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Icilin inhibits E2F1-mediated cell cycle regulatory programs in prostate cancer

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ABSTRACT

Aberrant expression of cell cycle regulators have been implicated in prostate cancer development and progression. Therefore, understanding transcriptional networks controlling the cell cycle remain a challenge in the development of prostate cancer treatment. In this study, we found that icilin, a super-cooling agent, down-regulated the expression of cell cycle signature genes and caused G_1 arrest in PC-3 prostate cancer cells. With reverse-engineering and an unbiased interrogation of a prostate cancer-specific regulatory network, master regulator analysis discovered that icilin affected cell cycle-related transcriptional modules and identified E2F1 transcription factor as a target master regulator of icilin. Experimental analyses confirmed that icilin reduced the activity and expression levels of E2F1. These results demonstrated that icilin inactivates a small regulatory module controlling the cell cycle in prostate cancer cells. Our study might provide insight into the development of cell cycle-targeted cancer therapeutics.

1. Introduction

Most prostate cancers regress after androgen-deprivation or anti-androgen therapy, but the cancers eventually progress into castration-resistant prostate cancer (CRPC) [1]. Because no effective therapeutic regimens are available for CRPC, the recurrence of prostate cancer remains a major challenge for improving clinical outcome [2]. Therefore, it is needed to develop new therapeutic approach for prostate cancer.

CRPC development is associated with relief from androgen deprivation-induced cell cycle arrest [3]. Indeed, many microarray studies revealed the association between prostate cancer progression and cell cycle regulation; over-expression of the cell cycle controlling gene signatures was observed in metastatic prostate cancer with the high proliferation rate [4]. These results suggest that aberrant cell cycle control is a crucial therapeutic bypass mechanism against androgen deprivation in prostate cancer [5]. Therefore, targeting cell cycle may be promising for prostate cancer treatment.

We previously reported that icilin (AG 3–5), a synthetic super-cooling compound, induces G_1 arrest in PC-3 prostate cancer cells and down-regulates the expression levels of several cell cycle regulators [6]. Therefore, icilin might be a promising chemical probe to produce knowledge about how to develop cell cycle-targeted therapy. However, the transcriptional regulatory effects of icilin on cell cycle control have not been thoroughly investigated.

In this study, we performed microarray experiments on PC-3 cells to facilitate system-level understanding of icilin actions. Gene set enrichment analysis (GSEA) confirmed that icilin markedly affected cell cycle gene signatures. We constructed prostate cancer-specific transcriptional regulatory network by integrating public microarray data sets and identified E2F1 as a crucial target of icilin. Our findings may provide a novel insight into the understanding of cell cycle control for prostate cancer treatment.

2. Materials and methods

2.1. Cell culture and reagents

PC-3 cells were supplied by ATCC and maintained under RPMI1640 medium containing 10% fetal bovine serum. All cell culture agents used were obtained from Invitrogen. All other reagents not specified were supplied by Sigma–Aldrich. Icilin was dissolved in DMSO and 0.1% DMSO was used as a vehicle.

2.2. Microarray experiment

Total RNA was extracted from PC-3 cells following treatment with vehicle or icilin at 200 μM for 24 h and microarray experiments were performed as described in an our previous paper [7].

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Our raw data are available through the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE45567.

2.3. Collection of public microarray data sets

We collected publically available microarray datasets of prostate cancer experimented on only PC-3 cells based on only the Human Genome U133A, U133 Plus 2.0, and 1.0 ST Affymetrix platforms because many microarray experiments were performed with these platforms and the three platforms contain many overlapped probe sets. Quality-control test and pre-processing steps for microarray data are described in Supplementary Text.

2.4. Unsupervised hierarchical clustering and significance analysis of microarrays (SAM)

Unsupervised hierarchical clustering was used to group samples and genes that had similar expression values; Pearson correlation coefficients were used as column and row distances, and the pairwise complete-linkage method was used to construct the hierarchies. Then, a heat map was generated using the GenePattern v3.7.0 software to enable visual inspection of the data [8]. SAM was used to identify statistically significant genes that were expressed differentially between icilin- and vehicle-treated PC-3 cells [9]. Significance was determined by permutation testing to allow for correlation between genes and to avoid the necessity of meeting parametric assumptions. SAM estimates a false discovery rate (FDR) to correct for multiple testing. We estimated our empirical null distribution using 1000 permutations and set an FDR threshold (q-value) of 0.001 for a stringent feature selection. Then, we performed gene set enrichment analysis (GSEA) to obtain better biological interpretations in the gene expression profile of icilin-treated PC-3 cells (see Supplementary Text for more details).

2.5. Algorithm for the reconstruction of accurate cellular networks (ARACNe) and master regulator analysis (MRA)

ARACNe algorithm was used to assemble a genome-wide repertoire of the transcriptional interactions from prostate cancer gene expression profiles of high-quality using icilin-treated PC-3 cell and public microarray data. ARACNe is an information theoretical approach to infer the direct interactions between TFs and their regulons from large sets of gene expression data [10,11]. The prostate cancer-specific interaction network constructed by ARACNe algorithm was applied to the Master Regulator Analysis-Fisher's exact test (MRA-FET) [12] and MAster Regulator INference algorithm (MARINa) method [13]. Then, they inferred master regulator candidates, which control the transition between the two phenotypes, and their targets from the global transcriptional activation. More detailed explanation for transcriptional interaction construction, MRA-FET and MARINa is described in Supplementary Text.

2.6. Biological experiments: RT-PCR, Western blot, cell cycle, and luciferase assay

RT-PCR experiments were performed using specific primers for E2F1, E2F2, or GAPDH as previously described [14,15]. The protein samples were resolved in 10% SDS–PAGE and analyzed with the antibodies to E2F1, E2F2, or GAPDH (Santa Cruz Biotechnology). The data shown here are representative of three independent experiments. Reporter gene construct containing E2F-binding elements was used to assess E2F transcriptional activity and luciferase assays were performed as previously described [16]. Cell cycle was analyzed using flow cytometry as previously described [6,17,18].

2.7. Statistical analysis of E2F1 expression level in different prostate cancer stages

Public microarray data of prostate cancer patients, GSE3325, were collected and pre-processed as described in this study. We analyzed the data to compare E2F1 expression level in different prostate cancer stages using ANOVA test. Statistical processing of data was performed using Excel and R (version 2.15.1) [19]. For all of the analyses, p-value 0.05 was considered as significant.

3. Results

3.1. Icilin modulates the expression of cell cycle-related gene signatures in PC-3 cells

To systemically understand pharmacologic actions of icilin, we performed a series of computational analyses. We performed unsupervised hierarchical clustering analysis for the gene expression profiles of all 11,877 genes in vehicle-and icilin-treated PC-3 cells (GSE45567). Hierarchical clustering algorithm clustered the microarray samples into two distinct types (Fig. 1A). The SAM analysis identified 2904 out of 11,877 genes as being differentially expressed between vehicle- and icilin-treated cells. Then, GSEA using GO biological process signatures found that icilin down-regulated 24 gene signatures and up-regulated three gene signatures in icilin-treated cells (FDR q-value < 0.2) (Table S1).

Because icilin is known to induce G_1 arrest [6], we noticed that 7 cell cycle-related gene signatures were significantly down-regulated in icilin-treated cells (Table S1). As an example, Fig. 1B (right) shows an enrichment plot of the distribution of up- and down-regulated genes in MITOTIC_CELL_CYCLE gene signature between icilin- and vehicle-treated PC-3 cells. The heat map in Fig. 1B (left) illustrates the gene expression patterns for the 49 leading edge subset (LES) genes. These results indicate that icilin affects the expression of various cell cycle-related genes. We also experimentally confirmed that icilin induced G_1 arrest (Fig. 1C).

We examined individual cell cycle-related gene signatures to identify LES genes. M_PHASE, MITOSIS, M_PHASE_OF_MITOTIC_CELL_CYCLE, MITOTIC_CELL_CYCLE, CELL_CYCLE_PROCESS, CELL_CYCLE_PHASE, and CELL_CYCLE_GO_0007049 have 32, 27, 28, 49, 55, 44, and 72 LES genes, respectively. The 72 LES genes of the CELL_CYCLE_GO_0007049 completely covered all LES genes in other cell cycle-related signatures (Table S2).

3.2. Collection of prostate cancer microarray data sets to construct prostate cancer-specific network

Based on computational and experimental results (Table S2 and Fig. 1C), we have questioned by which transcriptional regulatory mechanisms are involved in the G_1 phase signature changes inicilin-treated cells. We constructed prostate cancer-specific transcriptional regulatory network using publically available prostate cancer microarray data sets. Our strategy of computational analyses is summarized in (Fig. S1). The collected microarray data sets consist of various experimental phenotypes by treatment with various compounds (Table S3). After removing 11 of low-quality samples by quality control test (Table S3), 110 microarray samples were used for constructing transcriptional regulatory network. ARACNe algorithm inferred a consensus network of 405,315 prostate cancer-specific TF-target interactions from the 1066 human TFs.

3.3. Icilin affects the transcriptional modules linked to cell cycle regulation in PC-3 cells

From the prostate cancer-specific transcriptional network, MARINa algorithm identified 366 TFs as MR candidates that

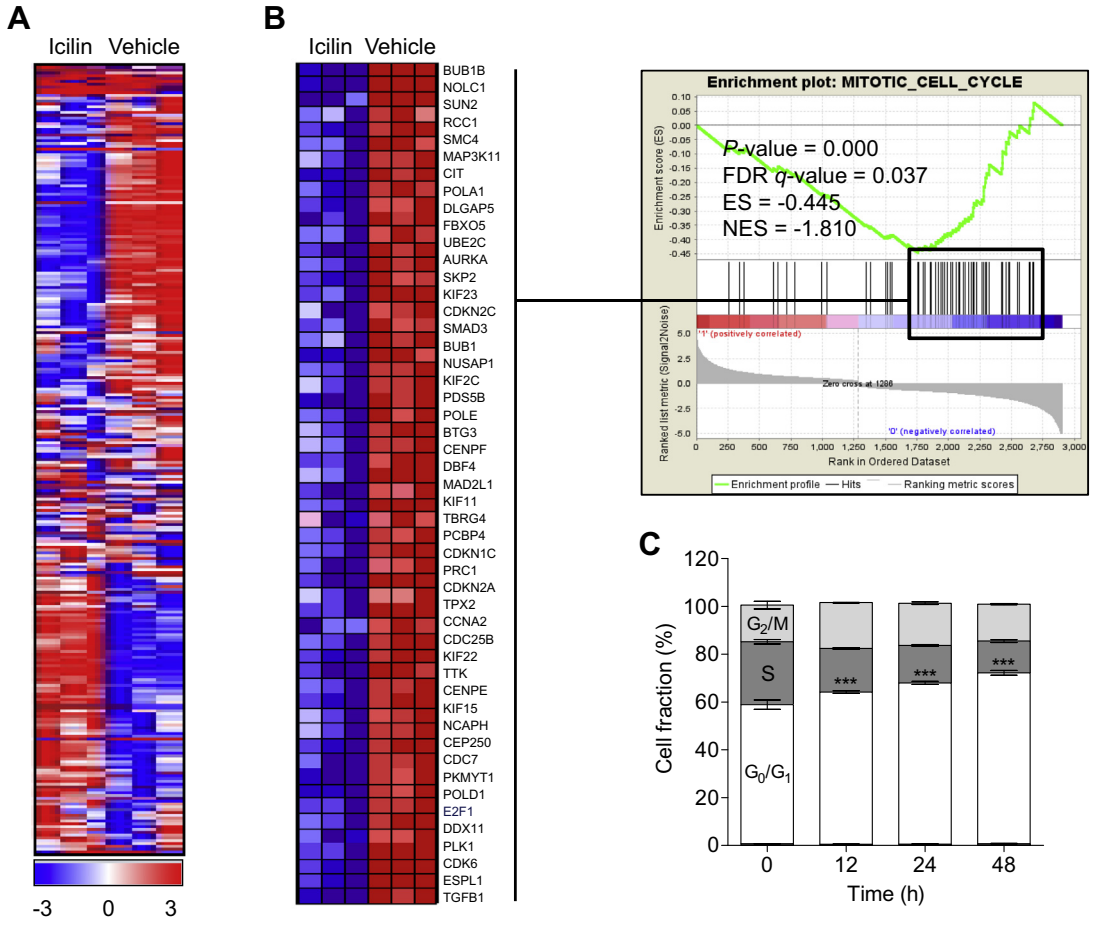

Fig. 1. Gene expression profile affected by icilin in PC-3 cells. (A) Heatmap illustrating large-scale differences in gene expression between vehicle- and icilin-treated PC-3 cells. (B) Enrichment plot of MITOTIC_CELL_CYCLE gene signature between icilin- and vehicle-treated PC-3 cells. (C) The cells were incubated with icilin at 200 μM for the indicated times prior to cell cycle analysis. Cell fraction is expressed as the percentage of cells in each phase of the cell cycle. The data were expressed as the mean ± SEM ($n = 4$) $^{***}p < 0.005$.

Table 1
The list of 23 MRs overlapping between MARINa and MAR analysis.

Gene ID	Gene symbol	NES[a]	*p*-Value of NES	Odd ratio[b]	Original MRA/ recovered_MRA[c]	FET *p*-value	Markers in regulon	Markers in intersection set	Mode
1063_at	CENPF	9.925	0.000	6.948	0	1.06E−24	685	35	–
4605_at	MYBL2	12.428	0.000	13.105	0	1.09E−23	676	34	–
3066_at	HDAC2	10.768	0.000	9.654	0	4.84E−21	692	32	–
865_at	CBFB	7.267	0.000	3.321	0	1.04E−19	766	32	–
2146_at	EZH2	8.629	0.000	3.807	0	1.78E−16	772	31	–
2305_at	FOXM1	13.983	0.000	9.856	0	2.67E−18	787	31	–
1786_at	DNMT1	11.693	0.000	10.433	0	5.08E−17	673	28	–
1870_at	**E2F2**	**9.438**	**0.000**	**16.761**	**0**	**1.39E−15**	**635**	**26**	–
2130_at	EWSR1	8.375	0.000	8.809	0	7.52E−17	507	25	–
1869_at	**E2F1**	**12.188**	**0.000**	**9.843**	**0**	**1.99E−14**	**522**	**23**	–
7022_at	TFAP2C	6.465	0.000	16.285	0	1.88E−14	464	22	–
140467_at	ZNF358	5.385	0.000	2.011	0	4.25E−10	553	21	+
3609_at	ILF3	11.859	0.000	12.178	0	2.50E−10	674	21	–
8914_at	TIMELESS	9.291	0.000	3.967	0	6.09E−09	649	19	–
10608_at	MXD4	−3.198	0.007	1.863	0	5.58E−08	665	18	+
7533_at	YWHAH	8.493	0.000	13.099	0	1.83E−09	534	18	–
10514_at	MYBBP1A	13.251	0.000	10.028	0	4.41E−06	716	16	–
1382_at	CRABP2	9.989	0.000	16.627	0	1.11E−06	644	16	–
221037_at	JMJD1C	−7.987	0.000	6.806	0	1.11E−06	644	16	+
26959_at	HBP1	−9.815	0.000	7.307	0	2.42E−07	501	15	+
7004_at	TEAD4	11.874	0.000	15.065	0	5.89E−07	537	15	–
25909_at	AHCTF1	8.354	0.000	6.512	0	8.25E−07	477	14	–
9569_at	GTF2IRD1	4.055	0.001	2.029	0	1.08E−06	488	14	+

Note: The 23 MRs overlapping between MARINa and MRA results were ranked on the basis of the number of markers in intersection set. NES values were rounded off to three decimal places.

[a] The normalized enrichment score (NES) for the regulon of the TF is calculated by normalizing the enrichment score (ES) across analyzed the regulon of the TFs.

[b] Odds of a regulon gene being in the GSEA leading edge set/odds of a regulon gene being in the GSEA trailing edge set.

[c] A value of 0 means that the TF was found to be enriched by GSEA and that it remained enriched even after the common regulons with the other TFs were removed from its regulon.

differentiate gene expression profile in icilin-treated PC-3 cells (Table S4). We found that 214 out of 366 MRs were negatively enriched and 152 MRs were positively enriched in icilin-treated PC-3 cells. On the other hand, MRA inferred 39 cell cycle-specific TFs as MRs at FET p-value 0.01 as the enrichment threshold (Table S5). We found the overlapped 23 MRs between 366 MRs by MARINa and 39 MRs by MRA (Table 1 and Fig. 2A). The 23 MRs are ranked on the basis of the total number of markers in intersection set, which counts the number of genes found in the intersection between the 72 LES genes of cell cycle signatures and the regulon of the MR.

The number of genes in the intersection set would give a better evaluation of the actual biological significance of the MR than p-value calculation because the number of regulons in each TF is different and p-values are not directly comparable to each other. We first focused on the top 13 MRs that regulate more than 21 cell cycle signature genes. The Spearman's correlation between the expression profile of the 13 MRs and their targets are indicated as color bar graph in (Fig. 2C). It showed that 12 out of the top 13 MRs have minus mode and one MR has plus mode. The expression profile of the MRs in the minus mode is positively correlated with their targets which have negative differential expression in the icilin-treated PC-3 cells. This means that the MRs of the minus mode were down-regulated by icilin-treatment, so the expression of MRs' targets were also down-regulated.

3.4. Icilin down-regulated E2F1, a master regulator for cell cycle regulation, in PC-3 cells and its clinical relevance

Because E2F family proteins regulate the expression of multiple genes involved in cell cycle transitions [20], we noticed that MARINa and MRA designated several E2F family members as MRs in prostate cancer (Tables S4 and S5). E2F1, E2F2 and E2F4 were found in MARINa (Table S4), whereas E2F1, E2F2 and E2F8 were predicted in MRA (Table S5). Only E2F1 and E2F2 were commonly found in both analyses (indicated by bold in Table 1). In fact, E2F1 and E2F2 are known to induce aberrant cell cycle regulation in cancer [21]. In the SAM, the mRNA levels of E2F1 and E2F2 were significantly down-regulated in icilin-treated PC-3 cells: E2F1 fold change 0.819, q-value < 0.001; and E2F2 fold change 0.895, q-value < 0.005.

To confirm that icilin down-regulates the expression of E2F1 and E2F2 in PC-3 cells, we first performed luciferase reporter assays. Icilin decreased luciferase activity by 60% in comparison to control (Fig. 3A). In addition, RT-PCR and Western blot analyses showed that icilin specifically reduced the mRNA and protein

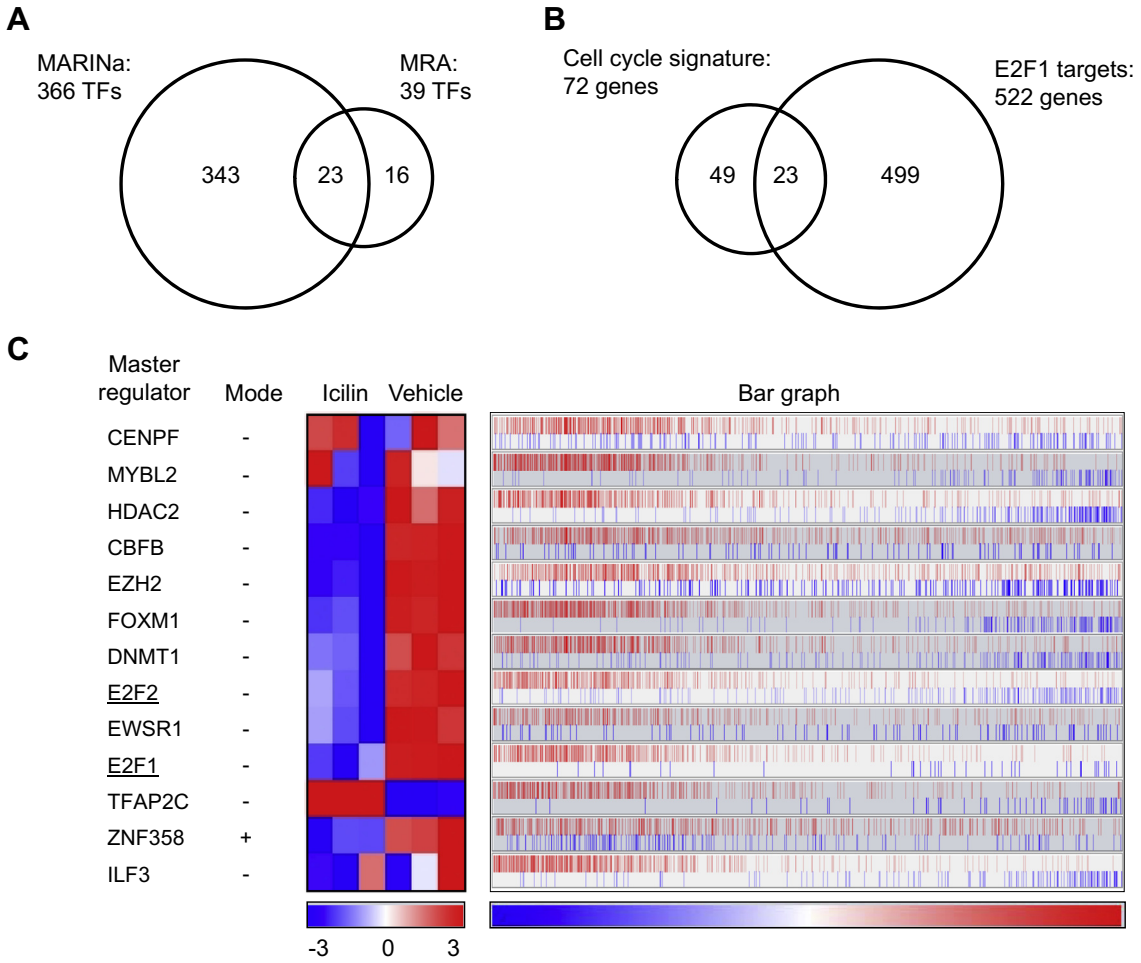

Fig. 2. Prostate cancer cell cycle regulatory master regulators (MRs). (A) Twenty-three MRs are overlapping between 366 TFs found by MARINa and 39 TFs found by MRA. (B) Twenty-three out 522 E2F1 target genes found in prostate cancer interactome are overlapping with cell cycle signature genes down-regulated by icilin-treatment in PC-3 cells. (C) The 13 MRs were ranked by the number of markers in the MR's regulon that are intersected with 72 LES genes. Mode explains how the expression of the TF is correlated with those target genes. The heat map in the middle shows the mRNA expression levels of the 13 MRs in icilin- and vehicle-treated PC-3 cells. Bar graph shows the distribution of positively (red) and negatively (blue) correlated target genes of the MR. The bar position corresponds to their rank which is calculated by '$-\log_{10}$ (p-value) $*$ sign (t-value)', and bar color indicates the sign of the Spearman's Correlation between the expression profile of the TF and its target genes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

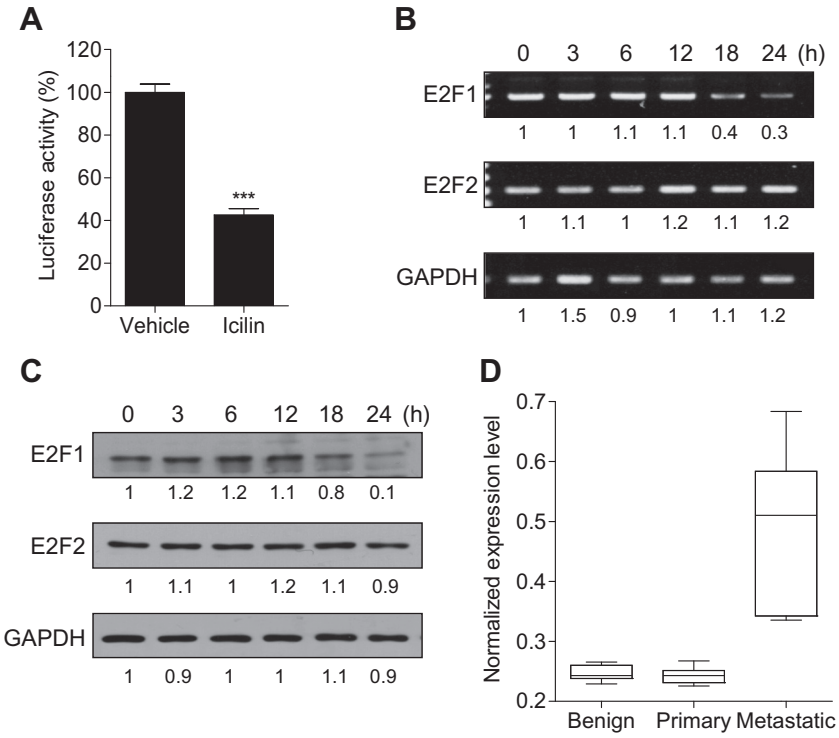

Fig. 3. Down-regulation of E2F1 in icilin-treated PC-3 cells. (A) The cells were transfected with the reporter construct containing E2F1-binding elements and treated with icilin at 200 μM for 24 h. The luciferase activity was expressed as a relative value compared to that of the untreated cells which was set to 100%. The data were expressed as the mean ± SEM ($n = 4$) $^{***}p < 0.005$. (B) The cells were incubated with icilin at 200 μM for the indicated times prior to RT-PCR. The GAPDH was used as an experimental control. (C) The cells were incubated with icilin at 200 μM for the indicated times prior to Western blot analyses. GAPDH was used as a loading control. Band intensity was densitometrically determined. (D) E2F1 expression level in prostate cancer progress was represented by box plot. The *x*-axis indicates three different prostate cancer stages and the *y*-axis represents normalized E2F1 expression level.

levels of E2F1 at 24 h after treatment, but those of E2F2 remained (Fig. 3B and C). These results demonstrate that icilin specifically reduced the expression and activity of E2F1 in PC-3 cells.

Then, we examined the cell cycle regulatory genes controlled by E2F1 transcription factor. MRA identified total 522 regulons of E2F1 (Table 1, column of 'Markers in regulon'), and computed 23 out of 522 targets are intersected with 72 cell cycle LES genes (Table 1, column of 'Markers in intersection set' and Fig. 2B). The 23 genes are listed in Table S6 with statistic significance. In addition, we constructed the sub-network of prostate cancer interactome between E2F1 and other cell cycle-specific TFs or E2F1 target genes which are identified by MRA (see Supplementary Text and Fig. S2 for more details).

To address the clinical relevance of our findings, we analyzed E2F1 expression levels in GSE3325 data of prostate cancer patients. Quality-control test result and phenotype data of microarray samples are described in the Table S7. The averages of normalized E2F1 expression levels in benign prostate tissue, localized primary and metastatic prostate cancer were 0.247, 0.245, and 0.489, respectively (Fig. 3D). This result suggests that high expression level of E2F1 is associated with prostate cancer progression.

4. Discussion

There are three main findings in the study: (1) icilin modulates the expression of key cell cycle regulatory genes in PC-3 cells; (2) icilin affects 13 MRs regulating more than 21 out of 72 cell cycle signature genes that were identified by GSEA in PC-3 cells; and (3) E2F1 was identified as a master regulator that can be an icilin's target to inhibit G_1-S phase transition in prostate cancer. These results facilitate system-level understanding of pharmacologic actions of icilin and provide insight into the development of cell cycle-targeted cancer therapeutics.

Our results suggest that icilin down-regulated cell cycle regulatory genes through targeting E2F1 master regulator in prostate cancer cells. Our previous study showed that icilin caused G_1 arrest in PC-3 cells and down-regulated the protein levels of G_1 phase regulatory genes, such as, cyclin A, cyclin D1, CDK1, and CDK2 [6]. This present study also showed down-regulation of the mRNA levels of G_1 phase regulatory genes which are registered in MSigDB (indicated by underline in Table S2). In addition, SAM analysis found that CDK1, CDK2 and CDK4 are significantly down-regulated in icilin-treated PC-3 cells (fold change 0.833, 0.852, and 0.773; *q*-values were less than 0.001).

The activation of E2F1 cell cycle pathway is characteristic of metastatic prostate cancer [22,23]. Our study suggests that icilin induced the growth inhibition in prostate cancer cells through the suppression of E2F1 expression levels and transcriptional activity. Out of the E2F family members, especially E2F1 is known to initiate G_1-S transition in CPRC and activate androgen receptor through physical interaction [24,25]. Recent studies revealed that the inhibition of E2F1 transcriptional activity caused G_1 arrest in prostate cancer cells [26,27].

A problem in many meta-analysis and cancer interactome construction studies is that they have overlooked rigorous pre-processing steps to ensure reliable analysis results [28]. Our study is the first to aggregate publically available and independent microarray data of prostate cancer and to perform intensive pre-processing steps, such as quality control test, batch adjusting and single-sample microarray normalization. These pre-processing steps certified high quality data for functional enrichment analysis and prostate cancer interactome inference. We expect that our rigorous pre-processing approach will serve as an example for gene-expression meta-analyses of other complex diseases.

In summary, this study clarified the molecular mechanism underlying cell cycle regulation in prostate cancer using genome-wide data. We demonstrated that icilin decreases E2F1 transcriptional activity in PC-3 cells. This study supports that targeting transcriptional regulators for cell cycle control is a promising strategy for prostate cancer treatment.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.015.

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