Teresa Maria Pereira da Silva

Multipotent mesenchymal stromal cells for Machado Joseph disease therapy

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Abbreviations

a.u. - arbitrary units

ADL - Activity of Daily Living Scale

aNSC - adult neural stem cells

BBS - Berg balance scale

BDNF - brain-derived neurotrophic factor

bFGF - basic fibroblast growth factor

BM-MSC - bone marrow-derived multipotent mesenchymal stromal cells

BSA - bovine serum albumin

CFU-Fs - colony forming unit fibroblastic cells

CNS - central nervous system

CNTF - ciliary neurotrophic factor

DAPI - 4, 6-diamidino-2-phenylindoline

DC - dendritic cells

DMEM - Dulbecco's Modified Eagle Medium

DMSO - dimethylsulfoxide

DPRLA - dentatorubral-pallidoluysian atrophy

DUB - deubiquitinating

EAE - experimental autoimmune encephalomyelitis

EEA - European Economic Area

EGF - epidermal growth factor

ERAD - endoplasmic reticulum-associated degradation

EU - European Union

FBS - fetal bovine serum

FGF - fibroblast growth factor

FITC - fluorescein isothiocyanate

GDNF - glial cell line-derived neurotrophic factor

GFAP - glial fibrillary acidic protein

GFP - green fluorescent protein

HA - hemagglutinin

HBSS - Hanks' Balanced Salt solution

HD - Huntington's disease

HGF - hepatocyte growth factor

HIV-1 - human immunodeficiency virus 1

HLA - human leukocyte antigen

Iba-1 - ionized calcium-binding adaptor molecule 1

IC - intracranial

ICARS - International Cooperative Ataxia Rating Scale

ILs - interleukins

IV - intravenous

MAP1b - microtubule associated protein 1b

MAP2 - microtubule-associated protein 2

MHC - major histocompatibility

MJD - Machado Joseph disease

MSA-C - Multiple system atrophy-cerebellar type

MSC - multipotent mesenchymal stromal cells

NES - nuclear export signal

NeuN - neuronal nuclei

NGF - nerve growth factor

NGS - normal goat serum

NIIs - neuronal intranuclear inclusions

NIs - neuronal inclusions

NK - natural killer

NLS - nuclear localization signal

NT-3 - neurotrophin-3

PBS - phosphate buffer solution

PE - phycoerythrin

PFA - paraformaldehyde

PolyQ - polyglutamine

RT - room temperature

SBMA - spinal bulbar muscular atrophy

Sca1 - stem cells antigen 1

SCA3 - Spinocerebellar ataxia type 3

SCAs - Spinocerebellar ataxias

SSEA-4 - stage-specific embryonic antigen-4

Treg - regulatory T cells

Ub - ubiquitin

UC-MSC - umbilical cord-derived multipotent mesenchymal stromal cells

UIMs - ubiquitin-interaction motifs

UPS - ubiquitin-proteasome system

VCAM-1 - vascular cell adhesion molecule 1

VEGF - vascular endothelial growth factor

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General Introduction

Scientific Paper

Resumo

A doença de Machado Joseph (MJD), também conhecida como ataxia espinocerebelosa do tipo 3 (SCA3), é a ataxia espinocerebelosa mais comum em todo o mundo. Esta doença é causada por uma repetição excessiva do trinucleótido CAG no gene *MJD1/ATXN3*, que se traduz num tracto de poliglutaminas na proteína ataxina-3. Até ao momento não existem terapias que sejam capazes de modificar ou atrasar a progressão desta doença fatal. No entanto, as células mesenquimatosas do estroma (MSC) têm demonstrado ser uma ferramenta promissora como terapia celular de doenças neurodegenerativas. Recentemente, o seu uso tem vindo a receber especial atenção em doenças que afectam o cerebelo, nomeadamente em ataxias espinocerebelosas. Estudos pré-clínicos e clínicos têm demonstrado que a utilização deste tipo de células é segura e que contribui para a melhoria de doentes com ataxias. No entanto, não existem ainda estudos que avaliem o uso das MSC em MJD usando modelos animais. Os estudos em humanos estão confinados a avaliação clinica e não permitem avaliar a neuropatologia antes e após a administração das MSC. Torna-se assim crucial o estudo do uso das MSC num modelo de murganhos portadores da MJD para investigar se a terapia pode aliviar o comprometimento motor e a neurodegeneração causada pela ataxina-3 mutante.

No presente estudo investigámos se o transplante local das MSC no cerebelo de murganhos transgénicos portadores de MJD poderia produzir um efeito neuroprotector e aliviar o fenótipo inerente a esta doença. Foi demonstrado que as MSC conseguem sobreviver no tecido cerebelar e produzir uma melhoria na coordenação motora. Além disso, as MSC conseguiram aliviar a neuropatologia neste modelo de MJD, preservando as células de Purkinje e as camadas molecular e granular do cerebelo. Em conclusão, estes resultados sugerem que a terapia com MSC pode tornar-se uma forte abordagem terapêutica para a doença Machado Joseph.

Palavras-chave: Ataxia espinocerebelosa do tipo 3; Doença de Machado Joseph; Células mesenquimatosas do estroma; transplante; modelo MJD.

General Introduction

1. Polyglutamine diseases

Polyglutamine (polyQ) diseases are a group of 9 inherited neurodegenerative disorders associated with the expansion of a CAG triplet repeat in the respective causative gene. This group of disorders includes Huntington's disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA), spinal bulbar muscular atrophy (SBMA), and the spinocerebellar ataxias 1, 2, 3, 6, 7 and 17. With the exception of SBMA which is X-linked, all other polyQ diseases are dominantly inherited disorders (Havel *et al.*, 2009). All polyQ diseases share several common features: they are progressive, typically striking in midlife, and cause increasing neuronal dysfunction and eventual selective neuronal loss usually 10–20 years after the onset of symptoms. The whole process is accompanied by severe physical and psychological complications (Zoghbi *et al.*, 2000). Other common aspect to all polyQ diseases is the inverse correlation between the age of onset and the number of CAG repeats, leading to earlier onset when there is a greater number of repeats (Dürr *et al.*, 1996, Maciel *et al.*, 1995, Matos *et al.*, 2011, Ranum *et al.*, 1995, Riess *et al.*, 2008, Zoghbi and Orr, 2000). The polyQ tract is highly polymorphic and the CAG expansion above a certain length threshold confers toxicity (James *et al.*, 2000, Paulson, 1999, Zoghbi and Orr, 2000). Besides the polyQ tract, proteins associated with different disorders share no homologies, being structurally and functionally unrelated to each other (Gatchel *et al.*, 2005, Zoghbi and Orr, 2000). This suggests that the polyQ stretch itself confers toxic properties to these proteins through a "toxic gain of function", thus indicating a possible common mechanism of pathogenesis (Nagai *et al.*, 2007b).

1.1. Spinocerebellar ataxias

Spinocerebellar ataxias (SCAs) are a heterogeneous group of typically late-onset (usually between 30 and 50 years of age) autosomal dominant inherited ataxias and consist on progressive neurodegenerative disorders that evolve to a fatal state (Carlson *et al.*, 2009, Dueñas *et al.*, 2006). These disorders are characterized by variable degrees of degeneration of the cerebellum, often accompanied by changes in the brainstem and spinal tracts. Patients present progressive gait and limb ataxia as the main clinical feature, which is usually accompanied by signs and symptoms that reflect damage beyond the cerebellum and including its afferent and efferent pathways (Paulson, 2009, Schöls *et al.*, 2004). SCAs are rare diseases, whose prevalence estimates vary from 0.3 to 2.0 per 100,000 (van de Warrenburg *et al.*, 2002).

2. Machado Joseph disease/Spinocerebellar ataxia 3

Machado-Joseph disease (MJD), also known as spinocerebellar ataxia type 3 (SCA3), is a polyQ disease whose CAGn trinucleotide repeat expansion is localized in the *MJD1/ATXN3* gene, encoding for a tract of glutamine repeats in the ataxin-3 protein. It was originally described in people of Portuguese Azorean descent by Nakano et al. (1972) who initially named this ataxia as "Machado disease" (Nakano *et al.*, 1972). Later, 3 more families were reported to have the same disease and this led to different designations: "nigro-spino-dendatal degeneration with ophtalmoplegia" (Woods *et al.*, 1972), "Joseph disease" (Rosenberg *et al.*, 1976) and "Azorean disease" (Romanul *et al.*, 1977). Later, it was proposed the name "Machado Joseph disease" for all the previously described clinical entities. It was defined as a single disorder accompanied by an unusually high degree of clinical variability (Coutinho *et al.*, 1978, Lima *et al.*, 1980).

At the moment, MJD is known to be the most common form of autosomal dominant ataxia worldwide (Schöls *et al.*, 2004). It has a higher relative frequency in countries such ias Brazil, Portugal, Singapore, the Netherlands, Germany and Japan, but within each country the geographic distribution is heterogeneous. For instance, in Portugal, MJD is relatively rare in the mainland, but highly prevalent in the Azores Islands, especially within Flores, where the highest worldwide prevalence occurs (Bettencourt *et al.*, 2011, Bettencourt *et al.*, 2008) (Fig. I).

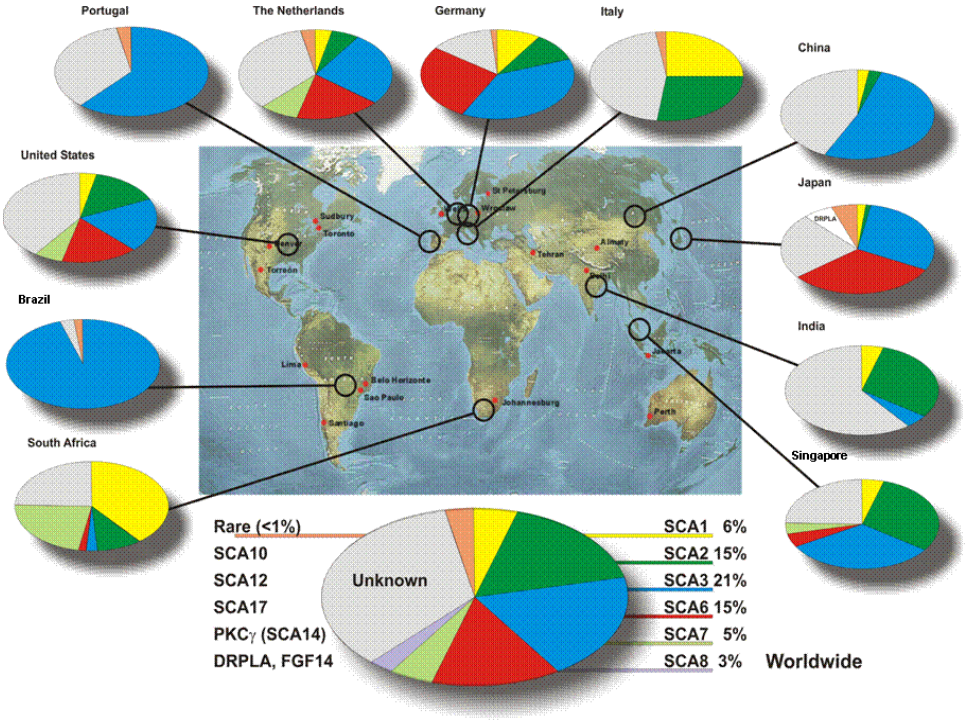


Figure I - Worldwide distribution of SCAs. **Abbreviations:** SCAs, spinocerebellar ataxias. Adapted from (Bird, 1993).

2.1. Molecular/genetics features and pathogenesis

2.1.1. The MJD1/ATXN3 gene

In 1993, Takiyama et al. first mapped the locus responsible for MJD in the long arm of chromosome 14 (14q32.1) and in the next year Kawaguchi et al. cloned the *MJD1/ATXN3* gene, previously mapped, and showed that an unstable CAG repeat expansion motif was present in all MJD patients (Kawaguchi *et al.*, 1994, Takiyama *et al.*, 1993). Later, Ichikawa and colleagues described the genomic structure of MJD gene (Ichikawa *et al.*, 2001). The gene was found to contain 11 exons, spanning about 48 kb, with the (CAG)n tract located at the exon 10. They also reported 4 different species of transcripts, ubiquitously expressed in neuronal and non-neuronal human tissues with different sizes, which were thought to result from differential splicing events of exons 10 and 11 and alternative polyadenylation of exon 11 (Ichikawa *et al.*, 2001). More recently, 2 additional exons - 6a and 9a - were described, and it is now known that a large number of splicing variants exist (about 50), which are generated by 4 types of splicing events occurring in a simple or combined way (Bettencourt *et al.*, 2010).

Currently named as *ATXN3* (but *MJD* and *MJD1* are still in use), this gene encodes for ataxin-3 protein, which contains a polyQ tract at the C-terminus encoded by the CAGn trinucleotide repeat. CAG repeats are unstable, so in healthy population the number range from 10 and 51 repeats, whereas in MJD patients it ranges from 55 to 87 repeats (Bettencourt and Lima, 2011, Kawaguchi *et al.*, 1994, Maciel *et al.*, 2001, Nóbrega *et al.*, 2012). Cases of homozygosity are rare in MJD, but when described patients show a more severe form of disease (Carvalho *et al.*, 2008, Lang *et al.*, 1994).

2.1.2. Ataxin-3 protein

Ataxin-3 is an intracellular deubiquitinating (DUB) enzyme belonging to the group of cysteine proteases, with an overall molecular weight of 42 kDa (Kawaguchi *et al.*, 1994).

This protein is widely expressed in various tissues and different cell types, displaying a ubiquitous expression throughout the brain, though different brain regions present variable levels of expression. Regarding its subcellular localization, ataxin-3 has been reported to exist both in the cytoplasm and in the nucleus, and even in mitochondria, but its localization is predominantly cytoplasmatic (Paulson *et al.*, 1997a, Pozzi *et al.*, 2008, Schmidt *et al.*, 1998, Trottier *et al.*, 1998).

2.1.2.1. Structure and domains

The human ataxin-3 was originally reported to be composed of 339 amino acid residues plus a variable number of glutamine repeats. This protein contains a conserved/structured globular N-terminal Josephin domain followed by an unstructured, flexible C-terminal tail containing 2 or 3 ubiquitin-interaction motifs (UIMs), depending on the protein, which binds polyadenylated proteins with preference for tetraubiquitin or polyubiquitin chain, and the polyQ region of variable length (Albrecht *et al.*, 2004, Burnett *et al.*, 2003, Goto *et al.*, 1997, Kawaguchi *et al.*, 1994, Masino *et al.*, 2003).

A highly conserved, putative nuclear localization signal (NLS) was found upstream of polyQ stretch (Albrecht *et al.*, 2004, Tait *et al.*, 1998). It was also found 2 nuclear export signals, NES77 and NES141 that coincide with the Josephin domain. There is the hypothesis that another NES (NES 174), localized following the Josephin domain, can also influence the nuclear export activity (Albrecht *et al.*, 2004, Antony *et al.*, 2009) (Fig. II).

Figure II - Schematic representation of ataxin-3 protein structure. Ataxin-3 contains a conserved Josephin domain followed by a flexible C-terminal tail containing 2 or 3 UIMs and a polyQ sequence of variable length. One functional NLS was described upstream of polyQ stretch and 3 NES were reported in the ataxin-3. **Abbreviations:** NES, nuclear export signal; NLS, nuclear localization signal; PolyQ, polyglutamine; UIMs - ubiquitin-interaction motifs.

2.1.2.2. Functions

Although the biological function of ataxin-3 has not yet been completely understood, this protein seems to participate in many cellular pathways. There is evidence that ataxin-3 can be involved in many cellular protein quality control mechanisms, operating through the ubiquitin-proteasome system (UPS) (Doss-Pepe *et al.*, 2003), i.e. ataxin-3 can edit the ubiquitin chain production by ubiquitin ligases and trim heavily ubiquitinated species, thus regulating the ubiquitination status of proteins before their presentation to the proteasome. (Paulson, 2012). After interacting with ubiquitined proteins, ataxin-3 interacts with others proteins, participating in substrate delivery to the proteasome (Doss-Pepe *et al.*, 2003).

Ataxin-3 was also associated with the endoplasmic reticulum-associated degradation (ERAD) system, which is responsible for the ubiquitination of misfolded proteins and their

exportation into the cytosol for degradation by the UPS (Albrecht *et al.*, 2004, Burnett *et al.*, 2003, Meusser *et al.*, 2005, Zhong *et al.*, 2006).

As a quality control protein, ataxin-3 has also been reported to regulate the aggresomes formation. Aggresomes are formed by misfolded proteins, which are transported to perinuclear localization, near to the microtubule-organizing center (MTOC). It was shown that endogenous ataxin-3 co-localizes with cytoplasmic aggregates/pre-aggresome particles and associates with components of the complex that transports misfolded proteins to the MTOC, histone deacetylase 6 and dynein. It has been proposed that ataxin-3 is recruited to bind and trim ubiquitin chains on misfolded ubiquitinated proteins, protecting them before they reach the MTOC for aggresome formation or it may use its DUB activity to stabilize proteins involved in the trafficking of misfolded proteins (Burnett *et al.*, 2005).

Ataxin-3 has been also associated with the cellular organization of the cytoskeleton, once in its absence cells display cytoskeletal and adhesion defects, with fewer projections, cytoskeletal disorganization, loss of adhesion and increased cell death; moreover, it can interact with components of the cytoskeleton, such as tubulin, MAP2 and dynein (Mazzucchelli *et al.*, 2009, Rodrigues *et al.*, 2010). Considering myogenesis, ataxin-3 appears to be critical for the regulation of integrin subunit levels and once more for the organization of the cytoskeleton (do Carmo Costa *et al.*, 2010, Mayer, 2003).

Other possible function of ataxin-3 is transcription regulation, as it can bind DNA and interact with transcriptional factors. It has been reported that ataxin-3 interacts with transcription activators and repressors, through its UIM and histones, at the N-terminal region (Evert *et al.*, 2006, Li *et al.*, 2002). In case of transcription activators, ataxin-3 seems to repress their activity by binding them through its C-terminal region. Histones are bonded to the N-terminal region of ataxin-3 and this represses acetylation, since histone acetylation sites are blocked. It was shown that ataxin-3 together with some transcription repressors can decrease histone acetylation and repress transcription (Li *et al.*, 2002, Nicastro *et al.*, 2005). Thus, the involvement of ataxin-3 in transcriptional regulation might be coupled to its DUB activity, since this activity may interfere with the turnover of transcriptional regulators, thereby influencing the repressor complex formation and subsequent activity (Evert *et al.*, 2006, Rodrigues *et al.*, 2007).

2.2. Clinical features

MJD is characterized by progressive ataxia, but ataxia by itself never occurs alone, since this disease involves various systems such as the cerebellar, pyramidal, extrapyramidal,

motor neuron and oculomotor systems. So, beyond cerebellar ataxia that can affect gaze, speech, gait, and balance, the typical signs include: progressive external ophthalmoplegia, dysarthria, dysphagia, pyramidal signs, dystonia, rigidity and distal muscle atrophies (Bettencourt and Lima, 2011, Lima and Coutinho, 1980, Paulson, 2007, Paulson, 2012, Taroni *et al.*, 2004). There is also clinical data showing that a non-motor involvement can also affect MJD patients including sleep disorders, cognitive, affective and psychiatric disturbances, olfactory dysfunction and peripheral neuropathy (Pedroso *et al.*, 2013).

The age of disease onset is highly variable. Though symptom onset usually occurs around 40 years, MJD was already reported either in young people (beginning as early as at 4 years old) or in old people (starting at 70's) (Bettencourt and Lima, 2011, Paulson, 2012).

2.3. Neuropathology

The MJD neuropathology involves neuronal loss in specific brain regions, such as basal ganglia, brainstem and the cerebellum. Dysfunction and degeneration have been observed in the whole cerebellum, cerebellar hemispheres and vermis (spinocerebellar pathways and dentate nucleus), whole brainstem (including midbrain, pons and medulla), basal ganglia (globus pallidus, caudate and putamen), thalamus, *substantia nigra*, *striatum*, pontine nucleus, spinal cord (including anterior horn cells and Clarke's column) and cranial nerves (III-XII), as well as the visual, auditory, vestibular, somatosensory and urination-related systems. Other structures such as cerebral and cerebellar cortex, inferior olive and Purkinje cells are relatively spared. Furthermore, metabolic abnormalities such as axonal dysfunction and glucose utilization deficits in cerebellum, brainstem and cerebral cortex were suggested to occur. Involvement of both the dopaminergic and the cholinergic systems have been also reported (Bettencourt and Lima, 2011, Costa *et al.*, 2012, Coutinho and Andrade, 1978, Dürr *et al.*, 1996, Matos *et al.*, 2011, Nóbrega and de, 2012, Paulson, 2012, Riess *et al.*, 2008, Rub *et al.*, 2008, Schulz *et al.*, 2010, Sudarsky *et al.*, 1995) (Fig. III).

The majority of the brains from MJD patients with advanced disease stage present low weight when compared with brains from individuals without any neurological or psychiatric disease (Iwabuchi *et al.*, 1999, Rub *et al.*, 2007).

A neuropathologic hallmark of MJD is the presence of neuronal intranuclear inclusions (NIIs) in brain, which results from the nuclear accumulation of mutant polyQ proteins. NIIs are spherical and eosinophilic structures and their size vary from 0.7 to 3.7 μm; they are non-membrane bound elements, heterogeneous in composition, and contain a mixture of granular and filamentous structures. Various proteins are found within the inclusion, both

normal and pathogenic ataxin-3, as well as ubiquitin, molecular chaperones, proteasomal components, transcription factors and other polyQ proteins. These NIIs can be present in both affected and unaffected brain regions, and the cytotoxicity of NIIs is controversial (Chai *et al.*, 1999a, Chai *et al.*, 1999b, Paulson *et al.*, 1997b, Schmidt *et al.*, 1998, Schmidt *et al.*, 2002, Yamada *et al.*, 2000). In addition, extranuclear accumulation in MJD brain and neuronal cytoplasmatic inclusions (NICs) containing mutant ataxin-3 have also been reported. These inclusions are composed by fine granules with about 1.5 μm in diameter, ubiquitin-negative and exhibit a similar distribution as the NIIs (Hayashi *et al.*, 2003, Yamada *et al.*, 2008, Yamada *et al.*, 2004, Yamada *et al.*, 2002).

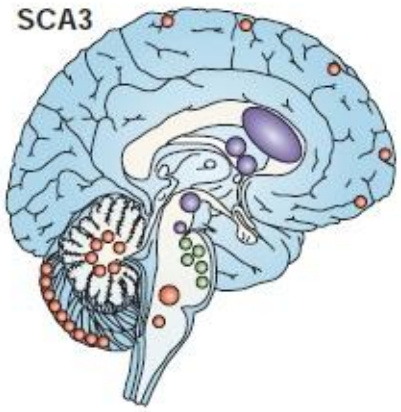


Figure III - Pathological features of SCA3. Schematic representation indicating the principal sites of neuronal loss and organ dysfunction in SCA3. Large dots indicate severe neuronal loss. Blue dots indicate involvement of extrapyramidal nuclei. Green dots indicate cranial nerve involvement. **Abbreviations:** SCA3, spinocerebellar ataxia type 3. Adapted from (Taroni and DiDonato, 2004).

2.4. Pathogenesis

The molecular basis of MJD is still poorly understood. However, several cellular and molecular mechanisms have been proposed for MJD pathology. It has been observed that expansion of the polyQ tract in ataxin-3 affects its properties through a conformational change, acquiring toxic properties, which can result in altered molecular interactions. The common feature among polyQ diseases is the presence of neuronal inclusions (NIs) containing the mutant protein, thus suggesting that misfolded proteins are pathological features. The abundance of these neuronal inclusions has been correlated to the CAG repeat size and disease severity and it has been proposed that they could also impair axonal transport and nuclear function (Nóbrega and de, 2012, Paulson *et al.*, 1997b). Moreover, NIs may compromise the recruitment of several key proteins such as transcription factors, proteasomal components and chaperones, thus affecting various cellular pathways (Chai *et*

al., 1999a, Chai *et al.*, 1999b, McCampbell *et al.*, 2000, Paulson *et al.*, 1997b, Schmidt *et al.*, 2002). Besides NIs, it has been shown that polyQ monomers of ataxin-3 acquire β-strand conformations and later assemble into oligomers. These β-rich ataxin-3 monomers and oligomers may be toxic for cells in MJD (Bevivino *et al.*, 2001, Nagai *et al.*, 2007a, Takahashi *et al.*, 2008).

It has been reported that the proteolytic cleavage of expanded polyQ ataxin-3 results in smaller toxic fragments containing an expanded polyQ tract, facilitating the entry from cytoplasm into the nucleus (Haacke *et al.*, 2006, Haacke *et al.*, 2007, Simões *et al.*, 2014) and contributing for aggregation and cytotoxicity (Simões *et al.*, 2012).

Mutant ataxin-3 could also contribute for the impairment of cellular quality control system, impairment of axonal transport, transcription deregulation, mitochondrial dysfunction (it was reported that mutant ataxin-3 decreases the activity of antioxidant enzyme causing damage of mitochondrial DNA); oxidative stress and calcium homeostasis deregulation (Costa and Paulson, 2012, Nóbrega and de, 2012, Yu *et al.*, 2009).

Another important aspect is neuroinflammation. It was demonstrated an upregulation of some inflammatory genes, such as the pro-inflammatory cytokine interleukin-1 β, when mutant ataxin-3 was present, suggesting that glia might also contribute to pathogenesis (Evert *et al.*, 2001, Evert *et al.*, 2003)(Fig.IV).

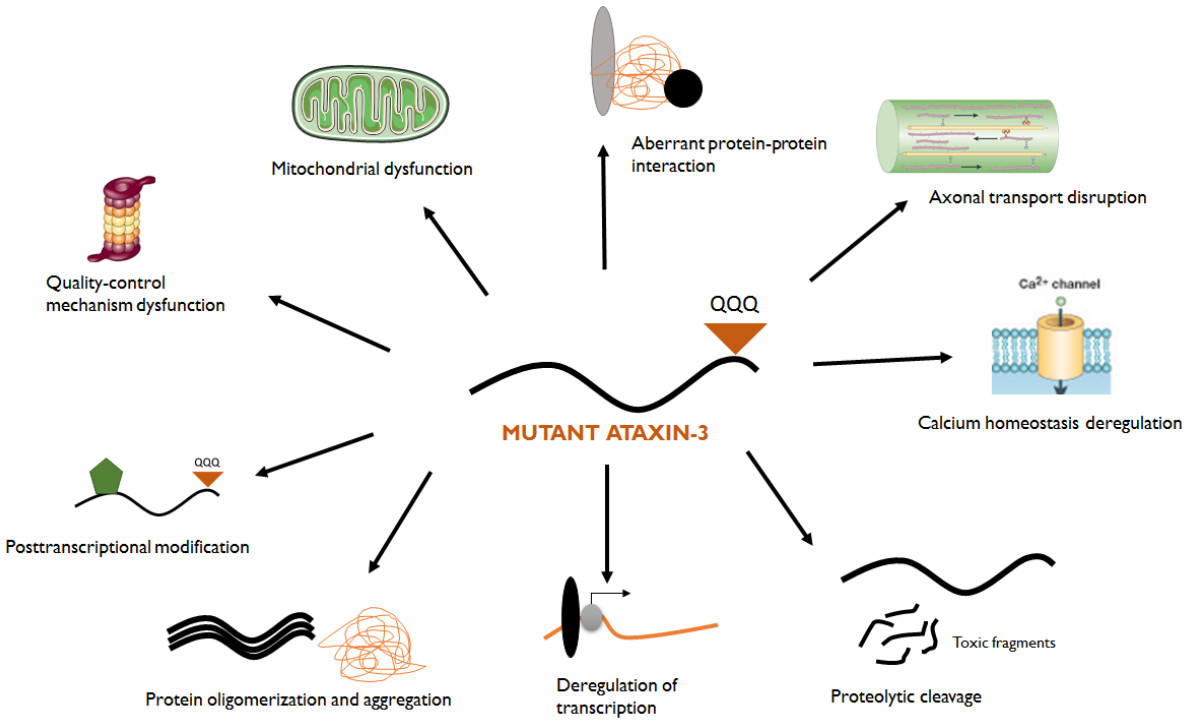


Figure IV - Mechanisms of pathogenesis in MJD. **Abbreviations:** MJD, Machado Joseph disease. Adapted from (Nóbrega and de, 2012)

3. Multipotent mesenchymal stromal cells

Multipotent mesenchymal stromal cells (MSC) are multipotent adult stem cells (Caplan, 1991) that were originally found in the stroma of bone marrow by Alexander Friedenstein in 1970s. He and his colleagues described it as a rare population of plastic-adherent cells (constituting only 0.01-0.001% of the total nucleated cells in the bone marrow) with a fibroblast-like appearance that could generate colonies called as CFU-Fs ("colony forming unit fibroblastic cells") (Friedenstein *et al.*, 1970, Friedenstein *et al.*, 1974).

These cells can also be isolated from other type of tissues, such as brain, liver, bone and adipose tissue, skeletal muscle, teeth, pancreas, lung, amniotic fluid, hair follicle, or umbilical cord, and they have been isolated from many species including human, mouse, rat, dog, and horse (Drela *et al.*, 2013, Rastegar *et al.*, 2010). In the bone-marrow, MSC are included into the stromal system and their functions are: to maintain a certain level of self-renewal, give rise to cells that can differentiate into various connective tissue lineages, produce extracellular matrix proteins and additional soluble molecules to promote haematopoiesis support. It is likely that MSC serve cellular functions similar to pericytes that surround bone marrow sinusoids (Bianco *et al.*, 2001, Deans *et al.*, 2000, Parekkadan *et al.*, 2010) (Fig. V).

Several names for the MSC have been reported, such as "CFU-F", "stromal stem cells", "marrow stromal cells", and "mesenchymal stem cells". To clarify the nomenclature of the MSC, the committee of the International Society for Cellular Therapy proposed that MSC should be termed "multipotent mesenchymal stromal cells" and "mesenchymal stem cell" should be reserved for cells that demonstrate stem cell activity (long-term self-renewing cells that are capable of differentiation into specific, multiple cell types from different lineages *in vivo*). The terminology should also include the source of MSC, for instance adipose-derived MSC, bone marrow-derived MSC, etc. (Horwitz *et al.*, 2005).

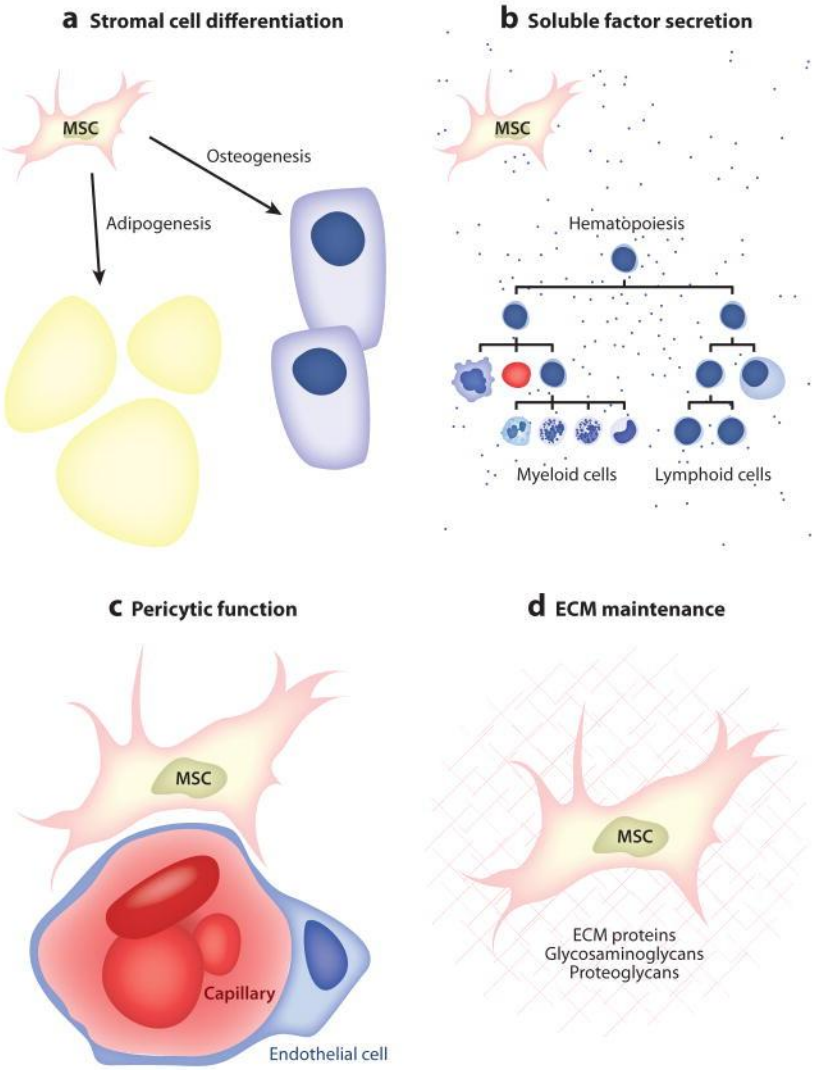


Figure V - Natural functions of MSC in the bone marrow. **Abbreviations:** ECM, extracellular matrix; MSC, multipotent mesenchymal stromal cells. Adapted from (Parekkadan and Milwid, 2010).

3.1. Phenotypic characterization and other key features

MSC are primitive cells originated from the mesodermal germ layer, characterized by their self-renewal potential and their potential for differentiation into multiples mesenchymal lineages, such as bone, adipose tissues, cartilage, tendon, muscle and other connective tissues (Baksh *et al.*, 2004, Caplan *et al.*, 2006, Salem *et al.*, 2010) (Fig VI).

Traditionally, MSC present a spindle-shaped, but it is know that there is MSC heterogeneity either in their morphology, containing cells ranging from narrow spindle-shape to large polygonal cells, or in terms of cell size, multipotency and marker expression (Chamberlain *et al.*, 2007, Mabuchi *et al.*, 2013).

Regarding phenotypic MSC characterization, there is lack of a unique marker to characterize these cells. Thus, The Tissue Stem Cell Committee of International Society for

Cellular Therapy proposed 3 minimum criteria to define human MSC: **1) plastic adherence** - when maintained in standard culture conditions; **2) specific surface antigen expression** - ≥95% of the MSC must be positive for the expression of CD105 (endoglin), CD73 (ecto-5'-nucleotidade), and CD90 (Thy-1 thymocyte differentiation antigen-1); and must lack expression (5/2% positive) of hematopoietic cell surface markers, CD45 (leukocyte common antigen), CD34 (primitive hematopoietic progenitors and endothelial cells antigen), CD11b or CD14 (monocytes and macrophages antigen), CD19 (B cells antigen), and HLA (human leukocyte antigen) or MHC (major histocompatibility) class II; **3) multipotent differentiation potential** - MSC must be able to differentiate into osteoblasts, adipocytes and chondroblasts (Dominici *et al.*, 2006).

Nonetheless, MSC isolated from other species do not express the same markers as those of human cells. For instance, CD34 is rarely expressed in human and rat MSC but their expression is variable on murine MSC. Furthermore, there are differences among murine MSC from different strains, for example in CD34, Sca-1 (stem cell antigen 1), and CD106 or VCAM-1 (vascular cell adhesion molecule 1) expression (Chamberlain *et al.*, 2007, Kolf *et al.*, 2007, Peister *et al.*, 2004). Nonetheless there is a list of markers that are more commonly used to characterize murine MSC isolated from C57BL/6 mice such as CD29, CD44, CD73, CD105, CD106, CD44, and Sca-1 (Baddoo *et al.*, 2003, Mabuchi *et al.*, 2013, Morikawa *et al.*, 2009, Sun *et al.*, 2003).

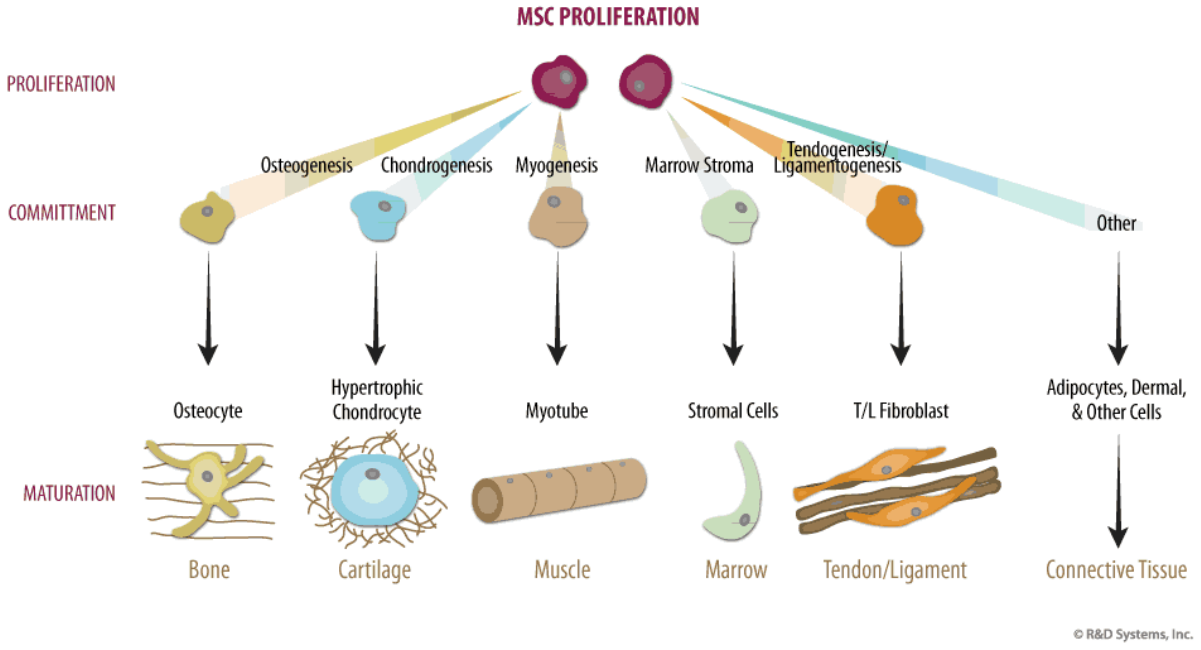


Figure VI - MSC have the potential to differentiate into a wide range of cell types mesodermal lineages. **Abbreviations:** MSC, multipotent mesenchymal stromal cells. Adapted from (Anissa S.H. Chan, 2007).

3.2. Localization, Homing and Trafficking

There is evidence that MSCs are not restricted to the bone marrow, but otherwise are localized in niches within various tissues. It has been proposed that MSC are identical to or are related to pericytes, because they share surface and intracellular protein expression patterns and differentiation capacity features. Pericytes are mural cells, with branched shape, located on the abluminal side of small blood vessels (arterioles, capillaries and venules) and they are connected with endothelial cells. This proximity to vessel would allow pericytes to quickly enter the bloodstream; thus, this perivascular localization of MSC within niches throughout the body allow them to easily access to all tissues and promote repair of local small lesions (Augello *et al.*, 2010, Kolf *et al.*, 2007, Parekkadan and Milwid, 2010, Paul *et al.*, 2013, Salem and Thiemermann, 2010).

MSC have a unique homing capacity, targeting injured or/and inflamed tissues. This process consists on cells migration to and engraftment in the injured tissues in which they will exert protective effects (Salem and Thiemermann, 2010). The exact mechanism by which MSC home to specific tissues is not yet fully understood, but it has been proposed that this mechanism is identical to the multistep process by which leukocytes migrate to inflamed sites, since MSC express some adhesion molecules in common with leukocytes, such as CD44 (homing cell adhesion molecule), CD49a-f (very late antigen-α1-6), CD29 (very late antigen-β chain) and CD18 (leukocyte function-associated antigen-1β chain) (Kavanagh *et al.*, 2014, Salem and Thiemermann, 2010, Sohni *et al.*, 2013). Homing is dependent on chemokines and chemokine receptors expressed by injured tissues; since MSC express various receptors for these chemokines, they answer to the gradient of inflammatory chemokines and home to the inflammation site (Salem and Thiemermann, 2010).

3.3. MSC application for neurodegenerative diseases

In the last years, MSC have been demonstrating promising results in regenerative medicine including in neurodegenerative diseases. There are many reasons why MSC have been used in cell therapy: they are easily isolated and can be expanded *ex vivo* to clinical scales in a relative short period of time. Besides that, they can be cryopreserved with minimal loss of potency and used later in transplants, allowing their use in the real clinical world (Parekkadan and Milwid, 2010, Teixeira *et al.*, 2013). Moreover, it has been reported that MSC possess low immunogenicity, allowing their use in both autologous and allogeneic transplants. They are also considered non tumorigenic, as they have been used in many in vivo

transplantation procedures without tumour formation (Parekkadan and Milwid, 2010, Sadan *et al.*, 2009). In addition to their safety features, MSC can home to injured tissues and secrete chemokines and growth factors among other molecules, which help the tissues to recovery from the lesion (Chamberlain *et al.*, 2007, Sohni and Verfaillie, 2013).

3.3.1. Possible mechanism of action

Several mechanisms have been proposed to explain the positive MSC effect in neurodegenerative diseases. The first mechanism is based on the transdifferentiation concept (differentiation of MSC into cells from the neural lineage), allowing the direct replacement of damaged cells. Another possible mechanism is the fusion between MSC and local endogenous neural cells in which MSC acquire the phenotype features of neural cells. The last possible mechanism is MSC secretion of soluble factors that stimulate resident neural cells to repair and/or enhance neurogenesis (Drela *et al.*, 2013, Maltman *et al.*, 2011) (Fig.VII).

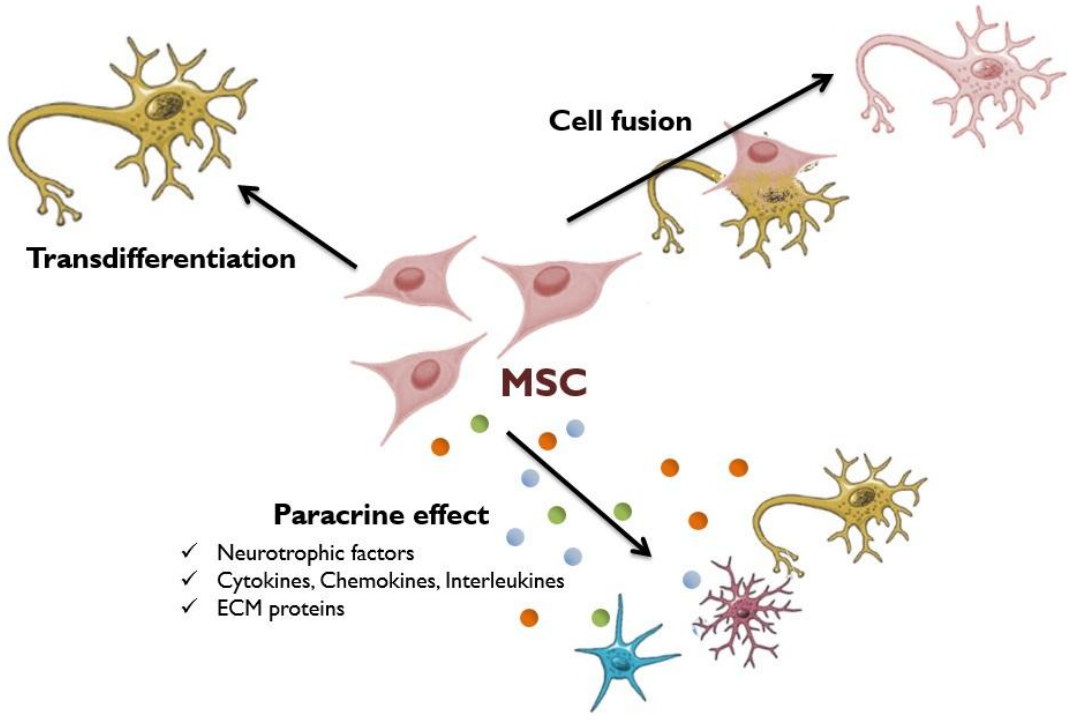


Figure VII - Possible mechanisms of MSC in neurodegenerative diseases. 1) Transdifferentiation – differentiation of MSC into neural/glial cells; 2) Paracrine effect – secretion of neurotrophic factors, cytokines, chemokines, interleukines and ECM proteins, which affect neuronal cells; 3) Cell fusion – fusion between MSC and neuronal cells, acquiring the neural phenotype. **Abbreviations:** ECM, extracellular matrix; MSC, multipotent mesenchymal stromal cells.

3.3.1.1. Transdifferentiation

It has been demonstrated that some adult stem cells are capable to differentiate into many cell types and tissue lineages of different germ layers from the one in which these stem cells reside. This plastic ability is commonly called "transdifferentiation" (Krabbe *et al.*, 2005). MSC have also been reported as being able to differentiate into cells of both endodermal (hepatocytes) and ectodermal lineages (neural cells) beyond their mesodermal origin, when in the presence of specific growth factors and very defined conditions (Fernández Vallone *et al.*, 2013).

There are many reports referring different *in vitro* protocols showing MSC transdifferentiation into neuronal and/or glial cells using chemical induction (supplementation of differentiation media with simple or cocktails of small molecules or chemicals), treatment with various growth factors and neurotrophic molecules, gene transfection with specific gene expression, or co-culture of MSC with neural cells (direct cell-cell interaction and/or interplay by soluble factors) (Maltman *et al.*, 2011, Ries *et al.*, 2012).

Although various reports demonstrated that MSC can give rise to neural-like cells - since they adopt the morphology of neurons, such as retractile cell bodies and long branching processes, and express both immature and mature neuronal markers, under differentiating conditions (Jiang *et al.*, 2010, Tropel *et al.*, 2006) - there are some doubts over MSC differentiation along neural lineage that result from specific in vitro differentiation protocols. The neuronal differentiation is a complex process that *in vivo* requires the temporal and spatial regulation of a wide array of genes and it is unlikely that it can occur in a short time, as is reported in various studies using the chemical induction (Lu *et al.*, 2004, Maltman *et al.*, 2011, Ries and Egea, 2012, Zurita *et al.*, 2008).

Furthermore, it has been described that MSC have predisposition to express neural markers. In basal conditions, undifferentiated MSC can already express neuronal genes, such as nestin, microtubule associated protein 1b (MAP1b), neuronal nuclei (NeuN), neuron-specific enolase, and β-III tubulin. This fact may contribute to the controversy about the differentiation capacity of MSC or may explain why it can be thought that MSC integrate the central nervous system (CNS) and differentiate into neural and/or glial cells when exposed to the neuronal environment (Blondheim *et al.*, 2006, Deng *et al.*, 2006a, Montzka *et al.*, 2009).

As conclusion, morphological features and neuronal marker expression analysis is not enough to evaluate MSC differentiation toward neural cells *in vitro*. Besides that, it is

important to simultaneously assess functional electrophysiological parameters and synaptic transmission (Jin *et al.*, 2003, Krabbe *et al.*, 2005, Wislet-Gendebien *et al.*, 2005).

In vivo MSC differentiation into neural and/or glial cells has also been reported. According to these studies, MSC injected into the CNS are able to survive, engraft and migrate. It has been reported that transplanted MSC express neural markers, leading to think that transdifferentiation can be the mechanism responsible for functional improvement exerted by MSC (Azizi *et al.*, 1998, Kopen *et al.*, 1999). However, *in vivo* transdifferentiation is unlikely to occur and it is not probable that this mechanism would be responsible by its one for the beneficial effect of MSC. Besides the number of MSC which engraft and express this neural markers being very low (Deng *et al.*, 2006b), these studies commonly describe transdifferentiation by immunohistochemical protocols, which is doubtful, since transplanted cells can already express this markers *in vitro*, under basal conditions. It is then essential to evaluate functional integration of MSC in the neural tissue and as a proof of concept, elucidate the mechanism by which transdifferentiation would occur (Maltman *et al.*, 2011).

3.3.1.2. Cell Fusion

Another alternative mechanism proposed for MSC's action is spontaneous fusion between transplanted MSC and host cells, although only a few studies have demonstrated this phenomenon.

Specifically considering the case of neurodegenerative diseases affecting the cerebellum, it was reported that upon human MSC intravenous (IV) injection into mice with experimental autoimmune encephalomyelitis (EAE) these cells migrate to the cerebellum and fuse with Purkinje cells. It was however reported that these fusion events are increased in the case of EAE, resulting as a response to neuroinflammation and stress microenvironment (Kemp *et al.*, 2011). In another study, the injection of bone marrow-derived MSC (BM-MSC) into the right retro-orbital sinus in a mouse model of SCA1, showed that MSC can be detected in the cerebellum and can fuse with host Purkinje neurons. Besides that, it was demonstrated that mice treated with BM-MSC presented a decrease in the number of nuclear inclusions, demonstrating the beneficial potential effects of MSC in this particular disorder (Chen *et al.*, 2011).

However, spontaneous cell fusion was reported as a phenomenon with very low frequency (2–11 clones per 10^6 bone marrow cells) (Terada *et al.*, 2002). Thus, although this is a possible mechanism by which MSC mediate neural functional recovery, its contribution is likely to be minor (Maltman *et al.*, 2011).

3.3.1.3. Paracrine effect

There is significant evidence that MSC secrete bioactive factors (including extracellular vesicles and soluble factors), which are associated with their regenerative effect. These molecules confer MSC their unique capacity to interact with cells from the immune system and/or directly or indirectly stimulate endogenous repair mechanisms (Dorronsoro *et al.*, 2013, Lavoie *et al.*, 2013, Paul and Anisimov, 2013) and include: neurotrophic factors, chemokines, interleukins or cytokines, and extracellular matrix proteins (Maltman *et al.*, 2011, Paul and Anisimov, 2013).

The neurotrophic factors that are most commonly secreted by MSC are: the proteins nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and ciliary neurotrophic factor (CNTF) (Chen *et al.*, 2005). These molecules/factors have been described as being involved in crucial mechanisms such as neuronal proliferation, survival and differentiation, neuritogenesis, and protection against apoptosis (Teixeira *et al.*, 2013). Additionally, several ILs such as IL-6, -7, -8, -11, -12, -14 and 15, have been reported as important elements during the neural development. Other molecules similarly associated to MSC are extracellular matrix proteins, including collagen, laminin and fibronectin (Nakayama *et al.*, 2003). These have been showed to be an important support for neurogenesis and play a direct trophic role in the neural niche (Maltman *et al.*, 2011).

On the other hand, MSC present a unique immunomodulatory capacity; they are capable of inducing immunosuppressive effects in innate and adaptive immune cells and to modulate the inflammatory response mediated by the action of ILs, chemokines, chemokine receptors or cytokines (Dorronsoro *et al.*, 2013, Kassis *et al.*, 2011). MSC were found to interact with almost all populations of immune cells: T and B cells, natural killer cells (NK), regulatory T cells (Treg) and dendritic cells (DC) (Dorronsoro *et al.*, 2013, Kassis *et al.*, 2011, Nauta *et al.*, 2007, Uccelli *et al.*, 2006).

Lastly, a special attention has been recently given to the secretion of exosomes by MSC. Exosomes are microvesicles that are released to the extracellular space which can contain, among other molecules, proteins and RNAs (Yu *et al.*, 2014). It is known that MSC can produce higher amount of exosomes than other cell types and because of that, they were suggested to become a good tool for drug delivery or to facilitate gene and cell therapy. However, this approach is still unexplored and untested (Lai *et al.*, 2013, Yu *et al.*, 2014).

In conclusion, the secretory features of MSC can be used to promote functional recovery of CNS injury or inflammation, making them an excellent candidate for neurodegenerative disease therapies.

4. MSC application for PolyQ diseases

MSC therapy has been commonly used in some polyQ disorders, namely in HD and SCAs.

Lin et al. used human BM-MSC (hBM-MSC) to investigate their therapeutic potential in an HD mouse model induced by quinolic acid injections in the striatum. HD mice were then transplanted in the striatum with hBM-MSC. Cells were capable to engraft into the compromised striatum, reduce the motor function impairment and increase mice survival rate, since BM-MSC-treated animals presented improvements in striatal volume, rotarod performance and surviral rates compared with non-treated controls. These authors also tested BM-MSC transplantation in R6/2-J2, a genetically-modified animal model of HD, and obtained similar outcomes, concerning the survival rates and striatum volume (Lin *et al.*, 2011).

In another study, rat BM-MSC were transplanted in the striatum after quinolinic acid injection in Wistar rats. The authors demonstrated that BM-MSC could be detected until 60 days after implantation in the injured striatum and they could reduce striatal neurodegeneration and decrease ventriculomegaly, demonstrating that MSC may be a promising therapy in HD (Moraes *et al.*, 2012).

Furthermore, Rossignol et al. compared the transplantation of BM-MSC, adult neural stem cells (aNSC), or co-transplantation of MSC and aNSC in a transgenic rat model for HD. They showed that either adult stem cells by itself or co-transplantation could retard the progression of motor deficits (demonstrated through rotarod test). It was also demonstrated that both MSC and aNSC could engraft as the cells were detected in the site injection, but the graft area was bigger in the case of the MSC-treated group. Interestingly, the co-transplantation enhanced the survival of the aNSC graft, suggesting that MSC can create a microenvironment capable to prolong aNSC survival and provide greater behavioural improvements (Rossignol *et al.*, 2014).

As shown above, the outcomes obtained in HD are very encouraging, as the results demonstrated that MSC can ameliorate the deficits present in HD mouse models.

4.1. MSC therapy in ataxias

In ataxias, MSC's effect is equally promising, as there have been reports suggesting safety and delay in disease progression in some SCAs, namely SCA 1 (Matsuura *et al.*, 2014), 2 (Chang *et al.*, 2011) and 3 (Jin *et al.*, 2013). Preclinical experiments demonstrated that MSC transplantation could alleviate motor function deterioration in SCAs. In a recent study, the authors observed an improvement in rotarod performance and neuroprotective effects on cerebellar Purkinje cells in hBM-MSC transplanted SCA2 transgenic mice. In this study, they compared intracranial (IC) and IV transplantation of hBM-MSC and concluded that IV was more effective than IC transplantation, as IC treatment did not demonstrate improvements in the rotarod performance (Chang *et al.*, 2011). Moreover, in another study where the authors used SCA1 transgenic mice, intrathecal injection of mouse BM-MSC suppressed the disorganization of the Purkinje cell layer and the atrophy of dendritic arborization, and treated mice presented better performances in rotarod than non-treated mice, reaching similar performances to wild type animals, which reveals a great improvement in motor coordination (Matsuura *et al.*, 2014).

In another report, Edalatmanesh and col. transplanted rat BM-MSC into Wistar rats after performing a unilateral lesion in the cerebellum with quinolinic acid, which affects memory and motor coordination. It was shown that MSC could alleviate the cerebellar dysfunction, decreasing the functional deficits as well as the neuronal loss in the injured area (Edalatmanesh *et al.*, 2011). Additionally, Jones et al. also reported the positive effects of MSC transplantation in cerebellar ataxias. The authors demonstrated that BM-MSC cells were capable of integrating into the cerebellar tissue, and could support survival of Purkinje cells, and ultimately improve the motor functions of mutant mice, evaluated by rotarod performances (Jones *et al.*, 2010). In another study, the authors used hMSC isolated from neonatal umbilical cord and transplanted them into a model of ataxic mice induced by cytosine beta-Darabinofuranoside (Ara-C). Zhang et al. showed that MSC can alleviate motor impairments and cerebellar atrophy, since treated mice presented better performances in rotarod and open field tests than non-treated mice; additionally animals exhibited increments on both body weight and cerebellar volume, and proliferation of Purkinje cells and internal granular layer cells (Zhang *et al.*, 2011).

To further support MSC therapeutic potential, several either completed or ongoing clinical trials have been using MSC for neurological disease therapies. According to the EU Clinical Trials Register, there are only 2 clinical trials using MSC presently ongoing in the European Union (EU) or/and the European Economic Area (EEA): 1 for multiple sclerosis, and

another for amyotrophic lateral sclerosis, and there is no registry of clinical trials in cerebellar ataxias (www.clinicaltrialsregister.eu). Nonetheless, in others parts of the world (namely in Taiwan, India and China) a few clinical trials have been evaluating the therapeutic potential of MSC in some SCAs, including MJD/SCA3 (www.clinicaltrials.gov; ClinicalTrials.gov Identifiers: NCT01489267, NCT01958177, NCT01360164, NCT01649687).

In the General Hospital of the Air Force (Beijing, China) the safety and effectiveness of umbilical cord MSC (UC-MSC) intrathecal transplantation in patients with SCA and multiple system atrophy-cerebellar type C (MSA-C) was evaluated. The study recruited a total of 24 patients, 10 diagnosed with MSA-C and 14 with SCA, and they were injected with both UC-MSC and dexamethasone intrathecally weekly, for 4 consecutive weeks (all patients received 1 course of treatment, except 3 who received 2). In this trial it was demonstrated that patients' symptoms, including unstable walking and standing, slow movement, fine motor disorders of the upper limbs, writing difficulties and dysarthria were improved, and the scores of International Cooperative Ataxia Rating Scale (ICARS) and Activity of Daily Living Scale (Haacke *et al.*) were decreased 1 month after the treatment. However, non-treated patient were not included in this study. This treatment did not report serious side effects, besides dizziness after the lumbar puncture, lumbago, and headache, which disappeared 1 – 3 days after MSC delivery without any special treatment (Dongmei *et al.*, 2011).

Likewise, Jin et al. reported a study that assessed the efficacy and potential toxicity of human umbilical cord MSC (hUC-MSC) therapy in patients genetically diagnosed with SCA1, SCA2, or SCA3 (ClinicalTrials.gov Identifier: NCT01360164). Sixteen patients receiving 4 UC-MSC treatments at 1week intervals were included. The first treatment consisted only on the IV injection of UC-MSC ($4x10^7$ cells), whereas the other 3 treatments included IV ($2x10^7$ cells) and intrathecal ($2x10^7$ cells) injection of UC-MSC. The majority of patients (63%) showed significant improvements on the Berg balance scale (BBS) scores (used to evaluate the balance of patients) from baseline, at 3 and 6 month after UC-MSC treatment. Similar results were obtained with ICARS, where patients presented amelioration of neural function at the third and sixth months after the treatment. Regarding side effects, no serious transplant-related adverse events were reported in 12 months of follow-up. This study suggested that UC-MSC transplantation is safe and might delay the progression of SCA (Jin *et al.*, 2013).

As conclusion, MSC have been proving to be a very promising therapy in SCAs. However, for most of the SCAs, and particularly in MJD, pre-clinical studies evaluating the potential of MSC are lacking. Furthermore, studies in human patients do not allow assessing for direct neuropathology evaluation prior and after MSC delivery. As such, it would be

crucial to study MSC cellular therapy in MJD models prior to its use in the clinics. In the present study, a MJD transgenic mouse model was used to investigate whether MSC can alleviate both motor behaviour and neurodegeneration in this fatal disorder.

Thereby, the main aim of our study was to investigate the *in vivo* therapeutic relevance of MSC for MJD therapy. Similarly to what it has been shown in other SCAs, we expect to provide evidences that MSC can become a valuable therapy for MJD.

Objectives

The main aim of the present work was to investigate the *in vivo* therapeutic potential of MSC for MJD therapy. For that, the specific aims were as follows:

- isolate and characterize a population of mouse MSC prior to transplantation;
- analyse whether MSC can engraft when injected stereotaxically in the cerebellum of a MJD transgenic mouse model (Torashima *et al.*, 2008);
- investigate whether MSC can alleviate MJD motor behaviour in MJD transgenic mice;
- evaluate whether MSC transplantation can rescue the neuropathology in MJD transgenic mice.

Scientific Paper

In preparation

Cerebellar transplantation of MSC alleviates motor impairments and neuropathology in a transgenic mouse model of Machado Joseph disease

Teresa Silva[1,2], Catarina Miranda[1], Clévio Nóbrega[1] and Luís Pereira de Almeida[1,2]

[1] CNC - Center for Neuroscience and Cell Biology, University of Coimbra, 3004- 504 Coimbra, Portugal

[2] Faculty of Pharmacy, University of Coimbra, Portugal

Correspondence to:

Professor Luís Pereira de Almeida,

CNC - Center for Neuroscience and Cell Biology and Faculty of Pharmacy,

University of Coimbra,

Rua Larga, FMUC, 2nd Floor, Office 212

3004-504 Coimbra, Portugal

E-mail: luispa@ci.uc.pt or luispa@cnc.uc.pt

Abstract

Machado Joseph disease or Spinocerebellar ataxia type 3 is the most common SCA worldwide, caused by an expanded CAG repeat in the *MJD1* gene, which translates into a polyQ tract within the ataxin-3 protein. Currently, there is no therapy able to modify or delay disease progression. Multipotent mesenchymal stromal cells (MSC) are extremely promising tools for therapy of neurodegenerative disorders and, more recently, this therapeutic approach became motif of interest with respect to cerebellar disorders, such as spinocerebellar ataxias (SCAs).

In the present study, we investigated whether the stereotaxic transplantation of MSC into the cerebellum of MJD transgenic mice can produce a neuroprotective effect, allowing rescue of the extremely severe phenotype of this mouse model. We found that MSC could engraft in the cerebellum and mediate a significant improvement of motor coordination. Furthermore, MSC alleviated the neuropathology of transgenic MJD mice, preserving the Purkinje cells and both molecular and granular layers. Therefore, our results suggest that MSC can become a strong candidate for disease-modifying MJD therapies, so far inexistent.

Keywords: Spinocerebellar ataxia type 3; Machado Joseph disease; Multipotent mesenchymal stromal cells; transplantation; MJD transgenic mice.

1. Introduction

Machado Joseph disease (MJD), also known as Spinocerebellar ataxia type 3 (SCA3), is an autosomal dominant neurodegenerative disorder reported as the most common SCA worldwide (Bettencourt and Lima, 2011). This disorder is caused by the over repetition of the CAGn trinucleotide in *MJD1* gene, translating into an expanded polyQ tract in the ataxin-3 protein. As result, ataxin-3 acquires an abnormal conformation with more propensity to aggregate, which triggers a degenerative process and accumulates forming neuronal intranuclear inclusions (Nóbrega and de, 2012, Paulson *et al.*, 1997b). Neuropathologically, not only the cerebellum is affected, but also other regions such as the *substantia nigra*, brainstem and the *striatum*. The clinical hallmark of MJD is the progressive gait and limb ataxia (Nóbrega and de, 2012).

Despite recent progresses regarding MJD putative therapies (Alves *et al.*, 2010, Nascimento-Ferreira *et al.*, 2013, Nóbrega *et al.*, 2013, Simões *et al.*, 2014), there is no effective therapy for MJD patients. Nonetheless, MSC have been reported as a promising therapy for several neurodegenerative disorders, including multiple sclerosis, amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease and HD (Trzaska *et al.*, 2008). Furthermore, MSC have already been used in ataxias either in pre-clinical and clinical studies and there is some evidence that this approach can become an effective therapy for SCAs (Chang *et al.*, 2011, Dongmei *et al.*, 2011, Jin *et al.*, 2013, Matsuura *et al.*, 2014). It has been also demonstrated that MSC are safe, as they are considered to be non-tumorigenic and have low immunogenicity, allowing both for autologous and allogeneic transplants (Parekkadan and Milwid, 2010, Sadan *et al.*, 2009).

MSC are multipotent adult stem cells with fibroblast-like appearance that can self-renew and differentiate into mesenchymal lineage cells, such as osteoblasts, adipocytes, chondrocytes and myocytes (Caplan, 1991, Friedenstein *et al.*, 1970). They are easily isolated from several tissues, namely from the bone marrow where they play an important function in the haematopoiesis support (Parekkadan and Milwid, 2010). Besides that, they exhibit unique therapeutic characteristics. They can "sense" the environment created by the "signals" emitted by the injured tissues and/or immune cells and then home to the lesion sites, where they secrete growth factors, ILs or cytokines, and extracellular matrix proteins thus exerting immunomodulatory and protective effects to promote the functional recovery of injury and/or inflammation (Salem and Thiemermann, 2010).

The evidence reported in the literature suggests that MSC could be an efficient approach for the correction of SCAs, particularly for MJD. Therefore, in this study we aimed at

evaluating whether MSC transplantation would alleviate the phenotype in MJD transgenic mice model and would improve the MJD associated neuropathology.

2. Materials and methods

2.1. MSC isolation and expansion

MSC were isolated from bone marrow of 6- to 8- weeks-old wild type mice with C57BL/6 background of both genders. For isolation, femurs and tibias were collected and transferred to culture medium Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100U/ml penicillin and 100µg/ml streptomycin (Invitrogen) in a sterile petri dish. Then, the contents of marrow was flushed with medium and collected into a sterile petri dish. The cell suspension was homogenized with a 20G syringe, centrifuged and the pellet resuspended in DMEM (not supplemented). Finally, mononuclear cells were separated in a Ficoll (Sigma) density gradient and resuspended in DMEM/F12 (Gibco) supplemented with 10% FBS, 100U/ml penicillin and 100µg/ml streptomycin (Gibco), 20 ng/ml epidermal growth factor (EGF, PeproTech), 20 ng/ml fibroblast growth factor (FGF, PeproTech) and 2% B-27 (Gibco) and were incubated at 37°C in 5% CO2/air atmosphere (Fig. S1-a). Forty-eigth hours later, nonadherent cells were removed and adherent cells were expanded for approximately 2-3 weeks, with medium changes every 2-3 days, until sub-confluence was reached. To eliminate hematopoietic contaminants in MSC cultures (Javazon *et al.*, 2004, Phinney *et al.*, 1999), the elimination of the CD45+ cells was performed by cell sorting (Fig. S1-b). MSC were expanded in the conditioned medium described above and frozen in FBS with 10% DMSO for later usage.

2.2. Flow cytometry

MSC were displaced by trypsinization, passed through a 70 µm filter (BD Falcon) and single cell suspension was incubated in 3% bovine serum albumin (BSA) for 15 min at 4°C to block non-specific binding of the primary antibody and labelled with specific antibodies (eBioscience) for 30 min at room temperature (RT) in the dark to analyse the expression of the following markers: fluorescein isothiocyanate (FITC) conjugated anti- mouse CD45 and anti-mouse CD106 (VCAM-1), phycoerythrin (PE) conjugated anti-mouse CD105 (endoglin),

anti-mouse/rat CD29 (integrin beta 1, Iß-1), anti-mouse CD73, and PE-Cy7 conjugated anti-mouse Ly-6A/E (Sca-1). For every condition, the appropriate isotype control antibodies (eBioscience) were used as negative controls. Moreover, every marker were analysed either alone or in double staining protocols (using 2 markers with different conjugated antibodies) in order to exclude acquisition of non-specific staining. Acquisition was done in a FacsCalibur flow cytometer (Becton Dickinson). Data was analysed with Cell Quest software (BD) and gating included single cells on the basis of forward and side light-scatter parameters (FSC vs. SSC); log fluorescence was collected and displayed as single parameter histograms.

2.3. Immunocytochemical procedure

MSC were washed with phosphate buffer solution (PBS, 0.1M) and fixed with 4% paraformaldehyde (PFA, Sigma). Afterwards, cells were permeabilized with 1% Triton X-100 (Sigma) followed by 1 hour blocking with 10% normal goat serum (NGS, Sigma) and overnight incubation with the primary antibodies at 4°C. The following primary antibodies were used: mouse anti-β tubulin III, (1:500, Invitrogen), mouse anti-MAP2 (microtubule-associated protein 2, 1:250 Sigma)g, rabbit anti-nestin (1:500 Millipore), rabbit anti-GFAP (glial fibrillary acidic protein, 1:500 Dako), rabbit anti-Iba1 (ionized calcium-binding adapter molecule 1, 1:500 Dako), rabbit anti-Nanog (1:400 Cell Signalling) and mouse anti-SSEA4 (stage-specific embryonic antigen-4, 1:500 Invitrogen). Subsequently, the incubation with the secondary antibodies was performed for 2 h at RT. The secondary antibodies used were as follows: goat anti-rabbit and/or mouse conjugated alexa 488 or alexa 594 (1:200 Invitrogen). Nuclei were stained with 4, 6-diamidino-2-phenylindoline, DAPI (1:5000, Applichem). Fluorescence images were acquired with a Zeiss Axiovert 200 imaging microscope.

2.4. MSC infection with lentivirus encoding for GFP

The lentivirus vector encoding for GFP (green fluorescent protein) was produced in 293T cells using a four-plasmid system as previously described (Alves *et al.*, 2008). The lentiviral particles were resuspended in 0.5% BSA in PBS and the viral particle content of batches was determined by measuring human immunodeficiency virus 1 (HIV-1) p24 antigen levels (RETROtek). Viral stocks were stored at -80°C until use.

MSC with eleven passages were displaced by trypsinization and 200,000 cells were plated in 6 multiwell plates. Twenty-four hours later, lentivirus encoding for GFP were added to cells (80ng of virus/100,000 cells) and 4 µl/ml of hexadimethrin bromid (Sigma). Cells were

incubated at 37°C in 5% CO2/air atmosphere overnight and then lentivirus were removed by replacing the culture medium. Eight days after infection, MSC were assessed for GFP expression with widefield fluorescence microscopy with a Zeiss Axiovert 200 imaging microscope and 12 days later the percentage of MSC infected with GFP were determined by analysing in a FacsCalibur flow cytometer (Becton Dickinson). Data were then analysed by Cell Quest software (BD).

2.5. *In vivo* experiments

2.5.1. Transgenic mouse model

A MJD transgenic mouse model (C57BL/6 background) expressing the N-terminal-truncated human ataxin-3 with 69 glutamine repeats together with an N-terminal hemagglutinin (HA) epitope in cerebellar Purkinje cell, driven by the L7 promoter was used (Oue *et al.*, 2009, Torashima *et al.*, 2008) (Fig. S2-a), and maintained in the animal house facility of the Centre for Neuroscience and Cell Biology (CNC) of the University of Coimbra by backcrossing heterozygous males with C57BL/6 females (Nascimento-Ferreira *et al.*, 2013). The animals were housed in a temperature-controlled room and maintained on a 12 h light/dark cycle. Food and water were available *ad libitum*. The experiments were carried out in accordance with the European Community Council Directive (86/609/EEC) for the care and use of laboratory animals. Genotype was confirmed by PCR.

The present study used 2 groups of animals: MSC-treated mice (stereotaxically transplanted with MSC, n=12), and the non-treated mice or control group (without injection, n=11). The 2 referred groups included animals of both genders.

2.5.2. Stereotaxic surgery

Mice were transplanted with 3-5 weeks of age and animals with the same age were used as controls (Fig S2-b). After anaesthesia of the mice by intraperitonial injection of a mixture of ketamine (100mg/kg, Clorketam 1000, Vétaquinol) with xylazine (10mg/kg, Rompun®, Bayer), the treated group was transplanted with a single injection of 300,000 MSC (300,000 cells/3µl HBSS) injected into the cerebellum at the following coordinates: -1.8 mm rostral to lambda, 0 mm midline, and 1.8 to 2.2 mm ventral to the skull surface, with the mouth bar set at -3.3 (Fig. S2-c). The cells were injected at a rate of 0.25 ml/min through an automatic injector (Stoelting Co.).

2.5.3. Behavioural assessment

Mice were subjected to motor tests every 2 weeks until 12 weeks after transplant (Fig. S2-b). All tests were performed after acclimatization. For each time point, to evaluate statistical significance unpaired student's t-test was performed in GraphPad software (La Jolla), and Welch's correction was performed when variances were significantly different. Data are represented as mean±SEM.

2.5.3.1. Rotarod

Fore and hind limb motor coordination as well as balance were evaluated in a rotarod apparatus (Letica Scientific Instruments). Mice were placed on the rotarod at a constant speed (5rpm), or at accelerated speed (from 4 to 40rpm in 5 minutes) for a maximum of 5 minutes, and the latency to fall (the amount of time they could stand in the rotated wheel) was recorded. Mice were subjected to 4 trials for each test at each time point, with a minimum of 15 minutes rest between trials. For statistical analysis, the mean latency to fall off the rotarod of 3 trials was used (the trial with the most distant value from the average was eliminated).

2.5.3.2. Swimming

Coordination of limbs used during voluntary locomotion was assessed by the swimming test. Mice were placed on one extremity of a tank filled with water at RT and allowed swimming along a 60-cm-long, 14-cm-wide tank until they achieve the platform (located at the other extremity and with the water level at the platform level, Fig. S2-d). The latency to navigate along the tank (time between they were dropped in the water and they reached the platform) was recorded. Mice were subjected to 4 trials for each test at each 3 point, with a minimum of 15 minutes rest between trials. For statistical analysis, the mean latency of 3 trials was used (the trial with the most distant value from the average was eliminated).

2.5.3.3. Footprint

Gait analysis was performed by the footprint test. Hind- and forefeet were coated with black and white nontoxic paints respectively, and the animals were allowed to walk along a 100-cm-long, 10-cm-wide runaway (with 15-cm high walls) over a fresh sheet of green paper.

The footprint patterns were analysed for hind base width, which was measured as the distance between the left and right hind footprints. These values were determined by measuring the perpendicular distance of a given step to a line connecting its opposite preceding and proceeding steps (Fig. S2-e). Six consecutive steps were used for evaluation (to perform 5 measurements), excluding the footprints at the beginning and end of the run. For statistical analysis, the mean of 4 measurements was used (eliminating the trial with the most distant value of the average).

2.6. Histological processing

2.6.1. Tissue preparation

Twelve weeks post MSC transplantation, animals were intracardiacally perfused with PBS followed by fixation with 4% PFA (Fig. S3-a). The brains were removed and post-fixed in 4% PFA for 24 h at 4°C. After a cryoprotective incubation in 20% sucrose/PBS for 48 h at 4°C, brains were frozen at -80°C and 35µm sagittal sections were cut at a cryostat-microtome (Leica CM3050S, Leica Microsystems). Sections of the entire cerebellum were collected and stored in 48-well plates as free-floating sections in PBS/0.05 µM sodium azide at 4°C.

2.6.2. Engraftment evaluation

To evaluate MSC engraftment, sections of one column of the 48-well plates were used. Sections of transplanted mice were mounted in gelatinized slides and were submitted to a 30 min dehydration period at 37°C, followed by 15min hydration in PBS. Nuclei staining was performed with DAPI incubation for 10 min. After drying, sections were mounted in Mowiol reagent (Sigma). Fluorescence images were acquired with a Zeiss Axiovert 200 imaging microscope.

2.6.3. Neuropathological evaluation

2.6.3.1. Immunohistochemical procedure

The immunohistochemical procedure was performed in a free-floating system, as referred elsewhere (Simões *et al.*, 2014). Briefly, sections were simultaneously blocked and permeabilized with 0.3 % Triton X-100 in PBS supplemented with 10 % NGS for 1 h at RT.

Sections were incubated overnight at 4°C with the following primary antibodies diluted in 0.3% triton/PBS supplemented with 10% NGS: rabbit anti-calbindin D-48K (1:1000 Millipore) and mouse anti-HA (1:1000 InvivoGen). After the incubation with the primary antibody, sections were incubated with the appropriate secondary antibodies: goat anti-rabbit or goat anti-mouse conjugated to alexa 594 (1:200 Invitrogen) for 2 h at RT. Afterwards, sections were incubated with DAPI for 10 min to perform the nuclei staining and then mounted in gelatinized slides. Once dried, slides were mounted in Mowiol reagent (Sigma). Fluorescence images were acquired with a Zeiss Axiovert 200 imaging microscope (Fig. S3-b).

2.6.3.2. Cresyl violet staining to determine cerebellar layer thicknesses

Cerebellar sections were mounted in gelatinized slides and stained with cresyl violet for 2 minutes, differentiated in 70% ethanol, dehydrated by passing twice through 95% ethanol, 100% ethanol and xylene solutions, and mounted with EukittH (Sigma) (Fig. S3-c).

2.7. *In vivo* imaging quantification

For all *in vivo* quantifications, we have addressed for sections nearby the localization of MSC grafts.

2.7.1. Quantification of granular and molecular layers thicknesses

To assess for the granular and the molecular layer thicknesses, quantifications were performed in 4 sections per animal, with an inter-section distance of 280 µm. In the control group the same localization on the 48-well plate used for treated mice was selected. The molecular layer size was determined by measuring the width of this layer (the Purkinje cell layer was also included); the width of the granular layer was assessed excluding the Purkinje cells. Measurements were all made in between lobules, in order to use precisely defined regions. Results were converted to µm using the Image J software.

2.7.2. Quantification of Purkinje cells and mutant ataxin-3 aggregates

The quantification of Purkinje cells was performed in the lobules II and III using 4 sections per animal (inter-section distance: 280 µm). The areas were scanned with a Zeiss PALM

Axiovert 200M microscope. To determine the number of Purkinje cells, we accounted for the cells immunoreactive for HA, as in our model, these cells have an N-terminal HA epitope (Torashima *et al.*, 2008). Additionally, optical densitometry of immunoreactivity for calbindin was also used to assess for Purkinje cell quantification (Nascimento-Ferreira *et al.*, 2013). Outliers were determined by average ± standard deviation and were excluded. The evaluation of the HA aggregates was performed in the whole cerebellum using 6 sections per animal, with an inter-section distance of 280 μm (also scanned with Zeiss PALM Axiovert 200M microscope). For all the quantifications, the image J acquisition and analysis software was used.

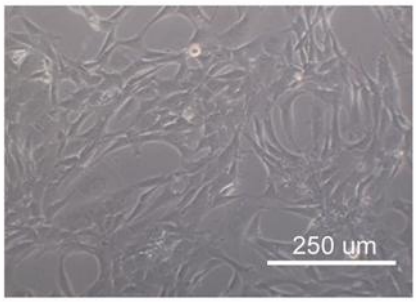
3. Results

3.1. The isolation method of BM-MSC generates a relatively pure MSC population and MSC express neuronal markers prior to transplantation

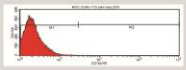
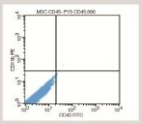
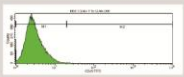
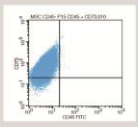
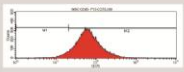
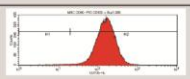
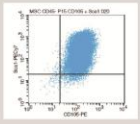
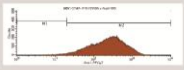
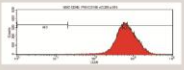
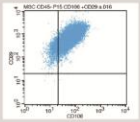
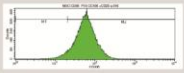
Given MSC heterogeneity and their likely contamination by hematopoietic cells (Javazon *et al.*, 2004, Phinney *et al.*, 1999), we performed MSC characterization to evaluate the purity of MSC population. For that, MSC were characterized by flow cytometry for characteristic MSC markers and by immunocytochemistry for neuronal/glial marker expression (Fig.1). In culture, MSC exhibited fibroblast-like morphology and grew as a monolayer, both typical features of MSC (Fig.1-a). Furthermore, MSC were negative for the hematopoietic markers CD11b and CD45 and highly positive (between 95%-100%) for the mesenchymal markers CD73, CD105, Sca-1, CD29 and CD106 (Fig.1-b). As such, a relative pure population of MSC can be obtained by our isolation/selection method.

Additionally, we could observe that *in vitro* MSC expressed both neuronal and glial markers (Fig.1-c), including nestin (a marker for immature neurons), β-tubulin III and MAP2 (markers for mature neurons), GFAP and Iba-1 (markers for astrocytes and microglia, respectively). MSC did also express Nanog and SSEA-4, two markers for pluripotency (Fig.1-c-panel D).

a)



b)

Marker	Cytometric analysis	Expression (gated events)	Plots	Phenotype
CD11b		0.01%		Negative
CD45		0.10%		Negative
CD73		94.5%		Positive
CD105		99.7%		Positive
Sca-1		98.6%		Positive
CD29		99.9%		Positive
CD106		95.4%		Positive

c)

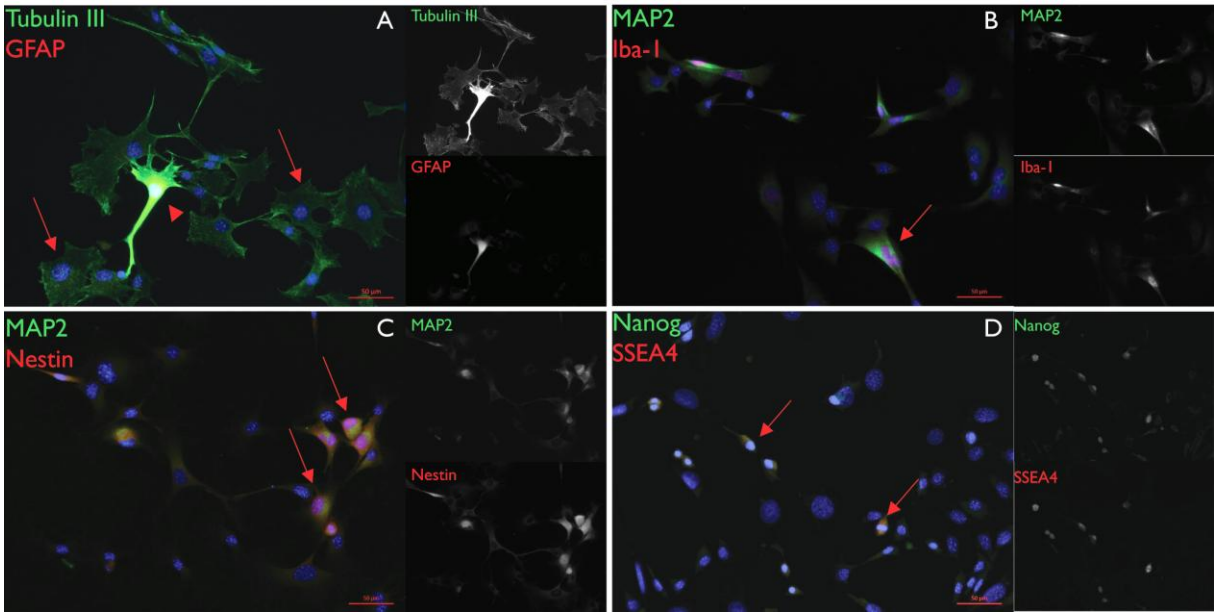


Figure 1 - MSC characterization. a) Brightfield photomicrograph showing that MSC exhibit a fibroblast-like morphology and grow in a monolayer in vitro cultures. **b)** Phenotypic characterization of MSC performed by flow cytometry demonstrating that MSC are negative for the hematopoietic markers CD11b and CD45 and

highly express the following markers: CD73, CD105, Sca-1, C29 and CD106. **c)** Immunocytochemistry reveals that *in vitro* MSC express markers for both immature (nestin, in red: arrows panel C) and mature neurons (Tubulin III, in green: arrows panel A; MAP2, in green: arrows panels B and C), astrocytes (GFAP, in red: arrowhead panel A), and microglia (Iba-1, in red: arrow, panel B). MSC did also express Nanog or SSEA-4, two markers for pluripotency (Nanog in green and SSEA4, in red: arrows, panel D). **Abbreviations:** GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adaptor molecule 1; MAP-2, microtubule-associated protein 2; MSC, multipotent mesenchymal stromal cells; Sca-1, stem cells antigen 1; SSEA-4, stage-specific embryonic antigen-4.

3.2. MSC are GFP-positive prior to transplantation and are able to engraft when injected stereotaxically in the cerebellum

In order to follow the cells after transplantation in the cerebellum of MJD transgenic mice, MSC were previously infected with lentiviral vectors enconding for GFP (Fig.2-a). To evaluate the efficacy of infection, flow cytometry analysis was performed on gated live cells. We could observe that a highly efficient infection (88% of cells expressing GFP – Fig2-b) was obtained, suggesting that this is an effective method to track MSC *in vivo*.

GFP-labelled MSC were stereotaxically injected in the cerebellum of transgenic mice. To investigate cells engraftment, animals were sacrificed at intermediate intervals and GFP signal was checked on sagittal brain sections of transplanted mice. We could detect MSC at 2 (n=1), 5 (n=1), 9 (n=3) - data not shown - and 12 weeks after transplantation (n=5) (Fig. 2-c). At 12 weeks post-transplantation MSC were mostly detected near the second or the third lobules of the cerebellum (Fig. 2-d). We can then conclude that MSC integrated into the cerebellar tissue and survive for an extensive period of time.

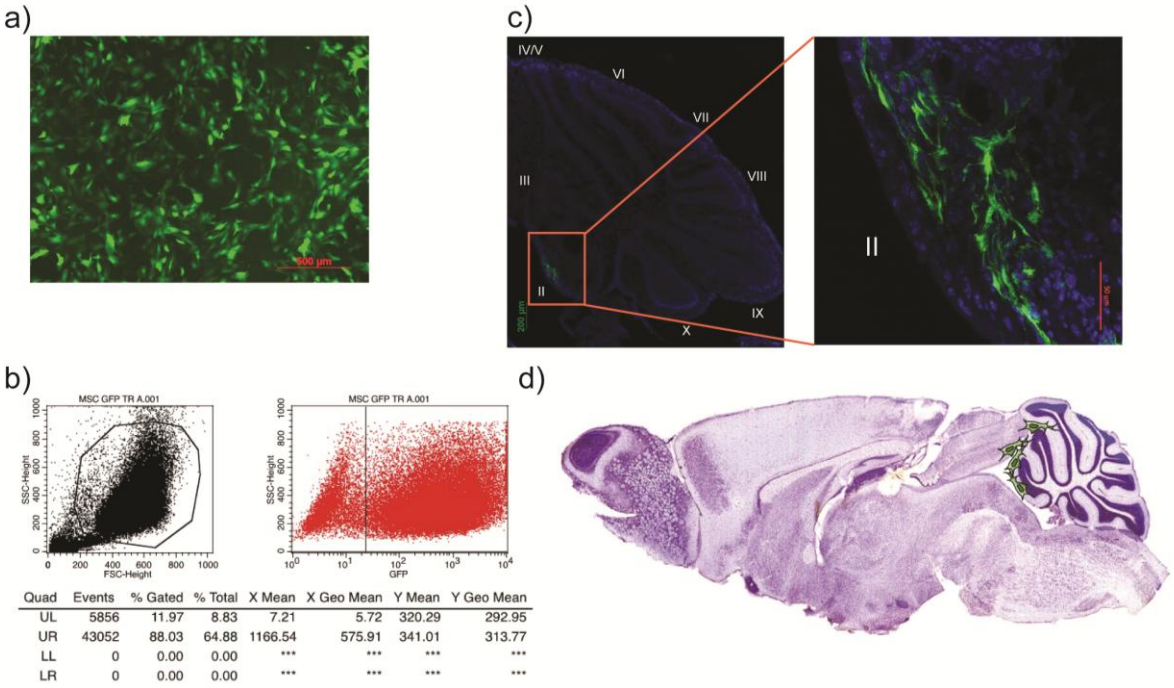


Figure 2 - GFP-labeled MSC can engraft within the cerebellum. a) Fluorescence microscopy and **b)** flow cytometry analysis, showing that MSC were robustly infected with a lentivirus encoding for GFP. Flow

cytometry analysis was performed on gated live cells. MSC were efficiently infected with GFP (88% of gated cells) **c)** Photomicrograph showing that GFP^+-MSC could engraft in the cerebellum as they were detected at 12 weeks after transplant. (GFP^+-MSC - green; DAPI - blue). **d)** Schematic representation of MSC localization in the cerebellum of transgenic mice at 12 weeks after transplant. **Abbreviations:** DAPI, 4, 6-diamidino-2-phenylindoline; GFP, green fluorescent protein; MSC, multipotent mesenchymal stromal cells.

3.3. MSC promote phenotype alleviation in MJD transgenic mice

To assess the therapeutic potential of MSC in promoting the functional recovery or phenotype improvement in MJD, motor behaviour was evaluated in transplanted MJD transgenic mice. As controls, non-treated (non-injected) MJD mice of the same age were used. Behavioural tests - constant and accelerated rotarod, swimming and footprint - were performed every 2 weeks after transplantation, to permit assessing the functional recovery of MJD mice over time.

Balance and motor coordination deficits were evaluated by constant and accelerated rotarod, as well as by the swimming test. At 4 weeks after transplantation, MSC mediated improvement of the performance of treated mice, presenting significant better performances when comparing to non-treated mice in both constant (NTMJD 4.038 ± 0.9347 sec vs MJD+MSC 10.45 ± 2.042 sec, p=0.012; Fig.3-a) and accelerated rotarod (NTMJD 7.499 ± 1.677 sec vs MJD+MSC 14.65 ± 2.869 sec, p=0.048; Fig.3-b), which measures the latency to fall. In swimming test, mice were placed at one extremity of a recipient filled with water and the time between they were dropped in the water and they reached the platform was registered. At the same time-point (4 weeks after the treatment), MSC transplantation significantly improved the performance of treated animals, reaching the plataform in less time as compared to non-treated mice (NTMJD 6.333 ± 0.6592 sec vs MJD+MSC 4.445 ± 0.3371 sec, p=0.016; Fig.3-c).

Additionally, the footprint test was used to evaluate the progression of gait and limb ataxia. We measured the distance between the left and the right hind footprint (hind base width). In our model, this distance is larger in MJD mice relatively to wild type mice, as a result of balance defects. Though not significant, MSC-treated mice showed a tendency to decrease in the hind base width, when compared to non-treated mice at 4 weeks after transplants (Fig.3-d; NTMJD 2.997 ± 0.07997cm vs MJD+MSC 2.768 ± 0.08569cm, p=0.065), suggesting that, in accordance to our previous results, MSC transplantation is mediating a tendency for improvement in this test as well.

Overall, a phenotypic alleviation was observed at 4 weeks after transplant. At later time points, MSC effect was not able to revert MJD phenotype. This may be explained by the fact that MSC transplantation has been performed at a late time point (3 to 5 weeks of age),

point at which the disease is already very severe in this transgenic mice model, showing dramatic motor impairments when compared to wild-type mice, as already demonstrated by our group (Nascimento-Ferreira *et al.*, 2013).

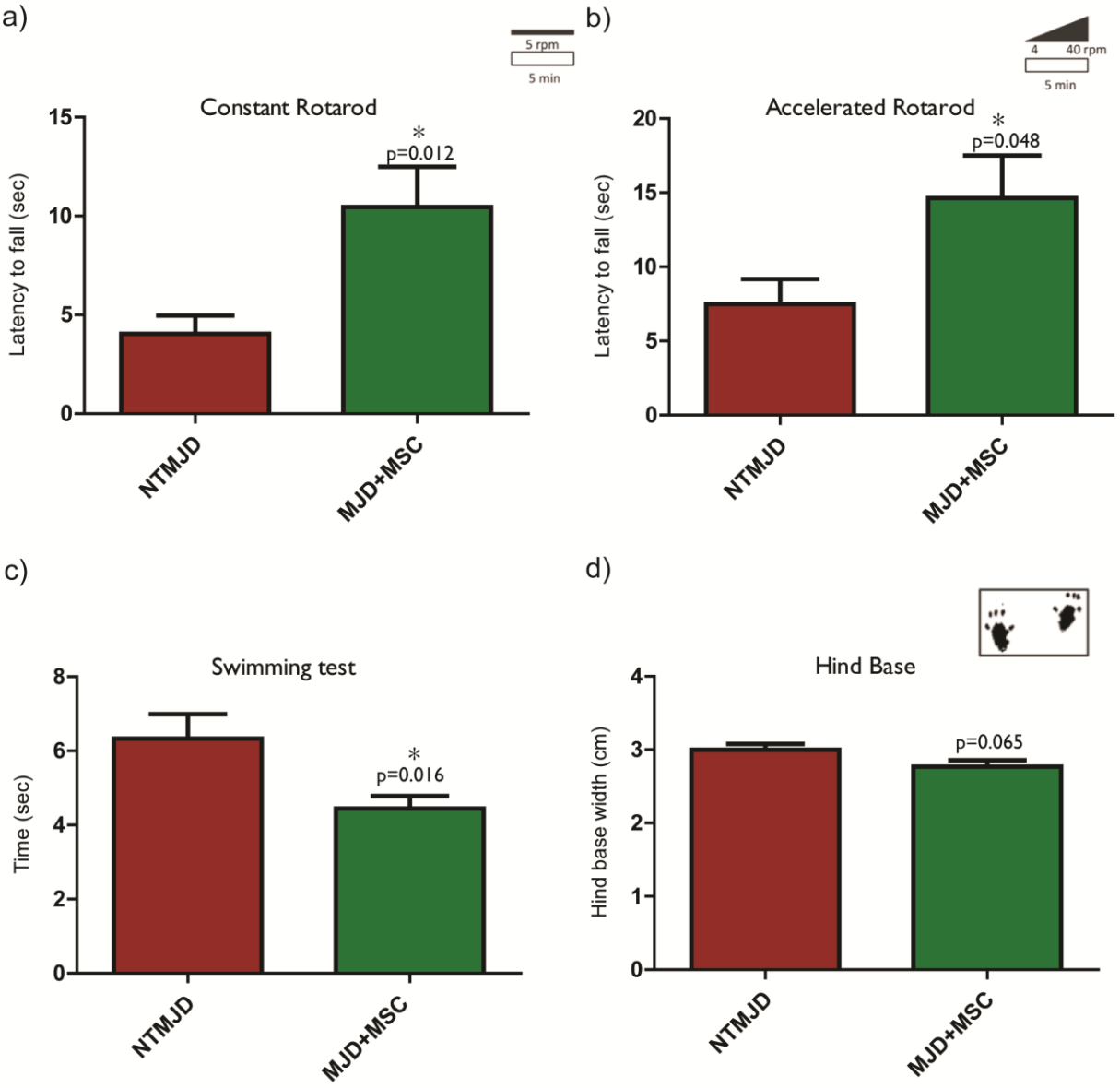


Figure 3 - Analysis of behaviour performance of MSC-treated and non-treated MJD transgenic mice. Mice were placed on the rotarod at **a)** a constant speed (5rpm, throughout 5 min) or **b)** accelerated speed (4 to 40rpm in 5 min), and their latency to fall was measured. Treated mice with MSC have shown significant better performances in both constant and accelerated rotarod when compared to non-treated mice, at 4 weeks after transplantation. **c)** Swimming test performance at 4 weeks after MSC transplantation, showing that treated animals significantly improved their performance comparatively to non-treated mice. **d)** Hind base quantitative analysis (footprint test) performed at 4 weeks after transplant. MSC-treated mice showed a tendency to decrease (non-statistical) on hind base width when compared to non-treated mice. Values are presented as mean ± SEM (Unpaired Student's test with Welch's correction when variances were significantly different, ***p <0.001,**p <0.01 and *p <0.05). Non-treated mice (NTMJD, n=11) and treated mice (MJD+MSC, n=12). **Abbreviations:** MJD, Machado Joseph disease; MSC, multipotent mesenchymal stromal cells.

3.4. MSC transplantation promotes neuropathological improvements in MJD transgenic mice

To evaluate whether MSC transplantation can mitigate the neuropathology associated with MJD motor function impairments, we analysed both the molecular and granular layer thicknesses, the number of mutant ataxin-3 aggregates and both the Purkinje cell number and immunoreactivity for the calbindin protein (a marker for Purkinje cells) by optical densitometry. All brain sections were examined at 12 weeks after transplant around the area where MSC were found.

The thickness of the cellular layers showed a tendency to increase in treated animals (Fig.4-a) when compared to non-treated controls, meaning that mice transplanted with MSC revealed a conservation of the molecular layer thickness, since these mice presented a significant thicker molecular layer than non-treated mice between lobules II and III (NTMJD=101.2 ± 2.029μm vs MJD+MSC=124.1 ± 5.135μm, p=0.0061), and between lobules III and IV (NTMJD=106.0 ± 6.306μm vs MJD+MSC=142.0 ± 10.93μm, p=0.0061; Fig.4–b). Regarding the granular layer, we also observed a tendency to increase the thickness of the layer between lobules III and IV in MSC treated mice relatively to non-treated animals though not reaching statistical significance (NTMJD= 59.07 ± 1.479μm vs MJD+MSC= 68.09 ± 3.568μm, p=0.0582; Fig.4-b).

Furthermore, since transgenic mice present marked defects in the Purkinje cells, we performed immunostaining for calbindin to assess for the immunoreactivity against this marker (Fig.4-c). Treated mice presented a significant increase of the optical densitometry of calbindin immunoreactivity when compared with non-treated mice in lobule II (NTMJD=22.56 ± 0.3592 a.u. vs MJD+MSC=26.23 ± 1.050 a.u., p=0.0349) and III (NTMJD=23.41 ± 0.3093 a.u. vs MJD+MSC=25.60 ± 0.5813 a.u., p=0.0311; Fig.4-d). Moreover, we measured the number of Purkinje cells by immunostaining for HA (Fig.4-e) as in our model these cells express mutant ataxin-3 tagged by an N-terminal HA epitope. Treated mice exhibited a non-significant tendency for increase in the Purkinje cell number (lobule II: NTMJD=78.67 ± 12.33 cells vs MJD+MSC=99.67 ± 4.485 cells, p=0.1848; and lobule III: NTMJD=58.00 ± 23.46 cells vs MJD+MSC=108.3 ± 10.73 cells, p=0.1451; Fig.4-f). As in this model Purkinje cells present aggregates of mutant ataxin-3, we measured the number of aggregates present in the whole cerebellum. However, no significant differences were detected between treated and non-treated mice (NTMJD=12.55 ± 2.359 aggregates vs MJD+MSC=11.88 ± 1.808 aggregates, p=0.8279; Fig.4-f).

Overall, MSC transplantation promoted the recovery of cerebellar atrophy and the survival of Purkinje cells, leading to neuropathological improvements.

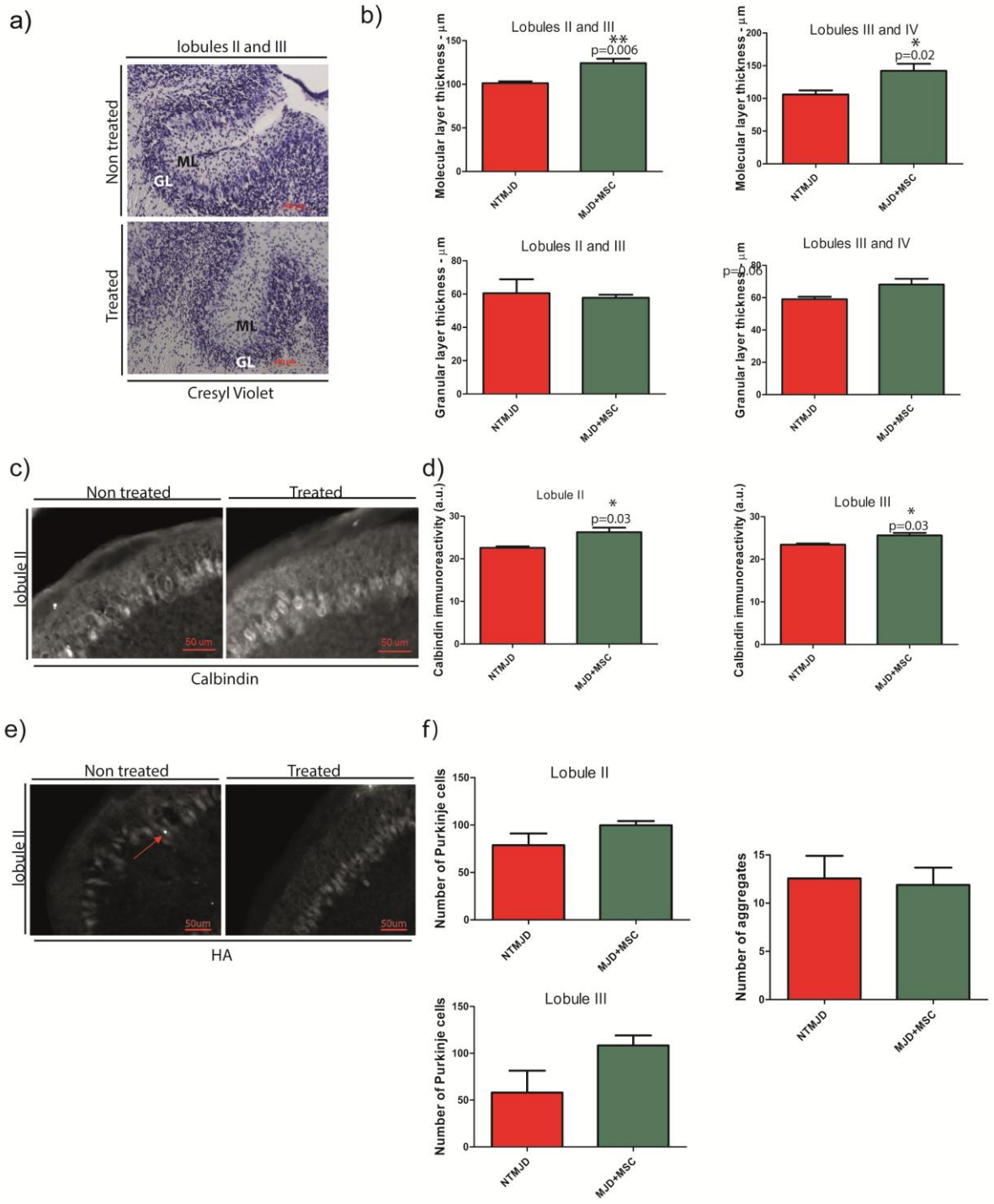


Figure 4 - Neuropathology of the cerebellum of MJD transgenic mice is protect by the MSC transplantation. **a)** Representative photomicrographs of sagittal sections of the cerebellum of both non-treated (upper panel) and treated (lower panel) MJD transgenic mice stained with cresyl violet, showing the molecular (ML) and granular layers (GL). **b)** Quantification of both molecular and granular layer thicknesses. A significant increase in the molecular layer thickness was observed in MJD transgenic mice treated with MSC between lobules II and III (NTMJD, n=4; MJD+MSC, n=4; p=0.006), and between lobules III and IV (NTMJD, n=5; MJD+MSC, n=5; p=0.02), when comparing with non-treated mice. Treated animals presented a tendency for increase in the granular layer thickness between lobules III and IV (NTMJD, n=4; MJD+MSC, n=4; p=0.058).

c) Representative photomicrographs of sagittal sections of the cerebellum of both non-treated (left panels) and treated (right panels) MJD transgenic mice immunostained with anti-calbindin. **d)** Quantification of optical densitometry of calbindin immunoreactivity. MSC transplantation significantly increased calbindin immunoreactivity in lobule II and III as compared to controls (NTMJD, n=3; MJD+MSC, n=4; p=0.03). **e)** Representative photomicrographs of sagittal sections of the cerebellum of both non-treated (left panels) and treated (right panels) MJD transgenic mice immunostained with anti-HA. **f)** Quantification of both Purkinje cell and aggregates (arrow) number in both treated and non-treated groups. A tendency to increase the Purkinje cells number was detected in the MSC-treated relatively to non-treated MJD transgenic mice (lobule II: NTMJD, n=3; MJD+MSC, n=3; p=0.18 and lobule III: NTMJD, n=4; MJD+MSC, n=3; p=0.15). There was no significant difference in aggregates number between treated and non-treated group (NTMJD, n=4; MJD+MSC, n=4; p=0.83). Values are presented as mean ± SEM (Unpaired Student's t-test, ***p <0.001, **p <0.01 and *p <0.05). **Abbreviations:** a.u., arbitrary units; GL, granular layer; HA, hemagglutinin; MJD, Machado Joseph disease; MJD+MSC, treated mice; ML, molecular layer; MSC, multipotent mesenchymal stromal cells; NTMJD, non-treated mice.

4. Discussion

In this work we investigated whether MSC transplantation in the cerebellum could alleviate the phenotype of MJD transgenic mice. Transplanted MSC engrafted in the cerebellum and rescued motor impairments and cerebellar neuropathology in this mouse model for MJD.

MSC-based therapies are promising therapies for neurodegenerative diseases, since it has been reported that they are capable to promote protection, tissue regeneration and/or modulation of the immune system in animal models for a number of neurological disorders (Trzaska *et al.*, 2008). More recently, this therapeutic approach has received considerable interest with respect to cerebellar disorders, such as SCAs. Chang and collaborators have shown that IV delivery of MSC can promote recovery in mice motor function as well as delay the disease onset in a transgenic mouse model of SCA-2 by rescuing cerebellar Purkinje cells (Chang *et al.*, 2011). Another group has shown that MSC treatment improved the morphology of the cerebellum and improved the motor behaviour when intrathecally injected in a transgenic mouse model of SCA-1 (Matsuura *et al.*, 2014).

Aligned with these results, clinical trials with MSC are already running and there have been reports that MSC are safe and may delay disease progression in some SCAs (Dongmei *et al.*, 2011, Jin *et al.*, 2013), including MJD (www.clinicaltrials.gov: ClinicalTrials.gov Identifiers - NCT01489267, NCT01360164). Nevertheless, clinical trials only allow evaluating the phenotype whereas the direct neuropathology evaluation prior and after MSC transplantation is not possible to perform in human patients. Moreover, how MSC mediate these effects remains a matter of controversy and to this contributes the lack of studies

evaluating MSC effect in animal models. In fact, studies in animal models are crucial to understand the pathways through which these cells mediate therapeutic effects, adjust delivery routes and frequency of intervention and anticipate safety issues. In this work, we show that MSC transplantation in the cerebellum of the MJD transgenic mouse model alleviates motor impairments and its associated neuropathology, providing evidences that MSC can become a therapy for MJD. Importantly this experimental model is expected to contribute to clarification of the mechanism of neuroprotection and technical aspects to be improved.

MSC are adult multipotent stem cells that can be isolated from various tissues, including the bone marrow, the first source described for MSC (Caplan, 1991, Friedenstein *et al.*, 1970). They are heterogenic cells and it is challenging to isolate a pure cell population (Mabuchi *et al.*, 2013). The most commonly used method for MSC isolation is based on their plastic adherence feature. Despite that, hematopoietic contaminants may persist in culture, especially in the case of MSC isolated from bone marrow of mice (which is the case of the present study) as it have been reported that in this species is difficult to remove hematopoietic contaminants (Javazon *et al.*, 2004, Phinney *et al.*, 1999). Thus, to isolate a more pure MSC population, we performed cell sorting to eliminate $CD45^+$ cells after the separation of mononuclear cells. Our results suggest that a relative pure population of MSC can be obtained by our isolation method as MSC exhibited a fibroblast-like morphology, could grow in a monolayer and accordingly, MSC were negative for the hematopoietic markers CD11b and CD45 and highly positive for all the other markers commonly used to characterize/identify MSC, such as CD73, CD105, Sca-1, CD29 and CD106 (Baddoo *et al.*, 2003, Caplan and Dennis, 2006, Morikawa *et al.*, 2009, Sun *et al.*, 2003).

We also assessed MSC expression of neural markers *in vitro* by immunocytochemistry and we could observe that MSC already express markers for neuronal/glial cells before transplantation, for both immature (nestin) and mature neurons (β-tubulin III and MAP2), astrocytes (GFAP), and microglia (Iba-1). This may result from the culture in conditioned medium with factors that can influence MSC phenotype. However, MSC kept their fibroblastic-like morphology and did not resemble neural/glial cells. These results are in accordance with previous observations, which demonstrated that in basal conditions, even in the absence of specialized induction agents, MSC already express specific neuronal markers (Blondheim *et al.*, 2006, Deng *et al.*, 2006a). Likewise, it has been reported that MSC express embryonic stem cells markers (pluripotency markers). Riekstina et al. reported that BM-MSC express Oct4, Nanog, alkaline phosphatase and SSEA-4 (Riekstina *et al.*, 2009). In accordance with this report, we found that *in vitro* culture conditions, MSC expressed

markers for pluripotency, namely Nanog and SSEA-4, which supports the idea that MSC are not committed into cells of the neuronal lineage.

It has been demonstrated that MSC can graft the nervous tissue and survive for long periods of time (Edalatmanesh *et al.*, 2011). In fact, in our study, we could detect MSC 12 weeks after transplant in the MJD cerebellum, demonstrating that MSC can survive in the cerebellar tissue and exert their effect in affected area.

The transgenic mouse model used is characterized by Purkinje cell expression of truncated form of human ataxin-3 with 69 glutamine repeats and driven by a Purkinje-cell-specific L7 promoter (Torashima *et al.*, 2008). The cerebellum has an important role in maintenance of balance and posture, coordination of voluntary movements and motor learning. Therefore, a functional defect in the Purkinje cells can trigger cerebellar ataxia, including balance and gait defects (Torashima *et al.*, 2008). Accordingly, at 3 weeks of age, transgenic mice already exhibit pronounced ataxia. In this study, animals were injected within 3-5 weeks of age. Four weeks after transplant, MSC treated mice present a significant alleviation of their motor impairments, with better performance in rotarod, swimming and footprint tests. Jones et al. demonstrated that mice injected with MSC in the cerebellum were capable of walking on the rotarod for longer periods of time than non-treated mice, demonstrating that MSC have the potential of alleviating the phenotype in cerebellar ataxias for long periods (Jones *et al.*, 2010). In our study, MSC were not able to revert MJD phenotype at later time points, which may be explained by the severity of this transgenic mice model at this time-point. In future studies, we will assess for MSC transplantation at earlier time points in order to investigate whether MSC can revert MJD phenotype at later ages. Moreover, multiple injections will also be assessed, as a single injection may not be sufficient to provide sustained effects. A clinical trial in China used UC-MSC transplantation in patients with MSA-C and SCAs (Dongmei *et al.*, 2011). UC-MSC were injected intrathecally weekly, for 4 weeks, meaning that a total of 4 injections were administered in each patient. This approach demonstrated to be very promising, as patients presented improvements in some MJD symptoms, including unstable walking and standing, slow movement, fine motor disorders of the upper limbs, writing difficulties and dysarthria. However, in some of the SCA patients (6 of 14), over the period of 1 to 14 months after transplantation the disease had progressed, demonstrating that probably repeated injections throughout a continuous therapy may be necessary (Dongmei *et al.*, 2011).

Importantly, in the future, IV injection of MSC will also be studied to assess the neuroprotective competence of MSC using this route of delivery, which would be translatable into clinical application within a short time frame. Zhang et al. demonstrated that

an ataxic mouse model treated intravenously with human UC-MSC presented better performances in rotarod test from 9 weeks until 12 weeks after transplant, proving that IV injection of MSC may be an effective approach for other cerebellar ataxias, including MJD (Zhang *et al.*, 2011). Besides that, Chang and collaborators compared the IV and IC delivery of MSC and concluded that IV transplantation effectively improved rotarod performance of SCA2 transgenic mice and delayed the onset of motor function deterioration, while IC transplantation failed to achieve such neuroprotective effect (Chang *et al.*, 2011). However, in our study we observed motor impairment alleviation, upon local transplantation of MSC. In the study of Chang and col. direct IC injection of MSC was performed through foramen magnum into cerebellum, while in our study MSC were stereotaxically injected into the cerebellum. MSC injections through foramem magnum (through the skull bottom hole) have associated risks such as possible surgical lesions, namely in the brainstem. Importantly, the authors have performed three injections per animal, accumulating the risks of the invasive procedure. Furthermore, this procedure does not allow precise delivery of the cells in a specific cerebellar region. Accordingly, the authors have reported that MSC were not detected over cerebellar white matter, molecular layer, or the Purkinje cell layer, but were limited to a few lumen of blood vessels (Chang *et al.*, 2011). This may explain the failure of the therapeutic effect of MSC local delivery in that study, whereas in our study stereotaxic cerebellar transplantation of MSC was effective. Nevertheless, we plan to address whether the systemic delivery of MSC would be an advantageous alternative in MJD context.

It has also has been reported that MSC can alleviate the neuropathology associated to cerebellar ataxias. In a study performed in the SCA1 transgenic mouse model, the authors demonstrated that MSC injected intrathecally mitigated the cerebellar neuronal disorganization. The cerebellum of these SCA1 transgenic mice had ectopically located PC bodies that resulted in a multilayer of PC; besides that, mice showed atrophy of the cerebellar molecular layer. In the referred study, MSC treated mice displayed a single layer of PC and the molecular layer was thicker than that of the untreated mice (Matsuura *et al.*, 2014).

In accordance with these previous reports, we also observed an improvement in the MJD associated neuropathology. The transgenic mouse model used presents mutant ataxin-3 inclusions and severe atrophy of the cerebellum with marked disarrangement of Purkinje cells (Torashima *et al.*, 2008). To evaluate the effect of MSC transplantation in the neuropathology, we measured both the molecular and the granular layer thicknesses, assessed for the number of Purkinje cells and for the number of aggregates at 12 weeks after transplants. Treated transgenic mice showed a conservation of both molecular and granular

layer, as well as a tendency to preservation of the number of Purkinje cells, however we have not observed significant differences in the number of mutant ataxin-3 inclusions. These results can indicate that despite the positive correlation between MJD phenotype alleviation and the neuroprotective competence of MSC, MSC transplantation was not able to down-regulate mutant ataxin-3. In future studies, we will assess for both mutant ataxin-3 mRNA and protein levels to confirm this paradigm. Inclusion bodies start forming at 40 days (Torashima *et al.*, 2008). As such, probably transplantation of MSC at 3-5 weeks may be too late to block aggregate formation. Therefore we intend to investigate whether MSC transplantation at earlier time points can prevent the formation of intranuclear inclusions. As expected, similarly to other reports, in the present study MSC mediated partial recovery of cerebellar tissue, supported the survival of the Purkinje cells, and ultimately improved the motor functions of mutant mice, after transplantation in cerebellum (Jones *et al.*, 2010).

In summary, our results demonstrate that MSC transplantated *in situ* in the cerebellum of MJD transgenic mice engraft and survive in cerebellar tissue. Moreover, MSC can exert a positive effect in this MJD model leading to a phenotype alleviation and neuropathological improvements. Therefore, transplantation of MSC can become a promising therapy for MJD.

5. Conclusion and future perspectives

In this work it was shown that local transplantation of MSC could alleviate motor impairments and cerebellar atrophy, proving that MSC is a good candidate for disease-modifying MJD therapies. Importantly this experimental paradigm allows thorough investigation of the pathways through which MSC transplantation alleviates MJD.

In order to deepen our knowledge on MSC's potential effect on MJD, we are presently quantifying ataxin-3 down-regulation either by Western blot (allowing assay of both soluble and aggregated forms of mutated ataxin-3) and qPCRs from tissue homogenates of the cerebellum. Furthermore, as a way of assessing both neuroprotective and immunomodulatory MSC potential, we will also measure neurotrophin and ILs levels by qPCR.

In future studies, we will investigate whether MSC´s effect can be even more effective and persist for longer periods of time by transplanting MSCs at earlier time points as well as by multiple injections. Moreover, systemic delivery of MSC will also be performed in order to assess the neuroprotective competence of MSC using this route of delivery, which would be translatable into clinical application within a short time frame.

6. Supplementary figures

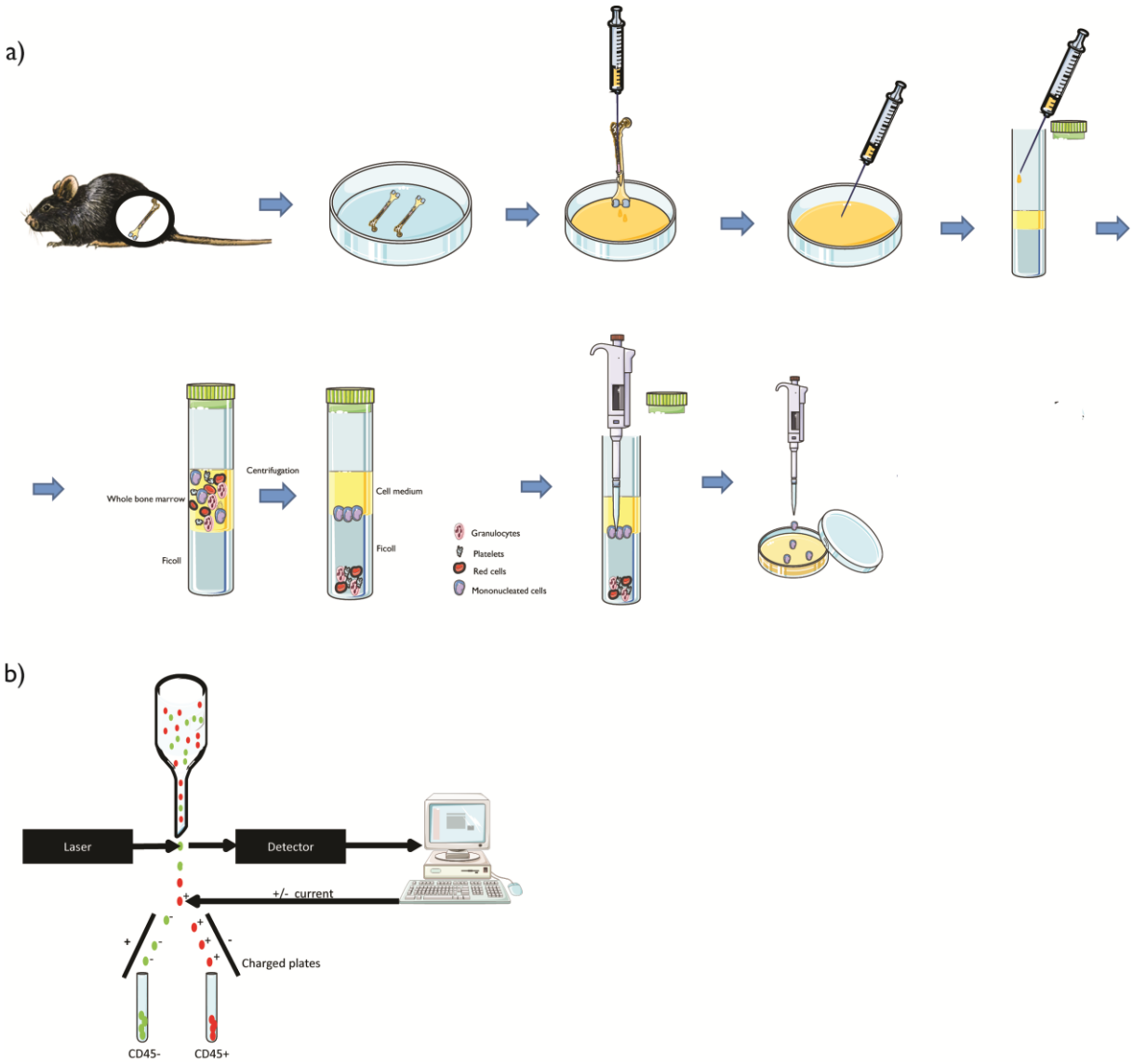


Figure S1 - MSC isolation. a) MSC were isolated from femurs and tibias of C57BL/6 wild type mice by bone marrow flush and mononuclear cells were separated in a Ficoll density gradient. MSC that did not adhere to the plastic were removed 48h after plating and cells were expanded until confluence. **b)** To get a more pure MSC population, the CD45+ cells were eliminated by sorter, using FACS. Adapted from (Willis, 2004). **Abbreviations:** FACS, fluorescence activated cell sorting; MSC, multipotent mesenchymal stromal cells.

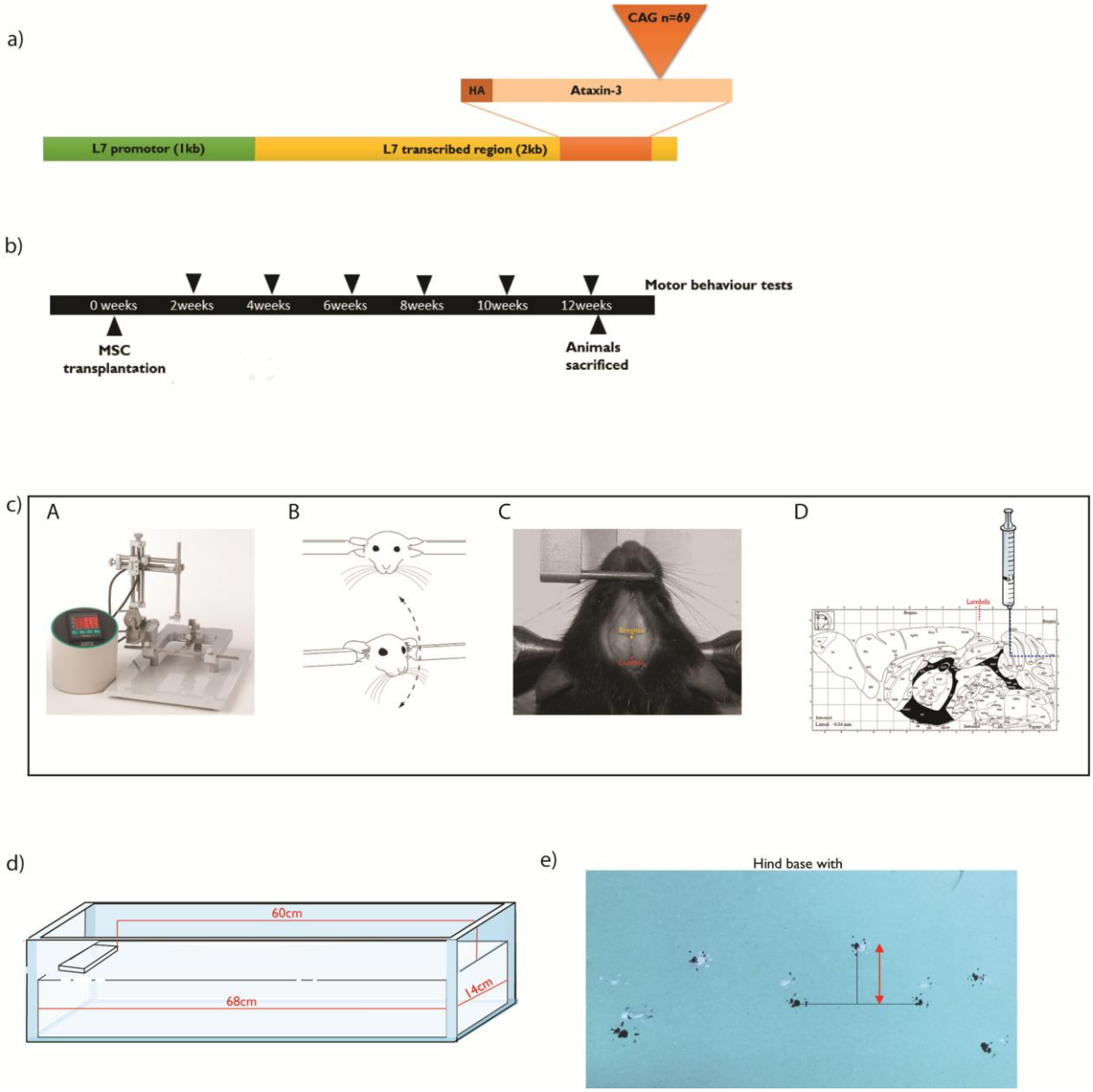


Figure S2 - *In vivo* experiments. a) Schematic representation of the transgene encoding mutant ataxin-3 under the control of the L7 promoter in the MJD transgenic mice model. Adapted from (Torashima *et al.*, 2008). **b)** Diagram illustrating the time line of MSC transplantation and behaviour tests in MJD transgenic mice. Mice were injected with 3-5 weeks of age and behaviour test were performed until 12 weeks after transplants. Brains were processed and analysed at 12 weeks post-injection. **c)** Representation of the stereotaxic surgery. Panel A - Picture representing the stereotaxic apparatus. The animal is correctly positioned and fixed in the stereotaxic apparatus (Panel B), with symmetric position of the head and the nose is able to move freely up and down, but lateral movement is constrained by fixation. Adapted from (Cetin *et al.*, 2006). Afterwards, the skin is spread apart and the surface of the skull is exposed and the main anatomical landmarks are highlighted: bregma and lambda (Panel C). Adapted from (Rynkowski *et al.*, 2008). The initial position is marked in lambda and correct coordinates (-1.8 mm rostral to lambda, 0 mm midline, and 1.8 to 2.2 mm ventral to the skull surface) are introduced to perform the injection in the cerebellum (Panel D). **d)** Schematic representation of the tank where mice are allowed swimming. **e)** Schematic representation of hind base width. **Abbreviations:** HA, hemagglutinin; MJD, Machado Joseph disease; MSC, multipotent mesenchymal stromal cells.

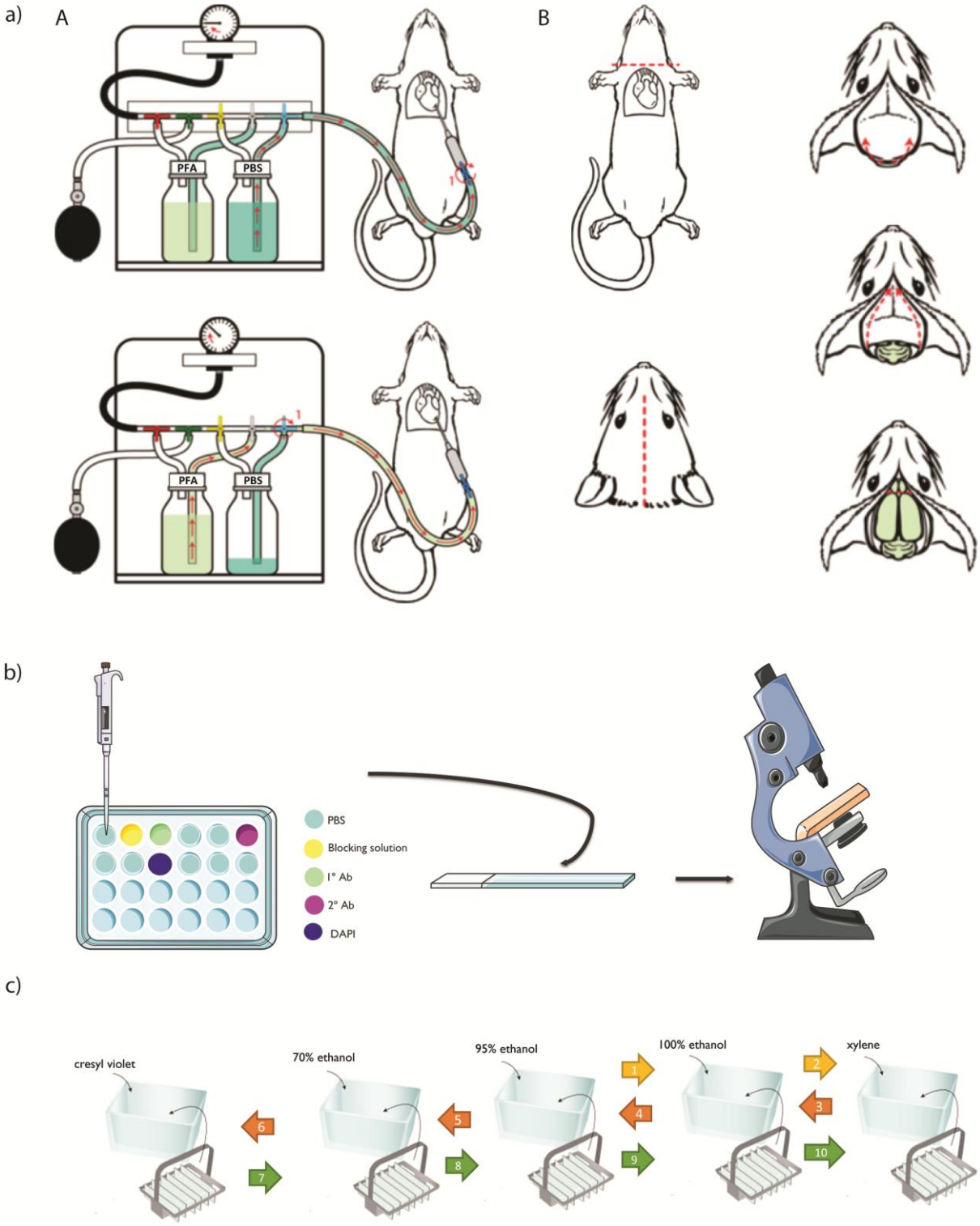


Figure S3 - Histological processing. **a)** Representation of intracardiac perfusion. The animals are intracardiacally perfused with PBS followed by fixation with 4% PFA (Panel A). Afterwards, the brain is removed (Panel B). Adapted from (Gage *et al.*, 2012). **b)** Representation of immunohistochemistry. **c)** Representation of cresyl violet staining. **Abbreviations:** AB, anti-body; DAPI, 4, 6-diamidino-2-phenylindoline; PBS, phosphate buffer solution; PFA, paraformaldehyde.

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