and Use Committee of the National University of Singapore. Male NOD SCID gamma mice (5–6 weeks old) were used in the study. LPS141 (0.5 $\times 10^6$ cells) and LP6 (0.25 $\times 10^6$ cells) were injected subcutaneously into the flank of the mice. Mice were monitored for tumor development and after 3 weeks, mice were sacrificed. Tumors were excised and weighed at the end of the experiment.

2.10. Immunoblot assay

Protein lysates were prepared by treating harvested cells with Mammalian-Protein Extraction Reagent (Thermo Scientific) for 10 min at room temperature with gentle shaking as described earlier [33]. Cell debris was removed by centrifugation and proteins were collected in clear supernatant and were quantified by BCA assay. Western blotting was performed according to standard protocols with antibodies listed in Supplementary Table ST3.

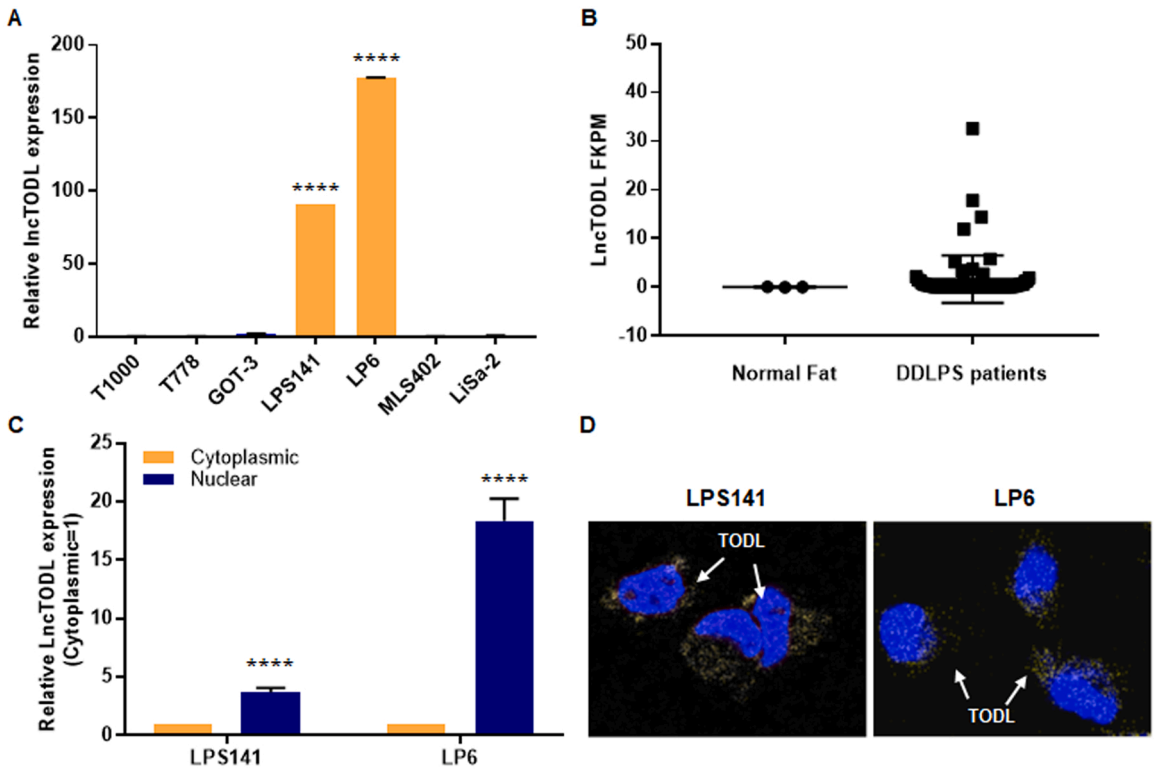

Fig. 2. Characterization of novel lncRNA *TODL*. (A) Expression of *TODL* transcript in liposarcoma cell lines compared with controls (ASC52telo and HAT) by qPCR. Data represent mean ± SEM; n = 3. * ** * denotes p values < 0.0001. (B) RNA sequencing analysis from TCGA database of dedifferentiated liposarcoma patient samples for *TODL* lncRNA expression levels (FPKM values) versus normal fat samples. (C) Subcellular distribution of *TODL* lncRNA in nuclear and cytoplasmic RNA fractions measured by real-time qPCR in dedifferentiated liposarcoma LPS141 and LP6 cells. Data presented as mean ± SEM; n = 3. * ** * denotes p values < 0.0001. (D) Cellular localization of *TODL* lncRNA in fixed and permeabilized LPS141 and LP6 cells detected by fluorescent RNA FISH probes using the confocal microscope. *TODL* lncRNA probe in orange-yellow and nuclear DAPI in blue color.

2.11. Cell cycle and apoptosis assay

Cells were harvested, washed with PBS, and fixed in cold 70% ethanol. Fixed cells were stained with propidium iodide/RNase solution and analyzed by flow cytometry for the cell cycle. For apoptosis, cells were harvested, washed with PBS, and stained with Annexin V-APC and Propidium iodide according to the manufacturer's protocol. Stained cells were analyzed by flow cytometry.

2.12. Cell cycle synchronization

LPS141 and LP6 cells were seeded a day before in 10 cm dishes. Cells were synchronized to the mitosis phase by treating cells with 50 ng/ml of Nocodazole for 18 h. The mitotic cells were washed again with PBS and grown in a fresh complete medium for 6 h to synchronize cells in the G1 phase. S-Phase synchronization was performed by double thymidine block. Briefly, cells were treated with media containing 2.5 mM thymidine for 24 h. After the first block, thymidine was removed and cells were released for 12 h in a fresh complete medium. Further, cells were incubated with 2.5 mM thymidine again for 24 h to synchronize cells at G1/S. The cells were back in a fresh complete medium for 4 h to harvest in the S phase and 8 h for the G2 phase. After harvesting cells at each phase, part of the cells were used for RNA extraction for real-time qPCR analysis, and the rest of the cells were fixed and analyzed for cell cycle analysis using Propidium iodide staining.

2.13. Adipogenesis induction assay and oil-red staining

Cells were seeded and grown in a normal complete medium till reached confluency. Next, the medium was replaced with Human Adipocyte Differentiation Medium (Sigma Aldrich) and cells grew for 3 weeks. Fresh adipocyte differentiation medium was added after every 3 days. After adipocytic differentiation, cells were washed gently with PBS, fixed with formalin, treated with isopropanol, and stained with oil-red stain. After staining for a few hours, cells were washed with water and imaged under a bright field microscope.

2.14. RNA pull-down assay

RNA pull-down protocol used here is adapted and modified ChIRP protocol [34]. *TODL* transcript of 308 nucleotides was PCR amplified containing T7 RNA Polymerase promoter in the correct orientation. The HiScribe T7 in vitro transcription kit (New England Biolabs) was used for the synthesis of large amounts of *TODL* RNA molecules using the template as a purified PCR product. Synthesized RNA was purified and used for labeling biotin at the 3′ terminal end using an RNA 3′ end biotinylation kit (Thermo Scientific Pierce) according to instructions. Cell lysates were prepared by sonicating 10 million cells in 0.5 ml of lysis buffer (50 mM Tris-HCl pH 7, 10 mM EDTA, 1% SDS, protease inhibitor, SUPERase In RNase Inhibitor). *In vitro* synthesized biotinylated *TODL* RNA probe (100pmol) was incubated with sonicated chromatin at 37^0 C for 16 h to bind RNA-protein complexes in hybridization buffer (750 mM NaCl, 50 mM Tris.HCl pH 7, 1 mM EDTA, 1% SDS, 15% Formamide, protease inhibitor, SUPERase In RNase Inhibitor). After hybridization, RNA-protein complexes were retrieved by Streptavidin magnetic C1 beads, followed by stringent washing with wash buffer (2X SSC and 0.5% SDS). Proteins associated with RNA were eluted in SDS Lamelli buffer and separated on SDS-PAGE for immunoblotting.

2.15. Statistical analysis

The statistical impact was calculated using a two-tailed Student's t-

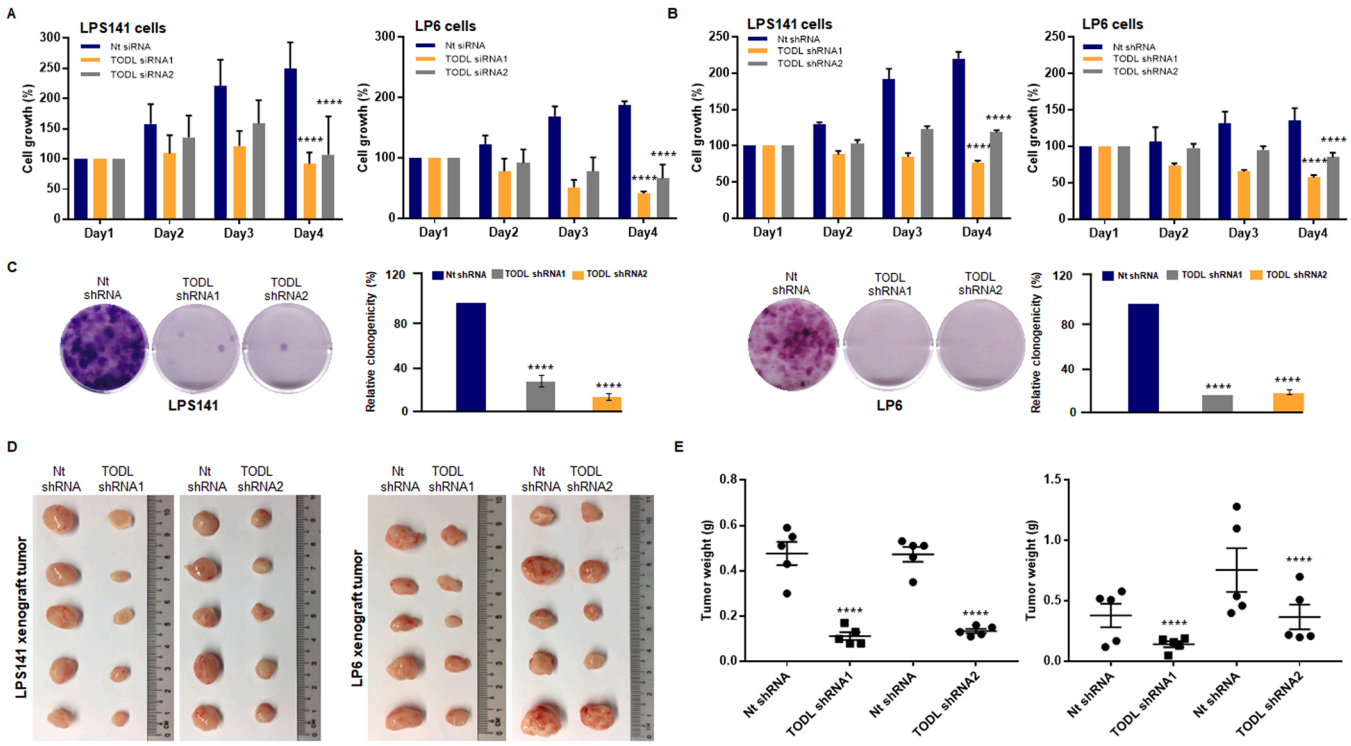

Fig. 3. Functional *in vitro* and *in vivo* studies of *TODL* lncRNA in dedifferentiated liposarcoma. (A-B) Effect of *TODL* silencing using two siRNAs and two shRNAs on cell viability of LPS141 and LP6 cells using MTT assay. Data are presented as mean ± SEM; n = 3. (C) Anchorage-dependent colony formation assay after silencing of *TODL* in LPS141 and LP6 cells. (D) Effect of shRNA-mediated downregulation of *TODL* lncRNA on tumorigenicity of LPS141 and LP6 cells *in vivo* xenografts. (E) The tumors were excised, weighed, and the effect of silencing of *TODL* lncRNA on tumor weight was measured. * ** * denotes p values < 0.0001.

test for two-group analysis and one-way analysis of variance for multiple-group analysis. The p-value of ≤ 0.05 was considered statistically significant. All *in vitro* experiments were performed three times in triplicates and data were expressed as mean ± standard error (SE).

3. Results

3.1. Transcriptomic analysis of liposarcoma cells

RNA sequencing analysis was performed on liposarcoma cells of well-differentiated (T1000, T778, GOT-3), dedifferentiated (LPS141 and LP6), myxoid (MLS402), and pleomorphic (LiSa-2) subtypes of liposarcoma along with control adipose-derived stem cell (ASC52telo) and human adipose tissue (HAT). We analyzed and identified known annotated lncRNAs (Supplementary Table ST4) and novel unannotated lncRNAs in liposarcoma based on coding ability, length, number of exons, and expression levels (Supplementary Table ST5). The novel unannotated lncRNA transcripts showed low expression and were comparable to known annotated lncRNAs in contrast to higher expression of protein-coding genes (Fig. 1A). Also, these novel lncRNAs revealed lower coding potential scores and shorter length density compared to protein-coding genes but are consistent with the known annotated lncRNAs (Fig. 1B and C). The novel lncRNAs had small exon counts compared to high counts of protein-coding genes (Fig. 1D). Our results displayed that several well-known annotated lncRNA transcripts were overexpressed in liposarcoma cells including *MALAT1*, *PVT1*, *SNHG15*, *LINC00152*, and *MIR210HG* (Supplementary Fig. S1A).

The expression profiles of identified novel lncRNAs were compared among the different liposarcoma subtypes and identified novel lncRNAs over-expressed only in either well-differentiated or dedifferentiated subtypes [Fig. 1E and F]. We focussed on a specific and novel lncRNA (TCONS_00064445) which we termed *TODL*, transcript overexpressed in dedifferentiated liposarcoma. The expression of *TODL* lncRNA was very high in dedifferentiated liposarcoma cells, comparable to levels noted in protein-coding genes (Supplemental Table ST2). *TODL* is in the amplicon of chromosome 12q15 [chr12: 69409275–69418806] containing two exons with a total size of 308 base pairs. Real-time qPCR analysis validated overexpression of *TODL* lncRNA exclusively in LPS141 and LP6 cells (dedifferentiated) relative to control cells (HAT and ASC52telo; Fig. 2A). Our data displayed that there is no correlation between the copy number change and expression levels of *TODL* lncRNA (Supplementary Fig. S1B). In addition, analysis of RNA sequencing data from the TCGA database showed *TODL* lncRNA overexpression in 17 out of 72 [23%] patients with dedifferentiated liposarcoma compared to control fat (Fig. 2B). After nuclear and cytoplasmic RNA fractionation followed by real-time qPCR, we noticed that *TODL* lncRNA was predominantly localized to the nuclear fraction (Fig. 2C). RNA-FISH using confocal microscopy highlighted the accumulation of *TODL* in both the nucleus and cytoplasm in LPS141 and LP6 cells [Fig. 2D].

3.2. TODL lncRNA promotes liposarcoma cellular progression

We have used both siRNA and shRNA to silence the expression of *TODL* lncRNA in LPS141 and LP6 cells. Our qPCR data confirmed approximately 90% depletion of *TODL* lncRNA at transcript levels in *TODL* siRNA1 and siRNA2 compared to non-target (Nt) siRNA in both LPS141 and LP6 cells (Supplementary Fig. S2A). Similarly, expression levels of *TODL* lncRNA were significantly repressed in *TODL* shRNA1 and shRNA2 compared to Nt shRNA in LPS141 and LP6 cells (Supplementary Fig. S2B). Further, silencing of the *TODL* lncRNA revealed significantly suppressed proliferation in liquid culture and soft agar colony formation as compared to Nt shRNA in LPS141 and LP6 cells (Fig. 3A-C).

Importantly, we stably overexpressed *TODL* in MLS402, myxoid liposarcoma cells (these cells do not express this lncRNA). Our real-time qPCR displayed significantly higher levels of *TODL* transcripts in TODL-

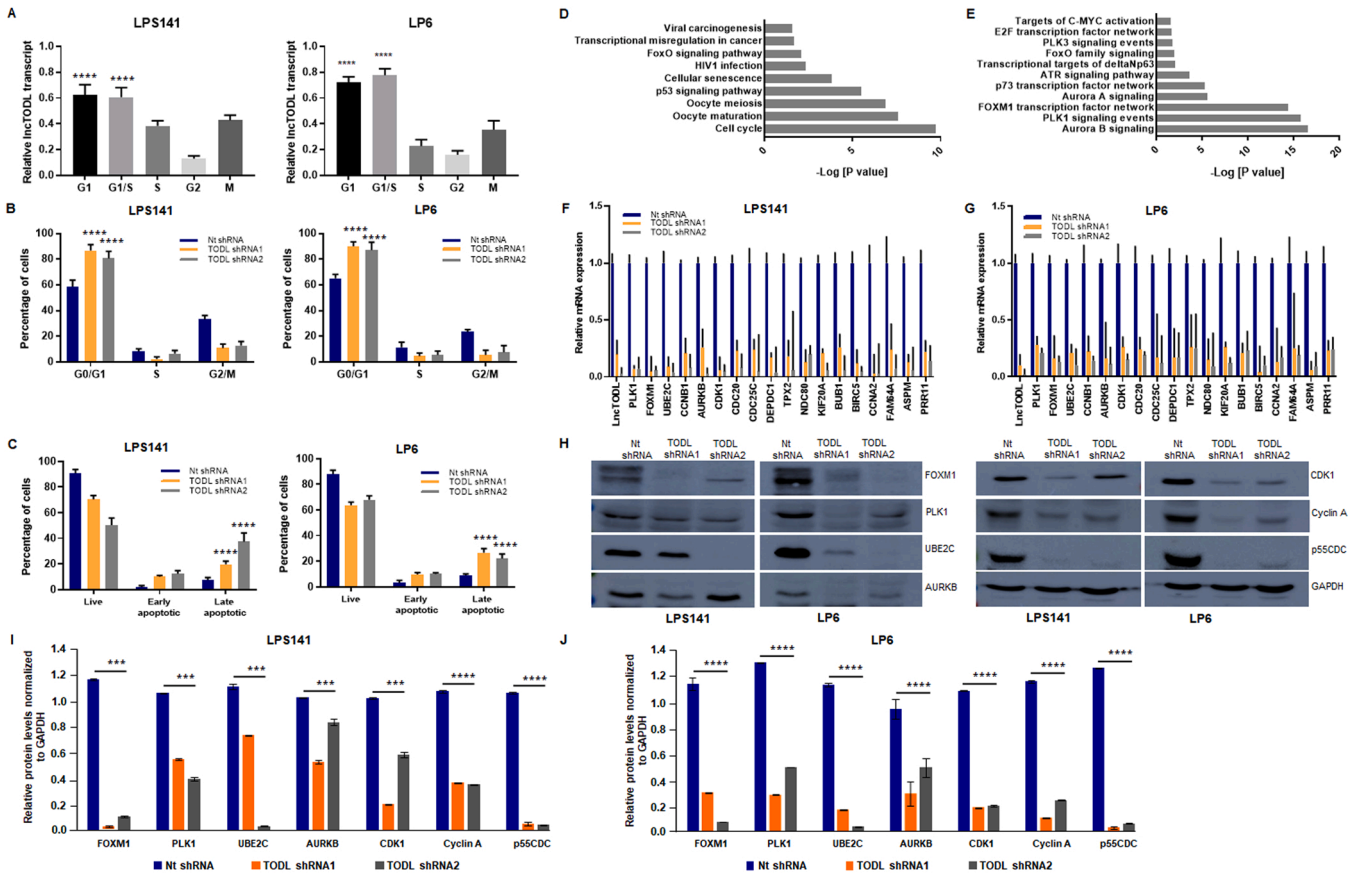

Fig. 4. Cell cycle regulation by lnc*TODL* of liposarcoma cells. (A) Real-time qPCR analysis of expression levels of *TODL* in cell cycle-synchronized LPS141 and LP6 cells. (B) Flow cytometric measurement of cell population in different phases of the cell cycle in *TODL* lncRNA silenced LPS141 and LP6 cells. (C) Early and late apoptotic cell population after downregulating *TODL* in LPS141 and LP6 cells using flow cytometry. (D) KEGG pathway analysis of downregulated genes after knockdown of *TODL* using siRNAs in LPS141 and LP6 cells. (E) Pathway interaction database analysis of RNA Seq data showing enrichment of pathways in down-regulated genes after silencing of *TODL* lncRNA. Real-time qPCR validation of cell cycle genes' downregulation identified from RNA seq data in *TODL* shRNAs using (F) LPS141 and (G) LP6 cells. (H) Effect of silencing of *TODL* on protein levels of cell cycle genes in LPS141 and LP6 analyzed by western blotting. (I-J) Densitometric analysis of western blots displayed significant reduction in cell cycle genes. GAPDH serves as the loading control. Data presented as mean ± SEM; n = 3. * ** * denotes p values < 0.0001.

OE (OE, over-expression) MLS402 cells as compared to GFP-OE [Supplementary Fig. S3A]. We observed a significant increase in cellular proliferation in *TODL* lncRNA overexpressing MLS402 cells (Supplementary Fig. S3B). The stimulating activity on cell growth with lncRNA overexpression mitigates against off-target effects of this lncRNA and confirms the oncogenic role of *TODL* in liposarcoma.

3.3. *TODL lncRNA affects in vivo tumorigenicity of liposarcoma in mice*

After validating, the growth inhibition and growth-promoting effects of knockdown and overexpression of *TODL*, respectively, tumor xenograft models were generated by injecting Nt shRNA and *TODL* lncRNA silenced LPS141 and LP6 cells subcutaneously in NSG mice. *TODL* lncRNA silenced liposarcoma cells exhibited smaller tumors compared to Nt shRNA cells (Fig. 3D). A significant decrease in tumor weight was observed in both LPS141 and LP6 xenograft mice with two different shRNAs against *TODL* (Fig. 3E). Interestingly, a tumor growth-promoting effect was noted with overexpression of *TODL* lncRNA in MLS402 xenografts (Supplementary Figs. S3C and S3D). However, no significant difference was noticed in the body weights of all mice during these experiments.

3.4. *TODL lncRNA controls cell cycle progression of liposarcoma cells*

Liposarcoma cells were synchronized and expression levels of *TODL* in specific stages of the cell cycle were measured using real-time qPCR. Significantly high levels of *TODL* were present in G1 and G1/S phases of the cell cycle compared to S, G2, and M phases (Fig. 4A). Further, we analyzed the biological effect of the down-regulation of *TODL* lncRNA expression on the cell cycle and apoptosis in liposarcoma cells. Interestingly, silencing of *TODL* induced G0/G1 arrest and resulted in a reduced population of the cells in the S and G2/M phases (Fig. 4B). In addition, a significant increase was observed in early and late apoptosis in the *TODL* lncRNA silenced LPS141 and LP6 cells in contrast to Nt shRNA cells (Fig. 4C). Taken together, the results obtained from flow cytometry assays indicated that silencing of *TODL* induced G1 cell cycle arrest and apoptosis of dedifferentiated liposarcoma cells.

RNA sequencing was performed on LPS141 and LP6 cells transfected with *TODL* siRNA1 and siRNA2 along with Nt siRNA to identify downstream targets of *TODL*. After analysis of the RNA sequencing data, a list of downregulated and upregulated transcripts are generated as provided in Supplementary Table ST6. Our data displayed that various essential signaling pathways were affected by the decrease in *TODL* expression. Moreover, the KEGG pathway analysis indicated, that cell cycle signalings are the top pathway regulated by the silencing of *TODL* lncRNA as well as other important pathways such as FoxO signaling, p53 signaling, and transcriptional misregulations (Fig. 4D). Interestingly, pathway interaction database (PID) analysis identified critical pathways from the research and treatment view of cancer and confirmed the down-regulation of FOXM1, PLK1, and Aurora signaling pathways (Fig. 4E).

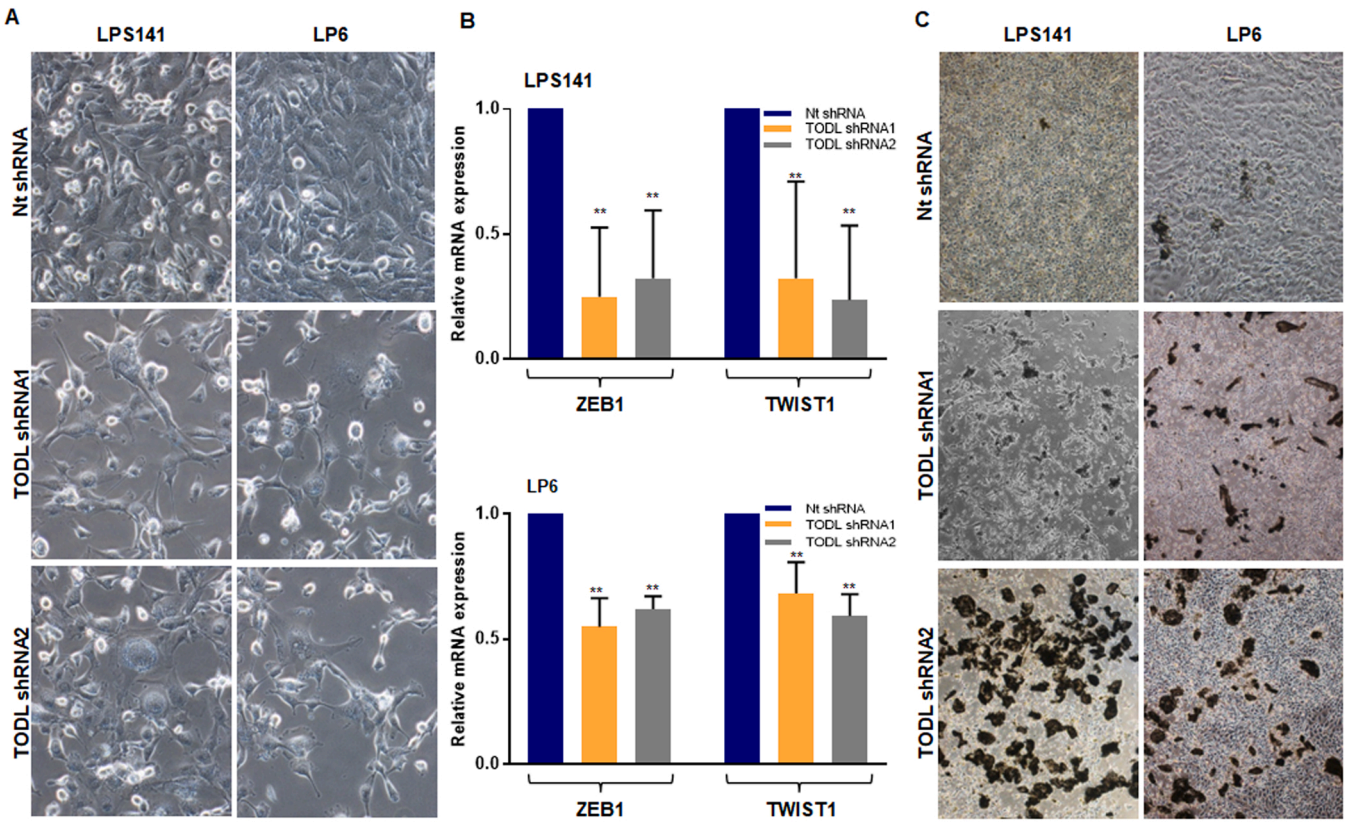

Fig. 5. Phenotypic changes after silencing of *TODL* in liposarcoma cells. (A) Bright-field microscopic images of LPS141 and LP6 cells grown for 2 weeks after the repression of *TODL*. Images were captured at 40x magnification. (B) Effect of morphological changes on the RNA expression of EMT inducer transcription factors in *TODL* silenced cells. (C) Oil red staining indicates adipogenesis in LPS141 and LP6 cells grown in the presence of an adipogenic induction medium for 3 weeks. Images were captured at 20x magnification. Data present as mean ± SEM; n = 3. * * denotes p values < 0.005.

Our qPCR results validated the downregulation of important cell cycle-related gene transcripts like *FOXM1*, *PLK1*, *UBE2C*, *AURKB*, *CCNB1*, and *CDK1* in the *TODL* lncRNA silenced group than Nt shRNA group in LPS141 and LP6 cells (Fig. 4F-G). Importantly, the western blot and densitometric analysis demonstrated that the downregulation of *TODL* lncRNA caused a significant inhibition of FOXM1 (major transcription factor) and its downstream pathway at protein levels (Fig. 4H-J). Further, we analyzed the publicly available FOXM1 ChIP-Seq database for epithelial stem cells (ESC; GSM2101199), HEK293T (embryonic kidney cells; GSM1464019) and U2OS (sarcoma cells; GSM936207) revealed distinct binding of FOXM1 on the promoter region of all cell cycle genes which were downregulated by silencing *TODL* (Supplementary Fig. S4). Interestingly, TODL-OE caused an increase in FOXM1 and its downstream cell cycle target genes validated by qPCR (Supplementary Fig. S5). These data suggest that *TODL* lncRNA regulates FOXM1, which in turn controls the cell cycle and proliferation of the liposarcoma.

3.5. Inhibition of TODL induces morphological changes in dedifferentiated liposarcoma cells

Morphological changes occurred when the expression of *TODL* lncRNA was silenced and cells were grown for 2 weeks. *TODL* silenced cells induced the establishment of islands of cells with epithelial-like morphology along with the decreased mRNA expression levels of TWIST1 and ZEB1, the crucial transcription factors associated with epithelial to mesenchymal transition (Fig. 5A-B). Also, *TODL* downregulated cells were grown in the presence of an adipogenic differentiation medium for 21 days. A remarkably high level of lipid-rich vacuoles and increased oil red staining of fat molecules was observed in *TODL* knockdown compared to Nt shRNA LPS141 and LP6 cells, indicating differentiation of LPS cells to adipocytes (Fig. 5C).

3.6. FOXM1 in liposarcoma contributes to the disease pathogenesis

FOXM1 expressed significantly high at both RNA and protein levels compared to normal controls in 32 human TCGA cancer types including sarcomas [35,36]. Analysis of alterations of *FOXM1* and its downstream targets in dedifferentiated liposarcoma patients in the TCGA database showed that the majority of patient samples displayed overexpression of *FOXM1* and its target genes that were downregulated upon silencing of the *TODL* in LPS141 and LP6 cells (Supplementary Fig. S6). Consistently, RNA expression of these FOXM1 downstream genes was upregulated in tumors compared to normal fat (Fig. 6A).

For further *in vitro* validation of *FOXM1* in liposarcoma, *FOXM1* was silenced using shRNA and achieved about 84–95% reduction in protein levels using two different shRNAs (sh2 and sh3) compared to Nt shRNA in LPS141 and LP6 cells (Fig. 6B). The silencing of the *FOXM1* significantly reduced cellular growth in both LPS141 and LP6 cells confirming the oncogenic role of FOXM1 in dedifferentiated liposarcoma cells (Fig. 6C).

3.7. TODL regulates oncogenic transcription factor FOXM1 in dedifferentiated liposarcoma

The possible mechanism by which *TODL* regulates FOXM1 was explored with the RNA pull-down of *TODL* and its associated proteins using a biotinylated *in vitro* synthesized RNA probe. MLS402 cells overexpressing exogenously *TODL* were used for this assay. Using this model system, lncRNA and attached proteins were pulled down using a biotinylated *TODL* probe (*TODL* B+) and non-specific controls; LacZ biotinylated RNA probe (LacZ B+), non-biotinylated *TODL* lncRNA

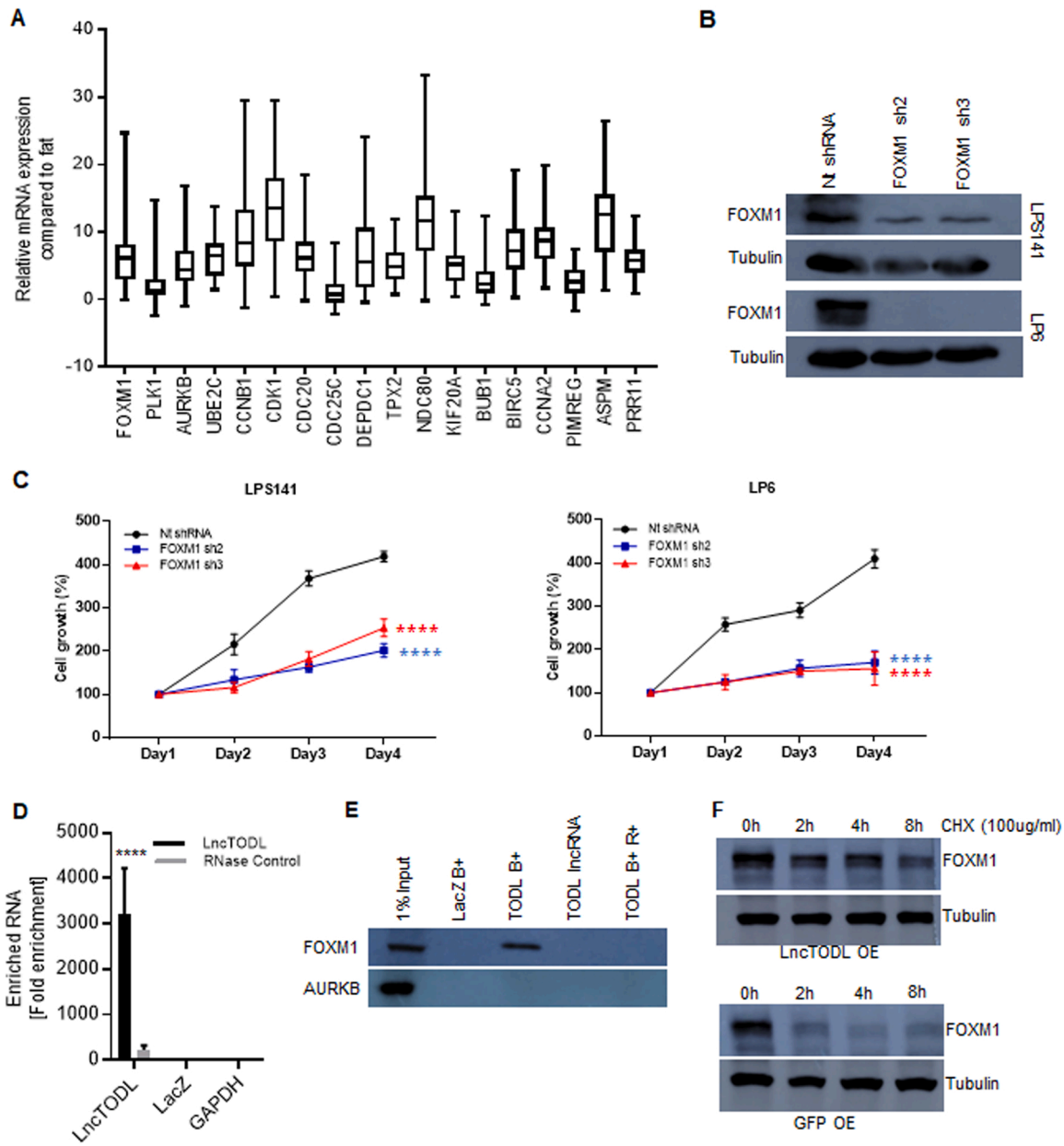

Fig. 6. *TODL* regulates oncogenic FOXM1 in liposarcoma. (A) RNA-seq expression analysis of genes regulated by silencing of *TODL* in TCGA database of dedifferentiated liposarcoma primary samples. (B) The efficiency of FOXM1 shRNA2 and shRNA3 in downregulating FOXM1 protein in LPS141 and LP6 cells as shown by western blotting. Tubulin serves as the loading control. (C) Effect of FOXM1 protein reduction on cell viability of LPS141 and LP6 cells (MTT assay). (D) RNA-pull down proficiency of biotinylated RNA probe to enrich *TODL as validated by real-time qPCR assay*. (E) Western blot of proteins retrieved by in-vitro transcribed biotinylated *TODL* RNA probe *(TODL B+)* from MLS402-*TODL* OE nuclear extracts with FOXM1 and AURKB antibodies. In vitro transcribed biotinylated *LacZ* probe *(LacZ B+),* in vitro transcribed non-biotinylated *TODL,* and RNase treatment *(TODL B+ R+)* was used as a negative control. (F) Western blot of FOXM1 protein stability during treatment with cycloheximide at different time-points in MLS402-*TODL* OE and -GFP OE cells. Tubulin serves as a loading control. Data presented as mean ± SEM; n = 3. * ** * denotes p values < 0.0001.

probe. Our qPCR data showed that *TODL* was pull-down in a significant amount using the *TODL* B+ probe compared to LacZ B+ (Fig. 6E). No RNA enrichment was found in samples treated with RNase before pull-down (*TODL* B+ R+) and for negative controls like LacZ and GAPDH. Functional interaction of *TODL* with FOXM1 protein was confirmed by western blotting demonstrating clear binding of *TODL* B+ with FOXM1 protein compared to all other controls (Fig. 6F). *TODL* B+ binding was also probed with another cell cycle protein AURKB, which showed no bands indicating the specific binding of *TODL* with FOXM1. *TODL*-OE cells were treated with a protein synthesis inhibitor, cycloheximide, and levels of FOXM1 protein were evaluated through western blotting. Results showed cycloheximide decreased FOXM1 protein expression in control cells. Importantly, *TODL* overexpression cells were able to rescue the protein degradation and enhanced FOXM1 protein stability (Fig. 6G). (Fig. 7).

4. Discussion

Long non-coding RNA (lncRNA) has emerged as a class of transcripts that play crucial roles in diverse cellular and biological functions [37]. Several investigations revealed important molecular functions of cancer-related lncRNAs emphasizing their potential as novel diagnostic and therapeutic targets [20]. Recently, a study identified various novel lncRNA transcripts across 18 tumor types and associated them with

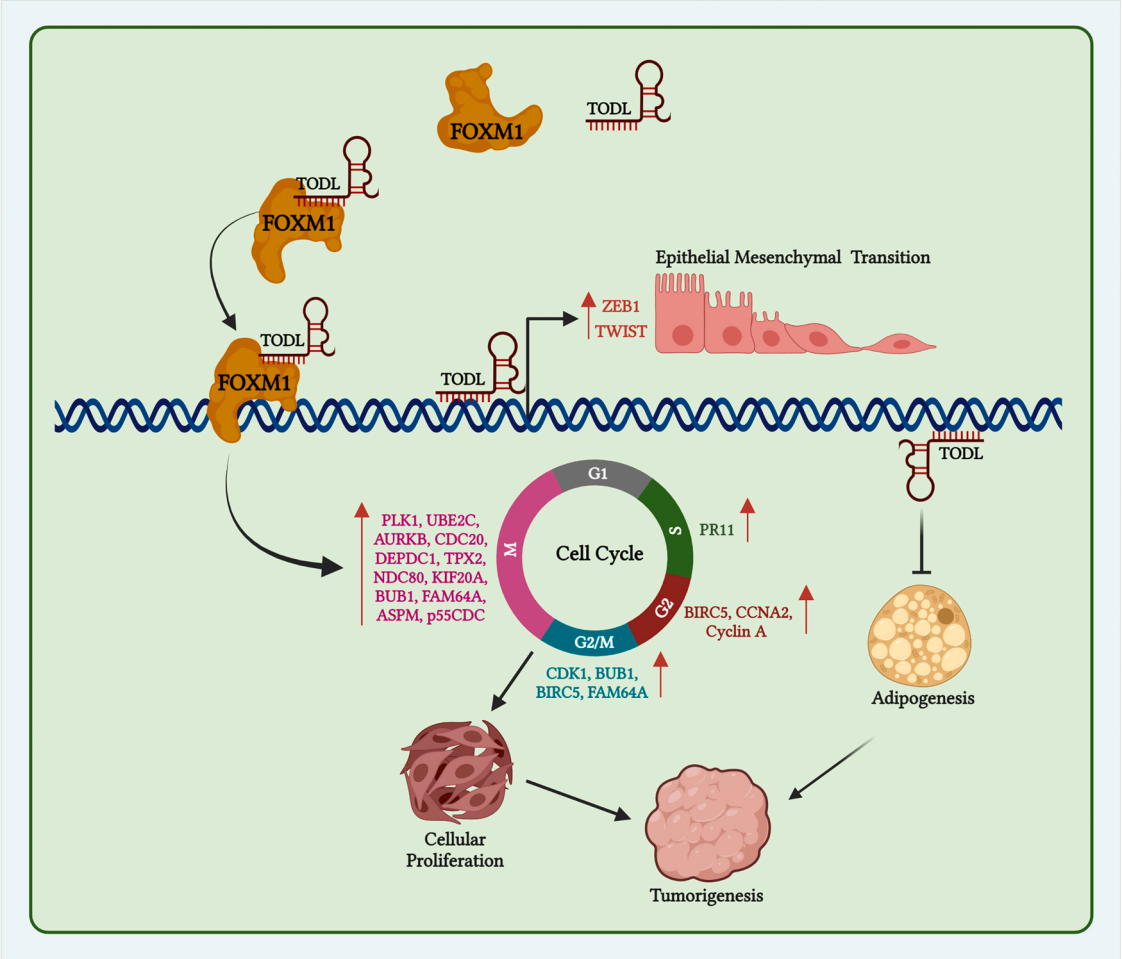

Fig. 7. Graphical representation of the *TODL* lncRNA mode of action in dedifferentiated liposarcoma. *TODL* lncRNA upregulation increased cellular proliferation by modulating cell cycle protein network, epithelial to mesenchymal transition, migration, and suppressed the adipogenesis of dedifferentiated liposarcoma cells through the FOXM1 axis.

physiological properties and cancer progression [38]. However, to the best of our knowledge, ours is the first comprehensive study reporting lncRNA transcripts in liposarcoma through RNA sequencing. Transcriptomic analysis identified various known lncRNA transcripts differentially expressed in liposarcoma including *MALAT1* [39], *PVT1* [40], *SNHG15* [41], *LINC00152* [42], and *MIR210HG* [43] that have been reported as potential oncogenes by other groups in human malignancies including osteosarcoma, and Ewing sarcoma [44,45]. In another study, Yong Zhang and colleagues identified the networks of non-coding RNAs in retroperitoneal liposarcoma (RLS) [46]. Their study focused on a downregulated Linc00423 in RLS tissue specimens. In functional assays Linc00423 forced expression suppressed cell growth and tumor growth of RLS, suggesting the tumor suppressor role of Linc00423 in RLS. Mechanistically, Linc00423 was found to be important for NFATC3/-MAPK cascade in RLS cells [46]. Interestingly, our study identified novel unannotated RNAs possessed the same characteristic features as most of the known lncRNAs i.e., low expression, low protein-coding potential, shorter length, and fewer exons. TCONS_00064445 lncRNA [termed as *TODL*] was identified as exclusively overexpressed transcript only in dedifferentiated liposarcomas which is an extremely aggressive, and metastatic subtype with a high mortality rate. *TODL* lncRNA is small (308 bases) and interesting lncRNA because of its extremely high expression in dedifferentiated liposarcomas.

Both *in vitro* and *in vivo* knockdown and overexpression studies demonstrated that *TODL* has potent oncogenic activity in liposarcoma. Functional assays show that expression of *TODL* is especially prominent in the G1 phase and plays a role in liposarcoma proliferation regulating its cell cycle progression. Several studies have indicated the role of several lncRNAs in the cell cycle regulation of cancer cells [47]. Additionally, the inhibition of *TODL* demonstrated significant enrichment of critical signaling cascades like transcriptional misregulation, FOXM1 network, cell cycle, FoxO signaling, PLK1 signaling, Aurora kinase pathway, and p53. Importantly, the cell cycle regulation by *TODL* lncRNA is manifested by its interaction with the major oncogenic transcription factor FOXM1 protein, which is known to control the proliferation of cancer cells in a variety of malignancies [35,36]. Similar to other tumors, *FOXM1* was observed as an upregulated transcription factor in dedifferentiated liposarcomas compared to fat and regulates its cell proliferation [48,49]. Silencing of *TODL* significantly decreased *FOXM1* transcript and protein levels leading to the downregulation of its target genes. Interestingly, we observed that downregulation of *FOXM1* using shRNAs did not affect the transcription of *TODL* transcript confirming *TODL* regulation of FOXM1 and not vice versa. Taken together, these results indicate a role of *TODL* in cell cycle regulation by stabilizing FOXM1 protein to enhance the proliferation of liposarcoma.

Interestingly, cells with silenced *TODL* transformed into a flat epithelial-like morphology indicating a transition that is less invasive and less metastatic. We could not observe any changes in E-cadherin levels since it is not expressed in liposarcoma cells. However, the expression of EMT-associated transcription factors like TWIST and ZEB1 were downregulated in *TODL*-silenced LPS141 and LP6 cells [50,51]. This phenotypic change also suggested that silencing of *TODL* lncRNA

could attenuate the aggressiveness of dedifferentiated liposarcomas. In addition, silencing of *TODL* induced the re-differentiation potential of dedifferentiated liposarcomas in the presence of adipogenic induction medium and initiated differentiation into mature adipocytes. Cell cycle arrest resulting in decreased cell proliferation assisted in the conversion of dedifferentiated liposarcoma cells into fat cells. This differentiation phenotype might be a consequence of the downregulation of *FOXM1* mediated by the silencing of *TODL*. There are no direct pieces of evidence of FOXM1 as a negative regulator of adipogenesis but a group investigated and showed that upregulated FOXM1 in epithelial stem cells induces enhanced proliferation ability, which can retard terminal differentiation [52]. Therefore, we hypothesized that *TODL* lncRNA regulates FOXM1 protein levels and facilitates liposarcoma cells a growth advantage, and inhibits adipogenic differentiation.

In summary, the present investigation characterized lncRNAs in liposarcomas and noted the overexpression of *TODL* lncRNA in the dedifferentiated liposarcoma. Our findings highlighted *TODL* lncRNA as a potential oncogenic lncRNA in regulating the progression of the cell cycle, uncontrolled proliferation, apoptosis, adipogenesis *in vitro,* and tumor growth using the xenograft model. Although a detailed exploration is further needed for establishing the therapeutic potential of *TODL* lncRNA but based on the demonstrated results *TODL* could be used as a novel diagnostic biomarker and pharmacological silencing of *TODL* may be a promising strategy for therapeutic management of dedifferentiated liposarcomas.

CRediT authorship contribution statement

DK, HPK, and MG conceived the idea and designed the study; DK, AK, NSVS, SPJ, PS, SSS, JY, HK, and MG performed the experiments; DK, PD, AM, and MG analyzed the data; DK, HPK, and MG wrote and revised the manuscript. All authors read and approved the final version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Data Availability

The data that has been used is confidential.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2022.106462.

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