Miguel Mitne Neto

Análise *in vitro* da esclerose lateral
amiotrófica tipo 8 e
Estudo genético da paraplegia espástica 4

In vitro analysis of amyotrophic lateral
sclerosis type 8 and
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Dedicatória

*À minha mãe
Ao meu irmão
À Tessa*

Epígrafe

*“...I am the master of my fate
I am the captain of my soul.”*

Invictus

William Ernest Henley

Epígrafe

"Pain is temporary. Quitting lasts forever"

Lance Armstrong

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Nota do Autor

O presente trabalho visa contribuir para o entendimento das doenças do neurônio motor, especialmente a Esclerose Lateral Amiotrófica e a Paraplegia Espástica Hereditária. A tese está dividida em cinco capítulos. No primeiro capítulo apresentamos uma introdução sobre os aspectos clínicos e moleculares envolvidos com as doenças mencionadas. O capítulo dois apresenta os resultados de estudos de expressão das formas selvagem e mutante (P56S) da proteína VAPB recombinante, bem como os achados sobre a interação destas formas com outras proteínas celulares, publicados no periódico *Protein Expression and Purification*. O Capítulo três traz os resultados da geração de células *iPSC* de pacientes com ELA8 e sua diferenciação em neurônios motores. Estes resultados estão apresentados sob a forma de um manuscrito que, no momento do fechamento da tese, está em fase final de preparação, a fim de ser submetido para publicação em periódico internacional. No capítulo quatro apresentamos os achados da avaliação de trinta indivíduos, de uma mesma família, portadores de uma nova mutação no gene *SPG4*, publicado no periódico *European Journal of Human Genetics*. Este capítulo contém uma figura suplementar (Fig. S.1) que não foi utilizada no manuscrito, mas está apresentada para demonstrar os pontos de quebra envolvendo a duplicação encontrada. O capítulo cinco é composto pela discussão geral e pelas conclusões do trabalho.

O material utilizado e os métodos estão apresentados nas respectivas seções de cada um dos manuscritos.

No anexo 1 encontra-se um trabalho publicado no periódico *Clinical Genetics*, no qual participamos com a avaliação do haplótipo em torno do gene *VAPB*, na descrição do primeiro paciente com ELA8 fora do Brasil.

O anexo 2 apresenta uma carta enviada por nós ao editor do periódico *Journal of Medical Genetics*, publicada no website da revista como *eLetter*. Nesta, tecemos comentários sobre um dos trabalhos apresentados neste periódico, que identificou a mutação c.C>T166 no gene *VAPB* em pacientes Nipo-descendentes.

As referências citadas nas seções de introdução e discussão estão listadas logo a seguir do capítulo 5.

Abreviações

ABrELA – Associação Brasileira de Esclerose Lateral Amiotrófica

AEP – Atrofia Espinhal Progressiva

BMAA – Beta-metil-amino-L-alanina

CG – Complexo de Golgi

DNM – Doença do Neurônio Motor

ELA – Esclerose Lateral Amiotrófica

Eph – receptor de epfrina

FFAT – Duas fenilalaninas em um trato ácido – motivo protéico com o qual o domínio MSP é capaz de interagir

FUS/TLS – Fused in Sarcoma / Translated in Liposarcoma

iPSC- *Induced Pluripotent Stem Cells* – células pluripotentes induzidas

JM – Junção Neuromuscular

MLPA – *Multiplex Ligation-dependent Amplification*

MSP – *Major Sperm Protein Domain*

NMDA – Receptor Ácido *N*-Metil-Aspártico – um tipo de receptor de glutamato

NMI – Neurônio Motor Inferior

NMS – Neurônio Motor Superior

RE – Retículo Endoplasmático

SMN – *Survival Motor Neuron Protein*

SOD1 – Super Oxide Dismutase 1

SPG4 – Spastic Paraplegia

TDP-43 - TAR DNA-binding protein 43

VAPB – Vesicle-Associated Membrane Protein-Associated protein-B/C

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Capítulo 1

I. INTRODUÇÃO

1 Doenças do Neurônio Motor

As doenças do neurônio motor (DNM) compreendem um grupo muito heterogêneo de afecções que comprometem a atividade neuromuscular. A atrofia e fraqueza muscular são decorrentes da morte dos neurônios responsáveis por conduzir a atividade nervosa para os músculos.

Os estímulos nervosos partem do córtex motor, de onde são conduzidos pelos neurônios motores superiores (NMS) até a região da medula espinhal. A partir deste local, esses sinais são levados pelos neurônios motores inferiores (NMI) até a placa motora, região de interação entre o neurônio e a fibra muscular, onde são liberados para controlar a contração dos músculos.

A classificação das DNMs depende, entre outros aspectos, do segmento motor afetado. A morte dos NMIs provoca doenças como a atrofia espinhal progressiva (AEP) ou atrofia espinobulbar progressiva (também conhecida como doença de Kennedy). No caso de morte dos NMS, podem ser observadas diversas formas de paraplegias espásticas ou ainda a esclerose lateral primária. Quando ambos os segmentos, superior e inferior, são afetados têm-se um quadro de esclerose lateral amiotrófica (ELA). A seguir estão descritos alguns dos aspectos envolvendo duas destas doenças.

1.1 Esclerose Lateral Amiotrófica

A Esclerose Lateral Amiotrófica (ELA), também conhecida como Doença de Lou Gehrig, é a forma mais comum de DNM, com início tardio (Goodall *et al.*, 2006). As características da doença foram, inicialmente, definidas por Jean-Martin Charcot, em 1874. O acometimento acontece pelo endurecimento (esclerose) das raízes laterais da medula espinhal (lateral), que acabam por levar à fraqueza e atrofia muscular (amiotrófica) (Oliveira & Pereira 2009).

Atualmente, o diagnóstico da ELA é estabelecido pela identificação de características presentes no *El Escorial* (Brooks, 1994) apoiados pela avaliação eletroneuromiográfica (de Carvalho *et al.*, 2008 e www.wfnals.org/guidelines). Para o fechamento do quadro clínico, faz-se necessária também, a exclusão de outras doenças que possam comprometer a função do neurônio motor, como neuropatia periférica, Síndrome Pós-Poliomielite ou Miastenia Grave entre outras (Radunovic *et al.*, 2007).

Os primeiros sintomas da doença aparecem, normalmente, entre a 4ª e 6ª década de vida e compreendem o declínio de força, geralmente com assimetria lateral, acompanhado de câimbras, e fasciculações (contrações rápidas e involuntárias da musculatura). Achados clínicos como espasticidade e hiperreflexia são devidos à degeneração dos NMSs, enquanto a perda dos NMIs provoca fasciculações, atrofia e fraqueza muscular. A disfagia, dificuldade na deglutição e a disartria, dificuldade de fala, são causadas quando há comprometimento dos neurônios motores da região bulbar.

A degeneração provocada pela ELA é irreversível e em estágios avançados são afetados os quatro membros, musculatura facial, cervical e torácica. Nesta etapa, os NMs apresentam-se com um quinto do diâmetro normal, o citoplasma e núcleo mostram-se extremamente condensados e o corpo celular adota formato fusiforme (Guégan & Przedborski 2003).

A evolução da doença é rápida levando os pacientes ao óbito entre 3 a 5 anos após o diagnóstico. Em geral, a morte é devida à falência respiratória (Donkervoort & Siddique 2001 – 2009).

Normalmente, transcorrem 11 meses do primeiro sintoma à confirmação do diagnóstico (www.tudosobreela.com.br). Infelizmente, até o momento, não existe um biomarcador que contribua para o diagnóstico da ELA nem qualquer elemento que permita diagnosticar indivíduos em fase pré-sintomática da doença. Esta situação agrava o já reduzido tempo de sobrevida do paciente.

O único medicamento aprovado para o tratamento da doença é o riluzol, que atua em uma das vias do glutamato (Mitchell & Borasio 2007), como discutido adiante. Contudo, mostrou-se que seu efeito é limitado, estendendo a sobrevida dos pacientes em apenas 2 – 4 meses (Bensimon *et al.*, 1994, Miller *et al.*, 2007).

O tratamento da ELA é paliativo, sendo essencial a atuação de uma equipe multidisciplinar capaz de assistir o paciente com fisioterapia motora e respiratória, orientação psicológica e nutricional, além de apoio de serviço social para o próprio paciente, seus familiares e cuidadores.

Na população mundial, calcula-se uma incidência de 1-2 casos em cada 100.000 e prevalência entre 4-13 pessoas em cada 100.000. Em nosso país, a

ABrELA (Associação Brasileira de Esclerose Lateral Amiotrófica) estimou em, aproximadamente, 1.5 novos casos por ano, a cada 100.000 indivíduos, dados de 1998.

Estudos recentes apontam para um aumento do número de casos de ELA. Entretanto, não se pode definir se este fenômeno ocorreu por uma maior atenção dada à doença, um melhor treinamento dos neurologistas para o diagnóstico, ou se algum fator ambiental estaria influenciando essa estatística (Associação Americana de ELA <http://www.alsa.org/>).

1.1.1 Formas

1.1.1.1 ELA Esporádica

Aproximadamente, 90% dos casos são ditos esporádicos. Nestes, desconhece-se uma causa específica para o desenvolvimento da ELA e especula-se que fatores genéticos somados a efeitos ambientais possam contribuir para a sua emergência. A exposição a neurotoxinas, como aquela sofrida por veteranos da Guerra do Golfo, o contato com inseticidas e pesticidas, bem como a interferência de campos eletromagnéticos foram propostos como fatores que aumentariam a predisposição à ELA. Além desses, a atividade física extenuante também estaria envolvida com o desencadeamento da doença. Esta hipótese foi levantada depois que um estudo italiano constatou uma alta incidência da ELA entre ex-futebolistas, que atuaram naquele país (Chiò *et al.*, 2005). Trabalhos relacionando injúrias repetidas na cabeça com o aparecimento da doença reforçam esta hipótese. (Dion *et al.*, 2009).

1.1.1.2 ELA Guam

Um tipo especial de ELA, associada a características de doença de Alzheimer e Parkinson, é encontrado na população Chamorro, da ilha de Guam, no Oceano Pacífico. Desde a década de 1950, o local chama a atenção pela altíssima prevalência da doença, cerca de cinquenta vezes maior quando comparada ao restante do globo (Cox *et al.*, 2003; Cox *et al.*, 2007). Após diversos estudos, verificou-se que o tecido cerebral dos pacientes da ilha apresentava altos índices do aminoácido neurotóxico, não-protéico, beta-metil-amino-L-alanina (BMAA). Uma explicação para este fenômeno viria da dieta da população local e da biomagnificação do BMAA (Cox *et al.*, 2003). Este composto é produzido por cianobactérias que vivem em simbiose com plantas do gênero *Cyca*. Morcegos conhecidos como “*Flying Fox*” alimentam-se das sementes de *Cyca sp.*, acumulando o BMAA em seus tecidos. Surpreendentemente, esses mamíferos constituem uma iguaria da culinária local e dessa forma, os habitantes locais estariam sujeitos a ingestão de grandes quantidades do BMAA. Entretanto, não se sabe, ao certo, se o BMAA é o fator determinante para que a ELA apareça em conjunto com características de doença de Alzheimer e Parkinson.

1.1.1.3 ELA Familiar

Dez por cento de todos os casos de ELA são transmitidos de uma geração para a outra e, portanto, denominados casos familiares. Clinicamente, não é possível distinguí-los dos casos esporádicos.

Dezessete regiões do genoma foram descritas como sendo responsáveis pelo aparecimento da ELA familiar, todavia, apenas 11 genes foram identificados (Tabela 1). Em algumas dessas formas, as características da ELA estão associadas

a achados de Demência Frontotemporal e doença de Parkinson (Dion *et al.*, 2009). As formas familiares podem apresentar padrão de herança autossômico recessivo, dominante ligado ao cromossomo X, ou na maioria dos casos, autossômico dominante.

Tabela1: Lócus e genes identificados como responsáveis pela ELA – adaptado de Beleza-Meireles & Brown 2009; Dion *et al.*, 2009. Os tipos ELA* e ELA** representam formas da doença que, apesar de estarem relacionadas com determinado gene, ainda não apresentaram consenso sobre o seu depósito, junto ao banco de dados Online Mendelian Inheritance in Man (OMIM).

Tipo	Cromossomo	Gene	MIM
ELA1	21q	<i>SOD1</i>	#147450
ELA2	2q	<i>ALS2</i>	#606352
ELA3	18q	?	#606640
ELA4	9q	<i>SETX</i>	#_602433
ELA5	15q	?	#_602099
ELA6	16q	<i>FUS/TLS</i>	#_608030
ELA7	20 <p>p</p>	?	#608031
ELA8	20 <p>q</p>	<i>VAPB</i>	#608627
ELA9	14q	<i>ANG</i>	#_105850
ELA10	1p	<i>TARDBP</i>	#_605078
ELA11	6q	<i>FIG4</i>	#612577
ELA12	10p	<i>OPTN</i>	#613435
ELA*	12q	<i>DAO</i>	Mitchell et al 2010
ELA**	2p	<i>DCTN1</i>	#601143
ELA-DFT1	9q	?	Hosler et al 2000
ELA-DFT2	9p	?	Morita et al 2006; Valdmanis et al 2007
ELA-X	Xcen	?	Siddique et al 1998

A seguir estão destacadas algumas delas.

1.1.1.3. A – ELA1 (SOD1)

A ELA1 é causada por mutações no gene *SOD1* (Rosen *et al.*, 1993). A proteína por ele produzida, superóxido dismutase 1 ou SOD1, é responsável pelo metabolismo do ânion superóxido (O_2^-), um dos mais reativos radicais livres encontrados nos sistemas biológicos. Vinte por cento dos casos de ELA familiar são causados por mutações no *SOD1*, sendo que 140 mutações já foram descritas ao longo deste gene (<http://www.hgmd.cf.ac.uk/ac/index.php>). Estudos apontam que as alterações no *SOD1* acabam por conduzir a um ganho de função tóxica do polipeptídeo, no lugar de levarem à perda da atividade enzimática, (Gros-Louis *et al.*, 2006; Pasinelli & Brown 2006; Dion *et al.*, 2009).

Camundongos transgênicos, que super expressam versões mutantes da *SOD1*, contribuíram muito para a compreensão dos mecanismos responsáveis pela doença. Assim como nos pacientes portadores de mutação no *SOD1*, os murinos apresentam agregados citoplasmáticos que seriam tóxicos (Deng *et al.*, 2006; Bruijn *et al.*, 1998). Nesses animais, o início dos sintomas ocorre em torno de 90 dias de vida e o óbito advém 5-6 meses após o nascimento (Hensley *et al.*, 2006). O alto percentual de casos familiares devidos ao *SOD1*, a sua ocorrência em diversas populações e a disponibilidade desses animais fizeram com que os transgênicos de *SOD1* se tornassem o principal modelo de teste de drogas no combate à doença.

O estudo das formas familiares tem ajudado a testar hipóteses que expliquem também o desenvolvimento da ELA esporádica. Ezzi *et al.* (2007) mostraram que a oxidação da *SOD1* faria com que o polipeptídeo adquirisse características semelhantes àsquelas apresentadas por cadeias protéicas portadoras de mutação.

Além disso, a SOD1 selvagem oxidada promove a morte de neurônios motores de forma dose-dependente, assim como é observado para a SOD1 mutante (Ezzi *et al.*, 2007). Na mesma linha de estudo, Bosco *et al.*, (2010^B) mostraram que a estrutura da SOD1 oxidada é muito semelhante àquela apresentada por polipeptídeos com a mutação G93A. Isto foi possível pelo desenvolvimento de um anticorpo específico, capaz de reconhecer apenas cadeias alteradas da proteína. Os autores observaram que 44% das amostras de medula espinhal de pacientes com ELA esporádica, apresentaram imunoreatividade para este anticorpo. Adicionalmente, eles mostraram que, assim como as formas mutantes, as versões oxidadas e aquelas purificadas a partir de tecidos de pacientes com ELA esporádica inibem o rápido transporte anterógrado nos axônios (Bosco *et al.*, 2010^B). Estes dados sugerem a ação de componentes da ELA familiar na patogênese das formas esporádicas, além de ilustrarem uma estreita relação entre os casos esporádicos e a ação da SOD1.

1.1.1.3. B – ELA6 (*FUS/TLS*)

Dois trabalhos independentes (Vance *et al.*, 2009; Kwiatkowski *et al.*, 2009) mostraram que mutações no gene *FUS/TLS* são responsáveis pela ELA6. A proteína codificada por esse gene apresenta funções muito semelhantes à TDP-43 (ELA10), sendo que a principal é a sua interação com o DNA e o RNA. A FUS nativa é encontrada no núcleo celular e seria um dos componentes do spliceossomo – maquinaria responsável pelo *splicing* (encadeamento) para a formação do RNA maduro. Assim como encontrado em outras formas da doença, células carregando versões mutadas da FUS apresentam agregados citoplasmáticos contendo esta proteína (Bosco *et al.*, 2010^A).

1.1.1.3. C – ELA10 (*TARDBP*)

As primeiras suspeitas da relação entre a TDP-43 e a ELA vieram da identificação de inclusões citoplasmáticas de TDP-43 nos neurônios de pacientes com formas específicas de ELA associada à Demência Frontotemporal (Neuman *et al.*, 2006 revisado Damme *et al.*, 2010). Posteriormente, mostrou-se que mutações no gene *TARDBP* segregavam junto com os pacientes pertencentes a famílias com histórico de ELA (Gitcho *et al.*, 2008; Sreedharan *et al.*, 2008; Kabashi *et al.*, 2008). Até o momento, mais de 25 mutações foram identificadas, quase todas na região que codifica a porção C-terminal da TDP-43 (Lagier-Tourenne & Cleveland 2009).

A TDP-43, assim como a FUS, é encontrada majoritariamente no núcleo (Nishimura *et al.*, 2010) e estaria envolvida com a transcrição de RNA mensageiro e com o mecanismo de *splicing* (Largier-Tourenne & Cleveland 2009).

Agregados de TDP-43 no citoplasma sugerem a ocorrência de um processo neurodegenerativo. Eles são identificados em pacientes com Demência Frontotemporal, em 25% dos casos de Doença de Alzheimer (Lagier-Tourenne *et al.*, 2010) em quase todos os casos de ELA esporádica, bem como nos casos de ELA familiar que não apresentam mutação no gene *SOD1* (Mackenzie *et al.*, 2007).

Os agregados de TDP-43 e *SOD1* seriam mutuamente exclusivos, pois não foram encontradas inclusões de *SOD1* em casos de ELA10, tampouco, agregados TDP-43 estariam presentes em pacientes com ELA1 (Maruyama *et al.*, 2010).

A identificação da TDP-43 e da FUS como causadoras da ELA permitiu propor uma nova via para a patogênese da doença: a alteração no metabolismo de RNA (Lagier-Tourenne & Cleveland, 2009). A hipótese de que o processamento e

transporte do RNA atuar na ELA é reforçada pelos achados relacionados a outras proteínas como a Senataxina e a SMN. Elas também atuam no metabolismo de ácidos nucleicos e são responsáveis por formas familiares de doenças do neurônio motor, como a ELA4 e a Atrofia Muscular Espinhal, respectivamente.

1.1.1.3.D – ELA8 (VAPB)

A ELA8 foi inicialmente mapeada, pelo nosso grupo, em 20q13, após o estudo de uma grande irmandade brasileira com ancestrais portugueses (Nishimura *et al.*, 2004^A). Em seguida, mostramos que a mutação c.C>T₁₆₆ no gene *VAPB/C* estava presente nesta e em outras 6 famílias. A transição pirimidínica leva à troca de uma prolina por uma serina na posição 56 (P56S) da proteína *vesicle-associated membrane protein-associated protein-B/C*, ou VAPB (Nishimura *et al.*, 2004^B). As famílias brasileiras analisadas apresentam um haplótipo comum em torno do *VAPB*, sugerindo um ancestral comum (efeito fundador). Este é calculado em torno de 500 anos atrás, concomitante com a colonização portuguesa em nosso país (Nishimura *et al.*, 2005).

Até o momento, foram encontradas 14 famílias apresentando ELA8, com mais de 100 pacientes confirmados, após avaliação genética, como portadores da mutação c.C>T₁₆₆ (dados não publicados). Contudo, essa mutação não está mais restrita ao Brasil e aos pacientes Luso-descendentes. A mesma mutação no gene *VAPB* foi encontrada na Alemanha (Funke *et al.*, 2010) e em pacientes Nipo-descendentes (Millecamps *et al.*, 2010). A análise do haplótipo mostrou que o paciente alemão não compartilha os mesmos marcadores que as famílias brasileiras, sugerindo que a mutação c.C>T₁₆₆ teria aparecido em dois eventos independentes (Funke *et al.*, 2010). Em estudo recente, foi identificada uma nova

mutação no *VAPB*. Ela provoca a alteração T46I, que foi encontrada em um paciente britânico e leva a efeitos moleculares semelhantes à P56S (Chen *et al.*, 2010), como discutido a seguir.

Existem três formas de VAP em humanos: VAP-A, B e C. A VAPA é codificada por um gene no cromossomo 18 e possui 63% de identidade com a VAPB. A VAPC é uma isoforma da VAPB gerada por splicing alternativo.

A VAPB é composta por três domínios: o *Major Sperm Protein* ou MSP é formado pelos primeiros 150 aminoácidos, ele é muito conservado entre todos os ortólogos da VAPB (Nishimura *et al.*, 2004^B) e é nele que se encontra a mutação P56S. A porção central é constituída por um domínio *coiled-coil* (rolo-enrolado) enquanto a porção C-terminal possui um domínio transmembrana (UniProt/Swiss-Prot—095292).

As proteínas da família VAP são expressas de forma ubíqua e localizam-se em porções do retículo endoplasmático (RE) e intermediários entre esta organela e o Complexo de Golgi (CG) (Skehel *et al.*, 2000). Elas estariam envolvidas com diferentes vias moleculares, incluindo algumas diretamente ligadas à atividade dos neurônios motores.

O seu envolvimento com o metabolismo de lipídios ocorre por meio da interação com proteínas contendo o motivo FFAT. Proteínas que interagem com lipídios e que possuem o FFAT ligam-se à VAPB para que possam ser ancoradas às vesículas do RE (Prosser *et al.*, 2008).

Em drosófilas, a proteína ortóloga dVAP-33, liga-se a microtúbulos e regula o número de junções neuromusculares (JM). Nestes modelos, a superexpressão da

dVAP-33 reduz o tamanho das JM e aumenta o seu número. Em contrapartida, em animais que não expressam esta proteína observa-se o efeito oposto (Pennetta *et al.*, 2002).

Assim como observado em outras formas de ELA familiar, a superexpressão da VAPB mutante (P56S) leva à formação de agregados citoplasmáticos. Eles são encontrados tanto em modelos *in vitro* (células de mamíferos) quanto *in vivo* (drosófilas e camundongos) (Nishimura *et al.*, 2004^B; Kanekura *et al.*, 2006; Teuling *et al.*, 2007; Tsuda *et al.*, 2008; Prosser *et al.*, 2008; Suzuki *et al.*, 2009; Fasana *et al.*, 2010; Tudor *et al.*, 2010). Além disso, os agregados de P56-VAPB são insolúveis em frações detergentes (Kanekura *et al.*, 2006; Teuling *et al.*, 2007; Suzuki *et al.*, 2009) e sofrem ubiquitinação (Kanekura *et al.*, 2006; Tsuda *et al.*, 2008; Tudor *et al.*, 2010).

O sistema Ubiquitina/Proteasoma é o principal responsável pelo controle de qualidade das proteínas nas células. Cadeias polipeptídicas mal-formadas, mesmo após a ação das Chaperonas no RE, acabam sendo poli-ubiquitinadas e degradadas pelo Proteasoma. Nos casos em que o sistema não consegue processá-las adequadamente, ocorre o acúmulo destas cadeias podendo levar à apoptose (Huang & Figueiredo-Pereira 2010).

A porção N-terminal da VAPB, contendo o MSP, é clivada e secretada. Utilizando a modelagem em *C.elegans* mostrou-se que a injeção da porção correspondente ao domínio MSP da VAPB humana é capaz de induzir efeitos semelhantes à proteína homóloga encontrada no verme: contração do ovário e maturação dos oócitos. Além disso, identificou-se uma interação entre o MSP e receptores do tipo *Eph* (ou receptores de efrina) (Tsuda *et al.*, 2008). Entre outras

funções, os receptores *Eph* promovem o agrupamento de outros receptores, como aqueles do tipo NMDA, sugerindo um envolvimento da VAPB com vias glutamatérgicas. (Susan & Cox 2008). Neste cenário, Tsuda e col. (2008) propõem que a ELA8 teria tanto um componente autônomo da célula, pela formação de agregados citoplasmáticos, quanto não-autônomo, pois estes agregados recrutam a VAPB selvagem diminuindo a sua disponibilidade para secreção e interação com receptores *Eph* (Tsuda *et al.*, 2008).

Acredita-se que a ação da VAPB não esteja limitada à ELA8, mas também com as formas esporádicas da doença. Mostrou-se que a VAPB está diminuída na medula espinal de pacientes com ALS esporádica (Teuling *et al.*, 2007; Anagnostou *et al.*, 2008) e em camundongos transgênicos com super-expressão de SOD1 mutante. No caso dos murinos, os níveis diminuem de acordo com a progressão da doença (Teuling *et al.*, 2007).

Tudor *et al.*, (2010) geraram linhagens de camundongos transgênicos com super-expressão da VAPB. Tanto a linhagem mutante (P56S) quanto a selvagem apresentaram desenvolvimento normal e não foram detectados sinais de doença do neurônio motor, mesmo após 2 anos de acompanhamento. Entretanto, os animais com expressão da proteína mutada, apresentam agregados citoplasmáticos da própria VAPB e da TDP-43. Os autores mostraram que os agregados de VAPB co-localizam com os de TDP-43, sugerindo uma contribuição de ambas as proteínas para a formação das inclusões (Tudor *et al.*, 2010). Entretanto, eles não apresentam uma explicação para a ausência de fenótipo nestes animais.

1.1.2 Mecanismos envolvidos

O estudo das diversas formas de ELA permitiu propor hipóteses para o desenvolvimento da doença. Entretanto, nenhuma delas foi capaz de explicar todo o espectro de características clínicas e moleculares.

Abaixo estão apresentados alguns dos mecanismos que estariam relacionados à doença:

1.1.2 A - Excitotoxicidade do glutamato

O glutamato é o principal neurotransmissor excitatório do sistema nervoso. Para a correta atividade sináptica entre os NMS e os NMI é necessário que o glutamato excedente seja recaptado pelas células da astrocíticas, por meio dos receptores GLT1. Nos pacientes com ELA observa-se uma diminuição da quantidade de GLT1 nas células da glia. Ainda, níveis aumentados de glutamato no soro e no líquido foram encontrados em amostras de pacientes com ELA (Silva, 2006). Foi proposto que o excesso de glutamato na fenda sináptica levaria a uma hiper-ativação de canais de Cálcio (Ca^{2+}), liberando grandes quantidades do íon no citoplasma. O Ca^{2+} é um mensageiro capaz de disparar diversas vias intracelulares. Em grandes quantidades no citoplasma, ele seria responsável pelo aumento na produção de radicais livres e a ativação de vias apoptóticas (Shaw & Ince 1997).

Apesar de ser o único medicamento liberado para o tratamento da doença, ainda não se sabe ao certo o mecanismo de ação do riluzol. Acredita-se que ele atue na recaptação do glutamato, diminuindo suas quantidades na fenda sináptica. Entretanto, pacientes tratados com riluzol não apresentaram redução dos níveis séricos de glutamato (Andreadou *et al.*, 2008)

1.1.2. B - Ação dos radicais livres

As primeiras evidências do envolvimento dos radicais livres na fisiopatologia da doença vieram da descoberta do gene *SOD1* como responsável pela ELA1. Inicialmente, fora proposto que a não metabolização do íon superóxido (O_2^-) por parte da *SOD1* mutada seria uma das causas da doença. Entretanto, mostrou-se que o efeito da *SOD1* está relacionado a um ganho de função e não à falta de atividade catalítica.

Shi *et al.*, (2010) propõem que o estresse oxidativo poderia ser gerado por uma disfunção das mitocôndrias, causado pelo recrutamento anormal de *SOD1* para determinados compartimentos desta organela. As evidências para esta são os estudos de Pasinelli *et al.*, (2004) que mostraram que camundongos transgênicos de *SOD1* apresentam neurônios motores com mitocôndrias vacuolizadas (Pasinelli *et al.*, 2004). Devido à falta da correta estrutura funcional, poderia haver vazamento de produtos metabólicos, sendo que os radicais livres os principais constituintes desta classe (Bento-Abreu *et al.*, 2010).

1.1.2.C – Formação de Agregados Protéicos

Corpos de inclusão são comumente encontrados em diferentes doenças neurodegenerativas, como Alzheimer, Parkinson, Huntington e na própria ELA (Kim *et al.*, 2010). Eles são formados por cadeias polipeptídicas sem estrutura que se aglomeram e geram, na maior parte das vezes, estruturas insolúveis. Se estes corpos de inclusão são efetores da degeneração, ou se eles constituem apenas uma cicatriz dos processos intracelulares ainda é uma questão em aberto.

Entretanto, o principal embasamento, para considerar os corpos de inclusão como causa da degeneração, está no processamento das proteínas. O retículo endoplasmático é o principal sítio de síntese de cadeias polipeptídicas. Nesta organela, as Chaperonas ajudam no dobramento de proteínas nascentes que posteriormente passarão por outros processos de modificação pós-traducional. As cadeias que escapam deste processamento não apresentam estrutura nativa e acabam sendo direcionadas para o citoplasma, onde possivelmente, serão marcadas com etiquetas de Ubiquitina para futura degradação pelo Proteasoma. Desestruturadas, as proteínas agregam formando corpos de inclusão. Nos processos neurodegenerativos haveria uma sobrecarga tanto do mecanismo das Chaperonas quanto do sistema Ubiquitina/Proteasoma que acabam por disparar vias apoptóticas. Outra proposta sugere que a intensa ocupação do Proteasoma, pelas cadeias mal-formadas, impede que este promova o correto processamento de outras proteínas celulares, levando a ineficiência do sistema de controle protéico (Pasinelli & Brown 2006).

Ainda, os agregados protéicos são capazes de seqüestrar cadeias com correto dobramento (estrutura nativa), diminuindo a disponibilidade delas para o metabolismo regular. Esta situação é mais evidente nos casos em que as proteínas atuam como dímeros ou multímeros, como a SOD1 ou a VAPB.

1.1.2.D – Efeito Autônomo X Não-Autônomo

Os mecanismos que desencadeiam a morte dos neurônios motores podem ser gerados a partir do próprio neurônio motor (autônomo) ou pelas células em seu entorno (não-autônomo), também denominadas de células da glia.

Apesar de a ação autônoma ser a mais estudada, recentemente, observou-se crescente interesse pelos efeitos não-autônomos. A glia corresponde a um grupo de células não-neuronais responsável pelo suporte aos neurônios. Entre outras atividades a glia está envolvida com a resposta a injúrias, stress pelo calor e recaptação de substâncias como o neurotransmissor glutamato. Com o objetivo de verificar o efeito destas células sobre a sobrevivência dos neurônios motores, dois grupos independentes utilizaram a estratégia de co-cultivo (neurônios motores sobre astrócitos – um tipo de célula da glia). Eles mostraram que quando os neurônios saudáveis eram cultivados sobre os astrócitos portadores de mutação no *SOD1*, havia maior morte celular do que quando os neurônios mutantes estavam sobre os astrócitos saudáveis (Marchetto *et al.*, 2008; di Giorgio *et al.*, 2008). Os astrócitos mutantes induzem vias inflamatórias nos neurônios motores e ainda ativam processos envolvendo o óxido nítrico – responsável pelo aumento de radicais livres (Marchetto *et al.*, 2008). Adicionalmente, Marchetto *et al.*, (2008) identificaram que a Apocinina (*Apocynin*) seria uma das moléculas capazes de inibir o estresse gerado pela glia mutada, sugerindo-a como futura alternativa terapêutica (Marchetto *et al.*, 2008).

1.1.3 Modelagem da ELA

Os modelos gerados para estudar a doença, até o momento, estão baseados principalmente na super-expressão de determinadas proteínas mutantes, seja ela a *SOD1*, *VAPB* ou *TDP-43*. Entretanto, diferenças entre as espécies e particularidades dos modelos impediram que um número substancial de drogas, efetivas nos estágios pré-clínicos, apresentassem o mesmo efeito quando testadas nos pacientes (Ludolph *et al.*, 2010).

A análise de neurônios obtidos diretamente dos pacientes permitiria uma avaliação mais fidedigna dos efeitos moleculares gerados pela ELA. Entretanto, essa estratégia esbarra em questões éticas e metodológicas. Um das opções para contornar tais obstáculos seria o emprego de células *iPSC* (*induced pluripotent stem cells*). A tecnologia que leva à formação das *iPSC* foi inicialmente proposta pelo grupo de Shinya Yamanaka (Takahashi *et al.*, 2006) e consiste na reprogramação de células somáticas para que apresentem características semelhantes as de células-tronco embrionárias. Para tanto, faz-se uso da expressão forçada de fatores de transcrição como *KLF4*, *Oct3/4*, *SOX2* e *c-Myc* (Takahashi *et al.*, 2007), conhecidos como fatores de Yamanaka; ou pelos fatores de Thomson, *Oct3/4*, *SOX2*, *Nanog* e *Lin28* (Yu *et al.*, 2007). A partir do estágio de pluripotência, as células podem ser diferenciadas em qualquer tipo celular.

O uso das *iPSC* passou, portanto, a ser uma alternativa para a modelagem de doenças, *in vitro*. Entretanto, um obstáculo à modelagem seria a interferência do tecido originalmente utilizado para a geração das *iPSC*. Já foi mostrado (Marchetto *et al.*, 2009) que as *iPSC* retêm uma certa “memória” do tecido do qual foram obtidas, mais especificamente, que estas memórias seriam marcas epigenéticas (Kim *et al.*, 2010). Entretanto, Polo *et al.*, (2010) apontam que a memória em questão é mantida apenas em passagens iniciais das células, quando analisadas em passagens mais avançadas elas são apagadas (Polo *et al.*, 2010).

Apesar da controvérsia a respeito das diferenças de acordo com o tecido de origem, as *iPSC* permitem capturar os genomas de indivíduos com doenças neurodegenerativas em células pluripotentes. Modelos baseados em *iPSC* evitam interferências de sistemas de super-expressão e diferentemente dos modelos animais, mantém o background genético humano. Dessa maneira, existe uma maior

similaridade entre o modelo proposto e a situação corrente nos pacientes. Essa foi a estratégia utilizada por diferentes grupos para obter neurônios de pacientes com doenças neurodegenerativas, como Atrofia Muscular Espinhal (Ebert *et al.*, 2009), Disautonomia (Lee *et al.*, 2009), doença de Parkinson (Soldner *et al.*, 2009) e a própria ELA (Dimos *et al.*, 2008). Contudo, ainda não foram observados fenótipos evidentes nas células reprogramadas para os dois últimos casos.

1.2 Paraplegias Espásticas Hereditárias

As paraplegias espásticas são causadas pela degeneração dos neurônios motores superiores, que partem do córtex motor e inervam o corno anterior da espinha dorsal, compondo o trato córtico-espinhal. A degeneração deste trato provoca tanto a espasticidade quanto a fraqueza muscular, ambas progressivas. A hipertonicidade e hiperreflexia dos membros afetados também são características da doença. Dependendo da porção cortical lesada a paralisia pode afetar membros inferiores e/ou superiores, podendo ainda ser unilateral (Reid, 2003).

As paraplegias espásticas hereditárias (PEH) correspondem a um dos principais e mais heterogêneos grupos de doenças do neurônio motor. Cerca de 45 *loci* gênicos já foram relacionados à sua manifestação, entretanto, apenas, 25 genes causadores puderam ser identificados (Dion *et al.*, 2009). A herança da doença pode apresentar padrão autossômico dominante, autossômico recessivo ou ligado ao cromossomo X (Depienne *et al.*, 2007).

Nas formas “puras” a espasticidade ocorre de maneira isolada, enquanto nas “complexas” ela está associada a outras manifestações neurológicas.

As duas principais formas de PEH estão descritas a seguir:

1.2.1 *SPG3A*

A *SPG3* (MIM 182600) é a segunda forma mais comum de PEH, e é responsável por 10% das famílias com PEH pura. O gene *SPG3A* está localizado no cromossomo 14 e codifica a proteína Atlastina. Ela possui alto grau de identidade com a GBP1, membro da superfamília das grandes GTPases de Dinamina (Scarano *et al.*, 2005). A Atlastina está localizada no RE e no CG e está envolvida com o tráfego de vesículas, além do crescimento de neuritos (Álvarez *et al.*, 2010).

A manifestação clínica da *SPG3* é quase indistinguível da *SPG4*, exceto pela idade de início mais precoce encontrada na *SPG3*, geralmente infância e adolescência (Depienne *et al.*, 2007).

1.2.2 *SPG4*

A *SPG4* (MIM 604277) é a forma mais comum de PEH, e compreende aproximadamente 40% dos casos de PEH pura (Fink *et al.*, 2003; Solowska *et al.*, 2010). Mais de 200 mutações foram descritas no gene *SPG4* (também anotado como *SPAST*) todas com padrão autossômico dominante (Fink, 2009).

O produto gênico do *SPG4* (ou *SPAST*) é a proteína Espastina (Spastin- UniProtKB= Q9UBP0). Ela possui 616 aminoácidos, está envolvida na dinâmica e estabilização de microtúbulos (Salinas *et al.*, 2005) e pertence à família das AAA ATPases (ATPases associadas a diversas atividades celulares). Os domínios de interação e ligação com microtúbulos, situados próximos à porção N-terminal, permitem que o domínio AAA, na porção C-terminal, defina o tamanho dos microtúbulos (Errico *et al.*, 2002; Schickel *et al.*, 2007; Solowska *et al.*, 2010).

Mutações de ponto, pequenas deleções e pequenas inserções já haviam sido descritas no *SPG4*. Grande parte delas está concentrada na porção que codifica o domínio AAA da Espastina (Errico *et al.*, 2002). Elas provocariam um mecanismo de perda de função, devido à sua possível interferência na separação dos microtúbulos (Solowska *et al.*, 2010).

O nosso grupo (Starling *et al.*, 2002) descreveu uma grande irmandade brasileira ligada ao locus *SPG4*. Entretanto, na época, nenhuma alteração foi encontrada na região codificadora ou nas sequências intrônicas que flanqueiam os exons componentes do *SPAST*. Destaca-se nesta família, o grande número de indivíduos do sexo masculino, clinicamente afetados pela doença e a presença de mulheres portadoras do haplótipo de risco, mas sem manifestação clínica (Starling *et al.*, 2002). Estas evidências sugerem a existência de agentes moduladores do fenótipo nesta família.

O desenvolvimento da técnica de MLPA (*Multiplex Ligation-dependent Amplification*) ampliou o espectro de mutações causadoras da *SPG4*. Por meio dela, tornou-se possível avaliar diversas sequências de DNA em uma única reação de PCR Multiplex. Os fragmentos gerados são analisados em eletroforese capilar e comparados de acordo com o seu tamanho. Através do MLPA, grandes deleções no *SPAST* puderam ser encontradas em famílias com ligação ao locus *SPG4*, nas quais, previamente, não havia sido encontrada nenhuma “pequena” alteração (Beetz *et al.*, 2007).

II. OBJETIVOS

Os objetivos deste trabalho são:

- Analisar, *in vitro*, os efeitos da mutação P56S na interação da VAPB com outras proteínas celulares.
- Desenvolver um novo modelo de ELA baseado na tecnologia de células *iPSC* derivadas de pacientes com ELA8.
- Identificar a mutação responsável pela Paraplegia Espástica Hereditária em uma grande genealogia brasileira e contribuir para o entendimento dos mecanismos responsáveis pela variabilidade de fenótipos identificados na mesma.

Capítulo 2

2. Análise *in vitro* dos efeitos da mutação P56S na atividade da VAPB

A mutation in human VAP-B–MSP domain, present in ALS patients, affects the interaction with other cellular proteins

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is the most common adult-onset Motor Neuron Disease (MND), characterized by motor neurons death in the cortex, brainstem and spinal cord. Ten loci linked to Familial ALS have been mapped. ALS8 is caused by a substitution of a proline by a serine in the Vesicle-Associated Membrane Protein-Associated protein-B/C (VAP-B/C). VAP-B belongs to a highly conserved family of proteins implicated in Endoplasmic Reticulum—Golgi and intra-Golgi transport and microtubules stabilization. Previous studies demonstrated that the P56S mutation disrupts the subcellular localization of VAP-B and that this position would be essential for Unfolded Protein Response (UPR) induced by VAP-B. In the present work we expressed and purified recombinant wild- type and P56S mutant VAP-B—MSP domain for the analysis of its interactions with other cellular proteins. Our findings suggest that the P56S mutation may lead to a less stable interaction of this endoplasmic reticulum protein with at least two other proteins: tubulin and GAPDH. These two proteins have been previously related to other forms of neurodegenerative diseases and are potential key points to understand ALS8 pathogenesis and other forms of MND. Understanding the role of these protein interactions may help the treatment of this devastating disease in the future.

Resumo

A Esclerose Lateral Amiotrófica (ELA) é a mais comum doença do neurônio motor (DNM) de início tardio. Ela é caracterizada pela morte dos neurônios motores no córtex, tronco cerebral e medula espinhal. Dez *loci* ligados a ELA familiar foram mapeados. A ELA8 é causada pela substituição de uma prolina por uma serina na proteína *Vesicle-Associated Membrane Protein-Associated Protein* – B/C (VAP-B/C). A VAP-B pertence a uma família de proteínas muito conservada que está envolvida com o transporte Retículo Endoplasmático (RE)–Complexo de Golgi e intra Golgi, além da estabilização dos microtúbulos. Estudos prévios mostraram que a mutação P56S abole a localização celular da VAP-B e que esta posição seria essencial para o mecanismo de resposta a proteínas não-dobradas (Unfolded Protein Response – UPR) induzido pela VAP-B. No presente trabalho, nós expressamos e purificamos tanto a forma selvagem quanto a mutante P56S do domínio VAP-B–MSP para a análise de sua interação com outras proteínas celulares. Nossos achados sugerem que a mutação P56S promove uma interação menos estável desta proteína do RE com pelo menos duas outras proteínas: Tubulina e GAPDH. As duas últimas foram previamente relacionadas a outras formas de doenças neurodegenerativas e são potenciais pontos-chave para desvendar a patogênese da ELA8 e de outras formas de DNM. Entender o papel destas interações protéicas pode ajudar, no futuro, no tratamento desta doença devastadora.

A mutation in human VAP-B–MSP domain, present in ALS patients, affects the interaction with other cellular proteins

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is the most common adult-onset Motor Neuron Disease (MND), characterized by motor neurons death in the cortex, brainstem and spinal cord. Ten loci linked to Familial ALS have been mapped. ALS8 is caused by a substitution of a proline by a serine in the Vesicle-Associated Membrane Protein-Associated protein-B/C (*VAP-B/C*). *VAP-B* belongs to a highly conserved family of proteins implicated in Endoplasmic Reticulum–Golgi and intra-Golgi transport and microtubules stabilization. Previous studies demonstrated that the P56S mutation disrupts the subcellular localization of *VAP-B* and that this position would be essential for Unfolded Protein Response (UPR) induced by *VAP-B*. In the present work we expressed and purified recombinant wild-type and P56S mutant *VAP-B*–MSP domain for the analysis of its interactions with other cellular proteins. Our findings suggest that the P56S mutation may lead to a less stable interaction of this endoplasmic reticulum protein with at least two other proteins: tubulin and GAPDH. These two proteins have been previously related to other forms of neurodegenerative diseases and are potential key points to understand ALS8 pathogenesis and other forms of MND. Understanding the role of these protein interactions may help the treatment of this devastating disease in the future.

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Keywords: Amyotrophic Lateral Sclerosis; *VAP-B*; Dimerization; Protein interaction

Amyotrophic Lateral Sclerosis (ALS)¹ the most common adult-onset motor neuron disease [1], is characterized by motor neurons death in the cortex, brainstem and spinal cord [2]. It results in muscle weakness and atrophy, leading to death by respiratory failure 2–5 years after the disease onset. There is currently no known treatment for this disorder, although some increase in life expectancy has been suggested with the use of the drug riluzole [3,4]. ALS is

classified into Sporadic Amyotrophic Lateral Sclerosis (SALS) and Familial Amyotrophic Lateral Sclerosis (FALS), accounting respectively for 90 and 10% of cases [5].

Ten loci linked to FALS have been mapped to date: ALS1 to ALS8, ALS with fronto-temporal dementia (ALS-FTD) and ALS with Parkinsonism and Dementia [1,6]. So far, only six genes have been identified in these regions that are related to ALS. ALS1 is responsible for 20% of all FALS cases and is due to mutations in *SOD1* gene, located at 21q22.21 [1]. It encodes the Cu/Zn superoxide dismutase 1 enzyme (SOD) responsible for superoxide anion (O₂[−]) metabolism. More than 100 mutations have been described in this gene causing a gain of toxic property rather than a “loss of function” [6,7]. However,

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¹ Abbreviations used: ALS, amyotrophic lateral sclerosis; MND, motor neuron disease; *VAP-B/C*, vesicle-associated membrane protein-associated protein-B/C; UPR, unfolded protein response; SALS, sporadic amyotrophic lateral sclerosis; TMD, transmembrane domain.

the mechanism through which mutated SOD1 exerts toxicity and results in selective motor neuron death remains uncertain [6].

Our group first mapped the ALS8 motor neuron disease at 20q13.3 [8], in a pedigree with more than 200 affected and at least 1300 family members [9]. Subsequently we identified a missense mutation, C₁₆₆T, in the gene coding for the vesicle-associated membrane protein-associated protein-B/C (VAP-B/C) that causes in some members a late-onset spinal muscular atrophy (MIM 182980) and in others amyotrophic lateral sclerosis (MIM 608627). The C₁₆₆T mutation changes a proline into a serine at the amino acid position 56 (P56S). The Proline 56 is present in a highly conserved region of VAP protein comprising 16 amino acids in its N-terminal region [9]. There are three forms of VAP in humans: VAP-A, B and C. The VAP-A (242 amino acid protein and 27.3 kDa) is encoded by a gene in chromosome 18 and has 63% identity at the amino acid level to its paralogous protein, VAP-B (243 amino acids and 27 kDa). The VAP-C isoform (98 amino acids and 11 kDa) is encoded by an alternative splicing product of VAP-B/C. VAP-B contains three different domains. The MSP (Major Sperm Protein) domain, conserved in all VAP family members, is formed by the first 150 amino acids [9,10] (Fig. 1). This domain has high identity with *C. elegans* MSP, a cytoskeletal element that also functions as a reproductive hormone in this organism [11]. The central portion of VAP-B is a probable coiled-coil domain that may be involved in protein–protein interaction. The C-terminal transmembrane domain (TMD) is responsible for VAP-B attachment to intracellular vesicles (UniProt/Swiss-Prot—095292) and the formation of homodimers and heterodimers with other VAP members [12,10].

VAP-B belongs to a highly conserved family of proteins (VAMP—Vesicle Associated Membrane Protein) implicated in Endoplasmic Reticulum—Golgi and intra-Golgi transport and microtubules stabilization. VAP-A has been demonstrated to interact with VAP-B via TMD and with SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins, such as syntaxin 1A, rbt1, rsec22, α SNAP and NSF [12,13]. VAP-A also interacts with microtubules [12,14,15], and OSBP (Oxysterol-binding protein) [16–18].

VAP-B has been demonstrated to interact with VAMP1 and VAMP2 [10,13], two proteins related to targeting and/or fusion of vesicle transport. VAP-B also interacts with

Nir2, a protein belonging to a highly conserved family (Nir/rdgb), implicated in the regulation of membrane trafficking, phospholipid metabolism, and signaling [12]. The FFAT (two phenylalanines in an acidic tract) motif was shown to be involved in the interaction with VAP-B [17]. The FFAT motif, present in Nir proteins (Nir1, Nir2 and Nir3), is responsible for Nir–VAP-B interaction, which affects the organization of the ER [12]. Kaiser et al. [19] solved the crystal structure of rat VAP-A–FFAT interaction motives and analyzed the specific molecular interactions between FFAT and VAP, and found them to be highly conserved from yeast to mammals. Moreover, VAP-B has been identified as a protein involved in hepatitis C virus (HCV) replication through the interaction of VAP-B coiled-coil and NS5A HCV protein [20]. Recently, VAP-B was demonstrated to participate on Unfolded Protein Response (UPR) [21], an Endoplasmic Reticulum process involved in the suppression of the accumulation of unfolded proteins in this organelle [22]. It was demonstrated that the proline at position 56 is essential for UPR induction by VAP-B [21]. Additionally, the P56S mutation disrupts the subcellular localization of VAP-B protein [9,21].

These data demonstrate that VAP-B participates in many cellular processes and that the mechanism responsible for the specific death of motor neurons in ALS8 patients may be due to a complex interaction of different metabolic pathways. Here, *in vitro* analyses of the consequences of P56S mutation in human VAP-B protein were performed, in an attempt to correlate them with the mechanisms responsible for ALS8. The results shown here indicate that P56S mutation may be related to the MSP–VAP-B interaction with other proteins.

Materials and methods

Cloning VAP-B's sequences

Total RNA was extracted by the Trizol™ method (Life Technologies) from lymphocytes of healthy control individuals (with informed consent). It was used as a template for cDNA synthesis with M-MLV Reverse Transcriptase (Invitrogen). Using For and Rev primers 5′-CGCGGATCCATGGCGAAGGTGG-3′ and 5′-CGGCTGGTTCGACCTACAAGGCAATC-3′, respectively (underlined and italic represent BamHI and SalI restriction sites) VAP-B coding region was PCR amplified and cloned into pGEM-T vector (PROMEGA). Recombinant plasmids were extracted using Eppendorf Fast Plasmid Mini Kit and sequenced using primers T7 and SP6 in a Mega Bace 1000 DNA Sequencer—Amersham Bioscience (GE-Healthcare).

pGEM-VAPB was used as a template to obtain the fragment corresponding to the MSP domain, using the primers MSPFor (5′-AGGATCCCATATGGCGAAGGTGGAGCAGG-3′—BamHI) and MSPRev (5′-GCGAATTCTTATGGCAATTCAAACACACATC-3′—EcoRI)—“reaction W”. MSP domain containing the P56S mutation was obtained

Fig. 1. VAP-B domains. The first region corresponds to the MSP domain and the arrow shows the P56S missense mutation present in ALS8 patients. The central region corresponds to a coil-coil domain and the C-terminal portion corresponds to a transmembrane domain.

by site directed mutagenesis of the wild-type sequence, using the primers MSPmutFor (5'-CTGTGTGAGGTCCAACAGCGGAA-3'), and MSPmutRev (5'-TTCCGCTGTTGGACCTCACACAGT-3'). Site directed mutagenesis was performed using three different PCR: reaction 1—utilized pGEM-VAP-B as template and the primers MSPFor with MSPmutRev; reaction 2—the template was pGEM-VAP-B and primers MSPmutFor with MSPRev; reaction 3—the template utilized was a mixture of reactions 1 and 2 at the same concentration, the primers for this reaction were MSPFor and MSPRev. The PCR products from reactions 1 and 2 were cloned into pGEM-T, generating pGEM-MSP and pGEM-MSP-P56S. All constructions were sequenced with T7 and SP6 primers using a Mega Bace 1000 DNA Sequencer—Amersham Bioscience (GE-Healthcare).

Expression and purification of recombinant proteins

The following steps were all performed in parallel with wild-type and mutant constructions and are summarized in Fig. 2a. DNA fragments corresponding to VAP-B–MSP domain (wt and mutant) were extracted from

pGEM-MSP and pGEM-MSP-P56S, using BamHI and EcoRI restriction enzymes and cloned into pAE vector [23], fused to a His-tag. BL21-DE3 RIL *Escherichia coli* strain was transformed with pAE-VAP-B–MSP and pAE-MSP-P56S plasmids and incubated overnight in 90 ml of LB medium. This culture was used to perform the expression of the recombinant proteins in 3 L of 2× Yeast Extract medium, 0.5% glycerol, 0.78% lactose. The cells were harvested after 7 h at 37 °C. The cultures were centrifuged at 3000 rpm and 4 °C. The pellet was collected and incubated for 14 h in denaturing buffer at room temperature (Table 1). It was subsequently sonicated and centrifuged at 10,000 rpm, 4 °C for 30 min. The supernatant was loaded onto a nickel column in a ÄKTA FPLC system (GE Healthcare).

Refolding of the proteins was performed while still bound to the nickel column, by slowly decreasing urea concentration (8–0 M) (Table 1—refolding buffer). The proteins were then eluted (elution buffer described in Table 1) and fractions were analyzed on SDS–PAGE. Sequentially they were dialyzed against the buffer presented in Table 1. A Western blot assay, with an anti-His antibody, was performed to confirm the identity of the proteins.

Fig. 2. (a) Scheme of protein expression and purification utilized for both recombinant proteins (VAP-B–MSP and MSP-P56S). (b) Twelve percent of SDS–PAGE gel showing the absence of VAP-B in the soluble fraction of cell extract. Soluble fraction was loaded on Ni-column, and flow through, wash and elution fractions were analysed on SDS–PAGE, although VAP-B was only present in the inclusion bodies. I, input (soluble fraction); FT, flow through (unbound); W, wash; S, soluble elute (bound to Ni-column); IB, inclusion bodies; M, MW marker.

Table 1

Summary of conditions used for refolding, elution, and gel-filtration

Denaturing buffer	8 M Urea, 50 mM Tris–HCl, pH 8.9, 0.5 M NaCl, 5 mM Imidazol, 2.5 mM β-mercaptoethanol, 0.1% Triton X-100
Refolding buffer	Decreasing urea concentration (8–0 M), 30 mM Tris–HCl, pH 8.9, 0.5 M NaCl, 5 mM Imidazol, 3 mM β-mercaptoethanol
Elution buffer	30 mM Tris–HCl, pH 8.9, 0.5 M NaCl, 500 mM Imidazol, 3 mM β-mercaptoethanol
Dialysis buffer	20 mM Tris–HCl pH 8.9, 50 mM KCl, 15 mM β-mercaptoethanol, 0.2% <i>N</i> -Lauroyl Sarcosine.
Gel-filtration buffer	20 mM Tris–HCl, pH 8.9, 50 mM KCl, 10 mM β-mercaptoethanol, 0.2% <i>N</i> -Lauroyl Sarcosine

Analysis by gel-filtration

Purified VAP-B–MSP and MSP-P56S proteins were subjected to gel-filtration through a Superdex-75 XK16 column on ÄKTA-FPLC using the buffer described into the Table 1.

Apparent molecular mass calculations were based on protein markers (Amersham Pharmacia), according to the elution volumes of each protein. The markers used for calibration were ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease (14 kDa).

Pull down and identification of interacting proteins

Human brain tissue was obtained according to the Brazilian Ethics Committee regulations. It was pulverized on dry ice to fine powder. One milliliter of extraction buffer (Table 2) was added to tissue powders and incubated for 30 min with repeated shaking and vortexing every 10 min. The mixtures were then centrifuged at 4 °C for 30 min and supernatants (total extracts) were then collected (~9 µg/µl).

The proteins corresponding to VAP-B dimers were collected after gel-filtration and concentrated with Millipore filtration system. Chelating Sepharose fast flow nickel column was equilibrated with buffer A (Table 2) and 1 mg of each recombinant His-tag protein was loaded. After washing the column with 50 volumes of buffer A, brain extract (~3.7 mg) was loaded on the column and incubated for 90 min at RT. The column was washed again with buffer A and proteins were eluted with buffer B.

Interacting proteins were analyzed through SDS–PAGE and bands with different intensities were extracted from the gel. The samples were analyzed by MALDI-TOF mass spectrometry using α -cyano-4-hydroxycinnamic acid as matrix on an Ettan Maldi-Tof/Pro instrument (Amersham Biosciences), according to Westermeier and Naven [24]. Briefly, gel spots were isolated, destained, and treated with DTT and IAA for reduction and alkylation of eventual disulfide bonds. Then, the treated spot was re-hydrated with a 40 ng/µL trypsin solution in 50 mM ammonium acetate and the reaction was carried out overnight at 30 °C. The tryptic peptides were extracted from the gel using an H₂O/ACN/TFA (1:1:0.05) solution incubated in an ultrasound bath. The peptide solution was concentrated and desalted with ZipTip C-18 (Millipore) prior to analysis. The sample was pre-mixed with the matrix solution (1:1, v/v) and approximately 0.4 µL were deposited on the

sample loader and let dry over the bench. Mass spectrometry was performed on reflectron mode and internal calibrants (trypsin autolysis peptide) were used. External calibration was also performed in case trypsin peptides were not present or clear. The obtained mass list was analyzed by peptide mass fingerprinting algorithms (MASCOT and/or ALDENTE) for matches with known proteins sequences deposited on the public databases.

Results

The fragments corresponding to VAP-B–MSP and MSP-P56S were cloned into the pAE expression vector [23] and the expression of the His-tagged proteins was obtained after incubation of *E. coli* cells in lactose-containing medium. Recombinant His-MSP and His-MSP-P56S were analysed on SDS–PAGE and showed similar levels of expression in *E. coli* (Fig. 2b). Soluble proteins and inclusion bodies were separated after centrifugation and soluble fraction was subjected to affinity chromatography on Ni-column. The results show that His-VAP-B–MSP and His-MSP-P56S were not present in the soluble fraction, but largely present in inclusion bodies (Fig. 2b—inclusion bodies). Proteins were solubilized with 8 M urea and refolding was performed using a decreasing urea gradient of 8–0 M while the recombinant proteins were still bound to the nickel column. After this step, the proteins were eluted with elution buffer (Table 1). Analyses of purified proteins on SDS–PAGE showed bands corresponding to 14 kDa, as predicted for VAP-B–MSP (Fig. 2b and data not shown). Using the strategy described in Fig. 2, the amount of recombinant proteins was the same for both His-VAP-B–MSP and His-MSP-P56S, approximately 0.8–1.0 mg proteins/liter of culture.

To analyze the dimerization state and to better purify these protein samples, a gel-filtration was performed. These analyses showed that both proteins (wt and mutant) are eluted with the same volume, indicating that they have similar mass and conformation. Interestingly, the main peak observed on gel-filtration corresponds to a mass a little smaller than 25 kDa (Fig. 3a). A secondary peak is also observed, which corresponds to a mass of 43 kDa. When analyzed by SDS–PAGE, the fraction corresponding to the main peak contains two protein bands, one stronger band corresponding to VAP-B–MSP (≈14 kDa), and other, less intense, corresponding to an *E. coli* contaminant (≈28 kDa; Fig. 3b). A Western blot assay was performed to confirm the identity of His-VAP-B–MSP and

Table 2
Summary of conditions used in the pull down assays

Extraction buffer	50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 10 g/L of NP-40 surfactant, 1 mM PMSF, 1 g/L of aprotinin, 1 g/L of leupeptin
Buffer A (column equilibration buffer)	20 mM Tris, pH 8.9, 50 mM KCl, 3 mM β -mercaptoethanol, 0.2% <i>N</i> -Lauroyl Sarcosine
Buffer B (elution buffer)	20 mM Tris, pH 8.9, 50 mM KCl, 10 mM β -mercaptoethanol, 0.2% <i>N</i> -Lauroyl Sarcosine, 300 mM Imidazol

Fig. 3. Gel-filtration and SDS-PAGE analysis of recombinant proteins. After elution from nickel column, followed by dialysis, the recombinant proteins were submitted to gel-filtration (a) and the principal peaks are analyzed in 14% SDS-PAGE (b). WT and mutant proteins have the same apparent molecular size (near 14 kDa). A minor contaminant can be seen near 30 kDa. The arrows and numbers above the curve indicate the molecular mass according to Amersham Pharmacia kit of calibration to gel-filtration Ovalbumin 43 kDa, Quimotrypsinogen 25 kDa e Ribonuclease 14 kDa.

His-MSP-P56S in the main peak from gel-filtration (data not shown). Through this assay it was also possible to determine that VAP-B was not present in the minor protein peak (43 kDa), containing only bacterial contaminants.

The refolding efficiency seems to be high since the majority of His-VAP-B-MSP and His-MSP-P56S obtained after refolding was present in one peak (the main peak) on gel-filtration and it was not possible to determine the presence of His-VAP-B-MSP or His-MSP-P56S in the secondary peak from gel-filtration (Fig. 3a).

VAP-B-MSP has a calculated molecular mass of 14.2 kDa and, therefore, the elution of this protein with a volume of 65 ml, very close to 59 ml of the 25 kDa marker indicates that a homodimer is being formed and is stable through gel filtration. The co-elution of a 28 kDa molecule with VAP-B-MSP and MSP-P56S in the gel filtration corroborates this hypothesis (Fig. 3a and b).

A second gel-filtration experiment was performed with a mixture of the same amounts of wt and mutant VAP-B-MSP showed the elution of the proteins as a single peak (data not shown), indicating that the dimers are either very stable or that wt and P56S VAP-B-MSP can form dimers with each other, regardless of the P56S mutation. It was not possible to observe a peak corresponding to monomeric VAP-B-MSP in any gel-filtration experiment.

In order to determine whether VAP-B-MSP and MSP-P56S could interact with human brain proteins *in vitro*, pull-down experiments were performed in which purified VAP-B-MSP and MSP-P56S were incubated with total brain extracts and co-purified proteins were analyzed through SDS-PAGE and identified by mass spectrometry.

The results show not only that wt and mutant MSP domains of VAP-B can interact with human proteins *in vitro*, but also that the mutation in this domain affects the efficiency of protein co-purification (Fig. 4). The P56S mutation, therefore, seems to affect the interaction of VAP-B with other brain proteins since less intense protein bands are observed in the pull-down with the mutant VAP-B-MSP.

Fig. 4. Pull down analysis with cytosolic human brain proteins. Left lane corresponds to proteins eluted from wild-type MSP-VAP-B interactions. Right corresponds to proteins eluted from mutant P56S-MPS-VAP-B interactions. Arrows indicate the identified proteins Tubulin (TUBB $\approx$ 50 kDa) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH $\approx$ 38 kDa). The spot corresponding to TUBB cannot be seen at mutant lane. Much less GAPDH was pulled-down with the mutant VAP-B.

The peptide mass fingerprinting algorithms (MASCOT and/or ALDENTE) diverged in the identification of one protein, β -tubulin. According to MASCOT, the most significant would be Class II β -tubulin isotype (Q8IWR2_HUMAN—ExPASy server) and according to ALDENTE it would be tubulin β -4 chain (TBB4_HUMAN—ExPASy server). Those entries belong to the same family proteins—Beta_tubulin IPR002453 InterPro code—and have 95% of identity (aligned with CLUSTAL W provided by SDSC—Biology Workbench). One of the proteins co-purified with VAP-B-MSP, but not with MSP-P56S is β -tubulin (Q8IWR2_HUMAN), a protein of approximately 50 kDa, which is consistent with the band extracted from the gel (Fig. 4).

Both programs coincided that the other identified spot would be the entry G3P_Human_Human on ExPASy Server glyceraldehyde-3-phosphate dehydrogenase. This protein

was found co-purifying more efficiently with MSP than with MSP-P56S. It has a molecular mass of 36–38 kDa consistent with the band extracted from the gel (Fig. 4). The other bands (Fig. 4) could not be identified because they did not present a minimal level of significance at ALDENT and MASCOT analyses.

Discussion

The present study aimed at the determination of whether the P56S mutation found in ALS8 patients leads to changes in VAP-B's interaction with other proteins. The proline at position 56 is highly conserved among VAP-B orthologues [9] and, therefore, may be essential for the correct VAP-B mechanism of action. Studies with the yeast VAP-B orthologue, SCS2, demonstrated that Ser51 (equivalent to P56S in VAP-B) does not destabilize the overall structure of the protein [25]. Moreover, the yeast SCS2 mutant protein maintained the property to bind to FFAT motifs, which are targeting signals, responsible for the localization of a number of proteins to the cytosolic surface of the ER [17,25].

Studies on the human VAP-B, however, have demonstrated that the P56S mutation leads to full-length protein misfolding and mislocalization in mammalian cells [21]. Expression of rat VAP-A–MSP in *E. coli*, on the other hand, has been shown to generate soluble proteins when fused to GST tag, although no interaction with cytosolic portions of SNARE proteins was detected [19]. Interestingly, analysis of human VAP-A interaction domains has shown that the dimerization depends on the three protein domains, with the decreasing order of importance: C-terminus—MSP—coiled-coil [13].

Our results showed that the VAP-B–MSP is insoluble when expressed in *E. coli*, with or without the P56S mutation, leading to the concentration of the recombinant protein in inclusion bodies. This insolubility might be related to the system of expression (prokaryote) and the absence of VAP-B's transmembrane domain that could increase its stability. The differences between our results and those on rat VAP-A–MSP [19] may be due to the different system of expression and protein tags used.

Deletion of the first 63 amino acids (N-terminal region of MSP domain) has been shown to have no effect on VAP-B oligomerization, suggesting that the C-terminal TMD domain would be critical for dimerization [12]. Interestingly, a VAP-B-ΔTMD deletion mutant has also been shown to form dimers [21], indicating that both MSP and TMD domains may be involved in protein-protein interactions. The results shown here indicate that the MSP domain alone, under the conditions used here, is able to form dimers. Although it was not possible to determine differences in the proteins dimerization between the two proteins (wild-type and mutant) due to the absence of recovered monomeric proteins, we can conclude that the P56S mutation does not eliminate the dimerization presented by the wild-type protein. The dimer formation con-

firms what has been reported [13], that the dimerization of VAP-B might depend not only on TMD, but that the MSP domain probably contributes to this mechanism as well.

We were also able to demonstrate that MSP domain interacts with other cellular proteins, such as tubulin and GAPDH, and that P56S mutation abolishes some of VAP-B interactions. Although MSP-P56S still co-purifies GAPDH, the interaction seems to be less stable than that with WT MSP.

Shekel et al. [16] showed that mouse VAP33, a VAP-B homologue protein, is associated with endoplasmic reticulum and microtubules. Microtubules are structures responsible for various cellular mechanisms and are formed by heterodimers of alpha and beta tubulin [26]. Nishimura et al. [9] and Kanekura et al. [21] showed that when P56S is expressed in mammalian cells intracellular aggregates are formed. In addition, VAP-B-P56S does not co-localize either with Golgi apparatus or with the ER, contrary to the wild-type protein. Here, we propose that the absence of VAP-B-P56S in those organelles and its presence in aggregates may be related to the lack of interaction between mutant P56S and tubulin.

Recent studies have demonstrated that GAPDH has multiple non-glycolytic functions dependent on its subcellular location [27,28], like regulation of the cytoskeleton, membrane fusion and transport, glutamate accumulation into presynaptic vesicles and binding to low molecular-weight G proteins. Nuclear functions of GAPDH may be related to transcription activation in neurons, nuclear export of RNA and DNA repair [28].

An increasing number of studies indicate that GAPDH may participate in neuronal death in some neurodegenerative diseases [28–31]. The participation of GAPDH in apoptosis *in vitro* suggests that it has a significant role in human pathophysiology. It was found that GAPDH might be involved with Huntington, Alzheimer, Parkinson diseases and Ischemia/Hypoxia either by its abnormal expression or by its intracellular localization [28]. Decreased nuclear and mitochondrial levels of GAPDH are detected in the spinal cords from mutant SOD1 G93A transgenic mice [27].

GAPDH and mutant VAP-B have been implicated in neuronal diseases, and the observation that the interaction of GAPDH with mutant VAP-B–MSP-P56S is less efficient than with wt VAP-B–MSP suggests that this could be an important pathway involved in ALS8 pathogenesis.

Further studies are necessary to find out whether the brain proteins co-precipitated with MSP bind directly to VAP-B or whether the interaction is indirect, with other proteins acting as intermediate. The identification of other proteins and mechanisms involved with VAP-B is essential to understanding the ALS8 phenotype. Such studies will certainly shed some light on the heterogeneous phenotype of amyotrophic lateral sclerosis and spinal muscular atrophy.

In summary our findings indicate that P56S mutation may lead to a less stable interaction of this endoplasmic

reticulum protein with other proteins. The proteins identified here interacting with VAP-B, β -tubulin and GAPDH, have been previously related to other forms of neurodegenerative diseases and are potential key points to understand ALS8 pathogenesis and other forms of Motor Neuron Disease. Understanding the role of these protein interactions may help the treatment of this devastating disease in the future.

Electronic-database information

ExPasy—UniProt/SwissProt <http://www.expasy.org>
 Clustal W provided by SDSC-Biology Workbench—<http://workbench.sdsc.edu/>
 MASCOT Peptide Mass Fingerprint—http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF
 ALDENTE: Peptide Mass Fingerprint—<http://www.expasy.org/tools/aldente/>

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Capítulo 3

3. Modelagem da ELA8 usando *iPSC*

Em preparação

Downregulation of VAPB expression in motor neurons derived from ALS8 patient human Induced Pluripotent Stem Cells

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Abstract

ALS is a devastating and incurable neuromuscular disease that leads to a profound loss of life quality in affected patients. Around 10% of the cases show a heritable trait. ALS8 is an autosomal dominant form of familial ALS caused by mutations in the *VAPB* gene. The VAPB protein is involved in many cellular processes and likely to contribute to pathogenesis in other forms of ALS besides ALS8. A substantial number of successful drug tests in ALS animal models could not be translated to humans demonstrating the need of novel ALS systems. The iPSC technology brings new hope in this area since it can be used to model diseases *in vitro*. Here we present a new ALS model based on ALS8-iPSC. Fibroblasts from ALS8 patients and their non-affected siblings could be successfully reprogrammed to a pluripotent state and differentiated into motor neurons. We found that VAPB is reduced in ALS8 samples but, in contrast to overexpression systems, intracytoplasmic aggregates could not be identified. Our results suggest that the correct amount of VAPB may be crucial for motor neuron viability and reveals the importance of ALS modeling in a human background.

Resumo

A ELA é uma doença neuromuscular devastadora e incurável que provoca uma profunda perda da qualidade de vida do paciente. Cerca de 10% dos casos são hereditários. A ELA8 é uma forma autossômica dominante de ELA familiar causada por mutações no gene *VAPB*. A proteína VAPB está envolvida com diversos processos celulares e provavelmente contribui para outras formas de ELA, além da ELA8. Um número significativo de drogas, testadas com sucesso em modelos animais de ELA, não pôde ser transferido para os pacientes, demonstrando a necessidade de novos sistemas de estudo da doença. A tecnologia das *iPSC* trouxe novas esperanças nesta área, pois elas podem ser utilizadas para modelar doenças *in vitro*. No presente trabalho, apresentamos um novo modelo de ELA baseado em células *iPSC*- ELA8. Fibroblastos de pacientes com ELA8, e seus irmãos não-afetados, puderam ser reprogramados a um estágio de pluripotência e diferenciados para neurônios motores. Encontramos uma redução da VAPB nas amostras de ELA8 e, em contraste com os modelos de super-expressão, não identificamos agregados intracitoplasmáticos. Nossos resultados sugerem que a correta quantidade de VAPB é crucial para a viabilidade dos neurônios motores e revelam a importância de modelar a ELA num *background* humano.

Introduction

Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig's disease, is the most common adult-onset motor neuron disease (MND) (Goodall *et al.*, 2006). There is no cure for this devastating disease that affects approximately 30,000 individuals at any time in United States (Lepore *et al.*, 2008). Clinical findings as muscular atrophy and weakness accompanied of fasciculation and spasticity result from the rapid and progressive degeneration of motor neurons in the cortex, brainstem and spinal cord (Rowland & Shneider, 2001). Patient's death occurs between two to five years from symptoms onset, usually due to respiratory failure (Beleza-Meireles and Al Chalabi, 2009).

Approximately 90% of all ALS cases are sporadic (SALS) and the remaining 10% comprise the familial forms (FALS), with autosomal recessive or, most commonly, autosomal dominant pattern of inheritance (Pasinelli and Brown, 2006). ALS8 is an autosomal dominant type of FALS that was first mapped in a Brazilian family (Nishimura *et al.*, 2004^A). Subsequently, the mutation c.166C>T in the *vesicle-associated membrane protein-associated protein-B/C* (VAPB/C) gene was identified on the original family and six additional ALS pedigrees in Brazil (Nishimura *et al.*, 2004^B). The pyrimidine transition leads to a substitution of a Proline by a Serine at position 56 (P56S) of the VAPB protein (Nishimura *et al.*, 2004^B). A founder effect is suggested to act in the Brazilian families, since they share the same haplotype (Nishimura *et al.*, 2005). Moreover, ALS8 is no longer restricted to South America. The same mutation was identified in Germany (Funke *et al.*, 2010) and in a Japanese descendent patient (Millecamps *et al.*, 2010). The German patient shows an haplotype that is different from the Brazilian families, suggesting that the mutation happened in at least two independent events (Funke *et al.*, 2010). Additionally, a recent study found a new mutation (T46I) in a British ALS patient that leads to similar molecular effects as the P56S alteration (Chen *et al.*, 2010). Together, these data provide strong evidence that ALS8 is not as rare as previously proposed (Hirano, 2008; Landers *et al.*, 2008).

VAPs are highly conserved proteins among species and are involved in many different cellular processes (Lev *et al.*, 2008). They are ubiquitously expressed and localize in the endoplasmic reticulum (ER) and pre-Golgi intermediates (Skehel *et al.*, 2000). VAPB action over ER (Amarilio *et al.*, 2005) is more evident by overexpression of P56S-VAPB modifying its structure (Teuling *et al.*, 2007) and forming a new type of organized smooth ER (Fasana *et al.*, 2010). Additionally, the mutated VAPB reduces the native interactions with other cellular proteins (Mitne-Neto *et al.*, 2007).

Protein aggregates are a hallmark for neurodegenerative diseases, such as Alzheimer, Parkinson and ALS (Kim *et al.*, 2010). Around 20% of FALS patients show *Superoxide Dismutase 1* (*SOD1*) gene mutations (ALS1). These alterations lead to a gain of toxic property rather than a “loss of function” (Gros-Louis *et al.*, 2006) and form cytoplasmic inclusions (Bruijn *et al.*, 1997). The recently identified ALS10 causing gene (*TARDBP*) is responsible for 3% of FALS cases (Lagier-Tourenne and Cleveland, 2009) and TDP-43 cytoplasmic aggregates are found in different forms of ALS, such as ALS10, ALS with dementia and in most cases of sporadic ALS (Mackenzie *et al.*, 2007). Similarly, P56S-VAPB forms cytoplasmic inclusions that, ultimately, comprise a common characteristic found in different overexpressing systems as mammalian cells, flies and mouse models (Nishimura *et al.*, 2004; Kanekura *et al.*, 2006; Teuling *et al.*, 2007; Chai *et al.*, 2008; Ratnaparkhi *et al.*, 2008; Tsuda *et al.*, 2008; Prosser *et al.*, 2008; Suzuki *et al.*, 2009; Fasana *et al.*, 2010; Tudor *et al.*, 2010; Kim *et al.*, 2010). Additionally, those inclusions are ubiquitinated (Kanekura *et al.*, 2006; Tsuda *et al.*, 2008; Tudor *et al.*, 2010) and insoluble (Kanekura *et al.*, 2006; Teuling *et al.*, 2007; Suzuki *et al.*, 2009).

The molecular mechanism by which VAPB induces ALS may be not restricted to ALS8. A reduction in VAPB levels was found in motor neurons from SALS patients (Teuling *et al.*, 2007; Anagnostou *et al.*, 2008) and transgenic *SOD1* mice (Teuling *et al.*, 2007). Moreover, VAPB levels decrease concomitantly with disease’s progression in the *SOD1* mouse model (Teuling *et al.*, 2007). While the most studied ALS model (*SOD1*^{G93A} mouse)

dies around 5-6 months (Hensley *et al.*, 2006), mice overexpressing WT-VAPB or the P56S mutant form, did not show any overt motor neuron disease or reduction in survival when compared to non-transgenic lineages (Tudor *et al.*, 2010). However, P56S-VAPB animals show signs of TDP43 pathogenesis, since TDP43 aggregates co-localized with VAPB inclusions in the cytoplasm (Tudor *et al.*, 2010).

For human studies purposes, the access of motor neuron cells, from live patients evokes ethical and methodological concerns. Additionally, analysis of postmortem tissue has limitations and would only show the disease end-stage (Marchetto *et al.*, 2010A). The induced Pluripotent Cell (iPSC) technology provides a promising approach as it allows the genomes of ALS patients to be captured in pluripotent stem cell lineages. Modeling ALS based on iPSC may also address human specific effects, recapitulating the genetic background, at the same time, avoiding overexpression systems (Takahashi *et al.*, 2007) and limitations of animal models, such as lifespan and cellular metabolism. iPSC lines from patients presenting different neurodegenerative diseases, such as Spinal Muscular Atrophy (SMA) (Ebert *et al.*, 2009), Familial Dysautonomia (Lee *et al.*, 2009), Parkinson's disease (Soldner *et al.*, 2009) and ALS (Dimos *et al.*, 2008) have been generated. However, in the last two cases, there is a lack of clear disease phenotypes.

Here, we demonstrate the successful generation of a new ALS *in vitro* model, based on cellular reprogramming of ALS8 cell patients. The reduction of VAPB protein levels in ALS8 samples indicates that reprogramming and motor neuron differentiation processes do not interfere in VAPB gene expression. Our work meets the need for a novel ALS model, to develop early diagnostic tools and possible drugs and targets for future therapies.

Results

Generation and characterization of ALS8 and control iPSCs

Skin biopsies were collected from affected patients and non-affected siblings from two ALS8 families. Family 1 was composed by two individual controls and two patients; and Family 2 by 1 individual control and 2 patients (Fig.1A). Fibroblast collection and culture implementation were performed in parallel for both control and patient samples following standard cell culture protocols. Cellular reprogramming was performed as previously described (Marchetto *et al.*, 2010^B), using the four Yamanaka Factors (*c-Myc*, *Oct3/4*, *Klf4* and *SOX2*) (Takahashi *et al.*, 2007). Human Embryonic Stem Cell-like cells were detected 10 – 15 days after virus transduction (Fig.1B, C). iPSC colony selection was based on hESC-like morphology and transfer to feeder-free culture conditions (Fig.1D, E). All seven samples (four patients; three controls) were successfully reprogrammed and expanded for several passages. The c.C>T166 mutation was accessed by *HaeIII* restriction enzyme fragments in iPSC carrying the mutation correspond to the original fibroblast used for reprogramming (Fig. S1). Here we show that ALS8-iPSCs can be derived and the presence of the mutation does not interfere in the iPSC generation or maintenance.

Immunocytochemistry assays were carried out in order to show that the generated iPSC colonies express pluripotent markers. As shown in Figure1F-I, colonies from control individuals and ALS8 patients express pluripotent markers, such as *Lin28* and *Nanog*. Moreover, RNA was extracted from fibroblasts and derived-iPSCs and used as a template for semi-quantitative RT-PCR using primers for the endogenous expression of *Oct4* and *Nanog*. These markers are present in iPSC but not in fibroblasts from the same individual (Fig. S2). Injection of iPSCs subcutaneously in nude mice induced the formation of teratomas. Tissues originated from the three germ layers were found, such as Gut-like cells from endoderm; cartilage and muscle from mesoderm; neural rosettes from ectoderm (Fig. 1J–M). Finally, karyotype analysis revealed a normal chromosomal number and lack of

gross chromosomal abnormalities (Fig. 1N–O). The iPSC characterization demonstrated that the reprogramming process successfully generated ALS8 and WT pluripotent stem cells.

Overexpression of VAPB P56S forms aggregates in mammalian cells

ALS8 overexpression models main feature is the presence of cytoplasmic aggregates containing VAPB protein (Nishimura *et al.*, 2004; Kanekura *et al.*, 2006; Teuling *et al.*, 2007; Chai *et al.*, 2008; Ratnaparkhi *et al.*, 2008 Tsuda *et al.*, 2008; Prosser *et al.*, 2008; Suzuki *et al.*, 2009; Fasana *et al.*, 2010; Tudor *et al.*, 2010, Kim *et al.*, 2010). In order to address the question of whether those aggregates could be identified in the ALS8 patients-derived motor neurons, we first test the ability of a monoclonal antibody (A1315F3G10) raised against a sequence to the VAPB N-terminal portion. When we overexpressed flag-tagged versions of the WT- and P56S-VAPB in HEK293T cells, the immunostaining pattern reveals the perinuclear localization of WT-VAPB and identifies specifically VAPB polypeptide chains into aggregates generated by the overexpression of P56S-VAPB in the cytoplasm (Fig. 2A). The specificity of the antibody was also tested in a western blot. Using both the VAPB and the Flag antibodies, we were able to detect both WT and P56S versions of the protein (Fig. 2B). Interesting, the amount of P56S-VAPB detected is clearly reduced when compared to the WT form, indicating that the WT form is likely more soluble. Additionally, only endogenous VAPB was identified on FLAG-VAPA transfected extracts. It shows that, besides the 63% of identity between VAPB and VAPA at amino acids level, there is no cross-reaction of this antibody with VAPA (Fig.2B). Next, we decided to check for the presence of cytoplasmic P56S-VAPB aggregates in human motor neurons derived from ALS8-iPSCs.

Absence of VAPB aggregates in iPSC-derived motor neurons

Motor neuron differentiation was based on previous protocol published by Marchetto *et al.*, (2008) with some modifications, described in details in the Methods section. To check for the presence of motor neurons, we used a reporter construct with GFP sequence under Hb9 promoter control (*Hb9::GFP*) (Marchetto *et al.*, 2008). After 4 weeks of EB differentiation in suspension, neuronal differentiation was confirmed by the expression of specific markers. A co-staining, using TDP-43 and Map2 markers, was used to distinguish cytoplasmic and nuclear areas (Fig. 2C). With 4 weeks of differentiation EBs were dissociated and plated on coated dishes. Three weeks later, GFP-expressing cells were observed on both control and ALS8-iPSC-derived cultures (Fig. 2D). After 7 weeks of differentiation, cell cultures containing GFP expressing motor neurons from controls and patients were analyzed for the presence of cytoplasmic aggregates containing VAPB protein. The distribution pattern of VAPB on motor neurons is very similar between controls and ALS8-derived cells, mainly localized in perinuclear sites (Fig. 2E). This data suggests that ALS8 motor neurons do not show the presence of aggregates *in vitro*. This result clearly contrasts with previous overexpression data reporting mutant VAPB aggregates by us (Fig. 2A) and others (Nishimura *et al.*, 2004; Kanekura *et al.*, 2006; Teuling *et al.*, 2007; Chai *et al.*, 2008; Ratnaparkhi *et al.*, 2008; Tsuda *et al.*, 2008; Prosser *et al.*, 2008; Suzuki *et al.*, 2009; Fasana *et al.*, 2010; Tudor *et al.*, 2010, Kim *et al.*, 2010).

Disease-related phenotypes in the specific cell type have been difficult to achieve, especially for late onset neurodegenerative diseases (Marchetto *et al.*, 2010^A). Challenging the neuronal cultures could be one of the ways to reveal phenotypes that would not be identified at normal common culture conditions. The Ubiquitin/Proteasome pathway comprises the main cell quality protein control system (Huang and Figueiredo-Pereira, 2010). Misfolded polypeptide chains tagged with ubiquitin are destroyed by the proteasome system, avoiding their accumulation inside the cell. Studies based on the overexpression of mutant VAPB have shown that the pathogenic variant is ubiquitinated, leading to an

accumulation of the tagging protein in the cytoplasm (Kanekura *et al.*, 2006, Ratnaparkhi *et al.*, 2008, Tsuda *et al.*, 2008, Tudor *et al.*, 2010, Kim *et al.*, 2010). For this reason, disturbing the Ubiquitin/Proteasome system could reveal nuances that would not appear under normal cell culture conditions. The proteasoma can be inhibited by the use of MG132 molecule (Joazeiro *et al.*, 2006). In this case, we predict an exacerbation of the amount of ubiquitin labeled proteins, since they would not be degraded. Based on overexpression studies, it is reasonable to expect the mutant VAPB accumulation caused by proteasome inhibition. Thus, we attempted to identify VAPB aggregates in the presence of MG132 in fibroblasts and motor neurons derived from control and ALS8 patients' iPSCs. In the presence of MG132, we detected an increase in ubiquitin aggregates both in fibroblasts and motor neurons, indicating a successful inhibition of the proteasoma complex (Fig. 3). However, we could not distinguish the pattern of VAPB and ubiquitin between normal and ALS8 treated fibroblasts (Fig. 3A). Similar results were reached for differentiated motor neuron cultures submitted to MG132 treatment (Fig. 3B). These results indicate that stressing ALS8 patient-derived cells by proteasoma inhibition was not sufficient to induce VAPB aggregates in the cytoplasm as seen in overexpressing models by ectopic expression of P56S-VAPB.

Reduced VAPB expression in ALS8 patient-derived cell types

VAPB is a ubiquitous expressed protein present in different levels depending on the tissue (Gkogkas *et al.*, 2008). However, it is unclear when VAPB expression starts during development and if the levels of VAPB expression is similar between controls and ALS8 patients. The late-onset and slow progression in ALS8 patients, could be the result of an imbalanced amount of VAPB. This hypothesis is supported by our data showing a reduction of P56S-VAPB soluble levels in ALS8 cells (Fig. 2B). Thus, we attempted to detect VAPB protein expression in different cell types.

VAPB expression is clearly detected on iPSCs. A co-staining with the pluripotent marker Nanog was performed to assure the pluripotent state of these cells (Fig. 4A). The intracellular pattern of VAPB is very similar between patients and control iPSC, i.e., perinuclear and without inclusion bodies in the cytoplasm. Cell reprogramming to a pluripotent state requires an uncountable number of modifying steps involving transcriptional and epigenetic factors. To exclude VAPB expression in iPSC as a “memory retention” phenomenon from reprogrammed fibroblasts, we also checked its expression on human embryonic stem cells lines HUES9 and HUES6, kept in the same culture condition, as the generated iPSC (Fig. 4B). This is the first demonstration that VAPB is expressed in pluripotent stages, indicating that VAPB protein is present since the beginning of development. Interestingly, there is a reduction of VAPB in the iPSC soluble protein extract from the ALS8 patient samples when compared to controls (Fig. 4C and D). Such protein levels reduction is independent of the iPSC clone used for comparison (Fig. S3). Next, we investigated whether the VAPB protein reduction in ALS8 iPSC would persist in differentiated cells. Immunoblot analyzes were performed comparing control and patient lineages, maintained in parallel during all steps of cellular reprogramming and the differentiation process. Figures 4E and 4F show that, in all stages of differentiation, VAPB in the soluble fraction is significantly reduced in ALS8 cells, suggesting that the iPSC generation and differentiation does not erase this disease’s hallmark. More importantly, the VAPB amount difference between control and ALS8 cells increase during the differentiation process (1.6-fold difference in iPSC to 4-fold in motor neurons), indicating that VAPB levels increase as cells progress towards the neuronal lineage (Fig. 4F). All together, our data revealed an unexpected increase in VAPB levels during neuronal differentiation that fails to take place in ALS8-derived cells.

Discussion

ALS is a fatal condition with poorly explained causes for motor neurons death. The generation of familial ALS mouse models has helped to clarify many aspects of the disease pathogenesis. Unfortunately, a substantial number of successful drug tests in ALS animal systems could not be translated to humans (Ludolph *et al.*, 2010). The lack of success in applying previous pre-clinical strategies in ALS patients may be due to the use of a single animal model – SOD1 mice carrying a high transgene copy number (Pizzasegola *et al.*, 2009; Ludolph *et al.*, 2010). ALS8 shows well-defined genetic mutations (Nishimura *et al.*, 2004^B, Chen *et al.*, 2010) and its neurodegeneration pathway may likely share common traits with other ALS forms (Teuling *et al.*, 2007). Here we show the development of a new ALS model, based on ALS8 patient-derived iPSCs and their non-affected siblings.

All samples were reprogrammed to a pluripotent status and cellular and molecular characterization showed that these are *bona fide* iPSC. It was not possible to identify a clear pattern of inclusions in ALS8-derived motor neurons even after the long time required for the neuronal maturation protocol (7-8 weeks). Instead, we found the VAPB distribution pattern to be very similar between control and ALS8 cells, which could not be distinguished with the use of MG132. This lack of aggregates detection is extended to other cell types – fibroblasts, iPSC and cells submitted to motor neuron differentiation. We then showed that VAPB is expressed since beginning of development, in pluripotent stem cells, and protein levels increase during motor neuron differentiation. Allied to previous studies from Dimos *et al.*, (2008) and Ebert *et al.*, (2008), our results suggest that mutations leading to motor neuron diseases are unlikely to impede cell reprogramming. This is highlighted specially in situations where the affected protein is expressed since the pluripotent stage, as it is the case of VAPB (this work) and for the SMN protein, responsible for Spinal Muscular Atrophy (Ebert *et al.*, 2008). Intracytoplasmic aggregates comprise a hallmark in different ALS forms, SOD1 (ALS1) (Bruijn *et al.*, 1997; Bergemalm *et al.*, 2010), TDP-43 (ALS10) (Gitcho *et al.*, 2008; Sreedharan *et al.*, 2008; Kabashi *et al.*, 2008), FUS/TLS (ALS6) (Vance *et al.*, 2009) and

VAPB itself (Nishimura *et al.*, 2004; Kanekura *et al.*, 2006; Teuling *et al.*, 2007; Chai *et al.*, 2008; Ratnaparkhi *et al.*, 2008; Prosser *et al.*, 2008; Suzuki *et al.*, 2009; Fasana *et al.*, 2010; Kim *et al.*, 2010). TDP-43 immunoreactive aggregates have a highlighted role in those inclusions, since they are found in most ALS cases that are SOD1-negative (Lagier-Tourenne and Cleveland, 2009) and in VAPB-P56S transgenic mice (Tudor *et al.*, 2010). However, in our *in vitro* analyzes we could not identify VAPB inclusions, contrasting this “one allele” model to the overexpression systems.

However, we show a dramatic reduction in VAPB protein levels in fibroblasts of ALS8 patients, compared to their normal siblings. Moreover, the difference persisted after cell reprogramming and further differentiation, but the ratio increases when iPSCs were induced towards the neuronal lineage. The reduction of VAPB ratio levels between patients and controls enforces the idea that iPSC technology can recapitulate the patients’ original cell status (Park *et al.*, 2008; Marchetto *et al.*, 2010A; Marchetto *et al.*, 2010^B). Our results that VAPB is reduced in motor neurons from ALS8 samples plus the fact that no other pathogenic situation was found in the patients-derived cells, boosts the hypothesis that the correct amount of VAPB is crucial for motor neuron viability. We hypothesize that, even in the presence of a smaller amount of VAPB, ALS8 carriers have a normal development until they reach the 4th or 5th decade of life, when those levels became critical for motor neuron survival. Our data reveals the importance of ALS modeling using human iPSC, avoiding possible misinterpretations from animal background and overexpression systems. Our data indicate that the iPSCs can recapitulate some aspects of neurodegenerative diseases and be used to extract new biological information relevant to human motor neuron neurodevelopment. We believe this cellular model has the potential to complement other human and animals models to lead to the discovery of new compounds that might rescue neuronal phenotypes to treat ALS and other forms of motor neuron neurodegeneration.

Material and Methods

Skin biopsy

All procedures were conducted with the approval of committees from University of São Paulo (USP - Brazil), Federal University of São Paulo (UNIFESP – Brazil) and University of California San Diego (UCSD). ALS8 patients were analyzed by experienced neurologists that defined the diseases based on clinical findings. Initially, DNA was extracted from lymphocytes. The mutation c.C>T₁₆₆ (P56S) was confirmed by *HaeIII* enzyme digestion and by the sequencing of the amplicon containing the exon 2 (Nishimura *et al.*, 2004^B). ALS8 families were selected according availability to meet the criteria of a minimum of 2 affected and 1 normal sibling from the same genitors. Skin biopsies were performed by a neurologist, using a 3 mm punch, after local anesthesia. Explants were collected in DMEM (1X) with 5% (v/v) antibiotics (Pen/Strep 10,000U/mL). Fibroblast cell culture was implemented using DMEM (1X) with 10% (v/v) Fetal Bovine Serum (FBS) and 2% (v/v) antibiotics.

Cellular reprogramming

Cellular reprogramming was performed as previously described (Marchetto *et al.*, Cell 2010^B). Briefly, the four Yamanaka factors (*c-Myc*, *Oct3/4*, *Klf4* and *SOX2*) (Takahashi *et al.*, 2007) in a retrovirus system were used to infect fibroblasts at low passage. After two days, infected human fibroblasts were moved to human embryonic stem cell (hESC) conditions on a feeder layer of irradiated mouse embryonic fibroblasts (Chemicon). Human Embryonic Stem Cell-like cells appears 10–15 days after virus transduction. iPSC colonies were selected by morphology and manually moved to a feeder-free condition in Matrigel-coated dishes (BD Bioscience) and mTeSR1 (Stem Cell Technologies). iPSCs were manually passed and maintained on feeder-free condition until the beginning of the

differentiation protocol. Three clones from one control and from two patients were selected for further characterization for each family.

Teratoma formation

Aproximattely 3×10^6 iPSCs were injected subcutaneously into the dorsal flanks of nude mice (CByJ.Cg-Foxn1 nu/J) anesthetized with isoflurane. Five to six weeks after injection, teratomas were dissected, fixed overnight in 10% formalin phosphate and embedded in paraffin. Sections were stained with hematoxylin and eosin for further analysis. Protocols were previously approved by the University of California San Diego Institutional Animal Care and Use Committee.

Motor Neuron Differentiation

Motor neuron differentiation was based on previous protocol published by Marchetto *et al.*, (2008) with some modifications. Briefly, the differentiation comprised a period of 7-8 weeks, with 5% efficiency at the end. Confluent iPSC on feeder free condition were moved to N2 media on F12/DMEM (Invitrogen, Carlsbad, CA), on day 0. On the following day a BMP signaling inhibitor, dorsomorphin – 1uM (Calbiochem) was added. On the third day, colonies were dissociated using dispase (0.5mg/mL) and kept under rotation at 37°C to form Embryonic Bodies (EBs). Cells were maintained in this condition for 4 more weeks with the gradual addition of neuronal survival and differentiation inducers as follows: day 9, addition of differentiation Retinoic Acid (RA – 1uM) and Ascorbic Acid (AC – 200ng/mL) were added; day 16, addition of Sonic Hedgehog (SHH – 500ng/mL: R&D Systems), Brain Derived Neural Factor (BDNF – 20ng/mL: Peprotech), Growth Derived Neural Factor (GDNF – 20ng/mL: Peprotech) with the simultaneous dorsomorphin removal. On day 30, cells were plated on poli-ornitine/laminin- coated plates and kept in culture for additional 3 – 4 weeks.

After cell plating, the previous neural induction media was modified by the reduction of SHH to 100ng/mL and addition of cyclic Adenosine Monophosphate (cAMP – 1uM). *Hb9::GFP* reporter construct was transferred to the cells using a lentivirus system (Marchetto *et al.*, 2008). The lentivirus was added to the culture either at the dissociation moment or 2 days after plating the cells on poli-O/L.

Proteasome Inhibition Assay

MG132 (Sigma) was used to block the proteasome system. Several attempts were made to optimize the MG132 concentrations. Fibroblasts were kept for 24h with 10uM of MG132. Motor Neurons were treated with 5uM of the inhibitor for 24h.

Karyotyping

Standard G-Banding chromosome analysis was performed by Cell Line Genetics (Madison, WI) and Molecular Diagnostic Services, Inc (San Diego, CA).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde, followed by permeabilization and blocking with 0.1% (v/v) Triton X in PBS containing 5% (v/v) donkey serum, for 1h. Primary antibodies were incubated overnight at 4°C. Samples were washed three times before secondary antibodies incubation (Jackson Laboratories and Alexa Fluor Dyes, Life Technologies) for 1h. Primary antibodies concentrations were: α-hVAPB mouse monoclonal A1315F3G10 1:500; α-Ubiquitin rabbit polyclonal 1:1000 (Dako); α-Nanog mouse monoclonal 1:100 (BD Bioscience); α-Oct3/4 mouse monoclonal 1:250 (Santa Cruz); α-Lin28 goat polyclonal 1:400

(R&D Systems); α -MAP2 mouse polyclonal 1:200 (Milipore); α -TDP-43 rabbit polyclonal 1:500 (Novus Biologicals); α -GFP chicken polyclonal 1:500 (Aves Lab).

Western Blot analysis

Cells were collected, re-suspended in 1X RIPA lyses buffer (Milipore) containing 1% (v/v) protein inhibitor cocktail (Sigma), triturated and centrifuged at 10,000g for 20min at 4°C. Ten micrograms of protein extract was separated in a 4-12% Pre-Cast Bis-Tris acrilamde gradient gel (Invitrogen, Life Tech), transferred to a nitrocellulose membrane and probed with primary antibody: α -hVAPB mouse monoclonal A1315F3G10 1:1000, followed by horseradish peroxidase-conjugated secondary antibody. Visualization was made by ECL chemiluminescence (Amersham). Membranes were striped and re-probed using antibodies against β -Actin 1:5000 (Ambion) and Tubulin 1: 5000 (Ambion) to generate a control for protein loading. Quantifications were performed by the use of Image J program and normalized according to control Tubulin and Actin signals.

RNA Extraction and RT-PCR

Total RNA was extracted using Trizol. Reverse Transcriptase reaction was performed by the use of Super Script III First-Strand Synthesis System RT-PCR from Invitrogen. cDNA synthesis was amplified by PCR using the following primers: *Nanog* F 5' cctatgcctgtgattgtgg3' and *Nanog* R 5' ctgggacctgtcttccttt3; *hOct4* F 5' gggaggggaggagctagg3' and *hOct4* R 5' tccaaccagttgccccaaac'3'.

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The authors declare no conflict of interest.

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Figure Legends

Fig. 1. Generation and characterization of controls and ALS8-patients iPSCs. (A) Heredograms of the two studied ALS8 families. ALS8 patients are represented in dark symbols and non-affected individuals in white. Skin biopsies were collected (*) from two affected and two control individuals of family 1; and one control and two affected individuals of family 2. (B) Phase contrast showing iPSC colony morphology after one week post-infection growing in mitotically inactivated mouse embryonic fibroblasts. (C) isolated iPSC colony with similar morphology to human embryonic stem cells. (D) iPSC colony on feeder free condition. (E) Representative image of an established human iPSC colony, with well-defined borders and compact cells. Bar=100um. ALS8-iPSC (F and G) and WT-iPSC (H and I) staining, showing the expression of pluripotent markers. Bar=20um. Derived iPSC clones were able to generate teratomas in nude mice. Tissues from the three germ layers could be identified: Mesoderm – Cartilage (J) and Muscle (L); Endoderm – Gut-like epithelium (K); Ectoderm – Neural Rosettes (M). Bar=200um. The karyotype from ALS8- (N) and WT-iPSCs (O) showed normal chromosomal number.

Fig. 2. VAPB localization in human cells. (A) Overexpression of WT and P56S forms of VAPB in HEK293T cells. While the WT form showed a perinuclear pattern, the mutant overexpression was mainly localized as cytoplasmic aggregates. Bar=70um (B) Western blot showing a reduction of the mutant VAPB (P56S) soluble form when compared to WT VAPB or VAPA. Anti-VAPB identifies endogenous VAPB on FLAG-VAPA transfected extracts. (C) A mature EB section revealing robust neuronal differentiation. Cells expressing the mature neuronal marker Map2 also express TDP43 in the nucleus (inset). Bar=20um. (D). Live image of motor neuron-like cells expressing GFP under the control of *Hb9* promoter. Bar=100um. (E). Perinuclear VAPB distribution on a GFP-positive motor neuron. Bar=20um.

Fig. 3. VAPB staining after MG132 treatment. (A) Fibroblasts treated with MG132 increased the number of ubiquitin puncta staining (arrows). Bar=25um. (B) VAPB perinuclear distribution is similar between control and ALS8-iPSCs derived *Hb9::GFP* motor neurons after MG132 treatment. Bar=10um.

Fig. 4. Reduction of VAPB protein levels in ALS8-derived motor neurons. (A) VAPB shows perinuclear staining on both WT and ALS8-iPSC expressing the pluripotent marker *Nanog*. Bar=50um. (B) Human embryonic stem cells also express VAPB. (C, D) VAPB levels on iPSC lines are reduced on ALS8 samples when compared to controls (Con). (E) Reduced levels of VAPB in ALS8 cells during differentiation. (D) VAPB increases during motor neuron differentiation of control but not from ALS8-iPSC.

Fig. S1. Cellular genotyping. *VAPB* exon 2 was amplified by PCR and digested using *HaeIII* restriction enzyme. The c.C>T166 mutation abolishes one of the sites for this enzyme revealing two fragments of 234 bp (mutated) and 194 (wt). Fibroblasts (A) and iPSC (B).

Fig. S2. Semi-quantitative PCR. Total RNA from primary fibroblasts and derived-iPSC respectively was collected and tested for the endogenous expression of *Oct4* and *Nanog*. *GAPDH* was used as an internal control.

Fig. S3. The difference of VAPB levels between control and ALS8-patient samples is independent of the iPSC used clone.

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Supplemental Figure 1

Supplemental Figure 2

Supplemental Figure 3

Capítulo 4

4. Paraplegia Espástica Hereditária Tipo 4

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A multi-exonic SPG4 duplication underlies sex- dependent penetrance of hereditary spastic paraplegia in a large Brazilian pedigree

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Abstract

SPG4 mutations are the most frequent cause of autosomal-dominant hereditary spastic paraplegia (HSP). SPG4 HSP is characterized by large inter- and intrafamilial variability in age at onset (AAO) and disease severity. The broad spectrum of SPG4 mutations has recently been further extended by the finding of large genomic deletions in SPG4-linked pedigrees negative for 'small' mutations. We had previously reported a very large pedigree, linked to the SPG4 locus with many affected members, which showed gender difference in clinical manifestation. Screening for copy number aberrations revealed the first case of a multi-exonic duplication (exon10_12dup) in the SPG4 gene. The mutation leads to a premature stop codon, suggesting that the protein product is not functional. The analysis of 30 individuals who carry the mutation showed that males have on average an earlier AAO and are more severely affected. The present family suggests that this HSP pathogenesis may be modulated by factors related to individual background and gender as observed for other autosomal dominant conditions, such as facio-scapulohumeral muscular dystrophy or amyloidosis. Understanding why some individuals, particularly women, are 'partially protected' from the effects of this and other pathogenic mutations is of utmost importance.

Resumo

Mutações no gene *SPG4* constituem a causa mais frequente de Paraplegia Espástica Hereditária (PEH) com padrão de herança autossômico dominante. A PEH causada por mutações no *SPG4*, é caracterizada por uma grande variabilidade inter e intra-familiar na idade de início dos sintomas assim como na severidade dos sintomas. O amplo espectro de mutações no *SPG4* foi, recentemente, estendido pela identificação de grandes deleções em pedigrees previamente ligados à região do *SPG4*, nos quais, entretanto, “pequenas” mutações não haviam sido encontradas. Anteriormente, havíamos reportado uma grande irmandade brasileira, ligada ao locus *SPG4*, que apresentava diferença na manifestação clínica de acordo com o gênero do paciente. A busca por alterações no número de cópias levou à identificação do primeiro caso de uma duplicação multi-exônica (exon10_12dup) no gene *SPG4*. Esta mutação leva à formação de um códon de parada prematuro, sugerindo que a proteína produzida não é funcional. A análise de 30 indivíduos portadores da mutação mostrou que os homens possuem, na média, idade de início antecipada e quadro clínico mais severo. A família em questão sugere que esta forma de PEH pode ser modulada por fatores relacionados ao background individual e ao gênero. Este padrão já havia sido observado para outras condições com padrão autossômico dominante, como a distrofia muscular fácio-escápulo humeral ou amiloidose. Entender porque alguns indivíduos, particularmente mulheres, são “parcialmente protegidos” dos efeitos desta e de outras mutações patogênicas é de extrema importância.

SHORT REPORT

A multi-exonic *SPG4* duplication underlies sex-dependent penetrance of hereditary spastic paraplegia in a large Brazilian pedigree

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SPG4 mutations are the most frequent cause of autosomal-dominant hereditary spastic paraplegia (HSP). *SPG4* HSP is characterized by large inter- and intrafamilial variability in age at onset (AAO) and disease severity. The broad spectrum of *SPG4* mutations has recently been further extended by the finding of large genomic deletions in *SPG4*-linked pedigrees negative for 'small' mutations. We had previously reported a very large pedigree, linked to the *SPG4* locus with many affected members, which showed gender difference in clinical manifestation. Screening for copy number aberrations revealed the first case of a multi-exonic duplication (exon10_12dup) in the *SPG4* gene. The mutation leads to a premature stop codon, suggesting that the protein product is not functional. The analysis of 30 individuals who carry the mutation showed that males have on average an earlier AAO and are more severely affected. The present family suggests that this HSP pathogenesis may be modulated by factors related to individual background and gender as observed for other autosomal dominant conditions, such as facio-scapulohumeral muscular dystrophy or amyloidosis. Understanding why some individuals, particularly women, are 'partially protected' from the effects of this and other pathogenic mutations is of utmost importance.

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Keywords: *SPG4*; duplication; sex-dependent penetrance; hereditary spastic paraplegia

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Introduction

The major feature of the heterogeneous condition hereditary spastic paraplegia (HSP) is progressive weakness and spasticity of the lower limbs. The most frequent cause is mutation of the *SPG4* gene, associated with autosomal-dominant inheritance.¹ *SPG4* HSP shows large inter- and intrafamilial variability in age at onset (AAO) and disease severity, suggesting the existence of modifying factors.² The broad spectrum of *SPG4* mutations has recently been further extended by the finding of large genomic deletions

in *SPG4*-linked pedigrees, which had screened negative for 'small' mutations.³ We previously reported on another such *SPG4*-linked but apparently mutation-negative pedigree, which, in addition, appeared unique in almost exclusively men expressing the phenotype.⁴

Subjects and methods

DNA of three definitely affected individuals was analyzed using *SPG4*-specific multiplex ligation-dependent probe amplification (MLPA – MRC Holland) as described previously.⁵ Long-range PCR on genomic DNA followed by sequencing was used to define the limits of the aberration identified. Mutation-specific PCR was used to establish the carrier status of 52 individuals.

The polymorphic variants c.131C>T or c.134C>A were screened as described previously.⁶ The samples were sequenced in a Mega Bace 1000 DNA Sequencer – Amersham Bioscience (GE-Healthcare). cDNA to be used in RT-PCR was prepared from peripheral blood of affected family members.

Fifty-two available family members (30 women and 22 men) from three generations were clinically re-investigated and classified by a team of neurologists, who were blind about the genotypes; these included most of the formerly reported⁴ as well as 14 additional individuals (Figure 1 and Table 1).

Results

Using MLPA, we identified a partial *SPG4* duplication (exon10_12dup) in DNA of three definitely affected individuals. Long-range PCR followed by sequencing of the junction revealed that this duplication was in tandem

(c.1246-2896_1493 + 523dup) with non-homology of the sequences contributing to the novel fusion (Supplementary Figure 1). None of the family members carried the polymorphic variants c.131C>T or c.134C>A. RT-PCR-based cDNA analysis showed the duplication to also be present at mRNA level. From the total of 52 individuals investigated, 30 (14 women) carried the duplication, 12 of whom (9 women) had no clinical complaints. AAO data compiled during the re-investigation showed a strong difference between men and women ($P = 0.017$, Figure 2).

Discussion

The molecular defect we identified corroborates the recent suggestion of copy number errors to underlie all cases of *SPG4* HSP negative for small mutations.³ However, only deletions have been reported so far, whereas we now present the first instance of a partial *SPG4* duplication. The lack of sequence identity at the novel junction in our case (Supplementary Figure 1) argues against non-allelic homologous recombination underlying the duplication event. The duplication is in tandem and results in an mRNA containing two adjacent exon 10–12 blocks. This arrangement causes a frameshift and creates a pre-terminal stop codon (PTC) in the second exon 10, thereby disrupting the enzymatic domain. Lack of enzymatic activity has been proposed as the disease-causing mechanism for numerous missense and truncating mutations showing the same primary effect.^{2,7}

The ascertainment of carrier status and AAO data by the present study allowed statistical confirmation of highly sex-dependent penetrance in our pedigree (Figures 1 and 2 and Table 1). Since the median AAO of *SPG4* HSP is approximately 30 years,⁸ we observed more women than

Figure 1 Pedigree for the 52 individuals included in this analysis. Symbol below individuals corresponds to molecular and neurological condition. N: no mutation detected; 0: asymptomatic at neurological examination; 1: asymptomatic with mild neurological abnormalities; 2: moderate neurological abnormalities, able to walk without support; 3: severe neurological abnormalities, only able to walk with cane or walker.

Table 1 Clinical data for 30 mutation carriers

Disability stage	Individual	Sex	Age (years)		Disease duration (years)	Disease severity	Pyramidal signs					
			At onset	At examination			LL reflexes	Plantar response	Vibration sense diminished in LL	Pes cavus	Spasticity	Sphincteric disturbance
0	IV-15	M		31		None	Normal	Flexor	No	No	No	None
0	IV-3	M		42		None	Normal	Flexor	No	No	No	None
0	III-6	M		55		None	Normal	Flexor	No	No	No	None
0	III-22	F		58		None	Normal	Flexor	No	No	No	Micturition urgency
0	III-41	F		55		None	Normal	Flexor	No	No	No	None
0	IV-10	F		38		None	Normal	Flexor	No	No	No	None
0	IV-5	F		38		None	Normal	Flexor	No	No	No	Micturition urgency
0	IV-6	F		32		None	Normal	Flexor	No	No	No	Micturition urgency
1	III-40	F		52		No motor complain	Normal	Extensor	No	No	No	Micturition urgency
1	III-33	F		62		No motor complain	Brisk	Extensor	No	No	No	Micturition urgency
1	III-43	F		45		No motor complain	Brisk	Extensor	No	No	Mild	None
1	III-10	F		65		No motor complain	Brisk	Extensor	No	No	Mild	None
2	IV-2	M	29	38	9	Mild	Brisk	Extensor	Yes	No	Mild	None
2	III-39	M	41	46	5	Mild	Brisk	Extensor	No	No	Mild	Micturition urgency
2	IV-14	M	27	32	5	Mild	Brisk	Extensor	No	No	Mild	None
2	III-9	M	50	67	17	Mild	Brisk	Extensor	Yes	No	Mild	Micturition urgency
2	V-1	M	1	10	9	Mild	Brisk	Extensor	No	No	Mild	Micturition urgency
2	III-29	M	12	46	34	Mild	Brisk	Extensor	Yes	Yes	Mild	Micturition urgency
2	III-35	M	54	58	4	Mild	Brisk	Extensor	Yes	Yes	Mild	Micturition incontinent
2	IV-11	M	1	33	32	Mild	Brisk	Extensor	Yes	Yes	Moderate	None
2	III-30	F	38	43	5	Mild	Brisk	Extensor	No	No	Mild	Micturition urgency
2	III-32	F	46	66	20	Mild	Brisk	Extensor	No	No	Mild	Micturition urgency
2	IV-1	F	48	49	1	Mild	Brisk	Extensor	No	No	Mild	None
2	IV-8	F	23	25	2	Mild	Brisk	Absent	No	No	Mild	Micturition incontinent
2	III-19	F	17	59	42	Mild	Brisk	Extensor	No	No	Mild	None
3	III-11	M	26	63	37	Moderate	Brisk	Extensor	Yes	No	Moderate	None
3	III-14	M	18	73	55	Moderate	Brisk	Extensor	Yes	No	Moderate	Micturition incontinent
3	III-18	M	25	67	42	Moderate	Brisk	Extensor	ND	No	Moderate	Micturition incontinent
3	III-16	M	49	68	19	Moderate	Brisk	Extensor	ND	No	Moderate	Micturition incontinent
3	III-4	M	38	63	25	Moderate	Brisk	Extensor	Yes	Yes	Moderate	Micturition incontinent

F, Female; LL, lower limbs; M, Male; UL, upper limbs; ND, not done.

Figure 2 Kaplan–Meier curves. AAO for women (green, $n=14$) and men (blue, $n=16$) mutation carriers. Outcome differs significantly between male and female mutation carriers ($P=0.017$, Log Rank Mantel-Cox).

men showing an unusual course of the disease (Figure 2). The presence of known intragenic disease modifiers,⁶ potentially contributing to the distribution of AAO, was excluded. Further efforts are needed to resolve why a seemingly special mutation leads to the ‘protection’ of a defined group of carriers in this pedigree. Interestingly, a similar gender difference in clinical course has been observed for other autosomal-dominant neuromuscular disorders, such as facioscapulohumeral muscular dystrophy⁹ and amyloidosis.¹⁰ Understanding why some individuals, particularly women, are ‘partially protected’ from the effects of this and other pathogenic mutations remains a great challenge that might open new avenues for treating these disorders.

Supplementary Information accompanies the paper on European Journal of Human Genetics website (<http://www.nature.com/ejhg>)

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junction: CTGAATCCAAATTGATTTTTTCAAATTTGTAAAATAACTTAACGAACATTTCACATAGTCTCTACCAGTTTACAGTGTTT

Fig.S.4 – Sequenciamento referente aos fragmentos de DNA (A) e cDNA (B) obtidos a partir de amostras de pacientes portadores da duplicação no *SPG4*. (A) junção dos introns 12 e 9 que evidencia a duplicação no DNA. (B) junção dos exons 12 e 10, mostrando que a duplicação também está presente no RNA. Os códons estão representados acima do eletroferograma de acordo com o primeiro *ATG* do *SPG4*, acima da “base intermediária” de cada trinca. O asterisco (*) representa o códon de parada prematuro produzido pelo transcrito mutante.

Capítulo 5

DISCUSSÃO

A relevância das doenças do neurônio motor é evidente não apenas pelas complicações geradas ao paciente, mas também à sua família, que muitas vezes constitui o grupo dos cuidadores. Afecções como a ELA levam a uma enorme perda na qualidade de vida, tanto pelas limitações motoras quanto pelas consequências psicológicas. Esta situação se agrava com o prognóstico finalista de uma doença incurável, progressiva e que na maior parte dos casos familiares tem chances de ser transmitida para cinquenta por cento dos filhos. Dessa forma, são essenciais os esforços para entender as suas causas a fim de propor novos tratamentos.

O estudo de doenças genéticas traz a vantagem de se saber, ao menos, um dos motivos para a manifestação da mesma. Neste aspecto, a descoberta de genes causadores de doenças do neurônio motor possibilitou elaborar hipóteses para o seu desenvolvimento, além de gerar modelos para testá-las.

A identificação da ELA8 trouxe novos elementos tanto na parte clínica quanto molecular: diferentes manifestações clínicas, como ELA típica, ELA atípica e Atrofia Muscular Espinhal de início tardio, podem ser causadas por mutações no mesmo gene. O crescente número de pacientes com ELA8 no Brasil, a presença da P56S em populações alemãs e japonesas, bem como o achado de uma nova mutação (T46I), sugerem que esta forma da doença não é tão rara quanto proposto anteriormente por Hirano *et al.*, (2008) e Landers *et al.*, (2008). Neste aspecto, contribuímos (Anexo 1: Funke *et al.*, 2010) para a demonstração de que a P56S

apareceu em dois eventos independentes, visto que o paciente alemão identificado não compartilha o mesmo haplótipo que os brasileiros (Funke *et al.*, 2010).

A troca P56S está presente em uma região muito conservada da proteína, que, provavelmente, é essencial para o seu correto funcionamento. Como mostrado no Capítulo 2, esta mutação não parece abolir o estado de dimerização do domínio MSP da VAPB humana. Entretanto, ela diminui a eficiência de interação deste domínio com outras proteínas. Conseguimos identificar dois desses ligantes, a GAPDH e a beta-Tubulina, ambas previamente relacionadas com mecanismos de degeneração (Mitne-Neto *et al.*, 2007^A).

Conhecida pelo seu papel no metabolismo de carboidratos, a GAPDH também possui funções não-glicolíticas e, dependendo da localização intracelular, estaria envolvida com doenças como Huntington, Alzheimer, Parkinson e processos de Isquemia/Hipóxia (Chuang *et al.*, 2005; Tatton *et al.*, 2000).

Microtúbulos são estruturas responsáveis por diversas atividades celulares e são formados por heterodímeros de alfa e beta-Tubulina. A dVAP33, ortóloga da VAPB em drosófila, está associada ao retículo endoplasmático e aos microtúbulos (Skehel *et al.*, 2000; Penetta *et al.*, 2002), sugerindo que a pouca interação entre a VAPB e a beta-Tubulina poderia ser uma das causas para perda de localização (formação de agregados) produzida pela super-expressão da proteína mutante. A idéia de que proteínas motoras exercem papel na neurodegeneração ganha cada vez mais força. Reduções na disponibilidade de microtúbulos têm efeitos deletérios sobre os neurônios motores e proteínas associadas aos microtúbulos causam doenças semelhantes à ELA, como doença de Charcot e Paraplegias Espásticas. Além disso, o transporte axonal, baseado em microtúbulos, é a primeira

anormalidade pré-sintomática identificada nos camundongos transgênicos para SOD1 (Carsten *et al.*, 2007).

Após a identificação do gene *SOD1* como causador da ELA1 (Rosen *et al.*, 1993), diversos estudos em conjunto desvendaram alguns dos mecanismos ligados ao ganho de função tóxica, gerado pelas mais de 140 mutações. Os animais transgênicos SOD1 tornaram-se a principal ferramenta para avaliações pré-clínicas. Entretanto, um número substancial de drogas que apresentaram êxito nesses modelos não pôde ser aplicado aos pacientes com ELA (Ludolph *et al.*, 2010). Alguns autores apontam que este hiato é devido, principalmente, ao uso de um único modelo – camundongos SOD1 carregando inúmeras cópias do transgene (Pizzasegola *et al.*, 2009; Ludolph *et al.*, 2010), demonstrando a necessidade do desenvolvimento de novos sistemas para avaliação das drogas.

A recente descoberta da TDP-43 e da FUS como causadoras da ELA mostrou que as vias que provocam a ELA são extremamente variadas. A relação entre o metabolismo de RNA e a degeneração dos neurônios motores ainda não é completamente compreendida. Entretanto, ela ganha relevância, pois quase todos os casos de ELA esporádica apresentam agregados citoplasmáticos contendo TDP-43 (Mackenzie *et al.*, 2007). Ressalta-se assim, a importância de se avaliar novas formas de ELA familiar que podem trazer luz também às formas esporádicas.

Apresentamos no Capítulo 3 um novo modelo para a ELA, baseado na tecnologia das *iPSC* derivadas de amostras de pacientes com ELA8. A modelagem por meio das *iPSC* permite abordar aspectos específicos da patologia humana e recapitular o *background* genético. Desta maneira, são evitados sistemas de super-

expressão, além de limitações dos modelos animais, como o tempo de vida e metabolismo celular.

Foram obtidas amostras de pacientes com ELA8 e seus irmãos sem a mutação, com a finalidade de minimizar o efeito de variações individuais. Assim como mostrado para outras doenças neurodegenerativas (Dimos *et al.*, 2008; Ebert *et al.*, 2009; Lee *et al.*, 2009; Soldner *et al.*, 2009), a presença da mutação não interfere na geração das linhagens de iPSC-ELA8 e não parece impedir a diferenciação destas em neurônios motores (Mitne-Neto, em preparação).

Contrastando com os resultados dos modelos de super-expressão (Nishimura *et al.*, 2004B; Kanekura *et al.*, 2006; Teuling *et al.*, 2007 Tsuda *et al.*, 2008; Prosser *et al.*, 2008; Suzuki *et al.*, 2009; Fasana *et al.*, 2010; Tudor *et al.*, 2010), não pudemos identificar agregados citoplasmáticos contendo a proteína VAPB (Mitne-Neto, em preparação) em nenhum estágio avaliado – Fibroblastos, iPSC ou Neurônios Motores.

Estressar o sistema seria uma das alternativas para exacerbar determinado fenótipo (Marchetto *et al.*, 2010^A). Dessa forma, lançamos mão do uso do agente MG132, um inibidor de Proteasoma, na expectativa de evidenciar os agregados de VAPB. Entretanto, essa estratégia também não permitiu identificá-los, sendo que a distribuição intracelular da VAPB mostrou-se muito semelhante entre as células de ELA8 e as células controle (Mitne-Neto, em preparação).

Para a realização desses experimentos, foi de fundamental importância o desenvolvimento de um anticorpo específico contra a VAPB. Como mostrado no Capítulo 3, o anticorpo A1315F3G10, gerado contra um peptídeo presente na porção N-terminal da proteína, é capaz de identificar a VAPB selvagem, bem como

os agregados gerados pela presença da mutação P56S. Mostramos ainda que, apesar de a VAPA e a VAPB apresentarem cerca de 60% de identidade, o anticorpo em questão reconhece apenas a VAPB.

Apesar da ausência de um fenótipo claro quanto à presença de corpos de inclusão, chamou-nos a atenção a redução da concentração de VAPB nos extratos protéicos das culturas celulares de pacientes quando comparadas com os controles. Observamos uma diminuição de VAPB nos extratos de fibroblastos, que se mantém durante a reprogramação celular (geração das *iPSC*) e também após diferenciação neuronal. A fim de estabelecer se a expressão da VAPB nas células *iPSC* seria uma “memória” dos fibroblastos reprogramados, mostramos pela primeira vez que esta proteína está presente em células-tronco embrionárias humanas normais, linhagens H9 e H6 permitidas para estudo nos EUA. Não se sabe ao certo quando a VAPB começa a ser expressa durante o desenvolvimento. Entretanto, nossos dados sugerem que os pacientes apresentam esta redução desde o início da vida (Mitne-Neto, em preparação).

Apesar do desenvolvimento dos pacientes ser normal, os níveis de VAPB seriam críticos para a sobrevivência dos neurônios motores próximos à 4.a. ou 5.a. décadas de vida, quando a doença se manifesta. Sabe-se que um desbalanço na quantidade de VAP em drosófilas provoca efeitos sobre o sistema neuromuscular. Neste modelo, a superexpressão da dVAP-33 reduz o tamanho das junções neuromusculares e aumenta o seu número. Em contrapartida, a redução da sua expressão apresenta o efeito oposto (aumento do tamanho das JM e redução do seu número) (Pennetta *et al.*, 2002). Ainda, Teuling *et al.*, (2007) mostraram que a redução de VAPB em células neuronais de mamíferos conduz à fragmentação do Complexo de Golgi e à morte celular, sugerindo um efeito dose-dependente.

Nossos dados apontam que a expressão da VAPB aumentaria de acordo com a diferenciação neuronal (Mitne-Neto, em preparação). Gkogkas *et al.*, (2008) mostraram que tanto a VAPA quanto a VAPB são expressas em diversos tecidos de camundongos. Os níveis de VAPB são semelhantes entre diferentes tecidos, entretanto, observam-se fragmentos de menor peso molecular apenas nos extratos de tecido nervoso (reproduzido na Fig.5.1A), apontando para um processamento diferenciado da proteína nestas células. Tsuda *et al.*, (2008) reportaram fragmentos de VAPB semelhantes aos identificados por Gkogkas *et al.*, (2008) em extratos proteicos de drosófilas e também no soro humano (Fig.5.1B). Entretanto, não foi mostrado se estas seriam isoformas geradas por *splicing*, como reportado por (Nachreiner *et al.*, 2010) ou por clivagem da VAPB como sugerido por Tsuda *et al.*, (2008). Adicionalmente, não se sabe, ao certo, qual/quais tecido/s contribuem, majoritariamente para a presença da VAPB no soro.

Fig. 5.1 – Fragmentos de VAPB, de peso molecular reduzido, identificados em camundongos (A – Reproduzido de Gkogkas e col. (2008)), drosófilas e humanos (B – Reproduzido de Tsuda e col. (2008)).

Resultados obtidos por nós (Mitne-Neto, em preparação) e os da literatura (Tudor *et al.*, 2010; Chen *et al.*, 2010; Tsuda *et al.*, 2008) sugerem que a proteína mutante ainda apresenta características semelhantes à selvagem: (1) parte da P56S-VAPB (Fig.5.2A , B) ainda é solúvel, assim como da T46I-VAPB (Fig.5.2C); (2) mesmo na presença da P56S, o domínio MSP da VAPB humana é capaz de estimular a contração do ovário e a maturação de oócitos de *C. elegans* (Tsuda *et al.*, 2008 - representado na Fig. 5.2D).

Dessa forma, parte do desenvolvimento tardio da ELA8 poderia ser explicada pela necessidade de níveis específicos de VAPB. Pode-se sugerir que o polipeptídeo contendo a P56S consegue suprir as necessidades de diversos tipos celulares durante parte da vida. Entretanto, após 45-50 anos, o peculiar metabolismo dos neurônios motores requer uma disponibilidade de VAPB que não pode ser suprida por apenas um alelo selvagem.

Figura 5.2 – Solubilidade das mutações na VAPB e seus efeitos. (A) VAPB expressa com cauda de FLAG. (B) expressão da VAPB nos camundongos transgênicos. (C) Comparação entre as duas mutações encontradas T46I e P56S. (D) Efeito da microinjeção do domínio MSP recombinante da VAPB humana (circulado em vermelho), quando da presença da mutação P56S ou do produto selvagem.

No Capítulo 4 descrevemos a identificação da primeira duplicação multi-exônica no gene *SPG4*, que codifica a Espastina, proteína envolvida na dinâmica e estabilização de microtúbulos. A mutação provoca um códon de parada prematuro, sugerindo que o polipeptídeo produzido não é funcional (Mitne-Neto *et al.*, 2007^B ; Fig.S.1).

A peculiaridade desse estudo reside na diferença de penetrância que a doença apresenta de acordo com o gênero do indivíduo. Os indivíduos portadores

da mutação do sexo masculino apresentaram idade de início inferior em relação aos do sexo feminino ($p=0.017$) sugerindo um efeito “protetor” sobre as mulheres (Mitne-Neto *et al.*, 2007^B).

A fim de verificar se a variabilidade clínica teria alguma relação com outras modificações no *SPG4*, foram testados os polimorfismos S44L e P45Q nesta família. Svenson *et al.*, (2004) mostraram que estas variantes genéticas, quando em heterozigose e em *trans* com mutações na região correspondente ao AAA, estariam relacionadas com uma diminuição na idade de início dos sintomas. Os polimorfismos em questão não foram encontrados em nenhum dos indivíduos portadores da duplicação, excluindo-os como modificadores do fenótipo na família (Mitne-Neto *et al.*, 2007^B).

Em trabalho publicado posteriormente, Simons *et al.*, (2009) demonstraram que camundongos sem a atividade enzimática da Fosfatidilinositol-4-quinase2 α (PI4K2 α) apresentam degeneração do trato córtico -espinal. Os sinais clínicos encontrados são semelhantes à PEH: incontinência urinária, andar espástico, além de fraqueza, inicialmente de membros motores superiores e posteriormente de membros motores inferiores. Esses sintomas aparecem de forma tardia, após 6 meses de vida. Chama a atenção o fato de os camundongos do sexo masculino apresentarem a doença mais precocemente (6 – 8 meses) que aqueles do sexo feminino (8 – 12 meses), de forma similar ao que acontece com os pacientes da família que estudamos (Mitne-Neto *et al.*, 2007^B). Assim, Simons *et al.*, (2009) sugerem uma interação funcional entre a PI4K2 α a Espas tina. Apesar de a PI4K2 α estar envolvida com mecanismos como controle do tráfego endossômico e regulação de canais iônicos, faltam evidências para suportar esta hipótese.

Uma das explicações para a diferença de penetrância gênero-dependente na SPG4 poderia ser o aumento do transporte retrógrado nos neurônios motores gerado pela ação do estrógeno (Murashov *et al.*, 2004). Entretanto, diferenças gênero-dependente no curso clínico também foram encontradas em outras doenças neuromusculares como Distrofia Muscular Fácioescapulohumeral (Zatz *et al.*, 1998 e Arashiro *et al.*, 2009) e Amiloidose (Bornebroek *et al.*, 1997), que não envolvem especificamente os neurônios motores. Estas observações sugerem que determinadas doenças neuromusculares podem ser moduladas por fatores relacionados ao *background* individual e ao gênero.

Em resumo nossos resultados apóiam a idéia de envolvimento dos microtúbulos com a patogênese das doenças neuromusculares, visto que tanto a VAPB quanto a Espastina interagem com estes elementos do citoesqueleto. De maneira geral, a disponibilidade de formas nativas destas proteínas, no ambiente celular, poderia ser um fator de disparo das doenças em questão. Ainda, apresentamos um novo modelo *in vitro* de análise da ELA e mostramos a necessidade de estudá-la num *background* humano.

Resumo

As doenças do neurônio motor (DNM) apresentam grande variabilidade clínica e genética. A Esclerose Lateral Amiotrófica (ELA) é a forma mais comum de DNM de início tardio. Sua manifestação devastadora e incurável leva a uma profunda perda da qualidade de vida do paciente. A ELA8 é uma forma autossômica dominante de ELA familiar causada por mutações no gene *VAPB*. A proteína VAPB está envolvida com diversos processos celulares. Nossos dados sugerem que a mutação P56S na VAPB diminui a interação dela com outras duas proteínas: Tubulina e GAPDH. Por terem sido previamente relacionadas a outras formas de doenças neurodegenerativas, essas proteínas são potenciais pontos-chave para desvendar a patogênese da ELA8 e de outras formas de DNM.

Um número significativo de drogas testadas com sucesso em modelos animais de ELA não pôde ser transferido para os pacientes, trazendo à tona a necessidade de novos sistemas de estudo da doença. A tecnologia das *iPSC* torna possível a reprogramação celular para um estágio de pluripotência, permitindo a modelagem de doenças *in vitro*. Apresentamos um novo modelo de ELA baseado em células *iPSC* derivadas de pacientes com ELA8. Este modelo apresenta uma redução dos níveis de VAPB quando comparado com os controles. Contudo, não identificamos os agregados intracitoplasmáticos, característicos dos modelos de super-expressão. Mostramos pela primeira vez que células-tronco embrionárias humanas normais expressam VAPB. Adicionalmente, nossos resultados sugerem que os pacientes com ELA8 teriam uma redução dos níveis de VAPB desde o início de suas vidas e revelam a importância de modelar a ELA num *background* humano. Dessa forma, sugerimos a hipótese de que quantidades específicas de VAPB sejam cruciais para a viabilidade dos neurônios motores.

A busca por alterações no número de cópias levou à identificação do primeiro caso de uma duplicação multi-exônica (exon10_12dup) no gene *SPG4*, ampliando o espectro de mutações encontradas nesse gene. Esta mutação leva à formação de um códon de parada prematuro, sugerindo que a proteína produzida não é funcional. A análise de 30 indivíduos portadores desta mutação mostrou que os homens possuem, na média, idade de início antecipada e quadro clínico mais severo. Esses dados sugerem que certas doenças neuromusculares podem ser moduladas por fatores relacionados ao *background* individual e ao gênero.

Em suma, apoiamos a ideia de que os microtúbulos estariam envolvidos na patogênese das doenças neuromusculares, visto que tanto a VAPB quanto a Espastina interagem com estes elementos do citoesqueleto. Ainda, apresentamos um novo modelo *in vitro* de análise da ELA e além de justificar os estudos num *background* humano.

Abstract

The motor neuron diseases (MND) show a huge clinical and genetic variability. Amyotrophic Lateral Sclerosis (ALS) is the most common late-onset form of MND. ALS's devastating and incurable manifestation leads to a profound loss of life quality. ALS8 is an autosomal dominant form of ALS caused by mutations in the *VAPB* gene. The VAPB protein is involved with many cellular processes and our data suggest that P56S mutation in this protein reduces its interaction with two other proteins: Tubulin and GAPDH. Since these proteins were previously related to other types of neurodegenerative diseases they potentially are key points to reveal the processes responsible for ALS8 and other MND.

A substantial number of successful drug tests in ALS animal models could not be translated to humans, showing the need of novel ALS systems. iPSC technology made possible cellular reprogramming. The iPSC technology brings new hope in this area since it can be used to model diseases *in vitro*. Here we present a new ALS model based on ALS8-iPSC. Compared to control samples, this model shows a reduction of VAPB levels. However, we could not identify intracytoplasmic aggregates, which characterize overexpression models. We show for the first time that human embryonic stem cells express VAPB. Combined with results showing a VAPB reduction in ALS8 samples, it suggests that ALS8 patients present diminished protein levels since the beginning of their lives and reveal the importance of modeling ALS in a human background.

The search for copy number variations has led to the identification of the first multi-exonic duplication (exon10_12dup) in *SPG4* gene, expanding the mutation spectrum in this gene. This mutation leads to a premature stop codon, which suggests that the expressed protein is not functional. The analysis of 30 individuals who carry the mutation showed that males have on average an earlier AAO and are more severely affected. These data suggest specific neuromuscular diseases can be modulated by factors related to individual background and gender.

In sum, we support the idea that microtubules can be involved with neuromuscular disorders pathogenesis, since both VAPB and Spastin interact with these cytoskeleton components. Additionally, we present a new *in vitro* model to ALS analysis and we justify the studies in a human background.

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Letter to the Editor

The p.P56S mutation in the *VAPB* gene is not due to a single founder: the first European case

To the Editor:

A dominant missense mutation p.P56S in the *vesicle associated membrane protein associated protein B* (*VAPB*) gene was described in eight Brazilian families of Portuguese descent showing a wide spectrum of motor neuron diseases (MNDs) including spinal muscular atrophy (SMA) and familial amyotrophic lateral sclerosis (ALS) (ALS8) (1, 2). Haplotype analysis indicated a common ancestor with a founding event 23 generations previously, when this ancestor was still living in Portugal (3). We report the first identification of the p.P56S mutation in the *VAPB* gene in a non-Brazilian patient.

A 43-year-old man (III-1) showed slowly progressive muscular weakness for 2 years and a family history of autosomal dominant neuromuscular disease through at least three generations (Fig 1). The patient's mother (II-1) suffered from slowly progressive muscular weakness over 30 years. No pyramidal tract signs had been observed. She was wheelchair bound 20 years after onset and died at the age of 67. The maternal grandfather (I-1) had a history of progressive muscular weakness, had died aged 57 and had six siblings. Three (I-5, I-6, I-7) suffered from muscular weakness. One cousin of the patient's mother (II-2) was diagnosed with SMA. There was no family record of Portuguese or Brazilian ancestors in at least four previous generations. All family members originated from northern Germany.

The index patient (III-1) showed paresis of the hip flexors and extensors (Medical Research Council (MRC) grade 4/5) and fasciculations in the proximal muscles of arms, legs on both sides. Deep tendon reflexes were normal except for absent Achilles tendon reflexes. There were no pyramidal tract signs. Needle electromyography showed

Fig. 1. Family tree. Family members who are deemed affected based upon family history are marked gray. It is not known if family members I-2, I-3, I-4, I-5, I-6, I-7, and II-2 are still alive.

fasciculations and signs of chronic denervation. Nerve conduction studies of tibial nerves revealed slightly reduced amplitudes on the left. Motor evoked potentials in both tibialis anterior muscles after magnetic stimulation of the motor cortex and the lumbar roots were normal.

Genomic DNA of the index case was extracted from peripheral blood and amplified using primer pairs flanking all exons and exon/intron boundaries of the *VAPB* gene. Amplicons were purified and sequenced directly on an ABI PRISM 310 Genetic Analyzer (PE, Applied Biosystems). The p.P56S mutation was screened in 100 German healthy controls. Haplotype analysis was performed using microsatellite markers D20S100, D20S171 and D20S173 from the ABI Prism Linkage Mapping Set kit version 2 (Applied Biosystems, Foster City, CA) as reported previously (3). The forward 5'AAGACAAGCAAACTAAAGAACTGC3' and reverse 5'TTCCCATTACCGGTTATCCA-3' primers were used to amplify part of 3' UTR sequence of the tubulin beta 1 (*TUBB1*) gene. A polymorphism in this region was used as intrafamilial marker. Polymerase chain reaction products were analyzed using a MegaBace 1000 DNA Sequencer (Amersham Bioscience, Little Chalfont,

Table 1. Haplotype comparison of the German index patient and the patients from three different Brazilian families with the p.P56S vesicle associated membrane protein associated protein B (*VAPB*) gene mutation^a

Position (Mb)	Marker	German index patient	Brazilian families		
			1	2	3
53.74	D20S100	222 232	224 230	226 230	232 230
57.0	TUBB1	G G	G C	G C	G C
57.24	D20S171	142 142	142 144	144 144	140 144
58.31	D20S173	176 178	178 178	176 178	178 178

^aThe haplotype given in boldface signifies the ancestral allele in the Brazilian families.

UK). DNA from the index patient was compared to three affected individuals from three different kindreds of the Brazilian families.

Sequencing revealed a heterozygous p.P56S point mutation in exon 2 of the *VAPB* gene. This mutation was not present in 200 German control chromosomes. Haplotype analysis revealed that this patient had a different haplotype compared to the Brazilian families (Table 1).

The phenotype of our index case and his mother represented late onset SMA as observed in one-third of the Brazilian patients carrying the p.P56S *VAPB* mutation (1). Although we cannot entirely exclude the possibility of Brazilian or Portuguese ancestors, haplotype analysis showed that our patient's mutation is not due to the same founder as in the reported Brazilian patients. Therefore, we assume that the p.P56S mutation happened in at least two independent events. Several studies failed to identify *VAPB* mutations in cohorts of patients with ALS (4–6)]. Mutations in the *VAPB* gene seem to be rare in familial MNDs. However, our case report demonstrates that the p.P56S mutation can be observed outside Brazil, and should be considered as a rare differential diagnosis in familial MNDs.

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Dr. Constantin Polychronakos,
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Journal of Medical Genetics

Dear Dr. Polychronakos

We read with great interest the recent publication from your journal entitled "*SOD1, ANG, VAPB, TARDBP, and FUS mutations in familial amyotrophic lateral: genotype – phenotype correlations*" Millecamps S., Salachas F., Cazeneuve C., et al. *J Med Genet* published online June 24, 2010 doi: 10.1136/jmg.2010.077180. This manuscript brings relevant information about the mutation frequencies and genotype-phenotype data regarding the main genes responsible for the familial forms of ALS (FALS). Additionally, as well pointed by the authors, this study is the first large scale evaluation of FALS in the French population.

However, we would like to call the authors attention for two important aspects:

- 1) According to the authors "... *The P56S carrier was of Japanese descent, and represents the first non-Brazilian patient carrying this mutation...*".

The ALS8 form was first mapped at 20q13 in a large Brazilian family in 2004, which was published in the Journal of Medical Genetics (Nishimura et al., 2004A). Later in the same year, the c.166C→T / p.Pro56Ser mutation in the *VAPB* gene was identified in this and six other families (Nishimura et al., 2004B) as responsible for this disorder. Additionally, microsatellites analyses around *VAPB* showed that these families share a common haplotype. It suggests a founder effect estimated to have occurred about 500 years ago, concomitant with Brazilian colonization by Portugal (Nishimura et al., 2005). Recently, in collaboration with our group, Funke et al. (2010) identified a German ALS8 patient, with no family record of Brazilian or Portuguese ancestry, carrying the c.166C→T mutation in *VAPB*, which showed that ALS8 is no more restricted to Brazil. Therefore, this German patient comprises the first case of an ALS8 patient out of Brazil and this should be corrected in Millecamps et al., MS.

- 2) The German patient, identified by Funke et al. (2010), carries an haplotype which is different from the one found in the Brazilian ALS8 families. This suggests that the c.166C→T (P56S) mutation is not due to the same founder and that, probably, it happened at least in two independent events. Millecamps et al., (2010) claims that "... *The finding of a P56S VAPB mutation in a patient of Japanese origin presumably reflects the Portuguese trading connection with the Far East and Brazil in the mid 16th century...*" suggesting that the Japanese and the Brazilian families could share the same genomic region. However, they do not show such haplotype analysis or present any historical evidence for this supposition.

We believe that such information is relevant for the researchers in the ALS field and for this reason an erratum should be considered in order to ensure the best information to the readers of the Journal of Medical Genetics .

We appreciate your attention
Sincerely

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