saKLK1-374 is More Difficult to Induce KLK1 Expression in Normal Cell Lines Than in Tumor Cell Lines And Inhibits the Growth of Prostate Cancer Cells Not via Induction of KLK1 Expression

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Research

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Abstract

Background: RNA activation, as a method of regulating gene expression at the transcriptional level, is far less widely used than RNA interference because of the insufficient understanding of the mechanism and the unstable success rate. It is necessary to analyze the failure cases of RNA activation to promote the application of RNA activation. When we validated the saRNAs designed to induce KLK1 expression, we found that saKLK1-374 can up-regulate KLK1 expression in prostate tumor cell lines, but failed in normal prostate cell lines. In addition, we also found that saKLK1-374 inhibited the growth of prostate cancer cells, which seems to be the opposite of the function of KLK1. This article is about experimental research and analysis of these two issues.

Methods: To determine whether the phenomenon that the RNA activation of normal cells is difficult to succeed is only valid when the target gene is KLK1, we used $p21^{WAF1/CIP1}$ as the target gene to perform RNA activation experiments in normal prostate cells and prostate cancer cells. Next, to determine whether the above phenomenon exists in other tissues, we also performed RNA activation experiments with KLK1 and $p21^{WAF1/CIP1}$ as target genes in normal cell lines and tumor cell lines derived from the bladder. We have also extended the time from transfection to the detection of target gene expression to evaluate whether a longer saRNA action time can change the phenomenon that saRNA fails to up-regulate target gene expression in normal cells. In terms of mechanism research, we used fluorescently labeled dsRNA to evaluate the transfection efficiency, and also detected the expression of Ago2 and IPO8 proteins. In another issue of saKLK1-374 inhibiting prostate cancer cells, we tested the ROS content and apoptosis levels of prostate cancer cells after saKLK1-374 transfection. We used recombinant KLK1 protein to directly interfere with prostate cancer cells as a positive control for KLK1 function research. In turn, we also used siRNA to inhibit the expression of KLK1 in prostate cancer cells to compare the growth of prostate cancer cells when KLK1 mRNA was up-regulated and reduced.

Results: The $p21^{WAF1/CIP1}$ gene could be significantly upregulated by saRNA in prostate cancer cell lines, but not in normal prostate cell lines. The expression of KLK1 in bladder-derived cell lines was extremely low and could not be induced by saRNA. The $p21^{WAF1/CIP1}$ gene could be up-regulated by saRNA to a higher extent in bladder cancer cell lines, while it was up-regulated by saRNA in normal urothelial cell line to a lower extent. Prolonging the action time of saRNA could not change that saRNA failed to induce the expression of target genes in normal cell lines. Compared with tumor cell lines, normal cell lines had lower transfection efficiency or lower expression of Ago2 and IPO8. After being transfected with saKLK1-374, prostate cancer cells had increased ROS and increased levels of apoptosis. The recombinant KLK1 protein did not increase ROS in prostate cancer cells, nor did it inhibit their growth. Even though saKLK1-374 up-regulated the expression of KLK1 in prostate cancer cells, siRNA still suppressed the expression of KLK1 below the baseline level, and in this case, the growth of prostate cancer cells was still at a suppressed level.

Conclusion: Normal cell lines may be more difficult to be successfully induced target gene expression than tumor cells due to low transfection efficiency or low Ago2 and IPO8 expression. In addition, although

saKLK1-374 is designed to up-regulate the expression of KLK1, the reason that it inhibits the proliferation of prostate cancer cells is irrelevant to the up-regulated expression of KLK1.

Background

Small double stranded RNA (dsRNA) partnering with Argonaute (Ago) proteins plays important roles in diverse biological processes by suppressing or up-regulating the expression of target genes[1]. RNA interference (RNAi) is a silencing mechanism in which small interfering RNA (siRNA) target specific mRNA sequences to inhibit mRNA translation or degrade them[2]. Pioneering observations on RNAi were reported in plants, but later on RNAi-related events were described in almost all eukaryotic organisms[3]. As a convenient tool for knocking down the expression of individual genes post transcriptionally, RNAi is well known among scientists and has been widely used to study the cellular function of genes[2]. In contrast, RNA activation (RNAa) is a currently discovered phenomenon that small activating RNA (saRNA) can activate target gene expression mainly via binding complementary sequences of the promoter[1,4]. Briefly, same as RNAi at first, saRNA exogenous introduced is loaded in the cytoplasm by an Ago protein which incorporates the guide strand in the saRNA to form an active RNA-Ago complex[1]. Next, the RNA-Ago complex is imported into the nucleus through a yet unknown mechanism[1]. In the nucleus, the complex may recruit key proteins for transcription initiation such as RNA helicase A (RHA), RNA polymerase-associated protein CTR9 homolog (CTR9), and RNA polymerase II-associated factor 1 homolog (PAF1) at the targeted promoter site[5], and finally leading to the transcription upregulation of target gene. Since its discovery in mid 2000s, improvements of saRNA design, synthetic chemistry and understanding of the biology have been maturing the way to apply RNAa[5]. However, there are still many uncertainties in the mechanism of RNAa, which causes that RNAa is not as widely applied as RNAi in biological research.

When researchers choose RNAa as a tool for up-regulating gene expression, they will need to consider whether saRNA can accurately activate target gene expression in the research object, the magnitude and duration of activation, as well as the off-target effects. Because the precise mechanism of RNAa is disputable and remains to be elucidated[6], the above problems are often more complicated in RNAa than RNAi. Until today, there have been many studies that have deeply explored the internal mechanisms of RNAa, and have also discovered many key factors and verified their functions. However, due to the complexity of RNAa itself and the other practical restrictions, there is still no comprehensive and precise theory to promote the convenient use of RNAa. To make RNAa truly a powerful molecular tool for gene expression manipulation, it is necessary to analyze the negative results in the RNAa experiment to find the reason for the unexpected results.

This article focuses on the different effects of the same saRNA in different cells, hoping to help the application methods of saRNA. When we tried to up-regulate tissue kallikrein 1 (KLK1) gene expression with saRNA in human prostate cells, we had some unexpected results, specifically: (1) KLK1 was more easily activated by saRNA in the prostate tumor cell lines than that in the normal prostate cell lines; (2) A large number of floating cells were observed in the medium of prostate tumor cells transfected with

saKLK1-374, an exogenous saRNA designed to activate KLK1 expression. Then two core questions were raised: (1) Are genes more easily to be up-regulated by exogenous saRNA in tumor cells? (2) Why did saKLK1-374, which up-regulated the expression of KLK1, promote the death of prostate tumor cells? (Note that bradykinin which is produced downstream of KLK1 has been reported to promote prostate tumor cell proliferation[7,8].) We designed experiments revolved around the two core questions (The research idea is shown in schematic illustration Fig. 1). We tried to draw some generalizable empirical conclusions based on the experimental results. In the parts where difficult to obtain deterministic results, we made restrictive explanations through rigorous analysis. In addition, it should be emphasized that all experimental results and conclusions in this study were based on conventional *in vitro* cell experiments of saRNA transfected by liposome.

Materials And Methods

Cell culture

All cells were cultured in the medium recommended by the supplier to maintain the correct phenotype. The normal human prostate epithelial cell line RWPE-1 was maintained in customized Prostate Epithelial Cell Medium (Catalog No. 4411, ScienCell, USA) with 1% Prostate Epithelial Cell Growth Supplement (Catalog No.4452, ScienCell, USA). The normal human prostate stromal cell line WPMY-1 was maintained in DMEM medium (Catalog No.11965092, Gibco, USA) with 10% fetal bovine serum (Catalog No.10099141, Gibco, USA). The human prostate cancer cell lines DU-145 and 22RV-1 were maintained in RPMI 1640 medium (Catalog No.31870082, Gibco, USA) with 10% fetal bovine serum. The human normal urothelial cell line SV-HUC-1 was maintained in F-12K medium (Catalog No.21127030, Gibco, USA) with 10% fetal bovine serum. The human bladder cancer cell lines T24 and 5637 were maintained in RPMI 1640 medium with 10% fetal bovine serum. Cells were incubated at 37 ℃ in a humidified incubator under an atmosphere of 5% CO_2. Cells were observed and imaged under a microscope (Olympus, Japan). All cell lines were purchased from the National Collection of Authenticated Cell Cultures (China).

saRNAs

We have designed six saRNAs according to the design rules for exogenous RNAa described previously[1,9]. The human KLK1 (Gene ID: 3816) promoter sequence was obtained from GENE BANK (NCBI Reference Sequence: NC_000019.10; Selected region from 50819146 to 50823787). The designed saRNAs targeted sites − 285, -374, -463, -498, -738, and − 919 bp relative to the transcription start site. Each saRNA was named according to its target within the KLK1 promoter (*i.e.*, saKLK1-285, saKLK1-374, *etc.*). After passing the experimental screening, the saRNA with the best activation effect was used in subsequent experiments. On the other hand, a dsRNA (saP21-322) targeting the $p21^{WAF1/CIP1}$ promoter at position − 322 relative to the transcription start site was used to activate $p21^{WAF1/CIP1}$ expression[10]. The saP21-322 has been confirmed to activate the expression of $p21^{WAF1/CIP1}$ on prostate cancer cell lines and bladder cancer cell lines in many published literatures[10,11]. Therefore, we used saP21-322 as a positive control to improve the credibility of the experimental operation in this study. A nonspecific dsRNA (dsControl) that

lacked significant homology to all known human sequences was also synthesized to serve as a negative control. All the dsRNAs mentioned above were generated by RiboBio (China). The specific promoter region of KLK1 within which we selected the targets, the schematic representation of the gene promoters and saRNA targets, and all saRNA sequences are available in Additional file 1.

Chemical modification of saRNA

Modification to the 2' position in the ribose backbone (e.g., 2'-fluoro) is utilized to improve nuclease resistance and reduce stimulation of the innate immune response[12]. In RNAa, restricting the 2'-fluoro modifications only to the guide strand does not affect gene activation efficacy and also improves the saRNA stability[5,13]. To rule out the failure of RNAa caused by the degradation of saRNA by nuclease, we modified saKLK1-374, saP21-322 and dsControl with 2'-fluoro only in the guide strand. They were named saKLK1-374-2'F, saP21-322-2'F and dsControl-2'F. The chemically modified saRNAs were generated by RiboBio (China).

siRNAs

To down-regulate the expression of KLK1 in cells, we purchased Single Gene siRNA Set which contained designed 3 pairs of siRNAs for KLK1 from RiboBio (China). The 3 pairs of siRNAs were named siKLK1-1, siKLK1-2 and siKLK1-3. After passing the experimental screening, the siRNA with the best inhibitory effect was used in subsequent experiments. The target sequences of the 3 pairs of siRNAs are available in Additional file 1.

Transfection

The day before dsRNA transfection, cells were plated in the growth medium without antibiotics at a density of 50%. Both saRNAs and siRNAs were transfected at a final concentration of 50 nM using Lipofectamine RNAiMax (Catalog No.13778150, Invitrogen, USA) according to the manufacturer's instructions. RNA or protein was extracted 72 hours after saRNA transfection or 48 hours after siRNA transfection to detect the change of the target gene.

Real-Time Quantitative PCR (qRT-PCR)

Total RNA was extracted with the TRIzol reagent (Catalog No.15596018, Invitrogen, USA) and quantitated at 260/280 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). One μg of each total RNA sample was reverse transcribed into cDNA using a PrimeScript™ RT Master Mix (Catalog No.RR036A, TaKaRa, China). QRT-PCR was performed to determine the mRNA levels of genes of interest based on TB Green® Premix Ex Taq™ II (Catalog No.RR82LR, TaKaRa, China) using a QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher, USA). The primers were provided by Tsingke Biotechnology (China) and were listed in Additional file 2. The mRNA expression levels of the examined genes were normalized to that of β-actin; the relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. All samples were independently repeated for analysis three times.

Western blot analysis

Cultured cells were lysed with RIPA protein extraction buffer (Catalog No.AR0105, Boster Biological Technology, China) containing phosphatase and protease inhibitors (Catalog No.AR1182-1, Boster Biological Technology, China). Equal quantities of protein were resolved by electrophoresis on sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA) by voltage gradient. After being blocked with 5% non-fat dry milk for 1 hour at room temperature, the membranes were incubated overnight at 4°C with primary antibodies and then incubated with horseradish peroxidase (HRP) conjugated secondary antibodies for 1 h. After washing three times with Tris-buffered saline with Tween-20, the bands were analyzed using an enhanced chemiluminescence detection system (Pierce, Thermo Fisher Scientific). The primary antibodies against KLK1 (Catalog No.32443) and β-actin (Catalog No.21800) were purchased from Signalway Antibody (USA). The primary antibodies against $p21^{WAF1/CIP1}$ (Catalog No.10355-1-AP) and Ago2 (Catalog No.10686-1-AP) were purchased from Proteintech (China). The primary antibodies against IPO8 (Catalog No.A14679) and Caspase-3 (Catalog No.A19654) were purchased from ABclonal Technology (China). The data were normalized using β-actin as an internal control. All samples were analyzed independently via three repetitions, and the mean values were determined.

Detection of the extracellular KLK1 protein

KLK1 is a serine protease that cleaves low molecular weight kininogen (LMWK) to produce the kinins, especially bradykinin[14]. KLK proteins are synthesized as inactive prepro-forms that are proteolytically processed to secreted inactive pro-forms[15]. Subsequently, pro-KLKs are activated to mature peptidases by specific proteolytic removal of their N-terminal propeptide either via autocatalytic activity or by another KLK or other endopeptidases[15]. To find out whether the cell after RNAa secreted more KLK1 protein to the extracellular medium, we collected the media from the 3rd day to the 5th day after saRNA transfection, and detected KLK1 using human KLK1 ELISA Kit (Catalog No.EK2486, Signalway Antibody, USA) according to the manufacturer's protocol.

Cell viability assay

Cells were transfected with dsRNAs or administered recombinant human KLK1 (Catalog No.AP70473, Signalway Antibody, USA), and seeded in 96-well Multiple Well Plates (Corning, USA) at a density of about 5000 cells per well. Cell viability was determined using Cell Counting Kit (CCK-8) (Catalog No.40203ES60, Yeasen Biotechnology, China) according to the manufacturer's protocol. The specific experiment grouping and detection interval are described in the results section.

Assessment of intake of dsRNA

Equal amounts of cells of different strains were seeded into six-well Multiple Well Plates (Corning, USA). We transfected siR Transfect Control (dsControl-5cy3) (Catalog No.siT0000002-1-5, RiboBio, China) which is a kind of nonspecific dsRNA labeled with fluorescent molecules to observe the uptake efficiency of the different cell lines. Observation and photographing were performed using ZOE™ Fluorescent Cell Imager (Bio-Rad Laboratories, USA) every 24 hours in a darkroom. The transfection efficiency of different cell lines was semi-quantitatively analyzed by the fluorescence intensity in 3 randomly taken photos. The

average fluorescence intensity of the cells (= optical density/number of cells) was analyzed by ImageJ software (National Institutes of Health, USA).

Assessment of reactive oxygen species (ROS) in cultured cells

Cells were seeded in 6-well Multiple Well Plates. After being transfected with saRNA, cultured cells were detected ROS every 24 hours using a Reactive Oxygen Species Assay Kit (Catalog No.50101ES01, Yeasen Biotechnology, China) according to the manufacturer's protocol. On the other hand, cells were administered recombinant human KLK1 every 6 hours, and finally ROS detection was performed at the 24th hour. Observation and photographing were performed using ZOE™ Fluorescent Cell Imager (Bio-Rad Laboratories, USA) in a darkroom.

Results

1. Regulatory effect of designed saRNAs on the expression of KLK1 in different prostate cell lines

To activate KLK1 expression through RNAa in human prostate cells, we have designed 6 saRNAs and each duplex was transfected into RWPE-1, WPMY-1, BPH-1 and DU-145 cells. KLK1 gene expression was evaluated via qRT-PCR and western blot 72 hours later. As shown in Fig. 1, we found that in the normal prostate cell lines RWPE-1 and WPMY-1, all saRNAs were difficult to up-regulate the expression of KLK1, while in the prostate tumor cell lines BPH-1 and DU-145, saKLK1-374 and saKLK1-463 had an up-regulation effect on the expression of KLK1. We chose saKLK1-374 with a higher up-regulation amplitude in BPH-1 and DU-145, for subsequent experiments.

It should be noted that BPH-1 was only used in the preliminary screening of effective saRNAs. After we discovered the unexpected results described above, the main purpose was to learn the difference between normal cells and tumor cells in RNAa. However, to reduce the difficulty of research, currently we only used malignant tumor cell lines as the representative of tumor cell lines rather than including the benign tumor cell line BPH-1 into the research. In subsequent experiments, DU-145 and 22RV-1 were used as representatives of prostate malignant tumor cell lines.

2. The chemical modification also failed to enable saKLK1-374 to activate KLK1 expression in normal prostate cell lines

To improve the stability of saKLK1-374 and rule out saKLK1-374 lost its function due to enzyme degradation, we created a localized modified saKLK1-374 (saKLK1-374-2'F) in which all cytidine and uridine nucleosides within the guide strand contained a 2'-fluoro-modified ribose sugar. The saKLK1-374

and saKLK1-374-2'F were transfected into cells by the same method as above. As shown in Fig. 2, saKLK1-374-2'F also could not up-regulate the expression of KLK1 in normal prostate cell lines RWPE-1 and WPMY-1. In prostate tumor cell lines, saKLK1-374-2'F had the same effect of up-regulating KLK1 expression as saKLK1-374. And it was worth noting that in this experiment, fluorine modification could not reverse the ineffectiveness of saRNA, nor could it significantly change the up-regulation amplitude of genes by saRNA. This meant that in this experiment, there was no failure of gene up-regulation due to the loss of activity of saRNAs.

3. In normal prostate cells, saP21-322 and saP21-322-2'F could not activate $p21^{WAF1/CIP1}$

Using another gene, $p21^{WAF1/CIP1}$, as the target of exogenous saRNA could help us eliminate many problems (but not absolutely). Under the same experimental operation, if the RNAa of other target genes was successful, then our conjecture was certainly wrong. But if RNAa for $p21^{WAF1/CIP1}$ also failed in normal prostate cell lines, then the answer becomes unpredictable. As shown in Fig. 3, the saP21-322 and saP21-322-2'F still could not up-regulate the expression of $p21^{WAF1/CIP1}$ in normal prostate cell lines RWPE-1 and WPMY-1, but they could up-regulate the expression of $p21^{WAF1/CIP1}$ in prostate tumor cell lines DU-145 and 22RV-1. The RNAa experiments with the two target genes of $p21^{WAF1/CIP1}$ and KLK1 yielded similar results, making us suspect that the key lay in the cell rather than the target gene.

The last step of RNAa is to bind the exogenous saRNA to the promoter of the target gene to promote the transcription of the target gene. The epigenetic characteristics of target gene promoters are uncertain, and their epigenetic characteristics may be different in different types of cells from the same tissue. The saP21-322 is a saRNA that has been successfully reported to activate the expression of $p21^{WAF1/CIP1}$ in prostate cancer cell lines. However, it is not certain that saP21-322 would bind to $p21^{WAF1/CIP1}$ promoter in normal prostate cells, because we did not detect the difference in the degree of methylation of the $p21^{WAF1/CIP1}$ promoter between normal prostate cells and prostate cancer cells. If you need to detect the methylation degree of the target gene promoter every time you try RNAa, it will undoubtedly significantly increase the cost of RNAa experiments. To answer the question " Are genes more easily up-regulated by exogenous saRNA in tumor cells? ", the core still needs to return to normal cells and tumor cells themselves. Therefore, in this article, we did not study the difference in the degree of methylation of KLK1 and $p21^{WAF1/CIP1}$ promoters in normal prostate cells and prostate cancer cells, or test any other genes via RNAa on these cells. Rather, it was assumed that the failure of RNAa occurs in the process before the exogenous saRNA binds to the promoter of the target gene.

4. RNAa experimental results of KLK1 and $p21^{WAF1/CIP1}$ in bladder cell lines

It was necessary to conduct RNAa experiments in cells derived from tissues other than the prostate to verify our conjecture. We used the bladder-derived cell lines available in our laboratory, which were the normal urothelial cell line SV-HUC-1, and the bladder cancer cell lines T24 and 5637. RNAa experiments

were performed for KLK1 and $p21^{WAF1/CIP1}$ in these cells. As shown in Fig. 5A, in all bladder cell lines, neither saKLK1-374 nor saKLK1-374-2'F up-regulated KLK1 expression. We speculated that this result was due to the extremely low expression of KLK1 in bladder cells. We detected the basic expression of KLK1 mRNA in prostate cells and bladder cells by qRT-PCR, and found that the expression of KLK1 in bladder cells was significantly lower than that in prostate cells (Fig. 5B, left). Therefore, only qRT-PCR rather than western blot was performed to detect changes in KLK1 expression. The extremely low expression in the basal state means that the KLK1 gene is inhibited by a powerful silencing mechanism in bladder cells. While RNAa is epigenetic regulation of a targeted promoter, and it cannot counteract powerful silencing mechanisms. Therefore, it was foreseeable that saKLK1-374 could not activate KLK1 expression in all bladder cell lines.

Different from KLK1, $p21^{WAF1/CIP1}$ was significantly up-regulated by saP21-322 and saP21-322-2'F in bladder cancer cell lines T24 and 5637 (about 3–4 times as much as mock; Fig. 5D&E), while the up-regulation rate is lower in normal urothelial cell line SV-HUC-1 (about twice as much as mock; Fig. 5C). Unlike KLK1, the expression of $p21^{WAF1/CIP1}$ in prostate and bladder cell lines is relatively normal. We detected the basic expression of $p21^{WAF1/CIP1}$ mRNA in prostate cells and bladder cells by qRT-PCR (Fig. 5B, right). Therefore, the activation effect of the $p21^{WAF1/CIP1}$ would not be affected by the gene itself. In the bladder, tumor cells had a higher magnitude of RNAa effect than normal cells, and it seemed that this phenomenon should also be explained in the process before the saRNA bonded to the target gene.

5. Prolonging the time that the exogenous saRNA acted on the cell did not change the effect of the saRNAs

RNAa usually takes effect more slowly than RNAi. RNAi usually has obvious gene down-regulation 24 hours after transfection, while the obvious gene up-regulation of RNAa appears 72 hours after transfection. The poor RNAa performance in normal cell lines might be caused by the insufficient duration of saRNA action. Therefore, we extended the time to 7 days and and detected the expression level of the target gene every 24 hours. To obtain more credible results and appropriately simplify the experiment, we only performed the RNAa experiments for KLK1 in prostate cells, and only performed the RNAa experiments for p21WAF1/CIP1 in the bladder cell line. As shown in Fig. 6A (upper), saKLK1-374 could not up-regulate the expression of KLK1 in normal prostate cell lines RWPE-1 and WPMY-1 for 7 days after transfection. However, in the prostate cancer cell lines DU-145 and 22RV-1, the expression of KLK1 reached a peak on the 3rd day after saKLK1-374 transfection, and fluctuated around this peak in the following 4 days (Fig. 6A, lower). In the 3 bladder cell lines, the expression of $p21^{WAF1/CIP1}$ peaked on the 3rd day after saP21-322 transfection, and fluctuated in the vicinity of the peak 4 days later (Fig. 6B). This meant that extending the time could not change the phenomenon that "normal cells were more difficult to achieve effective RNAa". And we could speculate that normal cells might lack certain components necessary for RNAa.

6. The difference in transfection efficiency of exogenous saRNA between normal cells and tumor cells

The process of RNA activation is that the exogenous saRNA crosses the cell membrane into the cytoplasm with the assistance of the carrier, and then enters the nucleus with the help of some key proteins such as Ago2 and targets the promoter to work. After excluding the target gene and saRNA, the cause of RNAa failure should be found in the process of exogenous saRNA entering the nucleus from the culture medium.

RNAiMAX has been the best cationic-lipid transfection reagent currently available for dsRNA. First, exogenous saRNA was combined with the cationic-lipid transfection reagent and added to the cell culture medium. Because of the cell membrane, only part of the saRNA-lipid complexes could enter the cytoplasm. The amount of saRNA-lipid complexes entering the cytoplasm could be assessed by fluorescently labeled dsRNA. As shown in Fig. 7, we detected the content of fluorescently labeled dsControl-5cy3 (red) entering the cytoplasm in four prostate cell lines and 3 bladder cell lines. After transfection with the same concentration of dsControl-5cy3, prostate cancer cell lines DU-145 and 22RV-1 took in more dsRNA-5cy3 than normal prostate cell lines RWPE-1 and WPMY-1 (Fig. 7A). After zooming in on the photo, it could be found that the nuclei of prostate cancer cell lines DU-145 and 22RV-1 have also taken in more dsRNA-5cy3 than normal prostate cell lines RWPE-1 and WPMY-1 (Fig. 7B). But the situation was different in the bladder cell lines. The intake of dsControl-5cy3 in bladder cancer cell lines T24 and 5637, and normal urothelial cell line SV-HUC-1 were similar (Fig. 7C). The intake of dsControl-5cy3 by the nucleus in the bladder cell lines was also similar (Fig. 7D).

Through semi-quantitative analysis, it could be found that the average single-cell intake of dsControl-5cy3 in prostate cancer cell lines was higher than that in normal prostate cell lines, while the average single-cell intake of dsControl-5cy3 in the 3 bladder cell lines was similar and also higher than that of normal prostate cell lines (Fig. 7E). It was worth mentioning that in this experiment, although the number of cells was the same in the initial procedure of seeding plate, the growth rate of different cell lines was still different, so the number of cells in each subsequent test was not equal. However, in our experiments, the growth rate of prostate cancer cell lines DU-145 and 22RV-1 was significantly faster than that of normal prostate epithelial cells RWPE-1, and was similar to the normal prostate stromal cell line WPMY-1. This meant that prostate cancer cells would have a larger number of cells in subsequent detections. In the presence of the same amount of dsControl-5cy3, assuming that the transfection efficiency of all cells was equal, the average single-cell intake of dsControl-5cy3 of prostate cancer cells could only be lower. However, the experimental results showed that the single-cell intake of dsControl-5cy3 of prostate cancer cells was higher than that of normal prostate cell lines, which means that the transfection efficiency of prostate cancer cell lines must be higher than that of normal prostate cell lines. It should be noted that the average single-cell dsControl-5cy3 intake obtained by analyzing the picture referred to the average fluorescence intensity of a single cell. However, limited by analytical methods, we could not calculate the fluorescence intensity in a single cell nucleus.

The above experimental results indicated that effective RNAa could not be achieved in the normal prostate cell lines RWPE-1 and WPMY-1 probably because they did not take in enough exogenous saRNA. However, the average single-cell intake of dsControl-5cy3 of the 3 bladder cell lines was similar, which meant that the up-regulation of $p21^{WAF1/CIP1}$ gene in normal urothelial cell lines was lower than that of bladder cancer cell lines T24 and 5637 was not due to the insufficient intake of exogenous saRNA.

7. The difference of RNAa indispensable accessory protein between normal cell line and tumor cell line

Currently, Ago2 and importin 8 (IPO8) have been found to play an indispensable role in the transport of dsRNA from the cytoplasm to the nucleus. To regulate gene expression, the exogenous saRNA absorbed into the cytoplasm needs to enter the nucleus with the help of Ago2 and IPO8. Similar to the role of Ago2 in RNAi, in RNAa, Ago2 serves the role of a navigator and a recruiting platform on which an RNAa effector complex is assembled. IPO8, a member of the karyopherin family, has been identified to interact with Ago2 and localize to cytoplasmic processing body which is a structure involved in RNA metabolism, and IPO8 has been demonstrated to play a critical role in mediating the cytoplasm-to-nucleus transport of mature micro RNAs[16,17].

As shown in Fig. 8, we detected the expression of Ago2 and IPO8 in all bladder and prostate cell lines by qRT-PCR and western blot. The qRT-PCR results showed that the expression of AGO2 and IPO8 was not different between untreated cells and cells transfected with dsControl, indicating that transfection of exogenous saRNA did not affect the expression of these two proteins. Among all cell lines, the expression of Ago2 and IPO8 of RWPE-1 was the lowest, which might explain the complete loss of function of RNAa in RWPE-1. In the bladder cell lines, the expression of Ago2 and IPO8 in the normal urothelial cell line SV-HUC-1 was lower than that of the two bladder cancer cell lines, was still also significantly higher than that of RWPE-1. Considering that the previous experimental results showed that the exogenous dsRNA in the cytoplasm of SV-HUC-1 was similar to the two bladder cancer cell lines, this could explain that the exogenous saRNA in the SV-HUC-1 could up-regulate the expression of $p21^{WAF1/CIP1}$ at lower amplitude, instead of completely ineffectiveness. In summary, to successfully use exogenous saRNA to up-regulate target gene expression, the transfected cells must absorb enough exogenous saRNA and express enough Ago2 and IPO8. However, due to lower transfection efficiency or lower expression of Ago2 and IPO8, or both, the normal cells may not be able to effectively activate target gene expression through exogenous saRNA or had a low amplitude of up-regulation. In contrast, tumor cells generally could absorb more exogenous saRNA and had higher expression of Ago2 and IPO8. It needs to be emphasized again that our experimental results and inferences were based on *in vitro* experiments using cationic-lipid transfection reagents to carry exogenous saRNA.

8. saKLK1-374 caused the death of prostate cancer cells instead of normal prostate cells

While conducting the above-mentioned RNAa experiments, we accidentally discovered that in prostate cancer cell lines DU-145 and 22RV-1, a large number of floating cells appeared in the culture medium after transfection with saKLK1-374 (Fig. 9C&D, left). However, there was no significant increase in floaters in normal prostate epithelial cell line RWPE-1 and normal prostate stromal cell line WPMY-1 (Fig. 9A&B, left). This seemed to indicate that saKLK1-374 had a targeted killing ability on prostate cancer cells. To more accurately determine the inhibitory effect of saKLK1-374 on prostate cancer cell lines, we conducted a cytotoxicity test. As shown in Fig. 9 (right), saKLK1-374 and saKLK1-374-2'F did not inhibit the growth of RWPE-1 and WPMY-1 within five days after transfection, while in DU-145 and 22RV-1, cell viability started to decrease on the first day after transfection.

9. saKLK1-374 increases intracellular ROS and promotes prostate cancer cell apoptosis

We started with common cell death mechanisms and briefly studied the causes of the death of prostate cancer cells caused by saKLK1-374. As shown in Fig. 10A&B, the intracellular ROS of prostate cancer cell lines DU-145 and 22RV-1 were significantly up-regulated after saKLK1-374 transfection. The qRT-PCR results showed that the ratio of BAX/Bcl-2 in DU-145 and 22RV-1 increased after saKLK1-374 transfection (Fig. 10C&D, left). The results of qRT-PCR and western blot showed that saKLK1-374 also up-regulated Caspase3 in DU-145 and 22RV-1 (Fig. 10C&D).

Up to now, our experimental results seemed to show that saKLK1-374 up-regulated the expression of KLK1 in prostate cancer cell lines DU-145 and 22RV-1 to cause cell oxidative stress and apoptosis. However, according to many previous reports, KLK1 often played an anti-oxidative stress role and its downstream bradykinin promoted the proliferation of prostate cancer cells.

10. KLK1 could not be detected outside the cell and recombinant KLK1 did not change ROS and cell viability.

We suspected that the cause of cell death by saKLK1-374 was not the increase in KLK1 gene expression. Considering that KLK1 usually played a role after being activated by other enzymes outside the cell, we first detected the content of KLK1 protein in the prostate cancer cell culture medium after transfection with saKLK1-374. But surprisingly, the KLK1 in the medium of all samples at 72 hours, 96 hours and 120 hours after transfection of saKLK1-374 were below the minimum detection limit (The KLK1 standard could be detected normally; The annotated detection range of the Human KLK1 ELISA Kit used in this experiment is “156pg/ml – 10000pg/ml”). We also used the recombinant KLK1 protein (concentration range: 10ng/ml to 10μg/ml) to directly interfere with prostate cancer cells, but no obvious oxidative stress or cell death was found. As shown in Fig. 11B&C, the recombinant KLK1 protein had no significant effect on the viability of prostate cancer cell lines DU-145 and 22RV-1. As shown in Fig. 11A, the recombinant KLK1 protein did not upregulate ROS in DU-145 and 22RV-1.

Since KLK1 protein did not be detected outside the cells transfected with saKLK1-374, and the recombinant KLK1 protein did not have the same effect as saKLK1-374, it was almost certain that saKLK1-374 did not increase the expression of KLK1 to cause the death of prostate cancer cells.

11. Interference with KLK1 mRNA expression could not completely reverse the inhibition of saKLK1-374 on the growth of prostate cancer cells

Through previous experiments, we ruled out the possibility of extracellular KLK1 inhibiting growth of prostate cancer cells. Next, we used RNAi to investigate whether intracellular KLK1 was the cause of prostate cancer cell death. As shown in Fig. 12A&B, we tested the inhibitory effect of the 3 purchased siRNAs on KLK1 expression, and found that siKLK1-1 has the strongest inhibitory effect on both DU-145 and 22RV-1, so we chose siKLK1-1 for subsequent experiments. When saKLK1-374 activated the expression of KLK1, siKLK1-1 could also suppress the expression of KLK1 below the baseline level (compared to the mock samples; Fig. 12C&D). In the cytotoxicity test, siKLK1-1 reduced the inhibition of saKLK1-374 on prostate cancer cell lines DU-145 and 22RV-1, but the inhibition could not be completely reversed (Fig. 12E)

Considering when both saKLK1-374 and siKLK1-1 were transfected into prostate cancer cells, KLK1 mRNA was lower than the baseline level, so it is difficult to entirely attribute the cell growth inhibition still existed in this case to the increase of intracellular KLK1. Therefore, there must be other reasons for cell death besides RNAa of the KLK1 gene.

Discussion

In the present study, we identified that a synthetic exogenous saRNA, saKLK1-374, could up-regulate the expression of KLK1 in prostate tumor cell lines BPH-1, DU-145 and 22RV-1. However, in the normal prostate epithelial cell line RWPE-1 and the normal prostate stromal cell line WPMY-1, due to the insufficient intake of saRNA carried by cationic-lipid transfection reagent and low expression of Ago2 and IPO8, saKLK1-374 could not up-regulate the expression of KLK1. For the RNAa of the $p21^{WAF1/CIP1}$ gene, it also failed in RWPE-1 and WPMY-1. In addition, among the cell lines derived from bladder tissue, the $p21^{WAF1/CIP1}$ gene expression up-regulated by saP21-322 in normal urothelial cell line SV-HUC-1 was lower than that of bladder cancer cell lines T24 and 5637. Different from prostate cell lines, the intake of exogenous saRNA carried by cationic-lipid transfection reagent of SV-HUC-1, T24 and 5637 was similar, but the expression of Ago2 and IPO8 of SV-HUC-1 was lower than that of T24 and 5637. Therefore, due to the poor permeability of the cell membrane to cationic-lipid transfection reagents or the low expression of Ago2 and IPO8, it could become a common problem in normal cell lines that saRNA cannot effectively up-regulate target gene expression and should be taken seriously by researchers who have corresponding

scientific research needs. On the other hand, we also found that saKLK1-374 could inhibit the growth of prostate cancer cell lines DU-145 and 22RV-1, but it was not due to the activation of KLK1 protein and it is more likely that saKLK1-374 changed some unknown signaling pathways in the cells.

Our research started from unexpected results and gradually explored the causes of these unexpected results. Our specific analysis process has been described in the results section, so they are not repeated here. Here we first discuss the shortcomings of our research. In our experiment, the normal prostate epithelial cell line RWPE-1, the normal prostate stromal cell line WPMY-1 and the normal urothelial cell line SV-HUC-1 were cultured in their dedicated medium respectively, which different from tumor cells (RPMI 1640 medium with 10% fetal bovine serum). The medium may affect the absorption of cationic-lipid transfection reagent or other unknown mechanisms. However, we cannot unify the culture medium of normal cell lines with tumor cell lines, because normal cells need to grow in a dedicated culture medium to maintain their normal phenotype. When normal cell lines are needed for research, they are usually required to maintain their normal phenotype. In fact, we have tried to culture RWPE-1 with RPMI 1640 medium contained 10% fetal bovine serum, but the result was that RWPE-1 lost the shape of normal epithelial cells and turned into a spindle shape, and the expression of KLK1 was still unable to be upregulated by saKLK1-374 (data not shown). Therefore, each type of cell line and their commonly used dedicated medium should be regarded as the same variable. On the other hand, we only used fluorescent dsRNA to observe the transfection efficiency rather than perform a quantitative analysis of saRNA absorbed into the cells via PCR. Because we think that the fluorescence difference between normal prostate cells and prostate tumor cells shown in the photo is sufficiently obvious and a quantitative analysis is not essential.

Experimental results from a total of 7 cell lines based on 2 tissue sources are not enough to draw a definite conclusion that normal cells are more difficult to activate target genes by exogenous saRNA than tumor cells. But to draw a definitive conclusion, all the cells need to be tested preferably. Our current research only inspires that the difference in the response of normal cells and tumor cells to RNAa can bring benefits in many situations. For example, saRNA targeting tumor suppressor genes may specifically kill tumor cells without affecting normal cells. Of course, the above content needs to be experimentally verified on primary cells and even living bodies. If only for proposing a novel conjecture, our experimental results on 7 cell lines from 2 tissues are sufficient, but if this mechanism needs to be applied further, the research subjects should be tested for transfection efficiency and expression of Ago2 and IPO8. Limited by early experimental plans and actual experimental costs, we did not explore novel RNAa mechanisms or other factors that are critical to RNAa. The results obtained in this article cannot fully explain the difference in RNAa between normal cells and tumor cells. It is necessary to design more rigorous experiments to study the difference between the specific process of RNAa in normal cells and tumor cells. The transport of exogenous saRNA to the nucleus and the formation of transcription initiation complexes still need to be studied. However, such experiments may be very complicated and costly.

Another important discovery of our research is that saKLK1-374 inhibited the growth of prostate tumor cells, but we have not completely explained the mechanism in our current study. The best way to study

the unknown mechanism is high-throughput sequencing, However, this research is only an exploration of unexpected results and is not included in our main project, so due to cost considerations, we did not use high-throughput sequencing. Initially, we tried to design saRNA to activate the expression of KLK1 in prostate cells because we found that KLK1 could protect the prostate from oxidative stress and fibrosis in KLK1 transgenic old mice, which meant that KLK1 might prevent benign prostatic hyperplasia[18]. To make KLK1 protect the prostate permanently, it is necessary to up-regulate the expression of KLK1 in the prostate as early as possible before the occurrence of benign prostatic hyperplasia, that is, to up-regulate KLK1 in normal prostate cells. But the results were unexpected again. After transfection with saKLK1-374, normal prostate cells had no response, but prostate cancer cells experienced cell death and oxidative stress. Theoretically, as a serine proteinase, KLK1 is synthesized and secreted as inactive forms. Subsequently, inactive KLK1 are activated to mature peptidases by specific proteolytic removal of their N-terminal propeptide either via autocatalytic activity or by another KLK or other endopeptidases[15]. KLK1 processes LMWK to produce vasoactive kinin peptides, such as bradykinin and Lys- bradykinin. The biological function of KLK1 is primarily mediated by kinin peptides and subsequent kinin receptor activation[19]. However, there have been many reports about bradykinin promoting prostate cancer cell proliferation and migration[7,8]. Therefore, it is hard to imagine that saKLK1-374 up-regulates KLK1 to cause the death of prostate cancer cells. We used the recombinant KLK1 protein as a positive control and proved that KLK1 itself does not cause cell death. Although we did not add LMWK to the medium when using recombinant KLK1 protein, this was consistent with the cells transfected with saKLK1-374. Moreover, the serum in the culture medium may contain substances similar to LMWK, and KLK1 itself has been reported to activate bradykinin receptors independently of bradykinin[20,21]. On the other hand, we used siRNA to silence the expression of KLK1. Even when saKLK1-374 promoted the expression of KLK1, siKLK1-1 also made the expression of KLK1 significantly lower than the level without intervention. After silencing the expression of KLK1 in the cells, the inhibited state of prostate cancer cells was still not lifted, indicating that saKLK1-374 also interfered with other signaling pathways to inhibit prostate cancer cells in an unknown way. All exogenously synthesized saRNA is essentially a kind of dsRNA. After transfection of saKLK1-374 into the cell, it can target the promoter of the target gene, or just like siRNA, it can target certain mRNAs to inhibit the expression of these genes. Through “Nucleotide Blast” on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi*)*, we searched for mRNAs that saKLK1-374 may affect. And found that the mRNAs of these genes may be targeted by saKLK1-374: SEC14L3 (ID: 266629), CAMTA1 (ID: 23261), ZNF283 (ID: 284349), LIG3 (ID: 3980), AAK1 (ID: 22848), PGRMC2 (ID: 10424), MAGED2 (ID: 10916), EPSTI1 (ID: 94240), BTD (ID: 686), HUWE1 (ID: 10075), DCAF12L2 (ID: 340578). None of these genes seem to be directly related to cell death so that we failed to find a breakthrough and had to end this study.

Finally, it needs to be emphasized again that our experimental results and conclusions were based on conventional *in vitro* cell experiments of saRNA transfected by liposome. At present, transfection via liposome is the most low-cost and convenient method. Therefore, our conclusions can still help most studies using liposome transfection reagents. There are still other ways to introduce exogenous saRNA into cells, such as adenoviral vector or lentiviral vector. Different transfection methods may bring

completely different experimental results. RNAa in normal cells using other transfection methods may also be successful. We searched a large number of articles about RNAa and found only 3 articles described activating the expression of target genes in non-tumor cells. Tao Wang reported RNAa-mediated activation of inducible nitric oxide synthase in cultured rat cavernous smooth muscle cells via adenoviral vector[22]. Bin Wang activated SOX2 in human lung diploid fibroblast via saRNA carried by lentiviral vector[23]. Chenghe Wang induced the expression of myogenic regulatory factor in adipose-derived stem cells via cationic-lipid transfection reagent RNAiMax which was consistent with the transfection reagent in our research[24]. Perhaps the virus could carry more exogenous saRNA into normal cells. Unfortunately, there is no data on transfection efficiency in their studies. On the other hand, referenced to Chenghe Wang's research, we also tried to use cationic-lipid transfection reagent to carry exogenous saRNA to activate the expression of brain-derived neurotrophic factor in adipose-derived stem cells, but we found that transfection efficiency in the cell was low and the target gene expression failed to be upregulated (data not shown). Therefore, the transfection reagent is not a decisive factor for RNAa, but it seems that viral vectors are more suitable for RNAa experiments in non-tumor cells.

Conclusions

Our research found that in prostate and bladder-derived cell lines, the same saRNA is more difficult to induce target gene expression in normal cell lines than in tumor cell lines because of the insufficient intake of saRNA carried by cationic-lipid transfection reagent and low expression of Ago2 and IPO8 in the normal cell line. Based on this difference, we have a conjecture that saRNA targeting tumor suppressor genes may specifically kill tumor cells without affecting normal cells. In addition, we also discovered a new saRNA that can inhibit the growth of prostate cancer cells by inducing oxidative stress and apoptosis. However, it is still unclear which signal pathways are affected by the saRNA. If this saRNA is used to treat prostate cancer, it is necessary to design more rigorous and detailed experiments to study the specific genetic changes.

Abbreviations

RNAa: RNA activation, RNAi: RNA interference, saRNA: small activating RNA, siRNA: small interfering RNA, dsRNA: double-stranded RNA, KLK1: tissue kallikrein 1, Ago2: argonaute 2, IPO8: importin 8, ROS: Reactive oxygen species, RHA: RNA helicase A, CTR9: RNA polymerase-associated protein CTR9 homolog, PAF1: RNA polymerase II-associated factor 1 homolog, SOX2: SRY-box transcription factor 2, SEC14L3: SEC14 like lipid binding 3, CAMTA1: calmodulin binding transcription activator 1, ZNF283: zinc finger protein 283, LIG3: DNA ligase 3, AAK1: AP2 associated kinase 1, PGRMC2: progesterone receptor membrane component 2, MAGED2: MAGE family member D2, EPSTI1: epithelial stromal interaction 1, BTD: biotinidase, HUWE1: HECT, UBA and WWE domain containing E3 ubiquitin protein ligase 1, DCAF12L2: DDB1 and CUL4 associated factor 12 like 2.

Declarations

Consent for publication

All authors have read and agreed to publish this manuscript.

Availability of data and material

The data of the materials and methods and results to support the conclusions are included in this article. If any other data are needed, please contact the corresponding author.

Competing interests

All authors report no conflicts of interest.

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Authors' contributions

ZC is responsible for the project development and manuscript editing, Ke Chen, Kai Cui, PW, CL and DL for the data collection and data analysis, and MZ for the project development, data collection, data analysis, and manuscript writing. All authors have contributed to and approved the final manuscript.

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Figures

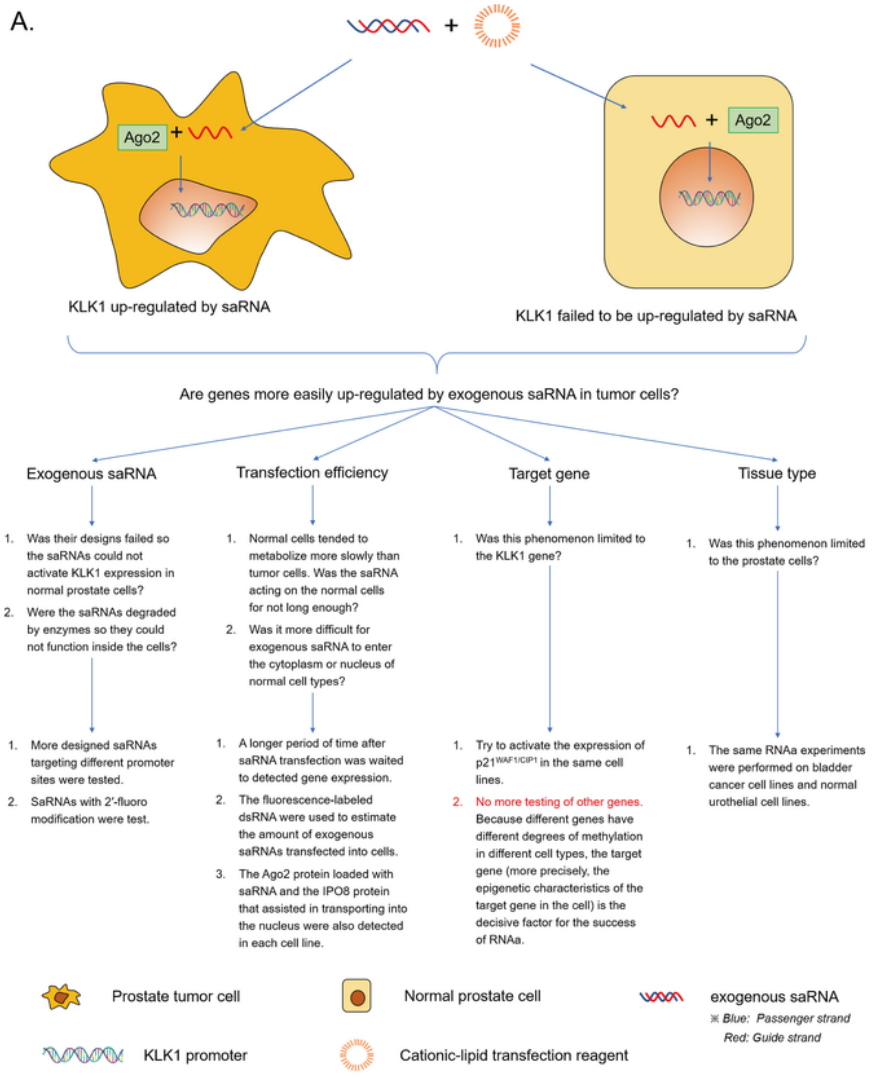

Figure 1

Schematic illustration of the research idea. (A) shows how we designed experiments to answer the question “Are genes more easily to be up-regulated by exogenous saRNA in tumor cells?”. (B) shows the possible pathways of saKLK1-374 leading to cell death.

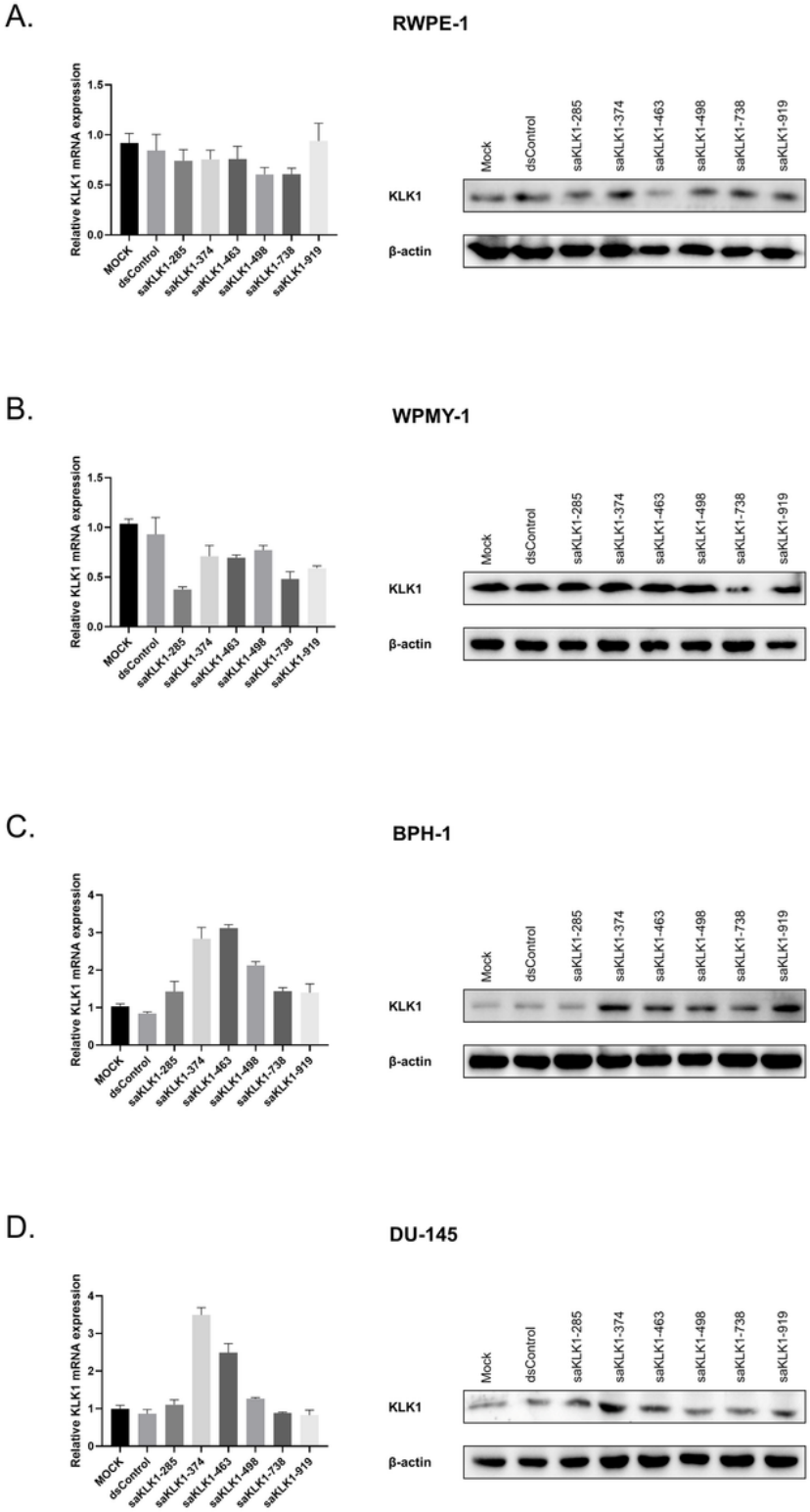

Figure 2

Preliminary screening of effective saRNAs for up-regulating KLK1 expression in prostate cells. (A) The 6 saRNAs and dsControl were transfected at a final concentration of 50 nM into normal human prostate

epithelial cell line RWPE-1. Mock samples were transfected in the absence of dsRNAs. Relative expression levels of KLK1 were evaluated by qRT-PCR (left; mean ± SD from 3 independent experiments) and western blot (right) 72 hours after transfection. β-actin served as a loading control. (B), (C) and (D) show the results of the same experiment as (A) of normal human prostate stromal cell line WPMY-1, benign prostatic hyperplasia cell line BPH-1 and prostate cancer cell line DU-145, respectively.

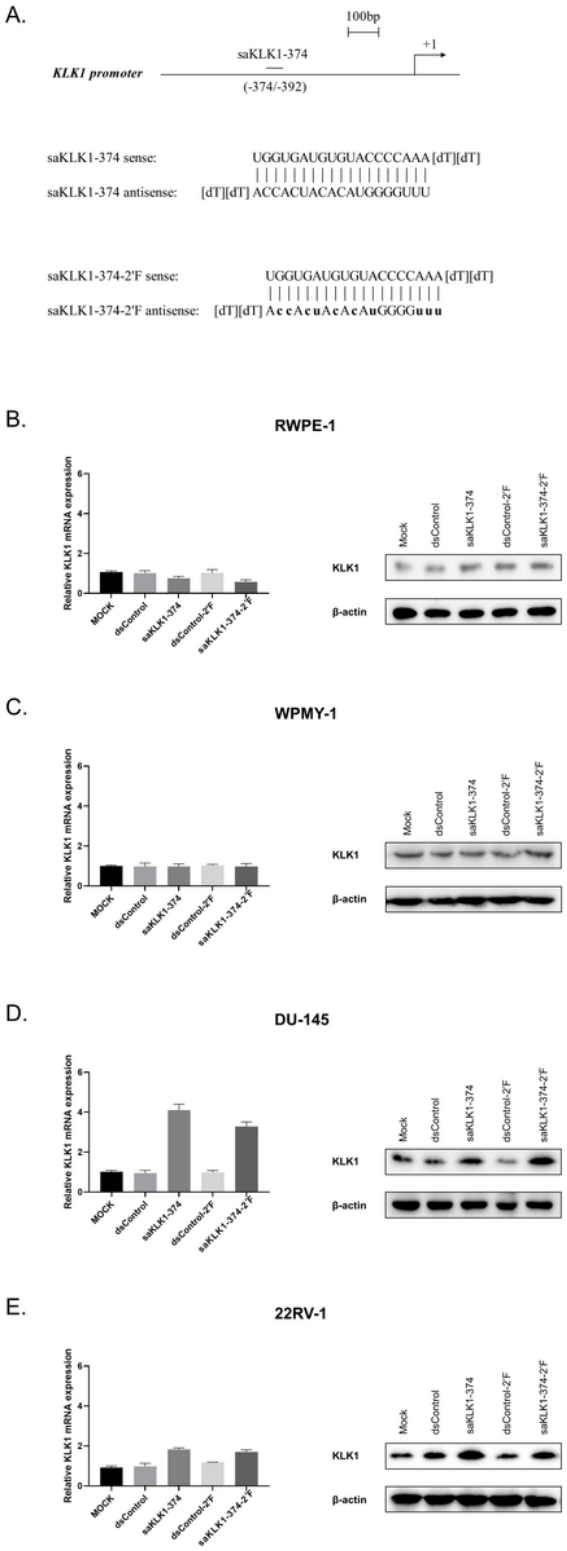

Figure 3

The effect of 2'-fluoro modification on RNAa in normal and malignant prostate cell lines. (A) Schematic representation of the KLK1 promoter and saKLK1-374 target site (upper). Sequence composition of saKLK1-374 (middle) and saKLK1-374-2'F (lower). Indicated are the sense and antisense strands, which possess dual deoxythymidine overhangs at their 3'-termini. Lowercase letters in bold correspond to 2'-fluoro-modified nucleotides. (B) Normal human prostate epithelial cell line RWPE-1 was transfected at a final concentration of 50 nM of dsControl, dsControl-2'F, saKLK1-374, or saKLK1-374-2'F for 72 hours. Mock samples were transfected in the absence of dsRNA. Relative expression levels of KLK1 were assessed by qRT-PCR (left; mean ± SD from 3 independent experiments) and western blot (right). β-actin served as a loading control. (C), (D) and (E) show the results of the same experiment as (B) of normal human prostate stromal cell line WPMY-1, prostate cancer cell line DU-145 and 22RV-1, respectively.

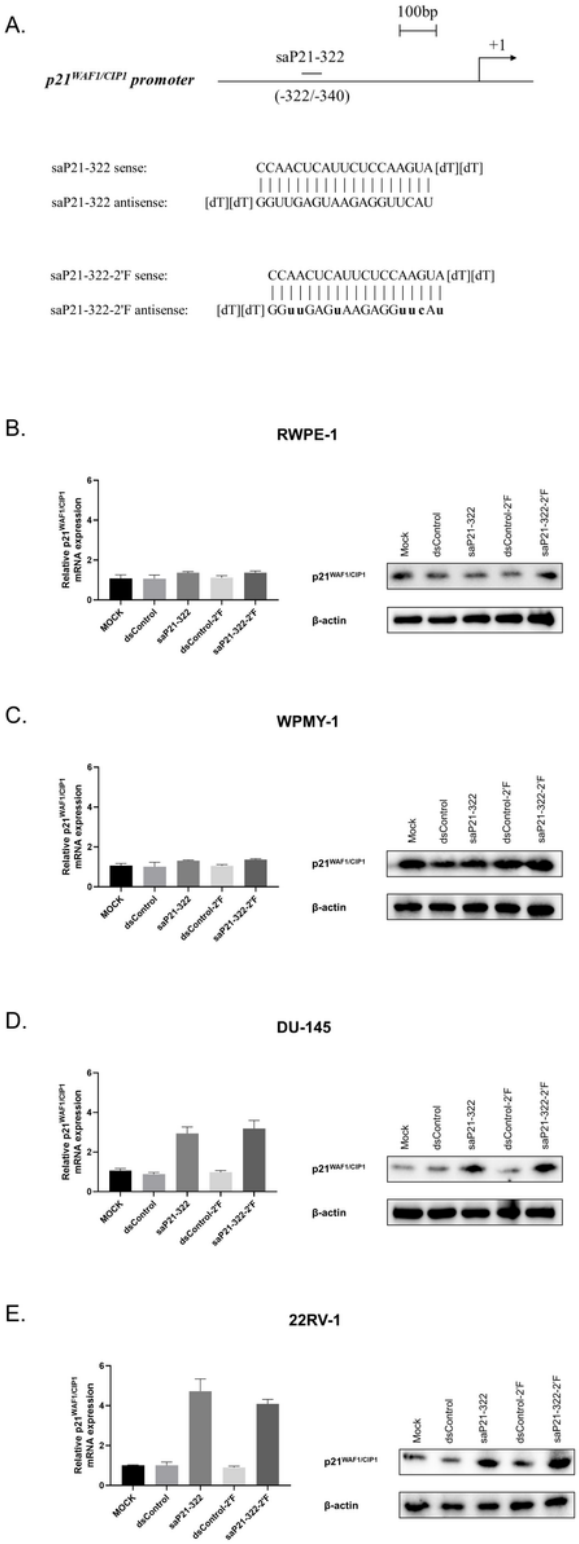

Figure 4

Activation effect of exogenous saRNA on p21WAF1/CIP1 in prostate cell lines. (A) Schematic representation of the p21WAF1/CIP1 promoter and saP21-322 target site (upper). Sequence composition of saP21-322 (middle) and saP21-322-2'F (lower). Indicated are the sense and antisense strands, which possess dual deoxythymidine overhangs at their 3'-termini. Lowercase letters in bold correspond to 2'-fluoro-modified nucleotides. (B) Normal human prostate epithelial cell line RWPE-1 was transfected at a

final concentration of 50 nM of dsControl, dsControl-2'F, saP21-322, or saP21-322-2'F for 72 hours. Mock samples were transfected in the absence of dsRNA. Relative expression levels of p21WAF1/CIP1 were assessed by qRT-PCR (left; mean ± SD from 3 independent experiments) and western blot (right). β-actin served as a loading control. (C), (D) and (E) show the results of the same experiment as (B) of normal human prostate stromal cell line WPMY-1, prostate cancer cell line DU-145 and 22RV-1, respectively.

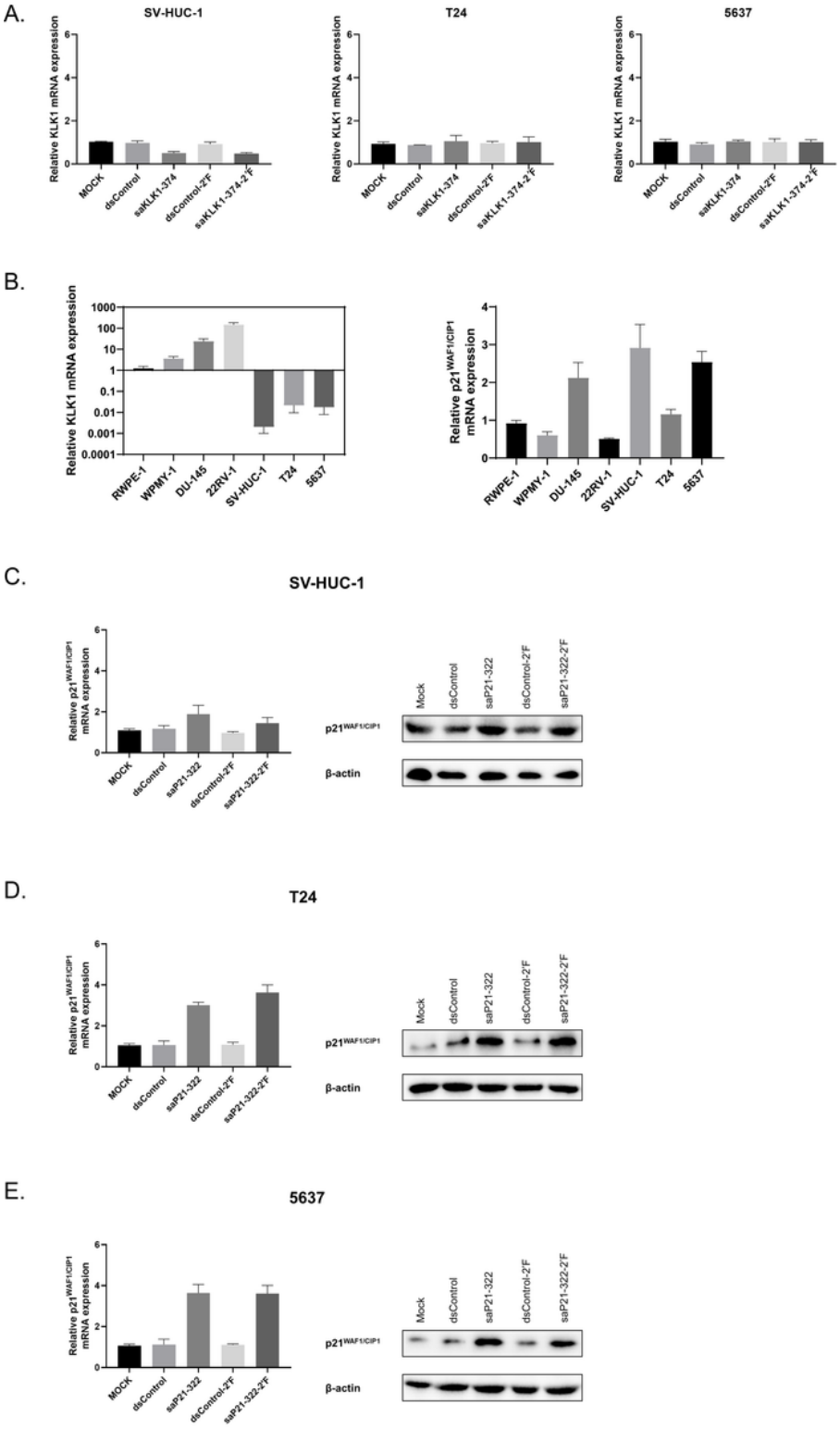

Figure 5

RNAa on KLK1 and p21WAF1/CIP1 in bladder cell lines. (A) Normal urothelial cell line SV-HUC-1, and the bladder cancer cell lines T24 and 5637 were transfected at a final concentration of 50 nM of dsControl, dsControl-2'F, saKLK1-374, or saKLK1-374-2'F for 72 hours. Mock samples were transfected in the absence of dsRNA. Relative expression levels of KLK1 were assessed by qRT-PCR (mean ± SD from 3 independent experiments). β-actin served as a loading control. (B) The 4 prostate cell lines and 3 bladder cell lines without intervention were extracted RNA under the same conditions, and the basic expression of KLK1 and p21WAF1/CIP1 was detected by qRT-PCR (mean ± SD from 3 independent experiments). (C) Normal urothelial cell line SV-HUC-1 were transfected at a final concentration of 50 nM of dsControl, dsControl-2'F, saP21-322, or saP21-322-2'F for 72 hours. Mock samples were transfected in the absence of dsRNA. Relative expression levels of p21WAF1/CIP1 were assessed by qRT-PCR (left; mean ± SD from 3 independent experiments) and western blot (right). β-actin served as a loading control. (D) and (E) show the results of the same experiment as (C) of bladder cancer cell lines T24 and 5637 respectively.

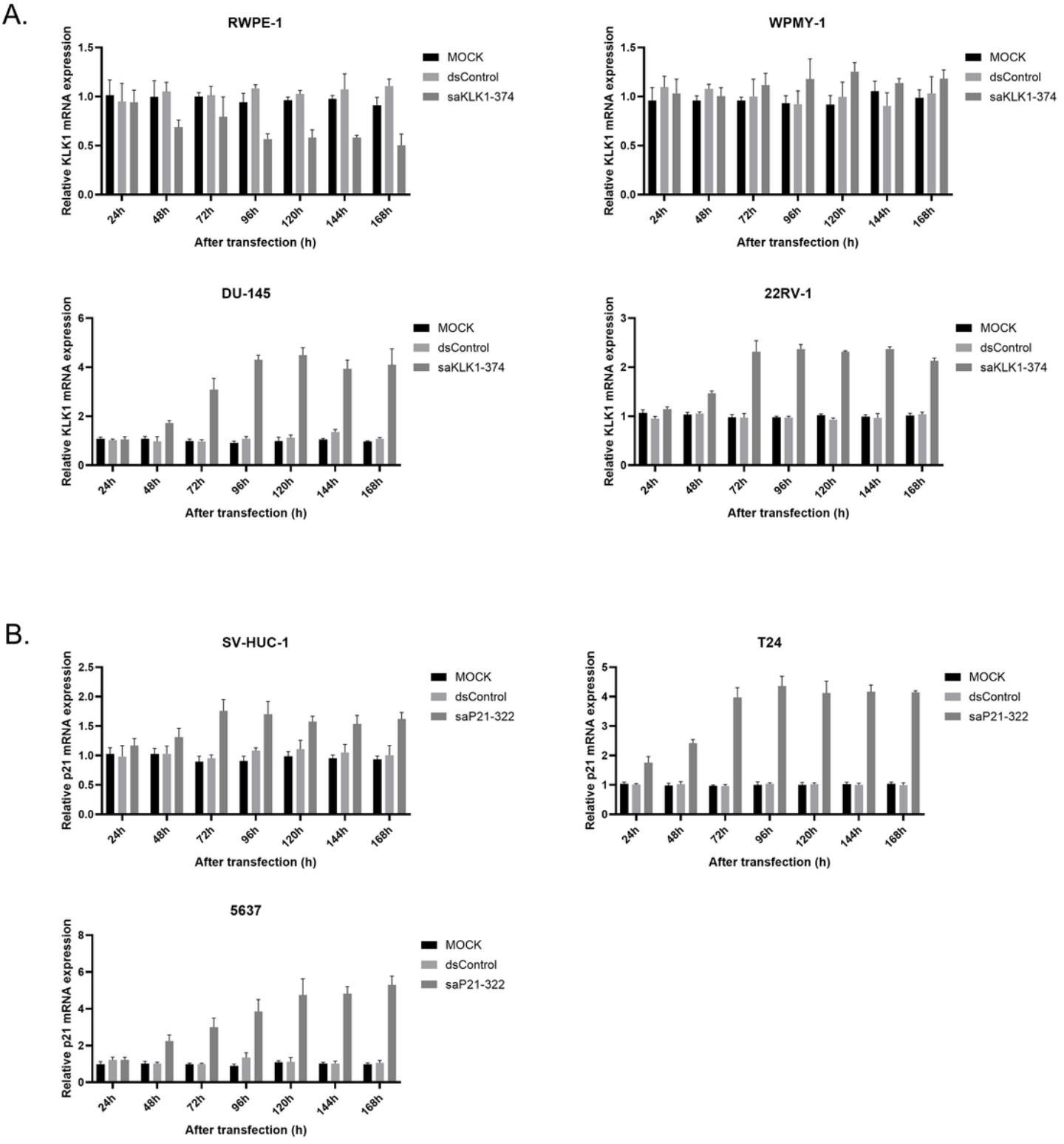

Figure 6

Target gene expression every 24 hours after transfection of exogenous saRNA. (A) Normal human prostate epithelial cell line RWPE-1, normal human prostate stromal cell line WPMY-1, and prostate cancer cell lines DU-145 and 22RV-1 were transfected at a final concentration of 50 nM of dsControl or saKLK1-374. Mock samples were transfected in the absence of dsRNA. The total RNA of cells was extracted every 24 hours for qRT-PCR to detect the expression of KLK1 (mean ± SD from 3 independent

experiments). β-actin served as a loading control. The data for every 24 hours was independently standardized. (B) Normal urothelial cell line SV-HUC-1, and the bladder cancer cell lines T24 and 5637 were transfected at a final concentration of 50 nM of dsControl or saP21-322. Mock samples were transfected in the absence of dsRNA. The total RNA of cells was extracted every 24 hours for qRT-PCR to detect the expression of p21WAF1/CIP1 (mean ± SD from 3 independent experiments). β-actin served as a loading control.

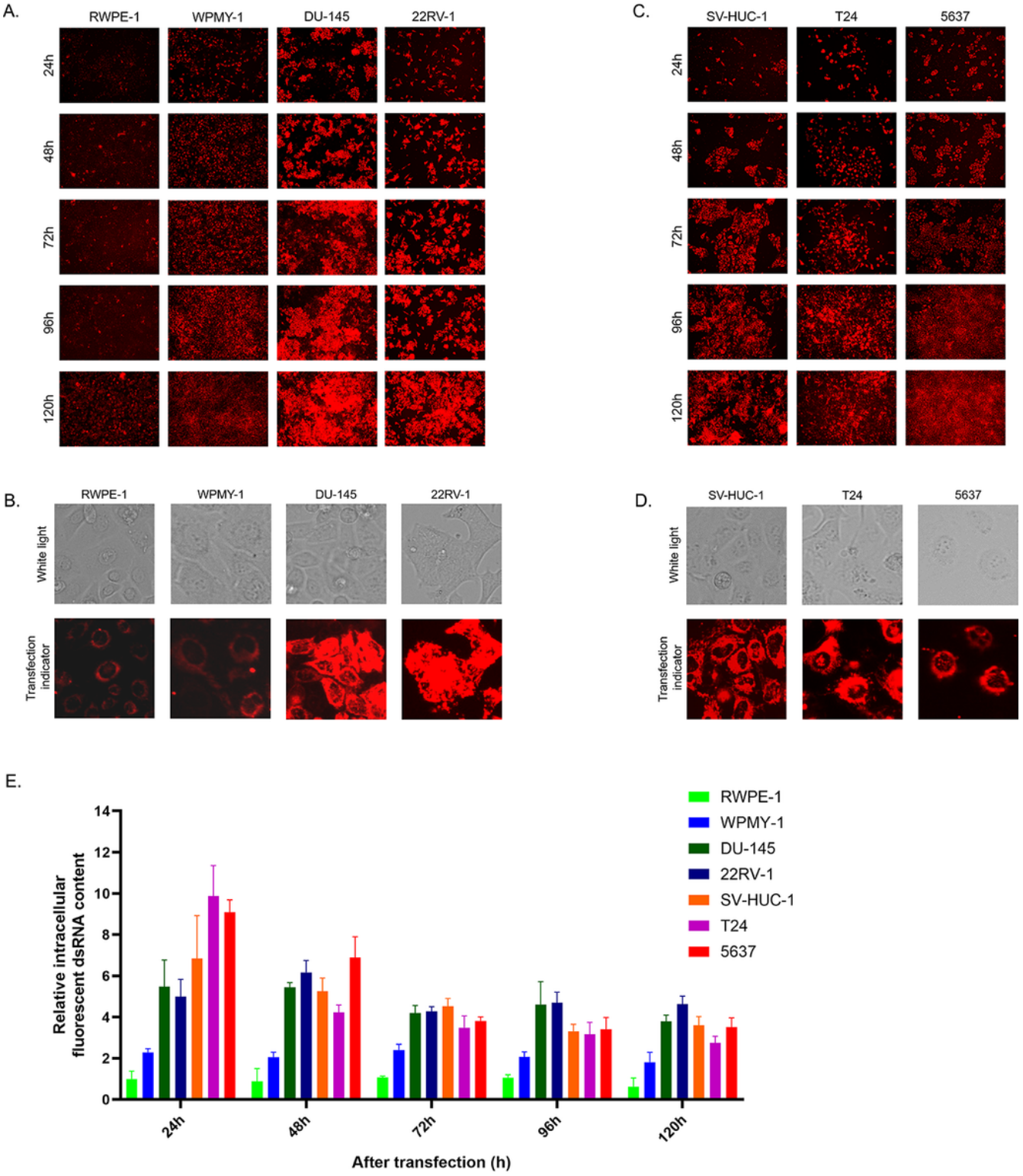

Figure 7

Analysis of the uptake of exogenous dsRNA by fluorescence photos. (A) The same amount of prostate cells were transfected with the same amount of dsControl-5cy3 24 hours after seeding the plate, and then fluorescent photos (magnification ×250) were taken every 24 hours. (B) The captured pictures described above were enlarged 100 times to show the difference of dsControl-5cy3 in the prostate cell nucleus. (C) The same amount of bladder cells were transfected with the same amount of dsControl-5cy3 24 hours after seeding the plate, and then fluorescent photos (magnification ×250) were taken every 24 hours. (D) The captured pictures described above were enlarged 100 times to show the difference of dsControl-5cy3 in the bladder cell nucleus. (E) The fluorescence intensity of dsControl-5cy3 was analyzed through red fluorescence photos, and the number of cells was calculated through white light photos. Then the average fluorescence intensity within a single cell was the ratio of the total fluorescence intensity to the total number of cells. The ordinate showed the data after normalization.

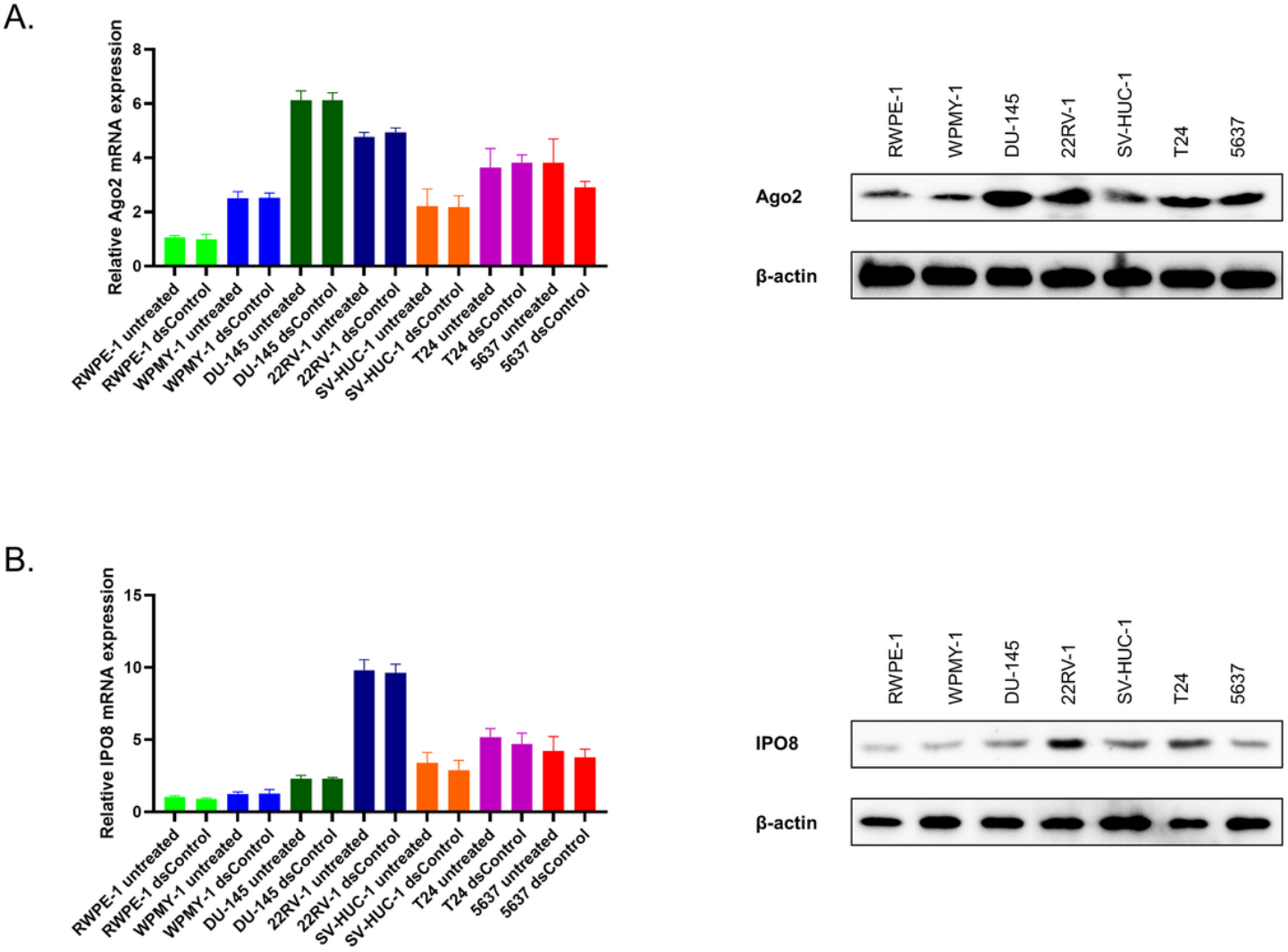

Figure 8

The expression levels of Ago2 and IPO8 in all cell lines. (A) The expression levels of AGO2 in 4 prostate cell lines and 3 bladder cell lines. The qRT-PCR results of untreated cells and cells transfected with dsControl are on the left, and the western blot results of untreated cells are on the right. (B) The expression levels of IPO8 in 4 prostate cell lines and 3 bladder cell lines. The qRT-PCR results of untreated

cells and cells transfected with dsControl are on the left, and the western blot results of untreated cells are on the right.

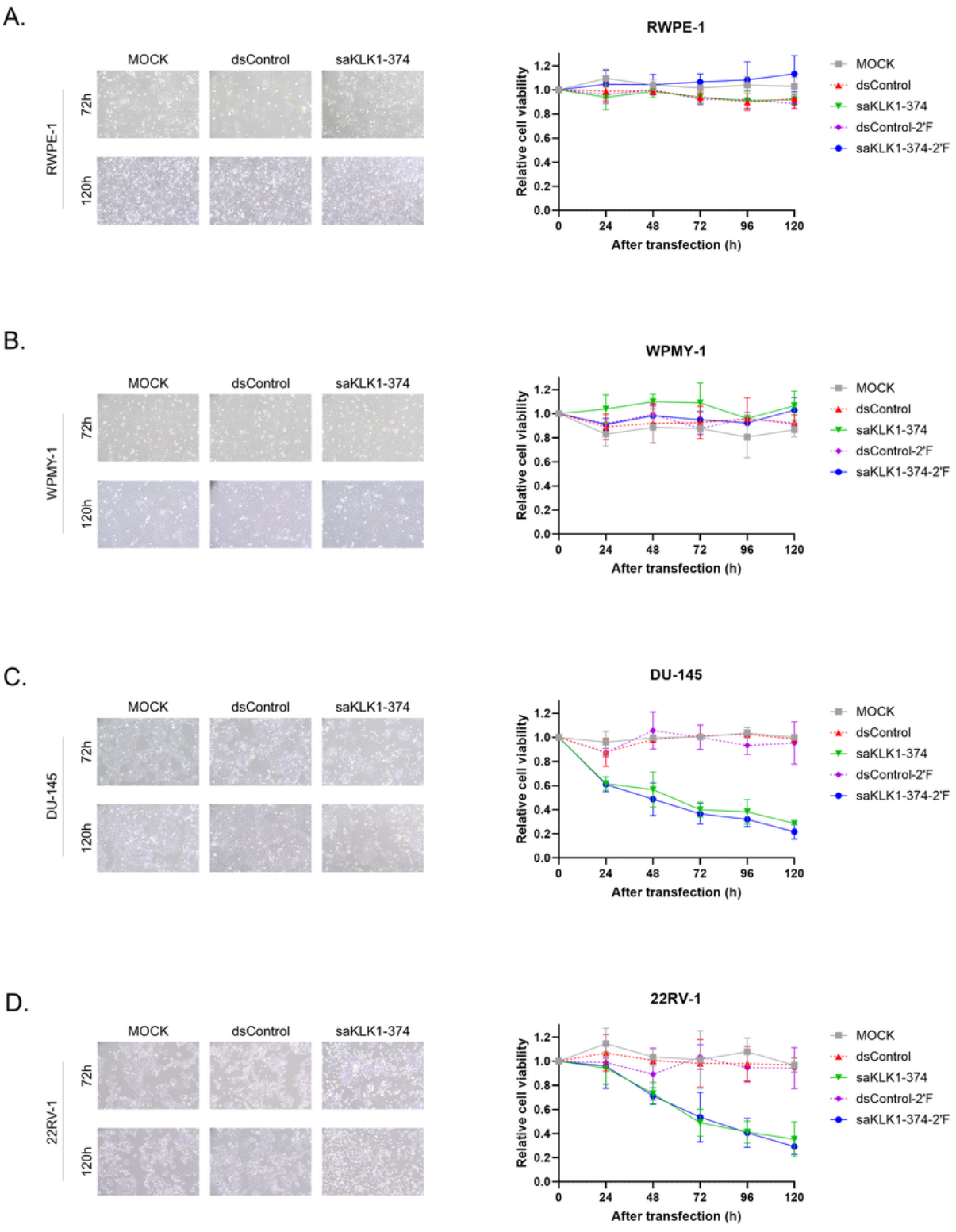

Figure 9

The effect of saKLK1-374 on the viability of prostate cell lines. (A) The photos on the left were taken in the above-mentioned experiments for activating KLK1 (magnification ×100). The line graph on the right showed the data of the cytotoxicity test. RWPE-1 cells were transfected with 50 nM concentrations of

dsControl, saKLK1-374, dsControl-2'F or saKLK1-374-2'F and seeded in 96-well microplates. Mock samples were transfected in the absence of dsRNA. Cell viability was quantified at each day utilizing CCK-8 reagent. Data are plotted as the mean ± SD of 3 independent experiments relative to untreated cells. (B), (C) and (D) show the results of the same experiment as (A) of normal human prostate stromal cell line WPMY-1, prostate cancer cell line DU-145 and 22RV-1, respectively.

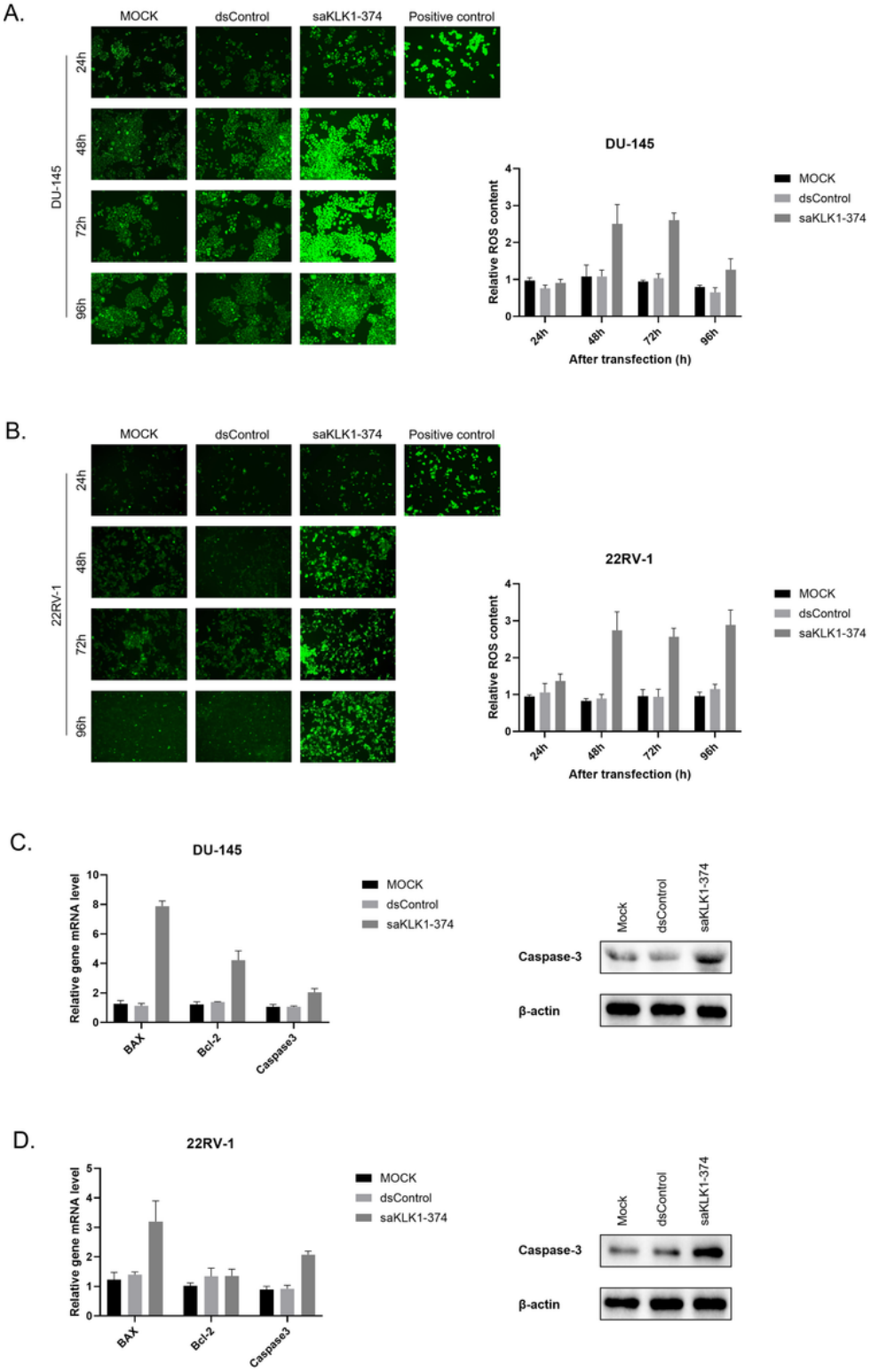

Figure 10

Prostate cancer cells undergo oxidative stress and apoptosis after transfection with saKLK1-374. (A) DU-145 cells were seeded in 6-well plates. After transfection of saKLK1-374, ROS detection was performed every 24 hours for 4 days, and fluorescence photos (left; magnification ×250) representing the ROS content in the cells were taken. "Positive control" sample was added to the "Rosup" which could significantly up-regulate intracellular ROS in the ROS Assay kit. The photos were used for semi-quantitative analysis (right). The fluorescence intensity of the ROS detection reagent was analyzed through green fluorescence photos, and the number of cells was calculated through white light photos. Then the average fluorescence intensity within a single cell was the ratio of the total fluorescence intensity to the total number of cells. The ordinate showed the data after normalization. (B) shows the results of the same experiment as (A) of 22RV-1. (C) & (D) show that RNA and protein were extracted from DU-145 and 22RV-1 respectively for analysis of apoptosis indicators BAX, Bcl-2 and Caspase3, 72 hours after transfection with saKLK1-374.

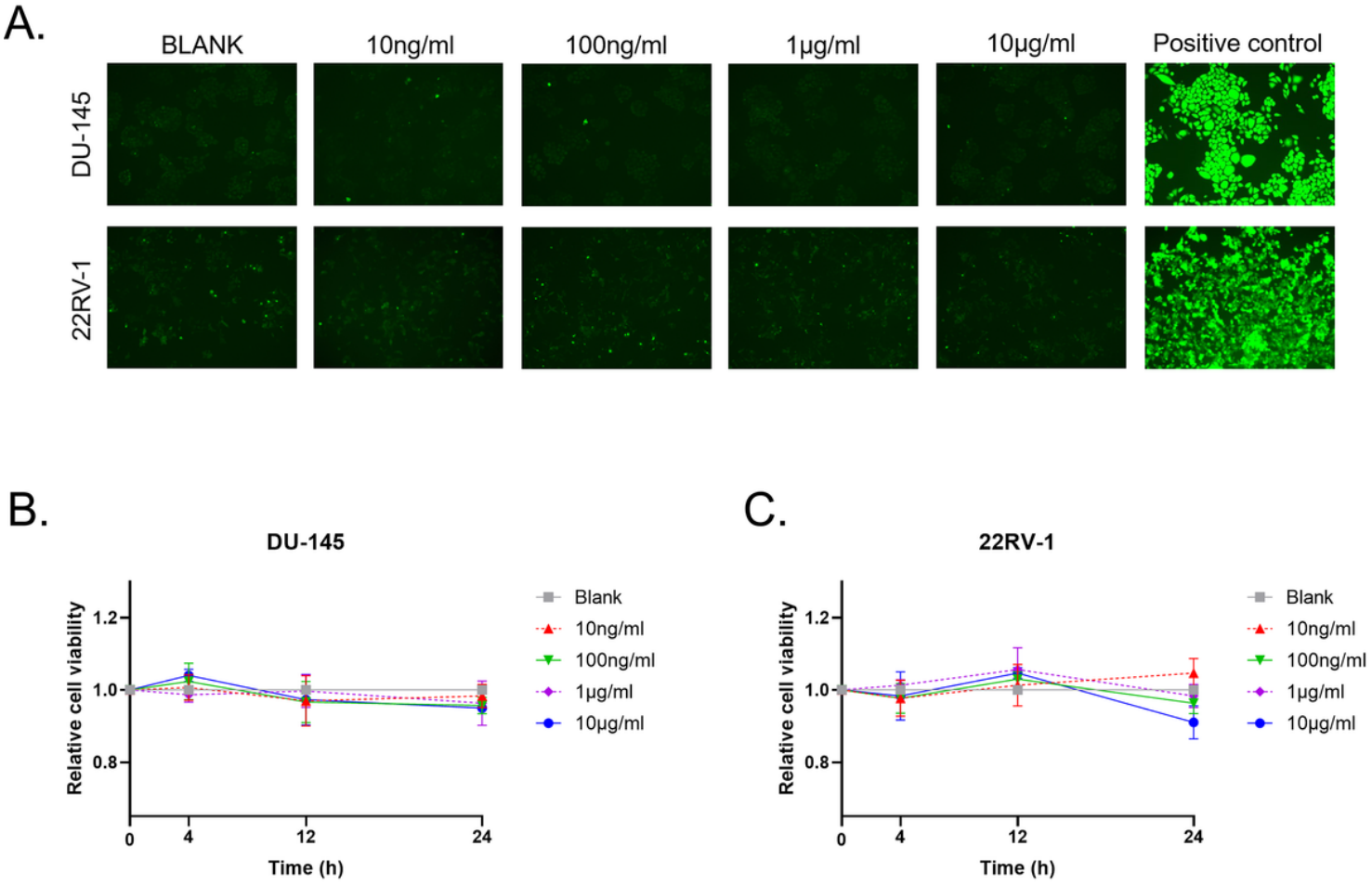

Figure 11

The direct effect of recombinant human KLK1 protein on prostate cancer cells. (A) The recombinant KLK1 protein at different concentrations was added to the DU-145 (upper) and 22RV-1 (lower) cells one day after seeding. "Positive control" sample was added to the "Rosup" which could significantly up-regulate intracellular ROS in the ROS Assay kit. ROS was detected after 4 hours of incubation at 37 ℃. (B) & (C) respectively show the cell viability of DU-145 and 22RV-1 cells at the 4th, 12th and 24th hour after the

intervention of recombinant KLK1. Data are plotted as the mean ± SD of 3 independent experiments relative to untreated cells.

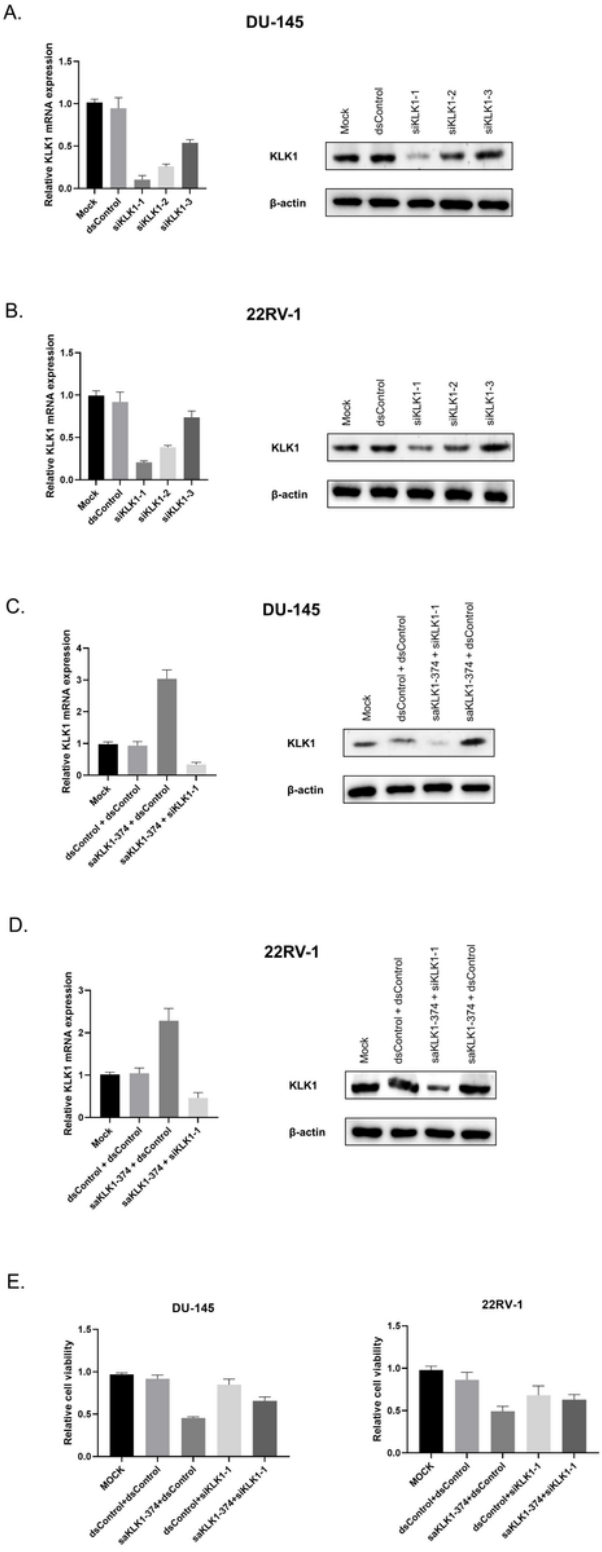

Figure 12

Interference of KLK1 expression by siRNA. (A) & (B) The 3 siRNAs and dsControl were transfected at a final concentration of 50 nM into prostate cancer cell lines DU-145 and 22RV-1. Mock samples were transfected in the absence of dsRNAs. Relative expression levels of KLK1 were evaluated by qRT-PCR

(left; mean ± SD from 3 independent experiments) and western blot (right) 48 hours after transfection. β-actin served as a loading control. (C) & (D) DU-145 and 22RV-1 cells were transfected with saKLK1-374 at a final concentration of 50 nM 24 hours after seeding, and then cells were transfected with siKLK1-1 at a final concentration of 50 nM 48 hours after seeding. Finally, RNA and protein were extracted for detection 96 hours after seeding. (E) Transfect saKLK1-374 and siKLK1-1 at the same time and concentration as above. Cell viability was detected 96 hours after seeding.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.pptx
- Additionalfile2.pptx