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(Versão Corrigida)

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(Reviewed Version)

Versão corrigida da dissertação apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Mestre em Ciências Biológicas, na Área de Biologia (Genética). A versão original se encontra à disposição na biblioteca do instituto.

Orientador(a): Profa. Dra. Mayana Zatz

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2016

Ficha Catalográfica

Rosa Olávio, Thiago

Estudo da proteína FUS em linhagens de células pluripotentes induzidas de uma família com esclerose lateral amiotrófica e mutação no gene *FUS*.

103 páginas

Dissertação (Mestrado) - Instituto de Biociências da Universidade de São Paulo. Departamento de Genética e Biologia Evolutiva.

1. Esclerose lateral amiotrófica 2. Células de pluripotência induzida 3. FUS I. Universidade de São Paulo. Instituto de Biociências. Departamento de Genética e Biologia Evolutiva.

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Dedicatória

Esse trabalho é dedicado à todos os pacientes de ELA e à todos os pacientes do Centro de Pesquisas do Genoma Humano e Células-Tronco da Universidade de São Paulo (CEGH-USP).

Agradecimentos

Eu gostaria de agradecer primeiramente à meus pais, que conseguiram me escolarizar e me educar mesmo frente à muitos desafios, que me amaram e me incentivaram mesmo em momentos incertos e que, ainda hoje, participam de maneira essencial da minha vida.

Agradeço também à minha noiva, Paula, que também me incentivou, me deu carinho, me compreendeu e me deu forças quando eu pensava em desistir. A sua participação nesse trabalho durante todos esses anos foi positivamente decisiva.

Não poderiam ser esquecidos todos os meus estimados familiares e amigos que, mais próximos ou mais distantes, acompanharam, torceram, ajudaram (e às vezes atrapalharam) e agora estão comemorando comigo o fim de mais um ciclo.

Com suma importância, tenho que agradecer aos meus queridos amigos do Colégio Stockler, que me apoiaram e ajudaram a tornar essa caminhada menos árdua.

Gostaria de destacar e agradecer a participação das pessoas do laboratório: Inês, Melinda, Luiz e Ernesto. Vocês participaram ativamente e foram decisivos para que esse trabalho fosse concluído. Não menos importantes, agradeço à todas as outras pessoas do laboratório e do departamento que contribuíram, de forma direta ou indireta, para este projeto.

Ainda, com participação protagonista, tenho que agradecer ao Dr. Fernando Kok, que me abriu as portas laboratório e me ensinou muito, e à minha orientadora Dra. Mayana Zatz, que também me acolheu em seu local de pesquisa, me deu condições para executar o projeto e que, com uma sensibilidade ímpar, soube me conduzir da melhor forma, ainda que por vezes me deixando contrariado.

Por fim, eu agradeço à todo o IB-USP, à toda Universidade de São Paulo, à CNPQ e à FAPESP que providenciaram toda estrutura e material necessário para que este trabalho ocorresse.

Obrigado à todos, espero retornar à altura todos os investimentos em mim feitos.

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INTRODUÇÃO

A esclerose lateral amiotrófica (ELA) é uma doença neurodegenerativa progressiva na qual a morte dos neurônios bulbares, corticoespinhais e espinhais levam à fraqueza na musculatura bulbar, dos membros, toráxica e abdominal, com relativa conservação dos músculos oculomotores e das funções dos esfíncteres. Como resultado da atrofia muscular o paciente geralmente se torna paralítico e a morte, que ocorre por insuficiência respiratória, normalmente acontece dentro de 2 a 5 anos após o início da doença(1).

A maioria dos casos de ELA não possuem causa definida, sendo denominados como casos de ELA esporádica (ELAe), porém aproximadamente 10% dos casos possuem transmissão familial (ELAf) e foram decisivos para identificar casos da doença com origem genética. Até o momento, mais de 30 genes(104) (uma lista atualizada pode ser encontrada em *http://alsod.iop.kcl.ac.uk*) já foram identificados como causadores e/ou como fatores de risco para o desenvolvimento da patologia, além de haverem indícios de que alguns aspectos ambientais também podem aumentar o risco de desenvolver a doença. Esses fatores evidenciam que um conjunto complexo de mecanismos deve estar por trás da via que leva ao aparecimento dos sinais clinicos observados nessa enfermidade, porém esse caminho ainda não é conhecido. A extensa sobreposição dos sintomas entre as formas esporádicas e familiais sugerem que o mecanismo que leva ao fenótipo de ELA seja comum para ambas as formas da doença.

O diagnóstico da doença é bastante laboroso e demorado, geralmente levando mais de doze meses, e por isso as medidas paliativas e o tratamento medicamentoso acabam por serem oferecidos ao paciente quando a doença já se encontra em estágios mais avançados. Com isso, a identificação de algum marcador que seja capaz de prever o início da doença ou capaz de oferecer um diagnóstico definitivo em um curto prazo de tempo representaria um avanço significativo para o estudo de novos métodos terapêuticos que possam retardar ou cessar a progressão da doença.

Aspectos históricos da ELA

Jean-Martin Charcot usa pela primeira vez o termo “Esclerose Lateral Amiotrófica” em sua publicação de 1874, porém casos da doença já haviam sido descritos anteriormente(2). Na publicação de Charcot, ele relaciona a fraqueza muscular e atrofia à perda celular nos cornos anteriores da medula espinhal (“amiotrófico” se refere à atrofia muscular resultante da perda de neurônios motores inferiores), e estabelece a ligação da rigidez e da espasticidade muscular com alterações na coluna lateral da medula espinhal (“esclerose lateral” diz respeito ao endurecimento do tecido da coluna lateral da medula espinhal). Neste trabalho, Charcot descreve a doença da seguinte forma:(1)

“1) Paralysis without loss of sensation of the upper limbs, accompanied by rapid emaciation of the muscles . . . At a certain time, spasmodic rigidity always takes over with the paralyzed and atrophic muscles, resulting in permanent deformation by contracture.

2) The legs are affected in turn. Shortly, standing and walking are impossible. Spasms of rigidity are first intermittent, then permanent and complicated at times by tonic spinal epilepsy. The muscles of the paralyzed limb do not atrophy to the same degree as the arms and hands. The bladder and rectum are not affected. There is no tendency to the formation of bedsores.

3) In the third period, the preceding symptoms worsen and bulbar symptoms appear. These three phases happen in rapid succession—6 months to a year after the onset, all the symptoms have appeared and become worse. Death follows in 2 or 3 years, on average, from the onset of bulbar symptoms. This is the rule but there are a few anomalies. Symptoms may start in the legs or be limited to one side of the body, a form of hemiplegia. In two cases, it started with bulbar symptoms.

At present, the prognosis is grave. As far as I know, there is no case in which all the symptoms occurred and a cure followed. Is this an absolute block? Only the future will tell."

Desde a época de Charcot o conhecimento sobre a ELA aumentou bastante, mas nenhum estudo foi capaz de modificar significativamente o curso e o prognóstico da doença. Geralmente os primeiros sintomas aparecem na quarta ou quinta década de vida e são marcados por fraqueza nos membros superiores e/ou inferiores (início espinhal), podendo afetar diferencialmente os lados dos corpo, ou por dificuldades na fala e na ingestão de alimentos (início bulbar). A morte de neurônios motores na medula espinhal levam aos sinais de fraqueza, enquanto que espasticidade muscular e reflexos exaltados são sinais de comprometimento dos NM no trato corticoespinhal. A rápida degeneração dos NM em questão leva à paralisia e perda muscular generalizada, apesar dos músculos oculomotores e dos esfíncteres anal e urinário permanecerem funcionando normalmente. A morte do paciente geralmente ocorre dentro de 3 à 5 anos desde o início dos sintomas, devido à problemas respiratórios.

Os primeiros avanços no entendimento da afecção vieram principalmente de observações feitas em cortes histológicos *post-morten* de pacientes. Em 1962, Bunina publica um artigo revelando a presença de inclusões intracelulares na medula espinhal e no tronco cerebral de dois pacientes com ELA. Inicialmente a autora acreditou que tais inclusões teriam origem viral mas falhou ao tentar provar a transmissibilidade do fenômeno.A descrição das inclusões que vieram posteriormente a ser chamadas de Corpúsculos de Bunina (CB) foi um passo decisivo para uma melhor compreensão da patogênese da ELA. (3) Nos anos que se seguem à publicação de Bunina, novos trabalhos utilizando marcações histoquímicas revelam outras formas de inclusões citoplasmáticas tais como inclusões hialinas "Lewy body-like"(4), acúmulo de neurofilamentos (5) e inclusões basofílicas em formas de ELA juvenil (6).

Em 1993, Okamoto et al., utilizando anticorpos anti-ubiquitina, observam uma forma anormal de inclusões citoplasmáticas proteicas ubiquitina-positivas e tau-

negativas em neurônios motores e células da glia, mais tarde estas inclusões também foram identificadas como TDP-43-positivas(7). Atualmente essas inclusões são consideradas uma característica marcante de ELA , estando presente em quase todos os casos.

Apesar dos estudos acerca da ELA terem se intensificado e se diversificado ao longo dos anos, houve pouco progresso no sentido de se encontrar uma cura ou um tratamento que retarde efetivamente ou cesse a progressão da doença. O gene *SOD1* (Cu/Zn superóxido dismutase) foi o primeiro a ser relacionado à formas hereditárias da doença(86), e um trabalho estudando este mesmo gene, publicado em 2015 por Dokholyan e colegas (8), é o primeiro a correlacionar espécies citotóxicas formadas pela proteína SOD1 portadora de mutações causadoras de ELA à um aumento da morte neuronal. O trabalho de Dokholyan et al. nos ajudam a elucidar um pouco mais sobre os mecanismos patogênicos de algumas formas ELA, porém os casos da afecção relacionados ao gene *SOD1* representam somente 2% dos casos de ELA. Outros genes como o *TDP-43* (*TAR-DNA binding protein 43*), *FUS* (*fused in sarcoma*), *VAPB* (*VAMP/synaptobrevin-associated membrane protein B*), e expansão da *C9ORF72*, além de outros, são associados à formas hereditárias e esporádicas da doença. A grande diversidade de funções exercidas por estes genes, e os casos da doença sem causas moleculares claras, dificultam a identificação do mecanismo que leva ao fenótipo de ELA.

Além das causas genéticas, alguns estudos apontam associações entre fatores ambientais, tais como exposição à metais pesados, radiação, campos magnéticos, prática de atividades físicas e alguns tipos de dietas com o aumento da propensão ao desenvolvimento da doença. Estabelecer a relação entre estes fatores e a doença tem se mostrado uma tarefa desafiadora, mas o indício de que fatores ambientais podem estar ligados ao aparecimento dos sinais clínicos de ELA aumentam ainda mais a complexidade dos estudos relacionados aos mecanismos envolvidos no desenvolvimento da doença e à procura de um tratamento ou cura para esta.(100)

Aspectos epidemiológicos

Alguns dos estudos epidemiológicos mais antigos acerca da ELA analisam casos desde a década de 1920, porém no começo deste tipo de estudo haviam problemas estatísticos e metodológicos que dificultavam a comparação dos dados entre diferentes regiões e países do planeta. A Classificação Internacional de Doenças ("International Classification of Diseases" ou ICD) sofreu diversas mudanças ao longo do tempo, inclusive no que diz respeito à ELA ser uma afecção independente ou se pertence às Doenças de Neurônio Motor (DNM), esse processo dificulta uma análise mais precisa da incidência da doença até a década de 1990. Além disso, somente em 1994 que pesquisadores da "*World Federation of Neurology (WFN) Research Group on Motor Neuron Diseases"* publicaram o "El Scorial", que estabeleceu um padrão de diagnóstico para identificar as DNM e distinguir as suas diferentes formas. Antes da publicação desse conjunto de critérios a probabilidade de um diagnóstico errado sobre o paciente era ainda maior. (9).

Estudos mais recentes sobre a distribuição da doença mostram que sua incidência na população global é de 2,7/100.000 (95% no intervalo de confiança [IC] 2,63-2,91) e a prevalência, em 2008, é de 0,32/100.000 (95% no IC 9,78-10,86). Muitos trabalhos apontam para uma incidência maior de ELA em homens do que em mulheres (homens:mulheres = 1,5:1), mas existem estudos que demonstram equilíbrio da distribuição da doença entre os sexos. (34)

A idade de início da doença varia em média dos 50 aos 65 anos de idade, sendo que a idade de início mediana é 64 anos. Geralmente em casos de ELAf a idade de início da patologia é uma década mais cedo em relação aos casos de ELAe. Situações onde pacientes manifestam os sintomas antes dos 30 anos são relativamente raras e representam cerca de 5% dos casos.

Em algumas populações de regiões como Guam, algumas ilhas do Japão e Nova Guiné, é observada prevalência da afecção 50-100 vezes maior do que no resto da população mundial. Estudos sugerem que a neurotoxina β-metilamina-L-alanina

(BMAA) produzida por cianobactérias simbióticas com as raízes de árvores do gênero *Cyca*, comumente encontradas nessas regiões, atinge as sementes dessa mesma planta. As sementes são usadas para a produção de farinha e, embora boa parte da neurotoxina seja retirada durante o processo de lavagem, uma parte do BMAA é retido no produto final e as pessoas que não conseguem evitar o acúmulo da substância desenvolvem o fenótipo de ELA.(100)

Existem poucos estudos epidemiológicos sobre a ELA na América Central, América do Sul e África, e seria de extrema importância o levantamento dos dados dessas regiões para ser feita a comparação com os dados das populações já estudadas, e assim traçar um panorama global da distribuição da doença e das populações de risco.

Aspectos moleculares

A Esclerose Lateral Amiotrófica é uma doença molecularmente muito complexa,.A maior parte dos casos da doença não possuem um fundo genético claro e, mesmo nos casos em que a afecção tem relação com alguma mutação, não é conhecida a via que leva à morte dos neurônios motores. Conhecer os mecanismos moleculares envolvidos na ELA é necessário para o desenvolvimento de diagnósticos mais rápidos e precisos, para pesquisas visando uma cura ou um tratamento que cesse ou ao menos diminua a progressão da doença.

Não há consenso entre os estudos sobre qual é a via que levam os neurônios motores de pacientes de ELA à degeneração. Alguns mecanismos hipotetizados para a patogênese da doença serão descritos a seguir:

Excitotoxicidade

Em neurônios saudáveis o glutamato é produzido no terminal pré sináptico e, em um processo de neurotransmissão, é liberado na fenda sináptica onde irá ativar os receptores pós sinátpticos. Após esse evento, o glutamato é retirado da fenda sináptica por células neuronais e da glia,

assim mantendo um gradiente de concentração estável e evitando degeneração neuronal por excitotoxicidade. Estudos demonstram que no córtex motor e na medula espinhal de pacientes com ELA há uma redução na quantidade de transportadores de glutamato EAAT2 (“excitatory amino acid transporters”) da astroglia, responsáveis por retirar o glutamato da fenda sináptica. Assim, com o aumento da concentraçãoo de glutamato extracelular há uma hiperestimulação dos receptores glutamatérgicos, que leva à deneração neuronal por excitotoxicidade. O excesso de glutamato extracelular ainda leva à um aumento no influxo de cálcio e à uma ativação excessiva dos neurônios motores, levando a célula a iniciar processos apoptóticos. (34)

Dois estudos demonstram que RNA’s anormais são a principal causa da queda no número de receptores EAAT2 em pacientes de ELA. (10, 11)

Alterações mitocôndriais

Alterações mitocôndriais aparentam ter um papel importante na degeneração de neurônios motores, tanto quanto a excitotoxicidade. As mitocôndrias são organelas essenciais ao funcionamento celular já que estão ligadas à produção de energia intracelular, à homeostase de cáclcio e ao controle de apoptose.

Estudos recentes relacionam o mal funcionamento da proteína Cu/Zn super óxido dismutase (SOD1), com disfunções mitocondriais relacionadas à neurodegeneração. Vande et al. (12) demonstraram que a SOD1 mutada se agrega na face citossólica da membrana externa da mitocôndria, o que pode justificar o defeito na produção de ATP, na homeostase de cálcio, no transporte axonal mitocondrial e pela ativação da apoptose observados na medula espinhal de pacientes de ELA. (13, 14) Mutações na SOD1 também mostram um queda na produção de ATP em biópsias da musculatura esquelética de pacientes de ELA, uma

desregulação na homeostase de cálcio levando ao dano neuronal e uma falha no transporte axonal de mitocôndrias. (15)

Os genes *VAPB* e *TDP-43*, relacionados à casos de ELA, também estão ligados à perturbações no metabolismo mitocondrial neuronal. Enquanto o gene da *VAPB* possui um papel importante no metabolismo de Ca^{2+} e na localização mitocondrial, o gene *TDP-43* afeta o metabolismo dessa organela interrompendo a ligação entre ela o retículo endoplasmático (RE) e a mitocôndria. Mutações nesses genes podem levar à problemas na homeostase de cálcio intracelular. (16,17,18)

Falha no transporte axonal

Os NM são células que podem atingir um comprimento de mais de um metro, e por isso o transporte de substâncias e organelas nesse tipo celular é essencial para o seu funcionamento correto. Achados no córtex motor e na medula espinhal de pacientes com ELA mostram o acúmulo de substâncias nos neurônios afetados, formando inclusões citoplasmáticas. Os agregados citoplasmáticos podem atrapalhar a circulação de substâncias, e com isso levar à perda da sinalização neurotrófica e ao transporte axonal defeituoso, como demonstrado em experimentos com camundongos mutantes para a SOD1. (19,20,21) A falha no transporte axonal causa o acúmulo de neurofilamentos, de mitocôndrias e de autofagossomos em NM degenerados, sendo por isso um possível promotor da morte neuronal. (22)

Defeitos no processamento de RNA

A descoberta da presença da proteína TDP-43 como o principal componente de inclusões citoplasmáticas em pacientes de ELAe e o posterior achado de pacientes de ELA portando mutações nesse gene, levou à descoberta subsequente de mais um gene associado à doença, o gene *FUS* (*FUsed in Sarcoma*). Estas descobertas, e a associação da afecção à outros genes ligados ao processamento de RNA, como o *ANG*,

SETX e outros, colocam em foco a possibilidade de defeitos nesta via estarem entre uma das possíveis causas da patogênese da ELA.

Corpúsculos nucleares são um grupo de estruturas localizadas no núcleo, que têm papel fundamental no processamento de RNA e controle da expressão gênica. O corpúsculo de cajal é um corpúsculo nuclear ligado à formação de snRNP's (pequenas ribonúcleoproteínas nucleares) spliceossomais, dentre outras funções, e em determinados momentos é possível observar gemas nucleares (GN) associados à ele.(23) As GN têm a proteína SMN (*survival of motor neuron*) como molécula chave do seu aglomerado, e mutações em tal proteína causam uma doença neurodegenerativa conhecida como atrofia espinhal progressiva (AEP). Assim como na ELA, na AEP são notáveis os sinais clínicos decorrentes da morte dos neurônios motores, e alguns estudos mostram que mutações patogênicas nos genes *SOD1*, *TDP-43* e *FUS*, ligadas à ELA e mutações no gene *SMN1*, ligadas à AEP, alteram a dinâmica das GN e também o perfil de RNA's expressos. (24,25,26,27,28) O crescente número de trabalhos evidenciando a importância do metabolismo de RNA's em processos neurodegenerativos coloca o processamento de RNA's em foco como possível explicação para a patogênese da ELA. (29)

As várias vias possíveis para explicar a o fenótipo observado na ELA são um reflexo da diversidade de genes associados à doença. Atualmente mais de 30 genes, com funções muito distintas, são conhecidos por estarem envolvidos em casos onde mutações aparentam ser responsáveis pela manifestação dos sinais da afecção, como mostrado na **figura 1**.

O gene *SOD1* foi o primeiro a ser associado com casos de ELA, e assim deu início à diversos estudos moleculares que levaram à descoberta de outros genes e à algum conhecimento sobre os mecanismos moleculares desta doença. Os principais genes associados à ELA estão caracterizados a seguir:

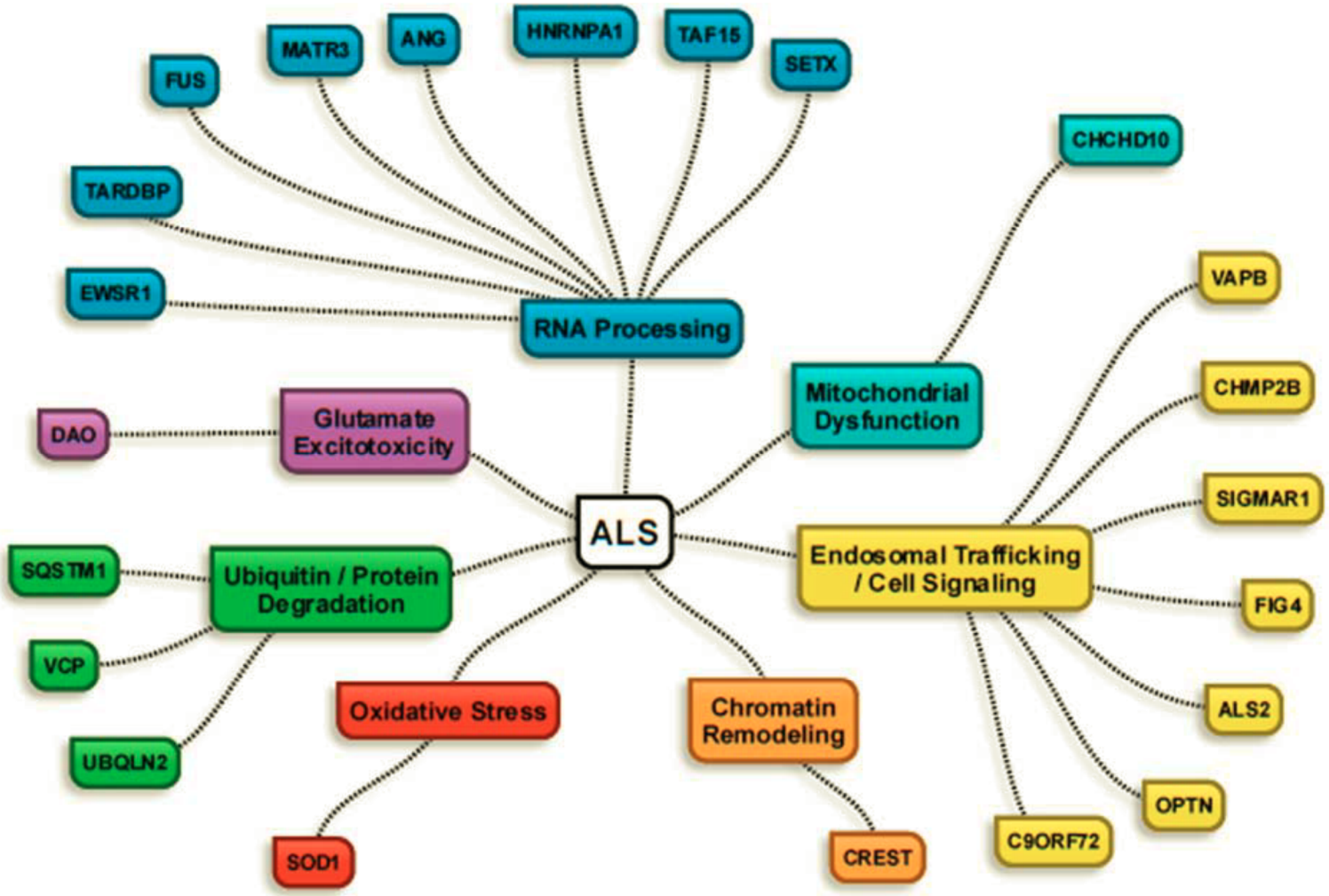

FIGURA 1: Genes atualmente conhecidos como associados à ELA, classificados pela via molecular comum envolvida na patofisiologia da doença. Retirado de Coatti e colaboradores. (30)

SOD1 (Cu/Zn superóxido dismutase 1)

O gene *SOD1* é um gene de expressão ubíqua que produz uma proteína, chamada superóxido dismutase 1, ligada ao metabolismo de quebra de espécies reativas do oxigênio. Atualmente 20% dos casos de ELAf e 5% dos casos de ELAe estão relacionados com mutações nesse gene, sendo que mais de 160 mutações diferentes já foram observadas.

Devido à função da proteína expressa por esse gene, inicialmente acreditou-se que estresse oxidativo poderia ser o motivo pelo qual os neurônios estariam morrendo nesta afecção, porém estudos posteriores sugerem que o estresse oxidativo seria uma característica secundária, e que a a formação de agregados proteicos citoplasmáticos contendo a proteína SOD1 talvez seja o motivo principal que leva à morte neuronal.(31).

A SOD1 mutante é mal dobrada e marcada para degradação por ubiquitilação, entretanto a proteína mal formada parece escapar deste processo regulatório e acaba por exercer um efeito tóxico sobre a maquinaria de degradação, prejudicando seus dois principais componentes: a via proteassomal e autofagia. Um aumento no número de fagossomos foi detectado em neurônios motores da medula espinhal de pacientes com ELA e em modelos murinos, o que sugere que o efeito da SOD1 sobre a degradação de proteínas é visto *tanto in vitro* quanto *in vivo*, e não apenas em roedores mas também em humanos. Em um recente estudo, Dokholyan e colaboradores demonstraram que a SOD1 mutada se aglomera em trímeros, que possuem estrutura terciária e quaternária diferentes da SOD1 selvagem, e a presença dessas espécies apresenta alta relação com a morte de células neuronais. A melhor compreensão acerca da toxicidade deste trímero pode nos ajudar a compreender melhor os mecanismos que levam à morte dos neurônio motores na ELA. (8)

C9ORF72 ("open reading frame 72" do cromossomo 9)

A expansão do hexanucleotídeo (GGGGCC) na região de "open reading frame 72" do cromossomo 9 foi associada à casos da doença através de estudos haplótipo de risco no locus *9p21*. A proteína produzida por esse gene ainda não possui uma função celular conhecida, mas um estudo indica que a proteína pode estar relacionda ao tráfego endossomal.(32) Os principais estudos acerca do mecanismo molecular que leva a expansão da ORF do cromossomo 9 a causar os sinais patológicos da ELA sugerem três possíveis mecanismos: Toxicidade do RNA, Toxicidade da proteína, ou haploinsuficiência.(33)

Essa alteração genética ganhou notoriedade quando estudos demonstraram que na Finlândia a expansão era encontrada em 61% dos pacientes com ELAf e 19% dos pacientes com ELAe, em outras populações européias. Nos EUA a incidência de casos da doença com a expansão é menor, mais ainda bastante grande. (33) Em aproximadamente 50% dos casos de ELA relacionados com mutações nesse locus o paciente também apresenta um quadro de demência fronto temporal (DFT), há também uma maior incidência de pacientes com início bulbar da doença em casos relaciondaos com a expansão. Estudos encontraram evidências de que a idade de início da doença geralmente ocorre entre 1,8 e 5,0 mais cedo do que a média dos pacientes de ELA como um todo. Casos da afecção relacionados com a expansão hexanucleotídica geralmente apresentam uma duração menor da doença, o que sugere que nesses casos a progressão da doença seja mais agressivo.

TARDBP (proteína de 43 kDa ligada ao "TAR-DNA", ou TDP-43)

A descoberta da relação do gene *TDP-43* com a ELA se deu ao analisar as inclusões ubiquitinadas citoplasmáticas presentes na medula espinhal de pacientes de ELA e verificar que a TDP-43, proteína produzida por esse gene, era o principal componente dessas inclusões. Posteriormente foram encontradas mutações neste gene em pacientes de ELA (ELA pura, ELA com DFT, e DFT pura), e associadas à outras doenças neurodegenerativas. Mutações nesse gene são responsáveis por 5-10% dos casos de ELAf(34), mas a presença de inclusões citoplasmáticas positivas para TDP-43 em células nervosas de pacientes com ELA é uma característica muito frequente, principalmente nos casos não ligados à mutações no gene *SOD1*.

A TDP-43 está relacionada à processos de transcrição, splicing, transporte e estabilidade de RNA's, e a sua relação com a ELA traz à tona a possibilidade dos mecanismos patogênicos desta doença estarem relacionados à falhas no metabolismo de RNA. Primáriamente a TDP-43 é uma proteína nuclear, que se desloca para o citoplasma em resposta à estímulos de estresse. Porém quando está mutada, a proteína em questão fica retida no citoplasma, e se acumula na forma das já citadas inclusões citoplasmáticas. As células com agregados citoplasmáticos de TDP-43 geralmente apresentam diminuição da concentração da proteína localizada no núcleo, o que sugere uma perda da função nuclear do TDP-43, e o acúmulo da proteína no citoplasma é sugestivo para ganho de função tóxica da proteína nessa região celular. Este sinais indicam que o mecanismo patológico da ELA ligada à TDP-43 deve ser uma combinação entre perda de função e ganho de função tóxica.

FUS ou TLS (Fused in sarcoma ou Translocated in liposarcoma)

Subsequentemente aos achados moleculares que relacionaram a TDP-43 com a ELA, ocorreu a descoberta de que a proteína FUS também

era encontrada em inclusões citoplasmáticas de pacientes com ELA. A proteína FUS possui 526 aminoácidos e é codificada por 15 éxons localizados no cromossomo 16. Sua estrutura está representada na **figura 2**. Esta proteína tem expressão ubíqua sendo localizada predominantemente no núcleo celular, apesar de também exercer funções importantes no citoplasma, e estudos já mostraram que ela se liga à DNA, RNA e proteínas atuando em vários estágios desde a expressão gênica à tradução proteica. (35)

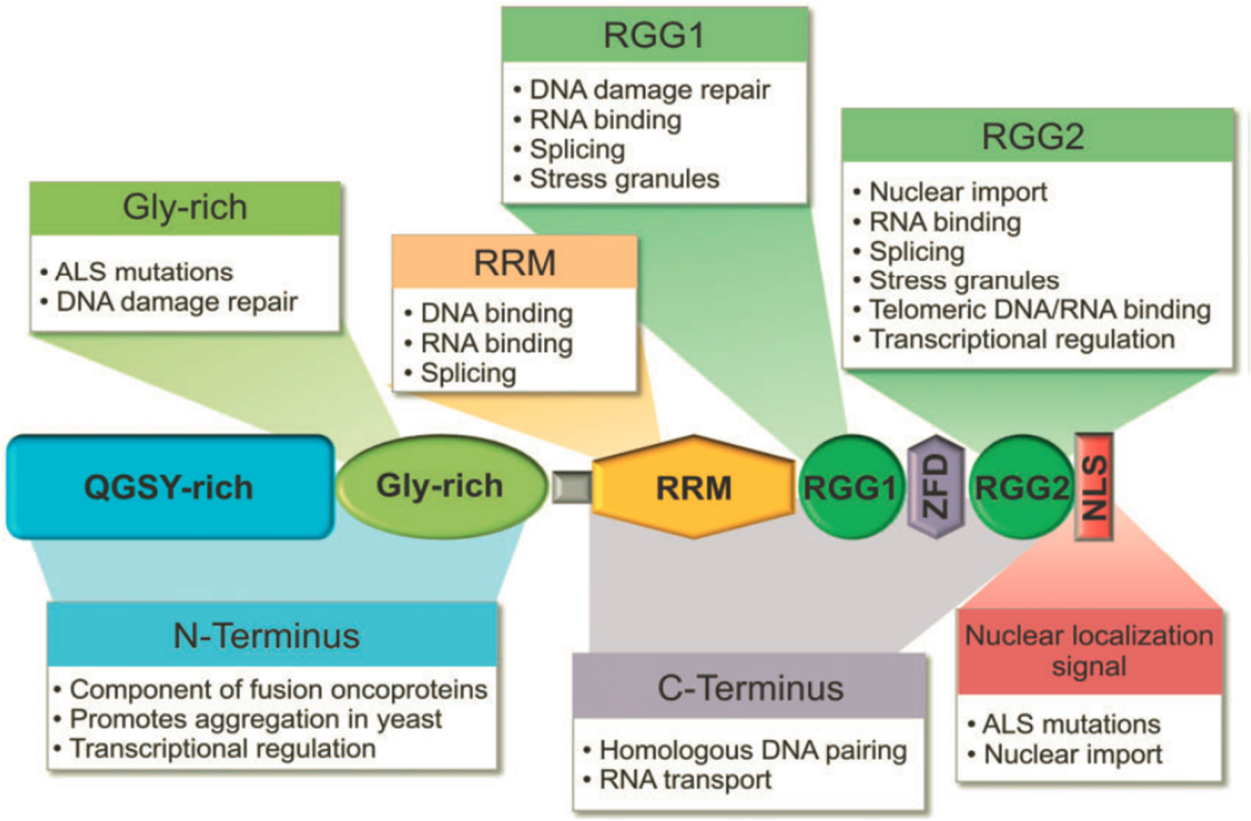

FIGURA 2: Os domínios funcionais dentro do gene FUS. FUS se liga à DNA, RNA e proteínas para executar uma grande variedade de funções, e nesta figura estão relacionadas as funções conhecidas para o gene de acordo com o domínio da proteína. Retirado de

Nota: QGSY-rich = domínio rico em glutamina-glycina-serina-tyrosina ou semelhante à príons; Gly-rich = rico em glycina; RGG = rico em arginina-glycina-glycina; RRM = motivo de reconhecimento de RNA; ZFD = domínio zinc-finger; NLS = sinal de localização nuclear; ALS = esclerose lateral amiotrófica. Retirado de Sama e colaboradores, 2014. (35)

A FUS possui domínios proteícos RGG, motivos de reconhecimento de RNA, e *zync-finger* que são domínios de ligação à ácidos nucleicos, que se mostraram capazes de se ligar ao DNA de fita simples e de fita dupla e ainda promover o pareamento homólogo de DNA(36). Estes achados, junto com estudos com camundongos knockouts para FUS(37,38) e com estudos dano de DNA induzido por laser (39), reforçam a ideia da ligação da FUS com o reparo do DNA celular.

Em 1994, um grupo de pesquisadores descobriu que a FUS age como ativador transcricional de proteínas de fusão oncogênica, levantando a hipótese de FUS estar ligada à regulação da transcrição celular(40). Posteriormente diversos estudos demonstraram a interação entre a FUS, RNA-polimerase II e TFIID (um fator de transcrição que está envolvido no complexo de iniciação da transcrição), sugerindo que FUS exerce influência sobre a transcrição celular de uma forma geral.(41,42,43,44,45,46,47)

Parte do papel da proteína FUS na regulação transcricional pode vir do fato desta proteína interagir com diversos RNA's. Estudos demonstraram que a proteína FUS interage com pré-mRNA's nos spliceossomos (48,49,50). Como o splicing do pré-mRNA acontece co-transcricionalmente e aparentemente FUS interage com a RNA polimerase II e com o spliceossomo, tem sido proposto que FUS atua na transcrição, no splicing e no metabolismo subsequente do RNA. (46,51) Dois trabalhos publicados em 2012 (52,53) sugerem que a FUS se liga aos transcritos originados durante a fase de elongamento da transcrição, devido à preferência de ligação dessa proteína com as extremidades 5' de íntrons longos. A diminuição dos níveis de FUS no sistema nervoso de camundongos causa mudanças em quase 100 transcritos, corroborando a

hipótese de que essa proteína tem um papel importante na regulação da abundância e do splicing de transcritos(101).

Consistente com o papel regulatório de *FUS*, esse gene apresenta uma regulação precisa, muito da qual é realizada na forma de autoregulação. Zhou et al., publicam em 2013 um artigo em que demonstram como a FUS realiza um *feedback* negativo ligando-se ao éxon 7 de seu próprio pré-mRNA causando a degradação do transcrito por *nonsense mediated decay*. (54) A proteína FUS também estimula a produção de dois micros-RNA's (miR-141 e miR-200a) que são responsáveis por reprimir a expressão do *FUS* ligando-se à extremidade 3'UTR do transcrito do *FUS*. (55)

A proteína FUS, apesar de ser predominantemente nuclear, alterna sua localização entre o núcleo e o citoplasma. Aparentemente esse processo está ligado à processos de transporte de mRNA (RNA's mensageiros) para o citoplasma, onde a proteína FUS aparece ligada à grânulos contendo RNA e proteínas. Estes grânulos compostos também por FUS aparecem tanto em processos ligados à resposta ao estress oxidativo quanto em processos de transporte de RNA's para serem traduzidos em locais celulares específicos. (56,57,58) Em neurônios, FUS interage com proteínas motoras e está presente em regiões do corpo celular e em grânulos dedríticos de RNA, o que sugere um controle no transporte e na tradução local de RNA. (59,60,61) Em neurônios piramidais do hipocampo, a proteína FUS responde à ativação neuronal deslocando-se para as espinhas dendríticas associada à mRNA codificadores para proteínas relacionadas com a actina. A actina é uma proteína relacionada à definição da morfologia celular, característica fundamental para o correto funcionamento de neurônios. Em 2005, Fuji e Takumi (62) publicaram um artigo relatando que, em camundongos, neurônios que não expressam a FUS têm o perfil de RNA's transcritos

diferente de camundongos selvagens. Nessa ocasião, dentre os RNA's diferencialmente expressos estão alguns ligados à produção de proteínas auxiliadoras da remodelagem da actina, dessa forma tem sido proposto que as alterações nas espinhas dendríticas de neurônios não expressando FUS são consequência do metabolismo anormal das proteínas modeladoras da actina nessa situação. A falha no metabolismo da FUS altera o funcionamento da actina e isso muda a morfologia neuronal, o que pode ser responsável pela falha dos NM na ELA.(62,63)

Apesar de diversos estudos acerca da função do gene *FUS*, o motivo pelo qual mutações nesse gene levam ao fenótipo de ELA e à outras doenças neurodegenerativas ainda permanece obscuro. Mais de 50 mutações relacionadas à ELA já foram descritas nesse gene, sendo que a maioria ocorre no sinal de localização nuclear (SLN) dessa proteína ou resultam na perda do SLN completamente. (1). As mutações que comprometem o SLN impedem a ligação com a transportina e a importação da proteína FUS para o núcleo, e um estudo realizado em 2010 relata uma forte relação entre o grau de comprometimento da localização nuclear da FUS com a gravidade da doença, sendo que os casos mais agressivos de ELA apresentam uma retenção citoplasmática da FUS maior que os casos mais amenos desta afecção. (64) Estudos relatam que a mutacão R521C, relacionados à casos típicos de início tardio da doença de , promovem uma retenção moderada da proteína FUS no citoplasma, enquanto que a mutação P525L, responsável por casos de início precoce e de progressão rápida de ELA, causam um retenção citoplasmática da proteína mutada bastante expressiva. (65,66,67,68)Ver a **figura 3**.

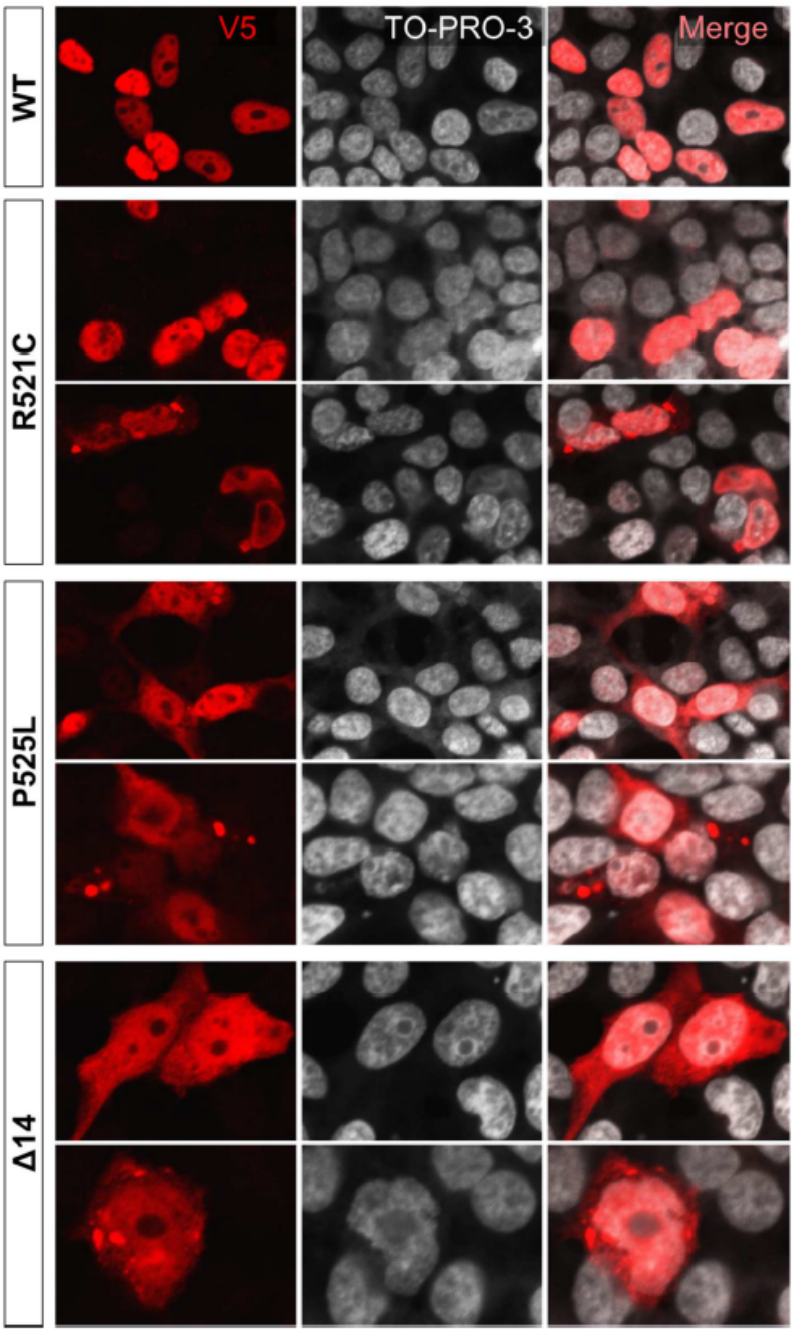

FIGURA 3: Efeito de mutações ligadas à ELA na localização subcelular da FUS. cDNA's da proteína FUS (selvagem e mutantes R521C, P525L e Δ14) marcada com V5foram expressos em células HEK 293 sob o controle do promotor CMV por transfecção transiente e detectadas 48 horas após com o anticorpo anti-V5. TO-PRO-3 foi usado para observar o núcleo. FUS WT apresenta FUS localizado exclusivamente no núcleo. FUS R521C apresenta localização predominantemente nuclear, mas 5% das células transfectadas apresentaram uma marcação fraca para localização citoplasmática da proteína com raros agregados citoplasmáticos. FUS P525L apresentou marcação difusa para a proteína no núcleo e no citoplasma, e agragados citoplasmáticos estavam presentes em 10% das células transfectadas. FUS Δ14 apresentou marcação difusa para a proteína no núcleo e no citoplasma, e agragados citoplasmáticos estavam presentes em 5% das células transfectadas. Retirado de Lyashchenko, 2015(1).

Inclusões citoplasmáticas de FUS estão presentes em neurônios e células da glia de pacientes de ELA relacionada à mutações no gene *FUS* (ELA-FUS), corroborando a ideia de que o acúmulo desta proteína no citoplasma pode ter um papel importante na patogênese da doença. Ainda é incerto se FUS deposita-se juntamente com TDP-43 em casos de ELAe. Alguns casos de ELA-FUS apresentam inclusões citoplasmáticas positivas para FUS mas negativas para SOD1 e TDP-43 e em alguns casos de ELA não relacionados ao gene *FUS* as inclusões citoplasmáticas não indicam a presença da proteína FUS (68). Porém outros estudos verificaram a presença da proteína nos agregados citoplasmáticos em casos esporádicos da doença. (69,70) As proteínas PABP-1 e eIF4G, relacionadas à grânulos de estresse celular, foram encontradas em inclusões citoplasmáticas positivas para FUS de pacientes de ELA e, aparentemente, mutações relacionadas à ELA aumentam a deposição de FUS nos grânulos de estresse, o que sugere que esses grânulos podem ser precursores dos agregados citoplasmáticos. (71,72) Em 2013, Buchan et al. publicaram um estudo que indica que mutações na proteína contendo valosina (VCP ou *valosin-containing protein*), também associada à casos de ELA, atrapalham o metabolismo celular de grânulos de estresse, reforçando a ideia de que estes grânulos podem estar envolvidos na patogênese da afecção (73). Aparentemente os grânulos de estresse positivos para FUS também apresentam ubiquitina e p62 (74,75) em sua composição, proteínas que marcam proteínas para serem degradadas via sistema proteassomo e via autofagia. O trabalho de Wang e colegas corrobora essas observações demonstrando um aumento da afinidade entre a proteína FUS com mutação relacionada à ELA e diversas proteínas ligadas à via ubiquitina proteassoma. (76) Mutações na ubiquitina, na *sequestome 1* (SQSTM1) e na VCP, proteínas que também

estão ligadas ao processo de degradação de proteínas são associadas à casos raros de ELA. (77) O trabalho de Almeida et al. de 2013 (78) associa mutações na C9ORF72, uma das principais causas conhecidas de ELA, ao metabolismo de degradação proteica, evidenciando ainda mais as vias de degradação de proteínas como possíveis responsáveis pela patogênese de ELA.

Outra via que merece destaque no estudo dos mecanismos responsáveis pela manifestação da ELA é a via de expressão gênica e do metabolismo de RNA no núcleo. Genes como *FUS*, *TDP-43*, *ANG* (angiogenina) e outros, estão ligados diretamente aos processos citados e apresentam variantes patogênicas relacionadas à ELA. Em 2012 foi publicado um trabalho (27) mostrando que a proteína codificada pelo gene *SOD1*, com mutação causadora de ELA, interfere no funcionamento da proteína SMN1 (*Survival motor neurons 1*), relacionada com a atrofia espinhal progressiva (AEP), doença também marcada pela morte de neurônios motores. A proteína SOD1 impede a formação das gemas nucleares, um corpúsculo nuclear que tem a proteína SMN1 como parte de sua composição e que está ligada à produção de pequenos RNA's ricos em uridina (U snRNA), um componente da maquinaria de splicing. Um trabalho subsequente (24) demonstra que a proteína FUS interage com a SMN1, e quando FUS apresenta mutações relacionada à ELA o número de gemas nucleares diminui consideravelmente. Este último trabalho demonstra também que mutações da TDP-43, proteína ligada à casos de ELA e que interage com FUS, levam à queda no número de gemas observadas em fibroblastos de pacientes com ELA com mutações no gene *FUS* ou *TDP-43*. Consistente com esses achados, Ishihara et al. (26) demonstraram que pacientes de ELA com mutações no gene da *TDP-43* apresentam níveis de U12 snRNA diminuídos em tecidos afetados pela doença, enquanto que nos tecidos não atingidos os níveis permanecem

normais. Desta forma, as vias de expressão gênica , de splicing e a dinâmica das gemas nucleares parecem estar relacionadas à neurodegeneração tanto em ELA quanto em AEP, e o gene *FUS* está diretamente envolvido nesses mecanismos.

Ferramentas de estudo sobre ELA

Devido ao fato da ELA ser uma doença neurológica o estudo *in vivo* da doença é extremamente difícil, sendo que gralmente os únicos tecidos disponíveis são colhidos após o falecimento do paciente. A utilização de material *post-mortem* trouxe diversos avanços no entendimento da doença, porém o viés de que os tecidos de um paciente que veio a falecer podem estar danificados e por isso é provável que apresentem alterações metabólicas consideráveis deve ser levado em conta. A utilização de tecidos *post-mortem* também inviabiliza o estudo dos mecanismos e sinais patológicos atuantes no início e durante o desenvolvimento da doença, além de não ser possível a utilização desse material para estudos clínicos que buscam um tratamento ou a cura para a afecção.

Desde a descoberta da associação entre o gene *SOD1* e a ELA, diversos modelos animais foram desenvolvidos para auxiliar no estudo desta patologia. Vários avanços sobre a nossa compreensão da doença vieram desses modelos, mas eles apresentam limitações no modo de recapitular aspectos da doença e na forma da utilização do conhecimento oriundo desses estudos. O modelo ideal deveria reproduzir os sintomas da doença e sua progressão, os mecanismos neurobiológicos e ainda servir como material de estudo para o desenvolvimento de novas terapias.

Até onde sabemos nenhuma espécie não humana desenvolve ELA, porém em cachorros há uma patologia conhecida como mielopatia degenerativa canina (MDC) que relembram diversos aspectos clínicos de ELA. Duas mutações no gene *SOD1* canino foram identificadas como relacionadas à MDC. A ELA e a MDC compartilham diversas características como estarem relacionadas ao início tardio,

ambas são doenças neurodegenerativas letais que levam à morte de neurônios motores, seguida de degeneração muscular. Nas duas doenças são encontrados, na medula espinhal, aglomerados proteicos citoplasmáticos positivos para a ubiquitina, sinais de estresse oxidativo e denervação neuromuscular. (79,80,81) Apesar das semelhanças a MDC difere da ELA por se iniciar com o comprometimento predominantemente dos NM superiores, enquanto na doença humana tanto o NM superiores quanto os inferiores são afetados no início da afecção. Grande parte das mutações patogênicas da SOD1 humana são dominantes, ao passo que as mutações que ocasionam a doença canina são rescessivas com penetrância incompleta, e na doença canina não existe diferença na incidência da doença relacionada ao sexo do indivíduo(79,82,83,84,85).

A similaridade com a ELA indica que informações sobre os mecanismos e a progressão da MDC pode trazer novas perspectivas para a compreensão da afecção humana. Grande parte das investigações histológicas de ELA é feita usando tecidos de pacientes que morreram em estágios avançados da doença, o que nos fornece poucas informações acerca dos estágios iniciais da doença. Na MDC os cachorros geralmente são eutanasiados no início dos sintomas da doença e portanto tecidos dos estágios iniciais da doença estão disponíveis.(86)

Modelos de ELA feitos com roedores têm sido os mais utlilizados nos estudos sobre esta afecção, principalmente devido ao seu sistema nervoso mais complexo, à fácil manipulação e ao curto período de tempo necessário para o início da manifestação dos sintomas relacionados à doença. Modelos de primatas não-humanos também foram desenvolvidos por apresentarem vantagens devidas à sua semelhança filogenética com os humanos, porém graves problemas como o tempo para estudo, espaço necessário para desenvolvimento do experimento, alto custo e problemas éticos podem se mostrar como desvantagens significativas.

O *Danio rerio*, ou *zebrafish*, é o único organismo que possui sistema nervoso vertebrado simplificado e conservado, com um curto período de vida e suscetível à manipulação genética e à testes de drogas. Além dessas características, estudos

demonstram que 70% dos genes humanos apresentam ao menos um ortólogo no genoma desse peixe, sendo que 82% dos genes associados com doenças humanas possuem um ortólogo no zebrafish. Particularmente em relação à ELA, o zebrafish tem se mostrado uma importante ferramenta de estudo, trazendo indícios relevantes sobre as vias sistêmicas, celulares e moleculares associadas à diferentes variantes genéticas. Apesar das vantagens da utilização desse modelo para o estudo de doenças neurodegenerativas humanas, os mecanismos bioquímicos e a as respostas fisiologicas observadas diferem entre o modelo e a espécie humana, fazendo com que por vezes os dados obtidos com zebrafish sejam irrelevantes para humanos. (86)

O modelo murino de ELA portando alterações no gene *SOD1* foi o primeiro modelo animal de ELA produzido. Mais de 20 modelos murinos de ELA relacionados ao *SOD1* já foram criados, sendo que a maioria superexpressa uma variante mutante da SOD1 humana (SOD1h). Camundongos com a variante G93A da SOD1h são os mais antigos e os mais utlizados para o estudo da ELA. Inicialmente, os modelos de SOD1 foram projetados para representar os casos de ELA como um todo. Uma vez que as formas familiais e esporádicas da doença apresentam manifestações clínicas iguais acreditou-se que o modelo murino de SOD1 seria capaz de representar todos os casos da doença. Apesar das semelhanças clínicas, o achado de que casos de ELAe e ELAf não ligados à mutações no gene *SOD1* apresentam agregados citoplasmáticos contendo TDP-43 nos neurônios e na glia, enquanto pacientes de ELA com mutações no gene *SOD1* apresentam agregados citoplasmáticos que não marcam para TDP-43, acabaram evidenciando diferenças moleculares entre as diversas formas da doença. Os modelos murinos com SOD1h portando a mutação G93A também não apresentam os agregados positivos para TDP-43, reforçando a ideia de que outros modelos murinos são necessários para que se investigue as diversas formas da doença, já que o os casos de ELAe e de ELAf não associados à SOD1 correspondem à aproximadamente 98% dos casos de ELA.

Modelos de roedores superexpressando a proteína TDP-43 mutante para simular os sintomas de ELA aparentam recapitular alguns sintomas da doença, tais como

inclusões positivas para ubiquitina, degeneração de NM e axônios, paralisia e morte. Mais recentemente, um modelo murino expressando níveis mais baixo da TDP-43 tentam recapitular os níveis de expressão da proteína em humanos. Apesar de alguns progressos, os modelos animais para estudo da ELA relacionada ao gene *TDP-43* são relativamente novos e necessitam de mais validação em relação ao fenótipo da doença humana.

Na tentativa de tentar entender os mecanismos pelos quais mutações no gene *FUS* levam ao fenótipo de ELA, diversos modelos foram desenvolvidos para o estudo desta afecção ligada ao gene *FUS*. Modelos usando leveduras, *C. elegans*, zebrafish e drosófilas recapitulam alguns sintomas da doença, e nos fornecem pistas sobre o mecanismo patogênico que leva a proteína FUS mutada à causar ELA. A partir desses modelos, foram observados indícios de que a proteína FUS atua posteriormente a proteína TDP-43 na via que leva à patogênese de ELA, e sugere-se que na proteína FUS mutante haja ganho ou perda de função.

Diversos modelos murinos simulando a ELA ligada ao gene *FUS* já foram desenvolvidos. Modelos de camundongos com o gene *FUS* deletado apresentam baixa viabilidade da prole, aumento da sensibilidade à radiação, instabilidade genética e defeitos na espermatogênese e no desenvolvimento de células B, reforçando a hipótese de FUS ser essencial para o correto metabolismo do DNA. Entretanto a deleção do gene *FUS* não produz camundongos com fenótipo semelhante à ELA, dificultando a utilização desse modelo para estudos acerca da doença.

Em 2012, Verbeeck e colegas (102) publicaram um trabalho onde camundongos foram modificados utilizando injeções de adenovírus com o FUS selvagem ou mutante (R521 ou Δ14, neste último o SLN está ausente) no sistema nervoso central de animais recém-nascidos. Enquanto a proteína selvagem permaneceu predominantemente nuclear e solúvel, as proteínas mutantes R521 e Δ14 demonstraram localização citoplasmática e insolubilidade proteica, leve e acentuada, respectivamente. No caso da mutação que elimina o SLN da proteína FUS, inclusões citoplasmáticas neuronais positivas para ubiquitina e para marcadores de estresse

celular, semelhantes às observadas em tecidos *post-mortem* de pacientes, foram encontradas.

Outros modelos murinos de ELA relacionada à FUS foram criados e, embora sejam capazes de recapitular alguns aspectos da doença, demonstram certas divergências entre os sinais observados em camundongos dentro de um mesmo grupo experimental ou possuem diversos problemas com o local de inserção e o nível de expressão do material transgênico. Dessa forma, a validade desse modelo para o estudo da afecção precisa ser fortalecida e corroborada por outros modelos de ELA.(1)

Modelos celulares utilizando células humanas oferecem um ambiente celular e genético mais próximo do encontrado em pacientes de ELA, e por isso são de extrema importância para avanços na compreensão da doença. A manipulação de células humanas afim de investigar a patologia da ELA utiliza duas linhas principais: linhagens de células imortalizadas (LCI) e células tronco com pluripotência induzida (iPSC, do inglês *induced pluripotent stem-cells*).

As linhagens de células imortalizadas são uma ferramenta importante para o estudo dos mecanismos patogênicos de ELA, pois são de fácil obtenção e manutenção. As linhagens mais utilizadas são as linhagens de células HeLa, derivadas de um câncer cervical, as células HEK, células derivadas do rim de um embrião humano abortado e que foram transformadas por adenovírus, e células SH-SY5Y, derivadas de um neuroblastoma.

Em geral, os trabalhos que utilizam LCI para estudos relacionados à ELA envolvem algum tipo de manipulação genética, como silenciamento gênico, superexpressão, entre outros métodos. Este fato, unido ao perfil de expressão gênica bastante peculiar encontrado nas LCI afim de permitir a divisão celular ilimitada, são considerados vieses importantes na utilização desse tipo de modelo.

Yamazaki e colaboradores publicaram em 2012(24) um trabalho em células HeLa mostrando que as células portando cópias o gene *FUS* com mutação relacionada à ELA exibem o fenótipo de retenção da proteína FUS no citoplasma, e ainda evidencia que as linhagens mutadas apresentam falhas nas formações das gemas

nucleares. Esse trabalho ainda mostra que a expressão da proteína TDP-43 mutada causa sinais celulares semelhantes aos causados pela mutação no gene *FUS*, e que ambas as proteínas interagem com a proteína SMN e com as gemas nucleares. Dessa forma, esse estudo aproxima a ELA da AEP, e coloca a via de funcionamento do FUS, do TDP-43 e a dinâmica de funcionamento das gemas nucleares como um importante foco de estudo para a melhor compreensão dos processos neurodegenerativos patogênicos.

Um trabalho de 2015(76), utiliza linhagens de células HEK superexpressando a preteína FUS mutada para mostrar, através de espectrometria de massas, o interatoma do gene mutado. Os autores demonstram a interação da proteína FUS mutada, e não da selvagem, com diversas proteínas metabólicas, com proteínas ligadas à processos neurodegenerativos e, principalmente, com proteínas ligadas à via de degradação de proteínas. A via de degradação de proteínas, juntamente com as vias de metabolismo de ácidos nucleicos têm sido frequentemente relacionadas como prováveis mecanismos responsáveis por processos neurodegenerativos.

Linhagens de neuroblastoma (SH-SY5Y) foram utilizadas para elucidar alguns mecanismos de atuação da proteína produzida pela C9ORF72, a maior causadora de ELA descrita até agora(87). Embora não tenham sido usadas linhagens contendo mutações na ORF relacionadas à ELA, a elucidação do envolvimento deste gene e de outros, como *TDP-43* e o *VAPB*, na via de transporte endossomal é um avanço no entendimento da patogênese da doença.

Infelizmente a manipulação genética das LCI é um viés forte que deve ser considerado na análise dos resultados obtidos através dessa ferramenta. Além do mais, alguns tipos de mutações, como as observadas nos casos de ELA relacionados à *C9ORF72*, são de difícil indução, o que pode dificultar a produção de modelos para diversas doenças. Com isso, os modelos em células imortalizadas de ELA relacionados à expansão da ORF do cromossomos 9 são insatisfatórios e, sendo os casos de ELA relacionados à esse gene a maioria dos casos com mutação descrita, os

modelos de LCI apresentam uma limitação grave em relação às mutações possíveis de serem estudadas.

Em 2007, Takahashi, K. et al.(88) publicaram uma técnica capaz de reprogramar células somáticas adultas humanas em células tronco pluripotentes. Com essa técnica inovadora é possível criar células neuronais derivadas de células dos pacientes de ELA, oferecendo assim um ambiente genético idêntico ao encontrado nas células afetadas pela doença e, por se tratarem de células adultas, a sua manipulação está livre de problemas éticos.

Inicialmente o processo de transformação das células somáticas em iPSC era feito através da utilização de vetores virais para a integração de fatores de desdiferenciação no genoma celular. Apesar de funcional, esse método cria o viés de que não se pode prever em qual local serão inseridos os fatores de desdiferenciação, podendo gerar problemas na cultura de iPSC e artificios celulares advindos da integração em locais críticos para o correto funcionamento celular. Atualmente têm sido desenvolvidos métodos não integrativos para a produção de iPSC, dessa forma é possível eliminar o viés de problemas que podem surgir por via da integração dos fatores de indução de pluripotência. Os métodos de transformação não integrativos têm sido os mais utilizados para a produção de iPSC atualmente. A **figura 4** apresenta os principais métodos de reprogramação de células somáticas.

Outro aspecto importante a ser considerado no processo de formação das iPSC é a variação clonal dentro das linhagens de iPSC. Este viés é especialmente importante quando as células são reprogramadas utilizando um método integrativo, já que alguns fenótipos celulares observados podem ser fruto da inserção dos fragmentos de reprogramação em locais críticos para o funcionamento correto da célula.

As iPSC apresentam diversas características em comum com células tronco embrionárias, como: capacidade de formar os tres folhetos embrionários, formar teratoma, atividade da telomerase restaurada, reativação do cromossomo X com reinativação aleatória do cromossomo X quando a célula é rediferenciada, marcas epigenéticas semelhantes, entre outras. Com isso é possível considerar que as iPSC

como células tronco embrionárias paciente-específicas e, consequentemente, podem ser usadas sem rejeição.

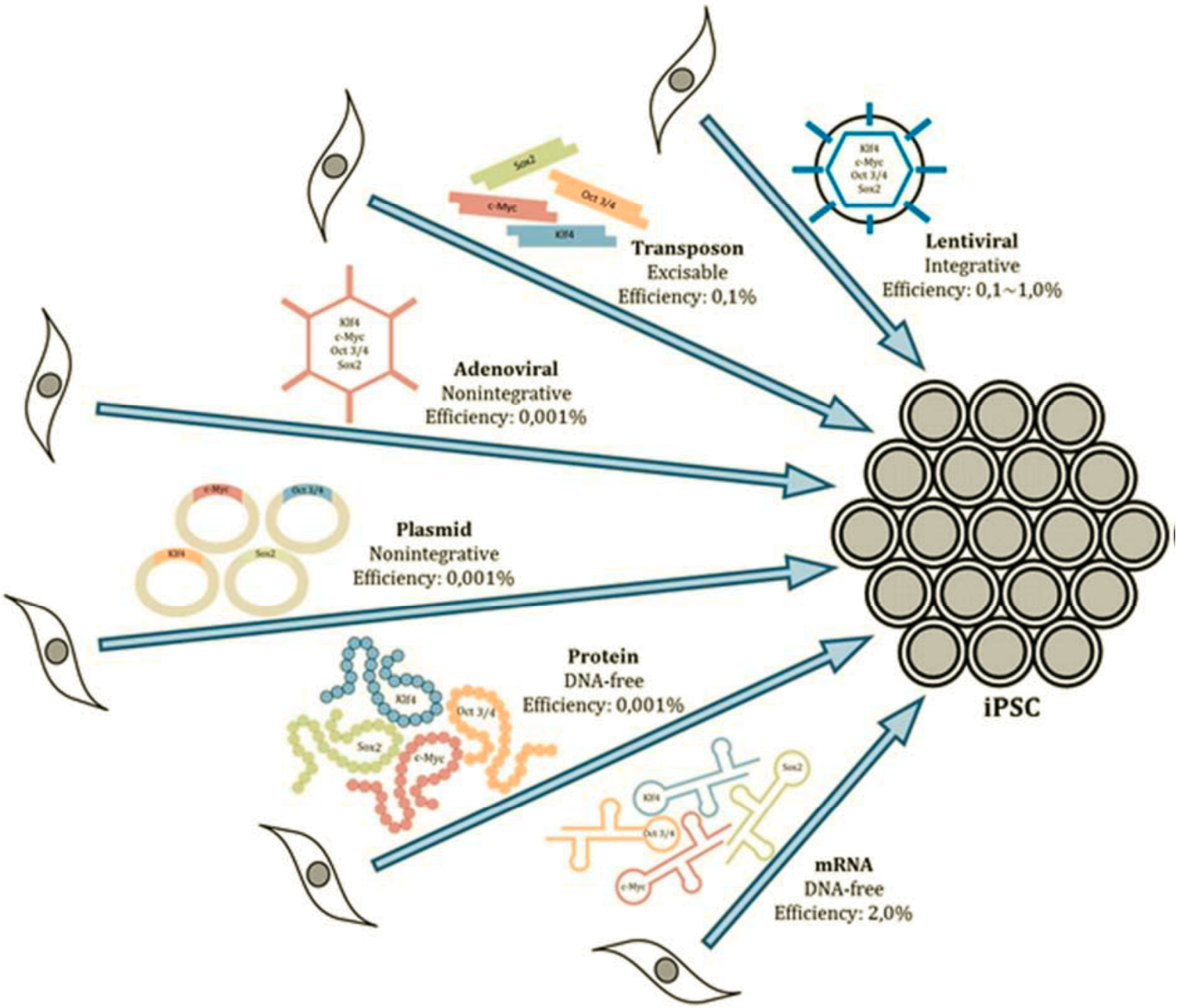

FIGURA 4: Principais estratégias para transformar células somáticas em iPSC. A eficiência pode variar de acordo com variações do protocolo. Retirado de Coatti, G. et al. 2015(30).

Os diversos estágios da diferenciação das iPSC em neurônios motores maduros são atualmente bem caracterizados. Após o estabelecimento das iPSC, as células são induzidas a formar corpos embrióides (EB) e subsequentemente a se transformar em progenitores neurais (NPCs). Fatores como o sonic hedhog e o ácido retinóico são geralmente utilizados para a diferenciação dos NPCs em neurônios motores maduros, que podem ser distinguidos de outros tipos neuronais pela expressão de marcadores, como o *Hb9* (gene homeobox 9).

O advento das iPSC trouxe novas perspectivas para os estudos acerca da ELA e Dimos et al. (98) desenvolveram a primeira linhagem de iPSC relacionadas à doença. As células pluripotentes derivadas de fibroblastos de um paciente foram usadas para obter neurônios motores portando mutação no gene *SOD1*. Entretanto nenhum fenótipo realcionado à ELA foi observado nessas células. Em 2011, Mitne-Neto e colegas (89) derivaram células iPSC obtidas de pacientes com mutação no gene *VAPB*. Observaram redução no nível dessa proteína durante a diferenciação de NM a partir das iPSC geradas de pacientes em comparação com controles normais, corroborando assim achados de outros estudos que relatam a queda nos níveis de VAPB em pacientes com ELAe e em camundongos portando mutação na SOD1. (90,91) Juntamente com outros achados (92,93) estes trabalhos reforçam a ideia de que NM derivados de iPSC podem recapitular o fenótipo da ELA.

No trabalho de Mitne-Neto (89), citado anteriormente, não foram observadas inclusões citoplasmáticas, encontradas em modelos de superexpressão da VAPB ou em pacientes de ELA com mutação em outros genes, como o *TDP-43*. Por outro lado, estudos usando NM derivados de iPSC de paciente com mutação na C9ORF72, e em outros genes relacionados à ELA, apresentaram correlação em diversos aspectos observados *in vivo* e *in vitro*, como hiperexcitabilidade neuronal. (92,94)

O co-cultivo de astrócitos e NM, derivados de pacientes com ELA.também tem sido investigado afim de caracterizar um possível efeito não autônomo na morte dos neurônios nessa afecção. Serio et al.(95)publicaram um artigo mostrando que os astrócitos derivados de iPSC de pacientes com ELA com mutação no *TDP-43* não

apresentam toxicidade para os NM derivados de iPSC do mesmo paciente, apesar dos astrócitos apresentarem menor tempo de duração em cultura quando comparado com os controles. Enquanto isso, um outro trabalho mostra que astrócitos derivados de células embrionárias e carregando mutação no *SOD1* apresentam um efeito tóxico sobre os neurônios motores(96). Os diferentes resultados obtidos em modelos celulares e animais demonstram a clara necessidade de continuar as pesquisas com o intuito de melhor compreender a patofisiologia da ELA.

Resultados aparentemente conflitantes podem surgir quando utilizamos modelos derivados de iPSC para estudos acerca da ELA devido ao fato de que essa doença geralmente possui início tardio, ocorrendo geralmente na quarta ou quinta década de vida. As iPSC se originam em um processo de "reinício celular", levando as células de volta para estágios embrionários, e o curto período de vida dos NM em cultura pode ser responsável por mascarar sinais patológicos observados em indivíduos afetados que poderiam aparecer futuramente. Uma solução para esse problema pode ser o enxerrto de NM ou NPCs derivados de iPSC em camundongos, o que poderia aumentar a sobrevivência dessas células que seriam recuperadas posteriormente e então analisadas em busca de sinais presentes em modelos *in vivo* ou em tecidos *post-mortem* de pacientes. Outra alternativa para solucionar esse impasse é tentar induzir características ligadas ao envelhecimento por meio da expressão de progerina, uma forma truncada da lâmina A associada com a doença conhecida como progeria, causadora de um envelhecimento prematuro. Esta última alternativa foi utilizada para estudos com neurônios dopaminérgicos, como modelo para o estudo da doença de Parkinson, e conseguiu recapitular algumas características fisiológicas da doença.

Atualmente, o uso das iPSC têm progredido para ensaios de teste de drogas. Um trabalho recente utilizou iPSC derivadas de fibroblastos de pacientes de ELA com mutação no gene *TDP-43* para a produção de NM. Os neurônios produzidos apresentaram neuritos curtos, menor concentração de mRNA de neurofilamentos, dentre outros aspectos da doença e então foram submetidos a testes para verificar a

eficiência do tratamento com ácido anacárdico. O tratamento resultou em uma atenuação do fenótipo da doença e, aparentemente, essa observação está relacionada à uma queda na expressão do gene *TDP-43* após a aplicação da droga.

Outro estudo recente, focado em alterações eletrofisiológicas em NM derivados de iPSC de pacientes com mutação nos genes *SOD1*, *FUS* ou *C9ORF72*, demonstraram que os NM apresentam uma hiperexcitabilidade que geralmente é observada *in vivo* e *in vitro*. Nesse mesmo estudo, os autores testaram o uso da retigabina (um anticonvulsivo já conhecido) para amenizar os sinais relacionados à doenças nos três tipos de NM produzidos. Este estudo é o mais amplo teste de drogas para ELA já feito até o momento com a utilização de iPSC.(94)

A maioria dos casos de ELA são esporádicos, e produzir iPSC destes pacientes pode nos trazer importantes informações sobre os mecanismos moleculares envolvidos nesses casos da doença. Em 2013, um grupo de cientistas publicou um trabalho utilizando NM derivados de iPSC de pacientes de ELAe, e testou 1757 compostos diferentes em um teste de droga de alta performance. Em 20% dos casos os NM gerados mostraram agregados de TDP-43 e, em um caso, foi confirmado posteriormente pelo tecido post-mortem de um paciente. Os NM com mutações na TDP-43 foram submetidos à testes de drogas, algumas já aprovadas pela FDA, e alguns compostos, como a Digoxina, amenizaram o fenótipo. Desta forma, os avanços na tecnologia de testes de drogas de alta performance aliados à uma melhor eficiência na produção e diferenciação de iPSC, aumentam a possibilidade de se tornarem reais os tratamentos personalizados para os casos de ELA. Os NM de pacientes seriam produzidos *in vitro* e testados para a droga mais efetiva, que então seria administrada ao paciente com o objetivo de cessar a progressão da doença.

CASUÍSTICA

Nosso laboratório identificou previamente um paciente de ELAf (P) com a mutação p.R521H (d.G1562A, éxon 15) no gene *FUS*. Posteriormente, descobrimos que um dos irmãos de P, aqui chamado de B-IV, possui a mesma mutação do paciente (d.G1562A) no gene *FUS*, porém não paresenta sinais clínicos de ELA. Outros dois irmãos do paciente (B-II e B-III) não possuem a mutação no gene *FUS*, e nem sinais clínicos de ELA. Todas as pessoas analisadas nesse trabalho são filhos de um mesmo casal. Foi colhido sangue e biópsia de pele do paciente e de três de seus quatro irmãos(B-II, B-III, B-IV).

OBJETIVOS

Objetivo geral

Gerar linhagens de iPSC a partir dos fibroblastos do paciente P e de seus irmãos, B-II, B-III e B-IV.

Objetivos específicos

1) Verificar se as linhagens de iPSC do paciente apresentam as alterações moleculares previstas em pacientes de ELA com mutações no gene *FUS*.

2) Comparar a localização da proteína FUS no paciente com a mutação no gene *FUS* (d.G1562A) e clinicamente afetado, com o paciente que apresenta a mesma mutação, porém sem sinais clínicos de ELA.

RESULTADOS E METODOLOGIA

Coleta de sangue e extração de DNA à partir do sangue.

O sangue dos participantes deste experimento foi coletado em tubos contendo EDTA. Após o processamento do material, o DNA foi extraído utilizando-se o aparelho *Autopure LS da Gentra System*, localizado no Centro de Pesquisas do Genoma Humano e Células-tronco da Universidade de São Paulo (CEGH-USP).

Biópsia e cultura de fibroblastos

Foram coletados fragmentos de pele dos participantes usando um *punch* de 3mm de diâmetro. Após extração, os fragmentos foram armazenados em criotubos contendo uma solução de DMEM high glucose (Life technologies), preparado de acordo com as instruções do fabricante, + 20% de SFB (Soro fetal bovino – Life Technologies) + 4% de ATB (Antibiotic-Antimycotic 100X – Life technologies), e transportados para a Universidade de São Paulo em gelo comum.

Os fragmentos foram processados e a cultura de fibroblastos foi implantada em garrafas T25 (Corning) contendo meio de cultura (DMEM high glucose + 20% SFB + 1% ATB). O meio de cultura foi trocado periódicamente. Quando as garrafas estavam com confluência de 80% aproximadamente, os fibroblastos foram colocados em suspensão com o uso de tripsina (Tryple Express – Life technologies) e então divididos em novas garrafas T25 vazias. Os fibroblastos foram congelados em passagem menor que 4. O meio de congelamento dos fibroblastos é uma solução de SFB + 10% DMSO (dimetisulfóxido). A **figura 5** mostra duas das quatro linhagens de fibroblastos produzidas neste estudo.

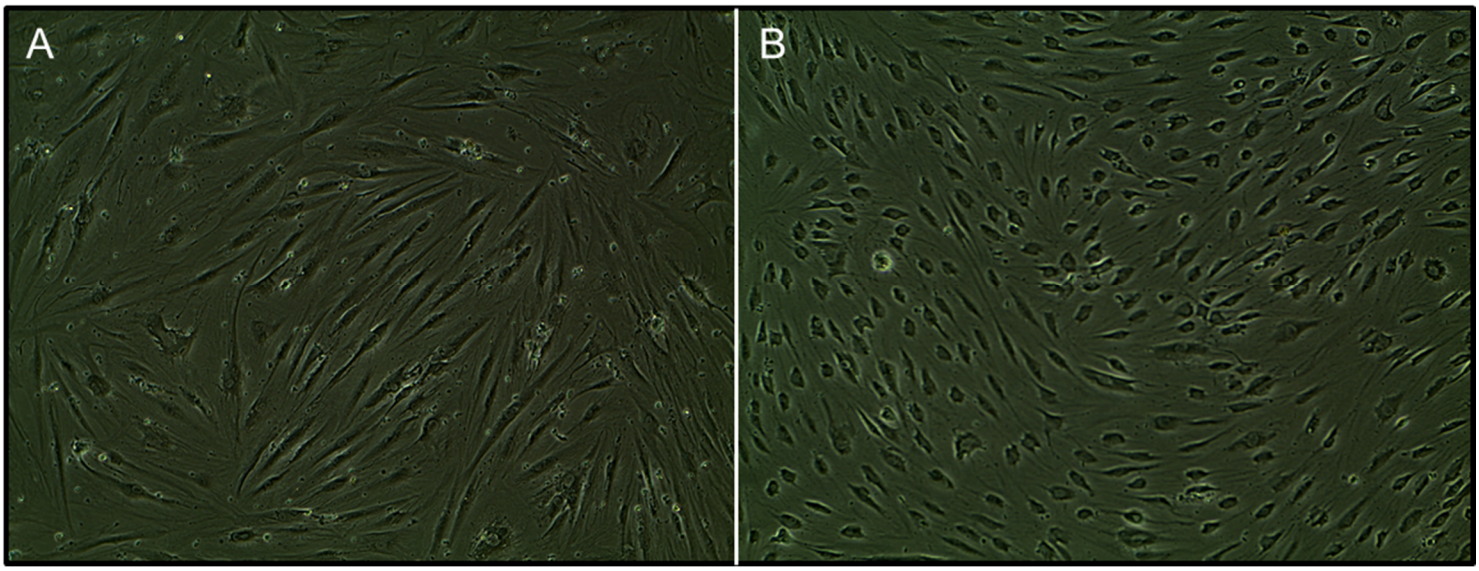

FIGURA 5: A e B são culturas de fibroblastos de dois dos participantes deste estudo. Posteriormente essas células foram submetidas à reprogramação em iPSC.

Reprogramação dos fibroblastos em iPSC

Foram produzidos 4 tipos diferentes de retrovírus e cada tipo contém material para expressar um dos quatro fatores de Yamanaka (88) (Sox2, Oct 3/4, c-Myc e Klf4). Após serem multiplicados em células HEK's, os diferentes tipos virais foram misturados e usados para infectar os fibroblastos dos pacientes. Os fibroblastos expostos aos vírus são então cultivados em placas contendo fibroblastos embrionários de camundongo (MEF's) com meio de cultura específico (mTeSR – Life technologies – condicionado em culturas de MEF's), até o aparecimento de colônias de iPSC. As colônias de iPSC foram transferidas para novas placas cobertas com Matrigel (Matrigel hESC-qualified – BD Biosciences), e cultivadas utilizando meio de cultura mTeSR + 0,2% Normocin™ (Invivogen). O meio de cultura foi trocado diariamente. Quando as colônias se tornavam muito grandes, ou quando a placa estava cheia, as iPSC eram passadas utilizando-se Acutase (Millipore) e raspagem da placa e divididas em novas placas cobertas com matrigel. Ao atingir a quantidade suficiente de células, as iPSC foram congeladas utlizando-se CryoStor™CS10 (Stemcell technologies) como meio de congelamento. Cada paciente possui 3 linhagens de iPSC

advindas de clones diferentes. Desta forma podemos excluir o viés de variação clonal, que pode surgir devido à diferenças celulares geradas pela transformação dos fibroblastos em iPSC. A **figura 6** mostra um exemplar de iPSC de cada participante.

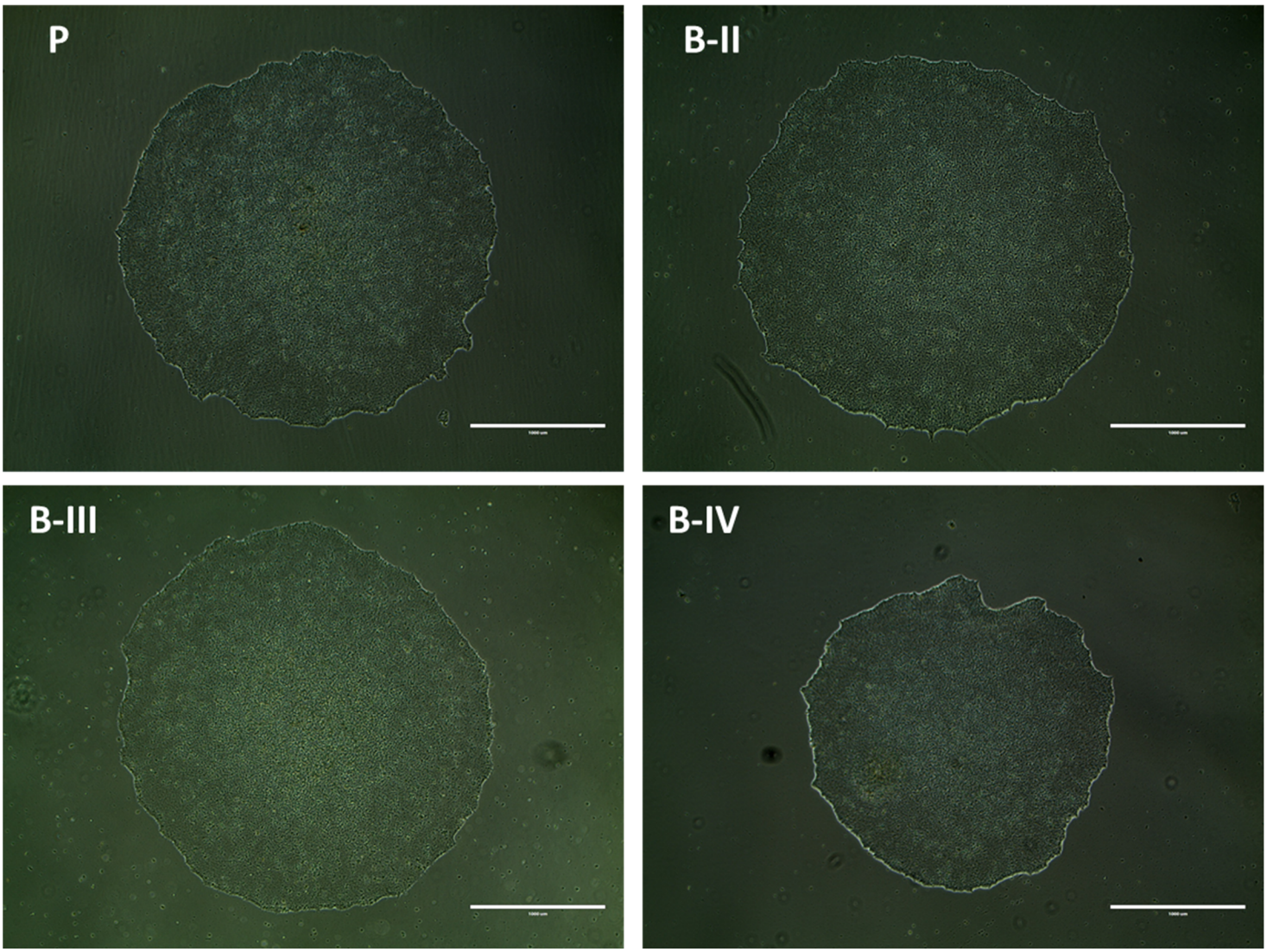

FIGURA 6: iPSC produzidas a partir dos fibroblastos dos participantes. A barra branca mede1000 μm.

Extração do DNA genômico das células cultivadas e análise do DNA de todas as amostras

Todas as linhagens e tipos celulares utilizadas neste estudo tiveram o DNA genômico (DNAg) extraído da seguinte forma: as células são colocadas em suspensão e precipitadas em um tubo, então o DNAg é extraído utlizando-se o kit DNAzol Reagent (Invitrogen – Life), seguindo as orientaçoes do fabricante. A quantidade e qualidade do DNA extraído das células cultivadas foram medidas em um espectofotômetro de massas (Nanodrop ND-1000, da Thermo Scientific) e, se em concentração adequada, o DNA é submetido à reação em cadeia da polimerase (PCR) utilizando-se primers específicos para amplificar o éxon 15 do gene *FUS*. O produto da PCR é purificado utilizando-se enzimas Exonuclease I e Shrimp Alkaline Phosphatase (USB-affymetrix). O produto purificado é submetido à reação de sequenciamento utilizando o kit *ABI Big Dye Terminator v3.1 cycle sequencing kit.* O resultado dessa reação é analisado no sequenciador *ABI 3730 DNA analyser* (Applied Biosystems, Carlsbad, CA, USA), localizado no CEGH-CEL. Os dados gerados são analisados computacionalmente utilizando o software *Sequencher*®(Gene Code Corporation).

Nenhuma das extrações de DNA das iPSC de B-III ficaram em concentração satisfatória e para contornar esse problema foi produzido cDNA dessa linhagem de iPSC. O sequenciamento do DNA realizado em todas as linhagens de iPSC utilizadas neste estudo foi feito com o intuito de confirmar a mutação previamente encontrada e os resultados estão na **figura 7**.

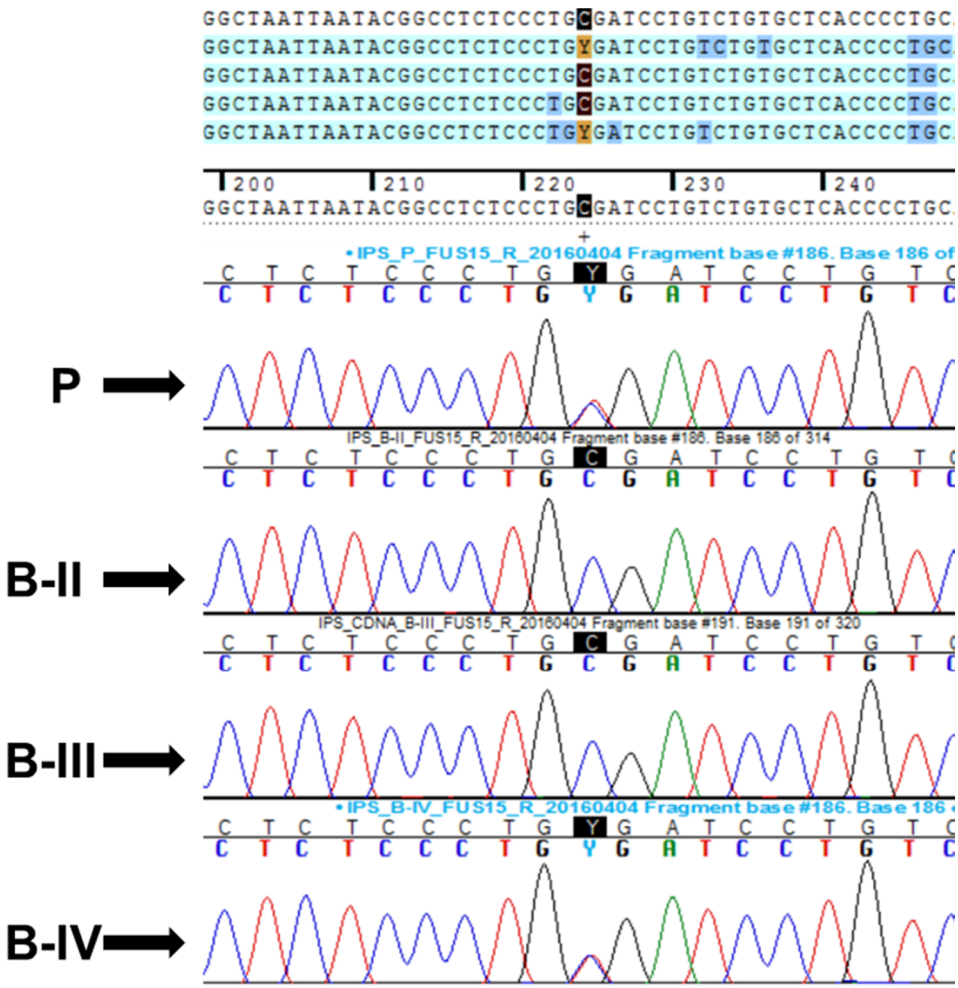

FIGURA 7: Resultado do sequenciamento do éxon 15 do gene FUS obtido a partir do DNA extraído das iPSC. Para o sequenciamento de B-III foi utilizado o cDNA das iPSC. A presença da mutação d.G1562A em P e B-IV foi confirmada, assim como a ausência da mesma nas linhagens de B-II e B-III.

Extração de RNA das células cultivadas e RT-PCR

As linhagens de iPSC produzidas foram submetidas ao protocolo de extração de RNA total usando-se o kit miRNeasy Mini Kit (Qiagen) e seguindo as normas do fabricante. Foi medida a qualidade e a quantidade de RNA no produto da extração utilizando-se um espectofotômetro de massas (Nanodrop ND-1000, da Thermo Fisher Scientific). As extrações de RNA do P não ficaram em concentração adequada e por isso os passos a seguir incluem somente B-II, B-III e B-IV.

Utilizando 1μg do RNA total extraído, foi feita uma reação de RT-PCR para a síntese de cDNA. Para a reação do RT-PCR foi usado o Superscript II (Thermo Fisher Scientific) e primers oligo-dT, o procedimento foi o indicado pelo fabricante. O cDNA foi utilizado para a realização de um Quantitative Real time PCR (PCR quantitativo em tempo real) a fim de verificar e a expressão dos genes endógenos de pluripotência *NANOG*, *OCT3/4* e dos indicadores de endoderme, mesoderme e ectoderme: *PAX2*, *BRACH* e *CXCR4*, respectivamente.

Extração de proteínas das células cultivadas

Todas as linhagens de fibroblastos foram cultivados até atingirem uma quantidade suficiente de células e entaõ foram submetidas à extração de proteínas por dois métodos:

1. Extração de proteína total da célula, utilizando-se o tampão RIPA (150 mM de cloreto de sódio; 1,0% Triton X-100; 0,5% de deoxicolato de sódio; 0,1% de dodecil sulfato de sódio; 1 mM ETDA; 50 mM Tris pH = 8,0) e protocolo padrão de extração proteica.
2. Extração de proteína utilizando-se o kit Nuclear/Cytosolic Fractionation Kit (Cell Biolabs), seguindo o protocolo indicado pelo fabricante. Neste tipo de extração são separadas a fração proteica nuclear e citoplásmica.

Os extratos proteicos 1 e 2 foram quantificados por métodos colorimétricos distintos, teste de BCA e teste de Bradford respectivamente. Foram utilizados diferentes métodos de quantificação devido à peculiaridades do tampão utilizado em cada método de extração.

Western Blotting

Os extratos proteicos previamente obtidos foram submetidos à ensaios de western blotting.

Primeiramente as proteínas foram separadas de acordo com o seu tamanho por processo de eletroforese em gel de poliacrilamida 14%, para subsequente transferência das proteínas para uma membrana de nitrocelulose pelo método semi-seco. As membranas contendo as proteínas são bloqueadas utilizando 5% de albumina de soro bovino em TBS-T(Tris-buffered saline, 0,1% Tween 20) como solução de bloqueio. A membrana bloqueada foi então incubada com anticorpo específico contra a proteína FUS (NB100-565 - Novus Biologicals) para se realizar a comparação entre as concentrações desta em cada extrato proteico. As membranas também foram marcadas com anticorpos anti-GAPDH (NB300-324 - Novus Biologicals), um marcador de citoplasma, e anti-Lâmina A/C (Anti-Lamin A + C antibody - ab40567 - Abcam), um marcador nuclear.

Por último, as membranas foram marcadas com anticorpos secundários específicos para cada anticorpo primário e então foram reveladas por ECL (Amersham ECL Prime Western Blotting Detection Reagent - RPN2232 - GE Healthcare Life Sciences) em filme fotográfico de acordo com o protocolo do fabricante.

Após diversas tentativas, não obtivemos resultados satisfatórios com essa técnica. Os problemas encontrados durante os ensaios com essa técnica se mostraram persistentes e não nos permitiu obter resultados confiáveis.

Imunofluorescência (IF)

As linhagens celulares utilizadas nesse trabalho foram submetidas à ensaios de imunofluorescência a fim de verificar a localização intracelular da proteína FUS. As iPSC foram também submetidas à IF para verificar a expressão dos fatores de pluripotência OCT-4 e LIN-28.

Primeiramente as células foram fixadas utilizando um solução de paraformaldeído 4%, por 20 minutos, sendo posteriormente permeabilizadas com uma solução de PBS 1X com 0,2% de Triton-X, por 30 minutos. Foi utilizada uma solução de 5% de BSA (*bovine serum albumine*) para bloqueio de ligações inespecíficas, por 60 minutos, e subsequentemente foram incubadas *overnight* com o anticorpo primário à 4ºC. Os anticorpos primários utilizados, diluídos em PBS em concentração 1:500, foram: FUS (NB100-565 - Novus Biologicals); LIN-28 (NBP1-49537 – Novus Biologicals); OCT-4 (NBP1-51664 – Novus Biologicals). Após a incubação com os anticorpos primários, as células lavadas 3 vezes com PBS 1x para retirada de excesso de anticorpos, e então, foram incubadas com o anticorpo secundário Alexa Fluor® 488 (A-21206 – Thermo Fisher Scientific) e/ou Alexa Fluor® 594 (A-21203 – Thermo Fisher Scientific). Ambos os anticorpos secundários utilizados foram diluídos em PBS na concentração de 1:1000. Após a marcação com os anticorpos, foi feita a marcação com DAPI (*4',6-diamidino-2-phenylindole, dihydrochloride* – Thermo Fisher Scientific) para a marcação de DNA e, quando necessário, foi realizada a marcação com PHAL (Rhodamine Phalloidin – R415 – Thermo Fisher Scientific) que marca a actina-F nas células. As placas foram analisadas utilizando o microscópio Nikon Eclipse Ti-U, e as imagens foram capturadas utlizando o programa NIS-Elements Advanced Research (Nikon).

As **figuras 8, 9, 10, 11 e 12** foram obtidas nesse ensaio. A **figura 8** mostra a expressão dos fatores de pluripotência das iPSC, enquanto que a localização da FUS em relação à OCT-4 ou à actina-F nos fibroblastos e nas iPSC estão expostas nas

figuras 9, 10, 11 e 12. O DAPI foi utilizado para corar o DNA em todos os ensaios. Os anticorpos secundários marcam especificamente o anticorpo primário.

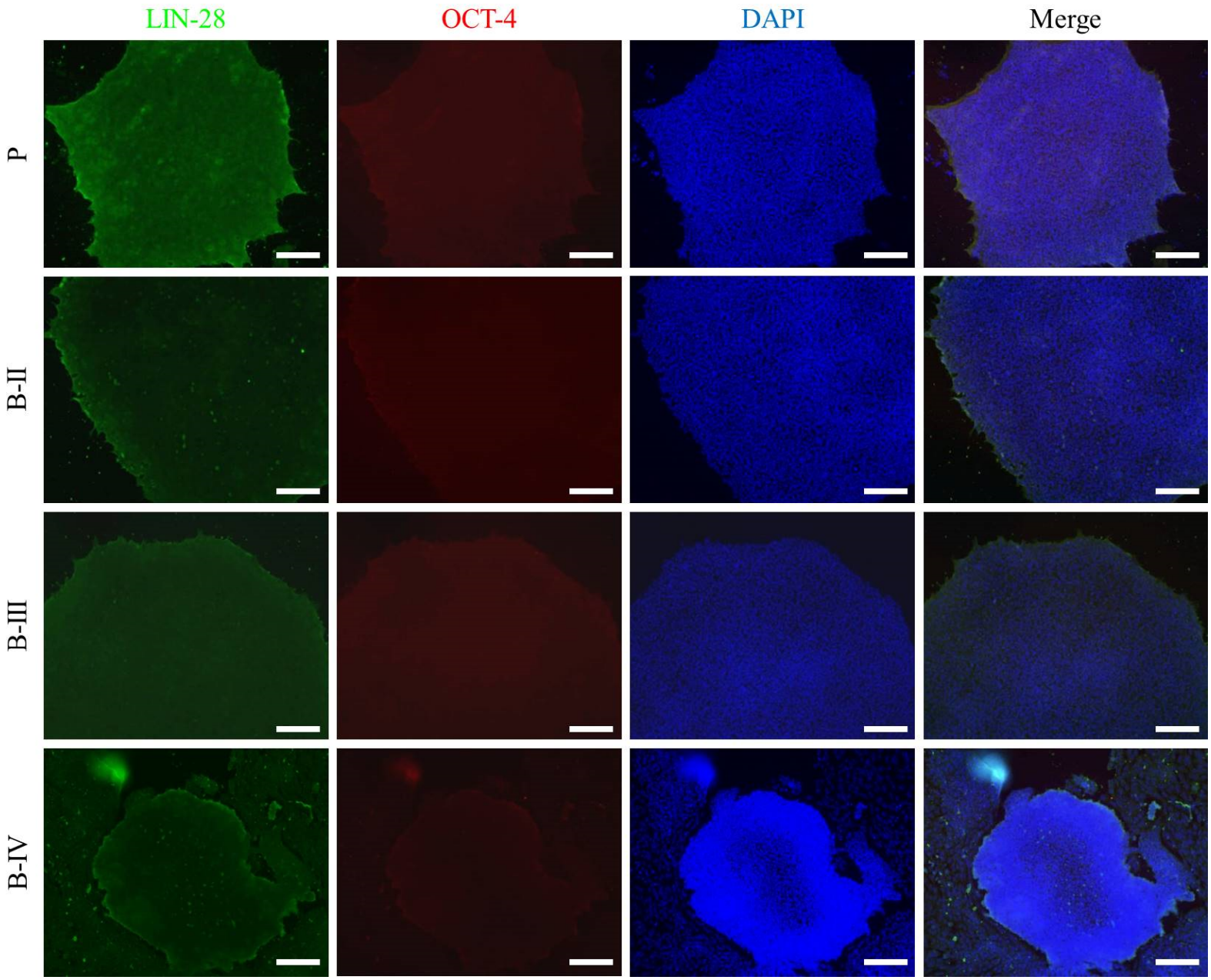

FIGURA 8: Ensaio de imunofluorescência em iPSC mostrando que as linhagens de iPSC derivadas nesse trabalho expressam os fatores de pluripotência LIN-28 e OCT-4. Imagens feitas utilizando o aumento de 20X. A barra branca mede 100 µm.

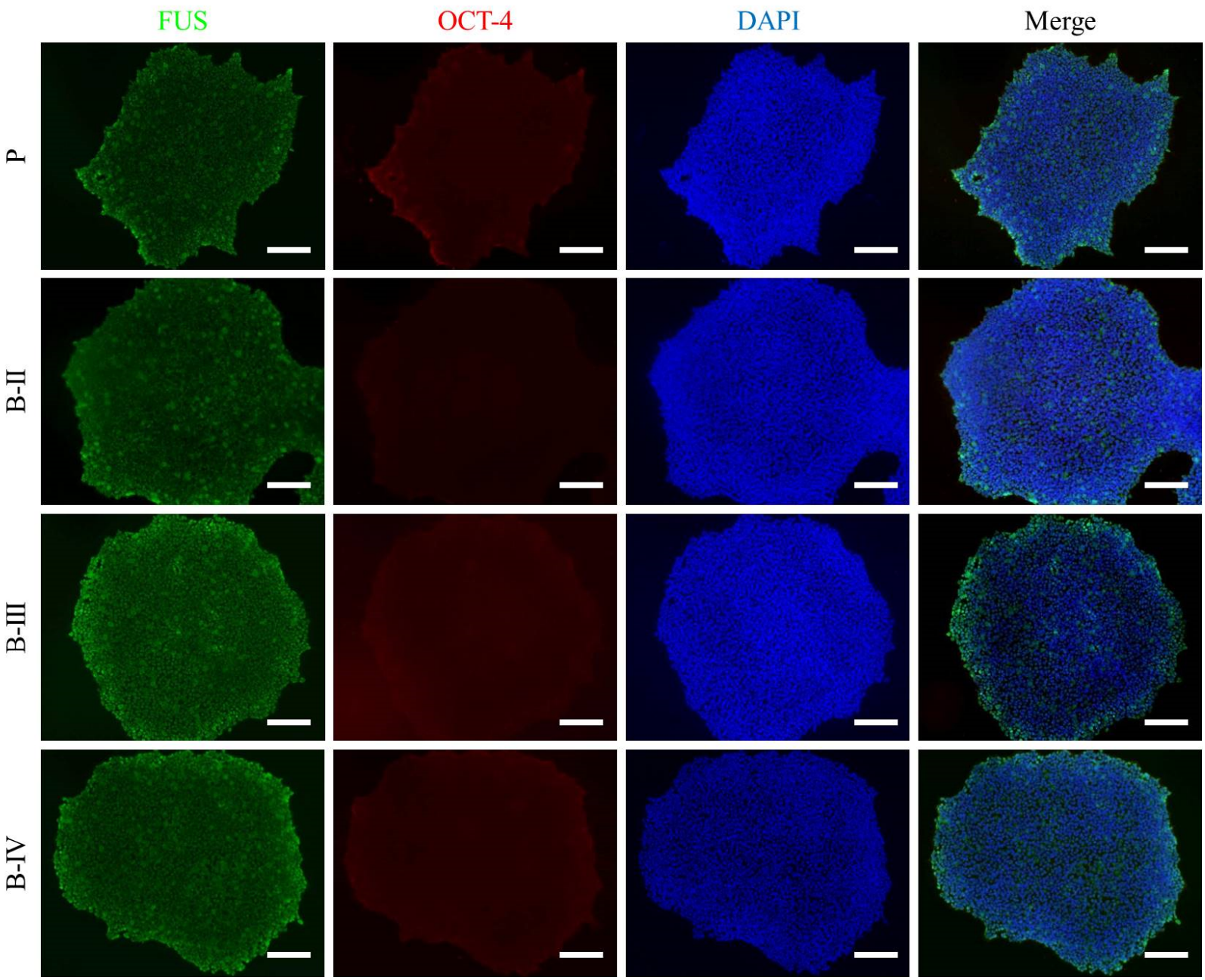

FIGURA 9: Ensaio de imunofluorescência em iPSC mostrando que as linhagens de iPSC derivadas nesse trabalho expressam o fator de pluripotência OCT-4 e a proteína FUS. A proteína FUS aparece mais retida no citoplasma das iPSC de P e de B-IV. Imagens feitas utilizando o aumento de 20X. A barra branca mede 100 µm.

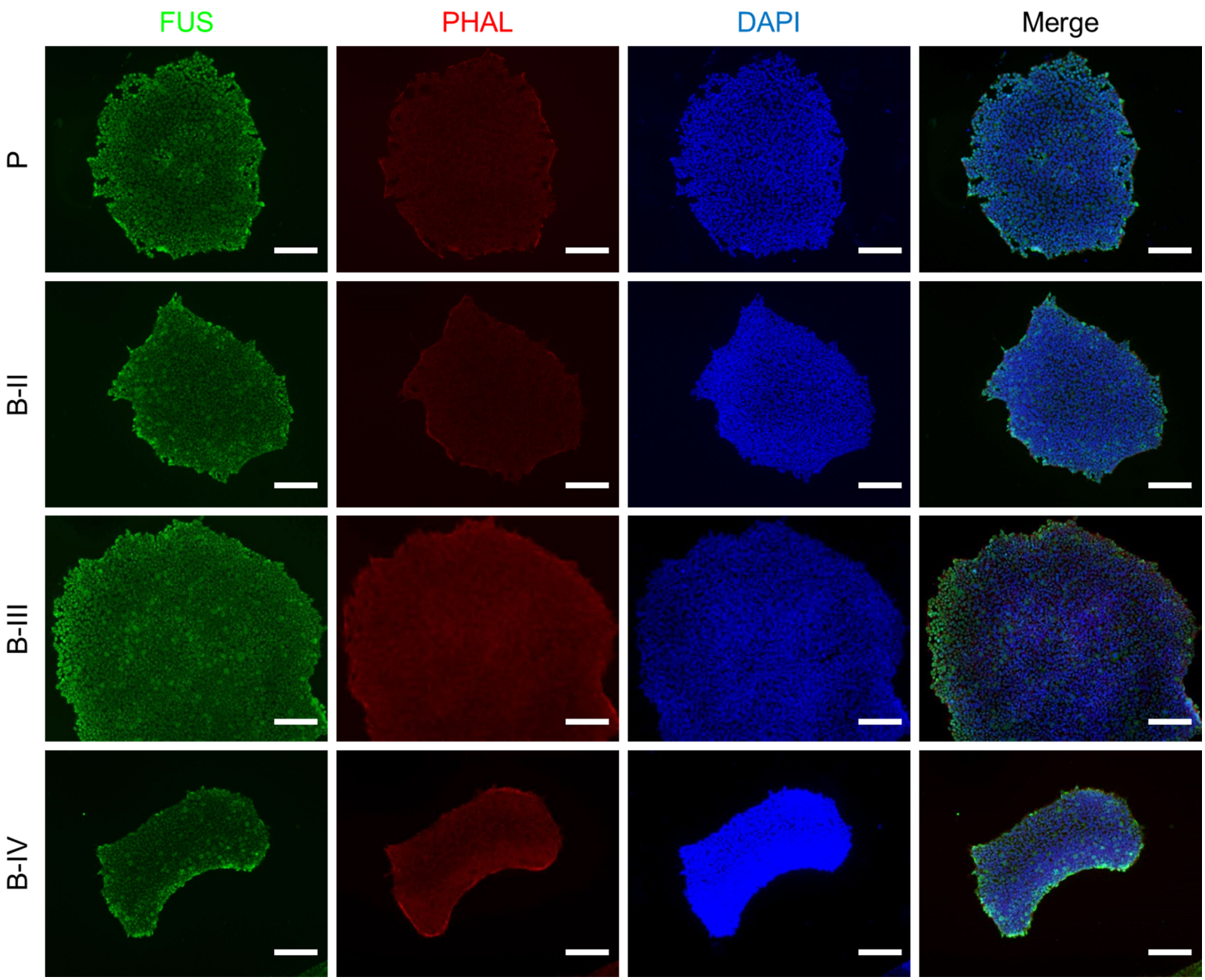

FIGURA 10: : Ensaio de imunofluorescência em iPSC mostrando a expressão da proteína FUS junto à um marcador de citoplasma, PHAL. A proteína FUS aparece mais retida no citoplasma das iPSC de P e de B-IV. Imagens feitas utilizando o aumento de 20X. A barra branca mede 100 µm.

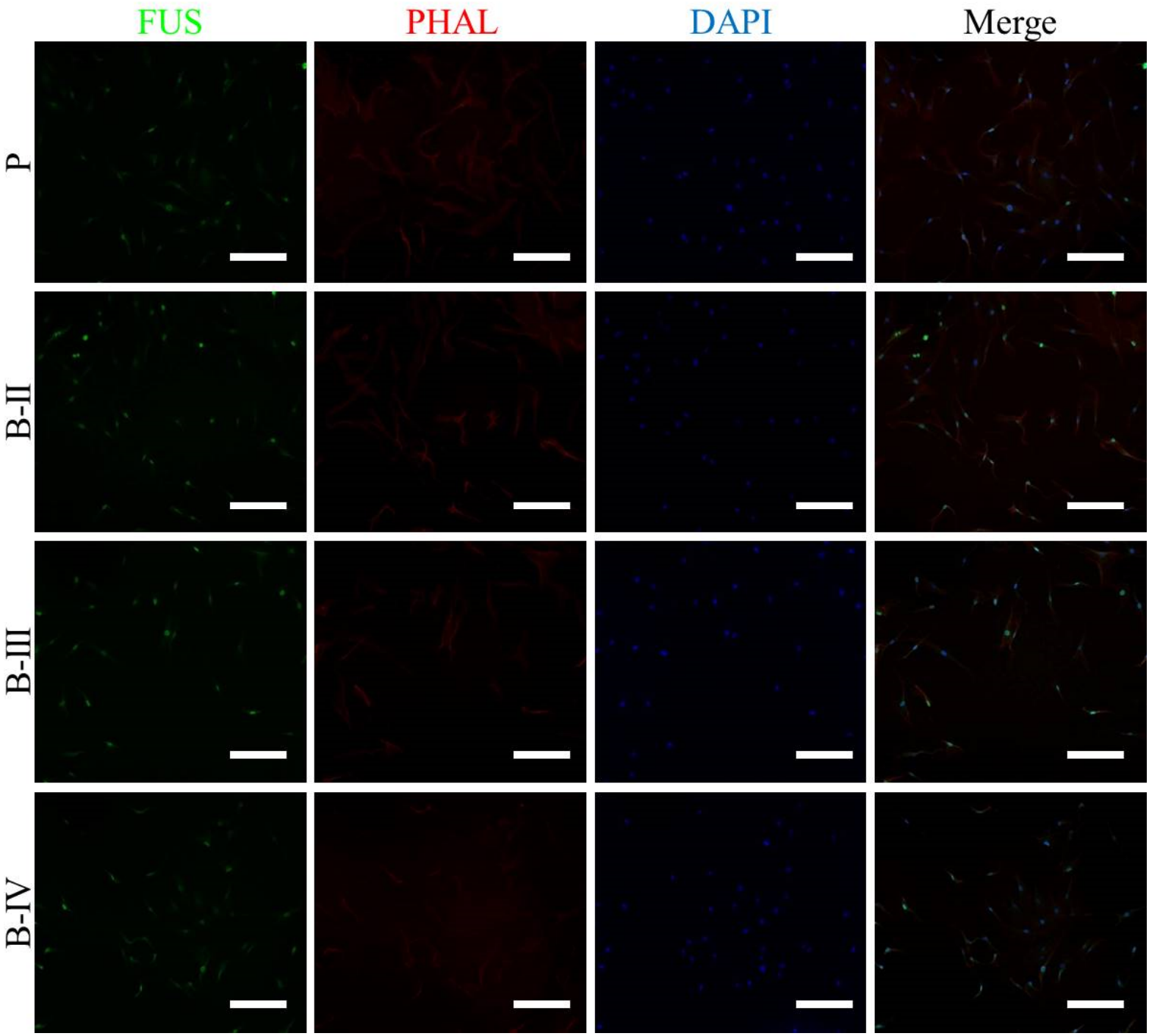

FIGURA 11: Ensaio de imunofluorescência em fibroblastos mostrando a expressão da proteína FUS junto à um marcador de citoplasma, PHAL. A proteína FUS aparece mais retida no citoplasma nas células de P e de B-IV. Imagens feitas utilizando o aumento de 20X. A barra branca mede 100 μm.

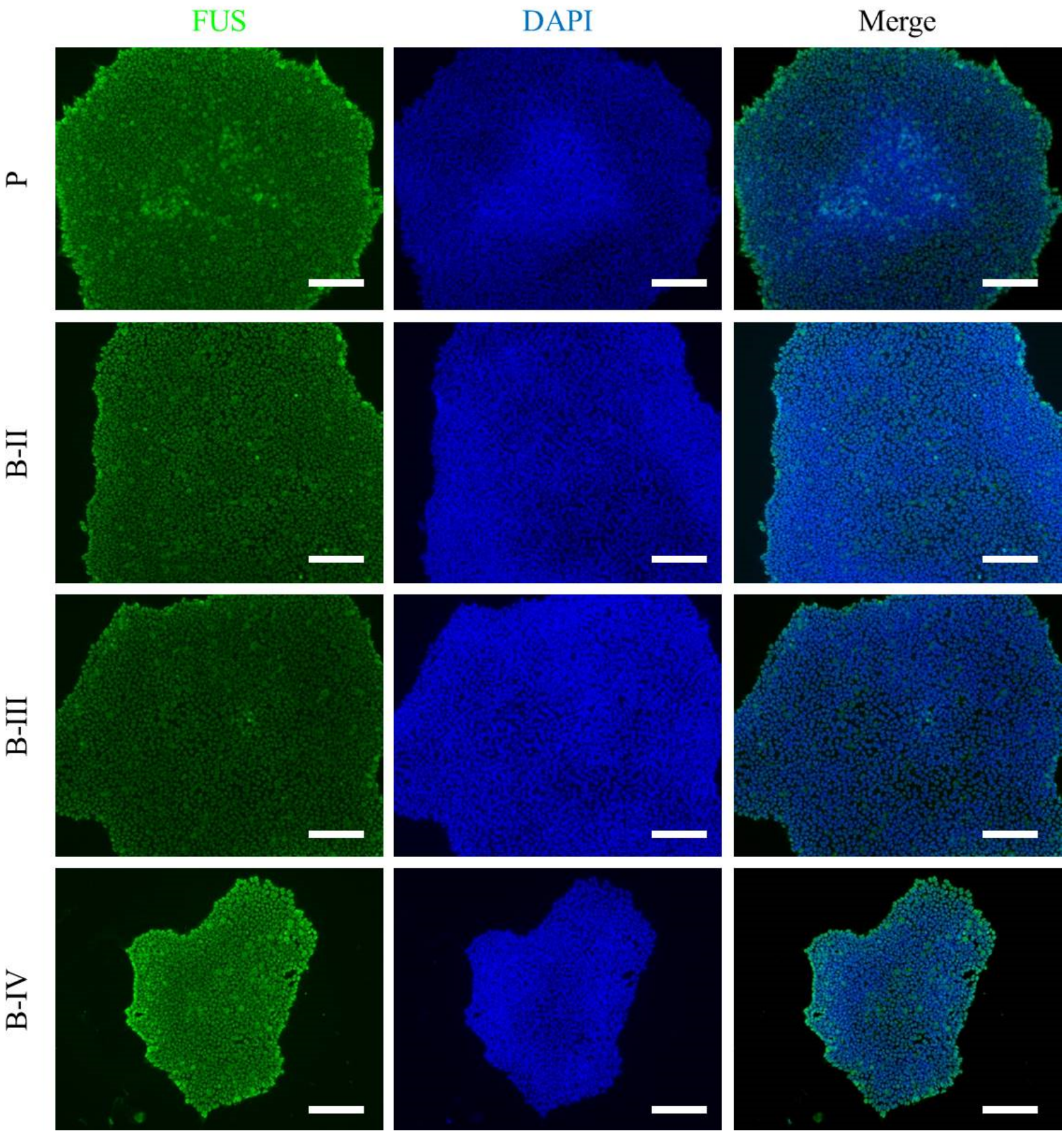

FIGURA 12: Ensaio de imunofluorescência em iPSC mostrando a expressão da proteína FUS. A proteína FUS aparece mais retida no citoplasma das iPSC de P e de B-IV. Imagens feitas utilizando o aumento de 20X. A barra branca mede 100 µm.

PCR quantitativo em tempo real

O ensaio de PCR em tempo real quantitativo foi realizado usando 2X Fast SYBR Green PCR Master Mix (Life Technologies) e 50nM–400nM de cada primer. Foi utilizado 7500 Fast Real-Time PCR System (Life Technologies) para detectar a fluorescência usando o protocolo de temperatura padrão. As seguintes ferramentas foram usadas para desenhar os primers: Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) ou retirados de (PrimerBank (http://pga.mgh.harvard.edu/primerbank/) .

Foi investigada a expressão dos seguintes genes: *NANOG*, *OCT3/4*, *PAX2*, *BRACH* e *CXCR4*. Os valores de expressão relativa obtidos foram apresentados no gráficos das **figuras 13 e 14**.

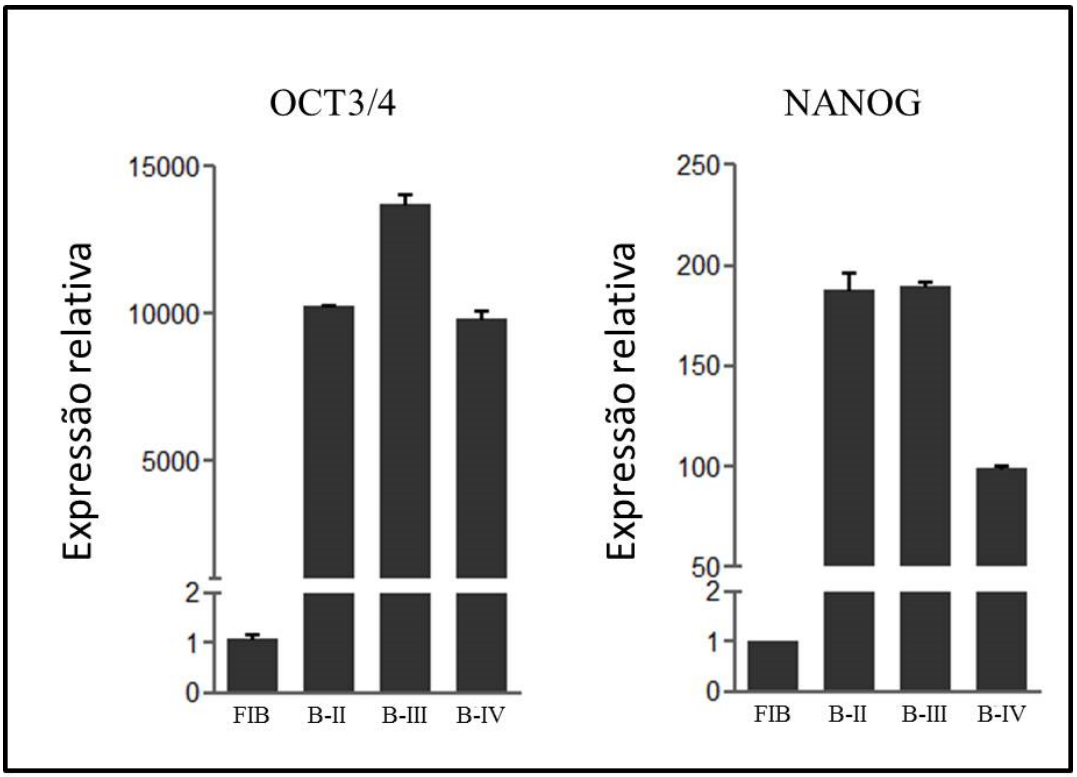

FIGURA 13: Valores de expressão relativa dos fatores de pluripotência OCT3/4 e NANOG nas iPSC de B-II, B-III e B-IV. Os valores foram obtidos através dos ensaios de PCR em tempo real e confirmaram a expressão dos fatores de pluripotência. FIB = fibroblastos.

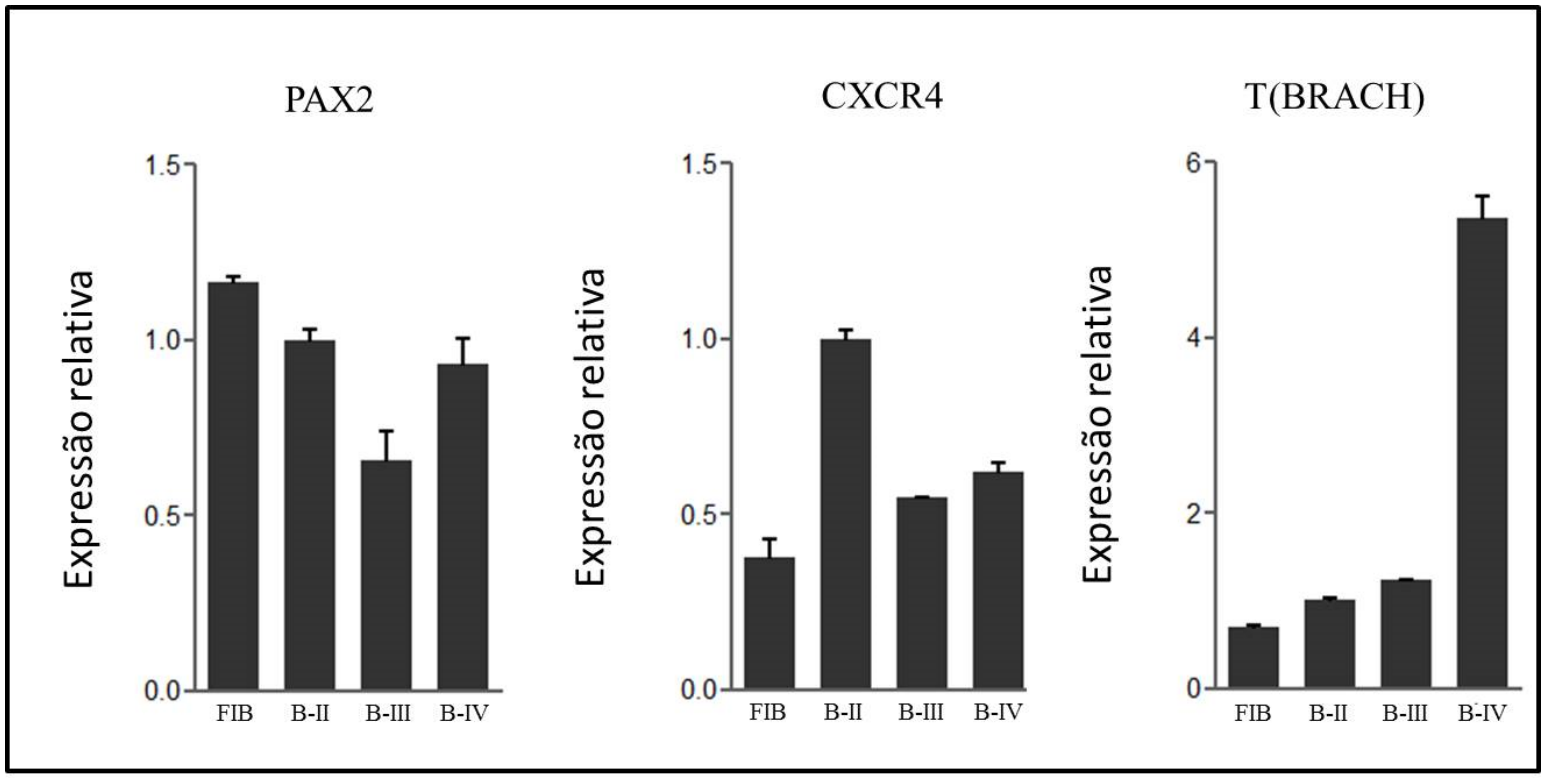

FIGURA 14: Valores de expressão relativa dos fatores de diferenciação em endoderme, ectoderme e mesoderme: PAX2, CXCR4 e BRACH [T(BRACH)], respectivamente. Foi usado o cDNA das iPSC de B-II, B-III e B-IV. Os valores foram obtidos através dos ensaios de PCR em tempo real e a expressão não significativa de nenhum dos fatores de diferenciação indica que as células estão em estágio embrionário.FIB = fibroblastos.

DISCUSSÃO

A ELA é uma afecção neurodegenerativa importante visto que, apesar de ser uma doença considerada rara (1 caso em cada 100.000 pessoas por ano), afeta também as pessoas mais próximas ao paciente (normalmente familiares), que tem que se adaptar para cuidar do doente. Com isso, segundo a AbrELA, 1 em cada 200 pessoas tem um membro familiar acometido pela ELA. No Brasil, acredita-se que a incidência de ELA seja de 2:100.000 pessoas, e no estado de São Paulo, são descritos 800 novos casos por ano(97).

Tratando-se de uma doença majoritariamente do sistema nervoso central, a investigação dos diversos aspectos da ELA em pacientes é bastante restrita. Como a confirmação do diagnóstico dessa patologia leva, de uma forma geral, mais de um ano, obter material dos pacientes em estágios iniciais da doença é extremamente difícil, e a retirada do tecido nervoso afetado na ELA de pacientes ainda vivo é eticamente questionável e perigoso para o paciente. Os problemas para a obtenção de tecidos e células afetadas pela ELA em pacientes dificultam a investigação de possíveis manifestações moleculares durante o aparecimento dos sintomas da doença, prejudicando também a busca para uma terapia que cesse ou regrida a progressão da doença.

Os processos envolvidos na degeneração neuronal na ELA são ainda desconhecidos, porém a descoberta de casos relacionados com mutações genéticas trouxe novas ferramentas para o estudo dessa patologia. O gene *SOD1* foi o primeiro que foi associado à casos familiais da doença, levando ao desenvolvimento do primeiro modelo animal de ELA. O camundongo transgênico portando a mutação G93A na SOD1 é um dos modelos murinos mais utilizados para o estudo desta afecção, pois recapitula diversos aspectos da doença humana. Os modelos animais não humanos são de extrema importância para o avanço das pesquisas acerca da ELA, porém ainda não foi produzido um modelo que apresente todos os sinais clínicos e

moleculares observados em humanos. Além dos modelos animais não expressarem todo o fenótipo da ELA, para simular a afecção os pesquisadores geralmente recorrem à métodos de superexpressão ou silenciamento gênico, tais manipulações não representam fielmente a expressão gênica no ambiente celular do paciente e podem influenciar os resultados obtidos.

Os modelos murinos têm sido muito utilizados para investigar possíveis terapias para pacientes de ELA, porem há uma pequena correlação entre os estudos pré-clínicos em camundongos e os testes terapêuticos em humanos. Essa discordância entre os estudos com modelo murino e os testes em humanos podem ser resultado de problemas na forma de administrar a droga, no estágio de evolução da doença em que a droga é aplicada, na forma de avaliação dos sinais patológicos, na dosagem utilizada no estudo, entre outros. O modelo murino de ELA portando a SOD1h com a mutação G93A é o mais utlizado na investigação de terapias para para a afecção, porém, como já citado anteriormente, esse modelo não é fidedigno à todos os casos da doença em humanos. Portanto. o desenvolvimento de modelos murinos, fiéis ao quadro clínico da doença em humanos, para as outras formas de ELA pode ajudar a expandir o alcance dos testes clínicos.

A tecnologia de produção de iPSC trouxe uma revolução no modo de estudo de doenças genéticas humanas, principalmente para aquelas nas quais os tecidos afetados no paciente são de difícil obtenção, como a ELA. Desde o primeiro modelo de ELA usando iPSC(98), os estudos recorrendo à essa técnica para tentar melhor compreender a doença têm se expandido consideravelmente. Wainger et al., em 2014,(94) desenvolveram linhagens de NM derivados de pacientes com ELA portando mutação patogênica no gene *SOD1*, *FUS* ou *C9ORF72*, e demonstraram a presença de hiperexcitabilidade nestas células, mesmo fenótipo observado nos NM de pacientes. Esse artigo evidencia o grande potencial da utilização das células iPSC em estudos sobre a ELA.

O presente trabalho traz a reprogramação de fibroblastos de um paciente de ELA com a mutação R521H no gene *FUS*, e de seus três irmãos (B-II, B-III e B-IV)

que, até o momento, são clinicamente saudáveis (apesar de B-IV ser portador da mesma mutação de P) A mutação encontrada no paciente já foi descrita anteriormente como patogênica. O supracitado B-IV, atualmente encontra-se com idade muito próxima à de P quando começou a manifestar os sintomas de ELA, e por isso ele talvez ainda esteja em um estágio pré-sintomático. Caso B-IV não desenvolva os sintomas de ELA nos próximos anos ele poderá ser considerado um portador assintomático da mutação no gene *FUS*.

As iPSC derivadas nesse estudo atendem à requisitos para serem chamadas de pluripotentes pois expressam fatores de pluripotência como demonstrado via IF (nas linhagens de todos os indivíduos) e via PCR quantitativo em tempo real (nas linhagens de B-II, B-III e B-IV). (**figuras 8 e 13**). O ensaio de PCR quantitativo em tempo real também mostra que as iPSC analisadas (B-II, BIII e B-IV) não expressam fatores de diferenciação para nenhum dos três folhetos embrionários.

O sequenciamento do éxon 15 do gene *FUS* de todas as linhagens de iPSC deste estudo confirmam a presença da mutação detectada em amostras de sangue de P e de B-IV, e o genótipo selvagem para B-II e B-III.

As iPSC produzidas não foram submetidas à testes para verificar a existência de aberrações cromossômicas, que podem surgir no processo de reprogramação. Recomenda-se que seja feitos um ensaio de MLPA (Multiplex Ligation-dependent Probe Amplification) ou de cariótipo para verificar a existência de anormalidades no material genético das iPSC. Recomenda-se ainda que sejam feitos futuramente ensaios de diferenciação aleatória das iPSC e/ou a injeção das iPSC em camundongos para verificar a formação de teratoma, a fim de atestar a capacidade de diferenciação destas células nos três folhetos germinativos. Somando os testes realizados para validar as características pluripotentes das iPSC com os testes sugeridos acima, será possível afirmar que as linhagens pluripotentes derivadas nesse estudo são adequadas para futura diferenciação neuronal afim de continuar os estudos com ELA.

A proteína FUS é de expressão ubíqua e localiza-se predominantemente no núcleo celular, apesar de também exercer funções no citoplasma. Um dos fenótipos

moleculares mais observados em casos de ELA relacionados com mutações no gene *FUS* é a retenção citoplasmática da proteína FUS. A FUS no citoplasma parece interagir com outras proteínas e com RNA's diversos, e estudos apontam para a ligação dessa proteína com mecanismos de transporte, de controle de tradução de RNA e de degradação de proteínas. (1).

Dormann et al, 2015, (103) expõem a correlação entre o grau de retenção da proteína FUS mutada no citoplasma e a idade de início dos sinais clínicos de ELA. A mutação R521H na proteína FUS causa uma retenção leve da proteína no citoplasma. A **figura** 15 compara imagens de IF marcando para a proteína FUS dos trabalhos de Kwiatkowski et al. (67) e de Vance et al. (68) à imagens produzidas nesse trabalho, e nela é possível notar uma maior concentração de células com marcação citoplasmática de FUS nos indivíduos portadores da mutação R521H (P e B-IV) em relação aos indivíduos com a forma selvagem da proteína.

O trabalho de Murakami e colaboradores (99) demonstram que a proteína FUS alterna entre o estado de gotículas livre de membranas e o estado semelhante à hidrogel, o equilíbrio desta mudança de estados físicos é alterado por mutações relacionadas à ELA, fazendo com que as formas patogênicas da proteína formem agregados estáveis de hidrogel que aparentemente afetam metabolismo proteico. O trabalho de Murakami corrobora o trabalho de Dormann et al. (103) e demonstra que mutações da proteína FUS que causam quadros mais graves da doença tendem a ficar retidos no citoplasma de forma mais intensa. Essa observação provavelmente se deve à menor capacidade das formas patogênicas da proteína em retornar do estado de hidrogel para o estado líquido, ocasionando os agregados citoplasmáticos característicos desta patologia. Aparentemente, quanto mais agressiva é a mutação no gene *FUS*, menor é a capacidade da proteína se converter do estado de hidrogel para o estado de gotículas. A proteína FUS na forma de hidrogel interage de maneira anormal com outras proteínas e deve alterar o funcionamento de FUS tanto no núcleo celular quanto no citoplasma, e possívelmente esse comportamento da FUS mutada deve ser responsável pelo desregulação celular que leva os NM à morte na ELA.

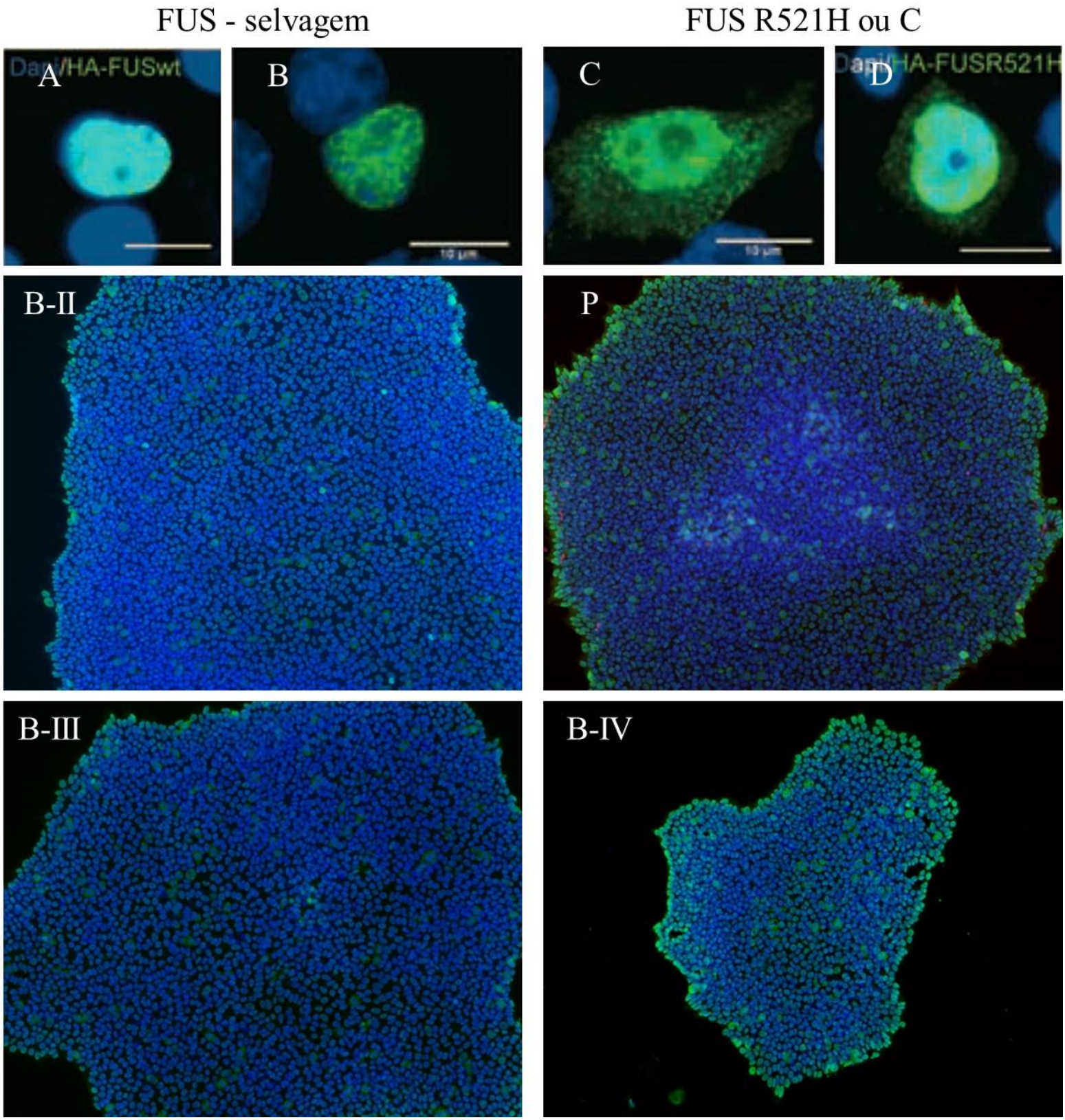

FIGURA 15: Comparação da posição da proteína FUS na forma selvagem ou portando mutação R521H ou C. **A** e **B** são, respectivamente, neurônios corticais de ratos e células SKNAS expressando a proteína FUS selvagem. **C** e **D** são, respectivamente, células SKNAS e neurônios corticais de ratos expressando a proteína FUS com a mutação R521C, em **C**, e R521H, em **D**. **P**, **B-II**, **B-III** e **B-IV** são iPSC dos respectivos indivíduos analisados nesse estudo. As imagens mostram a proteína FUS marcada em verde, e o DNA marcado em azul, por DAPI. A proteína FUS aparece mais retida no citoplasma das iPSC de **P** e de **B-IV**. As imagens **A** e **D** foram retiradas de Vance et al. (68) e as imagens **B** e **C** foram retiradas de Kwiatkowski et al. (67). Imagens **P**, **B-II**, **B-III** e **B-IV** feitas utilizando o aumento de 20X , onde a barra branca mede 100 μm. A barra branca mede 10 μm nas figuras **A**, **B**, **C** e **D**.

A retenção citoplasmática da proteína FUS observada entre o P e B-IV faz emergir o questionamento sobre qual a fonte da diferença fenotípica observada entre ambos os estudados. Se B-IV apresenta sinais moleculares característicos de ELA ligada à mutações no *FUS* assim como seu irmaão afetado, quais são os mecanismos que fazem com que ele seja clinicamente saudável? Entender como os sinais moleculares da doença se tornam sinais clínicos pode nos ajudar a prever e a prevenir os fatores responsáveis pelo início dos sintomas da doença e pela progressão da mesma.

O presente estudo traz dois indivíduos, P e B-IV, portadores da mutação R521H no FUS, com sinais moleculares semelhantes, porém com fenótipos clínicos totalmente discordantes. O trabalho baseado na análise iPSC e fibroblastos não oferece o ambiente celular ideal para a análise destes dados. As iPSC são células que simulam células embrionárias através de um processo de reinício celular e, apesar de estarmos observando células do próprio paciente e do portador da mutação (B-IV), não são o tipo celular afetado pela ELA. A diferenciação das iPSC deste estudo em NM é essencial para confirmar os resultados obtidos e para buscar mais informações acerca da discordância entre os sinais moleculares e clínicos do caso de ELA aqui apresentado. Visto que as células nervosas são afetadas na ELA, elas oferecem um ambiente celular muito mais próximo do observado no paciente, sendo os NM derivados das iPSC dos pacientes os mais indicados para estudos moleculares futuros acerca da doença nessa família e em casos relacionados.

A hiperexcitabilidade de NM é um sinal patológico típico da ELA, e o trabalho de Wainger e colaboradores(94) demonstra estre fenótipo em NM derivados de iPSC de pacientes de ELA. É sugerido que se façam ensaios para verificar a ocorrência da hiperexcitabilidade nos NM derivados de iPSC dos indivíduos deste estudo, assim como foi verificado no estudo citado.

É indicado usar NM derivados das iPSC produzidas nesse trabalho em ensaios verificando alterações na expressão gênica, o que pode trazer informações importantes. Conforme exposto na introdução, é sabido que mutações no gene *FUS*

levam à alterações dos genes transcritos(52,54,62), porém como os trabalhos geralmente recorrem à modelos celulares algumas perturbações podem ser causadas pela manipulação genética e pela variação genômica entre as células utilizadas. Devido ao fato de todos os indivíduos analisados nesse estudo serem irmãos, filhos de um único casal, eles possuem uma semelhança genômica muito grande e a verificação de genes diferencialmente expressos entre as linhagens pode evidenciar com maior clareza transcritos relacionados com a afecção, visto que as diferenças oriundas da variação populacional estão amenizadas. A diferença de expressão gênica observada nos casos de mutação do gene *FUS* pode ser estar ligada à alterações nas gemas nucleares advindas da proteína mutada(24), esse fato torna interessante analisar futuramente os metabolismo destas estruturas nucleares e compará-los entre os irmãos e entre casos externos de ELA.

O acompanhamento de B-IV ao longo dos próximos anos deve ser feito para verificar se o mesmo pode vir a manifestar os sintomas da ELA. Caso isso ocorra, seria interessante a nova coleta de material biológico e de dados clínicos para acompanhar a progressão da afecção. Caso B-IV não desenvolva a doença, ele passa a ser considerado assintomático. Neste caso, a investigação sobre quais mecanismos moleculares podem ser responsáveis por impedir o aparecimento dos sinais clínicos da doença nesse indivíduo pode trazer grandes avanços para a compreensão da patogênese da doença e para a tentativa de se encontrar um tratamento que cesse ou reverta a progressão da doença.

Por fim, este trabalho prepara um rico material para futuros ensaios sobre a ELA, e levanta indícios de similaridade molecular entre indivíduos possuindo a mesma mutação, porém fenótipos clínicos discordantes. A diferenciação das iPSC em NM deve ser o próximo passo para se avançar no estudo da ELA no contexto apresentado nessa família. Conforme discutido anteriormente, os mecanismos de formação de gemas nucleares e de expressão gênica podem ser promissoras áreas de investigação, assim como as vias de degradação celular. Diversos artigos apontam

para alterações nessas vias metabólicas como sinais patológicos de ELA comum à grande parte dos casos da doença.

CONCLUSÃO

I. As células iPSC desenvolvidas nesse trabalho expressam os fatores de pluripotência necessários para serem caracterizadas como tal.

II. As linhagens de células pluripotentes resultantes desse trabalho mantém o genótipo observado no DNA extraído do sangue dos indivíduos participantes.

III. Os ensaios de imunoflorescência corroboram os dados da literatura que demonstram que a localização da proteína FUS é ligeiramente maior no citoplasma de células portadoras da mutação R521H no gene *FUS*.

IV. O paciente e B-IV apresentam posicionamento celular da proteína FUS semelhantes entre si, porém são discordantes quanto ao fenótipo clínico. Ainda não é possível classificar B-IV como assintomático ou pré-sintomático.

V. As linhagens de iPSC geradas nesse trabalho podem ser utilizadas como ferramenta para estudos com essa família e com outros casos de ELA.

RESUMO

A esclerose lateral amiotrófica (ELA) é uma doença neurodegenerativa, progressiva de início tardio que afeta principalmente os neurônios motores (NM). As causas que levam os NM à morte são variadas e ainda sendo investigadas. A descoberta de alterações genéticas como uma possível causa de ELA deu início à uma nova era na investigação desta afecção. Atualmente existem mais de 30 genes associados com a doença, entre eles o *FUS*, um gene que frequentemente aparece mutado em casos familiais da doença. A proteína FUS normalmente se localiza predominantemente no núcleo, mas na maioria dos casos de mutações na FUS relacionadas à ELA, ela aparece retida no citoplasma. O presente estudo traz um paciente de ELA (P) portando a mutação p.R521H no gene *FUS* e três de seus irmãos (dos quais um é portador da mutação e não apresnta sinais clínicos de ELA, e os outros dois não apresentam mutações no *FUS*) dos quais foram obtidas amostras de sangue e biópsia de pele. O DNA extraído das amostras de sangue, foi submetido ao sequenciamento do tipo Sanger para verificar a presença, ou ausência, da mutação R521H na FUS. A partir dos fibroblastos dos participantes, foram derivadas linhagens de células tronco pluripotentes induzidas (iPSC). As iPSC produzidas passaram por ensaios a fim de indicar o estado de pluripotência e de indiferenciação destas linhagens. Nós investigamos a posição da proteína FUS nas linhagens de iPSC e de fibroblastos e há evidências que, assim como descrito na literatura, a proteína FUS aparece retida no citoplasma das linhagens do paciente e de seu irmão portador da mutação. Desta forma, o presente estudo associa dois irmãos com quadros clínicos discordantes mas que apresentam a mesma mutação e sinais moleculares patológicos semelhantes. As linhagens de iPSC obtidas são um rico material para o uso em pesquisas futuras sobre a ELA.

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a late onset, progressive, neurodegenerative disease that primarily affects motor neurons (MNs). The causes behind motor neuron death are diverse and still under investigation. The discovery of genetic alterations as possible causes of ALS initiated a new era for ALS research. There are currently over 30 genes associated with the disease, among which is *FUS*, one of the most frequently mutated in familial cases. The FUS protein is predominantly located in the nucleus, but in most of the ALS-related FUS mutations this protein is dislocated to the cytoplasm. The present work investigates the molecular aspects of a specific FUS mutation, p.R521H. An ALS patient (P) harboring the mutation and three siblings (of which one is a non-affected carrier and two present no mutations in FUS) were analyzed using blood samples and skin biopsies. We extracted DNA from blood samples and submitted it to Sanger sequencing for confirmation of the presence, or absence, of the R521H FUS mutation. The fibroblasts obtained from these biopsies were used for iPSC derivation. Assays were performed to confirm the undifferentiated state and pluripotency for the four strains obtained. We investigated the FUS location in these strains, and there is evidence for FUS retention in the cytoplasm of cells harboring the mutation (as seen in recent literature). Thus, this work associates two siblings with the same pathogenic mutation, showing the same molecular pathological signal but with discording clinical phenotypes. The iPSC strains obtained here are a valuable resource for further ALS investigation.

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ANEXOS: ARTIGOS PUBLICADOS

Cytometry PART A
Journal of the International Society for Advancement of Cytometry

Stem Cells for Amyotrophic Lateral Sclerosis Modeling and Therapy: Myth or Fact?

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Received 8 July 2014; Accepted 28 December 2014

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Published online 00 Month 2015 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.22630

1. Abstract

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease whose pathophysiology is poorly understood. Aiming to better understand the cause of motor neuron death, the use of experimental cell-based models increased significantly over the past years. In this scenario, much knowledge has been generated from the study of motor neurons derived from embryonic stem cells and induced pluripotent stem cells. These methods, however, have advantages and disadvantages, which must be balanced on experimental design. Preclinical studies provide valuable information, making it possible to combine diverse methods to build an expanded knowledge of ALS patho-physiology. In addition to using stem cells as experimental models for understanding disease mechanism, these cells had been quoted for therapy in ALS. Despite ethical issues involved in its use, cell therapy with neural stem cells stands out. A phase I clini-cal trial was recently completed and a phase II is on its way, attesting the method's safety. In another approach, mesenchymal stromal cells capable of releasing neuroregu-latory and anti-inflammatory factors have also been listed as candidates for cell therapy for ALS, and have been admitted as safe in a phase I trial. Despite recent advances, application of stem cells as an actual therapy for ALS patients is still in debate. Here, we discuss how stem cells have been useful in modeling ALS and address critical topics concerning their therapeutic use, such as administration protocols, injection site, cell type to be administered, type of transplantation (autologous vs. allogeneic) among other issues with particular implications for ALS therapy.

Advancement of Cytometry

Key terms: amyotrophic lateral sclerosis; stem cells; cell therapy; iPSC; ESC; MSC

Introduction

Amyotrophic Lateral Sclerosis (ALS), or Lou Gherig's Disease, is a neurodegenerative disorder characterized by the death of motor neurons, with symptoms includ-ing fasciculation, spasticity, muscle atrophy, and weakness. These signs emerge when axons retract, and denervation of muscles occurs. Initially, the retraction is compensated by more resistant neurons, which sprout and reinnervate some muscles; however, this mechanism eventually fails, and the neuronal cell bodies become visibly abnormal and die (1). In addition to motor neuron symptoms, 15% of patients pres-ent also Frontotemporal Dementia (ALS-FTD), with the neurons in the prefrontal and temporal cortexes being affected in the disease (2). The criteria used for diagno-sis are mainly clinical, as reported in the El Escorial, created in 1994 (3) and revised in 2000 (4). Electromyography data is now considered of equal importance as clinical features, and the signs required for an ALS diagnosis are revised in the Awaji-Shima electrodiagnostic criteria (5).

Most cases are characterized by an adult onset of symptoms, usually in the fourth decade of life. Some juvenile cases have, however, been described, including patients with mutations in ALS2 (6), SPG11 (7), SIGMAR1 (8), genes, and SETX (9).

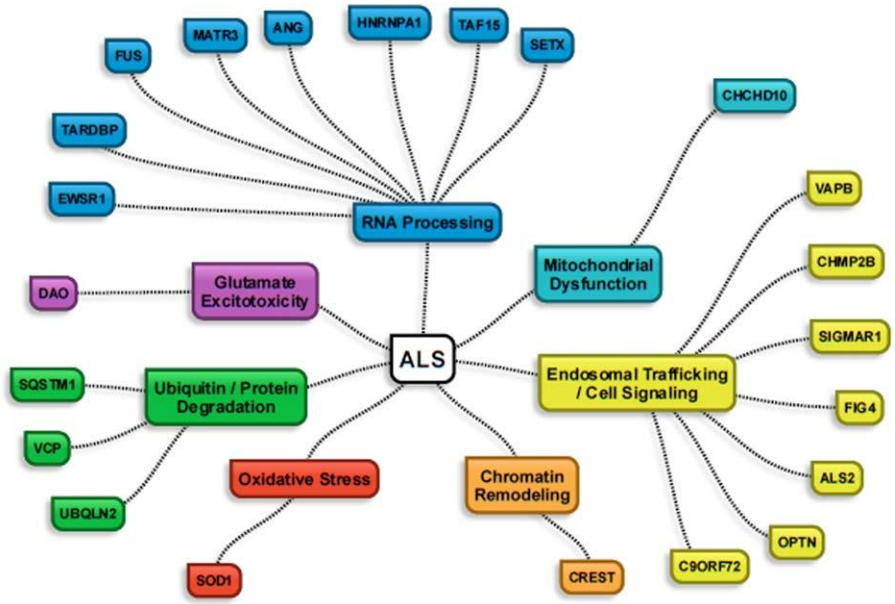

Figure 1. Genes currently known as associated with ALS classified by common molecular pathways involved in the disease pathophysiol-ogy. Adapted from Refs. (1) and (15). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The average survival ranges from 3 to 5 years after onset, but there is a great interfamilial and intrafamilial variability between patients, which suggests the existence of important disease modifiers that contribute to disease progression. Among them, the ephrin receptor EphA4 was described as one of the main modifiers in humans; the higher the expres-sion level of this receptor, the more vulnerable the motor neu-rons (10).

Another observed correlation with disease progression involves T-regulatory cells (T-regs), which are critically involved in suppressing the inflammation induced by neuro-toxic T-lymphocytes and microglia/macrophages in ALS. The analysis of affected patients' blood through flow cytometry (FC) recently showed that CD41CD25^{High} regulatory T-regs are reduced in patients with a rapid disease progression. Also, FoxP3 (a transcription factor required for T-reg function) lev-els were shown to be indicative of progression rates and thus could be used to identify rapidly progressing cases (11).

ALS is mainly classified into two groups: FALS (Familial ALS), for the inherited forms of the disease, and SALS for the sporadic cases, in which there are no affected relatives and where in the majority of cases it is not possible to identify a single causative agent. The familial cases have their historical importance in causative gene identification since through these discoveries much has been uncovered about ALS pathogenesis.

Around 33 genes or chromosomal regions have been linked to ALS, providing important clues on the pathophysio-logical mechanisms of the disease (ALSoD - http://alsod.iop. kcl.ac.uk). Recent studies using high-throughput technologies such as exome sequencing have identified not only novel mutations in these known ALS-causing genes, but also addi-tional genetic causes of ALS. A search for de novo mutations in SALS, for instance, revealed mutations in CREST (12), a protein that associates with FUS; deletions in SPG11, a gene associated with hereditary spastic paraplegia, have been found in familial ALS cases as well (13), highlighting the importance of the elucidation of disease modifiers; and last, but not least, mutations in MATR3, a DNA/RNA-binding protein that interacts with TDP-43, have been found in FALS cases (14).

The main pathways in which ALS-associated genes are involved are oxidative stress, RNA processing, endossomal trafficking, cell signaling, glutamate excitotoxicity, proteosta-sis, and cytoskeleton composition ((15); Fig. 1). However, lit-tle is understood about how these pathways converge into the ALS phenotype. Even though they are described as causative for ALS, a large amount of ALS-causing genes are also respon-sible for other types of neurodegenerative diseases; in other words, the same mutations are able to lead to various pheno-types. Such clinical variability suggests that ALS is indeed a syndrome, and not one single disease (1). Given the fact that the most frequent gene alteration in ALS, C9ORF72, is respon-sible for only _33% of familial cases and 8% of sporadic cases (16), environmental conditions could play an important role for disease development. Well-designed cohort studies are dif-ficult and costly, but could reveal important information on potential risk factors.

Despite the increasing amount of genes associated with ALS and the consequent elucidation of molecular pathways, other pathophysiological mechanisms contributing to the ALS phenotype are poorly understood. Besides the known neurodegeneration, recent findings also suggest the involve-ment of an aberrant innate immune system. The percentages of CCR2 and CD62L on CD141CD16- classical monocytes were significantly lower in ALS patients than in healthy con-trols (17).

This complex profile hinders progress for important clin-ical trials, which have been, in most cases, unsuccessful. How-ever, stem cells seem to be the new light at the end of the tunnel, providing important models for molecular pathway studies, drug screening, and even cell therapy, with two clini-cal trials currently in phase II: one by NeuralStem (18,19), and another by BrainStorm (NCT01051882).

In this review, we will discuss the importance of distinct stem cell types for disease modeling, summarizing new data from embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC) models, their uses, advantages and disadvan-tages, and how FC has significantly contributed to these cell-based in vitro studies. We also review and discuss important issues to be addressed on possibilities of cell therapy, including neural and mesenchymal stem cells.

FC as an Ally for Cellular and Molecular Studies

Among all available techniques for cellular marker evalu-ation, FC plays a major role in many aspects of cell-based ALS studies. It allows the search of characteristic ALS markers, such as mentioned above for CD4 lymphocytes and FoxP3, and may provide important insight into patient prognosis. This can help not only the design of a more personalized ther-apeutic approach, but also selection of patients for clinical tri-als based on similar progression rate and symptoms. A recent work from Marrali et al. (20) shows that NOX2 activity, eval-uated from fresh blood assayed through a special FC protocol, is a modifier of ALS survival. This kind of assay is of special interest for clinical trials due to the accessibility of the studied tissue and its applicability in a high throughput screening. Despite the motor neurons being the main affected cells in ALS, as described above, other cell types can take part in this disease, showing the need for a multicellular evaluation. In this case, not only the cell surface markers are the target of those studies, but also the evaluation of intracellular patterns. As presented by Pickles et al. (21) depositions of protein aggregates in mitochondria is age dependent in mouse models and are found in the lymphoblast fraction of ALS1 patients.

Regarding cell therapy, one great promise is the use of neural stem cells (NSCs) derived from the patient's own iPSC. By sorting the cells of interest, one can select and inject only the desired cell type, and exclude possible tumorigenic (or other harmful) cells.

In short, the importance of FC in this field of study, con-tributing not only to the understanding of ALS's pathophysio-logical mechanisms, but also to the elucidation of possible treatments will be discussed in this review as we describe cel-lular models and proposed cell therapies.

Cell-Based Models for Understanding ALS

Many transgenic murine models for ALS studies have been described, providing important information on the mechanisms behind motor neuron death. Even though they are very useful and informative, models that mimic human diseases are, biologically speaking, still very limited, since they present very distinct disease progression and drug responses. Consequently, to understand the precise molecular pathways behind ALS pathogenesis and the effects of different drugs and other potential therapies, recent efforts have been focus-ing on human cell-based disease models derived from pluri-potent stem cells. The difficulties of reaching the cells affected in neurological and psychiatric diseases, for studies in vitro or in vivo, highlights the importance of cell-based models. Pluri-potent stem cells provide models that are more flexible since they may generate different types of specialized neurons as well as supporting glial cells. Some disadvantages, however, accompany different sources of pluripotent stem cells. The pros and cons of ESCs and iPSCs as disease models are dis-cussed below.

Embryonic Stem Cells

ESCs, first isolated in 1998 by Thomson (22), have the capacity to self-renew indefinitely in culture while maintain-ing a potential to generate any of the hundreds of cell types in the body. The same plasticity that permits ESCs to generate so many different cell types also makes their control quite a chal-lenge (23). Many important and decisive differentiation fac-tors have been discovered, and simple protocols for ES differentiation into motor neurons are available. The first suc-cessful differentiation method for mouse ESC-derived motor neurons was described in 2002 (24), and the first protocol for human cells in 2005 (25), all of which turned possible due to previous discoveries of important factors such as Sonic hedge-hog (26), Shh factor, and the elucidation of the sequence of differentiation events that culminate in different neuronal populations (27). Later in this same year, in a well-designed study, a human ES lineage was transfected with plasmids encoding GFP placed under the control of a MN-specific enhancer within the 5^{0}-regulatory region of the gene encoding the transcription factor Hb9. After treatment with Shh and retinoic acid (RA), activated Hb9-driven GFP expression per-mitted the isolation of MN by fluorescence-activated cell sort-ing, excluding undifferentiated cells and thus making this differentiation protocol a more reliable process (28).

ESCs were firstly used to generate animal models. Clem-ent et al., used mouse ESCs to create chimeric mice composed of mixtures of normal cells and cells that express a human mutant SOD1 polypeptide. Interestingly, non neuronal cells that did not express mutant SOD1 presented delayed degener-ation and significantly extended survival of mutant-expressing motor neurons (29).

The differentiation of ES cells into motor neurons or other cell types such as glial cells have also been used as important models for ALS studies, elucidating the main dis-ease mechanisms (cell-autonomous and noncell-autonomous) involved in motor neuron death.

In 2008, two important studies were developed using ES-derived motor neurons. Di Giorgio et al. showed that other cells besides motor neurons play an important role in ALS dis-ease progression. Human motor neurons were cocultured with mice glial cells carrying the G93A mutation in the SOD1 gene, and were shown to be sensitive to the toxic, noncell-autonomous effect of these cells (30). Marchetto et al.

published a similar work simultaneously, coculturing human ESC-derived motor neurons with astrocytes carrying the G37R mutation in SOD1; this coculture caused a 50% reduc-tion in the number of motor neurons when compared with controls. The toxicity of these astrocytes is believed to be gen-erated by an increased inflammatory response, and also the activation of NOX2, consequently producing oxygen radicals. The addition of antioxidants was able to reverse this process and specifically, apocynin could rescue the motor neuron sur-vival in the presence of SOD1 mutant astrocytes (31).

In these studies, glial cells carried SOD1 mutations. Since most ALS cases are sporadic, Haidet-Phillips et al. sought to study the effects of FALS and SALS-derived astrocytes cocul-tured with motor neurons. These astrocytes were derived from neural progenitor cells (NPCs) obtained from postmor-tem tissues, and cocultured with mouse ESC-derived motor neurons. All patient-derived astrocytes showed great toxicity to these motor neurons, selectively killing them in this cocul-ture model system (32).

Supporting the idea of a toxic environment contributing to this motor neuron disease, the work published by Lopez_-Gonz_alez et al. in 2009 showed that transplanting mouse ESC-derived motor neurons into an ALS mouse SOD1 model (car-rying the G93A mutation) was not beneficial. Although the mice showed an initial recovery, the engrafted motor neurons died shortly after transplant, and the disease progression resumed its course (33).

Even though strong noncell autonomous effects are reported in diverse studies, cell autonomous effects also take place in ALS. Wada et al. (2008) described a human ESC-based model for ALS, in this work, the differentiated motor neurons expressed the mutated form of SOD1, and were not initially cocultured with other neuronal cell types. Interest-ingly, the G93A mutation does not affect the differentiation process. What the authors observed was a significantly higher death rate among terminally differentiated motor neurons, showing an intrinsic mechanism (cell-autonomous) behind motor neuron death in ALS. This work also involved the coculture of these differentiated cells with conditionated medium of astrocytes carrying the same mutation, confirming also a noncell-autonomous astrocyte-dependent cell survival mechanism (34).

ESC-based models can be of great importance not only for the understanding of pathophysiological mechanisms behind ALS, but also for the screening of potential therapeutic molecules. Recently Yang et al. (2013) have derived motor neurons from mouse ESCs, and performed a small-molecule screening comparing survival of wild-type motor neurons and those carrying a human transgene with the G93A SOD1 muta-tion. A compound named kenpaullone stood out, not only by keeping motor neurons alive for several weeks (in the absence of added trophic support), but also by maintaining neuritic processes, synapses, and normal electrophysiological charac-teristics (35). Olesoxime and Dexpramipexole (36,37), two drugs that recently failed a phase III trial, were also tested in this study, and were shown to be less effective. These two drugs had never been tested in in vitro models, only in vivo, which supports the concept that both types of study need to be performed to obtain a deeper knowledge of the drug's ther-apeutic potential, and avoid clinical trial failure.

In summary, cell models based on ES cells have shown that the mere substitution of motor neurons as a cell therapy would not be enough to circumvent the neurodegeneration; besides the cell-autonomous mechanisms, the toxic environ-ment, provided by glial cells, is harmful to motor neurons, and contribute to their death. Even though these findings rep-resent a great advance in ALS understanding, they have been limited to only one gene, SOD1. This disease involves multiple pathways, and most likely has multiple etiologies. Even in cases of ALS in which the causative mutation is unknown, proteins such as TDP-43, FUS, and others, are found in cyto-plasmic aggregates, suggesting an underlying convergence of cellular processes and pathological effects (38). ESC-based models for other frequent mutations, especially the expan-sions on C9ORF72, may brighten our knowledge as to other potential mechanisms to be aimed for therapy.

Despite these many advantages, the ethical issue related to the destruction of embryos still remains. Although ESC may be obtained for disease modeling and drug screening, a large-scale use of ESC still seems hardly feasible for treatment of all ALS patients

Induced Pluripotent Stem Cells

The development of iPSC in 2006 allowed remarkable changes in stem cell science (39). It made possible to obtain pluripotent stem cells directly from a patient's adult cells [mainly fibroblasts, although other sources have been used (40)], bypassing the large ethical issues behind ESCs and embryo destruction (39) and allowing the study of patient-specific ALS mutations, without the need for genetic engineer-ing cells. Although ESCs with ALS mutations could be obtained through preimplantation diagnosis in familial cases with known mutations, only iPSC obtained from patients allow the analysis of sporadic cases. Since then, many impor-tant discoveries with iPSC-derived motor neurons and glial cells have enhanced our (still currently little) understanding of ALS pathophysiology.

The diverse stages of iPSC differentiation into mature motor neurons are well characterized. After iPSC establish-ment, cells are usually induced to form embryonic bodies and subsequently neural precursor cells (NPCs). Factors such as Shh and RA are used to induce differentiation into mature motor neurons which can be distinguished by the expression of some markers such as Hb9 (Fig. 2).

Somatic cell reprogramming is a stressful process, since it goes against the natural course of a cell's life. The iPSCs gener-ated carry with them imprinting marks from their tissue of origin, and consequently present some altered genetic activity due to the reprogramming process. This possible genetic vari-ation raises concerns about the validity of these models. How-ever, researchers have shown that there is great similarity between iPSCs and ESCs, demonstrating telomere renewal during cell reprogramming into iPSCs and telomere shorten-ing on differentiation into somatic cells (41), teratoma

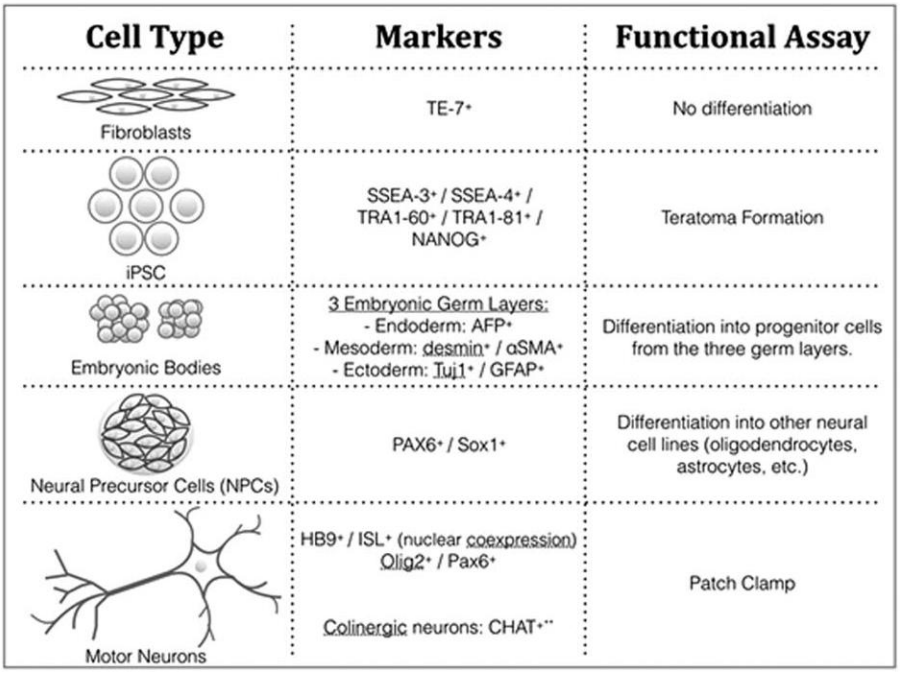

Figure 2. Characterization of cell type, markers, and functional assay of the stages of iPSCs differentiation into motor neurons. Adapted from Refs. (46,151, and 152).

formation, the possibility of derivation into the three kinds of embryonic tissues, epigenetic marks, and transcripts levels (42,43). This similarity suggests that iPSC cells could poten-tially be considered patient-specific ES cells, and consequently be used without rejection, and eliminate ethical issues.

Another important aspect to consider in the process of iPSC formation is the clonal variation inside iPSC strains, especially when the reprogramming technique used is integra-tive (44). Recently a shRNA mediated knockdown of the Mbd3 protein was reported (a regulator of gene expression and chromatin remodeling activities (45), generating a highly efficient, nonintegrative method for mouse cells. This repre-sents an important breakthrough which eliminates many important discussed issues. However, this strategy must be tested on human cells as well. The main techniques used for reprogramming somatic cells (e.g., fibroblasts) into a pluripo-tent state, and their respective reprogramming efficiency, are demonstrated in Figure 3.

The doors opened by iPSC models have brought many interesting discoveries for ALS. Dimos et al. (2008) developed the first strain of ALS-related iPSC. The pluripotent cells derived from an ALS patient's fibroblasts were used to obtain motor neurons with a SOD1 mutation (46). However, they could not identify any phenotype studying those cells. After-ward, an ALS (47) model developed in our laboratory by Mitne-Neto et al. (48), based on ALS8 patients' cells, has dem-onstrated that there is a reduction on the VAPB protein levels along motor neuron differentiation from iPSCs. These results complement other studies that show VAPB reduced levels in SALS patients and in SOD1 mice (49,50). These and other findings (51,52) strengthen the evidences that iPSC-derived motor neurons can recapitulate the disease phenotype. Inter-estingly, in this model the typical cytoplasmic inclusions found in overexpression VAPB systems or in patients carrying other mutations such as TARDBP (53,54) was not observed. Additionally, patient and control iPSC-derived motor neurons showed perinuclear localization of VAPB (Fig. 4), suggesting that late-onset disease models might not show a full pheno-type in these early stages (discussed below). Studies using MNs derived from iPSCs of ALS patients with C9ORF72 hex-anucleotide expansion, have correlated many aspects seen in vivo and in vitro as neuronal hyperexcitability (55). Besides the identification of intranuclear RNA foci, Sareen et al. (51) showed that antisense oligonucleotides against the expanded transcript could improve the expression profile of mutant cells.

Cocultivation of mutation-carrying astrocytes and motor neurons was also investigated. Serio et al. (2013) showed that, while astrocytes derived from ES cells and carrying a SOD1 mutation showed a toxic effect on motor neurons (previously discussed; (34)), astrocytes derived from iPSCs carrying a TDP-43 mutation presented no toxicity for patient iPSC-derived motor neurons (56). The authors reported that the astrocytes derived from iPSC of patients with ALS carrying a TDP-43 mutation die earlier than control iPSC derived astro-cytes. The different results observed in cellular and animal models show a clear necessity of understanding the ALS pathophysiology in deeper levels (56,57).

Some of the apparently conflicting results that arise when using iPSCs-derived models for ALS might be due to the fact that ALS is a late-onset disease, usually occurring between the fourth and fifth decades of life. iPSC originate from an

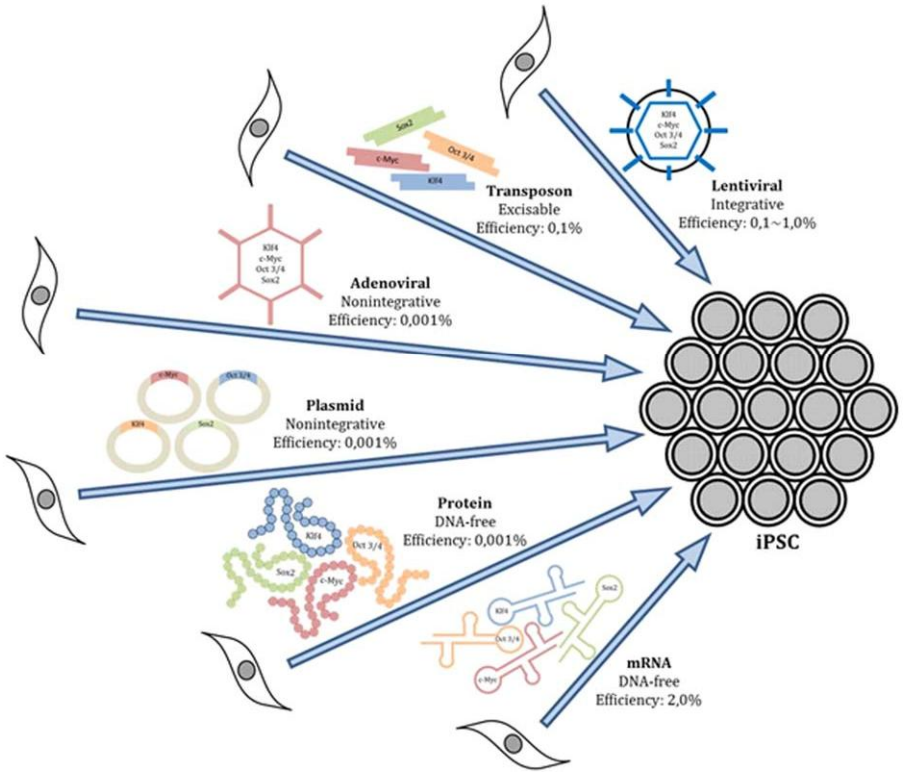

Figure 3. Main reprogramming strategies to transform somatic cells into iPSC, classified as integrative, excisable, nonintegrative, or DNA-free reprogramming vectors. Efficiency of transformation is also shown. Copyright CHS Press. Reproduced with permission (Ref 153).

"identity reset," taking the cells back to an embryonic stage. Given the short life span of motor neurons in vitro, this may disguise some of the signs observed in tissues from affected individuals and animal models that would appear afterwards. One possible solution to this issue is to graft motor neurons or even neuronal progenitor cells derived from iPSC in mice models, which could extend the survival of these cells; follow-ing these steps, a late recovery of the grafted cells from the mice might allow the visualization of ALS-related signs only seen in in vivo models and postmortem patient tissues. Another interesting (and possibly, more efficacious) strategy to induce aging-related features in iPSC-derived lineages is to

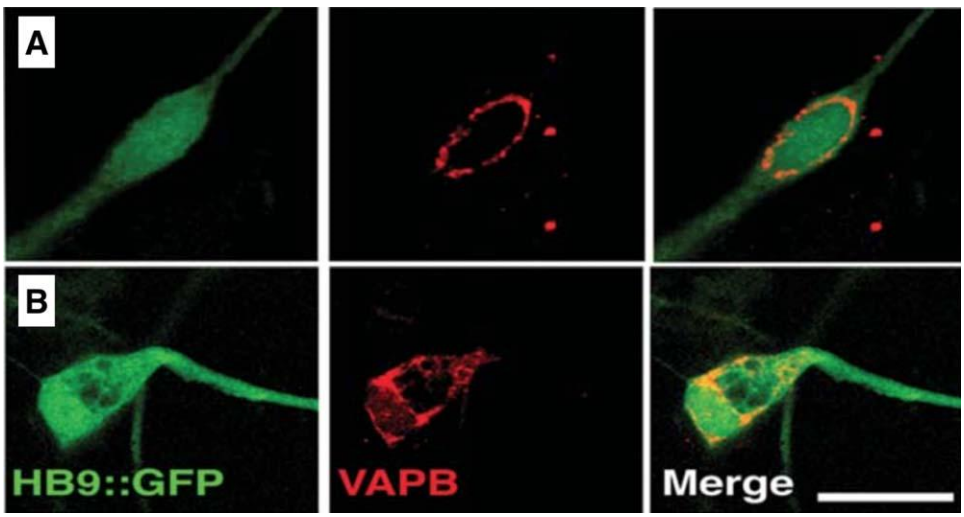

Figure 4. VAPB localization on iPSCs derived motor neurons (A) Perinuclear VAPB distribution on a GFP-positive motor neuron derived from a control iPSC clone and (H) from an ALS8. Bar 520 mm (48). Copyright Oxford University Press. Reproduced with permission (Ref 48).

express progerin, a truncated form of Lamin A associated with Progeria (premature aging). This was recently reported for dopaminergic neurons used as a model for Parkinson's disease generating many physiological signatures of the disease (58).

The progress in iPSC is currently extending to drug screenings. Recently, an iPSC-derived ALS model was gener-ated from fibroblasts of patients carrying a mutation in the TARDBP gene (52). The resulting motor neurons showed many correlated aspects of the disease, including shorter neu-rites and a decrease in neurofilament mRNA levels. These motor neurons were also used for testing the efficacy of treat-ment with anacardic acid. An overall attenuation of the phe-notype occurred and this result is most likely linked to the resulting down-regulation of TARDBP expression after drug administration (52).

Another recent iPSCs study focused on electrophysiologi-cal alterations observed in motor neurons derived from patients with mutations in SOD1, FUS, and C9ORF72 (55). These cells presented a neuronal hiperexcitability that is usu-ally observed in these ALS forms in vivo and in vitro. In this same work, the authors used retigabine (a drug already used as an anticonvulsant) to mitigate the disease-related signs in the three types of generated motor neurons, making it so far the study with the broadest drug screening in an iPSC-based model of ALS.

iPSCs could also be used to better understand the molec-ular pathways involved in sporadic ALS cases, which are the vast majority. Recently, iPSC-derived motor neurons derived from SALS patients were treated with 1757 different com-pounds in a high-throughput drug screening. In 20% of cases, MNs showed a de novo TDP-43 aggregation, and a corre-spondent postmortem tissue obtained from one of the patients validated this molecular phenotype. These TDP-43 cells were then used for drug screening, and some FDA-approved drugs such as Digoxin ameliorated this particular phenotype (59). With these high throughput screening advanced technology, and with an ever increasing efficiency of iPSC generation and differentiation, a personalized medicine approach for sporadic patients could become a real possibility. Patient MNs could be derived in vitro, analyzed for ALS hall-mark phenotypes, and consequently submitted to a drug screening that would reveal a possible effective treatment, which in its turn would hopefully stop disease progression.

Cell Therapy

Stem cells have been considered for therapy purposes in a number of different diseases for which no efficient treatments are available. For ALS therapy, in particular, the most studied types of cells are NSC and mesenchymal stromal cells (MSC).

Neural Stem Cells NSC

One of the most actively studied cells for therapy in ALS are the NSCs. When dividing, these cells give rise to other NSCs and/or progenitor cells which progressively differentiate into neurons, astrocytes, or oligodendrocytes (60). They are characterized by the expression of markers such as Sox2, GFAP, Nestin, and Musashin 1 and 2, but no specific combi-nation has been found to definitely distinguish NSCs from NPCs (78).

A great number of studies evaluating the therapeutic potential of NSCs in ALS models were performed (61,62). Recently, a well designed meta-analysis using the SOD1 (G93A) transgenic mouse model was performed by a consor-tium of 11 independent ALS investigators. In these experi-ments, transplanted NSCs (both mouse and human) were capable of slowing the onset and the progression of clinical signs. Prolonged survival was also seen in ALS mice. The ben-eficial effects of transplanted NSCs were mediated by a variety of processes, including the cells' ability to produce trophic fac-tors, preserve neuromuscular function, and reduce astrogliosis and inflammation, which differs from the primary hypothesis of motor neuron regeneration (63).

The NSCs have also been used in a recent phase I clinical trial by NeuralStem, completed in 2012. A total of 12 patients were divided in two groups receiving 5 unilateral or 5 bilateral (10 total injections) each, with _10.000 cells per injection at the lumbar level. These patients were followed for 18 months after treatment, and there was no evidence of disease progres-sion due to the intervention (18). The cell delivery apparatus was patented by NeuralStem. In this delivery procedure, a guide needle is inserted into the spinal parenchyma, followed by the insertion of an injection needle through the guide nee-dle horizontally into the spinal parenchyma; then, the injec-tion needle is withdrawn while delivering substrate into the spinal cord (NeuralStem patent # US2010198189A1). A sec-ond phase I trial was concluded in 2013, attesting the safety of cervical and thoracolumbar spinal cord injections with the same cells (64). The phase II dose escalating trial (NCT01730716) was recently concluded in July with an esti-mated publication of preliminary results in 2015.

iPSC Therapy–Can It Happen?

The idea of transforming adult cells into NSCs is very attractive for therapy. iPSCs can be obtained from the patients' cells, since they share the same MHC molecules as the patient's immune system the risk of rejection is greatly reduced, and the ethical issues concerning the source of ESCs is avoided.

A recent work generated and purified a specific NSC population from human iPSCs, based on high aldehyde dehy-drogenase activity, low side scatter, and integrin VLA4 positiv-ity by FC. After intrathecal or intravenous injections of these cells into SOD1-mutated mice, NSCs migrated and engrafted into the central nervous system of the treated ALS mice, which exhibited improved neuromuscular function and motor unit pathology and significantly increased life span. These positive effects are linked to multiple mechanisms, including produc-tion of neurotrophic factors and reduction of microgliosis and macrogliosis. According to this study, minimally invasive injections (intravenous) of iPSC-derived NSCs could exert a therapeutic effect in ALS (65).

Despite preclinical evidences, the therapeutic use of cells generated from iPSC is still a highly debated issue. First, the safety of this method should be ascertained, since these cells

have a well-known tumorigenic potential (39). Another important issue concerning iPSCs is that there are many reprograming protocols with different efficiency (66,67). Recent publications including novel transcription factors and chemical compounds (68,69) that substitute a reprogramming factor illustrate the lack of a gold standard protocol ensuring both reprogramming efficiency and safety for therapeutic uses. In addition, a protocol for separation of differentiated and nondifferentiated must be established, since nondifferen-tiated cells represent serious risk due to their oncogenicity.

Thus far, iPSCs have mostly been employed for the derivation of ALS models, which allow a better understanding of mechanisms behind motor neuron death, and also for drug screening, aiming the selection of compounds that might have the ability to reduce or stop disease progression and/or post-pone symptom onset (70). Aiming to circumvent the issues of therapeutic use of iPSCs, Wook Han and coworkers used a combination of transcription factors (Brn4, Sox2, Klf4, and c-Myc) to induce mouse fibroblasts to directly acquire a NSC identity. These induced NSCs (iNSCs), exhibit cell morphol-ogy, gene expression, epigenetic features, differentiation potential, self-renewing capacity, as well as in vitro and in vivo functionality similar to those of wild-type NSCs (71). Adult cells might now be used as potential sources of NSCs without the need of an iPSC reprogramming stage.

Mesenchymal Stromal Cells

The terms “stromal stem cells” and “MSCs,” established respectively by Owen in 1988 (72) and Caplan in 1991 (73), refer to a group of cells thought to have a common progenitor in the mesenchyme (74). In 1999, the capacity of these bone-marrow derived mesenchymal stromal/stem cells (MSCs) to differentiate into adipogenic, chondrogenic and osteogenic lineages was firstly demonstrated (75). Subsequently, some parameters had to be established for the correct classification of these cells.

According to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, there are three minimum criteria for defining human mesen-chymal stem cells: (1) MSCs must be plastic-adherent when maintained in standard culture conditions; (2) MSCs must express CD105, CD73, and CD90, and lack expression of CD45, CD34, CD14, or CD11b, CD79a or CD19, and HLA-DR surface molecules, and (3) MSCs must differentiate to osteoblasts, adipocytes, and chondrocytes in vitro (76,77). MSCs have been isolated from almost all adult tissues, includ-ing bone marrow (78), adipose tissue (79), umbilical cord tis-sue (80), and others. Since these cells are obtained from adult tissue sources, the application of MSCs does not involve ethical concerns (81,82), and because ALS does not affect MSC expansion and differentiation potential (83), cells can be obtained from the own patient, thus avoiding rejection.

Classic Model of Tissue Regeneration Versus MSC Transdifferentiation

The prominent regenerative potential of these adult stem cells in organs such as liver (84), muscle (85) and heart (86) has been widely studied. However, when it comes to neurodegenerative diseases, NSCs may have a therapeutic advantage over MSCs, since the latter have mesodermal origin and thus their ability to transdifferentiate into neuronal cells of ectodermal origin is questionable (87).

Neurogenin 1 (Ngn1) is a proneural basic helix-loop-helix transcription factor, reported to be sufficient for reprog-raming the mesodermal cell fate of human MSCs into a neu-ronal fate (88). In a recent and well-designed experiment, bone marrow-derived MSCs transduced with a retroviral vec-tor encoding Ngn1 (MSCs-Ngn1) were intravenously injected in an ALS murine model carrying a SOD1 mutation, and the effectiveness of non-neural (unprocessed) and neural-induced MSCs, Ngn1-expressing MSCs (MSCs-Ngn1) was compared, aiming future ALS treatment. In this study, the MSCs-Ngn1, but not the unprocessed MSC, exhibited a neuron-like mor-phology, and showed expression of the neuron-specific pro-teins NeuN, NF200, and MAP2 after induced terminal differentiation in vitro. Unlike the unprocessed MSC, the transplantation of MSCs- Ngn1, prevented the loss of ventral motor neurons, and increased cervical and lumbar neuron survival in 38 and 24%, respectively (89).

Furthermore, MSCs-Ngn1 delayed disease onset if trans-planted during preonset ages, whereas unprocessed MSCs failed to do so. If transplanted near the onset ages, a single treatment with MSCs-Ngn1 was shown to be sufficient to enhance motor functions during the symptomatic period, whereas unprocessed MSCs required repeated transplantation to achieve similar levels of motor function improvement (89), showing the importance of an early (and, if possible, pre-symptomatic) diagnosis.

Differently from the results obtained by a previous study of this same group (88), most grafted cells in the spinal cord of the SOD1 mice did not express neuronal, microglial or astrocyte markers, showing that transplanted MSCs-Ngn1 had the potential to efficiently migrate from the circulation into the CNS regions, but persisted as undifferentiated cells (89). These results suggest that the beneficial effect of the MSC-Ngn1 cells in SOD1 mice were due to a higher tropism for the affected areas in the neuroaxis, differently from what happens to unprocessed MSCs (demonstrated by a migration assay), thereby increasing the amount of released factors and thus extending neuroprotective effects.

Released Factors from MSCs

Besides tissue regeneration stem-cell based tissue repair is currently believed to occur through the paracrine secretion of factors. The list of these MSC-released factors increases con-stantly and it is known that they exert diverse biological func-tions, such as immunomodulation, antiscarring, chemoattraction, improvement and support of angiogenesis, antiapoptotic effects, or even proliferation/differentiation potential (90) The secretion of these biomolecules could pro-mote the regulation of postinjury tissue remodeling, activa-tion of endogenous repair/regeneration mechanisms, and attenuation of undesirable immune response (91–97).

It has also been shown that specific subpopulations of MSCs secrete some neuroregulatory molecules, such as brain

derived neurotrophic factor (BNDF), nerve growth factor (98), and other molecules that, despite not being classified as "neuroregulatory," act on neuronal cells (such as GLP-1; (99,100)). In the CNS, these molecules can promote beneficial effects such as stimulation of intrinsic autocrine survival, neu-rite outgrowth, and axon guidance. They also promote a cor-rect connectivity in the brain and spinal cord, influence dendrite, and synapse formation, and influence proliferation, migration, and differentiation of stem cells in the developing adult nervous system (reviewed in Ref. 101)).

The neurotrophic factor, BNDF, tested in preclinical studies, and clinical trials was shown to be safe for therapy, but it failed as a potential therapeutic drug (101). Some hypotheses were raised to explain these results. However, it is not clear whether BNDF is able to cross the blood-brain bar-rier; and on the other, it is possible that the BNDF molecule itself was not the ideal growth factor to be tested clinically (101).

Another MSC-secreted molecule was tested in three Phase III clinical trials. IGF-1 (also classified as neuroregula-tory) was injected subcutaneously in patients, but also failed to produce consistent and meaningful effects. After injections, the decaying of motor neuron function was reduced, but not bulbar function, and the patients did not show any vital capacity increase. One hypothesis to explain these results was the gravitational effect acting on the medication, since injec-tions were at the spinal level of intrathecal delivery (102).

Aiming a continuous release of the neurotrophic factor in the CNS, a recent work used an interesting strategy. After human MSCs were transfected with the GLP-1 gene, whose protein has neuroprotective (99,100) and antioxidant (103) properties in the brain, the encapsulation of these cells into a biopolymer material was performed to prevent rejection when transplanted into mice (104). The intracerebroventricular injection of GLP-1 producing MSC capsules in presympto-matic SOD1 (G93A) mice significantly prolonged survival, delayed symptom onset and weight loss, and led to significant improvements in motor performance tests compared to vehi-cle treated controls (105).

MSC Based Therapy–Clinical Trials

Based on the growing number of studies indicating the safety and feasibility of cell therapy with MSCs isolated from bone marrow (106,107), a Phase I clinical trial was performed with 10 ALS patients. Autologous bone marrow-derived MSCs were transplanted into the spinal cord at a high thoracic level and the patients were regularly monitored before and after transplantation by clinical, psychological, neuroradiolog-ical, and neurophysiological assessments. This study con-firmed that MSC transplantation into the spinal cord of ALS patients is safe and that MSCs might have a clinical use for future ALS cell based clinical trials (108). No severe morbidity was associated with the surgical procedure. However, to maxi-mize the safety of direct parenchymal injection, the authors suggest the use of a recently standardized microinjection plat-form (similar to the previously mentioned one patented by NeuralStem) to reduce eventual side effects in the surgery and facilitate MSC transplantation in the critical regions of the spinal cord (108,109). Hypothesizing that the main activity of the cells should be at the transplanted segments, the authors also suggest the application of cells in the cervical segments of the spinal cord to increase the potential benefit of MSCs ther-apy (108).

Another Phase I/II clinical trial was performed in the Hadassah Hebrew University Hospital (Jerusalem, Israel), with a total of 34 patients: 15 with intractable multiple sclero-sis (MS), and 19 with progressive ALS. To maximize the potential therapeutic benefits by accessing the CNS through the cerebrospinal fluid and the systemic circulation, the auto-logous injections were combined intrathecally and intrave-nously. In addition, nine patients received MSCs labeled with ferumoxide (Feridex) to track cell migration after local graft-ing. Only mild adverse effects were reported during the 6-month follow-up. Meningeal irritation and aseptic meningitis was observed in one patient and the researchers attributed this effect to residual dimethyl sulfoxide in the culture medium. The mean ALSFRS score, an instrument for evaluating the functional status of ALS patients, remained stable during the first six months of observation and the EDSS score (method of quantifying disability in MS) declined gradually, indicating functional improvement. Magnetic resonance imaging (MRI) of the labeled MSCs indicated their possible dissemination from the site of inoculation to the occipital horns, meninges, and spinal cord parenchyma. To assess the immunological effects of MSC transplantation, lymphocyte subsets and cyto-kine production were also evaluated. Stronger changes than those induced by the conventional immunomodulatory medi-cations were found in the treated patients, pointing to a down-regulation of activated lymphocytes and antigen-presenting cells after MSC transplantation

Amongst the initiatives with MSC trials, the leading trial is Brainstorm's, which uses NurOwn cells–bone marrow-derived MSCs taken from the patient and induced to produce large amounts of neurotrophic factors (www.brainstorm-cell. com). A phase I (NCT01051882) and a phase IIa (NCT01777646) trial have been recently concluded, and a phase II (NCT02017912) will be carried out with 48 patients in a multicentric study (www.clinicaltrials.org).

From Myth to Fact–Questions to be Addressed

In vivo function of MSCs and heterogeneity. The real in vivo function and physiological role of MSCs is still poorly understood. Recently, Waterman and coworkers observed that the stimulation of specific Toll-like receptors (TLRs) affects the immune modulating responses of human MSCs. The polarization of TLR4 or TLR3 receptors yielded two different MSCs lineages named MSC1 and MSC2, respectively. MSC1 mostly elaborate pro-inflammatory mediators, while MSC2 express mostly immunosuppressive ones (110). Taking these results into account, it is possible that MSCs might act differ-ently according to the microenvironment status of the patient. Indeed, this "priming" of MSCs before transplantation is under consideration for some complex diseases such as cancer. MSC1-based therapy was capable of attenuate tumor growth

in vitro and in vivo while both MSC2-based and unprocessed MSC-based were not (111). Besides evaluating different out-come of these distinct populations, the "priming" of MSCs prior to injection allows a more standardized cell therapy.

Another issue to be considered is that the MSC isolation process itself results in a heterogeneous population composed by distinct cell types with different morphologies, differentia-tion potential and expression of surface markers. These differ-ences are also related to their biological properties (112,113). Therefore, studies evaluating outcomes of MSC therapy with cells obtained from different donors are very important, espe-cially due to the possibility of using autologous transplants for future treatments.

The cell sorting technique has been used to identify and purify subpopulations of MSCs (114–117). One particular subset of cells, namely, pericytes, which express the CD146 surface marker, could be of great use for ALS. Pericytes sur-round endothelial cells in capillaries and microvessels (118) and adventitial cells around larger vessels (114). The therapeu-tic potential of pericytes was attested for some conditions, such as muscle-linked diseases (119,120) and myocardium infarction (121). In these experiments, pericytes were shown to migrate and exert paracrine functions.

In the central nervous system, pericytes assist the forma-tion and maintenance of the blood brain barrier, which, when impaired in neurodegenerative diseases, accelerates progres-sion of symptoms (122). In ALS patients, a 50% reduction of perycites is found in the blood-spinal cord barrier (123). The evaluation of these cells' therapeutic effect in ALS models is very important and is currently being carried out in our labo-ratory. These are examples where FC and other cell-sorting techniques are key tools that allow us to better understand the biology of determined cell types, helping to better design an efficient cell therapy.

Cell quantity and injection protocols. Protocols used for cell transplantations in SOD1 mice are quite diverse, and one of the main variations is the number of cells per injection. It was shown that, in SOD1 mice, a high-dose transplant (3.9×10^5 cells) of neuronal hNT cells does not provide any addi-tional benefit when compared with a lower-dose transplant (1.3×10^5 cells; (124), but when it comes to injecting mono-nuclear human umbilical cord blood cells into the same mouse model, a cell dose of 25×10^6 cells had the most bene-ficial effect on many therapeutic parameters than a lower (10×10^6) or higher dose (50×10^6) (125). Thus, the optimal amount of cells to be injected may vary according to the cell type.

Another important issue is the number of injections dur-ing the treatment period, allowing MSCs (or other cells used in therapy) to remain in contact with the damaged tissue for longer periods of time which could increase their beneficial effects. One previously mentioned study showed that two injections of 10^6 neural-induced MSCs in SOD1 mice led to a higher efficacy when compared with results obtained with a single dose (89). A triple injection of MSCs into the cerebro-spinal fluid of SOD1 mice yielded significant therapeutic effects, including attenuated weight loss, enhanced motor per-formance, decreased motor neuron loss, and increased sur-vival whereas a single transplantation did not alter disease progression (126).

Although there is increasing evidence that the optimal amount of cells and the transplantation method are crucial aspects for the therapy to succeed, only a few studies have been specifically designed to investigate these practical issues. Also, these studies illustrate that there are physiological differ-ences among cells obtained from different tissues emphasizing the need to better understand the influence of the original niche in cell interaction and its role in determining what type of cell is the best to be administered.

Site of injection. Some clinical trials involving neurological diseases have shown that intraspinal injection of cells is a safe procedure (108,127). In another scenario, several clinical trials with non-neurological diseases have indicated that intrave-nous administration of MSCs is also a safe procedure (128,129). New approaches, such as the phase II clinical trial led by Brainstorm Company are also considering intramuscu-lar injection of cells for ALS therapy. An important question to be addressed is which injection site for MSCs could yield the best results.

However, intraspinal (intrathecal) injections may facili-tate the migration of MSCs through the neuroaxis since cells would be placed near the damaged tissue. However, intrathe-cal injections are a risky procedure and can lead to several side effects. Microinjection platforms, like the one patented by NeuralStem, are being developed and standardized to reduce eventual side effects from surgery and optimize the procedure, allowing the delivery of cells along the entire length of the spi-nal cord with only one injection (109).

Intravenous injection is a simple technique with few risks involved. Despite the lower risks, many cells may eventually end up homing to other tissues such as lungs and lymph nodes (127), interfering with systemic homeostasis and reduc-ing the total number of cells available for engraftment in the damaged tissue (127). Even so, it has been shown that MSCs may migrate through the blood to the damaged areas of the CNS in response to inflammatory signals, and also that they are capable of exerting peripheral immunomodulating effects (130,131). This migration may be a consequence of the break-down of blood vessels in the region where the tissue degener-ates (89), or may occur through transiently formed intercellular gaps on the BBB (132).

Much attention has been given to spinal injections and intravenous injections, and only recently scientists are starting to show interest in injecting cells at the neuromuscular junc-tions. The motor neurons, before dying, retract, hampering these junctions, and, consequently, muscle function (133). If the neuronal protection is to start at the earliest stages possi-ble, it may be interesting to promote such preservation on the sites that suffer the earliest injuries. The delivery route adopted for cell injection has an important role in

determining whether MSCs will be susceptible to early immu-nological recognition and rejection (134), and influences ther-apeutic efficiency, determining the success or failure of a particular treatment. To help circumvent these issues, meth-ods for tracking the injected cells must be included in the research/therapy. MRI, positron emission tomography imag-ing and other cell imaging techniques are tools that could be extremely useful (135).

Donor age and autologous 3 allogeneic transplant. MSCs obtained from ALS patients' bone marrow have shown the same expansion and differentiation potential as cells obtained from healthy controls (83). Recently, possible correlations between certain biological markers and efficacy for autologous MSC transplant treatment was investigated. The levels of VEGF, ANG, and TGF-b were significantly higher in cultured autologous MSCs from ALS patients that responded to the therapy than in nonresponders patients (136).

However, in an autologous cell-based therapy for this late-onset disease, another factor must be taken into consider-ation: MSCs can suffer the effects of ageing (137), limiting the extension of autologous transplant, especially for late-onset diseases such as ALS. An overall decline in efficacy in cell ther-apy happens with the increase of both the age of the donor and the recipient (138,139). In addition, since a substantial number of ALS cases is genetic, the autologous transplant could be potentially harmful. Some of the proteins mutated in ALS are secreted (such as VAPB, for instance), raising the pos-sibility of an additional motor neuron damage.

Heterologous transplantation is under investigation for NSCs (obtained from an 8-week-old aborted fetus) in ALS patients as mentioned before. In these trials, the immunosup-pression protocol was designed based on a standard protocol used for whole organ transplantation, since there was no pre-vious experience with immunogenicity of transplantation of allogeneic fetal-derived stem cells into the nervous system. In these studies, the majority of nonserious adverse events were attributed to the toxicities of the immunosuppressant drugs. Besides of being toxic to patients, steroidal immunosuppres-sants were found to complete abolish the therapeutic effect exerted by MSCs on a mouse model of liver cirrhosis (140). This specific issue must be better analyzed since it may hinder these trials.

Interestingly, it has been hypothesized that MSCs may have a certain degree of "immunological privilege," since they have been effective for allogeneic cell therapies and xenogeneic transplants as well (141,142). If MSCs have such strong immunomodulatory capacities, they might even be obtained from a young, healthy donor, and administered without any immunosuppressants, benefiting patients significantly.

It was observed that MSCs can modulate both innate and adaptive immunity by releasing molecules such as the Factor H (inhibiting the complement, and thus avoiding acute and hyperacute rejection mechanisms normally mediated through this system); prostaglandins and indoleamine dioxygenase, which suppress the effector functions of NK cells (that play a key role in the initiation and regulation of adaptive immune responses after transplantation); last but not least, PD1/PDL1, which inhibits B cell activation with a role in chronic rejection (134,143).

MSC transformation after in vitro expansion. MSC trans-plantation, like all cell therapies, requires the expansion of cells in culture before injection. Although it has been shown that MSC are less prone to genetic abnormalities and malig-nant transformation during multiple passages in vitro (144,145), other studies show that MSCs can undergo malig-nant transformation after in vitro expansion (146). A recent study demonstrated that MSCs already show a significant increase in chromosomal aberrations after their fifth passage (147). Thus, routine procedures to check the possible trans-formation, chromosomal abnormalities and telomere shorten-ing of these cells should be included in preclinical and clinical trials as a quality control to ensure the safety of the method.

Since karyotype analysis before every round of injections is very laborious, focusing on a simpler method for observing DNA abnormalities would be very productive. Some attempts to do so are occurring in the field of cancer research with the high-resolution DNA FC (148), guaranteeing the safety of the cells to be injected in therapy. Cells containing DNA abnor-malities have a higher transformation potential.

Final Considerations

Finding a therapy for ALS is a great challenge. Different pathways culminating into the same symptoms point out to more than one solution. Even so, many important steps have been made, and there is some consensus regarding the types of therapy best suited for ALS. Cell replacement therapies for motor neurons seem highly unlikely, due to the necessity of the correct engraftment and innervation, the maintenance of a nontoxic environment and the control of the noncell-autonomous pathways that could culminate into motor neu-ron death. Preclinical studies have been performed transplant-ing motor neurons into mice with ALS, but without much success. However, neuronal protection seems a very promising therapy. With neurotrophic factors being released by trans-planted NSCs or MSCs, the disease onset might be delayed and/or disease progression might decelerate and, hopefully, stop altogether. With this in mind, it is necessary to plan care-fully injection protocols, immunosuppressant regimen, injec-tion sites and cell type to be administered to guarantee that these factors will not hamper the trials.

While the therapy with NSCs under investigation by the NeuralStem company seem promising for ALS treatment, one must not forget that the cells were obtained from an 8-week old aborted fetus. Before approval in clinical trials, some ethi-cal questions and discussions may probably arise again. Since the only source of native NSCs is the nervous system, will we be able to use them in a larger scale for all patients? The advantages of MSCs, like the facility to obtain them without ethical issues, their immunomodulatory properties and their production of important neurotrophic factors, turn them into

the best-suited cells for therapy. However, a more profound knowledge of the “black-box” of MSC secretion must be understood before assuming their beneficial effects. What do these cells secrete in vivo, and how do these molecules act on motor neurons and their surrounding cells? Do cells obtained from patients have a potential harmful effect?

Another important factor that directly impacts all clinical trials and studies is the fact that the diagnosis for ALS may take up to 14 months (149). Therefore, it is imperative to diagnose the disease in the earliest possible stages. As sug-gested for Parkinson’s disease and Alzheimer’s disease, iPSC models could provide excellent tools and opportunities to investigate the course of changes during the disease progres-sion, from the asymptomatic phases through the later stages when the pathology has become prominent (150). Early diag-nosis would increase the chances of more successful clinical trials, since neuron degeneration may be well advanced when the symptoms starts.

In summary, preclinical studies with MSCs and NSCs show exciting results. There are, however, parameters that must be reconsidered when it comes to clinical trials in ALS patients, as mentioned above. If overlooked, they may delay therapeutic progress, and even contribute to the lack of positive results reported for most ALS trials. To eventually trans-form cell therapy for ALS from myth to fact, important issues addressed in the present review should be taken into consideration.

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Human Molecular Genetics, 2015, Vol. 24, No. 24 6877–6885

doi: 10.1093/hmg/ddv388
Advance Access Publication Date: 18 September 2015
Original Article

O R I G I N A L A R T I C L E

Overexpression of KLC2 due to a homozygous deletion in the non-coding region causes SPOAN syndrome

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Abstract

SPOAN syndrome is a neurodegenerative disorder mainly characterized by spastic paraplegia, optic atrophy and neuropathy (SPOAN). Affected patients are wheelchair bound after 15 years old, with progressive joint contractures and spine deformities. SPOAN patients also have sub normal vision secondary to apparently non-progressive congenital optic atrophy. A potential causative gene was mapped at 11q13 ten years ago. Here we performed next-generation sequencing in SPOAN-derived samples. While whole-exome sequencing failed to identify the causative mutation, whole-genome sequencing allowed to detect a homozygous 216-bp deletion (chr11.hg19:g.66,024,557_66,024,773del) located at the non-coding upstream region of the KLC2 gene. Expression assays performed with patient's fibroblasts and motor neurons derived from SPOAN patients showed KLC2 overexpression. Luciferase assay in constructs with 216-bp deletion confirmed the overexpression of gene reporter, varying from 48 to 74%, as compared with wild-type. Knockdown and overexpression of klc2 in Danio rerio revealed mild to severe

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Received: June 14, 2015. Revised and Accepted: September 14, 2015

curly-tail phenotype, which is suggestive of a neuromuscular disorder. Overexpression of a gene caused by a small deletion in the non-coding region is a novel mechanism, which to the best of our knowledge, was never reported before in a recessive condition. Although the molecular mechanism of KLC2 up-regulation still remains to be uncovered, such example adds to the importance of non-coding regions in human pathology.

Introduction

Hereditary spastic paraplegias (HSPs) are common neurodegen-erative genetic disorders in which patients present progressive spasticity and lower limbs weakness. Up to date, more than 70 loci had been associated with HSPs and at least 50 genes have been identified (1). In 2005, our group identified in a geographic isolate in the backlands of Northeastern Brazil, 26 Caucasian in-dividuals belonging to consanguineous families with an auto-somal recessive (AR) complicated form of HSP, which associates spastic paraplegia, optic atrophy and neuropathy (SPOAN syn-drome, OMIM #609541) (2). This condition is characterized by onset of progressive spastic paraplegia in infancy, and progres-sive motor and sensory axonal neuropathy in late childhood/ early adolescence leading to severe motor disability. All patients are wheelchair bound after 15 years old, with progressive joint contractures and spine deformities. Patients also have sub nor-mal vision secondary to apparently non-progressive congenital optic atrophy, dysarthria starting in the third decade of life and exacerbated acoustic startle response. Patients show no intellec-tual impairment. Ten years after the gene mapping, more than 70 individuals from this cluster, three unrelated affected individuals from Southern and Southeast Brazil, and a pair of Egyptian sib-lings were diagnosed with SPOAN. Although, all patients share the same haplotype spanning 2.3 Mb into chromosome region 11q13, Sanger sequencing of candidate genes failed to reveal the causative gene (3). Here we describe the SPOAN causative mu-tation, a small deletion in the non-coding region that causes gene overexpression. Gain of function in a recessive condition is a novel mechanism that, to the best of our knowledge, was never reported before.

Results

Next-generation sequencing and SPOAN mutation

Whole-exome sequencing (WES) was performed in genomic DNA from one Brazilian and one Egyptian patient diagnosed with SPOAN syndrome. We identified six homozygous variants at the critical region, but population frequency and segregation analysis excluded four variants, while the remaining two were SNPs located in non-coding region, suggesting that these two were unlikely to be associated to the clinical phenotype (Supple-mentary Material, Table S1). Although WES failed to reveal the SPOAN mutation, the sequencing allowed us to refine the critical interval on chromosome 11q13 to 1.77 Mb, between markers rs508548 (A>G at 65,626,289 position in CFL1) and an undescribed variant located at 67,395,410 (G>C in NUDT8). Next, using whole-genome sequencing (WGS), we identified a homozygous 216-bp deletion (chr11.hg19:g.66,024,557_66,024,773del), located at the non-coding upstream region of kinesin light chain-2 (KLC2) (Supplementary Material, Fig. S1). This variant was detected in homozygosity in all affected Brazilian individuals (n = 73), and in the Egyptian affected siblings, while it was not present in homozygosity in 111 healthy Brazilian relatives. This 216-bp deletion was also absent in 474 Brazilian healthy controls and is not described in the 1000 genomes database.

Gene expression analysis

To verify if the deletion affects the expression level of genes lo-cated in SPOAN critical region, we performed expression array using cDNA from fibroblasts. Several genes (n = 23; Supplemen-tary Material, Table S2) showed differential expression in pa-tients compared with controls ($P < 0.01$). Unexpectedly, this assay revealed KLC2 overexpression. Quantitative reverse tran-scription PCR (RT-qPCR) performed using fibroblast cDNA sam-ples confirmed the expression array results (Fig. 1A). We next generated induced pluripotent stem-cells (iPSC) which were differ-entiated into motor neurons (MN). RT-qPCR using MN samples revealed KLC2 up-regulation in SPOAN patients compared with healthy controls, confirming the over expression observed in the previous experiments (Fig. 1C). Also we investigated KLC2 expres-sion in blood, using a larger number of cDNA samples from healthy controls, heterozygotes and affected individuals. This assay did not reveal any difference in expression levels between heterozy-gotes compared with SPOAN's and to healthy controls (Fig. 1E).

To investigate if the 216-bp deletion is the cause of KLC2 up-regulation, we performed luciferase gene reporter assay using three cell lines (HEK293T, U87MG and MN), which were trans-fected with two constructs: a KLC2 wild-type promoter and KLC2 216-bp deleted regulatory region driving the Luciferase gene. In the three cell lines, the construct with the 216-bp dele-tion produced a luciferase activity increment compared with wild-type promoter, varying from 48 to 74% (Fig. 1F).

Klc2 knockdown and overexpression in Danio rerio

We then used Danio rerio as an animal model to study the 'in vivo' effect of klc2 knockdown and overexpression. Knockdown regula-tion was achieved by microinjecting zebrafish embryos with two different klc2 morpholinos (translation blocking morpholino [$MO^{klc2\text{-}TB}$] and splice morpholino [$MO^{klc2\text{-}SP}$]), each one at doses of 4 and 6 ng. Mild phenotype was defined for embryos showing curly-tail and circular swimming whereas severe phenotype for embryos with dramatically shortened and twisted tail and that were unable to swim. Both phenotypes became evident at 48-h post fertilization (hpf) (Fig. 2A). In all cases, statistically significant differences were observed between mismatch-MO and specific-MO injected embryos. For both morpholino strategies when comparing to the respective mismatch-MO controls, an increase in lethality and/or frequency of phenotypes was mainly observed in detriment of normal phenotype. Furthermore, this difference was more evident when higher amount of either $MO^{klc2\text{-}TB}$ or $MO^{klc2\text{-}SP}$ was injected (Fig. 2B). Phenotype rescue assays were performed by co-injection of 100 pg of $mRNA^{klc2\text{-}eGFP}$ and splice morpholino at 6 ng (Fig. 2C), and an improvement of ~33% ($P < 0.01$), from severe to mild phenotype, was consistently observed (Fig. 2D).

As SPOAN syndrome seems to result from KLC2 up-regulation, we mimicked this condition in zebrafish by microinjecting $mRNA^{klc2\text{-}eGFP}$ in specific concentrations in embryos. Fluorescent embryos displayed similar phenotype to klc2 morphants (Fig. 3A). A high lethality (over than 70%) was observed in embryos micro-injected with $mRNA^{klc2\text{-}eGFP}$ at 200 pg at 24-hpf stage and we ex-cluded this concentration data in phenotype analysis (Fig. 3B). We observed higher frequency of curly-tail phenotype in

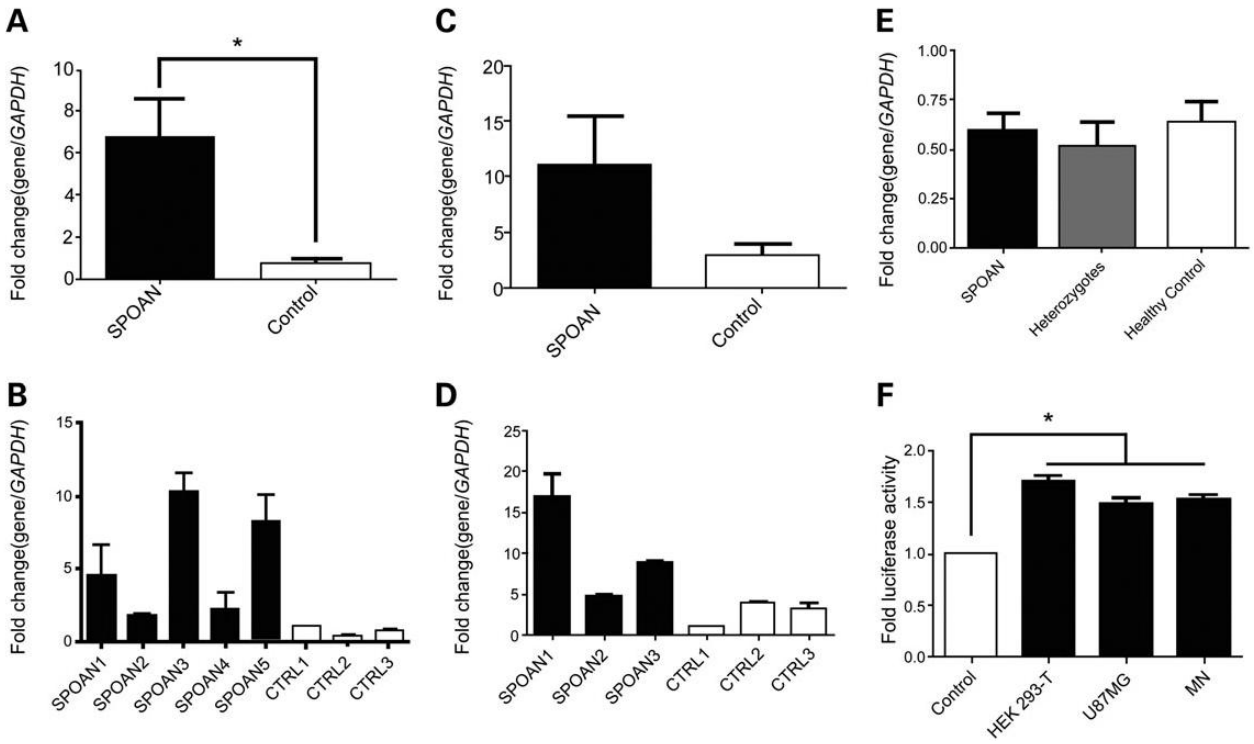

Figure 1. Effect of 216-bp deletion on KLC2 expression. (A) Relative expression of KLC2 measured by RT-qPCR performed on fibroblast cDNA isolated from SPOAN patients and healthy controls ($P < 0.05$; Nonparametric test [Mann–Whitney]). (B) KLC2 relative expression measured on fibroflast samples from individual patients and healthy controls. (C) Relative expression of KLC2 measured by RT-qPCR using MN. (D) KLC2 relative expression measured on MN samples from individual patients and healthy controls. (E) KLC2 relative expression measured on whole-blood cDNA samples from affected (homozygotes), heterozygotes and healthy controls. Each RT-qPCR experiment was performed in triplicate and each sample was replicated twice. (F) Expression of luciferase reporter gene controlled by the 216-bp-deleted KLC2 regulatory region relative to the expression controlled by the wild-type KLC2 regulatory region measured in HEK293 T, U87MG and MN cells. Each experiment was performed in triplicate and each cell type was replicated twice ($P < 0.05$; One-way ANOVA).

embryos microinjected with $mRNA^{klc2\text{-}eGFP}$ compared with con-trol ($mRNA^{eGFP}$), being statistical significant in embryos microin-jected at 150 pg mRNA concentration ($P < 0.05$) (Fig. 3C).

Discussion

We previously mapped the SPOAN gene, responsible for a syn-dromic form of AR spastic paraplegia, at 11q13 (2,3). Based on next-generation sequencing, we were able to uncover a new causative mechanism for this condition. We observed that a small deletion in KLC2 non-coding region is responsible for the gene up-regulation and SPOAN phenotype. Additionally, BSCL2 and FLRT1, two genes previously associated with HSP and located nearby but outside the 11q13 critical region, were excluded as candidates (4,5). The Egyptian patients reported in this study as SPOAN carried the c.T2023C (stop loss) homozygous mutation in FLRT1, and were previously assigned by Novarino et al. (5) (Family 709) as SPG68. However, here we suggest that 216-bp deletion, shared by all SPOAN patients, is probably the causative mutation in both Egyptian siblings, rather than the reported FLRT1 mutation.

KLC2 codes for KLC2, a protein involved in anterograde axo-plasmatic transport of organelles and macromolecules cargoes (6–10). KLC2 is a part of kinesin protein-1 complex (11), which binds to kinesins heavy chain in a stoichiometric ratio of 1:1 (12), being highly expressed in neurons. Several neurodegenera-tive diseases show impairment in axonal transport (13,14) and some kinesins heavy chains (KIF5A, KIF1A and KIF1C) have been associated with HSP (15–18). Animal models have also shown that disturbance of axonal transport proteins cause neurodegen-erative disease and axon degeneration (10,19–21). Although the disease mechanism described here involves a homozygous dele-tion in a non-coding region, all these observations strongly suggest that KCL2 is the causative gene for SPOAN.

According to the RepeatMask database, KLC2 upstream region was generated by a non-LTR retrotransposon (L3/CR-1) insertion. DNA footprint and alignment of L3/CR-1 did not show conserva-tion among distant species, but the high conservation observed among primates suggests it was inserted during the divergence of primates from other mammals. In several human populations, KLC2 surrounding region (10-kb up- and downstream) and three described SNPs surrounding the mutation location have low fix-ation index (F_{ST}) (Supplementary Material, Fig. S2) (rs116801155, rs190099601 and rs76627914 with F_{ST} of 0.0044, 0.0002 and 0.0427, respectively), indicating a high conservation in humans.

Surprisingly, the small deletion in its non-coding upstream region causes KLC2 overexpression, suggesting a novel molecular mechanism never report before, a gain of function in recessive condition. Intriguingly, the 216-bp deletion overlaps 9-bp of 5′-untranslated region (5′-UTR) of the largest KLC2 transcript (NM_001134775.1), which means that this mutation is located at KLC2 promoter region (upstream of the transcription start site [TSS]) and it should cause gene downregulation instead gain of function. Although this region has characteristics of a promoter (enrichment of H3Kme3, DNase I hypersensitive sites [DHS], RNA pol II binding sites, etc.), transcription factors complexes that bind at this region may act as transcriptional repressor, which could explain the gene up-regulation. Additionally, this

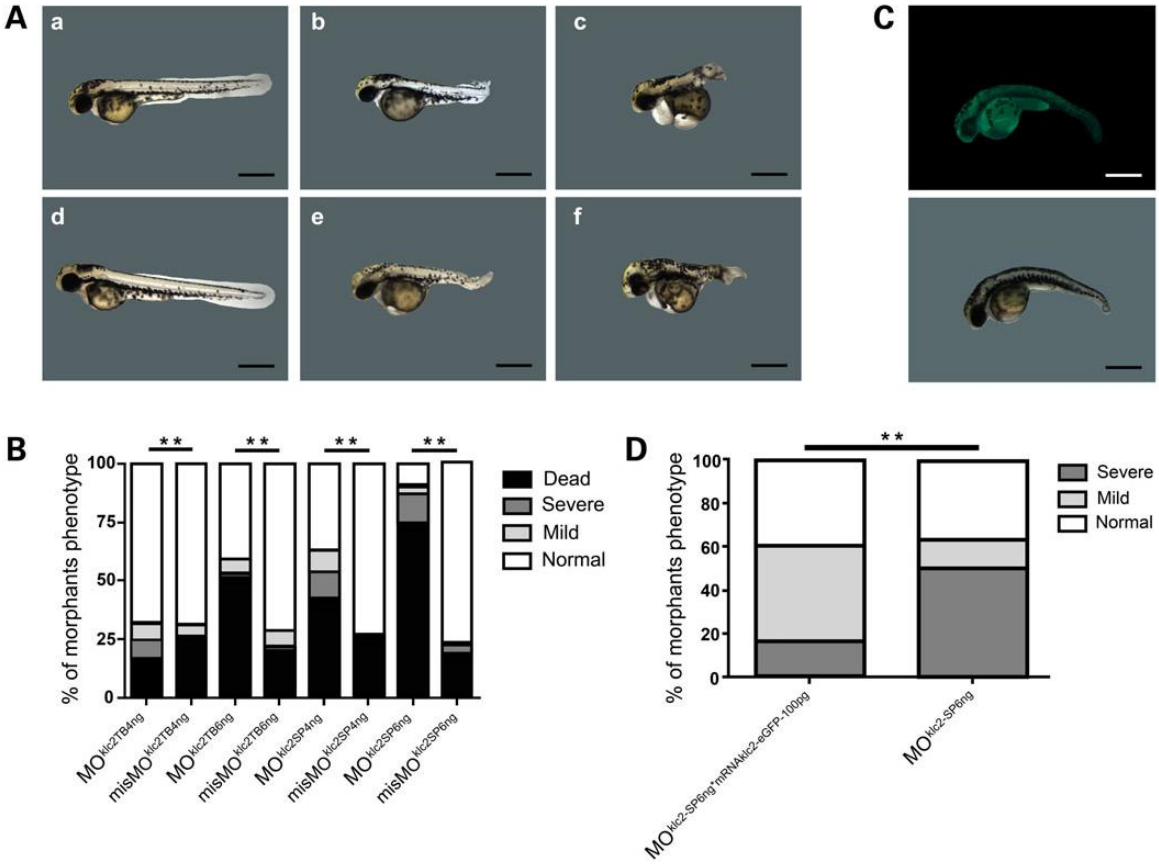

Figure 2. Effect of klc2 knockdown in zebrafish. (A) (a and d) Embryos microinjected with control splicing blocking morpholino: (a) misMO$^{klc2\text{-}SP4ng}$ (d) misMO$^{klc2\text{-}SP6ng}$. (b, c, e and f) Embryos microinjected with splicing blocking morpholino: (b and c) MO$^{klc2\ -SP4ng}$ (e and f) MO$^{klc2\ -SP6ng}$. Normal (a and d), mild curly-tail (b and e) and severe curly-tail (c–f) phenotypes were recorded at 48-hpf. (B) Frequencies of observed phenotypes among morphants. Number of microinjected embryos: MO$^{klc2\text{-}TB4ng}$ (292); misMO$^{klc2\text{-}TB4ng}$ (305); MO$^{klc2\text{-}TB6ng}$ (103); misMO$^{klc2\text{-}TB6ng}$ (249); MO$^{klc2\text{-}SP4ng}$ (283); misMO$^{klc2\text{-}SP4ng}$ (234); MO$^{klc2\text{-}SP6ng}$ (423); misMO$^{klc2\text{-}SP6ng}$ (370). $P < 0.01$, χ^2 test. (C) Fluorescent embryo coinjected with 6 ng MO$^{klc2\text{-}SP}$ and 100 pg mRNA$^{klc2\text{-}eGFP}$ (selected by fluorescence at 24-hpf) showing mil-curly tails was recorded at 48-hpf. Scale bar 200 µm. (D) Embryos coinjected with 6 ng MO$^{klc2\text{-}SP}$ and 100 pg mRNA$^{klc2\ -eGFP}$ (n = 70 embryos) showed a partial rescue of morphant phenotype compared with MO$^{klc2\text{-}SP6ng}$ (n = 30 embryos). $P < 0.01$, χ^2 test.

deletion overlaps an unspliced antisense long non-coding RNA (lncRNA, AU311830.1) and regulatory elements: DHS, several transcription factors binding sites (TFBS), histone marks and DNA methylation (Supplementary Material, Fig. S1). Thus, a dis-ruption of this non-coding and regulatory region might alter the expression level of downstream genes, which can explain SPOAN gain of function.

Expression analysis showed an unexpected KLC2 overexpres-sion from fibroblast and MN SPOAN samples. Because SPOAN is a recessive condition, we tried to check the KLC2 expression pat-tern in heterozygous samples. Whole-blood samples collected from a large number of heterozygotes did not reveal increased KLC2 expression, when compared with homozygotes and healthy controls. These results suggest a tissue-specific effect since 216-bp deletion causes KLC2 up-regulation in fibroblast and MN cell-lines, but does not in blood. Also, luciferase assay showed that reporter constructs with 216-bp deletion have increased luciferase activity when compared with the wild-type. These results support the hypothesis that the 216-bp deletion located at non-coding region is likely the responsible for the KLC2 overexpression.

Zebrafish has been an interesting animal model used in gen-etic studies due to its fast embryonic development and the fact it carries several human orthologues genes. The percentages of lethality and animals with curly-tail phenotype observed in morphants in this study were similar to those reported in several reports that employed zebrafish for other HSP (22–28). Microinjec-tion of mRNA$^{klc2\text{-}eGFP}$ in zebrafish embryos showed a similar phenotype of klc2 morphants, which reinforces our hypothesis that klc2 is an essential gene for MN function and development. Thus, we hypothesize that imbalance of KLC2 gene expression results in neurodegenerative phenotype in humans.

Gene overexpression had been associated with several neuro-logical disorders but none of them have AR inheritance. For example, duplication or triplication of PLP1 cause Pelizaeus-Merz-bacher disease (OMIM #312080) (29–33) and PMP22 duplication causes Charcot-Marie-Tooth disease type 1A (OMIM #118220), a hereditary demyelinating neuropathy (34,35). Variants detected upstream APP region were associated with up-regulation of APP protein in Alzheimer disease and Down syndrome patients (36). Additionally, downregulation or complete disruption of protein synthesis is usually the common mechanism in HSP in which functional studies have been conducted. For instance, this is the case in X-linked [e.g. L1CAM (37)], autosomal dominant [e.g. ATL1 (38) and SPAST (39)] and AR conditions [as SPG20 (40) and FA2H (41)].

In short, several unexpected and surprising results were observed during SPOAN syndrome molecular investigation. Although the molecular mechanism of this up-regulation still

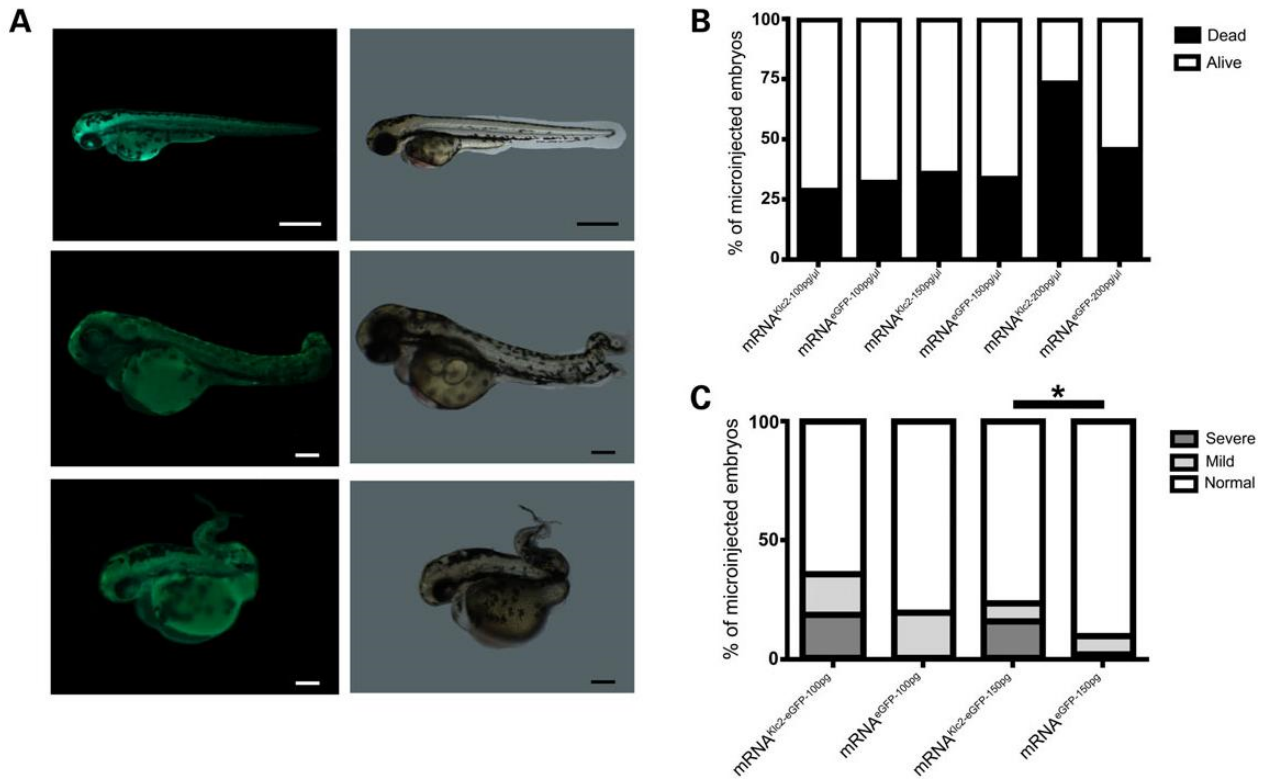

Figure 3. Effect of klc2 overexpression in zebrafish. (A) (a and b) Embryos microinjected with 100 pg of $mRNA^{eGFP}$ and (c–f) embryos microinjected with 100 pg of $mRNA^{klc2\text{-}eGFP}$ were selected by fluorescence at 24-hpf, and recorded at 48-hpf. (a and b) Normal Embryos, (c and d) mild curly-tail and (e and f) severe curly-tail phenotypes. Scale bars: (a and b) 200 µm; (c–f) 100 µm. (B) Lethality frequency observed in embryos at 24-hpf stage. Embryos microinjected with 200 pg $mRNA^{klc2\text{-}eGFP}$ showed high lethality and were excluded form phenotype analysis. (C) Frequencies of observed phenotypes among GFP-fluorescent embryos. Numbers of embryos selected by fluorescence: $mRNA^{klc2\text{-}eGFP\text{-}100pg}$ = 32; $mRNA^{eGFP\text{-}100pg}$ = 16; $mRNA^{klc2\text{-}eGFP\text{-}150pg}$ = 43; $mRNA^{klc2\text{-}eGFP\text{-}150pg}$ = 52. $P < 0.05$, χ^2 and Fisher's exact tests.

remains to be uncovered, it adds another example of the import-ance of non-coding regions in human pathology.

Materials and Methods

Patients

Clinical information regarding SPOAN patients in the geographic cluster detected in northeastern Brazil was detailed elsewhere (2,3). Additionally, we evaluated another three Brazilian patients, with different ancestors from northeastern Brazil and two Egyp-tians siblings with the identified 216-bp deletion and same clin-ical symptoms. Blood samples were used for DNA extraction from all patients, from several obligated carriers and from unaffected siblings. Fibroblasts were obtained from dermal biopsies from five patients, one heterozygote and four Brazilian healthy con-trols, following informed consent under protocols approved by the Biosciences Institute, University of São Paulo (Protocol CEP 010/2003).

Molecular analysis

Previous studies conducted by our group using Sanger sequen-cing did not identify deleterious variants in exons of candidates genes located in the critical region for SPOAN (LRFN4, KLC2 and CCS) (3). To have a more comprehensive and detailed view over this region, WES was performed using DNA samples from two SPOAN subjects using Agilent SureSelect Human All Exon 50 Mb Kit and sequenced in Illumina HiSeq2000 (Illumina, San Diego, CA, USA). Alignment against reference GRCh37 was performed with BWA (42); genotyping with GATK (43); SNP and InDel annotation with Annovar (44) and CNV detection with the R package ExomeDepth (45). The WES coverage achieved at the candidate region was 40× and 77× in the Egyptian and Brazilian samples, respectively. The 216-bp deletion was not detectable by WES. Variants detected in the mapped linkage region were filtered by their frequency, compared with 1000 Genomes data-base, NHLBI GO Exome Sequencing Project (ESP), Exome Aggrega-tion Consortium (ExAC) and with sequences obtained from 1484 Brazilian controls.

Whole-genome sequencing was performed in DNA from a third affected patient (a distant cousin from Brazilian series) using Illumina TruSeq DNA kit. Alignment against reference GRCh37 was performed with BWA (42); genotyping with GATK (43); SNP and InDel annotation with SnpEff (46) and CNV detec-tion using R package ExomeDepth (45) restricted to exon regions (using bedfile template of the Agilent V4Plus kit), Pindel and add-itional manual screening in the target linkage region. The achieved coverage at the candidate region was 26×. Variants were filtered by comparison with 1000 Genomes. SPOAN muta-tion (chr11.hg19:g.66,024,557_66,024,773del) was checked for co-segregation in affected and family health controls (also checked in 474 unrelated health controls) by PCR followed by agarose gel electrophoresis using primer ID 1 (Supplementary Material, Table S3).

Induced pluripotent stem-Cells (iPSC)

Retrovirus vectors containing the Oct4, c-Myc, Klf4 and Sox2 human cDNAs were obtained from Muotri's group and the

protocol is described elsewhere (47). Embryoid bodies (EBs) were formed by mechanical dissociation of cell clusters (pre-treated with dorsomorphin, 1 nM, for 2 days) and plating onto low-adher-ence dishes in NB media (DMEM/F12 plus 0.5X N2 and 0.5X B27 supplements) plus dorsomorphin for 2 days and in the next 5 days in NB media plus FGF and EGF. After that, mature EBs were dissociated with accutase for 5 min at 37°C and plated in matrigel in NB media plus FGF 20 ng/ml and EGF 20 ng/ml. Rosettes were visible for collection after 7 days and were then dissociated with accutase (Chemicon, EMD Millipore, Darmstadt, Germany) and plated onto poly-ornitine/laminin-coated dishes (Sigma) with NB media plus FGF and EGF. Homogeneous populations of neural progenitor cells (NPCs) were achieved after 1–2 passages with accutase in the same condition. To improve cell differenti-ation, brain-derived neurotrophic factor (20 ng/ml), glial cell-derived neurotrophic factor (20 ng/ml), insulin-like growth factor-1 (20 ng/ml), Ri (5uM) and SHH (100 ng/ml; neuronal mat-uration medium) were added to neuronal cultures for 5 weeks. NPCs were differentiated in MN following a protocol modified from study described elsewhere (48).

Human RNA extraction and cDNA synthesis

RNA extraction from fibroblasts (n = 5 affected; n = 1 heterozygote; n = 4 healthy controls) and MN (n = 3 affected; n = 1 heterozygote; n = 3 healthy controls) was performed with TRIZOL® reagent (Invitrogen) and Norgen Biotek RNA/DNA/Protein Purification Kit (Norgen Biotek Corp., Ontario, Canada); RNA from whole-blood (n = 7 affected; n = 7 heterozygotes; n = 6 family healthy controls +1 unrelated healthy control from the same region) were extracted using PAXgene Blood RNA Kit (Qiagen); RNA was reverse-transcribed with oligo(dT) primers using SuperScript™ III First-strand Synthesis System (Life Technologies).

Expression array

Fibroblast cDNA samples were submitted to array expression assay using GeneChip® Scanner 3000 7G System (Affymetrix, Santa Clara, CA, USA). The results of expression array were nor-malized by Robust Multi-array Average (49) and statistical meth-od (test-T) was performed using CLCbio Genomics Workbench, adjusted by Bonferroni and false discovery rate (FDR). Data were-submitted to GEO (accession number: GSE67527).

Quantitative reverse transcription PCR (RT-qPCR)

KLC2 primers for RT-qPCR were detailed in Supplementary Material, Table S3 (primer ID 2). RT-qPCR was normalized to GAPDH and was performed using LightCycler® 480 (Roche Diag-nostics). KLC2 expression data were calculated using 2^{-CT} meth-od (50). Mann–Whitney test (Nonparametric) was performed using GraphPad Prism version 5.00 (San Diego, CA, USA). Each experiment was performed in triplicate and each sample was replicated twice.

TaqMan Gene Expression Assay probes: MNX1/HB9 (Hs00907365_ m1), CHAT (Hs00252848_m1) and ISL1 (Hs00158126_m1) were used to validate the neurons derived cells from iPSC as MN (Applied Biosystems, USA). RT-qPCR was normalized to Human ACTB (β-actin; Hs01060665_g1). RT-qPCR was performed using the Applied Biosystems® 7500 Fast Real-time PCR System.

Immunofluorescence and MN validation

For immunofluorescence evaluation of MN, cells were fixed with 4% paraformaldehyde, followed by permeabilization and blocking with 0.05% (v/v) Triton X in PBS containing 5% (v/v) don-key serum. Primary antibodies were incubated overnight at 4°C. Samples were washed three times before secondary antibodies incubation (Alexa Fluor Dyes, Life Technologies). Dapi was added in the last 20 min of secondary antibody incubation. Pri-mary antibody concentrations were: a-NeuN mouse monoclonal 1:500 (Millipore); a-Hb9 mouse polyclonal 1:500 (DSHB) and a-Islet 1 rabbit polyclonal 1:1000 (BD Bio-science). Images were obtained through Axio Observer.A1 immunofluorescence microscope (Zeiss). cDNA obtained from fibroblasts, NPC and MN were used for MN validation using TaqMan probes described above. RT-qPCR using fibroblast samples did not show expression of MN probes. RT-qPCR of MN samples showed expression of MNX1/ HB9 probe, which was not amplified in NPC samples. MN samples showed higher significant ($P < 0.05$) expression of CHAT compared with NPC (Supplementary Material, Fig. S3B). We con-firmed the presence of 216-bp deletion in DNA extracted from MN patient samples (Supplementary Material, Fig. S3C).

Gene reporter assay

The full-length (3,313-bp) and deleted 216-bp (3,097-bp) KLC2 up-stream region was synthesized (Genone) and cloned into promo-terless firefly luciferase vector pGL4 (Promega). pShuttle/RL was used for transfection normalization, which expresses the report-er gene Renilla luciferase (51). Assays using HEK239T, U87MG and MN about 1×10^4 cells were plated in 96-well dishes in triplicate for each point. In HEK239T and U87MG a total of 200 ng of plas-mids (180 ng pGL4 and 20 ng pShuttle/RL) were used for transfec-tion using Lipofectamine 2000 Transfection Reagent (Invitrogen). In MN we used 480 ng pGL4 and 20 ng pShuttle/RL. Two days after DNA transfection, the luciferase activities were measured in Glo-max luminometer (Promega) with the Dual-Glo Luciferase Assay System (Promega) according to manufacturer's instructions. One-way ANOVA was performed using GraphPad Prism version 5.00 (San Diego, CA, USA).

Zebrafish animal model

Adult zebrafish were maintained at 28°C on a 14 h light/10 h dark cycle and the embryos were obtained by natural mating. Zebra-fish presents only one klc2 gene in its genome (ZFIN ID: ZDB-GENE-030131-2670), which turns appropriate the use Danio rerio as animal model in this study. The use of Danio rerio in this study was approved by the Committee on the Ethics of Animal Experiments of Pharmacology and Biochemistry Sciences depart-ment of National University of Rosario, Argentina (No. 429/2014).

Zebrafish RNA extraction and cDNA synthesis

Total RNA was extracted from whole embryos at different embry-onic stages (6, 24, 48 and 72-hpf). RNA extraction was performed using TRIZOL® reagent (Invitrogen), following the manufac-turer's protocol. First-strand cDNA was synthetized using Super-Script Reverse Transcriptase (Invitrogen) with a specific primer (primer ID 3) for Danio rerio klc2 gene transcript (Ensembl EN-SDARG00000075485). The complete klc2 CDS was amplified by PCR using primers ID 4, forward including EcoRI and reverse in-cluding SacI restriction sites.

Plasmids and DNA constructs

The complete CDS sequence from klc2 ($mRNA^{klc2}$) was cloned using EcoRI and SacI sites into an engineered version of pCS2 +MT as described elsewhere (52). This plasmid was used to

transcribe mRNA$^{klc2\text{-}eGFP}$ coding for KLC2 fused to eGFP. Plasmid without klc2 insert was used to transcribe mRNA^{eGFP} as a control. For mRNA$^{klc2\text{-}eGFP}$ and mRNA^{eGFP} transcription, plasmids were lin-earized by NotI and the SP6 promoter was used for in vitro transcription using mMESSAGE mMACHINE® Kit (Ambion, Applied Biosystems). The mRNA$^{klc2\text{-}eGFP}$ was used to perform the overexpression assay and for rescue of morphant's phenotype.

Knockdown and overexpression assays

Microinjection of morpholino oligonucleotides (MO) in the yolk of embryos at one- to two-cell stage were performed in specific concentrations (4 and 6 ng). Translation blocking morpholino (MO$^{klc2\text{-}TB}$) sequence was 5′-GGTGGACATCACCCACTGACACACA-3′ (misMO$^{klc2\text{-}TB}$ was 5′-GGaGcACATgACCCAgTcACACACA-3′) and splicing blocking morpholino (MO$^{klc2\text{-}SP}$) sequence was 5′-CGTGT GTGTTTCACCTGTGCTTCCC-3′ (misMO$^{klc2\text{-}SP}$ was 5′-CGTcTcT GTTTgACCTcTcCTTCCC-3′). MO$^{klc2\text{-}SP}$ target exon 2 of klc2 gene. The rescue of phenotype was performed by co-injecting 6 ng MO$^{klc2\text{-}SP}$ and 100 pg mRNA$^{klc2\text{-}eGFP}$ in the yolk of embryos staged at one- to two-cells. Chi-square and Fisher's exact tests were performed using GraphPad Prism version 5.00 (San Diego, CA, USA).
Overxpression of klc2 gene in zebrafish was performed by microinjecting mRNA$^{klc2\text{-}eGFP}$ at specific concentrations (100, 150 and 200 pg), as described in previous study (53). Same concentrations of mRNA^{eGFP} were microinjected in zebrafish embryos to be used as controls. Both microinjected embryos (mRNA$^{klc2\text{-}eGFP}$ and mRNA^{eGFP}) were selected by fluorescence at 24-hpf stage and evaluated at 48-hpf under MVX10 Olympus Microscope, and recorded with MVXTV1XC Olympus digital camera. Chi-square test was performed using GraphPad Prism version 5.00 (San Diego, CA, USA).

Supplementary Material

Supplementary Material is available at HMG online.

Acknowledgements

We are grateful to H. Miranda for her help in conducting MN differentiation. We also thank R. Moura for his contribution in bioinformatics analysis.

Conflict of Interest statement. None declared.

Funding

This work was supported by Propesq/UEPB, PPSUS/FAPESQ/PB, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Instituto Nacional de Ciência e Tecnologia (INCT), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP)/ Centro de Pesquisa, Inovação e Difusão (CEPID) and National Counsel of Technological and Scientific Development (CNPq).

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J Neurol (2016) 263:821–822
DOI 10.1007/s00415-016-8065-8

LETTER TO THE EDITORS

A novel GFAP mutation in a type II (late-onset) Alexander disease patient

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Received: 14 January 2016 / Revised: 9 February 2016 / Accepted: 10 February 2016 / Published online: 25 February 2016

Dear Sirs,

Alexander disease (AxD) is a rare neurodegenerative disorder caused by mutations in GFAP gene, encoding glial fibrillary acidic protein, and affects primarily astrocytes. Two clinical subtypes are described: an early onset one characterized by failure to thrive, macrocephaly and a predominantly frontoparietal leukodysthrophy—named type I; and a late-onset phenotype, type II, characterized by spastic gait, cerebellar ataxia and atrophy of the medulla [1].

A 36-year-old woman presented to our service with a slowly progressive complaint with loss of balance, frequent falls, mild dysarthria, and urinary incontinence initiated after her second uneventful pregnancy, when she was 25-year-old. By the age 32, she developed weakness on her left lower limb. Two years later, the left arm was also affected, and she became wheelchair-bound when she was

She became depressed, apathetic and experienced insomnia. Her past medical history was remarkable by tobacco and alcohol abuse. Her mother died at age 46 due to breast cancer, her father died at age 68 after ischemic stroke complications, and she had eight siblings—one brother died ate age 34 of sudden death, one sister died at age 8 years (she was diagnosed with mental retardation, but no further information were available). The six remaining siblings were healthy. On neurologic exam she scored 23/30 on the Mini Mental State Examination, and presented an asymmetric spastic quadriparesis, worst on the left hemibody, predominantly axial cerebellar ataxia, hypometric horizontal saccades, gaze-evoked nystagmus to the left, absent gag-reflex, dysarthria and dysphagia. There was no palatal tremor. Ancillary tests, CSF analysis and nerve conduction studies were unremarkable. MRI disclosed abnormal periventricular white matter hyperintensity and ‘‘tadpole atrophy’’, suggesting AxD (Fig. 1). Molecular analysis of GFAP gene disclosed a novel pathogenic variant chr12:42,989,137 C[G, c.809G[C (p.Arg270Pro), on exon 5. Algorithms of pathogenicity prediction (SIFT, PolyPhen, and Mutation Taster) are in agreement that this variant is deleterious or probably damaging. She died of acute respiratory failure at 39, presumably by pulmonary embolism—necropsy was declined.

This is a challenging case and the diagnosis was not suspected until a detailed review of the MRI was done by an experienced neuroradiologist (L.T.L). The adult-onset asymmetric presentation without a clear family history might divert the clinician away from a possible genetic etiology [2, 3]. The tobacco and alcohol abuse could also be misleading, raising the possibility of a Marchiafava–Bignami disease, although this one has a quite distinct MRI pattern, with demyelination of the corpus callosum [4]. Another important clinical clue for the diagnosis of late-onset AxD that was lacking in this patient was palatal tremor. The presence of that sign in a patient with spastic-ataxia syndrome would have narrowed the differential diagnosis to AxD and progressive ataxia and palatal tremor syndrome. The presence of atrophy of medulla and upper cervical spinal cord prompted to a target molecular analysis

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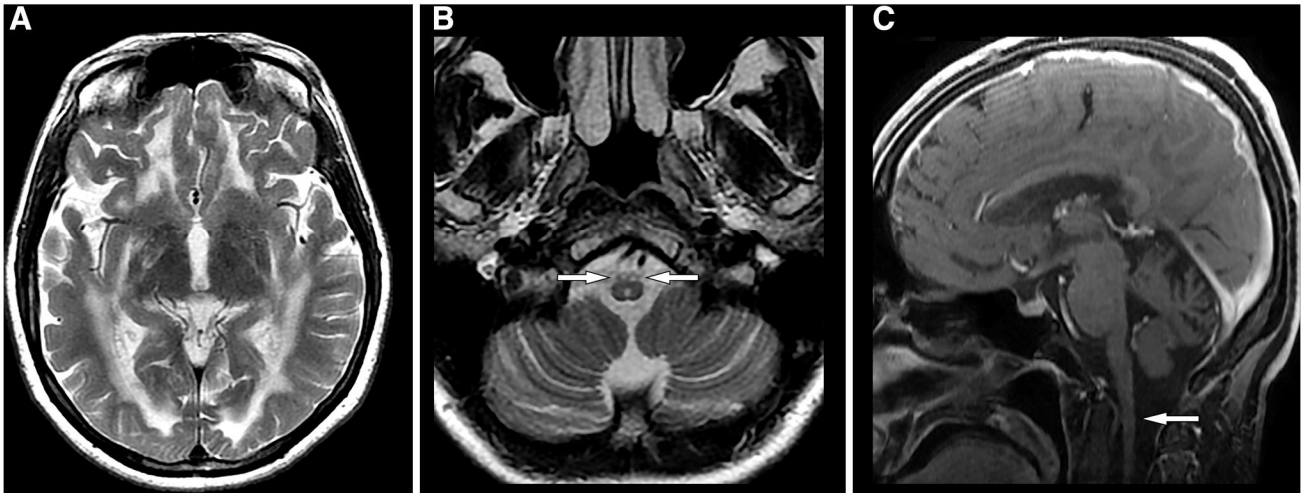

Fig. 1 Axial T2-weighted images (a, b) demonstrate symmetric periventricular hyperintensity in periventricular white matter (a) and hyperintensity in both corticospinal tracts (arrows in b). Sagittal postcontrast T1-weighted image (c) demonstrates atrophy of the medulla and upper cervical spinal cord (arrow), with relative sparing of the pons, the so-called ‘‘tadpole atrophy’’

that made the correct diagnosis. This is a missense mutation and involves an arginine residue, like the majority of the 110 previously reported pathogenic variants.

We highlight that, despite being an autosomal dominant condition, the majority of the cases are due to de novo mutations and a high index of suspicion is needed to not miss this diagnosis.

Compliance with ethical standards

Conflicts of interest The authors report no disclosures relevant to the manuscript.

Ethical approval This study was approved by the Institutional Ethical Committee In Research (Comissao˜ de Etica para Analise´ de Projetos de Pesquisa).

Informal consent Written informed consent was obtained from the relatives according to ethical committee standards—available in Portuguese.

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BIOGRAFIA

Thiago Rosa Olávio foi aluno de graduação da Universidade de São Paulo nos cursos de Bacharelado em Ciências Biológicas (entre 2006 e 2012) e Licenciatura em Ciências Biológicas (entre 2006 e 2013). Iniciou sua iniciação científica em 2009 sob a orientação do Dr. Fernando Kok, passando para a orientação da Dra. Mayana Zatz em 2011. Em 2013, ingressou no curso de mestrado do Departamento de Genética e Biologia Evolutiva do Instituto de Biociências da Universidade de São Paulo, e neste momento deposita a sua dissertação como parte dos requisitos para obtenção do título de mestre neste curso.