MARIANNA TEIXEIRA DE PINHO FAVARO

"DESENVOLVIMENTO DE VETORES NÃO-VIRAIS PARA ENTREGA GÊNICA BASEADOS NA CADEIA LEVE DE DINEÍNA RP3"

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"DESENVOLVIMENTO DE VETORES NÃO-VIRAIS PARA ENTREGA GÊNICA BASEADOS NA CADEIA LEVE DE DINEÍNA RP3"

"Development of nonviral vectors for gene delivery based on dynein light chain Rp3"

Dissertação apresentada ao Instituto de Biologia para obtenção do Título de Mestre em Genética e Biologia Molecular na área de Genética de Microrganismos

Orientador: Prof. Dr Adriano Rodrigues Azzoni
Co-Orientadora: Profa. Dra. Anete Pereira de Souza

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“Humanity's first sin was faith; the first virtue was doubt.”
(Carl Sagan)

OFEREÇO

Aos meus pais, Lucy e Sergio, por terem tolerado desde os meus primeiros anos de vida a minha insaciável curiosidade pelo mundo.

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Prefácio

Os resultados obtidos durante o desenvolvimento do presente trabalho são apresentados na forma de um artigo científico e de um capítulo de resultados complementares. No artigo intitulado “A non-viral gene delivery vector based on the dynein light chain Rp3 and TAT peptide” encontra-se a parte principal do trabalho desenvolvido durante esta dissertação e descreve a produção de uma proteína recombinante, produzida em *E. coli,* que age como vetor de entrega gênica em um modelo de cultura de células de mamíferos. Tal manuscrito foi submetido à revista Journal of Controlled Release. O resumo deste trabalho também foi apresentado no 15° Encontro Anual da Sociedade Americana de Terapia Gênica e Celular (ASGCT) na forma de pôster e publicado em um suplemento da Molecular Therapy em maio de 2012.

Como resultados complementares, são apresentados os experimentos preliminares de expressão da proteína TcTex1, uma outra cadeia leve de dineína, fusionada a um outro peptídeo membrano-ativo, Penetratina cujo andamento dos trabalhos foi finalizdo a favor da proteína mais promissora T-Rp3 visando a conclusão desta dissertação.

Em anexo, encontra-se um artigo, no qual participei como co-autora, desenvolvido pelo grupo de pesquisa onde realizei esta dissertação. Esse artigo, publicado em 2012 e intitulado “Development of a recombinant fusion protein based on the dynein light chain LC8 for non-viral gene delivery” (Journal of

Controlled Release, 159 (2), 222–231), descreve a utilização de uma outra cadeia leve de dineína, Lc8, para aumentar a eficiência da entrega gênica.

Finalmente, ao final desta dissertação, apresenta-se a discussão e as conclusões baseadas nos resultados obtidos no estudo da proteína T-Rp3. Incluiu-se também uma breve descrição das perspectivas futuras e sugestões para próximos trabalhos relativos a este tema a serem desenvolvidos no grupo.

Resumo

Entrega gênica é uma estratégia muito promissora com grande potencial médico, que consiste na introdução de ácidos nucléicos exógenos, e pode ser aplicada tanto para terapia gênica quanto para vacina de DNA. Contudo seu uso ainda é limitado pela falta de um vetor de entrega ideal, que seja ao mesmo tempo eficiente e seguro.

Embora muito mais eficientes, os vetores virais ainda despertam preocupações a respeito de sua segurança. Por outro lado, vetores não-virais são muito mais seguros e facilmente manipuláveis, ainda que menos eficientes. Neste contexto, “vírus artificiais” são uma opção interessante, uma vez que são vetores não-virais desenvolvidos para explorar a arquitetura celular de uma forma eficiente, superando uma série de barreiras físicas, enzimáticas e difusionais, mas mantendo a segurança do DNA plasmidial (pDNA). O principal objetivo da abordagem estudada é explorar os motores moleculares, como dineína, para transportar cargas da periferia para o centrossoma de células de mamíferos através da rede de microtúbulos. Para isso, a cadeia leve Rp3 da dineína foi fusionada a um domínio de interação com DNA (DNA-binding) no N-terminal, e ao peptídeo membrano-ativo TAT no C-terminal. A proteína, nomeada T-Rp3, tem ainda um His-Tag. Esta proteína recombinante construída contém então diferentes domínios para promover condensação do pDNA (DNA binding), para facilitar a entrada na célula e no núcleo (TAT) e para aumentar o escape endossomal (His-Tag), além da própria Rp3 que deve assistir no tráfego intracelular, agindo assim

diretamente na maioria dos principais obstáculos intracelulares enfrentados pelos vetores. Estudos de expressão indicam que a proteína recombinante é corretamente expressa em *E. coli* BL21(DE3). Experimentos de mobilidade em gel de agarose ("gel retardation assay") combinados com estudos de espalhamento de luz e potencial zeta indicam que a proteína efetivamente interage com o pDNA, formando complexos que são pequenos (~95 nm) e positivamente carregados (+28 mV na relação molar de pDNA:proteína 1:8000). Ensaios de transfecção em cultura de células HeLa indicam que T-Rp3 atinge uma eficiência de transfecção muito maior que a proteína nuclear Protamina (aqui usada como controle), chegando a ser 900 vezes maior a expressão relativa do gene repórter na relação molar de pDNA:proteína 1:8000. Na comparação com Lipofectamina 2000^{TM}, um reagente bem caracterizado de transfecção aqui usado como controle positivo, a T-Rp3 demonstrou atingir níveis similares de eficiência, com a vantagem adicional de ser menos citotóxica, conforme evidenciado em ensaios de viabilidade celular. Transfecções realizadas na presença da droga Nocodazol indicam que a eficiência da T-Rp3 depende fortemente da rede de microtúbulos, uma vez que a eficiência é reduzida em 92% quando os microtúbulos estão despolimerizados. A partir das transfecções na presença da droga Cloroquina, pudemos observar que o aprisionamento no endossomo ainda é um fator limitante. Finalmente, ensaios de cromatografia de afinidade realizados com o domínio da cadeia intermediária de dineína imobilizado indicam que a cadeia leve recombinante T-Rp3 mantém a capacidade de interagir com o complexo da dineína. Analisados em conjunto, os resultados apontam para uma grande participação da rede de microtúbulos na eficiência de transfecção de T-Rp3, objetivo inicial deste trabalho.

Abstract

Gene delivery is a promising technique with great medical potential that consists in the introduction of exogenous nucleic acids, and can be applied for gene therapy as well as DNA vaccination. However, its use is still limited by the lack of an ideal delivery vector, which is both safe and efficient.

Although much more effective, viral vectors still raise several concerns about its safety. On the other hand, non-viral vectors are safer and easier to manipulate, but less efficient. In this context “artificial viruses” are an interesting option, since they are non-viral vectors intended to explore the cell’s architecture in an efficient way, to overcome a series of physical, enzymatic and diffusional barriers, while still preserving the safety of plasmid DNA (pDNA) vectors. The main objective herein is to exploit molecular motors, like dynein, to transport cargoes from the periphery to the centrosome of mammalian cells via the microtubule network. For that, human dynein light chain Rp3 was fusioned to a N-terminal DNA binding domain and a C-terminal membrane active peptide, TAT. The protein, named T-Rp3, has additionally a His.Tag. The shuttle protein built contains therefore different domains to promote pDNA condensation (DNA binding), to increase cell and nucleus penetration (TAT) and to enhance endosomal escape (His.Tag), besides the Rp3 to assist in the cytosol trafficking, thus covering most of the major obstacles to the vectors in intracellular level. Expression studies indicate that the fusion protein was correctly expressed in soluble form using *E. coli* BL21(DE3) strain. Gel retardation assays, dynamic light scattering and zeta

potential studies indicate an efficient complex formation between pDNA and the fusion protein, resulting in a particle that is both small (~95 nm) and potivelly charged (+28 mV in the molar ratio of pDNA:protein 1:8000) Transfection of cultured HeLa cells indicates that T-Rp3 has a much higher transfection efficiency when compared to the nuclear protein Protamine (here used as a control), reaching a 900-fold increase in expression of transfected reporter gene, both in the same molar ratio of pDNA:protein 1:8000. When compared to Lipofectamine 2000^{TM}, a well-known transfection reagent here used as a control, T-Rp3 showed to reach similar levels of efficiency, but with the further advantage of being less cytotoxic, as observed in cell viability assays. Transfections performed in the presence of the drug Nocodazole indicate that T-Rp3 efficiency largely depends on the microtubule network, since its efficiency is reduced by 92% when microtubules are depolymerized. From transfections in the presence of Choroquine we can deduce that endosomal entrapment remains a limiting factor. Finally, affinity chromatography experiments performed with the immobilized domain of dynein intermediate chain demonstrate that the recombinant light chain T-Rp3 retains the ability to interact with the dynein complex. Taken together, these results point to a strong participation of the microtubule network in the enhanced efficiency of T-Rp3.

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1. Introdução

Com o advento das tecnologias de DNA recombinante e a crescente gama de estratégias de manipulação genética, surgiu o interesse em aplicar este conhecimento na área de saúde, através da inserção de moléculas de ácidos nucléicos em células de mamíferos. O objetivo, entre outros, poderia ser a correção de um processo patológico de base genética, bem como a inserção de genes exógenos visando a imunização. Em ambos contextos, a entrega gênica surge como uma estratégia de grande potencial médico.

Mas apesar do potencial da entrega gênica, o DNA não possui a capacidade natural de entrar nas células, tornando necessário o uso de vetores que o carreiem. Neste contexto, dispõe-se atualmente de duas classes de vetores: virais e não virais. A escolha de um vetor de entrega passa pela questão de *eficiência x segurança*, uma vez que vetores virais são mais eficientes porém menos seguros, enquanto vetores não virais são mais seguros porém menos eficientes.

Assim sendo, uma abordagem de suma importância é o desenvolvimento de vetores que sejam ao mesmo tempo seguros e eficientes, permitindo assim uma ampla aplicação *in vivo* da entrega gênica. Explorando a segurança e facilidade de manipulação dos vetores não virais baseados em plasmídeos (pDNA), é preciso se aprofundar em quais são os obstáculos que limitam sua eficiência e dificultam sua chegada ao núcleo. Sabe-se que o ambiente intracelular é muito "congestionado" e que quanto maior o tempo de residência de um vetor no citosol, maior é a possibilidade de que seja degradado por nucleases ou capturado em vesículas.

Portanto, a abordagem adotada se baseia na redução do tempo de residência no citosol e direcionamento do plasmídeo, utilizando a rede de microtúbulos. Uma vez que são as dineínas os motores moleculares responsáveis pelo transporte da periferia para o centrossoma, utilizamos a cadeia leve Rp3 da mesma como vetor de entrega não viral. A proteína recombinante T-Rp3, formada pela cadeia leve Rp3, um peptídeo membrano-ativo e um domínio de interação com o DNA pode ser considerada uma proteína modular multifuncional que promove o encapsulamento e age em diferentes etapas do tráfego intracelular, explorando a própria arquitetura celular e aumentando assim a eficiência da entrega gênica. Além de inovadora, essa estratégia permite a obtenção de um vetor não viral seguro, eficiente e pouco citotóxico.

2. Revisão bibliográfica

2.1. Terapia Gênica

A idéia central que embasa o conceito de terapia gênica é o fato de que ao mesmo tempo que doenças podem ser determinadas geneticamente, a manipulação genética pode então reverter ou prevenir o advento de doenças. A terapia gênica promove a inserção de ácidos nucléicos através de um vetor visando prevenir, alterar ou reverter um processo patológico. A estratégia pode ser a inserção de um gene faltante, a correção ou o nocaute de um gene já presente, ou uma combinação delas [1], sendo a adição a forma mais comumente usada nos atuais testes clínicos e pré-clínicos, para suprir alguma proteína faltante devido a mutação. Já a abordagem de nocaute frequentemente envolve a utilização de microRNAs e RNA de interferência. A administração pode ser *in vivo* ou *ex vivo,* e dependendo do vetor pode ocorrer a integração do DNA terapêutico no cromossomo da célula, ou este pode existir na forma de episssoma.

A terapia gênica, que já está em sua terceira década, começou nos anos 90, com um teste clínico em pacientes com deficiência de adenosina deaminase. Atravessando uma história de altos e baixos, principalmente devido a reações adversas inesperadas, que incluem leucemia e até morte [2], a terapia gênica hoje se consolida como uma opção terapêutica realista e em expansão. Até 2007 havia 1340 testes clínicos já haviam sido aprovados no mundo [2], número este que subiu para 1786 em 2012.

Entre as muitas aplicações da terapia gênica, lideram ensaios com câncer, representado 64,7% do total de testes clínicos, seguido por doenças monogênicas e cardiovasculares (figura 1).

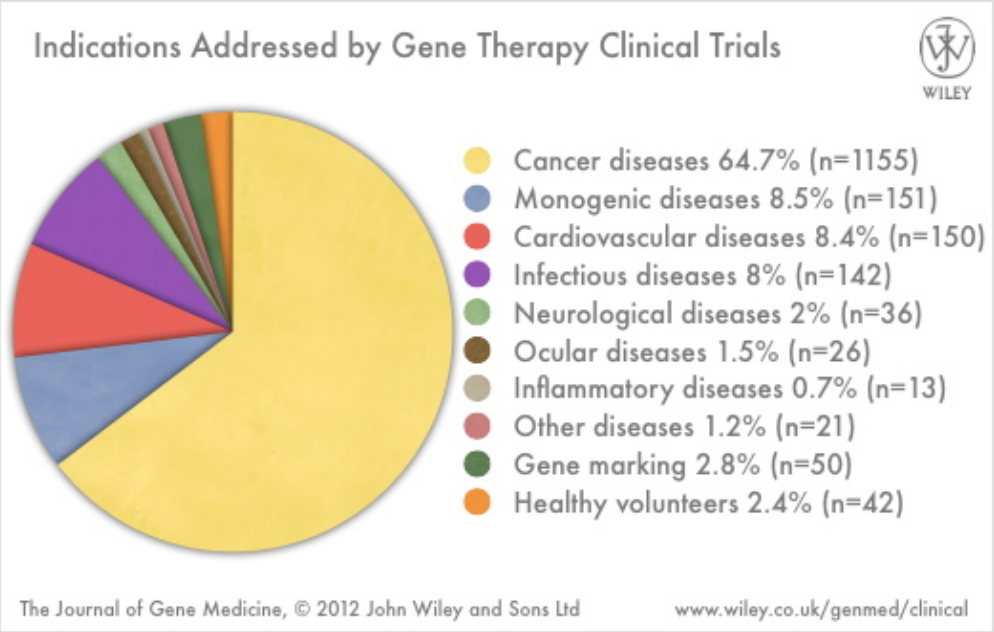

Figura1: Aplicações da terapia gênica em testes clínicos até o ano de 2012.

2.2. Vacina de DNA

Vacinas de DNA consistem, na maioria dos casos, em plasmídeos contendo um gene responsável por codificar um antígeno, um promotor e um terminador para controlar a expressão em células de mamíferos. Essa é uma estratégia promissora para elicitar resposta imune através de linfócitos T citotóxicos, T helper e anticorpos. Além de segura, essa é uma abordagem que permite fácil manipulação [3], especialmente comparado ao elaborado processo de atenuação de patogenicidade e virulência enfrentado nas vacinas clássicas. Vacinas de DNA vão de encontro à crescente necessidade por vacinas que ainda não foram bem

sucedidamente produzidas, como contra malaria e HIV. Segundo Liu e colaboradores [3], a segunda geração de vacinas de DNA depende de estratégias adicionais para aumentar a eficiência de entrega gênica através de, entre outros, sistemas de encapsulamento do pDNA, no que se inclui a proteína T-Rp3 desenvolvida no presente trabalho. Descritas desde 1992, as vacinas de DNA se tornaram uma realidade, com 4 produtos já aprovados para uso veterinário [4].

Nas vacinas de DNA o pDNA é entregue por via intradermal, subcutânea ou intramuscular, através de diversos métodos, e o plasmídeo chega ao núcleo das células do tecido transfectadas, no que se incluem células apresentadoras de antígenos (APCs). A expressão do antígeno pode então ocorrer tanto diretamente nas APCs, quanto nas células locais do tecido (como miócitos), que vão apresentar o antígeno via complexo de histocompatibilidade (MHC) para outras APCs, que migram para o sistema linfático e promovem ativação do sistema imune, através da ativação e expansão de células T, ou B com produção de anticorpos [4].

2.3. Barreiras intracelulares

Uma vez que tanto para terapia gênica quanto para vacina de DNA o desafio é sempre aumentar a eficiência de entrega gênica, é preciso avaliar os obstáculos que limitam a chegada do DNA exógeno ao núcleo. Embora haja diversos fatores no nível extracellular que afetem a eficiência, o foco da nossa abordagem reside nas etapas intracelulares, uma vez que apenas a entrada na célula não garante a eficiênca.

Começando pela interação eletrostática do pDNA com a membrana celular negativamente carregada, ocorre a entrada na célula por endocitose, incluindo as rotas dependentes de clatrina e caveolina. Apos a internalização, deve ocorrer o escape endossomal antes que ocorra fusão da vesícula com o lisossomo, o qual promove degradação por acidificação do compartimento. Do citosol, o DNA deve ser transportado para o núcleo e entrar, onde será transcrito em mRNA, o qual deve ser exportado para o citoplasma e traduzido [5,6], conforme ilustrado na figura 2.

É sabido que o pDNA deve ser liberado de seu carreador para ser transcrito, mas uma liberação prematura pode prejudicar o desempenho do vetor uma vez que o empacotamento protege o pDNA da degradação. Outro fator que afeta determinantemente o tráfego intracelular e a internalização é o tamanho da partícula, sendo que a endocitose mediada por proteoglicanos ocorre para partículas com menos de 200 nm de diâmetro. A carga da particula é outro fator determinante, uma vez que a repulsão eletrostática entre pDNA e a superfície celular, ambos carregados negativamente, é um fator limitante à entrada na célula [7].

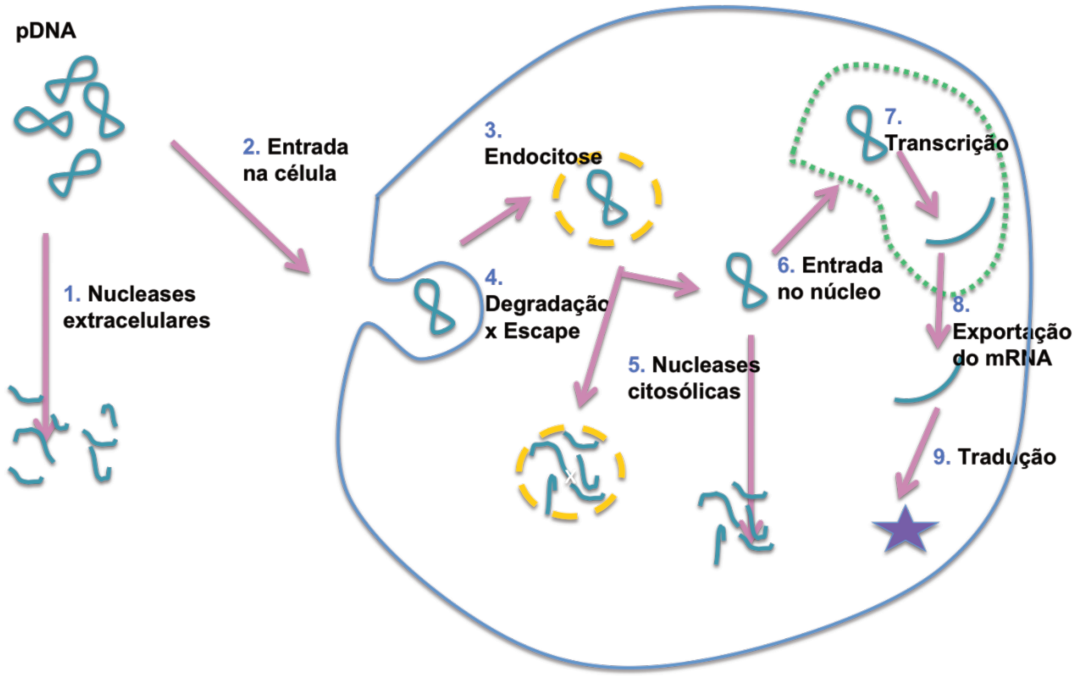

Figura 2: Barreiras intracelulares à entrega gênica em células de mamífero.

2.4. Vetores de entrega

Para mediar a entrega gênica, é indispensável o uso de vetores que carreiem o ácido nucléico exógeno até o núcleo da célula. Neste contexto, há duas classes de vetores: virais e não virais, que diferem substancialmente entre si.

2.4.1. Vetores virais

Os vetores virais são uma classe de vetores que utiliza vírus modificados para realizar a entrega gênica. São a estratégia mais comumente utilizada, compreendendo aproximadamente 67% dos testes clínicos (Figura 3). Sua utilização depende de atenuá-los e de remover sua capacidade replicativa, e se baseia em sua habilidade natural de transfectar células hospedeiras. Contudo o sistema imune possui a capacidade de reconhecer e destruir patógenos invasores,

de modo que o vetor viral pode elicitar respostas imunes inatas e adaptativas, que além de danosas podem bloquear o efeito terapêutico [8].

Os mais utilizados vetores virais se enquadram em duas categorias principais: os que promovem integração cromossomal do gene exógeno (oncoretrovírus e lentivirus) e os que persistem nas células como um epissoma extracromossomal (virus adeno-associados, adenovirus e herpes virus) [9]. Essas distinções determinam as diferentes aplicações de cada tipo de vetor viral.

2.4.2. **Vetores não virais**

Os vetores não virais são compostos por dois elementos básicos: o DNA plasmidial (pDNA) e o constituinte de entrega. A principal vantagem desta classe de vetores é não conter contaminantes virais, não estimular imunidade, e não ter limitações ao tamanho ou à quantia de DNA entregue. Embora bastante útil para aplicações *ex vivo*, vetores não virais ainda não são suficientemente eficientes *in vivo,* o que limita seu uso.

Para driblar a baixa eficiência, que limita a aplicação *in vivo* e faz com que menos de 25% dos testes clínicos já realizados envolvessem vetores não virais (Figura 3), o DNA pode ser complexado a diferentes macromoléculas como polimeros, polissacarídeos, peptídeos, lipídeos catiônicos e nanopartículas [1,10]. Uma das principais etapas é promover a condensação via interações eletrostáticas entre os grupos fosfato aniônicos do DNA e os carreadores catiônicos [11].

Embora lipídeos e polímeros sejam os mais utilizados, polipeptídeos quiméricos com domínios selecionados podem cumprir a mesma função. As chamadas proteínas modulares são uma opção versátil e podem incorporar diferentes funções para aumentar a eficiência de entrega [12].

Uma vez que vetores virais são considerados pouco seguros e vetores não virais pouco eficientes, foi criado o conceito de "vírus artificial", que seria um sistema capaz de aliar a segurança de vetores não virais à eficiência de vetores virais, o que pode ser obtido adicionando-se (i) ligantes de diferentes naturezas que estimulam o direcionamento celular e facilitam a entrada na célula, (ii) peptídeos fusogênicos que evitam a degradação endossomal, (iii) sinais de localização nuclear que tornam a entrada no núcleo mais eficiente, sempre associados em diferentes combinações com o pDNA em busca de mimetizar os vírus verdadeiros [13]. Os vírus artificiais devem cumprir alguns requerimentos funcionais, tal qual transporte estável de DNA, proteção contra nucleases, habilidade de atravessar membranas, expressão elevada e estável do gene terapêutico, baixa toxicidade celular e sistêmica e baixa ativação do sistema complemento [14,15].

Em geral, os efeitos das moléculas e ligantes utilizados para aumentar a eficiência de vetores não-virais são estudados isoladamente, de modo que o desenvolvimento de vírus artificiais bem sucedidos tem sido comprometido pela falta de estudos sistemáticos e comparativos [13,16]. Uma vez que o vírus artificial deve anexar pequenos peptídeos e proteínas além de polímeros ou lipídeos

catiônicos ao plasmídeo, é importante que estas moléculas sejam incorporadas sem inibir ou dificultar a função do vetor em si [16].

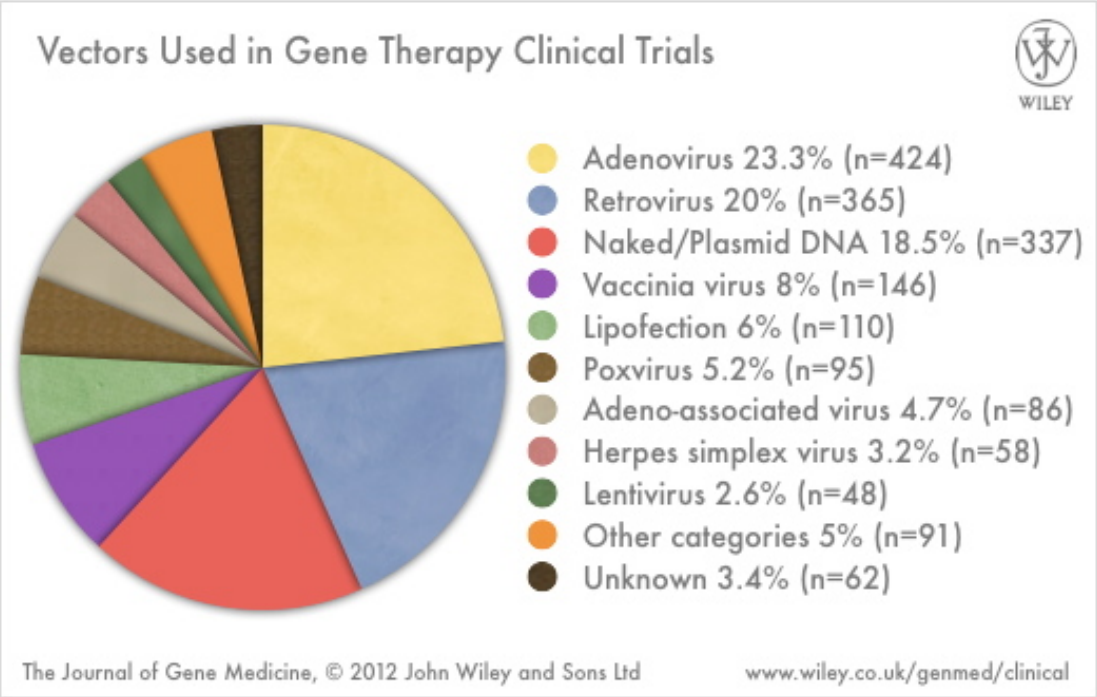

Figura 3: Distribuição dos testes clínicos já realizados de acordo com o tipo de vetor utilizado, o que evidencia como a baixa eficiência dos vetores não virais limita sua aplicação.

2.5. Tráfego intracelular

É sabido que o tráfego intracelular é uma etapa limitante da entrega gênica, e o ataque de nucleases citosólicas é diretamente proporcional ao tempo de residência do pDNA neste ambiente. Na tentativa de mimetizar as funções virais que aumentam sua eficiência, a rede de microtúbulos desponta como uma alternativa promissora. Diversos estudos já indicaram que vetores virais possuem a capacidade natural de explorar a rede de microtúbulos para chegar ao núcleo,

inclusive o herpes vírus, que interage diretamente com as cadeias leve Rp3 e TcTex de Dineína [17]. A interação com a rede de microtúbulos já foi também evidenciada para citomegalovírus humano, HIV, adenovirus, parvovirus, simian vírus 40, influenza vírus, hepatite B vírus [18].

Um exemplo adicional da utilização da rede de microtúbulos é a proteína supressora de tumor p53, que explora as dineínas através da cadeia leve Lc8 para se acumular ao redor do núcleo [19]. Também foi enfatizado recentemente o papel crucial das dineínas como facilitadoras do translado da proteína P do vírus da raiva e da poliproteína Gag do vírus M-PMV, através da associação das proteínas com as cadeias leves de dineína LC8 e TcTex-1, respectivamente [20, 21].

O potencial da interação pDNA-motores moleculares para o aumento da eficiência de vetores não-virais foi sugerido por Mesika e colaboradores [22], que mostrou que complexos formados pelo fator de transcrição p50 com pDNA aumentam significativamente o acúmulo de pDNA junto ao envelope nuclear ou no interior do núcleo. Visto que a inativação química da cadeia de MTs ou uso de anticorpos anti-dineínas reduziu a eficiência do complexo, os autores sugerem a interação da p50 com Dineínas como um dos principais fatores envolvidos. Hasegawa [23] reportou o envolvimento da rede de microtúbulos no transporte de lipossomas, e Suh [24] relatou o envolvimento da rede de microtúbulos no transporte de vetores não virais basedos em pDNA e PEI (polietilenimina), contrariando o conceito corrente de que vetores não virais dependiam apenas de difusão para se deslocar no citoplasma.

2.5.1. A rede de microtúbulos e as dineínas

Os microtúbulos (MTs) são filamentos polarizados do citoesqueleto formados por heterodímeros igualmente orientados de α e ß-tubulina, e também proteínas associadas ao MT [18]. Sua extensa rede é utilizada para o transporte de uma variedade de cargas (organelas membranosas e proteínas) para regiões celulares específicas, a uma velocidade relativamente alta, em torno 1μm/s [25]. Dentre as famílias de proteínas motoras já descritas, são as dineínas citoplasmáticas as responsáveis pelo transporte intracelular de cargas pela rede de MTs no sentido da periferia para o centro de organização de microtúbulos/centrossoma [26], localizado próximo ao núcleo de células não polarizadas. As dineínas são complexos protéicos (20S) constituídos por duas cadeias pesadas (CP, ~530 kDa), duas cadeias intermediárias (CI, ~74 kDa), algumas cadeias leves intermediárias (CLI, 52-61 kDa), e várias cadeias leves (CL) das famílias LC8, TcTex/Rp3 e LC7/roadblock (CL, 10-25 kDa) [18, 27] (Figura 4). As cadeias pesadas são responsáveis pela ligação ao MT, hidrólise de ATP e movimento, enquanto as cadeias CI, CLI e CL são responsáveis pela ligação às cargas e regulação da atividade motora. Variações na composição das subunidades de ligação (CL e CLI) acarretam interações com diferentes tipos de cargas [18].

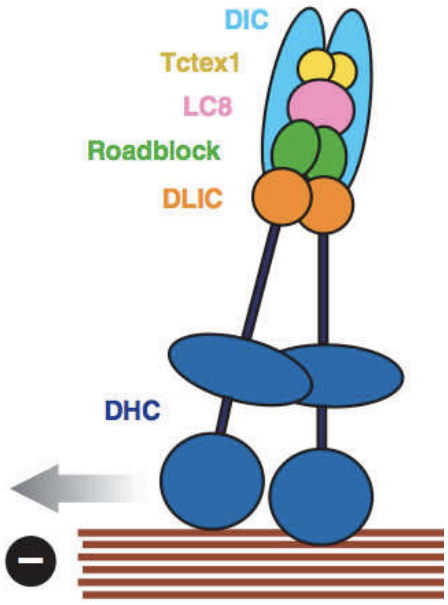

Figura 4: Ilustração representando o complexo da dineína. Observam-se as cadeias leves das famílias TcTex, Lc8 e Roadblock, as cadeias leves intermediárias (DLIC), as cadeias intermediárias (DIC) e as pesadas (DHC) [46].

2.5.2. As cadeias leves Rp3 e TcTex

Uma vez que são as cadeias leves que interagem com a carga a ser transportada pelo complexo dineína, foram selecionadas Rp3 e TcTex-1. Ambas cadeias leves pertencem à mesma família e apresentam elevada identidade (52%) e similaridade (75%). Contudo, ambas apresentam afinidade por diferentes moléculas carreadas pelo complexo dineína, indicando que tais cadeias estão envolvidas em diferentes funções do complexo [28]. É digno de nota que em células epiteliais polarizadas de rim canino Madin-Darby foi demonstrado que as cadeias leves Rp3 e TcTex competem pela mesma cadeia intermediária no complexo dineína, uma vez que há apenas um sitio para a família TcTex (Figura

4), porém a superexpressão de Rp3 foi capaz de deslocar cadeias TcTex do mesmo [29].

Embora TcTex-1 e Rp3 compitam por serem da mesma família, há um sitio de interação com o N-terminal da cadeia intermediária para cada uma das três famílias de cadeia leve (TcTex1, Lc8 e Lc7, todas homodiméricas quando ativas pelo C-terminal), e a presença de uma cadeia leve ligada à cadeia intermediária aumenta a afinidade de ligação da segunda cadeia leve [30].

A observação de que diferentes isoformas das subunidades de dineína têm distintas distribuições celulares indica que talvez existam complexos de Dineína com diferentes composições de subunidades [29].

Entretanto as cadeias leves de dineína não se encontram apenas associadas ao complexo, podendo ser observadas livres no citoplasma, sugerindo assim uma função independente. Rp3, por exemplo, foi reportada no núcleo onde funciona como um modulador transcricional e possivelmente um co-fator de SATB-1 (*nuclear matrix binding transcription factor*), promovendo a supressão de diversos genes [31]. Seu papel no núcleo é regulado de acordo com o ciclo celular e fatores externos. TcTex1 e Lc8 já foram reportadas no núcleo também por imunocitoquímica.

A estrutura da proteína homóloga a TcTex-1 em Drosophila foi elucidada, buscando identificar as regiões responsáveis pela interação com as diferentes moléculas descritas na literatura [32]. Já a Rp3 não tem sua estrutura resolvida ainda.

2.6. Peptídeos membrano-ativos

A observação do fator de trans-ativação TAT de HIV-1 e do fator de transcrição Antennapedia de Drosophila revelou a capacidade de entregar material gênico, atravessando membranas intactas [33]. A partir disso emergiu um novo conceito, o dos peptídeos membrano-ativos (CPP – *cell penetrating peptides*). Trata-se de uma nova classe de compostos, hoje amplamente estudada, visando até mesmo a síntese artificial de peptídeos que mimetizem as características de TAT e Antennapedia (também chamada penetratina), embora estes ainda sejam os mais utilizados [33]. São amplamente usados na entrega de moléculas bio ativas, especialmente na entrega gênica [34], e devido a sua composição rica em argininas e lisinas, possuem um efeito adicional de condensar a partícula formada com o pDNA [14].

O TAT é um peptídeo de onze aminoácidos (YGRKKRRQRRR) [35] capaz de condensar o DNA e carreá-lo tanto através da membrana plasmática quanto nuclear, provavelmente devido ao fato de ser rico em argininas [36]. A entrada no núcleo ocorre independente de ATP e por uma via diferente da usada pelas seqüências de localização nuclear, ainda que não esteja propriamente elucidada a via e os mecanismos utilizados [35]. Já a penetratina (RQIKIWFQNRRMKWK) [37] é um peptídeo anfifílico que possui diversos resíduos de arginina e lisina em sua cadeia, que interagem e condensam DNA, além de atuarem ativamente na transposição de membranas, facilitando assim a internalização na célula e também o escape de vesículas endossomais [16].

Há ainda grande discrepância nos estudos sobre a forma de entrada dos peptídeos membrano-ativos na célula, principalmente se ela ocorre por endocitose ou por uma via independente de endocitose. Isso é provavelmente acentuado pelo fato da entrada de TAT, por exemplo, variar de acordo com a concentração e o tipo celular [16, 38, 39]. Mas acredita-se que a primeira etapa consiste em uma forte interação eletrostática entre o CPP positivamente carregado e a célula negativamente carregada [40].

Entre os dois CPPs, TAT se mostra ainda mais promissor, uma vez que possui a capacidade adicional de penetrar a membrana nuclear, com uma cinética mais rápida que sinais de localização nucleares [35], e ao contrario da penetratina, não desencadeia resposta imune [38].

2.7. Condensação e escape endossomal

Uma vez que a condensação do complexo de entrega é um fator determinante para a entrada e deslocamento dentro da célula [41], em nosso grupo foram desenvolvidas previamente a essa pesquisa seqüências de ligação ao DNA capazes de elevar a capacidade de ligação de proteínas de fusão ao pDNA. A seqüência de ligação ao DNA usada, desenvolvida com base na literatura [14, 42] é uma seqüência pequena e rica em resíduos de aminoácido arginina, conforme segue: WRRGFGRRR. Além dos dois clusters de arginina, ela é ainda intercalada por um aminoácido aromático que favorece a condensação e aumenta a estabilidade do complexo pDNA-proteína. A organização dos resíduos em clusters se baseou em histonas, que na natureza possuem motivos de condensação de DNA, o que de fato se mostrou mais eficiente do que a utilização dos aminoácidos

dispersos [42]. A utilização deste pequeno domínio afeta não só a condensação da partícula, bem como facilita a interação da proteína recombinante com o pDNA.

Adicionalmente, vencer a barreira endossomal é considerado um desafio de suma importância no aumento de eficiência da entrega gênica [33, 43]. Embora sugira-se que o escape endossomal pode ocorrer através de interação direta da membrana negativamente carregada com as cargas positivas do adjuvante catiônico, a hipótese mais aceita é a “esponja de prótons”. Membranas endossomais possuem ATPases que transportam ativamente prótons do citosol para o interior da vesícula, resultando em acidificação do compartimento [44]. Assim sendo, peptídeos ou proteínas contendo muitos resíduos de histidina são capazes de tamponar o pH interno da vesícula através da protonação de grupos imidazol, obrigando assim a ATPase a transportar ainda mais prótons para atingir o pH ácido. O acúmulo de prótons na vesícula resulta no influxo de íons, o que causa um inchaço osmótico, levando assim ao rompimento da membrana endossomal [42, 45]. Lo e Wang [44] reportaram em 2008 que a adição de 10 resíduos de histidina promoveu um aumento de 7000 vezes na eficiência de transfeccção, tornando promissora a utilização de resíduos de histidina, na busca por um maior escape endossomal.

3. Objetivos

3.1. Objetivos gerais

Desenvolver um vetor de entrega não viral, baseado em proteínas recombinantes, aplicável tanto para terapia gênica quanto para vacina de DNA que possa oferecer uma eficiência de transfecção mais elevada em cultura de células.

3.2. Objetivos específicos

Desenvolvimento de um vetor não viral de entrega cujo componente peptídico seja baseado na cadeia leve de Dineína Rp3 ou TcTex1, fusionada a um peptídeo membrano ativo (TAT ou Penetratina) e uma sequência de DNA binding. A proteína recombinante deve possuir as características físico-quImicas adequadas a um vetor de entrega gênica e apresentar eficência elevada de transfecção. A Figura 5 representa a construção da proteína e o desempenho esperado pode ser observado na Figura 6. A partir da Figura 7 compreende-se a estratégia utilizada para cumprir os objetivos.

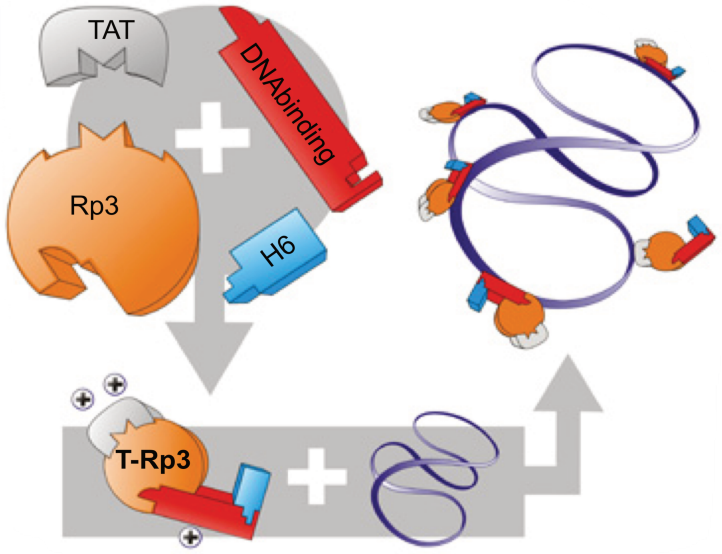

Figura 5: Diferentes sequências são utilizadas no desenvolvimento da proteína recombinante T-Rp3, que deve interagir com o pDNA formando um complexo pequeno e positivo. O mesmo padrão é seguido para P-TcTex.

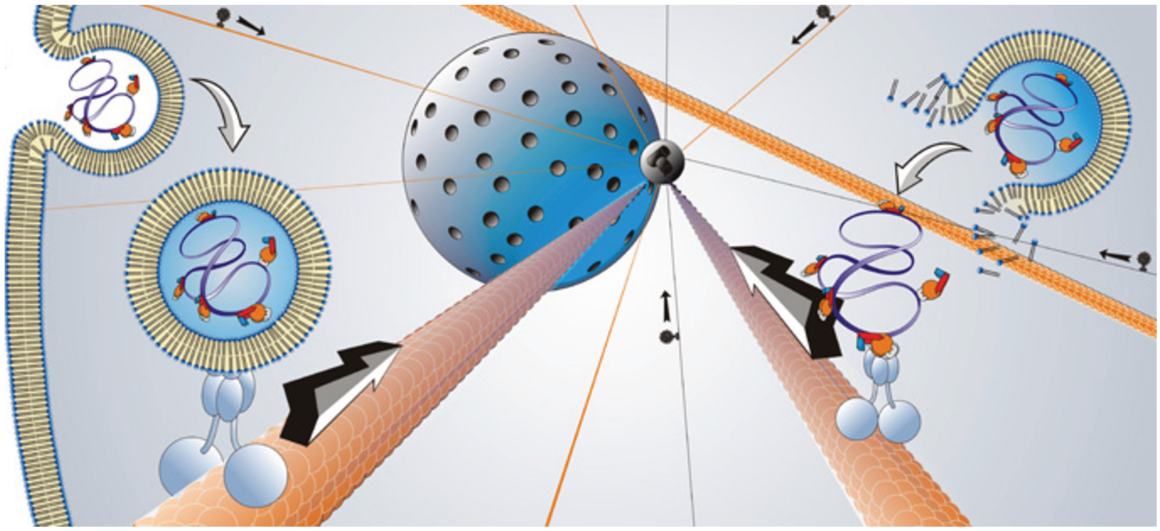

Figura 6: Representação das etapas da entrada na célula de um vetor baseado em cadeias leves de dineína. A partir da entrada do complexo (formado pelo pDNA e pela proteína recombinante) na célula através da membrana, ocorre o aprisionamento em um endossomo, o qual naturalmente explora a rede de microtúbulos. Entretanto, espera-se que após a liberação do complexo no citosol, este possua a capacidade de explorar as dineínas independentemente para viajar em direção ao centrossoma da célula.

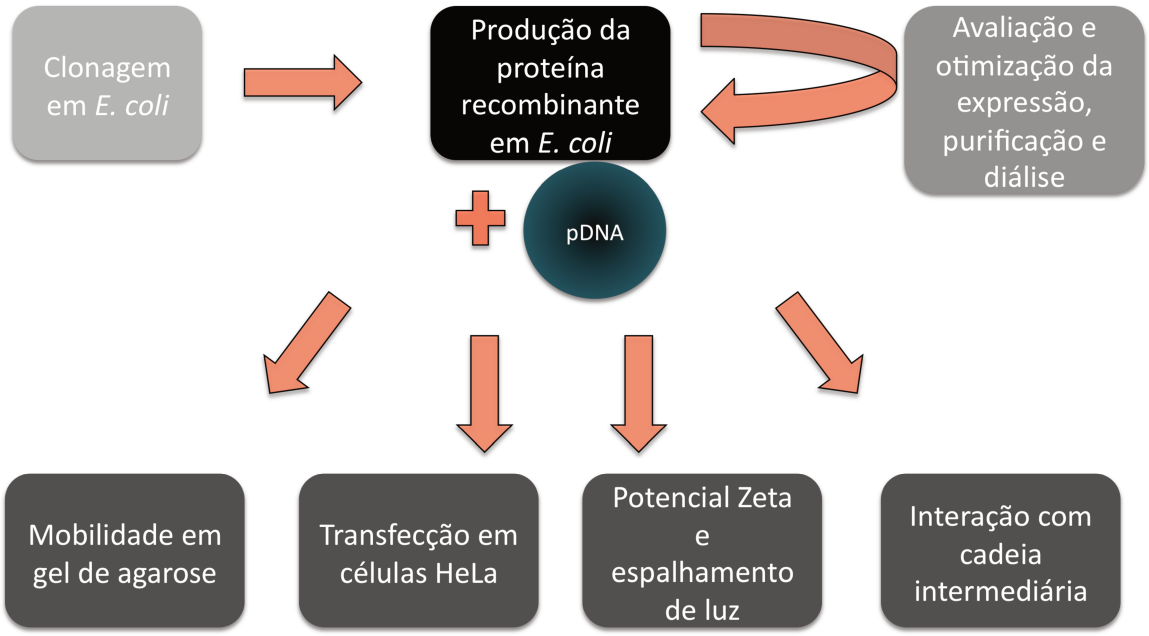

Figura 7: Esquema da estratégia utilizada para analisar o vetor de entrega gênica desenvolvido. Após a clonagem das seqüências em *E. coli*, a produção da proteína recombinante pode ser otimizada. Uma vez obtida a proteína recombinante, esta é combinada com o plasmídeo (pDNA) para avaliar as principais características do complexo proteína:pDNA.

4. Manuscrito

A NON-VIRAL GENE DELIVERY VECTOR BASED ON THE DYNEIN LIGHT CHAIN RP3 AND TAT PEPTIDE

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Keywords: gene delivery; microtubules; shuttle protein; dynein; Rp3; TAT

ABSTRACT

One of the major challenges faced by gene therapy and DNA vaccination studies is the development of a delivery vector that combines efficiency and safety. Although much more effective, there are still several concerns regarding the safety of viral vectors. Therefore, non-viral vectors that mimic the strategies used by viruses to infect mammalian cells are an interesting approach to combat these problems. These vectors are capable of exploring the cell's architecture in an efficient way to overcome a series of physical, enzymatic and diffusional barriers while still preserving the safety feature of non-viral vectors. The main purpose of this work is to exploit the molecular motor dynein to transport plasmid DNA-protein particles from the periphery to the centrosome of mammalian cells, improving gene delivery to the nucleus. The approach is based on the use of the recombinant human dynein light chain Rp3, which has been fused to a N-terminal DNA-binding domain and a C-terminal membrane active peptide, TAT. This recombinant protein, named T-Rp3, is expected to simultaneously interact with plasmid DNA (pDNA) and the dynein molecular motor with an enhanced capacity to penetrate membranes, thus facilitating pDNA intracellular trafficking toward the nucleus. Expression studies indicated that the fusion protein was correctly expressed in *Escherichia coli* BL21(DE3) strain and is able to interact with the dynein intermediate chain *in vitro*. Gel retardation assay, dynamic light scattering and zeta potential indicate the formation of a positive and stable complex between pDNA and the protein. Finally, transfection of cultured HeLa cells indicated that T-Rp3 enhanced the transfection

efficiency when compared to the positive control protamine. The transfection efficiency of T-Rp3 was almost similar to efficiencies seen when utilizing Lipofectamine2000^{TM}, but T-Rp3 yielded lower cytotoxicity.

1. INTRODUCTION

Gene therapy, in a wide definition, is the introduction of nucleic acids into cells with the intention of altering gene expression to prevent, halt or reverse a pathological process [1]. Despite the great potential of these new therapies, major challenges are still faced by gene therapy since DNA requires a vector to protect and transport it to the interior of the cell. An ideal vector for gene therapy should be safe, efficient and reproducible; however, finding such a vector remains a major challenge [2,3]. Viral vectors, that are biological carriers that have naturally evolved to transfer genetic materials into host cells [4], are widely more efficient, although are associated to several adverse effects that raise doubts about its safety, ranging from inflammation to death [5,6]. Non-viral vectors are less efficient but are considered safer, cheaper to produce and do not have limitations on the size of DNA sequence delivered [1].

DNA delivery systems must overcome multiple intracellular barriers before reaching the nucleus; barriers include binding to the cell surface, cell entry/endocytosis, endosomal escape, evasion of cytosolic nucleases and nuclear entry [2,7,8]. To assist in the difficult task of traversing the cell's barriers, multifunctional non-viral vectors have been developed and are composed of

several different molecules, including polymers, peptides, liposomes, nanoparticles and combinations thereof. These additional molecules associate with DNA and function as gene carriers [9,10].

The improvement of non-viral vectors non focuses on the fine-tuning of its functional components and should ultimately mimic viral properties. These are referred to as ‘artificial viruses’ [11,12]. Among the properties of these vectors are the ability to condense and protect DNA from nuclease degradation, low systemic and cellular toxicity, membrane crossing abilities, and steady expression of the therapeutic gene [13]. The ligands or molecules usually added to these particles aim to facilitate endocytosis, improve DNA condensation, promote endosomal escape and enhance trafficking to the nucleus [14].

However, one important obstacle that is often neglected in strategies to mimic viral trafficking is transport throughout the cytoplasm. Plasmid DNA cannot depend solely on diffusion during trafficking to the nucleus, as indicated by microinjection studies [15,16]. Additionally, free pDNA in the cytosol is also rapidly degraded by endonucleases [11]. Therefore, researchers have suggested the use of dyneins to improve the transport of pDNA toward the minus-end of microtubules (MT), which are usually located close to the cell´s nucleus [17-20]. Dynein is a multisubunit protein complex formed by two dynein heavy chains (~530 kDa, responsible for ATP hydrolysis and binding to microtubules), two dynein intermediate chains (~74 kDa), several dynein light intermediate chains (~52-61 kDa) and a number of dynein light chains (~10-25kDa). The dynein light chains are from the LC8,

TcTex/Rp3 and LC7/roadblock families and are responsible for cargo binding and the regulation of motor activity [20,21].

Numerous viruses explore microtubules via dynein motors to translocate to the nuclear periphery, including the herpes simplex virus type 1, that binds to dynein light chains Rp3 and TcTex1 to reach the nucleus [7,22,23]. The relevance of the microtubule network to the transport of pDNA complexes has been demonstrated [16] and the protein p53 also exploits this machinery to accumulate in the nucleus [24]. Recently, Moseley and collaborators [19] demonstrated that dynein light chain association sequences enhance nuclear accumulation of exogenous proteins by exploring the MT network.

Regarding gene delivery, our group recently demonstrated that the dynein light chain LC8 can be modified by insertion of a synthetic DNA binding motif while still maintaining the ability to interact *in vitro* with the dynein intermediate chain [25]. This protein (LD4) was able to interact with and condense pDNA and form positive particles that efficiently transfected HeLa cells *in vitro.* This process was dependent on MT transport and was minimally cytotoxic. The studies also demonstrated that the pDNA-LD4 particle could be further optimized, as there was a tendency toward particle aggregation due to low positive zeta potential values. Additionally, transfection studies using the drug chloroquine indicated an extensive entrapment of the pDNA-LD4 particles inside late endosomes and lysosomes, contributing to the reduction in trafficking efficiency.

In the present work, we used a similar approach to modify the dynein light chain Rp3 to function as a gene delivery carrier. The Rp3 has approximately

13kDa and is a member of the Tctex dynein light chain family. It can be found in homodimeric and also in heterodimeric forms and is associated with Tctex1, although heterodimers are unable to bind to the dynein intermediate chain [26]. Rp3 and Tctex1 share 74% sequence similarity and 55% sequence identity [27]. However, the proteins bind different cargo and are differentially expressed [28,29]. In our work, the Rp3 was fused to a N-terminal DNA binding domain and to a C-terminal TAT peptide, a cell penetrating peptide [9]. Cell penetrating peptides (CPPs) are also known as protein transduction domains (PTDs) and are short peptides usually rich in basic amino acids [30]. These peptides originated from proteins that are naturally capable of crossing membranes. CPPs have been widely used in the delivery of bioactive molecules, especially for gene delivery [31]. The better-known CPP is TAT, an arginine-rich peptide derived from HIV-1 trans-activator protein [9]. The mechanism of TAT entry into the cell is still the subject of some debate, but entry has been shown to be concentration and cell type-dependent [13,32,33]. TAT is also promising for gene delivery because it has the additional capacity of entering the cell nucleus with much faster kinetics than nuclear import mediated by the nuclear localization signal (NLS) [34], and unlike Penetratin (another known CPP), TAT does not elicit any immune response [32].

2. MATERIAL AND METHODS

2.1. Plasmid DNA vector

The plasmid DNA used is this study was described by Toledo and collaborators [25]. Named pVAX1-Luc, the plasmid was constructed by replacing

the GFP-encoding sequence of the pVAX1-GFP plasmid [8], with the Luciferase gene obtained from the pGL3-Luc control vector (Promega). Purification of the pVAX1-Luc plasmid used in all transfection studies was performed as described by Freitas and colleagues [35].

2.2. Recombinant proteins expression and purification

TAT DNA sequence was first cloned into the pET28a expression vector (Novagen). The TAT sequence was synthesized as two complementary single strands, containing the amino acid sequence YGRKKRRQRRR [34] optimized for expression in *E. coli*. The complementary sequences were annealed and phosphorylated prior to cloning in a pET28a, which was previously digested with *EcoR*I and *Xho*I. The vector was then transformed into *E. coli* strains.

The fusion protein DNA binding domain WRRRGFGRRR, named DNAb4, was designed by Toledo and collaborators [25] for peptides and protein domains with high DNA binding and condensing capacity. The DNAb4 domain was fused to the Rp3 sequence amplified from HeLa cell cDNA as described previously [25].

The Rp3 protein sequence containing the DNA binding domain sequence was amplified and inserted in the pET28a plasmid encoding the TAT sequence described above with specific primers (forward: 5´-GATAATGCTAGCTGGCGTCGCCGTGGTTTTG-3' and reverse: 5´-GCAAGCCTTAAGAAGAACAATAGCAATGGCAA-3´). The fragment was cloned into the pET28a vector using the *Nhe*I and *EcoR*I restriction sites. The fusion protein (DNA4-Rp3-TAT) produced here was named T-Rp3.

Recombinant T-Rp3 was expressed in *E. coli* BL21(DE3). Briefly, cells were grown in 1 L of LB media at 37ºC, 300 rpm and up to an optical density of 0.8 AU (600 nm). Protein expression was induced with 5.6 mM lactose for an additional 20 h at 28ºC and 200 rpm. After centrifugation, the cell pellet was resuspended in 50 mM Tris (pH 7.0), 1 M NaCl, 0.1 mM EDTA, 15 mM β-mercaptoethanol and 1 mM PMSF (phenylmethylsulfonyl fluoride). Cell lysis was performed by sonication, T-Rp3 was successfully purified by a single Ni-NTA affinity chromatography step and eluted with an imidazole gradient in suspension buffer. The protein was then dialyzed in 40 mM HEPES buffer (pH 7.3), a condition that favored protein stability and was still suitable for further assays.

2.3. *In vitro* interaction of recombinant dynein light chain T-Rp3 and Lc8 with dynein intermediate chain 2, isoform C.

To assess the interaction of the recombinant T-Rp3 with the dynein complex, we used an *E. coli* strain kindly supplied by Toledo and collaborators [25], which contains the N-terminal (first 300 amino acids) of the human dynein intermediate chain DYNIC2, isoform C, that was expressed and purified as described. The protein was then immobilized in a CNBr-activated Sepharose resin (GE Healthcare, Sweden) as described by the manufacturer for affinity chromatography. The extract was composed of an equimolar mix of T-Rp3 and Lc8 (produced as previously described [25]), and is based on the premise that the presence of one light chain enhances the affinity of another light chain to a dynein

intermediate chain. The elution was performed with an increasing amount of salt and adsorption buffer at pH 3.0 in the final step.

2.4. Evaluation of DNA-protein interaction by gel retardation assay

To evaluate the ability of T-Rp3 and protamine to interact and condense pDNA, we performed a gel retardation assay. Proteins were dialyzed in 40 mM HEPES (pH 7.3) and incubated with 1 µg of pVAX-Luc vector (previously in PBS) at various pDNA:protein molar ratios (1:100, 1:200, 1:500, 1:1000, 1:4000 and 1:8000) in a final volume of 50 µL. Protamine sulfate powder was resuspended in PBS. The samples were incubated at room temperature for 1 hour, which was followed by the addition of 50 µL of non-supplemented F-12 media and an additional incubation for 20 min. Samples were run on a 0.8% agarose gel and visualized by ethidium bromide staining.

2.5. Zeta potential and dynamic light scattering assays

Zeta potential measurements were performed to comparatively evaluate the surface charge of complexes formed by [T-Rp3]-pDNA at different molar ratios. Complexes were formed as previously described for the gel retardation assay but without addition of F-12 media. Each sample was measured six times using the Zetasizer 3000 (Malvern, England). Particle size measurements were performed by dynamic light scattering to evaluate the behavior of the pDNA:delivery vector size over time. pDNA:[T-Rp3] was measured at a molar ratio of 1:8000. Complexes were formed with 1 µg of pDNA and the corresponding amount of protein in a final

volume of 800 µL. Each sample was subjected to multiple readings during a 60 min period. Particle diameter was plotted against time for each sample.

(a) 2.6. Culture and transfection of HeLa cells

HeLa cells were grown in a F-12 (Ham) nutrient mixture (Gibco, UK) containing 10% (v/v) fetal bovine serum (growth medium, Gibco, UK). All cell cultures were performed in 75 cm^2 culture flasks and incubated in a 5% CO_2 humidified environment at 37ºC. Following growth to confluence, cells were trypsinized and seeded in 24-well culture plates (5 x 10^4 cells per well). The cells were incubated for 48 hours (to 70% confluence) and then transfected with pDNA:protein complexes formed as described previously for gel retardation assays, using the same molar ratios. When indicated, transfection was carried out using the Lipofectamine 2000^{TM} reagent (Invitrogen, USA) according to the manufacturer's instructions (1 µg pDNA plus 1.5 µL reagent in 100 µL of medium per well) or protamine sulfate (Sigma Aldrich, Germany). The medium containing the transfection solution remained on the transfected cells for 6 hours and was then replaced with fresh growth medium. Cells were collected 24 hours post-transfection, and luciferase activity was determined by utilizing the Luciferase Assay System (Promega, USA) according to the manufacturer's instructions. Luminescence intensity was normalized against the protein concentration in each transfection sample, determined by the Micro BCA Protein Assay Kit (Thermo Scientific, USA).

To evaluate the contribution of the microtubule network in the intracellular trafficking of the complexes, cells were pre-incubated for 2 hours with nocodazole (25 μM) to disrupt the microtubules. The drug was dissolved in DMSO, and an equal volume of drug-free DMSO was used as a control (0.4% for nocodazole). Transfections in the presence of chloroquine were performed to evaluate the contribution of lysosomal degradation of protein-pDNA complexes. Cells were pre-incubated for 4 hours with chloroquine (100 μM). For all assays with the mentioned drugs, pre-treated cells were incubated in the presence of the different complexes for 4 hours, after which the medium was replaced with fresh growth medium. After 24 hours, cells were collected, and the luciferase activity was measured as described above.

2.7. Cytotoxicity assay on HeLa cells

Cytotoxicity assays of delivery vectors were performed using the Cell Proliferation Reagent WST-1 (Roche Applied Science, USA) according to the manufacturer's instructions. Briefly, HeLa cells were grown in 96-well plates to a confluence of 70% and then transfected as described above with pDNA:protamine (pDNA:protein molar ratio of 1:8000) or pDNA:T-Rp3 (pDNA:protein molar ratio of 1:8000) complexes with and without Lipofectamine2000^{TM}; pDNA only served as a control. Finally, 10 μL of WST-1 reagent was added to each well, and cells were incubated for an additional 2 hours. Absorbance was recorded at 440 nm in an ELISA reader.

3. Results and Discussion

3.1. T-Rp3 fusion protein was successfully produced in *E. coli*

T-Rp3 cloning into the pET28a vector was performed so that the DNA binding domain was fused to the N-terminus of Rp3 to prevent the blockage of domains involved in the interaction with the Dynein motor complex. This approach is based on the available structural data for TcTex (no structural data are available for Rp3) and Dynein complex formation data [36]. The TAT peptide was fused to the C-terminus of Rp3 in order to increase the probability that this membrane active domain would be exposed following complex formation with pDNA. With the described methodology, T-Rp3 was successfully cloned and expressed in *E. coli* BL21 (DE3) strain. The protein was obtained in the soluble fraction and purified in a single step using Ni-NTA affinity chromatography. Unstable in the elution buffer, T-Rp3 requires specific conditions to prevent aggregation and precipitation. Its stability was improved after dialysis in 40 mM HEPES buffer at pH 7.3 (Figure 1).

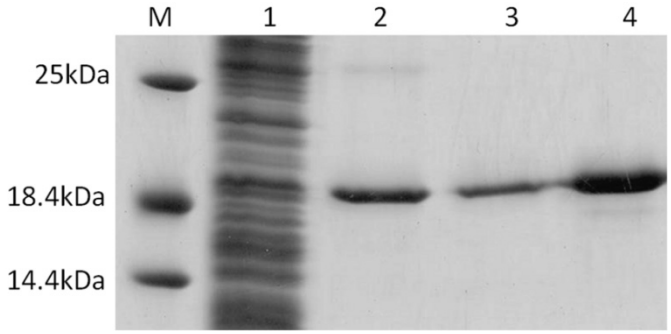

Figure 1 – SDS-PAGE showing the T-Rp3 expression in *E. coli* BL21 (DE3) and purification by Ni-NTA affinity chromatography. The protein can be visualized in the soluble fraction (Lane 1) and was eluted in 200 and 500mM imidazole (Lanes 2 and 3), as well as dialyzed, and concentrated in 40 mM HEPES buffer at pH 7.3 (Lane 4).

3.2. T-Rp3 retains the ability to interact *in vitro* with dynein intermediate chain

After the successful purification of T-Rp3, we evaluated whether the recombinant T-Rp3 retained the ability to interact with dynein intermediate chain *in vitro*. As discussed above, T-Rp3 contains a TAT peptide sequence in its C-terminal region, the same region that is involved in light chain dimerization and the interaction with the dynein complex [37]. Therefore, we analyzed the interaction of recombinant T-Rp3 with human dynein intermediate chain DYNIC2 (isoform C) *in vitro*. In this approach, we also used Lc8, another dynein light chain. It is known that the presence of one type of light chain bound to the intermediate chain enhances the binding of a second type of light chain and stabilizes the complex [38]. The results of the affinity chromatography assay indicated a strong interaction between T-Rp3 and the immobilized intermediate chain (Figure 2). T-Rp3 and Lc8 were only eluted under strong acidic conditions (pH 3.0). Additionally, this interaction could not be disrupted by a salt gradient, which also pointed to a specific interaction between recombinant light chains and the immobilized intermediate chain. As a control, we used activated CNBr-Sepharose blocked with Tris and observed only nonspecific interaction; the majority of the light chains were eluted during the salt gradient step (Figure 2B).

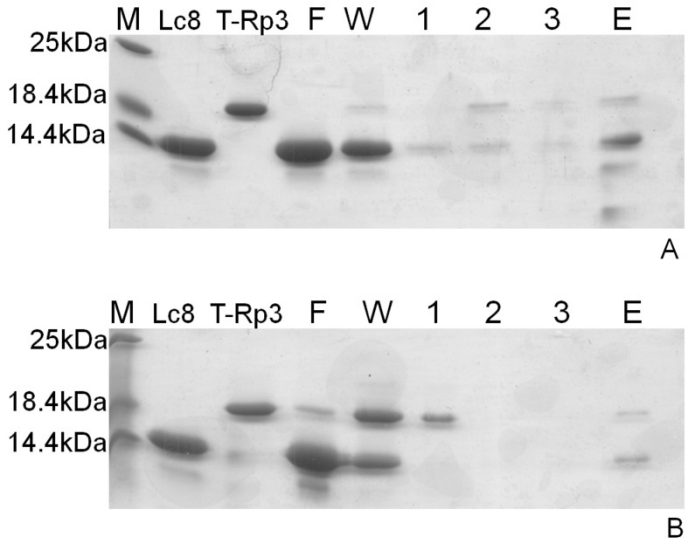

Figure 2 – The T-Rp3 interaction with the intermediate chain was evaluated in the presence of Lc8 by affinity chromatography in CNBr-sepharose. We observed a much stronger interaction in the resin with immobilized intermediate chain (top) than in the control (below). After flow-through (lane F) and washing (lane W), three further washing steps were performed with an increasing salt gradient (lanes 1-3), and a final step in pH3,0 (lane E)

3.3. T-Rp3 is able to interact and condense pDNA

We evaluated the ability of the T-Rp3 protein to interact and condense pDNA by gel retardation assay, where the migration of the complex depends on its size and net charge. An increasing gradient of the pDNA:protein molar ratio was used for T-Rp3 as well as for the protamine control. Protamine is an arginine-rich protein important in spermatogenesis and is responsible for inducing torus formation and DNA packaging [39]. In Figure 3, results indicate that the interaction of T-Rp3 with pDNA is molar ratio dependent and yielded similar results to protamine, a well-established pDNA condenser. This effect can be attributed to the addition of DNA-binding and TAT domains, as both are positively charged. In

concordance with this observation, wild-type Rp3 exhibits no significant capacity to interact with pDNA, even at higher molar ratios (data not shown).

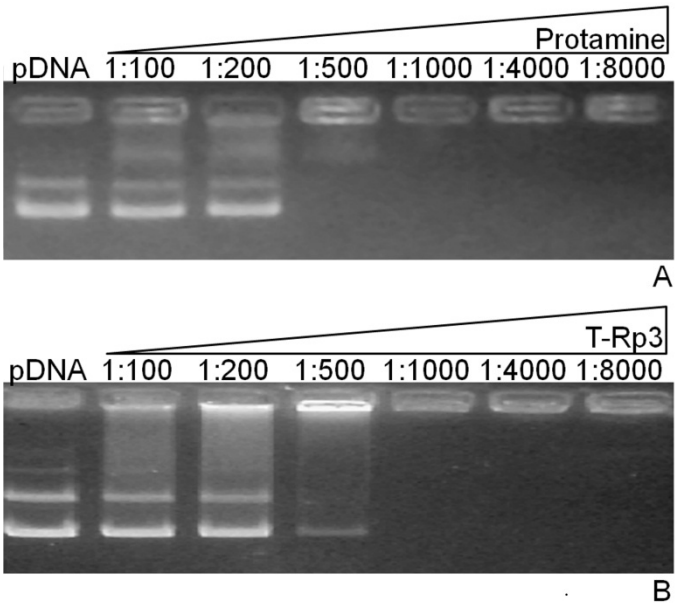

Figure 3 – Protein–pDNA interaction analysis by gel retardation assay. The ability of protamine (A) and T-Rp3 (B) to interact and condense plasmid DNA was analyzed by gel retardation assay. Six pDNA:protein molar ratios were studied, as shown in the gel legends.

3.4. T-Rp3 is capable of condensing pDNA to produce a small and positively charged particle

Considering the fact that both pDNA and cell surface are negatively charged, a required feature of a non-viral adjuvant is the necessary charge switch of the pDNA particle, which is accomplished by T-Rp3 (Figure 4). Zeta potential measurements taken in six replicates indicate that in higher pDNA:protein molar ratios, the negative charge is switched to a positive charge and is dependent upon the amount of protein added. Using T-Rp3, the zeta potential changed from -25.98 mV (at 1:500 pDNA:protein molar ratio) to +28.55 mV (at 1:8000 pDNA:protein

molar ratio). Utilizing the same molar ratio, protamine's net charge was +14.34 mV. Furthermore, dynamic light scattering assays indicate that at a molar ratio of 1:8000, pDNA:T-Rp3 particles were smaller and more stable when compared to protamine under the same conditions (Figure 5). While pDNA:protamine particle size increased from 213 nm to 575 nm in one hour, T-Rp3 formed a smaller particle (77 nm) that did not increase much in size after one hour (95 nm).

As previously studied by Toledo and collaborators [25], the formation of ternary particles formed by pDNA, T-Rp3 and the cationic lipid Lipofectamine 2000^{TM} was studied. The particle formed by the addition of Lipofectamine 2000^{TM} to a 1:100 pDNA:T-Rp3 complex, lead to the formation of a particle with size ranging from 102 to 179 nm, which is considerably smaller than pDNA:Lipofectamine 2000^{TM} (150 to 370 nm) particles reported by Toledo and collaborators [25]. Cumulatively, these results indicate that T-Rp3 surpasses protamine in two specific features: net charge and size. T-Rp3 forms a smaller, positively charged and stable particle, even when compared to the previously described LD4 protein based on the LC8 dynein light chain [25]. The presence of the TAT sequence in the T-Rp3 protein probably leads to the formation of particles with higher net charges, reducing the tendency of particles to aggregate. Regarding TAT, it has been described that this peptide can assist in nuclear delivery of cargo of up to 90 nm [34], which is within the size range of the pDNA:T-Rp3 particles obtained in this work.

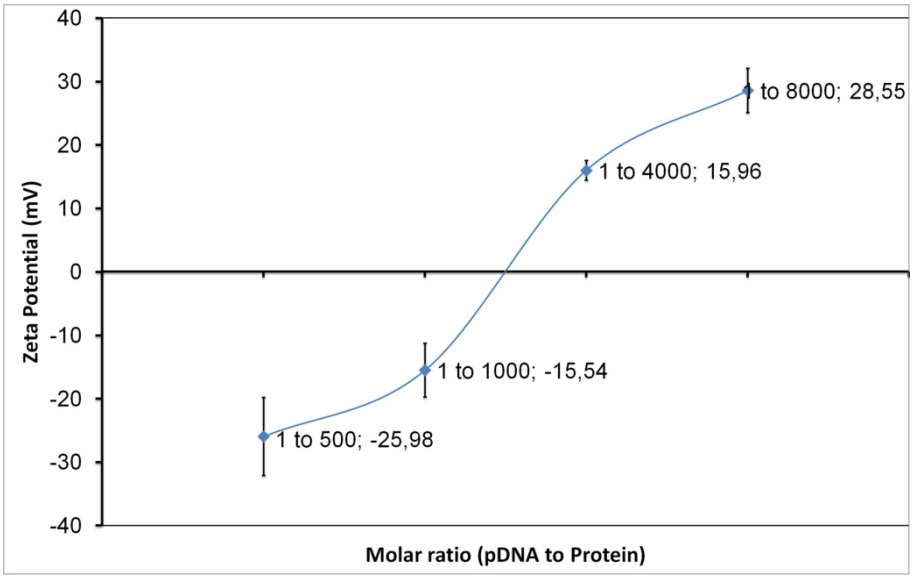

Figure 4 – Net surface charge of pDNA:T-Rp3 complexes for different molar ratios.

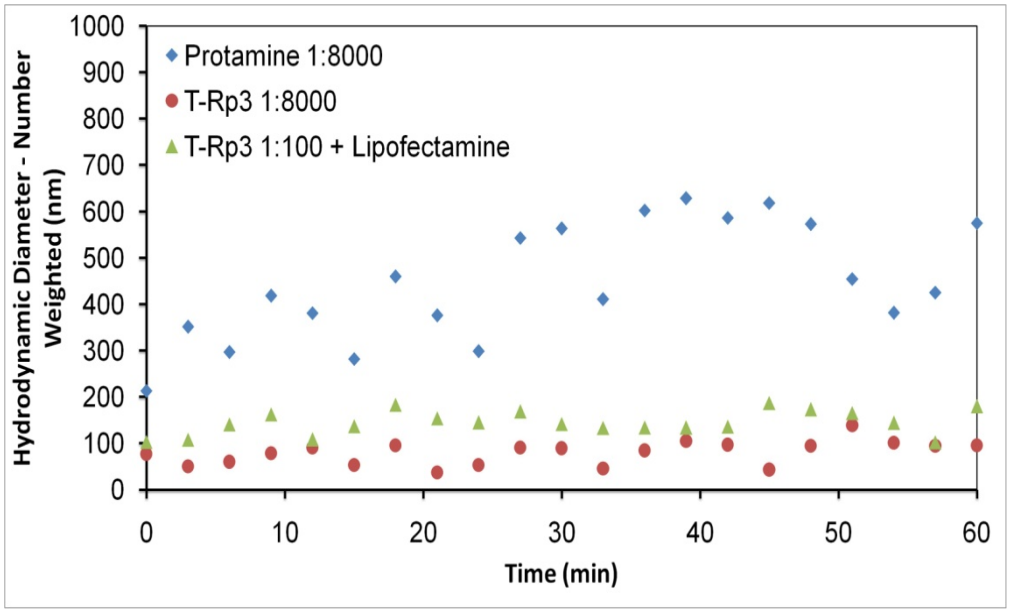

Figure 5 – Time course of hydrodynamic number-weighted diameter for various pDNA:delivery vector complexes studied.

3.5. T-Rp3 efficiently delivers pDNA to cultured HeLa cells

The efficiency of pDNA delivery mediated by T-Rp3 in cultured HeLa cells was evaluated and compared to the efficiency of protamine, a well characterized nuclear protein with a natural ability to condense DNA molecules, facilitate DNA uptake by cells, and traverse the nuclear barrier due to the presence of a nuclear localization signal. We were also interested in comparing T-Rp3 to wild-type Rp3 (wtRp3) with respect to the efficiency of delivering naked pDNA. As seen in Figure 6, wtRp3 had a very limited efficiency, similar to the one observed for naked pDNA. This can also be explained by the low theoretical isoelectric point of wtRp3 (pI of 6.0), which results in no electrostatic interaction with the pDNA as verified by the gel retardation assay (data not shown). These results indicate that the addition of the TAT and DNA-binding sequences to Rp3 promotes nearly 2000-fold increase in transfection efficiency.

As previously reported by Toledo and collaborators [25] we also studied the transfection efficiency of multicomponent particles formed by pDNA, protein and Lipofectamine 2000^{TM}, a well-known pDNA delivery cationic lipid-based vector that is highly efficient *in vitro*. The results indicate that T-Rp3 has an efficiency 13 times lower than that obtained by using pDNA:Lipofectamine 2000^{TM} (Figure 6).

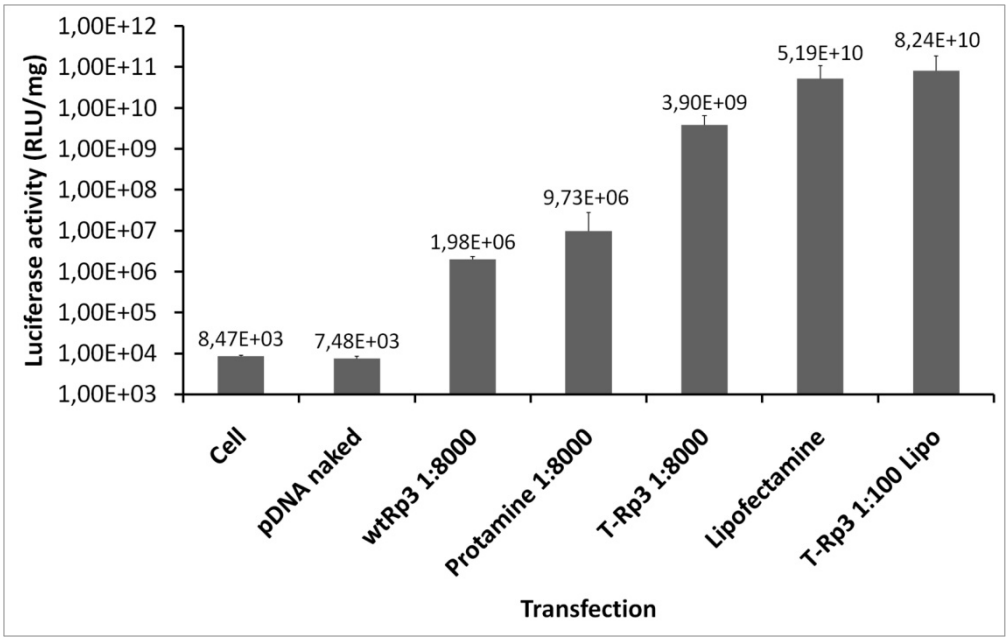

Figure 6 – Transfection efficiency of HeLa cells comparing naked pDNA, pDNA:wtRp3 (wild type Rp3) 1:8000, pDNA:T-Rp3 1:8000, and the controls pDNA:protamine 1:8000 and Lipofectamine 2000™. Transfection efficiency was assessed by measuring the activity of the luciferase reporter gene. Error bars indicate the standard deviation of triplicate measurements. In the case of Protamine, T-Rp3 and Lipofectamine 2000™ values, error bars indicate standard deviation between six replicates. The y-axis is presented using a Log_{10} scale.

The addition of Lipofectamine 2000™ to create a multi-component complex (pDNA:T-Rp3:Lipofectamine 2000™) produced an efficiency slightly higher than Lipofectamine 2000™ alone, particularly for the pDNA:protein molar ratio of 1:100. This could be an outcome of the different roles played by the protein and lipid parts of this complex. Lipofectamine 2000™ is known [40] to improve endossomal escape and delivery to the cytosol, while T-Rp3 could assist in the intracellular traffic of the pDNA. The increase of T-Rp3 content in the ternary complex (1:8000) resulted in a decrease of transfection efficiency, as previously reported [25], probably due to charge repulsion of the cationic lipid by the already positively charged pDNA:T-Rp3 binary particle. However, we should note that T-Rp3

presented a 400-fold increase in transfection efficiency compared to protamine, as well as a 13-fold increase in transfection efficiency compared to the previously reported LD4, the LC8 based shuttle protein [25]. Finally, we compared the transfection efficiency of protamine and T-Rp3 at different molar ratios (Figure 7).The experiment was conducted in triplicate and as expected, the results indicate that the efficiency of pDNA delivery increases as the protein molar ratio increases. Best results were observed at a molar ration of 1:8000, where T-Rp3 presented a 900-fold increase in Luciferase expression when compared to protamine, for this specific set of experiments. This result is consistent with the fact that the particle formed by protamine in not only less positive but also larger than T-Rp3 ones, which may contribute to the observed difference in transfection efficiencies.

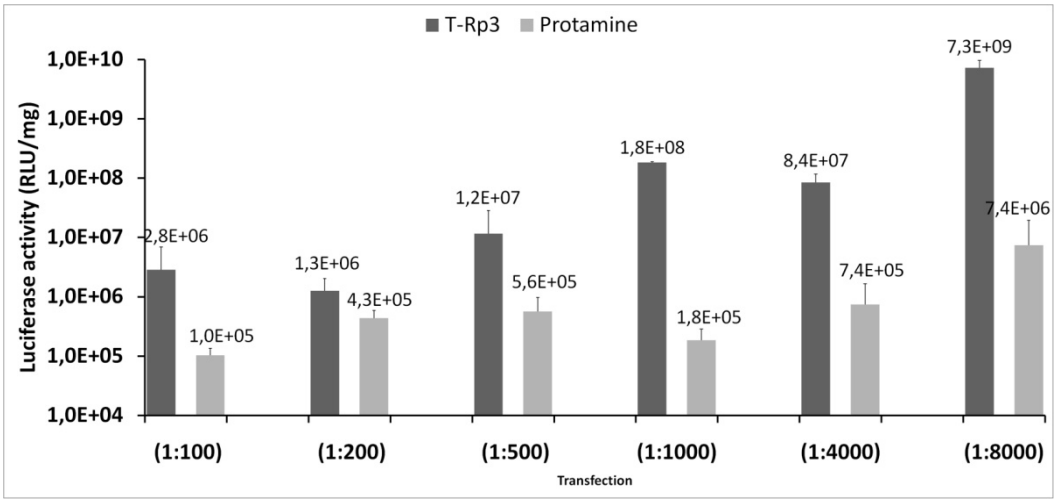

Figure 7 – Transfection efficiency of HeLa cells assessing different molar ratios of protamine and T-Rp3 complexes. Transfection efficiency was evaluated by measuring the activity of the luciferase reporter gene. Error bars indicate the standard deviation of triplicate measurements. The y-axis is presented using Log_{10} scale.

3.6. T-Rp3 relies on the participation of microtubules for intracellular trafficking

The main expected feature of pDNA:T-Rp3 particles is the interaction with the microtubule network and an improved endosomal escape. To evaluate the dependence of these particles on the microtubules, we performed transfections in the presence of nocodazole, a microtubule-depolymerizing agent that is expected to decrease the delivery efficiency of T-Rp3. In fact, in the presence of this drug, Luciferase expression was reduced by 92%, suggesting that T-Rp3 is highly dependent on microtubules for pDNA delivery (Figure 8). This is a promising result given that poor intracellular trafficking is part of the reason why non-viral vectors are less efficient [2], and it is a strongly indicates that T-Rp3 accomplishes its expected function: interaction with dyneins. For comparison, transfections conducted with protamine and Lipofectamine 2000^{TM} exhibited much smaller decreases in Luciferase expression in the presence of nocodazole (56% and 41%, respectively). However, these last results were not statistically significant at a significance level of 0.05 (data not shown).

Additionally, we assessed the level of entrapment of pDNA:T-Rp3 particles inside endosomes and lysosomes, which is also considered to be a major limiting step in efficient gene delivery. We performed transfections in the presence of chloroquine, a weak base that is often used to investigate endosomal entrapment. Chloroquine accumulates in acidic organelles such as late endosomes and lysosomes, raising the luminal pH of the organelles and preventing enzymatic degradation of non-viral vectors. Under the conditions tested, a 366% increase in

T-Rp3 transfection efficiency was observed (Figure 8). This result indicates that endosomal entrapment remains as an important limiting factor despite the presence of a N-terminal His-Tag in the recombinant protein, which was expected to increase endosomal escape based on previous reports [7]. The N-terminal location of the His-Tag may have implications that limit its effect. Because the DNA-binding sequence is also located in the N-terminal region, the His-Tag may be inaccessible and not exposed once the complex is formed with pDNA, which may result in reduced endosomal/lysosomal escape.

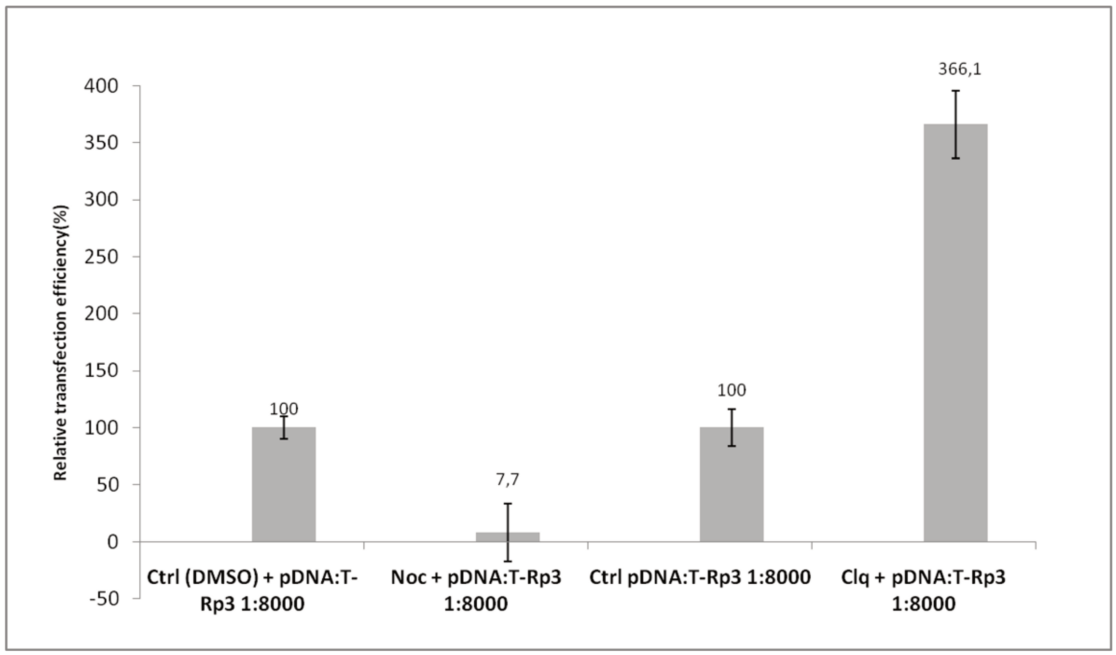

Figure 8 – Involvement of microtubules and endosomes in transfection efficiency of HeLa cells using T-Rp3:pDNA complexes. The involvement of microtubules was studied using the drug nocodazole, and chloroquine was used to investigate the effect of the endosomal/lysosomal entrapment as a barrier to gene delivery. Experiments were performed in triplicate as described. Error bars indicate the standard deviation of triplicate measurements.

3.7. Cytotoxicity assay

T-Rp3 showed increased transfection efficiency when compared to protamine and had similar efficiency to that of Lipofectamine 2000™, which has limited use *in vivo* due to high toxicity [41]. Proteins are naturally expected to be less toxic than cationic lipids [42]. This was confirmed for T-Rp3, and the protein was shown to have a significantly lower level of cytotoxicity than Lipofectamine 2000™ (Figure 9). Moreover, multicomponent complexes, formed by pDNA:protein:Lipofectamine 2000™ exhibit a higher cytotoxicity when compared to pDNA:protein particles. These results point to the potential use of these binary complexes, particularly pDNA:T-Rp3 complexes, for *in vivo* protocols.

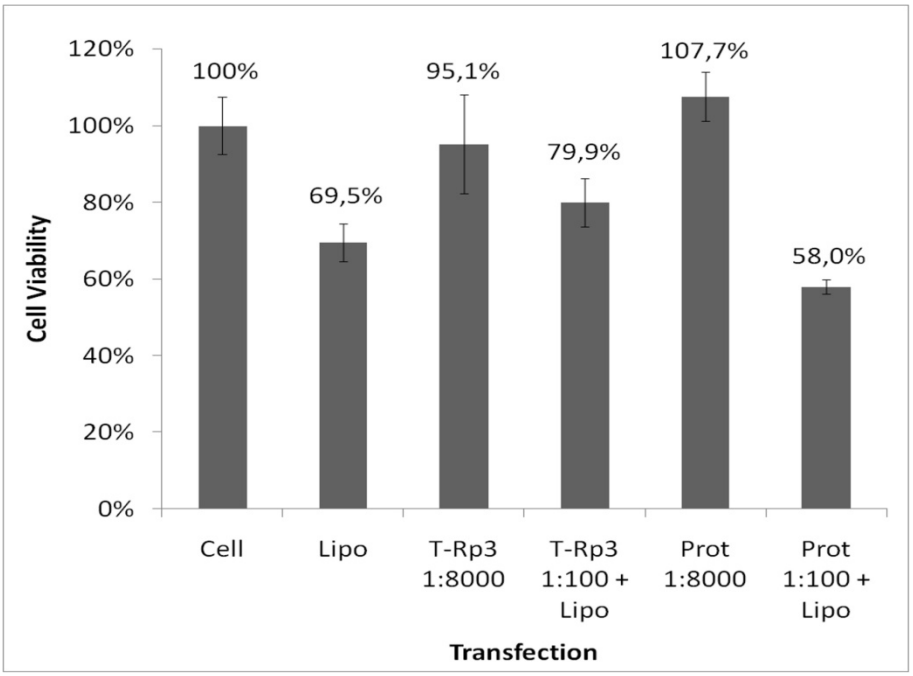

Figure 9 - Evaluation of the cytotoxicity for different delivery vectors in HeLa cells, which was performed using the WST-1 reagent (Roche Applied Science - Germany). We assayed cell viability following transfection with complexes formed by pDNA and Lipofectamine 2000™, protamine and T-Rp3 as already described. Error bars indicate standard deviation of six replicate measurements.

4. CONCLUSIONS

The recombinant protein T-Rp3, designed as a modular shuttle protein, was successfully produced in *E. coli* and retained its ability to interact with the dynein intermediate chain *in vitro*. The protein also was shown to efficiently interact with and condense pDNA, forming a small and positively charged particle. These are regarded as crucial features for effective non-viral gene delivery vectors and may contribute to the T-Rp3 performance during transfection. The fact that the pDNA:T-Rp3 complex is smaller than pDNA:protamine complexes indicates that the positively charged TAT and DNAbinding domains highly influence the complex architecture; this not only determines the formation of the complexes but also increases complex stability over time. Transfection assays using HeLa cells showed that the T-Rp3 delivery efficiency is 400 times higher than that of protamine and is only 13 times lower than that of Lipofectamine 2000^{TM}, presenting a reduced cytotoxicity as a further advantage. Finally, the results strongly suggest that the cell microtubule network plays a crucial role in the trafficking of pDNA:T-Rp3 particles, probably due to the recruitment of dynein motors, as T-Rp3 was able to interact with the dynein intermediate chain *in vitro* and transfection efficiency was severely affected by the use of nocodazole. The results obtained here indicate that the strategy of exploiting molecular motors through modular recombinant proteins is promising and may significantly contribute to the development of more efficient non-viral vectors.

5. ACKNOWLEDGEMENTS

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5. Resultados complementares

5.1. Clonagem de TAT e Penetratina em pET28a

A primeira etapa realizada neste trabalho foi a clonagem das sequências de TAT e Penetratina em um vetor pET28a (Novagen), para que as etapas seguintes de clonagem pudessem ser realizadas. Os fragmentos de TAT e penetratina foram sintetizados, na forma de duas fitas complementares de DNA, com as seguintes seqüências:

Tabela 1: Seqüências complementares sintetizadas de TAT e penetratina. Em azul observa-se as extremidades coesivas que permitiram clonagem usando-se as enzimas EcoRI e XhoI.

TAT forward	5´ AA TTCTAT GGC CGC AAA AAA CGC CGC CAG CGC CGC CGC TAA C 3´
TAT reverse	5´ TC GAG TTA GCG GCG GCG CTG GCG GCG TTT TTT GCG GCC ATA G 3´
Penetratina forward	5´ AA TTC CGC CAG ATT AAA ATT TGG TTT CAG AAC CGC CGC ATG AAA TGG AAA AAA TAA C 3´
Penetratina reverse	5´ TC GAG TTA TTT TTT CCA TTT CAT GCG GCG GTT CTG AAA CCA AAT TTT AAT CTG GCG G 3´

As duas fitas foram aneladas, fosforiladas e então ligadas ao plasmídeo pET28a previamente digerido com EcoRI e XhoI. Os plasmídeos contendo TAT ou Penetratina foram usados para transformar *E. coli* DH5α. As colônias selecionadas foram testadas através da digestão dos plasmídeos purificados com HindIII (Figura 8), uma vez que o sitio de restrição desta enzima encontra-se entre os sítios de EcoRI e XhoI, e o padrão de digestão é capaz de revelar o sucesso na inserção do fragmento.

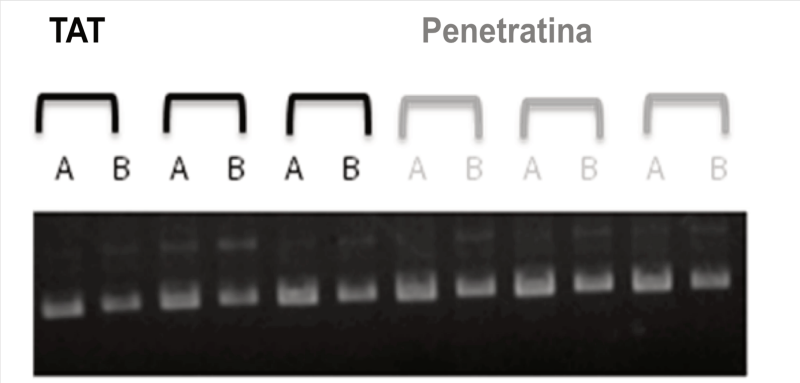

Figura 8: Padrão de bandas obtido na digestão de pET28a com TAT e penetratina, respectivamente, com HindIII. Observa-se que não houve digestão, garantindo que o sitio da enzima de restrição foi excluído na clonagem com os peptídeos.

5.2. Expressão de P-TcTex

Visando trabalhar com diferentes combinações de cadeias leve de dineína e peptídeos membrano-ativos, a sequência da cadeia leve TcTex1 foi clonada em um vetor pET28a contendo previamente a sequência de penetratina. A proteína recombinante obtida foi chamada P-TcTex, e foi expressa e *E. coli* BL21(DE3). Usando-se o tampão TrisHCl 50 mM, NaCl 500 mM, EDTA 0,1 mM, ß-mercaptoetanol 15 mM a pH 7,0 na extração da fração proteica solúvel, não se conseguiu observar a expressão de P-TcTex em gel de SDS-PAGE. Entretanto, após o carregamento do extrato protéico em coluna Ni-NTA, observam-se bandas de tamanho compatível (19,2 kDa) nas eluições das frações contendo 200 mM e 500 mM de Imidazol (Figura 9).

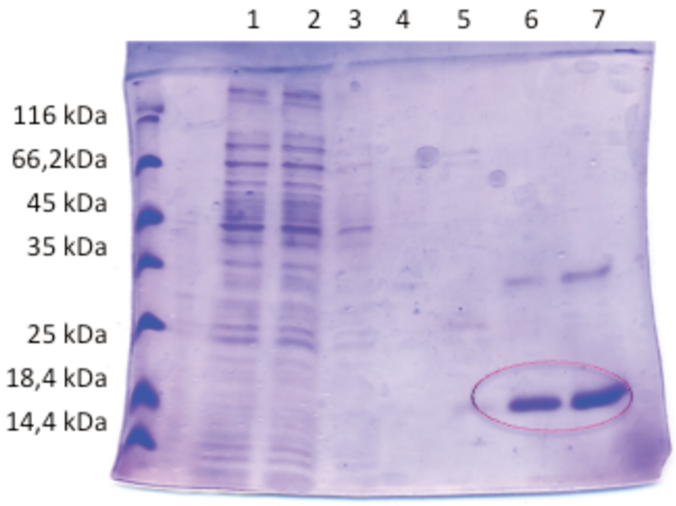

Figura 9: Eletroforese em gel de poliacrilamida 12% da expressão de P-TcTexa 28˚C/200rpm com 20 horas de indução em *E. Coli* BL(21DE3). 1- Fração solúvel; 2- Flow-Through; 3 a 7- Eluição com 20mM, 75mM,100mM, 200mM e 500mM de Imidazol, respectivamente. A proteína foi eluída nas frações com 200 e 500mM de Imidazol.

5.3. Estudo de estabilidade de T-Rp3

Nos primeiros ensaios de expressão a proteína T-Rp3 se mostrou bem expressa em *E. coli* (vide manuscrito do artigo submetido), porém muito instável, o que dificultava seu manuseio devido à precipitação. O processo de precipitação era ainda mais acentuado em tampão PBS, que é comumente usado em experimentos com cultura de células. Avaliamos então o perfil de instabilidade de T-Rp3 através de programas de predição a partir da estrutura (Figura 6).

Observamos que T-Rp3, quando comparada à Rp3 contendo apenas a sequência de DNA binding, apresenta um aumento da instabilidade (Figura 10). Uma análise das proteínas pelo programa ProtParam indica que o coeficiente de instabilidade aumenta de 53,8 para 65,7, sendo que a partir do valor 40 a proteína

já é considerada instável. Os dados de predição das estruturas das proteínas pelo programa PONDR indicam que a inclusão da seqüência de TAT causa aumento no grau de desordem da estrutura (aumento de 14% para 30% na porcentagem de desordem), o que poderia facilitar agregação e precipitação.

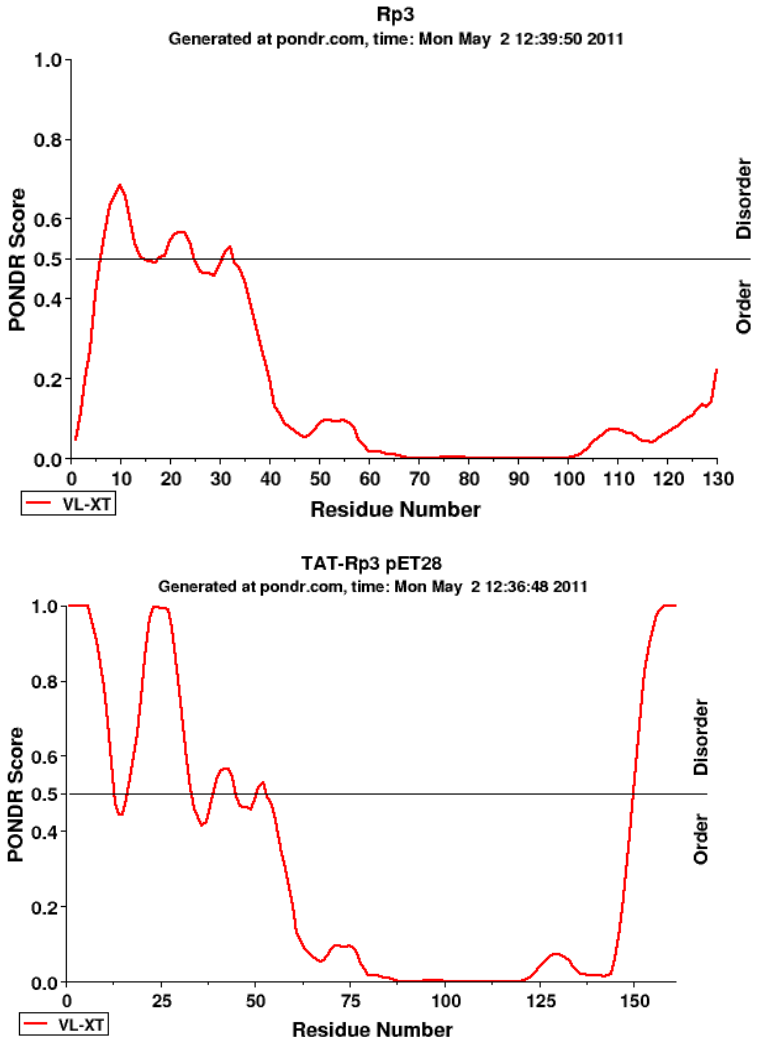

Figura 10: Comparação das predições de regiões de desordem de Rp3-DNAbinding (A), T-Rp3-DNAbinding e (B), sendo que as proteínas fusionadas aqui foram analisadas na forma como são extraídas, antes da clivagem da His-Tag. Observa-se que, assim como o DNA binding na região N-terminal, a adição de TAT acrescenta uma região desordenada na porção C-terminal da proteína Rp3. Fonte: PONDR – www.pondr.com.

Na tentativa de encontrar um tampão de diálise apropriado, fizemos um *screening* de diversas condições, variando pH, força iônica em diferentes soluções tampão, entre eles PBS, Acetato, Tris-HCl e Hepes. Observamos que a melhor condição para diálise é em tampão Hepes 40 mM pH 7,3 à temperatura ambiente, o que permite à proteína se manter estável por diversos dias. Essa foi a condição utilizada na preparação da proteína para os estudos de interação com pDNA e transfecção de células HeLa apresentados no manuscrito.

6. Discussão

A proteína recombinante T-Rp3 foi eficientemente produzida em *E. coli* e manteve sua habilidade de interagir com a cadeia intermediária de dineína. A T-Rp3 pode ser considerada uma proteína modular recombinante que promove a condensação do pDNA para entrega gênica, formando uma partícula pequena e positivamente carregada. O fato do complexo formado por pDNA:T-Rp3 ser menor e mais positivo que o formado por pDNA:protamina é um indicativo de que as sequências TAT e DNAbinding, ambas positivamente carregadas, têm grande influência na arquitetura do complexo, podendo inclusive afetar sua estabilidade ao longo do tempo.

As transfecções em células HeLa indicam que a eficiência de entrega gênica da proteína T-Rp3 é 400 vezes maior que a da protamina, e apenas 13 vezes menor que a da Lipofectamina 2000^{TM}, com a vantagem adicional de ser menos tóxica que esta última. A utilização da droga Nocodazol nas transfecções reduziu drasticamente a eficiência de entrega promovida pela T-Rp3, indicando uma forte dependência da rede de microtúbulos para sua eficiência. Além disso, complexos ternários formados por pDNA, T-Rp3 e Lipofectamina 2000^{TM} apresentaram os mais altos níveis de eficiência, inclusive mais altos que a Lipofectamina 2000^{TM}, porém com toxicidade reduzida. A T-Rp3 cumpre, portanto, a função esperada de realizar a entrega gênica de maneira eficiente e pouco citotóxica, provavelmente explorando a rede de microtúbulos através da dineína.

Embora a proposta inicial deste projeto fosse explorar diferentes combinações entre cadeias leves (Rp3 e TcTex) e peptídeos membrano-ativos

(TAT e penetratina), os desafios encontrados para manipular as proteínas recombinantes dificultaram os estudos com diferentes construções. Além disso, os resultados obtidos com a primeira construção testada (T-Rp3) requeriam uma caracterização mais profunda para atingir uma melhor compreensão do seu desempenho. Assim sendo, adaptamos nossa proposta para um estudo mais completo com a T-Rp3, o que nos permitiu observar que o seu desempenho é realmente promissor, além de possuir as carcterísticas físico-químicas adequadas a um vetor de entrega gênica.

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7. Conclusões

Facilmente manipulável através de clonagens, o uso de proteínas recombinantes permite abordar as diferentes etapas que limitam o tráfego intracelular, de forma direcionada, combinando diversas estratégias. O uso de proteínas modulares como vetores de entrega gênica tem despontado no âmbito das abordagens inovadoras como uma alternativa bastante promissora. Diferentemente de outras classes de vetores, proteínas e peptídeos permitem um ajuste fino das funções e características conferidas pelos mesmos, se adaptando a condições específicas. Associa-se a estes vetores uma baixa citotoxicidade como característica geral, tornando-os ainda mais vantajosos.

Além do uso de proteínas ser uma estratégia bastante nova e ainda pouco explorada, a abordagem utilizada pelo nosso grupo envolvendo proteínas motoras para facilitar o tráfego intracelular é anda mais diferenciada. Embora muitos grupos sugiram a importância das proteínas motoras, inclusive como uma forma de se aproximar do desempenho dos vetores virais, nossa abordagem destaca-se uma vez que pouco foi feito até o momento no sentido de efetivamente ampliar a utlização da rede de microtúbulos.

Além de conferir características físico-químicas apropriadas para um vetor de entrega gênica, como discutido no Capítulo 6, os resultados aqui apresentados indicam que a proteína T-Rp3 promove o direcionamento do complexo através da rede de microtúbulos. As seqüências de TAT, DNAbinding e HisTag adicionadas à proteína recombinante modficaram-na o suficiente para converter uma cadeia leve

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de dineína em um promissor vetor de entrega gênica, porém mantendo sua habilidade de explorar proteínas motoras e o transporte em direção ao centrossoma. A eficiência observada em transfecções com T-Rp3 confirma as sugestões freqüentes na literatura de que o tráfego intracelular é um fator determinante no sucesso da entrega gênica. Pode-se considerar a T-Rp3 um adjuvante promissor para promover a entrega gênica de maneira eficiente e pouco tóxica. Assim sendo, esse estudo abre ainda espaço não só para estudos futuros de entrega gênica, raros no Brasil, mas também para uma abordagem diferenciada e inovadora entre as pesquisas na área.

8. Perspectivas futuras

Os resultados apontam para T-Rp3 como um vetor muito promissor de entrega gênica que cumpre a proposta de favorecer o direcionamento no citosol via microtúbulos. A indicação de que a maior eficiência alcançada foi através de complexos ternários torna este um campo a ser explorado, especialmente através de lipídeos modificados e menos tóxicos que a Lipofectamina 2000^{TM}.

Além disso, o escape endossoal se manteve como uma barreira relevante, indicando que o uso de uma TAT modificada como a proposta por Lo e Wang [44], que contenha mais resíduos de histidina, pode ser uma alternativa promissora.

Sabe-se que os resultados obtidos em transfecção de células pode variar muito de acordo com o tipo celular, de modo que será indispensável testar em outros modelos. Finalmente, o futuro do desenvolvimentos destes vetores deverá incluir testes *in vivo* com um modelo de doença já bem estabelecido, como distrofia de duchenne ou fibrose cística. Uma estratégia promissora seria utilizar os complexos ternários *in vivo* uma vez que o lipídeo pode conferir uma proteção adicional ao complexo.

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10. Anexo

GENE DELIVERY

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Development of a recombinant fusion protein based on the dynein light chain LC8 for non-viral gene delivery

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ABSTRACT

The low efficiency of gene transfer is a recurrent problem in DNA vaccine development and gene therapy studies using non-viral vectors such as plasmid DNA (pDNA). This is mainly due to the fact that during their traffic to the target cell's nuclei, plasmid vectors must overcome a series of physical, enzymatic and diffusional barriers. The main objective of this work is the development of recombinant proteins specifically designed for pDNA delivery, which take advantage of molecular motors like dynein, for the transport of cargos from the periphery to the centrosome of mammalian cells. A DNA binding sequence was fused to the N-terminus of the recombinant human dynein light chain LC8. Expression studies indicated that the fusion protein was correctly expressed in soluble form using *E. coli* BL21(DE3) strain. As expected, gel permeation assays found the purified protein mainly present as dimers, the functional oligomeric state of LC8. Gel retardation assays and atomic force microscopy proved the ability of the fusion protein to interact and condense pDNA. Zeta potential measurements indicated that LC8 with DNA binding domain (LD4) has an enhanced capacity to interact and condense pDNA, generating positively charged complexes. Transfection of cultured HeLa cells confirmed the ability of the LD4 to facilitate pDNA uptake and indicate the involvement of the retrograde transport in the intracellular trafficking of pDNA:LD4 complexes. Finally, cytotoxicity studies demonstrated a very low toxicity of the fusion protein vector, indicating the potential for *in vivo* applications. The study presented here is part of an effort to develop new modular shuttle proteins able to take advantage of strategies used by viruses to infect mammalian cells, aiming to provide new tools for gene therapy and DNA vaccination studies.

1. Introduction

Gene therapy and DNA vaccination protocols demand efficient and safe mechanisms to deliver therapeutic genetic material to the patient cell nucleus. So far, viral-based vectors have been preferred as delivery vehicles since they are naturally efficient in receptor-mediated recognition and cell internalization, endosomal escape, nuclear transport and DNA integration [1,2]. Nevertheless, the use of viruses as gene delivery systems continues to rise safety concerns and the future development of viral gene therapy continue to generate intense scientific debates [2,3]. The alternative approach, non-viral vectors, is considered safer and has been also attracting significant attention of the scientific community. Efforts have been made to increase the delivery efficiency of non-viral vectors, including the creation of sophisticated vehicles able to mimic some of the viral properties regarding both size and biological properties [2]. These vectors are called "artificial viruses" and include polymeric constructs [4–6], protein-only shells and virus-like particles [4]. Among the properties of these vectors are the ability to condense and protect DNA from nuclease degradation, low systemic and cellular toxicity, membrane crossing abilities, and steady expression of the therapeutic gene [7]. However, the inability of these vectors to efficiently traverse the target cell cytoplasm and reach the nucleus has largely been overlooked [8]. It has been also reported that non viral vectors face several extra- and intracellular barriers before DNA can be delivered to the cell's nucleus [9] and that cytosolic proteins may bind to the delivery complex acting as another barrier [10].

The majority of the non-viral vectors studied so far rely on passive diffusion or non specific transport for trafficking within the cytoplasm, and this limited mobility represents a significant barrier to gene delivery [11]. Since it has been shown that diffusion of DNA

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fragments larger than 2000 base pairs through the crowded cytoplasmic environment is greatly restricted [12], an ideal vector should, like most viruses, include the ability to exploit the host cell machinery to rapidly traverse the cytoplasm. Many authors have suggested that an ideal form to actively transport drugs, including transgenes, from the site of cytoplasmic entry to the nuclear periphery would include recruitment of the minus end-directed motor dynein [8,13–15]. Cytoplasmic dynein is a multisubunit protein complex (~1.2 MDa) composed of two heavy chains (~530 kDa) responsible for microtubule attachment and ATP hydrolysis [16], two 74-kDa intermediate chains (IC74), four light intermediate chains (52–61 kDa), and several light chains (10–25 kDa) which are responsible for cargo attachment to the dynein motor complex and hence, its transport through the cytosol toward the nucleus [17,18]. However, so far no successful strategies to exploit motor proteins' retrograde transport for efficient gene delivery have been demonstrated. In an attempt to design synthetic gene carriers that display dynein-binding peptides for enhanced intracellular transport, Bergen and Pun (2007) studied the use of a peptide that binds to the dynein light chain LC8 subunit, as the first potential dynein-binding peptide [8]. It was demonstrated that, while the peptide readily bound free LC8, it could not bind to dynein-associated LC8, emphasizing the need to identify or design peptides that could mediate binding to the intact dynein motor complex. More recently, Moseley and collaborators (2010) reported that protein transduction can be enhanced by attachment to a dynein light chain association sequence [15]. These sequences were able to enhance nuclear accumulation of GFP-fusion proteins, with dependence on the LC8/microtubule (MT) network. This work provided the first successful evidence that dynein/MT-association can be exploited for DNA or drug delivery approaches.

Here, we propose the use of the dynein light chain LC8 itself as a cargo adaptor for plasmid delivery into mammalian cells, taking advantage of the dynein retrograde transport *via* the MT network. LC8, also called DYNLL1, is a small (10 kDa) and highly conserved globular protein reported as an essential component of the dynein and myosin V molecular motors [19,20]. The LC8 binds as a dimer directly to specific sites on the dynein intermediate chain IC74 or myosin V heavy chain, while some studies indicate additional roles for LC8 in multiple protein complexes unrelated to the motor proteins such as p53-binding protein 1 [21], neuronal nitric oxide synthase [22], the proapoptotic member of the Bcl-2 family proteins Bim and Bmf [23,24], the product of the *Drosophila* swallow gene [25], and a number of proteins with unknown functions [26]. We envision that LC8 could be modified with short DNA-binding sequences, rich in positively charged amino acids, which would interact and condense pDNA and facilitate its transport toward the nucleus periphery *via* interaction with the dynein complex. Finally, the work presented here intends to generate new information on plasmid delivery and presents a new strategy for the development of modular non-viral vectors potentially useful for gene therapy and DNA vaccination.

2. Materials and methods

2.1. Plasmid DNA vector

In the present study, a plasmid DNA model named pVAX1-Luc was constructed based on the previously reported pVAX1-GFP plasmid [27]. Using XbaI and EcoRI restriction endonucleases, the sequence coding for GFP was replaced by the luciferase gene obtained from the pGL3-Luc control vector (Promega) using the same restriction enzymes. The success of the reporter gene replacement was confirmed by the expression of the reporter enzyme after transfection of HeLa cells, as described in this work. Purification of the pVAX1-Luc plasmid used in all transfection studies was performed as described by Freitas and co-workers [28].

2.2. DNA binding domains design

The fusion protein DNA binding domains were designed based on the scientific literature available [2,29] for peptides and protein domains with high DNA binding and condensing capacity. We designed four domains to be cloned upstream of human dynein light chain LC8: DNAb1, WRRRGHGKKK; DNAb2, WRRRGFGKKK; DNAb3, WRRRGHGRRR; and DNAb4, WRRRGFGRRR. The corresponding single strand oligonucleotides were synthesized with optimized codons for *E. coli* expression and annealed before cloning. The DNA binding domains were cloned in the *NdeI* and *BamHI* sites in the pET28a expression vector (Novagen, Darmstadt, Germany). The clones containing the DNA binding domains were further used for LC8 cloning in the *BamHI* and *XhoI* restriction sites. In this work we define as LD4 the recombinant human LC8 fused to the DNA binding domain 4 (DNAb4), described above.

2.3. Recombinant proteins expression and purification

The human dynein light chain LC8 was amplified from HeLa cDNA with specific primers (forward:5′-ATAGGATCCATGTGCGACCGAAAG-3′, reverse: 5′ATACTCGAG TTAACCAGATTTGAACAGAAGA-3′) and cloned into pET28a with and without DNA binding domains previously cloned at the N-terminal. Recombinant LC8 with or without DNA binding domains were expressed in *E. coli* BL21(DE3). Briefly, cells were grown in 1 L LB medium at 37 °C, 300 rpm and up to an optical density of 0.8 AU (600 nm). Protein expression was induced with 5.6 mM lactose or 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for further 12 h at 25 °C, 200 rpm. After centrifugation, the cell pellet was suspended in 50 mM sodium phosphate pH 8.0, 500 mM NaCl, 0.1 mM EDTA, 15 mM β-mercaptoethanol and 1 mM PMSF (phenylmethylsulfonyl fluoride). Cell lysis was performed by sonication and the recombinant LC8 was purified by a single Ni-NTA affinity chromatography step. This method was successfully used to purify recombinant LC8 with and without the four different DNA binding domains.

For *in vitro* interaction of recombinant dynein light chain LC8 and LD4 with human dynein intermediate chain DYNIC2 its N-terminal domain (the first 300 amino acids) and the dynein light chain TcTex were cloned using the same methodology as described for LC8. The DYNIC2 domain was amplified with specific primers from HeLa cDNA and cloned into pET28a using *NdeI* and *BamHI* restriction sites (forward, 5′-ATTCATATGATGTCAGACAAAAGTGAATT-3′, reverse, 5′-ATTGGATCCTTAGTTATAGGAAGCCAC-3′). The TcTex light chain was inserted in *BamHI* and *XhoI* restriction sites (forward, 5′-ATAGGATCCATGGAGGAGTACCATCG-3′, reverse, 5′-ATACTCGAGTTAAAGAACAATAGCAATGG-3′). Both recombinant proteins were expressed in *E. coli* BL21 (DE3) Rosetta strain. The expression of the N-terminal DYNIC2 was induced with 0.2 mM IPTG at 20 °C and 200 rpm for 12 h. For all recombinant proteins, purity was evaluated by SDS-PAGE and concentration was measured by absorbance at 280 nm.

2.4. Circular dichroism studies

Circular dichroism (CD) spectra of the purified recombinant proteins were obtained using a Jasco J-810 Spectropolarimeter dichrograph (Japan Spectroscopic, Tokyo, Japan). The far-UV CD spectra were generated at 20 °C using 10–20 μM of each protein in 10 mM sodium phosphate buffer pH 8.0. The assays were carried out using a quartz cuvette with a 1 mm path length. Ten accumulations within the 185–260 nm range at a rate of 50 nm/min were recorded. Data was processed using OriginLab 8.0 software.

2.5. Size exclusion chromatography

To assess the oligomeric state of purified LC8 with and without DNA binding domains, size exclusion chromatography was performed using a Superdex 200 GL10/300 or Superdex 75 GL10/300 prepacked column

(GE Healthcare, Uppsala, Sweden). After equilibration of the column with extraction buffer or PBS (8.0 g NaCl, 0.2 g KCl, 1.4 g Na_2HPO_4, 0.24 g KH2PO_4, pH 7.4 per liter), oxidized and reduced (10 mM DTT) samples (250 μl) were loaded at a flow rate of 0.75 ml/min. The calibration curve was prepared using High and Low molecular calibration kits (GE Healthcare). Gel permeation chromatography was also used to evaluate LC8-TcTex-Intermediate chain complex formation.

2.6. In vitro interaction of recombinant dynein light chains LC8 and TcTex with dynein intermediate chain 2, isoform C

In order to evaluate the ability of recombinant LC8 and LD4 to interact *in vitro* with the human dynein intermediate chain DYNIC2, isoform C, the N-terminal of this protein (first 300 amino acids) was cloned, expressed and purified as described. We also used in this assay the TcTex light chain since its presence in the complex may enhance the interaction between LC8 and the intermediate chain, as discussed in the Results and discussion section. LC8 (19.2 μM) or LD4 (8.9 μM) and TcTex (6.3 μM) light chains were incubated with purified intermediate chain domain, IC_{DOM} (7.23 μM), at 8 °C for 20 h under reducing conditions (10 mM DTT). The purified proteins were used immediately after purification procedure and complex was formed in 50 mM sodium phosphate pH 8.0, 500 mM NaCl, 0.1 mM EDTA and approximately 200 mM imidazole. Complex formation was evaluated by gel permeation chromatography using a Superdex 75 GL10/300 prepacked column (GE Healthcare, Uppsala, Sweden) and collected samples corresponding to the complex elution peaks were concentrated (10-fold concentration) using a Amicon Ultra Centrifugal Filter (3 kDa) (Millipore, Ireland) and visualized by SDS-PAGE.

2.7. Evaluation of DNA-protein interaction by gel retardation assay

To evaluate the ability of LC8, LC8 with different DNA binding domains and protamine to interact and condense pDNA, we performed a gel retardation assay. Proteins were dialyzed in PBS (protamine sulfate powder was ressuspended in PBS) and incubated with 250 ng of pVAX-Luc vector (previously in PBS) at different pDNA:protein molar ratios (1:400, 1:800, 1:1000, 1:2000, 1:4000 and 1:8000) in a final volume of 50 μL. The samples were incubated at room temperature for 1 h following by the addition of 50 μL of non supplemented F-12 media and additional incubation for 20 min. Samples were run in a 0.8% agarose gel and visualized by ethidium bromide staining.

2.8. Zeta potential and particle size assays

Zeta potential measurements were performed to comparatively evaluate the surface charge of complexes formed by pDNA:LC8 and pDNA:LD4. Complexes were formed as described for gel retardation assay but without F-12 media addition and using the same pDNA:protein molar ratios. Each sample was measured in triplicate using Zetasizer 3000 (Malvern, England). Particle size measurements were performed to evaluate the behavior of different complexes regarding its size along time. pDNA:protamine (pDNA:protein molar ratio of 1:8000) and pDNA:LD4 (pDNA:protein molar ratio of 1:100 and 1:8000) complexes with and without Lipofectamine™ and pDNA:Lipofectamine™ complexes were formed by 1.0 μg of pDNA and the corresponding amount of protein in a final volume of 800 μL. When indicated, 1.5 μL of Lipofectamine™ was added to the complex. Each sample was submitted to multiple readings during a 60 min period. Particle diameter was plotted against time for each sample.

2.9. Atomic force microscopy (AFM)

Plasmid DNA:protein complexes at different molar ratios (1:400, 1:1000 e 1:2000 for LC8 and 1:100, 1:200 e 1:500 for LD4) were prepared in 10 mM Tris–HCl buffer pH 7.4 in a final DNA concentration of 600 pg/μl by incubation at room temperature for 1 h. After complex formation, a final concentration of 5 mM $MgCl_2$ was added to each sample and 60 μl of the solution was physisorbed for 1 min on freshly cleaved muscovite mica (Ted Pela, California, USA). After adsorption, the surface was washed for 10 s in ultrapure water and dried in a weak nitrogen stream. The AFM imaging was performed in air a room temperature in acoustic mode at a scanning speed of 300 nm/s with an Agilent 5500 (Agilent, Santa Barbara, USA) using commercial silicon cantilevers (MicroMash, NSC-14/ALBS) with a tip radius of approximately 10–20 nm. The topography images were treated using the Open-Source software Gwyddion (www.gwyddion.net).

2.10. Culture and transfection of HeLa cells

HeLa cells were grown in F-12 (Ham) nutrient mixture (Gibco, UK) containing 10% (v/v) fetal bovine serum (growth medium, Gibco, UK). All cell cultures were performed in 75 cm^2 culture flasks and incubated in 5% CO_2 humidified environment at 37 °C. Following growth up to confluence, cells were trypsinized and seeded in 24 well culture plates (5×10^4 cells per well). The cells were incubated for 48 h (to a 70% confluence) and then transfected with pDNA:protein complexes formed as described for gel retardation assays and using the same molar ratios. When indicated, transfection was carried out using the Lipofectamine 2000™ reagent (Invitrogen, USA) according to the manufacturer instructions (1.0 μg pDNA plus 1.5 μL reagent in 100 μL of medium per well), protamine sulfate (Sigma Aldrich, Germany) or PEI (polyethylenimine, branched, MW ~25,000) (Sigma Aldrich, Germany). The medium containing the transfection solution remained on the cell containing wells for 6 h and was then replaced by regular growth medium. Cells were collected after 24 h post-transfection for determination of luciferase activity using the luciferase Assay System (Promega, USA), following the manufacturer's instructions. Luminescence intensity was normalized against protein concentration in each transfection sample, as determined by the Micro BCA Protein Assay Kit (Thermo Scientific, USA).

To evaluate the contribution of the microtubule network and actin filaments in the intracellular trafficking of the complexes, cells were pre-incubated for 2 h with nocodazole (25 μM) or cytochalasin D (25 μM) for microtubule and actin filaments disruption, respectively. Both drugs were dissolved in DMSO and an equal volume of drug-free DMSO was used as control (0.5% for cytochalasin D and 0.4% for nocodazole). Transfections in the presence of chloroquine were performed to evaluate the contribution of lysosomal degradation of protein-pDNA complexes. Cells were pre-incubated for 4 h with chloroquine (100 μM). For all assays with the mentioned drugs, pre-treated cells were incubated afterwards in the presence of the different complexes for 4 h, when the medium was replaced by freshly growing medium. After 24 h, cells were collected and the luciferase activity was assayed as described above.

Cytotoxicity assays of delivery vectors studied in the present work were performed using Cell Proliferation Reagent WST-1 (Roche Applied Science, USA) following manufacturer's instructions. Briefly, HeLa cells were grown on 96 wells plates to a confluence of 70%. Transfection was performed as described before with pDNA:protein complexes (molar ratio of 1:8000) with and without Lipofectamine™, with pDNA:Lipofectamine™, and also with naked pDNA as control. Finally, 10 μL of the WST-1 reagent was added to each well, and cells were further incubated for 2 h. Absorbance was read after 60 s agitation at 440 nm using a Spectramax 384 Plus UV/VIS Microplate Reader (Molecular Devices, USA).

3. Results and discussion

3.1. Recombinant human dynein light chain LC8 was successfully produced with N-terminal DNA binding domains

Using the methodology described above we were able to produce recombinant LC8 and recombinant LC8 fused to DNA binding domains

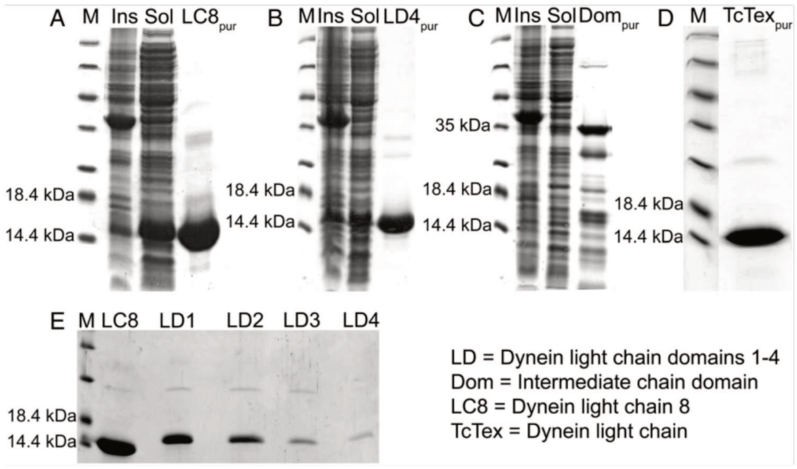

Fig. 1. SDS-PAGE analysis of purified recombinant proteins used in the present study. A: Purified LC8. B: Purified LD4. C: Purified intermediate chain N-terminal domain. D: Purified TcTex. E: LC8 and LC8 with the four different DNA binding domains at the N-terminal after dialysis against PBS. M: Broad Range molecular weight marker (Fermentas). Ins: Insoluble fraction after cell lysis. Sol: Soluble fraction after cell lysis. $LC8_{pur}$: purified LC8. $LD4_{pur}$: purified LD4. Dom_{pur}: purified N-terminal domain of DYNIC2 intermediate chain. $TcTex_{pur}$: purified TcTex. LC8, LD1,LD2,LD3,LD4: LC8 without and with each DNA binding domain.

(DNAb1, DNAb2, DNAb3 and DNAb4), all proteins efficiently expressed in *E. coli* BL21(DE3) strain. All constructs were obtained in the soluble fraction after cell disruption and purified by a single Ni-NTA affinity chromatography step (Fig. 1). Circular dichroism analysis of the secondary structures of the proteins showed that addition of the DNA binding domains had little effect on the secondary fold of LC8 probably due to the addition of the domain to its N-terminal (Fig. 2 and Supplementary Data). The oligomeric state of LC8 and LC8 fused with DNAb4 (LD4) was evaluated in phosphate saline buffer (PBS) by gel permeation chromatography under reduced and non reduced conditions. We could observe that the human LC8 is dimeric under reducing conditions, as reported for the *Drosophila* homologue [30], and appears as a tetramer under non reducing conditions (results not shown). The LD4 presented a higher propensity to aggregate under low ionic strength (such as in PBS), as judged by the fact that a significant portion of the purified protein precipitated during dialysis against PBS, even under reducing conditions. Gel permeation chromatography shows that LD4 in PBS under reducing and non reducing conditions is polidispersed in diverse oligomeric subpopulations. However, analysis of small angle X-ray scattering (SAXS) data for Lc8 and LD4, collected in 50 mM sodium phosphate, 500 mM NaCl, 0.1 mM EDTA and 500 mM Imidazole, allowed us to assume that the recombinant proteins were folded in solution under reducing conditions. A bell shaped Kratky plot was observed, which is characteristic of folded proteins (Supplementary Data). Therefore, the observed aggregation for LD4 may be due to the relative low ionic strength of PBS. In summary, our results indicate that the recombinant LC8 and its variations with different DNA binding domains were successfully and correctly expressed and purified (Fig. 1) allowing us to proceed with pDNA interaction and transfection assays. It is noteworthy that LD4 (as well with other DNA binding domains), despite appearing as a population of aggregates in gel permeation assays, was successfully used in pDNA condensation assays, indicating that upon DNA addition, the protein aggregates were disrupted allowing protein interaction with the pDNA.

3.2. Recombinant LC8 and LD4 are able to interact in vitro with human dynein intermediate chain

The human LC8 dynein light chain interacts with the dynein intermediate chain and some other proteins not related to the dynein motor complex as mentioned before (see Introduction). Additionally, the interaction between LC8, TcTex and the intermediate chain IC74 is well characterized for the *Drosophila* homologues. In a recent work, Hall and collaborators (2009) showed that when one of the light chains (LC8 or TcTex) is previously bound to the intermediate chain IC74, the binding of the second light chain is enhanced by 50-fold [31]. In the context of the gene delivery mechanism envisioned in

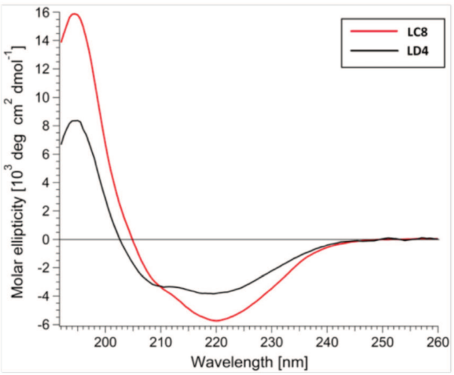

Fig. 2. Circular dichroism curves for dynein light chain LC8 and for LC8 fused with DNA binding domain 4. LC8: LC8 dynein light chain. LD4: LC8 dynein light chain fused with DNAb4 binding domain. Little variation between the spectra can be observed probably due to the addition of the DNA binding domain.

the present work, the recombinant dynein light chain LC8, whether fused or not to DNA binding domains, should be able to interact with the dynein machinery. Thus, in order to analyze the functionality of recombinant LC8 and LD4, we assayed their interaction with the N-terminal domain (300 first amino acids) of the human dynein intermediate chain 2, isoform C (DYNIC2), in combination with TcTex dynein light chain. We could observe that both, LC8 and LD4 were able to interact *in vitro* with dynein intermediate chain by gel permeation chromatography and subsequent SDS-PAGE analysis of the eluted peak (Fig. 3). This result shows that the recombinant LC8 light chain, with or without the N-terminal DNA binding domain retains its functionality once it is able to interact with human intermediate chain *in vitro*.

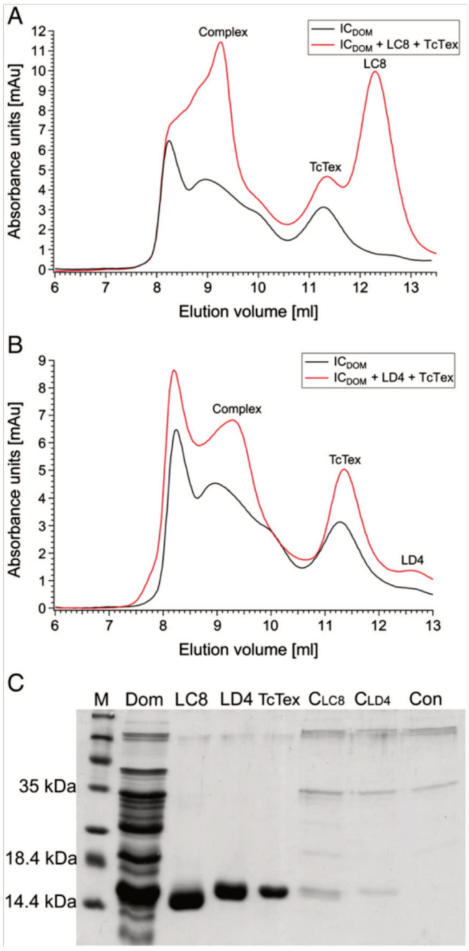

Fig. 3. Interaction of recombinant LC8 and LC8 with DNA binding 4 with the N-terminal domain of DYNIC2 and with the dynein Light Chain Tctex. A: Gel permeation chromatography elution curves for LC8-DYNIC2-TcTex. B: Gel permeation chromatography elution curves for LD4-DYNIC2-TcTex. C: SDS-PAGE 15% for elution samples from gel permeation chromatography showing that DYNIC2 and Light Chains are eluted together indicating interaction. Dom: purified N-terminal domain of Intermediate Chain (premature translation end can be observed for this protein). LC8: purified LC8. LD4: purified LD4. TcTex: purified TcTex. C_{LC8}: elution peak collected at 9 ml and concentrated 10 fold for the LC8-DYNIC2-TcTex complex. C_{LD4}: elution peak collected at 9 ml and concentrated 10-fold for the LD4-DYNIC2-TcTex complex. Con: Control experiment, elution peak collected at 9 ml and concentrated 10-fold for DYNIC2 alone.

3.3. Addition of DNA binding domains at the N-terminal of LC8 light chain leads to enhanced interaction and condensation of plasmid DNA

A gel retardation assay was performed to evaluate the effect of DNA binding domains on the LC8 ability to interact and condense pDNA. We tested the same gradient of DNA:protein molar ratio for all DNA binding constructs, for LC8 alone and used protamine as control. Despite being non quantitative, this assay clearly shows an enhanced capacity of LC8 with DNA binding domains to interact and condense pDNA when compared to LC8 alone or to protamine (Fig. 4). At low pDNA:protein molar ratios (1:400), shifted bands can be observed for all constructs with DNA binding domains whereas only at higher molar ratios (1:2000) some shift can be observed for LC8 alone and for protamine. In addition, at higher molar ratios (1:4000), uncomplexed pDNA can still be observed for LC8 whereas for LC8 with DNA binding domains, all pDNA is complexed and has its migration retarded.

3.4. LC8 with DNA binding domain 4 interacts with pDNA generating positively charged complexes

An efficient vector for gene delivery must mediate the condensation and uptake of the genetic material by the cell. Specifically, the delivery vector must help to overcome the charge incompatibility between the negatively charged cell surfaces and pDNA molecules, by generating complexes with a positive net charge. We thus evaluated the surface charge of complexes formed between pDNA and LC8 or LD4. Zeta potential of pDNA-protein complexes was measured at different pDNA:protein molar ratios (Fig. 5A). The plot of zeta potential against each molar ratio shows that the addition of the DNA binding

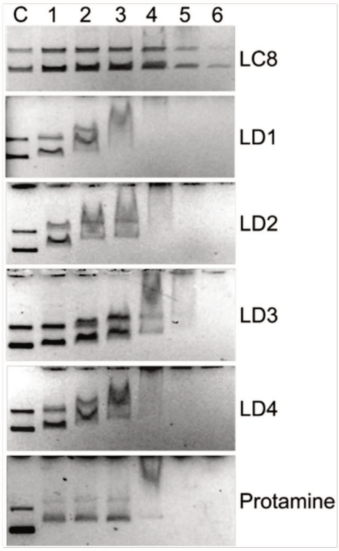

Fig. 4. Protein–DNA interaction analysis by gel retardation assay. The ability of protamine, LC8 and LC8 fused to DNAb1 (LD1), DNAb2 (LD2), DNAb3 (LD3) and DNAb4 (LD4) binding domains to interact and condense plasmid DNA was analyzed by a gel retardation assay. Six pDNA:protein molar ratios were used (lanes 1 to 6): 1:400, 1:800, 1:1000, 1:2000, 1:4000 and 1:8000. C: control pDNA with no protein.

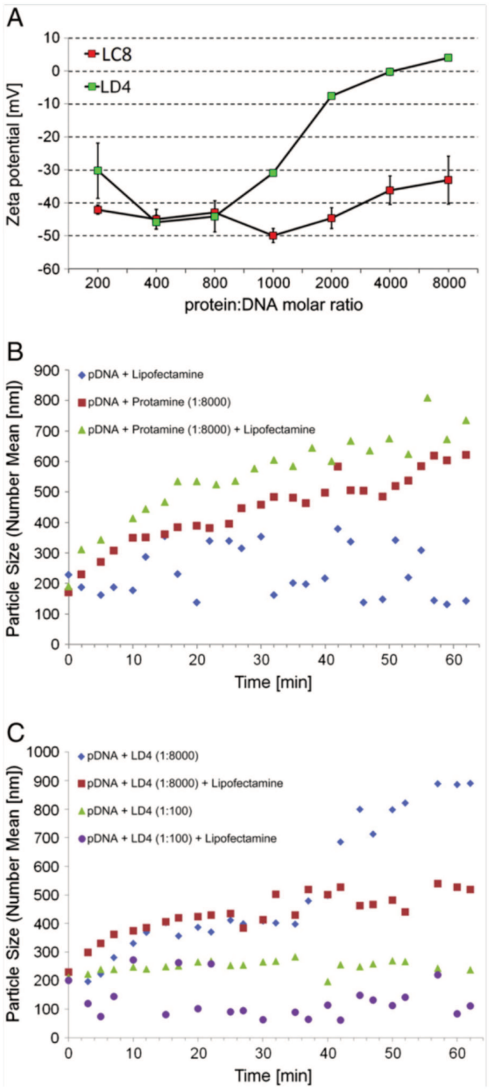

Fig. 5. A: Net surface charge of pDNA complexes formed by LC8 and LC8 fused to DNA binding domain 4 (LD4). The zeta potential of pDNA:protein complexes was measured at seven different molar ratios (1:200, 1:400, 1:800, 1:1000, 1:2000, 1:4000 and 1:8000). LD4 clearly shows higher ability to form positively charged complexes at molar ratios above 1:4000, while LC8 alone generated negatively charged complexes in all conditions tested. B and C: Time course of particle size for the different pDNA:delivery vector complexes studied.

sequence 4 to LC8 enabled the formation of complexes with positive net surface charge when a molar ratio above 1:4000 was used. For complexes formed with LC8 without DNA binding sequence, the net surface charge remained negative in all molar ratios tested.

We also monitored the particle size of pDNA:protamine (1:8000 molar ratio) and pDNA:LD4 (1:8000 and 1:100 molar ratio) with and without Lipofectamine™ and pDNA:Lipofectamine™ complexes during 60 min of incubation at room temperature (Fig. 5B and C). This analysis showed that addition of Lipofectamine™ to pDNA:protein complexes (LD4 or protamine) slightly increased the particle size, especially in the first 40 min of incubation. After that, pDNA:LD4 complexes, in especial, presented a sharp increase in size, probably due to aggregation caused by the lack of electrostatic repulsion, as indicated by the low values of zeta potential. Interestingly, the smallest complex which also showed no significant variation during the analysis was pDNA:LD4 (1:100) with Lipofectamine™. Particle size and particle surface charge are two main factors that dictates internalization of the delivery complex by the cell and is directly correlated to transfection efficiency [32,33]. Thus, by monitoring both variables we are able to correlate and perhaps explain the results found during transfection.

In order to evaluate the pDNA condensation, we performed atomic force microscopy (AFM) assays. The methodology allowed us to visualize the effect of the DNA binding domain 4 on the LC8 light chain affinity for the pDNA molecule. As it can be observed (Fig. 6), LD4 presented enhanced capacity to interact and condense plasmid DNA in contrast to LC8. In excess of both pDNA binding proteins, the pDNA:protein complexes form nearly spherical particles. In comparison, LD4 condenses pDNA to compact particles with sizes in the range of 75 ± 8 nm at lower molar ratios (1:500) than LC8 (1:2000) with size in the range of 101 ± 9 nm. However, it is important to note that the complexes observed by this method were formed at a lower ionic force and different buffer salt (10 mM Tris–HCl buffer) than those shown in gel retardation and Zetasizer experiments (PBS). The excess of salt in PBS buffer strongly affects the AFM imaging procedure. Despite the difference in ionic strength that probably lead to DNA condensation at lower pDNA:protein ratios, the AFM images clearly indicate the effect of the DNA binding domain.

3.5. Transfection experiments demonstrated that LD4 is able to deliver pDNA to HeLa cells

After the interaction tests *in vitro*, we assayed the ability of the fusion protein LD4 to deliver pDNA to HeLa cells in culture. Firstly, we tested complexes formed by pDNA and recombinant LC8 with and without the four different DNA binding domains. These preliminary transfections assays showed that LD4 was the most efficient shuttle protein among all the constructs (results not shown). Therefore, we focused our efforts on LD4, and used LC8 and the arginine rich protamine as controls. The results show that LD4 is more efficient in cell transfection when compared to protamine (Fig. 7A), a well characterized nuclear protein that is known for its ability to condense DNA molecules, facilitate DNA uptake by the cells, and transposition of the nuclear barrier due to presence of a nuclear localization signal [34,35]. LD4 mediated transfection presented a 30-fold higher luciferase expression compared to protamine at the same pDNA:protein molar ratio (1:8000), while LC8 presented even lower efficiency (4-fold lower than protamine). It is noteworthy that pDNA:LD4 (1:8000) complexes formed particles with bigger size than pDNA:protamine (1:8000) complexes as shown by size measurements and a mildly positive surface charge, as shown by Zeta potential measurements. Therefore, the observed enhanced transfection efficiency presented by pDNA:LD4 complexes compared to protamine complexes might be related to an additional property of the recombinant LD4 that facilitates pDNA delivery. Interestingly, when pDNA:LD4 complexes were formed at a molar ratio of 1:16,000, the transfection efficiency dropped 3-fold. It is possible that the decrease in transfection efficiency was caused by protein saturation and formation of bigger complexes by non-specific protein–protein interaction leading to a lower complex uptake by the cell [36].

We also tested multicomponent complexes formed by pDNA and protein (LC8, LD4 and protamine), Lipofectamine™ or PEI (Fig. 7B). Cationic lipids like Lipofectamine™ are known as efficient pDNA delivery vectors in culture cells, enabling the internalization of several thousand pDNA copies per cell during *in vitro* transfection [34]. As expected, binary complexes formed by pDNA and Lipofectamine™

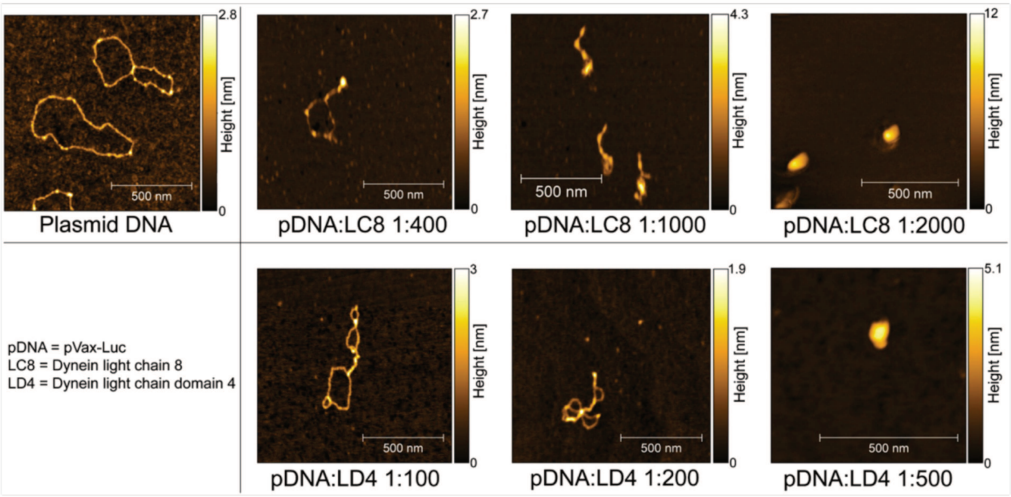

Fig. 6. Visualization of pDNA:LC8 and pDNA:LD4 complexes by atomic force microscopy (AFM). pDNA:protein complexes formed at different molar ratios (1:400,1:1000 and 1:2000 for LC8 and 1:100, 1:200 and 1:500 for LD4) allowed us to visualize the condensation process mediated by the protein. This methodology clearly shows that addition of a DNA binding domain 4 to the LC8 Dynein Light Chain enhanced its ability to interact and condense plasmidial DNA.

promoted higher transfection efficiencies than pDNA:protein complexes (using either LD4 or protamine). Interestingly, ternary complexes formed by pDNA, Lipofectamine and LD4 presented even more efficient pDNA delivery. In this case, we observed that in the presence of Lipofectamine™, complexes formed at 1:100 pDNA:protein molar ratio were more efficient than those formed at 1:8000. One possible explanation is that addition of Lipofectamine™ to the already compact complex formed by pDNA:LD4 (1:100) lead to the formation of more stable and even smaller complexes of approximately 100 nm, as can be seen in Fig. 5.

It is interesting to note that the addition of protamine to the ternary complex (1:8000:8000 pDNA:LD4:protamine), resulting in the formation of quaternary complexes, restored the transfection efficiency, indicating that synergic effects between the lipid and proteins are responsible for the increase in transfection efficiency. The increase in transfection efficiency promoted by protamine in multicomponent complexes have already been reported and was credited to its nuclear localization signal [35,37]. Despite this may also be the case of LD4, the observed increase in pDNA delivery of complexes containing LD4 at a low molar ratio (1:100) can also be explained by changes in complex stability, size, and the ability to overcome different barriers to the intracellular trafficking, including the recruitment of dynein motors. Synergic effects resulted from the combination of proteins and the cationic polymer PEI were also observed (Fig. 7B). Therefore, these multicomponent complexes may combine the high capacity of pDNA internalization of the cationic lipids or polymers with the enhanced capacity of transport through the cytoplasm and nuclear entrance of the protein vectors.

3.6. Cytoskeleton and endossome/lysosome involvement in the transfection efficiency of pDNA:LD4 complexes

Our expectation on the study of LD4 protein for pDNA delivery is set on the possibility that this protein could mediate pDNA trafficking throughout the cytoplasm *via* dynein retrograde transport following escape from endosomes. The insertion of a DNA binding domain at the protein N-terminus was decided based on the structural data of the LC8 protein, since interaction with the dynein motors occurs *via* C-terminus and dimer interface [38]. Since the dynein motor complex relies on the microtubule network to mediate the cellular transport, the use of microtubule depolymerizing agents, such as nocodazole, would abort the active transport resulting in the decrease of transfection efficiency.

Therefore, to evaluate the role of microtubules on pDNA:LD4 complexes (1:8000 pDNA:protein molar ratio) mediated gene transfer into HeLa cells, nocodazole was used. By using this drug, we observed a 49% decrease in the transfection efficiency (Fig. 8). Similarly, cytochalasin D was used to evaluate the effect of actin filaments disruption on transfection efficiency (Fig. 8). The disruption of the actin filaments lead to a decrease in transfection efficiency of 99%. The results indicate that pDNA:LD4 complexes strongly rely on the cells cytoskeleton for intracellular trafficking and pDNA delivery. However, the decrease in transfection efficiency caused by the disruption of the cytoskeleton has already been described in the literature using pDNA:Lipofectamine™ and pDNA:PEI (polyethyleneimine) complexes [39,40]. Despite microtubules tend to facilitate intracellular trafficking of vectors *via* active retrograde transport of the vesicles formed after endocytosis, they can also contribute to their degradation since most of the vectors remain entrapped inside late endosomes and lysosomes and are finally destroyed in these vesicles [41,42]. On the other hand, the observed decrease in transfection efficiency when cells were pre-treated with cytochalasin D can be credited to the role attributed to actin filaments in the early steps of complex entry in the cell [40] as they are important to receptor-mediated endocytosis and might be involved in other pathways of internalization [43]. Disruption of actin filaments tends to decrease the internalization of complexes and hence lower transfection efficiency.

In order to further investigate the endosomal entrapment, which stands as a limiting step for gene delivery efficiency [44] of the pDNA:LD4 complexes, we performed transfections of HeLa cells treated with the lysosomotropic agent chloroquine (Fig. 8). Cloroquine is a weak base that accumulates in acidic organelles such as late endosomes and lysosomes, raising the luminal pH of the organelles and avoiding enzymatic degradation of non-viral vectors [45]. This drug is frequently used to investigate the effect of the endosomal/lysosomal entrapment as a barrier to gene delivery, since its action causes a

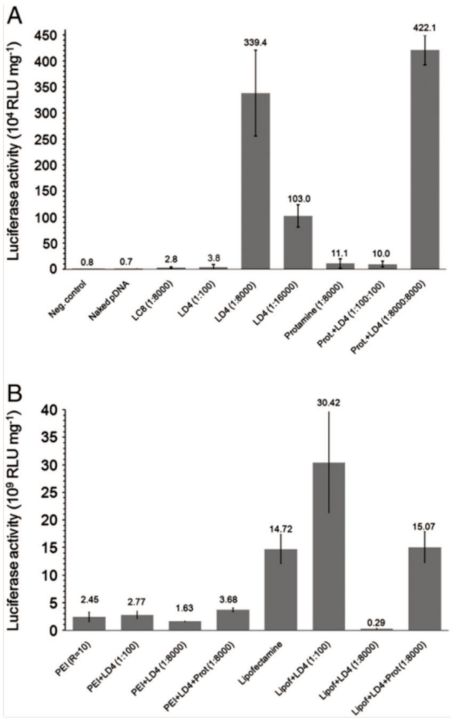

Fig. 7. A: Gene transfer to HeLa cells mediated by pDNA:LC8 and pDNA:LD4 complexes. Transfection efficiency was assessed by measuring the activity of the luciferase reporter gene. B: Comparison of the transfection efficiency of the LD4 fusion protein with the traditional delivery vectors PEI and Lipofectamine™. The combination of proteins (LD4 and protamine) with Lipofectamine™, resulting in ternary vectors, was also evaluated. Error bars indicate standard deviation between triplicates.

rise in intraendosomal osmolarity and its eventual lysis [46,47]. In our case, pre-treatment of HeLa cells with chloroquine (100 μM) enhanced the LD4 mediated transfection efficiency by 383%. Considering

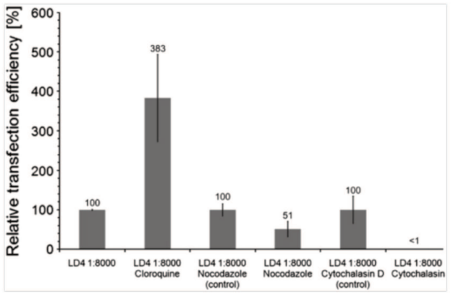

Fig. 8. Involvement of microtubules and endosomes/lysosomes on transfection efficiency of HeLa cells using LD4:pDNA complexes. The involvement of microtubules and actin were studied using the drugs nocodazole and cytochalasin D, respectively. The lysosomotropic agent chloroquine was used to investigate the effect of the endosomal/lysosomal entrapment as a barrier to gene delivery. Experiments were performed in triplicate as described in the Materials and methods section. Error bars indicate standard deviation between triplicates.

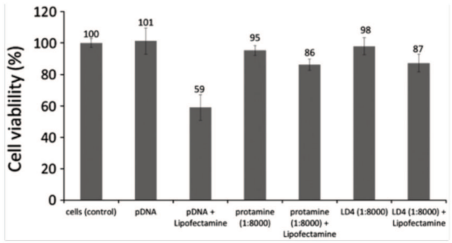

Fig. 9. Evaluation of the cytotoxicity of the different delivery vectors for HeLa cells performed using WST-1 reagent (Roche Applied Science). We assayed the cell viability after transfection with complexes formed by pDNA with lipofectamine, protamine and LD4 according to the procedure described in the Materials and methods section. Error bars indicate standard deviation between six replicates.

this data, escape from endosomes and lysosomes seems critical for the efficiency of LD4 mediated gene delivery and also for the understanding of the mechanisms of the intracellular trafficking of these pDNA:protein complexes. Beside this, we should also consider that an early release of pDNA:LD4 complexes in the cytosol promoted by chloroquine has another positive effect, since the complex may still rely on the retrograde transport to get closer to the cell nucleus *via* direct interaction between LD4 and dynein. In fact, we performed transfections using pDNA:protamine complexes in the presence of chloroquine and we found that the drug promoted an increase of 11-fold in Luciferase expression (Supplementary Data). Taking this result into account, LD4 seems to be more efficient in endosomal/lysosomal escape than protamine.

Finally, since both, pDNA:LD4 and pDNA:protamine, complexes used in this study have similar physical-chemical characteristics (size, charge ratio, nature, etc.), we believe that the differences found in transfection efficiency between LD4 and protamine mediated transfection (30-fold) may also be related to the natural ability of the LD4 to interact with the dynein motor and facilitate the intracellular trafficking. A complete study of the differences in intracellular trafficking of the different complexes studied here is still in progress by our research group. However, due to the complex nature of the vectors and the diversity of cellular entry pathways and intracellular trafficking, this is a very challenging task.

3.7. Plasmid DNA:LD4 complexes presented reduced cytotoxicity when compared to pDNA:Lipofectamine™ complexes

Cytotoxicity of the delivery vectors used in the present work was evaluated using the Cell Proliferation Reagent WST-1 (Roche Applied Science, USA) and cultivated HeLa cells. As expected, protein vectors proved to be far less toxic (98% and 95% cell viability for LD4 and protamine, respectively) than Lipofectamine™ (59% cell viability) (Fig. 9). Interestingly, the combination of LD4 and Lipofectamine™ lead to a reduced cell mortality comparing to the complex formed by cationic lipid and pDNA. So far, it is not clear the mechanisms behind the reduction of toxicity caused by the presence of the proteins in the ternary complexes. A possible explanation could be the reduction of the cationic lipid content in these complexes caused by competition with the proteins for the negatively charged plasmid backbone. Anyway, an important attribute of a gene delivery vehicle is the low toxicity, opening the possibility for a secure *in vivo* utilization, and the results indicate that pDNA:LD4 is a promising vector.

4. Conclusion

We presented here an innovative approach for the non-viral delivery of plasmid DNA. By combining the recombinant dynein light chain LC8 with a synthetic N-terminal DNA binding domain we were able to

construct a modular fusion protein specifically designed for gene delivery. The modified LC8, named LD4, was able to: i) interact and condense pDNA *in vitro* forming positively charged complexes and ii) to interact *in vitro* with dynein intermediate chain, confirming its functionality. Despite still far from being an optimized vector like the commercial lipids Lipofectamine™, LD4 proved to be 30-fold more efficient in transfection than protamine and 485-fold more efficient than naked DNA – this one the delivery method used in most of the non-viral clinical trials conducted so far. Transfection and cytotoxity assays also indicated that LD4 can be associated to cationic lipids to generate even more efficient delivery vectors for *in vitro* applications. Pre-treatment of the transfected HeLa cells with different drugs showed a major involvement of the cell's cytoskeleton in the intracellular trafficking, indicating the involvement of the dynein molecular motor in the transport of the pDNA:LD4 complexes. Despite the transfection results found using the LD4 vector are promising, the results also indicate the possibility of different forms of vector optimization, particularly particle charge and the ability to escape from endosomes/lysosomes. Finally, we believe that the work presented here add new information on the development of recombinant modular proteins specifically designed for gene delivery. By taking advantage of strategies used by virus to infect mammalian cells these vectors may, in the near future, increase the efficiency of non viral vectors and provide new tools for DNA vaccination and gene therapy studies.

Supplementary materials related to this article can be found online at doi:10.1016/j.jconrel.2012.01.011.

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