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Abstract

Prostate cancer is the most common cancer in men of the western world. To date, no effective treatment exists for metastatic prostate cancer and consequently, there is an urgent need to develop new and improved therapeutics. In recent years, the therapeutic potential of RNA interference (RNAi) has been extensively explored in a wide range of diseases including prostate cancer using numerous gene delivery vectors. The aims of this study were to investigate the ability of a non-viral modified cyclodextrin (CD)vector to deliver siRNA to the highly metastatic PC-3 prostate cancer cell line, to quantify the resulting knockdown of the two target genes (RelA and SRF) and to study the effects of the silencing on metastasis. Data from a Matrigel *in vitro* invasion assay indicated that the silencing of the target genes achieved by the CD vector resulted in significant reductions (P=0.0001) in the metastatic potential of these cells. As the silencing of these target genes was shown not to have a negative impact on cell viability, we hypothesize that the mechanism of invasion inhibition is due, in part, to the significant reduction observed (P= <0.0001) in the level of pro-inflammatory cytokine, MMP9, which is known to be implicated in the metastasis of prostate cancer.

Introduction

Prostate cancer is the most prevalent cancer in men worldwide. In 2014 alone, it is estimated that nearly a quarter of a million men (233 000) will be diagnosed with prostate cancer in the US with almost 30 000 men dying from the disease (www.cancer.gov). Nearly 85% of diagnosed cases of prostate cancer will be localized to the prostate gland itself[1]. When the prostate cancer is localized, several forms of treatment can be administered, including active surveillance, surgery (prostatectomy) and radiation therapy [2]. When the disease becomes invasive and spreads beyond the capsule of the prostate, alternative treatment options are required such as androgen-deprivation therapy (ADT) or chemotherapy (such as Docetaxel). Although initially effective, ADT will eventually fail leading to castration resistant prostate cancer (CRPC)[3], which is far more difficult to treat, with established (Docetaxel) and newly developed drugs (Sipuleucel-T, Abiraterone Acetate, Denosumab, Cabazitaxel and MDV-3100) offering only a modest survival time (approximately 2.4 - 4.8 months depending on the treatment used) [4, 5]. Resistance to current therapies has been associated with a number of mechanisms including the expression of transcription factors NF-κB and SRF [6].

Since its discovery by Fire and Mello in 1998, RNAi has shown potential as an effective treatment for a wide range of disease states [7]. One of the major challenges to translating this discovery into an effective RNAi therapeutic is the design of a delivery system that is able to encapsulate and protect the siRNA while simultaneously facilitating delivery to the intended target site [8]. In 2010, systemically delivered small interfering RNA (siRNA) was shown to induce a RNAi response in humans when complexed with targeted nanoparticles, highlighting its therapeutic potential [9]. Modified cyclodextrin (CD) molecules have been shown to effectively transfect a wide range of different cell types including colorectal [10] and neuronal cells [11]. A CD modified with a hepta-guanidino group on one side and a PEG-anisic acid moiety targeting the sigma receptor on the other has been synthesised for use in prostate cancer. These targeted CD molecules were shown to be capable of knocking down the expression of target genes in the PC-3 prostate cancer cell line and resulted in reduced tumour growth in a TRAMP-C1 induced mouse model of prostate cancer [12].

A recent study identified 11 potential transcription factors associated with CRPC, among these, was the central transcription factor SRF[13]. The serum response factor (SRF) is a key transcription factor that regulates the expression of genes associated with cellular growth and differentiation [14]. The target genes of SRF are identified by the presence of a consensus sequence known as the CArG box [15]. SRF has been implicated in prostate cancer progression via its role in the activity of the androgen receptor through FHL2. FHL2 is frequently over-expressed in prostate cancer and is implicated with a poor patient outcome [16]. The fibroblast growth factor receptor 4 (FGFR-4) Arg388 protein activates the extracellular-signal-regulated kinases (ERK) pathway which in turn leads to an increased expression of SRF and thus a more aggressive prostate cancer phenotype. Increased levels of SRF have also been implicated in a biochemical recurrence of prostate cancer following prostatectomy [17].

NF-κB is a transcription factor with a role in the establishment of an effective immune system as well as being integral to other developmental processes [18]. NF-κB has been defined as having a critical role in the initiation and progression of many different cancer types including prostate, breast and colorectal [18, 19]. The degree of nuclear localization of NF-κB in primary prostate cancer has been shown to be an effective prognostic marker in determining the probability of the cancer becoming metastatic [20]. The transfection of PC-3 cells with a mutated form of the NF-κB inhibitor protein (IκB) essentially blocked NF-κB activity. When the transfected cells were transplanted into nude mice, resulting tumours were slow growing compared to the controls which displayed rapid growth and metastasised readily to the lymph nodes [21].

Cancer metastasis is a complicated process with many events, the underlying mechanisms of which are not completely understood [22]. In order for a prostate cancer cell to become metastatic it has to acquire the ability to disrupt cell-cell interactions, become motile and gain the ability to enzymatically degrade the extracellular matrix (ECM)[23].

A proposed initiator of prostate cancer metastasis is epithelial to mesenchymal transition (EMT). In EMT, epithelial cells lose their inherent characteristics and become more mesenchymal-like. This transition results in cells losing cell-cell adhesion and becoming more motile. This facilitates the escape of the cell from the primary tumour and allows it to become metastatic[24]. A critical EMT event is the down regulation of the transmembrane protein E-cadherin, which functions in binding intracellular catenin and binding cadherins on adjacent cells[25]

Another critical event in prostate cancer is the breakdown of cell-matrix binding. This has been shown to be facilitated in malignant tumours by the production of matrix metalloproteinases (MMPs), which function in cancer metastasis through the enzymatic degradation of ECM[23, 26]. Twenty four different MMPS have been identified to date [27].

The main aim of this study was to investigate the effect that silencing two transcription factors (SRF and the RelA subunit of NF-κB) would have on the ability of prostate cancer cells to invade. This was tested as either a single therapy (silencing one gene), or alternatively as a combination therapy approach (silencing both genes). The genes were silenced using the commercially available Lipofectamine2000 (LF2000) vector and a well-established modified CD delivery vector. The mechanism behind the invasion inhibition was also investigated by quantifying the expression of various proinflammatory cytokines in the treated PC-3 cells.

Results

CD.siRNA is capable of significant knockdown of target gene expression *in vitro*

Optimal MR (MR; ratio of mass of CD to mass of siRNA) was decided by assessing uptake of nanoparticles and determining toxicity for a range of MRs with MR 20 chosen as the optimal formulation (Supplementary figure 1). To determine the ability of the CD vector (Figure 1) to silence the expression of the target genes *in vitro*, PC-3 cells were treated with CD.siRNA complexes. 48 hours following transfection total RNA was isolated for RT-PCR and total protein for western blot analysis. RT-PCR results show that the CD.siRNA reduced the expression of RelA and SRF mRNA (Figure 2a and 2c) with 100 nM siRNA identified as the optimal dose (Supplementary figure 2). At MR20 levels of RelA and SRF mRNA were reduced by 50 % and 35 % respectively relative to the untreated control ($p<0.001$). Compared to the CD, significantly greater levels of target gene knockdown (approximately 80 % for both RelA and SRF) were achieved with LF2000. However, the *in vivo* application of this vector is limited due to enhanced toxicity[28]. Results from the western blot analysis (Figure 2b and 2d) mirrored the mRNA results with a 31 % and 40 % knockdown of SRF and RelA respectively after 48 hours (supplementary figure 3)

Silencing multiple genes has no additional inhibitory effect on the level of target gene expression

To investigate potential synergistic or antagonistic effects when both genes (RelA and SRF) were silenced using a combination of siRNAs, RT-PCR was carried out on cells transfected with CD.siRNA complexes. The results (Figure 2) show no significant difference in the relative expression of RelA following the singular versus the combination treatment (50 % versus 33 % respectively). Similar results were observed for the relative expression of SRF (31 % vs 36 % respectively).

Figure 2

Gene silencing with CD.siRNA reduces the metastatic potential of PC-3 cells

The ability of the optimised CD.siRNA complexes to reduce the metastatic potential of PC-3 cells was assessed using the Matrigel *in vitro* transwell invasion assay (Figure 3). The assay was first optimised using LF2000 as a positive control (Supplementary figure 4). Relative to the untreated controls, knockdown by CD.RelA siRNA reduced the levels of invasion to 69.07 ± 6.06% ($p<0.01$), while knockdown by CD.SRF siRNA reduced the levels to 70.56 ± 4.05% ($p<0.01$). The highest level of inhibition (reduced to 58.43 ± 2.20%) was detected with the CD-combination (siRNA for RelA + SRF) group, however, this was not significantly different to the other treatment groups ($P=0.16$).

Figure 3

Modified cyclodextrin is non-toxic to PC-3 cells and knockdown of target genes has no negative effect on cell proliferation

An MTT assay was used to determine the level of toxicity of the CD vector on PC-3 cells: three different MRs were tested (MR10, 20 & 30). The CD complexed with 100 nM of scramble siRNA at these MRs were shown to be non-toxic when compared to untreated PC-3 cells over both a 24 and 48 hour period ($P > 0.05$, $N=3$). In contrast, when LF2000 was used, cell viability fell to 69.23 ± 3.56 at 24 and 66.77 ± 6.98 at 48 hours respectively relative to untreated controls (Figure 4a). The effect of gene knockdown by CD.siRNA complexes on cell proliferation was assessed by the CCK-8 assay (Figure 4b). Knockdown of the target genes (in both the singular and combination treatment groups) was shown to have no negative effects on cell proliferation ($p>0.05$) over a 48 hour period.

Figure 4

Silencing of target genes reduced expression of MMP-9 at both protein and mRNA levels, with no change in the level of E-Cadherin.

Forty eight hours following the transfection of cells with CD.siRNA complexes, total cellular protein was extracted and western blot analysis was used to determine if knockdown of RelA or SRF upregulated the expression of E-cadherin and lead to a less motile and invasive cell. Western blot analysis determined that there was no difference in the levels of E-cadherin seen when silencing RelA (figure 5a) or SRF (figure 5b).

MMP-9 has previously been implicated in the metastasis of prostate cancer [29]. Forty eight hours following treatment of the cells with CD.siRNA complexes, RT-PCR was used to analyse the levels of MMP-9 mRNA for the different treatment groups. Significant reductions in the levels of MMP-9 mRNA for the RelA ($p<0.01$), SRF ($p<0.01$) and combination (RelA + SRF) ($p<0.001$) treatment groups were detected versus the untreated controls (0.69 ± 0.05 for RelA, 0.65 ± 0.04 for SRF and 0.33 ± 0.05 for combination) (Figure 5c).

MMP-9 protein secretion was assessed using the MSD MMP-9 Ultra-Sensitive Kit. A significant reduction in the levels of the MMP-9 protein was detected following treatment with RelA ($p<0.001$), SRF ($p<0.01$) and combination ($p<0.001$) treatments versus untreated controls (Figure 5d). The combination group showed the greatest level of reduction in MMP-9 secretion (266.94 pg/mL ± 60.10 pg/mL). In the case of the RelA and SRF groups the levels of MMP-9 protein decreased to 460.64 pg/mL ± 148.98 pg/mL and 762.99 pg/mL ± 74.58 pg/mL respectively.

Figure 5

Discussion

An effective treatment for metastatic prostate cancer is still an unmet clinical need. RNAi offers potential as an effective therapeutic in many disease states, with the main barrier to development being the lack of a suitable delivery vector. Since 2004, there has been a surge in the number of RNAi therapeutics for cancer entering clinical trials. In the early stages, these were mainly modified free siRNA, with the first actively targeted cyclodextrin-containing nanoparticle incorporating siRNA developed by Calando Pharmaceuticals[30, 31]. This demonstrated the *in vivo* potential of treating cancer in humans with CDs. In contrast to the previous studies ([30, 31]) where the CD is incorporated as part of a polymer backbone, in the current work the CD itself acts as a scaffold allowing direct modification by various functional groups to produce, for example, the cationic amphiphilic CD used in this study[32]. In this case, we are investigating the potential of a modified cyclodextrin-based delivery vector to deliver siRNA for the treatment of metastatic prostate cancer.

The choice of target gene is integral to the development of an effective RNAi therapeutic[33]. Several candidate gene targets have been silenced by siRNA utilising different delivery vectors, and their knockdown inhibits the metastasis of prostate cancer[8]. Examples include CD31[34], PKN3[35], EZH2 and P110-α [36]. In the present study, two transcription factors that are implicated in the metastasis of prostate cancer, (i.e. RelA and SRF) were chosen as targets. High SRF nuclear expression in bone metastasis has been negatively associated with patient survival[37]. A novel inhibitor of SRF transcriptional activity has been developed that inhibits the invasion of PC-3 cancer cells using a Matrigel invasion assay[38]. A quinazolinone derivative has shown inhibition of metastasis in DU145 cells by blocking the activity of NF-κB and AP-1 (downstream target of SRF)[39]. Similarly, protein kinase D3 (PKD3) phosphorylates ser535 on RelA leading to the transactivation of urokinase-type plasminogen activator (uPA), the activity of which aids in invasion[40]. These studies indicate that targeting of RelA and SRF may offer a beneficial inhibition of prostate cancer invasion.

In this study, the CD vector was shown to be capable of delivering siRNA and inducing RNAi in PC-3 cells. The level of knockdown of RelA and SRF (at both the mRNA and protein level) was shown to be significant and similar to levels of knockdown previously observed in other cell lines[41].

The knockdown of the two genes resulted in a significant reduction in cell invasion, analysed using the Matrigel invasion assay. This is a well-established and accepted assay for measuring metastatic potential in cancer cells *in vitro.* As well as silencing each of the target genes individually, the study aimed to assess the potential synergistic effect (if any) when both genes were knocked down together. The RT-PCR and cell invasion assay indicated that the combination treatment did not afford a significant additional effect in reducing target gene expression when compared with either of the singular gene knockdown treatments (i.e. RelA and SRF) alone ($p > 0.05$) (Figure 2 + 3). These results highlight that in the case of the two target genes under investigation, there is no benefit to the use of a combination RNAi approach for suppressing metastasis.

It was important that the reduction in invasion observed was not merely attributed to a reduction in cell viability due to the knockdown of the gene itself. In order to analyse this, the toxicity attributed to the vector was determined by MTT and proliferation was assessed using CCK-8 assays. The CD vector was shown to be non-toxic to the cells up to 48 hours and up to mass ratio 30, in contrast to LF2000 (figure 4a). The SC_{12}-click-propyl amine cyclodextrin used in this study has previously shown a low toxicity profile in many different cell types [10, 42]. The results from the CCK-8 assay confirmed the silencing of RelA, SRF and a combination of both using CD was not detrimental to the viability of the PC-3 cells (figure 4b). These results mirror a recent study where the knockdown of eight target genes followed by the assessment of cell viability indicated that the silencing of SRF had little influence on the viability of three prostate cancer cell lines (LNCaP, PC-3 and DU-145) [43]. In addition, knockdown of RelA alone by siRNA was reported to have no negative effect on the viability of different cell lines, including LNCaP cells[44, 45]. These results support the hypothesis that the significant reduction in invasion (Figure 3), is not attributable to a reduction in cell viability, but rather, via a different mechanism (Figure 5).

EMT has been shown to be a key step for the initiation of metastasis with loss of E-cadherin known to be a hallmark of epithelial cancer metastasis. In this study the effect of silencing the target genes (RelA and SRF) on levels of E-cadherin was investigated. However the data indicate that invasion is independent of the EMT pathway (figure 5a and 5b), and thus implies the involvement of alternative mechanisms.

While it is important to note that many factors are necessary for the invasion of cancer cells, many crucial components of successful cancer metastasis (such as tumour angiogenesis) have been shown to be dependent (to some degree) on the activity of MMPs [46]. In this study, we hypothesise that the reductions in the levels of invasion of the PC-3 cells following treatment (Figure 3) may to some degree be explained by a reduction in the levels of this pro-invasion cytokine (Figure 6). In particular, MMP9 seems to be the downstream target of many genes that aid in prostate cancer invasion and numerous studies have been carried out to establish the role that MMP9 plays in prostate cancer metastasis. For example, the knockdown of Osteoactivin (OA) by siRNA reduced the metastatic potential of two prostate cancer cell lines (PC-3 and DU-145) and the activities of MMP9 and MMP2 were shown to be strongly inhibited[47]. The immune cell population of the prostate tumour microenviroment (pTME) has been hypothesized to play a role in prostate cancer progression[48]. Infiltrating CD4 (+) T cells have been shown to enhance prostate cancer invasion via MMP9 activation (through the androgen receptor). Inhibition of MMP9 reversed this enhanced invasion[49]. Filamin A (FlnA) is an actin-binding protein with a role in Pca metastatic progression[50]. Transfection of PC-3 cells with a plasmid encoding FlnA reduced cell proliferation, invasiveness and MMP9 protein expression, highlighting a possible mechanism for the role of FlnA in degrading MMP9[51]. The CD44 antigen plays a role in the activation of MMP9[52]. The knockdown of MMP9 by RNAi alters the isoform expression of CD44 and promotes a non-invasive cellular phenotype[53]. MMP9 plasma concentrations have been shown to be elevated in more aggressive and invasive phenotypes of prostate cancer and the induction of MMP9 expression in a usually non-metastatic cell line (in this case the prostatic LNCaP cells) has been shown to increase the invasion potential of that cell line [54] [55].

These studies illustrate that the role of MMP9 in prostate cancer is complicated and manifests via various mechanisms, all promoting invasion.

In this study, we have shown that silencing two transcription factors significantly reduced the level of MMP9 at the protein and mRNA level. Figure 6 highlights how the transcription of MMP genes occurs in cells via NF-κB and SRF. The canonical activation of NF-κB is usually stimulated by TNF-α, lipopolysaccharide (LPS) or IL-1β via various receptors such as TLR, TNRF and IL-1R[56] . Activation of these receptors leads to the phosphorylation of the NF-κB inhibitor complex (IκBα), leading to its proteasomal degradation[57]. This allows the free heterodimeric NF-κB to translocate to the nucleus where it transcribes a wide range of target genes including IL-6, IL-8 and MMPs [58]. Similarly, IL-1 and TNF-α (as well as certain growth factors such as CSF-1) have been shown to stimulate the P38 MAP kinase pathway, which activates SRF [59]. One of the targets of SRF, c-fos, is a component of the transcription factor AP-1 which has been shown to be implicated in the transcription of MMP genes [60] [61].

Numerous studies have hypothesized a link between NF-κB/SRF and MMP9 activity. The inhibition of NF-κB resulted in the suppression of MMP9 expression in smooth muscle cells [62] and human peripheral blood mononuclear cells (PBMC)[63]. NF-κB activation by Bcl-2 interacting cell death suppressor (Bis) induced the expression of MMP9 mRNA in glioma cells, thereby contributing to an invasive phenotype[64]. Increased epidermal growth factor receptor signalling results in the nuclear localisation of RelA to the promoter region of the MMP9 gene in pancreatic progenitor cells[65]. In a hepatocellular carcinoma cell line, the overexpression of SRF led to an increase in the activity and protein levels of several MMP types, including MMP-9 [66].

These identified links between the central transcription factors and MMP9 expression in different cell types and disease states is supported by our data whereby the silencing of RelA and SRF via RNAi has led to a significant reduction in MMP-9 at both the protein and mRNA level in metastatic prostate cancer.

Materials and Methods

Synthetic siRNAs. Synthetic siRNA duplexes were obtained from Qiagen (Hilden, Germany) or Dharmacon (Lafayette, Colorado). RelA siRNA was custom made from Dharmacon with the following sequence: (sense 5'3') CCAUCAACUAUGAUGAGUUdTdT, (anti-sense) AACUCAUCAUAGUUGAUGGdTdG. Non-silencing scramble siRNA (N/S siRNA) and SRF siRNA were obtained from Qiagen.

Figure 1

Figure 1: The chemical structure of the cationic amphiphilic cyclodextrin [42]

Preparation of CD.siRNA Complexes and LF2000.siRNA Complexes. A cationic amphiphilic CD was synthesized as described previously and used to formulate the siRNA [42]. Briefly, CD was dissolved in chloroform to 1mg/mL concentration. The solvent was removed under a stream of nitrogen gas for 30-40 minutes. The resulting CD film was reconstituted in 0.1 μm filtered water (for a final concentration of 1mg/mL), followed by sonication at room temperature for 1 h in order to reduce vesicle size. Equal volumes of CD and siRNA (diluted in RNAse-free water) were mixed together at room temperature for 20 minutes in order to allow for the formation of electrostatic interactions. LF2000 was purchased from Invitrogen (Carlsbad, California) and complexes were prepared as per manufacturer's instructions. Briefly, the required amount of LF2000 was dissolved up to 50 μL in OptiMEM and left at RT for 5 minutes. Similarly, the required amount of siRNA was also dissolved up to 50 μL in OptiMEM and was then added to the diluted LF2000. The LF2000 and siRNA were mixed gently and incubated at RT for 20 minutes. 1 μL of LF2000 was used per 20 pmol of siRNA.

Cell culture. PC-3 cells were obtained from the European Collection of Cell Cultures (ECACC). PC-3 cells were maintained in RPMI 1640 medium (Life Technologies™) supplemented with 10 % FBS and 2 mM L-glutamine unless otherwise stated. PC-3 cells were cultured in T175 flasks (SARSTEDT, Nümbrecht, Germany) unless otherwise stated. PC-3 cells were detected for the presence of Mycoplasma contamination using MycoProbe® detection kit (R&D Systems, Minneapolis). All cells were grown in the Forma Series II Water Jacketed CO_2 incubator (Thermo Electron Corporation, Waltham Massachusetts) at 37 °C with 5 % CO_2 and 95 % relative humidity.

Quantitative real-time PCR. Total RNA was extracted using GenElute™ Mammalian Total RNA Mini-prep Kit (Sigma-Aldrich, St. Louis, Missouri). RNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham Massachusetts). cDNA was synthesised from 300ng of total RNA using High-capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, California). Real-Time PCR was carried out in triplicate, using Applied Biosystems TaqMan® Gene expression assays for human MMP-9. RelA (P65), SRF (FAM labelled) and β-actin (VIC labelled) and Applied Biosystems TaqMan® Universal PCR Master Mix. Quantitative real-time PCR was carried using the Applied Biosystems 7300 Real-Time PCR system. Average CT values were used to determine gene expression. For all PCRs, β-actin was used as an endogenous control and CT values were normalized to levels of β-actin expression [11].

Western Blot Analysis. 48 hours following transfection cells were washed twice in cold PBS and were lysed in 0.5 % NP-40 lysis buffer (10 mM Tris pH 8.0, 60 mM KCl, 1 mM EDTA pH 8.0) supplemented with Protease Inhibitor cocktail (P8340)(Sigma) and PMSF (Sigma). Samples were left on ice for 10 minutes, following which cells were spun at 13,000 rpm for five minutes at 4°C and the supernatant was collected. Total cellular protein was quantified using DC™ Protein Assay (Bio-Rad). 30ųg of total cellular protein was loaded onto 10 % SDS polyacrylamide gels and run at 120 V. Following this, proteins were transferred onto Immobinin P (Millipore) membranes at 100 V for 80 minutes. Western blotting was carried out using antibodies to SRF (1:1000, Santa Cruz, sc-13029), RelA (P65) (1:1000, Santa Cruz, sc-372), E-Cadherin (1:1000, Millipore, MAB3199) and β-actin (1:5000, Sigma) followed by incubation with either anti-mouse or anti rabbit horseradish peroxidase-conjugated secondary antibodies (Cell Signalling). Signals were detected using ECL™ (Pierce). Bands were quantified using ImageJ (NIH) and results were normalised to β-actin levels.

***In vitro* Transwell Invasion Assay.** A matrigel-based cell invasion assay was performed following the method of Albini [67]. Matrigel, originally extracted from a tumour, has all the components found in the basement membrane extracellular matrix (ECM) and is highly biologically active [68]. Matrigel (Sigma-Aldrich) was diluted to 1 mg/mL using serum-free RPMI 1640 medium. Due to the nature of the Matrigel to polymerise above 4°C [69], it was kept on ice at all times. 8.0 μM inserts (Falcon 3097) were placed into a 24-well plate and 100 μL of the 1 mg/mL Matrigel was added to each insert. The plate was then incubated overnight at 4°C and then for one hour at 37°C to allow the gel to polymerise. Excess media was removed from the inserts, and they were rinsed with serum-free RPMI 1640 prior to use. Cell suspension containing $5x10^4$ PC-3 cells was added to the upper chamber of the inserts. 500 μL of RPMI 1640 media supplemented with 10 % FBS was added to the well beneath the insert. The invasion assay was maintained in the Forma Series II Water Jacketed CO_2 incubator (Thermo Electron Corporation) at 37 °C with 5 % CO_2 and 95 % relative humidity for 48 h. Following this time, any non-invading cells were scraped from the upper surface of the insert with a cotton swab and any invading cells were stained using 0.25 % crystal violet (Sigma-Aldrich) for 10 minutes. The inserts were then placed in a 10 % acetic acid solution and a consistent volume of the mixture was transferred to a 96-well plate for colorimetric reading of OD at 560 nm.

Cell viability and proliferation assays;

MTT Assay. The MTT assay is a well-established indicator of cytotoxicity that has been used in prostate cancer cells [70]. This assay measures the enzymatic reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) by mitochondrial dehydrogenase in living, metabolically active cells. 10 000 PC-3 cells were seeded into a 96 well plate 24 h prior to transfection. siRNA (100 nM) was complexed with either LF2000 or else CD as described above. Complexes were diluted in OptiMEM® and then added to the cells for either a 24 h or 48 h period. Following this time, medium was removed and replaced with 200μL of fresh medium and 20 μL of MTT reagent (5 mg/mL solution) for four hours. Following this time, formazon crystals were dissolved in 100 μL of dimethyl sulfoxide (DMSO). Absorbance was measured at 590 nm using a UV plate reader.

CCK-8 Assay. Cell proliferation was assessed using Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies In, Kumamoto, Japan). 10 000 PC-3 cells were seeded into a 96 well plate 24 h prior to transfection. siRNA (100 nM) was complexed with CD as described above. Complexes were diluted in OptiMEM® and then added to the cells for a 48 h period. Following this time, medium was removed and replaced with 100 µL of fresh medium and 10 µL of CCK-8 solution. Absorbance was measured at 450 nm using a UV plate reader.

Meso Discovery Plates. 200 µL of 1 mg/mL Matrigel (Sigma) was added to each well on a 24 well plate. The plate was then incubated overnight at 4°C and then for one hour at 37°C to allow the gel to polymerise. $5x10^4$ PC-3 cells were added to each well 24 h prior to transfection. siRNA was complexed with CD as described above. Complexes were diluted in OptiMEM® and then added to the cells for a 48 h period. Following this time, the cell supernatant was harvested and samples were analysed for MMP-9 using the Item Human MMP-9 Ultra-Sensitive Kit (catalogue number Catalogue Number K151HAC-1) from Meso Scale Discovery (Rockville, Maryland) as per the manufacturer's instructions. The plate was read using SECTOR® Imager 2400 (Meso Scale Discovery).

Statistical analysis. Data were expressed as mean ± SEM of three independent experiments (N=3). For all *in vitro* experiments, Gaussian distribution and equal variance was assumed. One-way Analysis of Variance (ANOVA) was used to test the significance of differences in three or more groups followed by a Post-Hoc test (in this case, Tukey) for all experiments. In all cases, $P < 0.05$ was considered to be statistically significant. All graphs and statistical calculations were prepared using GraphPad Prism 5 (San Diego, California).

Conclusions

The SC_{12} click-propyl amine cyclodextrin used in this study has previously exhibited efficacy as an siRNA delivery vector in *in vivo* models of inflammatory bowel disease[10] and Huntington's disease [11]. The focus on this study was metastatic prostate cancer. The results from the *in vitro* invasion assay have indicated the potential of using a CD-based, non-viral gene delivery vector complexed with siRNA to combat metastasis of prostate cancer. In terms of designing a successful RNAi therapeutic, the choice of target gene is vital, and this study shows there is no significant benefit (in this case) in using a combination gene therapy approach as a means of reducing metastasis. The results demonstrate that knockdown of two central transcription factors (RelA and SRF) using the modified cyclodextrin resulted in a significant reduction in a cytokine well known to be involved in cancer metastasis and we hypothesise that this reduction in part, explains the mechanism of invasion inhibition. To our knowledge this is the first report of a CD-based combination siRNA approach for the treatment of metastatic prostate cancer and the results presented indicate the potential for future development of an RNAi-based therapeutic.

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Conflict of interest

The authors declare that there are no conflicts of interest

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Figure Legends

Figure 1: Chemical structure of cationic amphiphilic CD [42]

Figure 2: RT-PCR results from 48 h transfection of PC-3 cells using SC_{12} click-propyl-amine CD at MR20 targeting RelA (a) and SRF (c). For both transfections, LF2000-siRNA was used as a positive control. The CD treatment groups were shown to significantly reduce the expression of the target gene when compared with the untreated controls both singularly ($p<0.001$) or in the combination groups ($p<0.001$). There was no significant difference in the level of target gene knockdown between the singular and combination treatments ($p>0.05$). RelA and SRF gene expression was normalized against the expression of β-actin. Western blot analysis from 48 hour transfection of PC-3 cells using SC_{12} click-propyl-amine CD at MR20 targeting RelA (b) and SRF (d). There was a significant reduction in the level of RelA (<0.001) and SRF ($p<0.001$) proteins when compared to the untreated controls (Supplementary figure 3) (***$p<0.001$, N=3) (UT=untreated, N/S= cyclodextrin with scramble siRNA control).

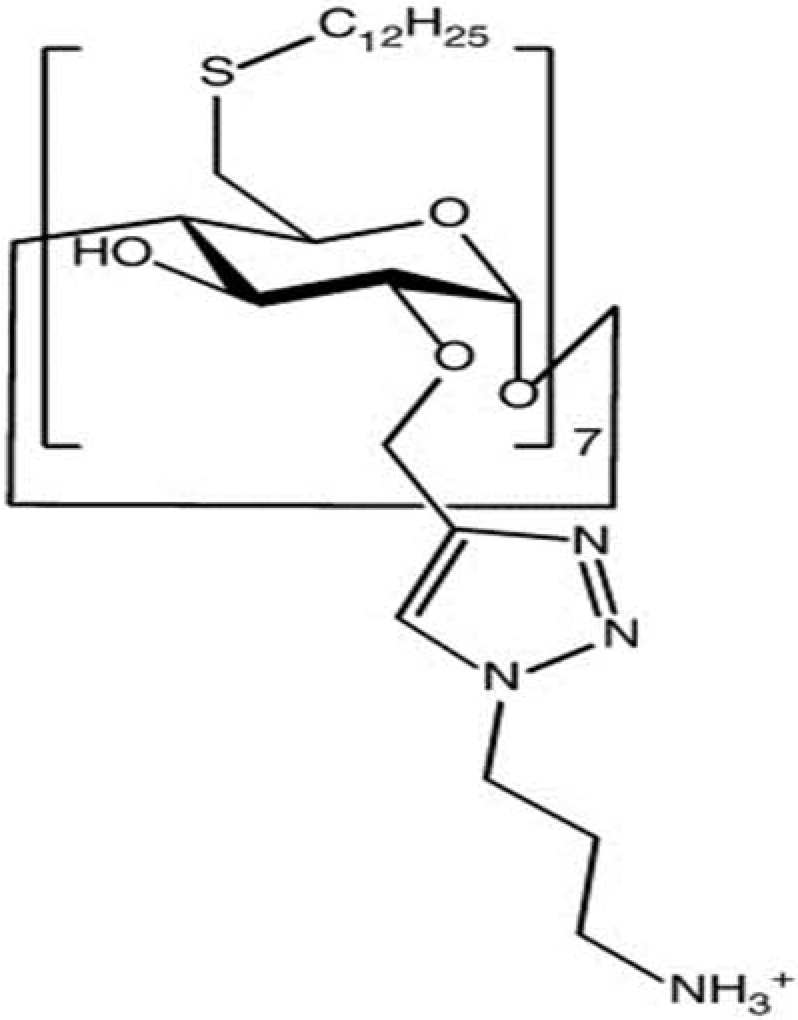
Figure 3: The effect of silencing SRF, RelA and SRF+RelA (Comb) on the invasion capacity of PC-3 cells using CD.siRNA (MR20). There was a significant reduction in the levels of invasion observed in all of the treatment groups when compared with the UT controls (***$p<0.001$, **$p<0.01$, N=3) (UT=untreated, N/S= cyclodextrin with scramble siRNA control).

Figure 4: a) Levels of cell viability in PC-3 cells following treatment with CD.siRNA at three different MRs and LF2000.siRNA complexes. Toxicity was determined by means of an MTT assay. Levels of cell viability were normalised to the untreated controls (*$p<0.05$, N=3). **b)** Levels of cell viability in PC-3 cells following treatment with CD.siRNA (MR20) complexes for 48 hours. Viability was assessed using CCK-8 assay. Levels of cell viability were normalised to the untreated controls (ns=non-significant, N=3) (UT=untreated, N/S= cyclodextrin with scramble siRNA control).

Figure 5: Western blot analysis for E-cadhein following knockdown of a) RelA and b)SRF. There was no significant difference in the levels of E-Cadherin versus the untreated controls ($p>0.05$).

Levels of MMP-9 mRNA expression (c) and secretion (d) following treatment with CD-siRNA. mRNA levels were quantified by RT-PCR (N=3 **p <0.01, ***P <0.001 versus the untreated control). MMP-9 gene expression was normalized against the expression of β-actin. Levels of MMP-9 in the cell supernatant were quantified using MSD kit (N=3, **p<0.01, ***p<0.001 versus the untreated control) (UT=untreated, N/S= cyclodextrin with scramble siRNA control).

Figure 6: Overview of the effects of SRF and NF-κB on the expression of MMPs and subsequent metastasis. Inflammatory mediators and cytokines lead to the phosphorylation of Iκ-Bα and the transportation of the NF-κB dimer into the nucleus where it binds to NF-κB binding sites leading to the expression of MMP genes. Similarly, many growth factors, cytokines and hormones activate MAPK. This leads to the activation of SRF, which in turn activates the central transcription factor AP1, leading to the transcription of MMP genes. MMPs genes are transcribed and translated into proteins which are secreted by the cells into the extracellular environment. Once in the extracellular space, MMPs work by degrading the extracellular matrix - this can facilitate the "release" of cells from the primary tumour site and can promote their metastasis to distant site.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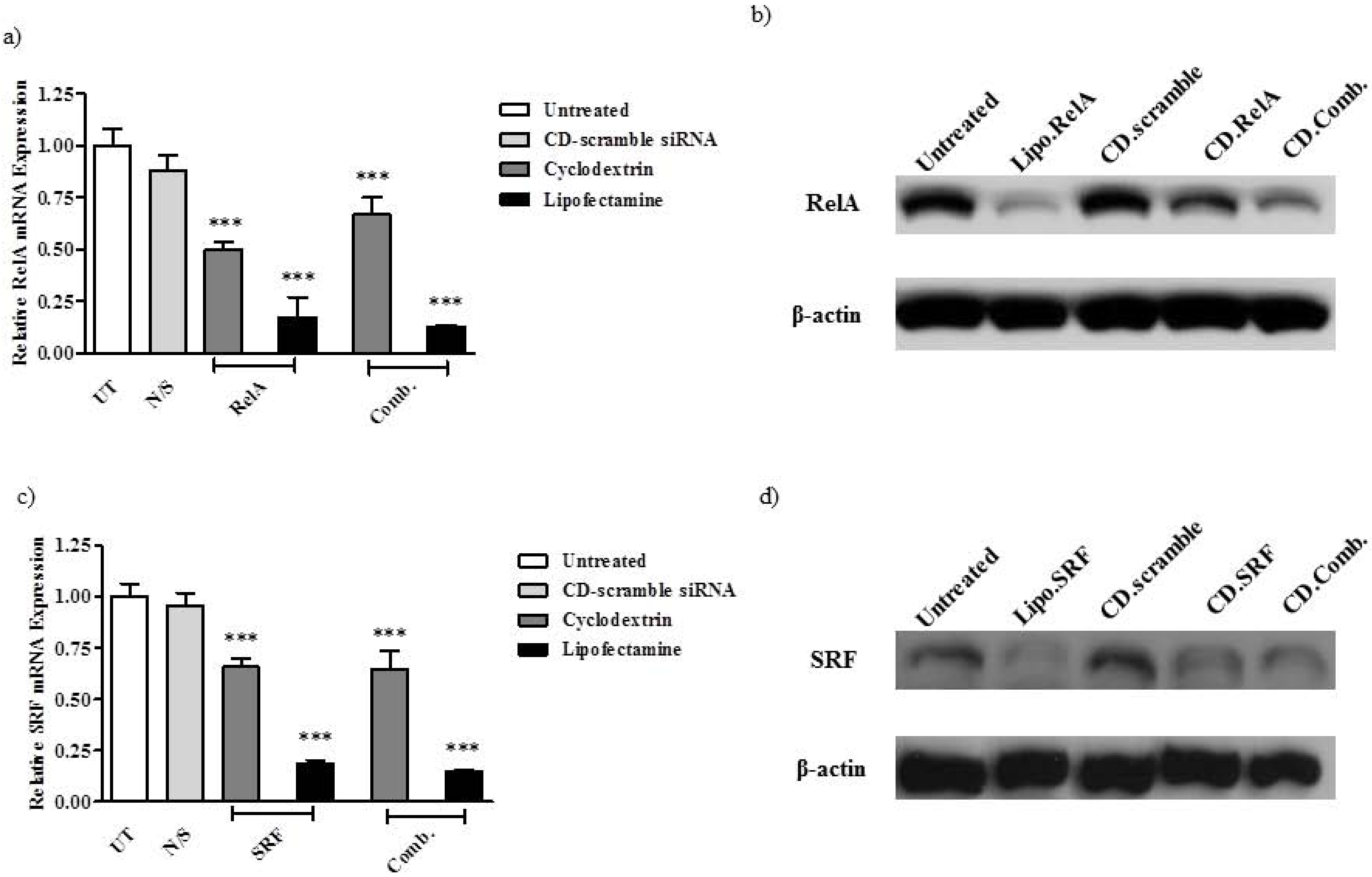
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Lipofectamine
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CD.scramble
CD.RelA
CD.Comb.
RelA
β-actin
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Relative SRF mRNA Expression
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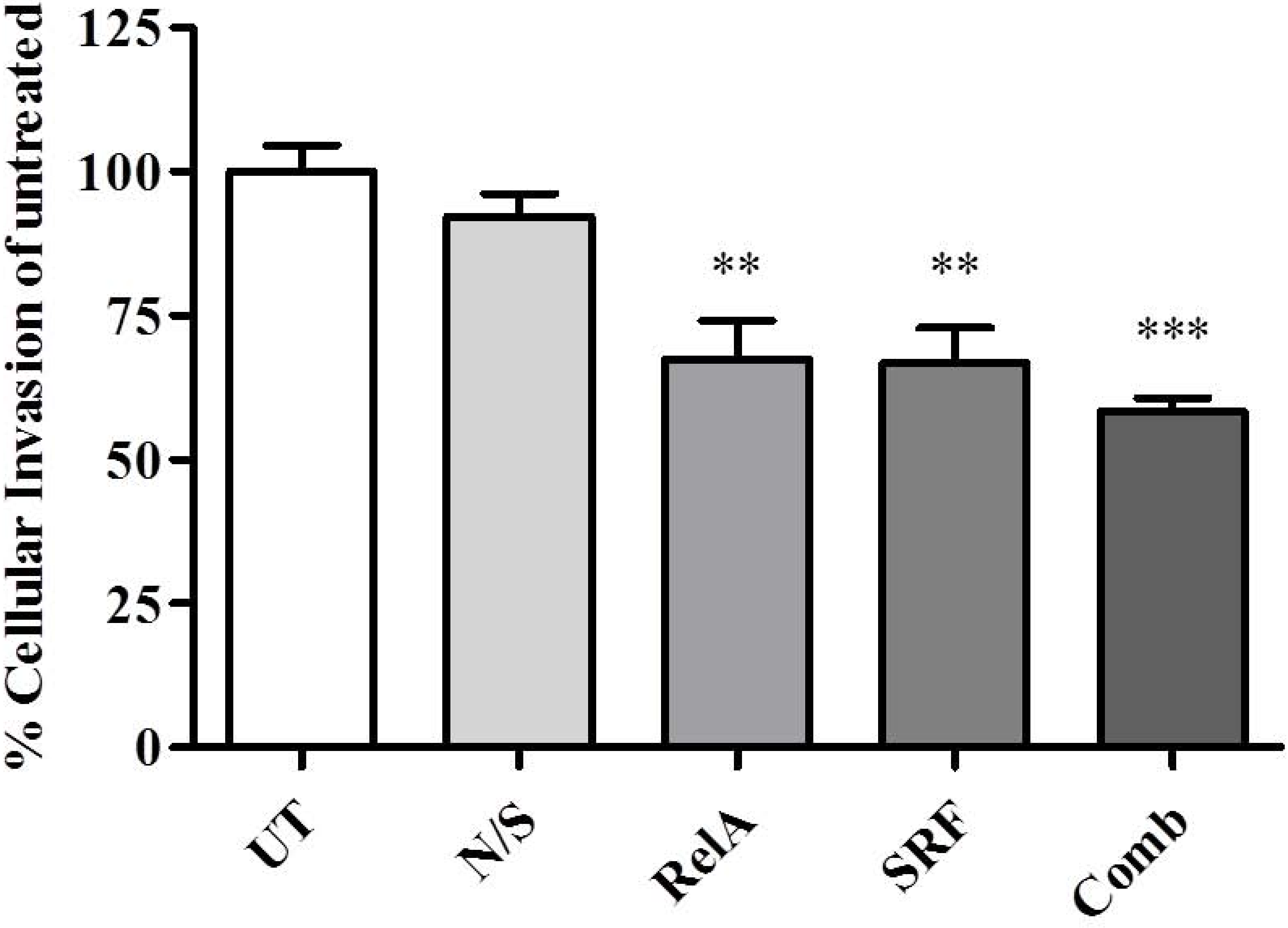
CD.SRF
CD.Comb.
SRF
β-actin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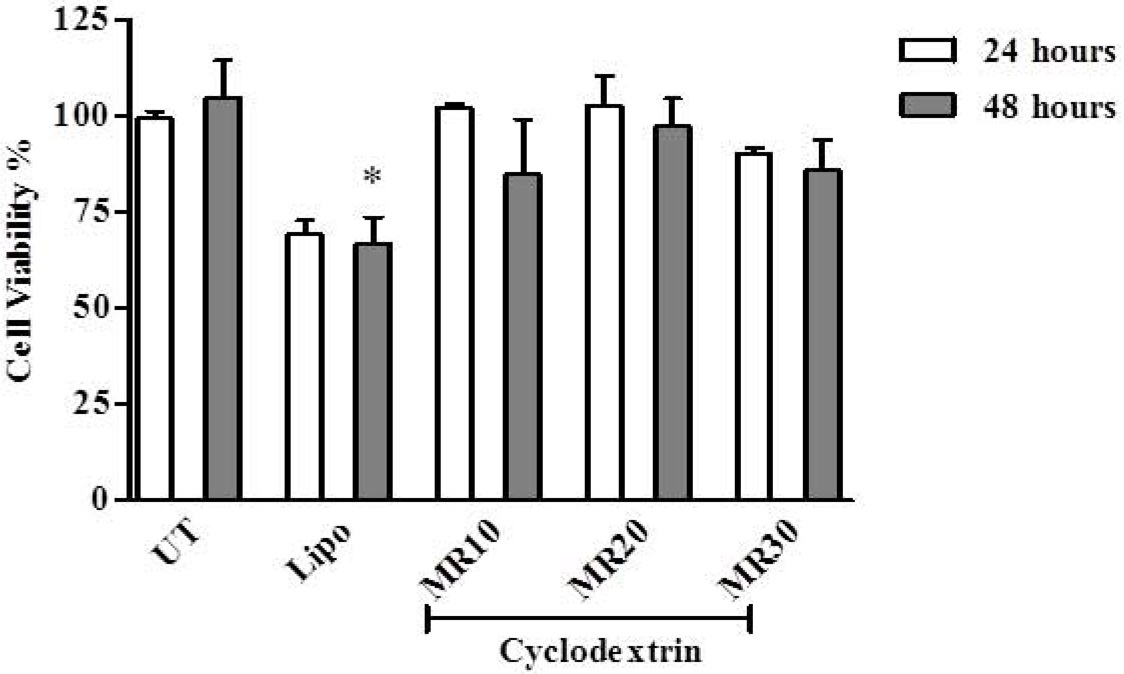
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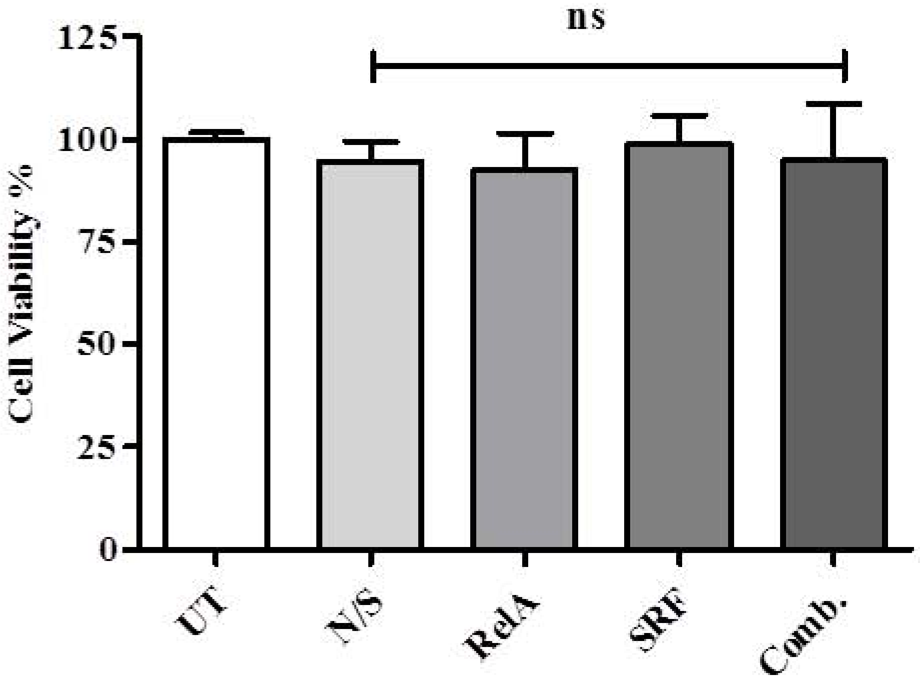
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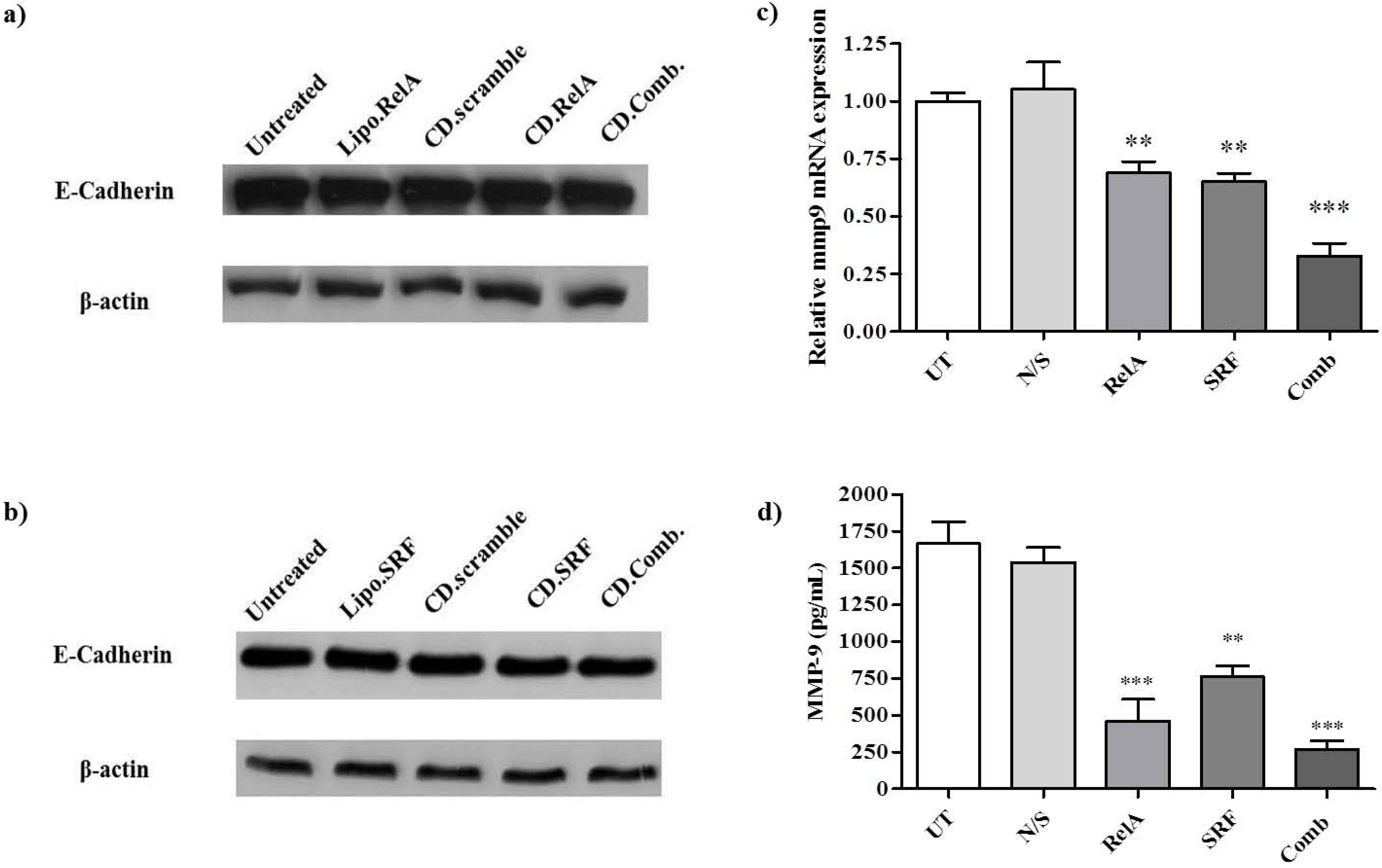
a)
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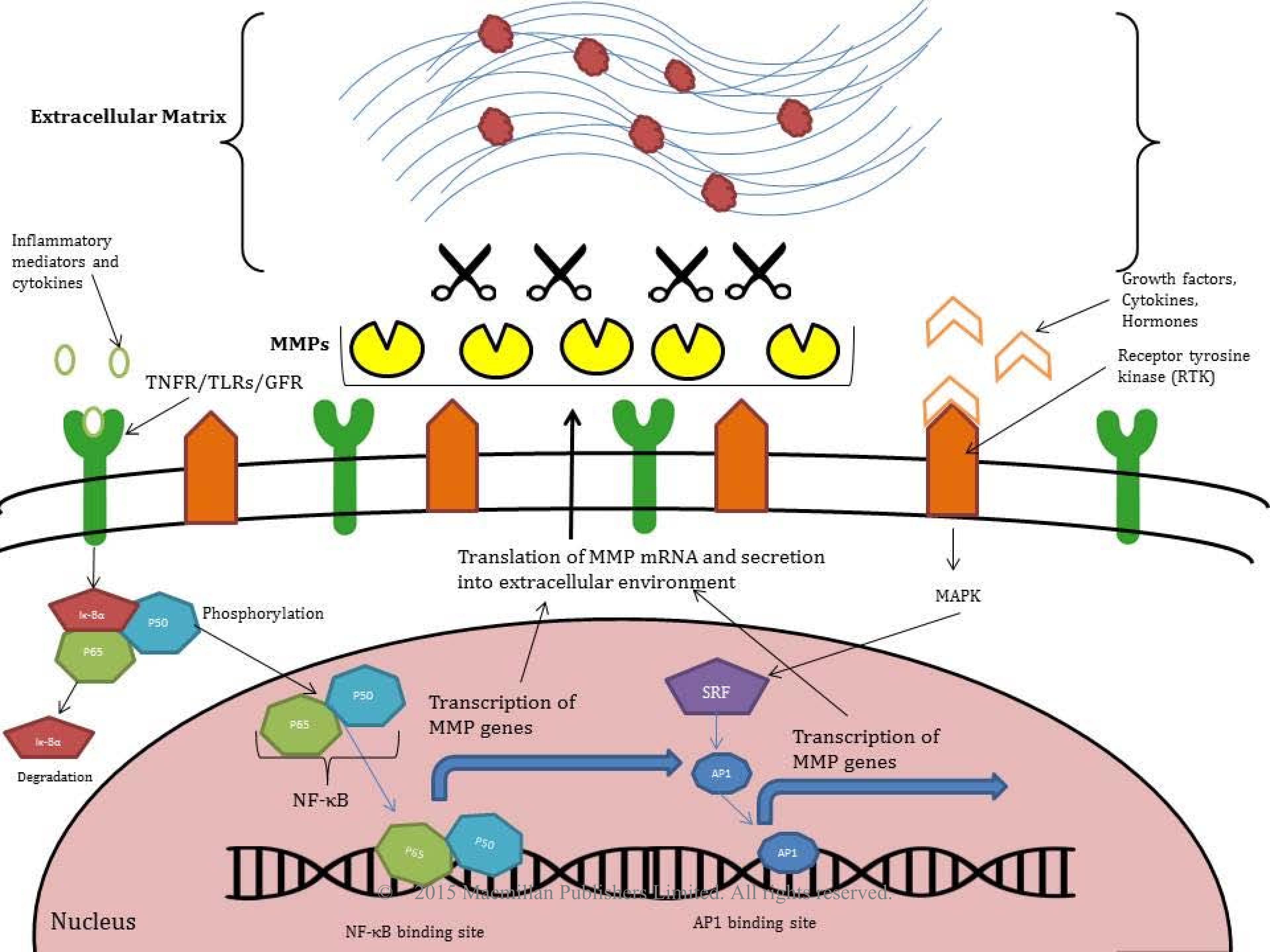
Extracellular Matrix
Inflammatory mediators and cytokines
MMPs
Growth factors, Cytokines, Hormones
Receptor tyrosine kinase (RTK)
TNFR/TLRs/GFR
Translation of MMP mRNA and secretion into extracellular environment
MAPK
Iκ-Bα
P50
P65
Phosphorylation
Iκ-Bα
Degradation
P65
P50
NF-κB
Transcription of MMP genes
SRF
AP1
Transcription of MMP genes
P65
P50
AP1
Nucleus
NF-κB binding site
AP1 binding site