

Cell-Replacement Therapy with Stem Cells in Neurodegenerative Diseases

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Abstract: In the past few years, research on stem cells has expanded greatly as a tool to develop potential therapies to treat incurable neurodegenerative diseases. Stem cell transplantation has been effective in several animal models, but the underlying restorative mechanisms are still unknown. Several mechanisms such as cell fusion, neurotrophic factor release, endogenous stem cell proliferation, and transdifferentiation may explain positive therapeutic results, in addition to replacement of lost cells. The biological issue needs to be clarified in order to maximize the potential for effective therapies. The absence of any effective pharmacological treatment and preliminary data both in experimental and clinical settings has recently identified Amyotrophic Lateral Sclerosis (ALS) as an ideal candidate disease for the development of stem cell therapy in humans. Preliminary stem transplantation trials have already been performed in patients. The review discusses relevant topics regarding the application of stem cell research to ALS but in general to other neurodegenerative diseases debating in particular the issue of transdifferentiation, endogenous neural stem cell, and factors influencing the stem cell fate.

Key Words: Amyotrophic lateral sclerosis, Neurodegenerative disease, Stem cell, Transplantation.

INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS), after Charchot's first definition and in relation to Lou Gehrig's centennial birthday in 2003, still remains a lethal disease. Due to the absence of any effective remedy and supporting preliminary data, both in experimental and clinical settings for treating ALS, the possibility of developing stem cell therapy in humans using ALS as a candidate disease has been recognized recently by the scientific community. Over the past two decades, extensive experiments, beginning with fetal neuron transplantation in Parkinson's disease (PD) animal models, have provided the basic proof of principle for cell replacement (Brundin and Hagell, 2001). The observed positive results have spearheaded the development of this therapeutic approach even in humans. Patients affected by PD, Huntington's (HD) and, more recently, ALS have been approached. Despite these promising results, significant constraints still hamper the use of embryonic cells for neurotransplantation (Bjorklund and Lindvall, 2000). Besides, the ethical concerns related to the use of material, the viability, purity, and phenotype final destiny of the fetal cells have not been completely defined. The recent breakthroughs in SC research have opened up new possibilities for cell-replacement therapy since these cells can be indefinitely expanded in number and cryopreserved even for long periods without losing their potentiality. The use of SCs overcomes

the need of synchronization between donation and transplant, the problem of limited donor cell number and, at the same time, it discloses the novel possibility of employing autologous sources.

ALS AS A CANDIDATE DISEASE FOR CELL-REPLACEMENT THERAPY

ALS is a lethal disease characterized by the degeneration and death of both upper and lower motor neurons. The course of the disease is relentless and progresses without remissions, relapses, or even stable plateaus. Its pathogenesis is probably multifactorial and includes the interaction of several susceptibility genes, undetermined environmental factors, and the physiological cellular aging (Rowland and Scheider, 2001).

In the last few years, several drug therapies based on known or suspected etiopathogenetic mechanisms have been translated into clinical trials. However, they failed to arrest degeneration and restore motor function. Therefore, an alternative strategy has been sought for effective treatment. Neural transplantation in the past and SC therapy now seem to be two of the most promising answers to this problem (Silani *et al.*, 2002).

The large majority of ALS patients are sporadic cases (SALS) and only 5-10% of patients show familial inherited forms (FALS) associated to mutations in the Cu/Zn superoxide dismutase-1 (SOD1) gene in a small proportion (15-20%) (Gaudette *et al.*, 2000). This gene encodes the copper- and zinc-dependent SOD1 with a possible cytotoxic activity in the mutant form due to an abnormal protein folding. Mutant SOD1-G93A mice exhibit progressive degeneration of lower motor neurons, decreased stride, and muscle strength with death occurring at 4-5 months of age.

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Received: February 4, 2004; Revised: March 5, 2004; Accepted: March 11, 2004

Thus, this FALS model became a convenient tool not only for understanding the pathogenesis, but also for developing new therapeutic strategies (Wong *et al.*, 2002). Positive effects on the onset of motor dysfunction and on the average lifespan were observed after human NT neurons transplantation into the ventral horn spinal cord of SOD1G93A mice (Garbuzova-Davis *et al.*, 2002). Also human umbilical cord blood mononuclear and bone marrow SC infusions were reported to substantially increase mice lifespan with a dose dependent effect, although no human DNA was detected in the host tissues (Chen and Ende, 2000; Ende *et al.*, 2000). Similar results were obtained by Garbuzova-Davis *et al.* (Garbuzova-Davis *et al.*, 2003) where human umbilical cord blood cells, administrated intravenously to presymptomatic SOD1G93A mice, were shown to migrate preferentially towards degenerated sites, but also to peripheral organs. In addition, expression of neural markers by few human transplanted cells was demonstrated and an overall neuroprotection effect was suggested to be the main cause of the observed benefits.

Recently, two preliminary papers reported a limited series of ALS cases grafted with blood SCs. Janson *et al.* (Janson *et al.*, 2001) intrathecally injected peripheral blood SCs in three ALS patients. FACS purified CD34⁺ cells were administrated and after 6-12 months none of the patients reported side effects; but no significant clinical efficacy was reported during following evaluations. More recently, Mazzini *et al.* (Mazzini *et al.*, 2003) injected autologous bone marrow derived cells after expansion *in vitro* in 7 patients affected by ALS. The transplantation procedure was performed after resuspension of the cells in the autologous cerebrospinal fluid and direct injection into the surgically exposed spinal cord at T7-T9 levels. According to the authors, none of the patients manifested severe side-effects; only minor adverse effects were reported, such as reversible intercostal pain and reversible leg sensory dysesthesia. Neuroradiological examinations were normal and no significant psychological status or quality of life modifications were reported. However, the clinical efficacy of the transplant is still under evaluation. The authors concluded that these procedures were "safe and well tolerated by ALS patients" (Mazzini *et al.*, 2003).

Even if additional data related to clinical efficacy are not available, cell transplantation into the human cord seems to be feasible and other grafting programs in ALS have just been started (Vastag, 2001). Nevertheless, while patients are desperately trying to enroll in SC clinical trials, caution and careful evaluation of the preliminary results, especially considering the issues of transdifferentiation and transplantation, are necessary before widely applying such a pioneer technique (Silani and Leigh, 2003). More recently, two papers demonstrated, in two different animal models of motor neuron disease, the efficacy of human pluripotent cells to restore functions in paralyzed rats (Kerr *et al.*, 2003) and the influence of adjacent cells in the long-term survival of SOD1G93A motor neurons (Clement *et al.*, 2003). In particular, this last evidence provides the strongest support to the stem cell implementation therapy, sustaining the possibility of inducing clinical recovery by increasing the number of unaffected non-neuronal (astroglial) cells.

STEM CELL POTENTIALS

SCs are undifferentiated multipotent cells capable of both self-renewal and generation of several differentiated cell types (Potten and Loeffler, 1990; Morrison *et al.*, 1997; Labat, 2001). It is proved that they are present until adulthood in almost all tissues and organs (i.e. hematopoietic, neural, muscle, intestinal crypt, and skin SCs) where they preserve homeostasis of cell number (Weissman *et al.*, 2001; Verfaillie *et al.*, 2002). SCs are also characterized by extensive plasticity, (a feature reported repeatedly in recent pieces of literature), by which a tissue-specific cell can either dedifferentiate or transdifferentiate into a novel unrelated phenotype. These two processes are thought to occur mainly through DNA transcriptional activation and through repression due to chromatin structure modifications (i.e. histone methylation and acetylation) determined by intrinsic and extrinsic growth factors (Tada and Tada, 2001). SC proliferation kinetic is tissue-specific and it is influenced by genetic and environmental clues, such as developmental stage and mitogen supply (Monna *et al.*, 2000; van Heyningen *et al.*, 2001; Peterson, 2002). As a matter of fact, during embryogenesis, SCs contribute at first to organ formation through a massive symmetric cell division mode aimed at increasing the cell number. Later, there is a switch towards a preferential asymmetric division in order to give rise to slightly differentiated cells or "progenitors", which in turn, can originate fully differentiated progeny (Temple, 2001). Progenitors, in opposition to SCs, show a limited self-renewal capacity and are often unipotent (Seaberg and van der Kooy, 2003). SC number decreases in favor of progenitors and functional, fully differentiated cells, during an organism's lifespan, since at least in animals, stem cell proliferation reduces with age (Fallon *et al.*, 2000). In the long term, this process diminishes the rate of neurogenesis, and seems to be strictly related to telomere shortening and stem cell cycle extension (Taupin and Gage, 2002). In the adult, the SC compartment may be present in a relatively quiescent state in relation to the tissue, specifically, in a finely tuned and dynamic balance between the proliferative and the resting conditions. Perturbation of this equilibrium by environmental factors, such as severe lesions or injuries, may induce the exit of SCs from the quiescent state and their proliferation in order to restore the lost/damaged cell population(s). SC flexible sensitivity to the surroundings is maintained in culture and, for example, can be used to generate nearly pure neuronal populations from human fetal neural SCs for transplantation into adult rat central nervous system (CNS) (Wu *et al.*, 2002).

Several kinds of SCs were shown to integrate into the blastocyst and give rise to almost all differentiated tissues (Geiger *et al.*, 1998; Clarke *et al.*, 2000; Jiang *et al.*, 2002). Therefore, SC seems to be a complex biological entity in continuous evolution in relation to its genetic program and the surrounding microenvironment rather than a discrete, independently existing cellular type (Leminska, 2002; Vernig and Brustle, 2002).

Dedifferentiation occurs when a cell reverts to an earlier and more immature state with the expression of primitive markers, usually detected during the differentiation pathway. This dedifferentiative capacity is normally absent in mam-

malian cells, but it has been proved that at least myotubes possess this property in a permissive environment (Odelberg *et al.*, 2001). When the cell fate change is rapid and doesn't involve a passage back to early progenitor cells, the process is referred to as transdifferentiation. This may occur both within the same tissue (i.e. a glial cell reverts to a neuron) and in different tissue derivatives (i.e. a hematopoietic cell acquires a neural phenotype). It has been shown that hematopoietic SCs are able to transdifferentiate into neural cells (Brazelton *et al.*, 2000; Mezey *et al.*, 2000) and vice versa (Bjorson *et al.*, 1999). Moreover mesenchymal and skin SCs may convert to a neural phenotype (Eglitis and Mezey, 1997; Toma *et al.*, 2001). Such transdifferentiation event seems to be restricted not only to SCs, but has also been observed in progenitors and differentiated cells (Kondo and Raff, 2000; Malatesta *et al.*, 2000).

All these recent reports have created much excitement in scientific community due to the possibility of exploiting the transdifferentiation mechanism combined with SC properties (capacity of unlimited self-renewal plus potential to exponentially generate several types of differentiated progeny) for cell replacement therapy. As a matter of fact, easily accessible autologous SCs could then represent a source for therapeutical transplantation when a specific cell population is lost or damaged. Therefore, investigation on this topic has been boosted in the last few years but, as we will discuss in the following paragraphs, the results are still controversial.

STEM CELLS AND NEURODEGENERATIVE DISEASES

In neurodegenerative diseases, drugs can alleviate symptoms and relieve pain, but up to now they have not been able to permanently repair damaged tissues. It has been proposed that SC persistence in adult organs could be a compensatory mechanism which contributes to self-repair under normal conditions, but fails in particular circumstances or tissues, such as in the case of a wide neurodegeneration (Armstrong and Barker, 2001; Kuhn *et al.*, 2001). Even though plastic adult neurogenesis has been demonstrated in specific brain regions (i.e. subventricular zone and hippocampal dentate gyrus) even in humans, the CNS demonstrates a remarkably limited capacity for self-repair. This phenomenon is probably due to the lack of suitable signals able to activate a sufficient number of endogenous neural stem cells and then to instruct appropriate cell differentiation (Peterson, 2002). Focalized extensive neuronal cell death caused by stroke in adult brains triggers proliferation/recruitment of neuroblasts, and newly generated neurons are reported to migrate from subventricular zone to damaged area (Arvidsson *et al.*, 2002). Additional papers demonstrate proliferation/differentiation of endogenous neural SCs (Fallon *et al.*, 2000; Peterson, 2002; Nakatomi *et al.*, 2002; Schmidt and Reymann, 2002), even in normally non-neurogenic regions (Johansson *et al.*, 1999). Unfortunately, the physiological action of the newly generated neurons has not been completely proved and, just the same, it is not sufficient to produce a functional recovery (Yamamoto *et al.*, 2001; Magavi and Macklis, 2002).

Adult neurogenesis can be stimulated «*in situ*» by intraventricular infusions of basic Fibroblast Growth Factor (bFGF) and Epidermal Growth Factor (EGF) following

global ischemia (Nakatomi *et al.*, 2002) and in intact brain (Palmer *et al.*, 1999; Gould *et al.*, 1999). Similarly, the expansion of brain neuroprecursor cells can be enhanced by Brain Derived Nerve Growth Factor (BDNF); (Benraiss *et al.*, 2001), Ciliary Neurotrophic Factor (CNTF); (Shimazaki *et al.*, 2001) and Noggin gene activation (Lim *et al.*, 2000). These observations suggest that a limited availability of appropriate growth factors combined with the presence of repressive signals practically restricts the brain regeneration potential.

Since reduced neurotrophic support is likely to be correlated to the pathogenesis of neurodegenerative disorders such as Alzheimer disease (AD), PD, HD, and ALS, neurotrophin administration in clinical trials is actually under study, but with no apparent success so far (Zuccato *et al.*, 2001; Dawbarn and Allen, 2003). The only exception is the insulin growth factor 1 (IGF-1) adenovirus associated delivery in an ALS animal model, which was reported to prolong survival (Kaspar *et al.*, 2003). On the other hand, riluzole (2-amino-6-trifluoromethoxy-benzothiazole), an antiexcitotoxic agent partially effective in the treatment of ALS patients, was also shown to induce neurotrophic effects in animal models of PD, HD and brain ischemia (Doble, 1996; Palfi *et al.*, 1996; Guyot *et al.*, 1997). It has been proved that it stimulates Nerve Growth Factor (NGF), BDNF, and Glial Cell Line-Derived Neurotrophic Factor (GDNF) release in cultured mouse astrocytes and *in vivo* in the rat hippocampus (Mizuta *et al.*, 2001; Katoh-Semba *et al.*, 2002).

A hypothetical scenario for adult human brain cell repair would therefore combine transplantation of donor stem/progenitor cells, purposely purified and committed towards the differentiated requested phenotype, with growth factors/drugs administration and eventually biomatrices in order to stimulate axon development and synaptogenesis (Steindler and Pincus, 2002).

MECHANISMS OF ACTION AND TROUBLES IN TRANSPLANTATION/ TRANSDIFFERENTIATION

Experience with human fetal cell transplantation, especially in PD, has demonstrated that results of clinical trials are quite variable among groups of patients receiving the same cell preparation. Several different parameters (such as patient conditions, graft location, type of transplanted tissue/cells, etc.) seem to affect the positive integration and survival of dopaminergic neurons in the host brain (Dunnet *et al.*, 2001). For example, cell suspension was demonstrated to be more effective for reconstitution of lost connection and recovery in comparison to small tissue pieces in the case of fetal grafts (Isacson *et al.*, 2003).

Identification and isolation of pure SCs is hard to achieve because of the absence of specific antigens. Although some markers have been identified so far, they cannot be used to univocally separate stem population from progenitors, which possess a limited proliferative self-renewing capacity and are often even unipotent (Seaberg and van der Kooy, 2003). This practical problem has to be carefully considered if SCs are to be used for transplantation purposes and clinical applications. The optimal cell therapy should exploit a pure and well-characterized population in order to achieve the best results (Rossi and Cattaneo, 2002), since both experimental

and clinical trials are actually limited by mixed cellular composition and low neuronal yield, i.e. dopaminergic neurons (Isacson *et al.*, 2003).

Nevertheless, some antigens allow for discriminating among tissue-specific stem populations (i.e. cell surface markers CD133 and CD34 are mainly used for hematopoietic SCs) (Weissman *et al.*, 2001; Verfaillie *et al.*, 2002), but in several cases we are still far from a clear identification and tracking of SCs, and this issue is particularly relevant for neural stem cells. As a matter of fact, antigens such as musashi1, nestin, Sox1, Sox2, SSEA-1/LeX have been proposed as possible markers for neural SC selection, but they only enrich stem ratio instead of providing a pure stem population (Sakakibara and Okano, 1997; Cai *et al.*, 2003). Nestin, an intermediate filament protein, is also expressed in myoblasts and endothelial cells (Wroblewski, 1997; Johansson *et al.*, 1999) and therefore may not be considered a reliable marker for NSC selection. Fine separation techniques based on negative fluorescence-activated cell sorting (FACS) (in order to exclude lineage-restricted cells) and immunomagnetic beads have also been recently developed in order to obtain purified and homogeneous stem populations (Cai *et al.*, 2003).

Recent gene expression profile studies show that SCs of different origins (ES, hematopoietic, neural stem cells) share a common genetic program together with a tissue-specific gene expression (Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002). These oligonucleotide microarray data may help elucidate the key genes or regulatory pathways that may be necessary to maintain the stem condition and, at the same time, to obtain a switch in SC fate for clinical applications. Experimental data reveal that both adult mouse bone marrow and neurally derived SCs injected into blastocyst can generate chimeric mice (Geiger *et al.*, 1998; Clarke *et al.*, 2000), thus demonstrating a complete nuclear reset. On the other hand, a precise sequence of epigenetic signals can modulate gene expression and is required for both tissue specification during normal embryogenesis and acquisition of a novel cell fate, as has been reported for hematopoietic lineage development induced from human muscle and neural tissue (Jay *et al.*, 2002). Similar regulatory mechanisms are known to be active also *in vitro*: the use of Leukemia Inhibitory Factor (LIF) cytokine, in fact, is reported to exert a positive effect on neural SC cultures increasing their growth rates and prolonging their self-renewal capacity (Wring *et al.*, 2003). The molecular data collected from microarray studies on LIF-treated NSC may be useful to discriminate genes/pathways that are important for maintaining long-term NSC cultures.

Presently, the transdifferentiation event is viewed with criticism: reports are continuously published in favor or against the future clinical exploitation of this possibility. Recently, for example, it has been published that marrow stromatal cells can generate neurons *in vitro* (Munoz-Elias *et al.*, 2003), that umbilical cord blood SC infusion ameliorates functional defects in spinal cord injury (Saporta *et al.*, 2003) and that fetal hematopoietic SCs can give rise, sequentially, to neurons and then astrocytes in culture (Hao *et al.*, 2003). Transdifferentiation process requires activation of specific genes as was demonstrated for myelin genes in transfor-

mation of murine melanoma into glial cells (Slutsky *et al.*, 2003). These observations coincide with the findings of bone marrow-generated neurons after transplantation into human leukemic patients (Mezey *et al.*, 2003) and of improvements of neurological defects by bone marrow grafts after ischemia (Zhao *et al.*, 2002). A novel functionality has also been found to be acquired after transdifferentiation of endothelial progenitor cells into active cardiomyocytes (Badoff *et al.*, 2003).

On the other hand, several cases and reports against the natural occurrence of cellular change of fate are continuously published. After many contradictory reports, Jlang and colleagues (2002) described the existence of a rodent and human bone marrow sub-population, co-purifying with mesenchymal stem cells, termed multipotent adult progenitor cells or MAPCs. These cells were able to differentiate, at the single cell level, in cells with mesoderm, endoderm, and neuroectoderm characteristics *in vitro* and *in vivo* after transplantation, showing extensive proliferation without loss of differentiation potential, similarly to embryonic SC (ES). Even better results have been achieved *in vitro* with human hematopoietic SCs obtained from the umbilical cord, demonstrated to differentiate sequentially into neuronal SCs and then astrocytes after exposure to suitable microenvironment (Hao *et al.* 2003).

Recently, however, transdifferentiation has been debated and a number of other biological explanations have been put forward. Cell fusion, specifically, has been heavily advocated as an alternative to transdifferentiation (Terada *et al.* 2002). Perhaps, the most knowledgeable conclusion to draw is that transdifferentiation is an uncommon phenomenon that takes place only under very peculiar circumstances. In fact, adult hematopoietic SCs are reported to reconstitute a lost leukocyte population, but they are not appreciably integrated into other tissues such as muscle, brain, gut, liver and kidney (Wagers *et al.*, 2002). In one recent report, hematopoietic potential of muscle SCs was due to contamination of the donor cells instead of real transdifferentiation (McKinney-Freeman *et al.*, 2002). Conversely, no apparent neuronal differentiation was found in a significant number of animals, either lethally irradiated or presenting neuronal injury, after whole bone marrow cell transplantation (Castro *et al.*, 2002). Finally, bone marrow cells, migrating in the adult brain 4-18 weeks after transplantation, fail to transform into neural cells and preserve their hematopoietic properties (Ono *et al.*, 2003) and transdifferentiation of freshly dissociated brain cells into blood was not proven (Magrassi *et al.*, 2003). Cell fusion was also considered the main source of bone marrow-derived hepatocytes (Wang *et al.*, 2003; Vassipoulos *et al.*, 2003).

All this data suggests that plasticity is rare and needs an important cellular deficit and/or niche pushing for proliferation and localized accumulation of donor cells in the lesioned site (Wagers *et al.*, 2002). An «ideal» condition combines the generation of appropriate chemokine signals, specific adhesion proteins, and the depletion of endogenous SC population in order to maximize the transplanted cell contribution. These conditions are usually achieved in experiments with host irradiation; but this procedure induces endogenous neural progenitor dysfunction due to a mutated

microenvironment with an increase in glial cell number at the expense of new neurons, as has been recently demonstrated (Monje *et al.*, 2002) and therefore, may have limited applications in patients.

How is it possible to conciliate these apparently opposing data on transdifferentiation? First we have to keep in mind that the experimental conditions vary a lot among different laboratories and that even a sorted purified SC population is heterogeneous «*per se*». For example, Ianus *et al* (Ianus *et al.*, 2003) have recently demonstrated the existence of a candidate pancreatic progenitor in bone marrow, which can be activated and made to proliferate in response to circulating signals, giving rise to functional active pancreatic β cells. Therefore, in the absence of specific selective markers, the purification of a SC population is impossible to achieve and contamination by different tissue stem/progenitors cannot be excluded. Probably the transdifferentiation process and/or the clinical improvements derive even from interactions of different cellular subsets, reciprocally providing cytokines, growth factors, and intercellular signals. The neural SC transplantation in an animal model of multiple sclerosis determines a clinical improvement that is accompanied by remyelination. This remyelination is directed by transplanted precursors and probably by a reduced reactive astrogliosis – a negative regulator of endogenous oligodendrocyte proliferation (Pluchino *et al.*, 2003). Cooperation and/or synergistic action between endogenous stem and transplanted cells could therefore be indispensable for a functional recovery. Dissection of such complex interacting mechanisms could be very difficult to gain.

Cell fusion has been advocated and even demonstrated in several reports in order to explain an apparent transdifferentiation event (Terada *et al.*, 2002; Wurmser and Gage, 2002; Ying *et al.*, 2002), but it has been recently proposed that this mechanism could represent a normal pathway towards differentiation and repair in damaged tissues (Blau, 2002).

Several recent reviews have tried, with limited success so far, to clarify and set the precise requirements, such as functional integration and specific antigen acquisition, necessary for a transplanted SC to be considered differentiated and/or transdifferentiated (Joshi and Enver, 2002; Alison *et al.*, 2003; Collas and Hakelien, 2003; Greco and Recht, 2003; Liu and Rao, 2003; Moore and Quesenberry, 2003).

Finally, since so many mechanisms and pathways could be involved during and after transdifferentiation/clinical transplantation, it's not too surprising that a complex mass of opposite results derives from different experiments and that therefore a clear scenario on these processes is still far from being achieved.

CONCLUSIONS

Only after SC differentiation mechanisms are fully understood, promising treatment strategies can be designed to redirect adult SCs to regenerate motor neurons in a clinically relevant manner in ALS patients. Furthermore, ALS requires, for the regeneration of the lower and upper motor neurons, a specific strategy for the cell replacement approach. In fact, the functional integration in the neuronal circuitry must be obtained after having restabilized the

complex and precise connectivity of the implanted cells. In light of the above considerations, even if ALS is a desperate disease that, more than others, is able to induce the patient to seek innovative therapeutical strategies, at the same time it also provides a real challenge to the clinician and the neurobiologist who need to define a new approach to simultaneously restore the degenerating cortico-spinal tract and the lower motor neurons. Finally, it is crucial that the medical-scientific and the medical-media communities work together to keep the general public well informed, but also realistically appraised as to the significance of scientific breakthroughs in the development of new treatments related to ALS (Silani and Leigh, 2003).

ACKNOWLEDGEMENTS

The authors would like to thank John P. Hemingway for critically reading the manuscript. Supported in part by a grant from IRCCS Istituto Auxologico Italiano, Dr. Angelo Mauri's donation, and Fondazione Italo Monzino.

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