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The Use of Human Samples to Study Familial and Sporadic Amyotrophic Lateral Sclerosis: New Frontiers and Challenges

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by death of upper and lower motor neurons, which results in muscle wasting and death from respiratory failure typically within 2-5 years from diagnosis.

ALS is a multifactorial disease [1] where different cell types, i.e. astrocytes, microglia and oligodendrocytes, contribute to the pathologic mechanism [2, 3]. For a long time ALS was thought to be a pure motor neuron disease, however, thorough pathological investigations and recent findings linking mutations in transactive response DNA-binding protein gene (TARDBP) to familial and sporadic cases of ALS have relocated this disease within a spectrum of neurological disorders, ranging from pure motor neuron disease to frontotemporal dementia [4, 5].

Since 1993, when the first mutation in the Cu/Zn superoxide dismutase (SOD1) enzyme was linked to familial forms of ALS, researchers have tried to unravel the mechanisms underlying this disease by interrogating *in vivo* and *in vitro* models overexpressing human SOD1. Although these models have highly contributed to understanding the pathogenic mechanisms involved in motor neuron degeneration, they only account for less than 2% of all cases. Hence, the ALS field is still lacking effective therapies and a deep understanding of the etiology of the sporadic disease.

For 15 years the SOD1 models have been the only available, until, in 2008, mutations in TARDBP were found to be responsible for familial and sporadic forms of ALS [6, 7]. This led to the discovery that mutations in a second RNA/DNA-binding protein called fused in sarcoma (FUS) or translocated in liposarcoma (TLS) were also cause of the disease [8, 9]. More

recently, the field of ALS has seen a breakthrough with the association of GGGGCC-hexanucleotide repeat expansion in chromosome 9 open reading frame 72 (C9ORF72) to 35-40% of familial cases and 5-7% of sporadic cases [10-12].

In the same years, from 2007 to present, *in vitro* technologies to model neurological disorders have also undergone an impressive development.

With the discovery that adult human fibroblasts could be reprogrammed to induced pluripotent stem (iPS) cells with the use of selected transcription factors [13], the field of ALS saw the opportunity to finally model not only the familial, but especially the sporadic disease *in vitro*. In fact, in 2008, the first human iPS-derived motor neurons from patients were cultured in a petri dish [14]. Since then, several iPS lines have been produced from patients and healthy individuals and they have been made commercially available (<http://www.coriell.org/stem-cells>).

Moreover, in 2011, neural progenitors cells (NPCs) were isolated from post-mortem spinal cord samples of ALS patients and successfully cultured and differentiated into motor neurons, astrocytes and oligodendrocytes *in vitro* [15]. This technology provided for the first time the possibility to model all forms of ALS *in vitro* without inducing major epigenetic alterations in the cells used.

In this chapter we will give an overview of how human tissues have been used so far, what discoveries they have led to since 2007, and how the recent advances in technology combined with the recent genetic discoveries, have tremendously widened the horizon of ALS research.

2. Latest genetic discoveries

Even though Jean-Martin Charcot initially described ALS in 1869, it took more than a century until the first disease-causing gene – the Cu/Zn superoxide dismutase (SOD1) - was identified [16]. Mutations in this gene account for approximately 10-20% of the familial ALS (FALS) cases (Anderson 2006) and about 1% of the sporadic (SALS). Up to now, mutations in 21 different genes have been linked to ALS, although some of them present with atypical disease characteristics [17]. FALS is usually inherited in an autosomal-dominant manner, but in rarer cases, it appears also recessive or X-linked. Along with the rising number of genes and mutations involved, the differentiation between sporadic and familial ALS becomes increasingly difficult and depends on the definition applied. With the most stringent classification, a patient is considered to suffer from the familial form if he/she has at least one first- or second-degree relative affected by ALS [18]. However, other studies define FALS when at least one relative is affected by motor neuron disease, i.e. ALS, primary lateral sclerosis (PLS) or progressive muscular atrophy (PMA) [19]. In addition, several indications exist that mutations leading to ALS are also involved in the development of other neurodegenerative diseases such as different types of dementia or Parkinsons disease. This further broadens the range of possible familial linkage [19]. Missing family history data and

the existence of mutations with incomplete penetrance, thus masking inherited genetic forms of the disease as sporadic, further contribute to complicate the discrimination between FALS and SALS.

In the last 20 years, most efforts were concentrated on studying the effect of SOD1 mutations, resulting in the generation of over 30 different animal models including *Drosophila*, *C. elegans*, *D. rerio*, mice, rats and dogs [20]. In most cases, expression of human mutant SOD1 in the animal models led to astrogliosis, inflammation and degeneration of motor neurons in a similar manner as observed in patients.

The generated SOD1 animal models highly contributed to the understanding of SOD1 functions in the central nervous system (CNS) leading to the development of potential therapeutic strategies targeting these pathways. Unfortunately, most of the therapeutics that show an effect in rodent models, fail in human clinical trials. Overall, SOD1 only accounts for about 2% of all ALS cases, therefore the question arose how applicable the findings from these models really are for other familial cases and especially for the huge majority of sporadic ALS cases.

In 2006, the transactive response DNA-binding protein (TDP-43) was identified as a major component of intraneuronal inclusions, a form of protein aggregates representing a hallmark of SALS and non-SOD1-FALS cases [21]. Soon after, researchers found ALS causing mutations in this gene [6, 7]. One year later, mutations in a second RNA/DNA-binding protein called fused in sarcoma (FUS) or translocated in liposarcoma (TLS) were published [8, 9]. While TDP-43 mutations account for 4% of FALS, FUS mutations are less frequent and account for approximately 1-2% [22]. The discovery of the involvement of these two genes can be considered a milestone in ALS research, not necessarily because of the mutation frequency, but rather because of the wide presence of these proteins in the aggregates characterizing tissues from sporadic ALS cases. Mutations in TDP-43 and FUS can also be found in some forms of frontotemporal dementia (FTD), while aggregates of the non-mutated protein seem to be an even more common feature for neurodegenerative diseases including Huntington's, Alzheimer's and Parkinson's [23]. As both proteins are involved in RNA metabolism, a common disease mechanism underlying sporadic and familial forms of ALS might exist. This link rises hope that a common therapeutic strategy could be developed benefiting a broad patient population.

The TARDBP gene encoding TDP-43 lies on chromosome 1p36.2. The TDP-43 protein consists of 414 amino acids and is highly conserved among species [7]. The expression pattern is almost ubiquitous with high levels during development. Loss of TDP-43 is detrimental in rodents as knockouts in mice are lethal in both cases, either when performed during embryonic stages, or also as conditional knockouts in the adult mouse [24-26]. As mentioned above, the protein is involved in RNA metabolism, but therein, various functions including regulation of alternative splicing, transcription, miRNA levels, RNA stabilization, as well as formation of stress and RNA granules have been described. TDP-43 seems to preferentially bind RNAs with unusually long introns and/or such that are involved in neuronal function like synaptic activity and neuronal development. Some of these RNAs encode proteins which have previously been shown to be involved in neurodegenerative diseases [27]. Al-

though TDP-43 depletion affects the expression and splicing of many different RNAs in the CNS, the vast majority of the more than 40 ALS causing mutations identified so far, lie within the C-terminal domain encoding a glycine rich stretch that is important for protein-protein interaction. While in healthy individuals TDP-43 is mainly localized in the nucleus, it gets mislocalized and trapped in cytoplasmic aggregates in ALS patients, leading to reduced levels in the nucleus [22]. The trapped TDP-43 seems to be heavily modified displaying ubiquitination, phosphorylation and cleavage and in some cases, misfolding. The exact meaning of TDP-43 mislocalization in ALS and other neurodegenerative diseases remains to be elucidated, as so far, it is unclear whether the inclusions actively participate in the disease development and progression or rather represent a mere indicator of other dysregulated cellular mechanisms. The observed changes in RNA regulation could arise from both, the mislocalization itself, leading to a reduced abundance in the nucleus, or function altering mutations or modifications of the protein. In addition, it was reported that certain mutations increase the stability of the protein and therefore its overall abundance in the cell [28], which might lead to the visible accumulations and alterations in RNA metabolism.

Until now, a plethora of different animal models including *Drosophila*, mouse and rats has been generated. Unfortunately at present, TDP-43 models have originated controversial results. In fact, overexpressing wild type TDP-43 in the CNS appears to be toxic by itself, while the effects of the mutant protein vary broadly ranging from no symptoms to severe neurodegeneration in different regions of the mouse brain [20]. Mostly, it is unclear whether the toxicity is due to the mutation or the simple presence of the transgene.

The gene encoding FUS lies on chromosome 16 in a region that was already linked to familial ALS before the first mutations were identified. After the discovery of TDP-43 mutations, the focus on genes encoding RNA/DNA-binding proteins increased leading to the fast discovery of mutations in FALS patients by two independent groups [8, 9]. Similar to TDP-43, more than 40 different FUS mutations have been identified in FALS patients or patients suffering from FTD. The FUS protein consists of 526 amino acids and like TDP-43, it is widely expressed amongst different tissues. Knockout of FUS in different mouse strains led to differing results, indicating that the genetic background of the used mouse strain plays an important role as disease modifier. In the inbred strains (C57BL/6 and 129), the knockout causes death at birth, whereas outbred strains survive until adulthood. In all cases, FUS depletion seems not to induce classical neurodegeneration as a primary effect. Interestingly, unlike TDP-43, the ALS related mutations in the FUS gene do not cluster in the glycine-rich region of the protein, but rather at the very end of the highly conserved C-terminus of the protein that contains the nuclear localization signal [22]. FUS is also mainly localized in the nucleus of cells in healthy individuals, but its mislocalization and aggregation in cytoplasmic granules in ALS patients leads to a less severe reduction in the nucleus than TDP-43 [22]. Up to now, only few binding partners of FUS (RNA or proteins) are known, making it further challenging to speculate about the function of the protein, which remains mostly unknown. In general, FUS is thought to be involved in regulation of gene expression, transcription, RNA splicing, RNA transport, translation, miRNA processing as well as DNA damage repair [29]. Interestingly, TDP-43

and FUS might directly interact with each other as they were detected in the same complex in cultured cells [28, 30]. Even though further data from patients and animal models are needed to confirm this finding, it is an interesting observation, as TDP-43 does not seem to be mislocalized in ALS cases that have accumulation of FUS containing aggregates in the cytoplasm [28].

Following TDP-43 and FUS, the thorough analysis of the cytoplasmic aggregates found in ALS patients led to the identification of additional common components such as optineurin and ubiquilin-2. Several of these proteins were afterwards identified to be mutated in a smaller portion of ALS patients as well [31].

In 2011, the identification of a new ALS gene harboring a different type of mutation was achieved. The association of the chromosomal locus 9p21.2 with ALS and FTD had already been described in 2006 [32]. The improvement of sequencing techniques and continuous research finally led to the identification of the disease causing gene: C9ORF72. While the function of this widely expressed protein is unknown, the type of mutation differs from other ALS related genes. It consists of a massive GGGGCC-hexanucleotide repeat expansion in intron 1 between two non-coding exons. Whereas healthy individuals carry up to about 23 repeats, affected patients have at least 30, but in some cases, many hundred copies of it [11, 12].

The first reports about the mutation in this gene came from two independent studies analyzing relatively small cohorts respectively of sporadic and familial ALS cases in Finland and Europe and ALS/FTD cases in the USA. Both studies started off with re-sequencing of the 9p21.2 locus from well defined families, in which the linkage of the disease to this chromosome 9 location had been previously demonstrated [11, 12]. After the detection of the repeat, the analyses were expanded to larger cohorts.

The most striking discovery of these studies is the high frequency of mutations in this gene in the analysed cohorts. Between 9 and 20% of American patients suffering from familial FTD and up to 38% of familial ALS cases from different European countries resulted positive for the new mutation. For the sporadic cases, the percentage lies around 7% for the American FTD patient population and 21% of sporadic ALS patients in the genetically homogenous Finnish population. With these initially published percentages, C9ORF72 mutations appeared to be the so far most frequent known cause of ALS and FTD. However, the cohorts were recruited through only few institutions and were rather small. Recently, a cross-sectional study including more patients from various different countries and with differing genetic background has been published. In this study, a total of 588 familial ALS cases and 403 familial FTD cases were screened for the mutation. For FALS, 37.6% of patients were identified to carry the pathological repeat, for FTD the percentage was 25.1% [33]. Although further studies are needed to confirm these findings, they are truly exciting, considering the fact that together with SOD1, which accounts for approximately 10-20% of FALS, almost 50% of the familial disease cases can now be explained by mutations in one of these two genes.

The observation that C9ORF72 mutations not only cause ALS, but also FTD and clinically mixed syndromes such as ALS-FTD, is in line with the clinical spectrum caused by TDP-43 and FUS mutations as well as wild type aggregations in both diseases. This further strengthens the indications that these two clinically distinct syndromes share a common pathogenic link [19, 34].

Interestingly, the pathological features of C9ORF72 related ALS seem very unusual and distinct up to the point that the mutation itself can actually be predicted from the observed pathology [35]. While the spinal cord shows the typical neuronal loss and TDP-43 positive cytoplasmic inclusions, other regions of the brain seem to accumulate aggregates that are widely devoid of TDP-43 and contain p62.

The mechanism through which this expansion repeat conveys toxicity to neurons still has to be elucidated. Two major hypotheses can be distinguished: 1) The expansion repeat alters or abolishes expression of all or certain C9ORF72 protein isoforms leading to reduced protein levels and a loss of functionality, 2) the expanded repeat itself conveys toxicity by sequestration of other RNA binding proteins and aggregate formation inside the nucleus, thus inhibiting proper functionality of the bound proteins. To date, it is not known which hypothesis applies in the case of C9ORF72 expansions, but the experience from various other toxic expansion repeat diseases like Huntington's disease are favoring the second. While Renton et al demonstrate the presence of aggregates in the nucleus of fibroblasts from affected patients, the results from the second study by DeJesus et al are less clear concerning accumulation of RNA granules. The latter study also lies more emphasis on a change in the expression levels of different C9ORF72 mRNA isoforms, which would support the first hypothesis rather than the second. Currently, the tools for a detailed analysis of protein expression and function are still lacking, but considering the importance of the mutation, huge efforts are put into the development of better antibodies, probes and assays.

Overall, the finding that mutations in TDP43, FUS and C9ORF72 might cause ALS by altering the normal interaction of these proteins with RNA or might cause a toxic gain of function leading to unexpected protein-RNA interaction opens new avenues for ALS research. These recent genetic discoveries have shifted the attention to cellular processes, i.e. RNA metabolism, transport and processing, that were not under investigation in the SOD1 models. These pathways might represent a common mechanism for different forms of ALS, as well as creating a link between ALS and a wider spectrum of neurodegenerative conditions.

3. The role of wild type SOD1 in ALS

In 1993 for the first time mutations in SOD1 were identified as cause of familial ALS and were found to be responsible for about 20% of familial cases [16]. Since that discovery nearly twenty years ago, more than 160 mutations in SOD1 have been identified (<http://alsod.iop.kcl.ac.uk/>) and cellular and animal models of the disease carrying different forms of mutant SOD1 have been generated [36, 37]. Experiments using animal models revealed that the toxicity of mutant SOD1 is not related to a loss of function of the enzyme [38], but

rather a gain of toxic function. In the past twenty years different forms of mutant SOD1 have been characterized for their biochemical properties, however no common characteristics between mutations have been found. In fact, different mutations seem to cause different changes in enzymatic function or no change at all [39-41], leading to the conclusion that SOD1 dismutase activity is not responsible for protein toxicity. Although the nature of the toxic function gained by mutant SOD1 is still obscure, there is clear evidence that the mutant enzyme undergoes conformational changes leading to its misfolding and subsequent aggregation [41, 42]. SOD1 aggregates are, in fact, one of the histological hallmarks of SOD1-related FALS, as well as sporadic cases carrying SOD1 mutations.

Although SOD1 mutations are responsible for less than 2% of ALS cases and this disease is mainly of sporadic origin, sporadic and familial cases are clinically undistinguishable. Moreover, with the exception of patients carrying C9ORF72 mutation, which seem to define a specific clinical subgroup [35], other genetic mutations do not determine different clinical characteristics. This observation has led to the conclusion that familial and sporadic ALS must share common pathogenic mechanisms [1]. Consequently, in recent years, efforts have been made in understanding whether the genes causing familial ALS can be responsible or can be involved in the pathophysiology of sporadic cases. Recently, strong evidence has been gathered suggesting that SOD1 might play a crucial role also in SALS.

Wild-type human SOD1 is a 32KDa homodimer known to be one of the most stable proteins with a melting temperature around 90°C. However, its stability is highly dependent upon post-translational processes including binding of copper and zinc ions and the formation of an intramolecular disulfide bond. Impairment or retardation of these post-translational processes can disrupt SOD1 stability, causing the formation of misfolded structures and aggregates. Indeed, in 2007, it was shown that oxidised wild-type SOD1 could acquire *in vitro* aberrant properties leading to association with poly-ubiquitin, Hsp70 and chromogranin B, similarly to the mutant enzyme [43]. The same year, another group used covalent chemical modification to show that spinal cord samples from both familial and sporadic ALS cases displayed a form of SOD1 that was absent in non-neurological controls as well as in spinal cord samples from patients affected by other neurodegenerative disorders [44]. Recently, Guareschi et al [45] managed to immunoprecipitate SOD1 from sporadic and familial ALS patients' lymphoblasts and then analysed the presence of oxidized carbonyl groups. A form of over-oxidized wild-type SOD1 was indeed found in a subset of SALS patients. This post-translationally modified form of the wild-type enzyme recapitulates some of the toxic properties attributed to mutant SOD1, i.e. the ability to cause mitochondrial damage through interaction with Bcl-2 [45]. Altogether, these findings supported the hypothesis that SOD1 could be a link between familial and sporadic ALS.

On another front, the use of the SOD1 mouse model also provided important clues as to whether normal SOD1 can play a role in the disease. Surprisingly, overexpression of wild-type human SOD1 accelerated disease onset in several transgenic mouse models of ALS [46, 47], supporting the involvement of wild type SOD1 in the disease mechanism. However, these results have to be interpreted with caution, as they might derive by the toxicity of transgene accumulation rather than a specific SOD1-related mechanism.

In order to determine whether *in vivo* wild-type SOD1 can undergo misfolding and can be detected without altering the original sample, recent studies have focused on the production and investigation of new antibodies able to distinguish mutant/misfolded/monomeric SOD1 as opposed to its wild-type form. Although some of these antibodies have been tested only on limited samples and their ability to discriminate between aberrant conformations of SOD1 is debatable, their use has led to potentially interesting findings. One of the first antibodies produced to detect abnormalities in SOD1 post-translational processing was the SOD1-exposed dimer interface (SEDI). This antibody was prepared with the peptide at the dimer interface of SOD1. When SOD1 is folded as a homodimer in its active state, this site is inaccessible, while it is exposed upon monomerization [48]. This antibody successfully stained inclusions in motor neurons from SALS samples, however it did not detect positive inclusions in SALS spinal cords where no SOD1 mutations were detected [49]. Similarly, an antibody developed against the region Leu⁴²-His⁴⁸, which specifically recognizes SOD1 in which the beta barrel is unfolded, failed to detect misfolded SOD1 in SALS spinal cord samples, but succeeded in recognizing aggregates in the FALS samples [50]. Despite these results, it could not be concluded that wild-type SOD1 does not contribute to the pathogenic mechanisms occurring in SALS. Indeed, Forsberg et al. have produced a series of polyclonal antibodies against several SOD1 peptides that react with the denatured enzyme, but not with the wild-type form. Using these antibodies, small inclusions were detected in the motor neurons of SALS patients [51] as well as in the nuclei of astrocytes, microglia and oligodendrocytes [52]. These studies supported the hypothesis that wild-type SOD1, although not involved in the formation of Lewy body-like inclusions in SALS, is likely to undergo conformational changes, thus contributing to the pathologic mechanism.

Another antibody that has been used to detect misfolded SOD1 is C4F6. This peptide was raised against metal depleted (apo) SOD1 with G93A mutation [53]. Although this antibody was raised against a specific mutant form of SOD1, it successfully recognised skein-like inclusions in FALS spinal cord samples, as well as inclusions in SALS [54].

Recently, a monoclonal antibody, called 3H1, was used to detect misfolded SOD1 in a subset of SALS cases displaying TDP-43/FUS-positive inclusions. This antibody recognizes a peptide corresponding to a structurally disrupted SOD1 electrostatic loop, detectable only when the protein is misfolded. Spinal cord immunocytochemistry showed that, in some SALS samples, TDP-43/FUS-positive inclusions were also positive for 3H1 antibody, suggesting that the pathologic mechanisms involved in ALS might trigger SOD1 misfolding, thus triggering toxic pathways common to both sporadic and familial ALS [55].

Besides the efforts to generate antibodies able to detect misfolded SOD1, no consensus has been reached on which antibodies, if any, can reliably and consistently detect the different forms of misfolded SOD1. Other studies have, therefore, used a different approach, trying to understand whether normal SOD1 shares common characteristics with the mutant form of the enzyme. Recently, a novel rare mutation in SOD1 (L117V) was identified in two Syrian ALS families [39]. Unusually, the disease showed uncommon low penetrance and slow progression. Biochemical analysis of L117V SOD1 showed that its properties were indistinguishable from the wild-type form and yet causing the disease. This study highlights that

normal SOD1 is in the range of protein stability that can cause disease and suggests that mutant forms of SOD1 with high stability might be related to low penetrance and be therefore categorized as sporadic forms of ALS. The authors suggest that, similarly, other complex genetic, environmental and lifestyle factors can influence the stability of normal SOD1 causing its misfolding in SALS cases. However, this does not exclude that the toxicity related to L117V mutation could derive by the interaction with other proteins and not by its stability.

The importance of finding common pathways or players between sporadic and familial ALS is crucial for therapeutic approaches. One recent study explored this possibility [15]. The authors assessed the toxicity of astrocytes derived from neural progenitors isolated from the spinal cord of sporadic and familial ALS patients. This study showed for the first time that astrocytes from sporadic cases of disease are as toxic to motor neurons as astrocytes carrying mutations in SOD1. As expected, the shRNA mediated reduction of mutated SOD1 led to a complete rescue of motor neurons in this co-culture system. Of particular interest however, was the finding that even the knock down of wild type SOD1 in astrocytes from sporadic patients markedly attenuated toxicity towards motor neurons.

The data summarized in this section provide strong evidence for a pathologic role of wild-type SOD1 in sporadic disease. This hypothesis opens new frontiers for future therapeutic approaches in the treatment of ALS.

4. Human samples to study ALS

In ALS the cells mainly affected by the disease, the motor neurons and the glia, are located in the motor cortex and the spinal cord, which are accessible only post-mortem. The scarce availability of CNS samples, along with post-mortem delay and different preservation techniques that can affect the quality of the tissue and limit its use, are great challenges when studying this disease. Moreover, post-mortem material is only representative of the end stage of disease and, although used in microarray studies to unravel the mechanisms of neurodegeneration, it is unlikely to help identify early biomarkers. For these reasons, peripheral tissues, i.e. blood, fibroblasts and cerebrospinal fluid (CSF) have been preferentially used in high-throughput screening assays for biomarkers identification as well as gene expression profiling.

4.1. Gene expression profiling

Multiple research groups have used post-mortem samples to identify the pathways involved in the neurodegenerative process of ALS. The studies utilizing complex tissues, representative of a mixed cell population, i.e. motor cortex [56] or spinal cord [57], have mainly recorded gene expression changes indicating the presence of an aggressive inflammatory reaction and active astrogliosis. These processes are prevalent in the spinal cord of ALS patients and have masked the transcriptional changes occurring in motor neurons. However, the motor cortex seems to be affected to a lesser extent by astrogliosis and this enabled Lederer and colleagues to identify important changes in transcripts involved in the cytoskele-

tal, mitochondrial and proteasomal functions, as well as ion homeostasis and glycolysis [56], in agreement with other lines of research in ALS [1].

In order to determine those genes differentially expressed in the cell type most affected in ALS, i.e. spinal motor neurons, laser capture microdissection (LCM) has been used to isolate single cells from human post mortem spinal cord samples.

Gene expression profiling of motor neurons has been performed on sporadic ALS cases [58], as well as ALS cases carrying mutations in the *SOD1* and chromatin modifying protein 2B (*CHMP2B*) genes [59, 60]. The three studies highlighted the activation of different pathogenic pathways. The motor neurons isolated from sporadic cases showed decreased expression of genes associated with the cytoskeleton and transcription, whilst cell death-associated transcripts were increased. Moreover, genes involved in cell cycle activation and progression were found to be upregulated, supporting the theory that inappropriate activation of the cell cycle in these post-mitotic cells can lead to cell death [58].

In contrast, microarray analysis of motor neurons isolated from *SOD1*-related ALS cases highlighted the activation of a cell survival pathway in the motor neurons that were spared by the disease. The study, in fact, revealed differential expression of genes involved in the protein kinase B/phosphatidylinositol-3 kinase (AKT/PI3K) pathway, along with decrease in phosphatase and tensin homologue (*PTEN*) gene, a negative regulator of AKT [60]. The authors also showed that inhibition of *PTEN* led to increased activation of the AKT/PI3K pathway, with beneficial effects on primary motor neuron survival. Thus, activation of the AKT/PI3K pathway is a potential candidate for future therapeutic strategies.

Finally, the transcriptional profiles from motor neurons isolated from the *CHMP2B*-related ALS cases showed dysregulation of genes involved in p38 MAPK signalling pathway, reduced autophagy and repression of translation [59]. The significant impairment of the autophagy pathway reflects the function of *CHMP2B*, the gene mutated in these patient samples.

In spite of the differences between the pathways described above, dysregulation of calcium handling and cell cycle, as well as transcription, cytoskeleton assembly and metabolism, were common between the different genetic subtypes and SALS. Taking into account that these results derive from end-stage tissues, they support the evidence that etiologically diverse forms of ALS converge into common mechanisms involved in motor neuron death.

4.1.1. Results from use of human peripheral tissue

Gene expression profiling has also been conducted on blood cells from ALS patients in order to identify biomarkers and/or achieve a better classification of disease subtypes through the identification of common transcriptional patterns [61-63]. In the study conducted by Saris and colleagues, microarray analysis of SALS and control whole blood samples was followed by hierarchical clustering of all differentially expressed transcripts [61]. This approach successfully identified different clusters that were able to differentiate between ALS and control samples. Interestingly, this study showed that peripheral blood can be used to investigate the pathways activated during disease, as the blood from ALS patients reveals decrease in

transcripts involved in RNA processing as well as upregulation of inflammatory genes. This suggests that the mechanisms affecting motor neurons, also strike other cell type. However, because of their post-mitotic characteristics and their unique function, motor neurons are the most susceptible.

A more recent study performed gene expression profiling on peripheral blood mononuclear cells (PBMCs) from patients with SALS [62]. The results show upregulation of LPS/TLR4-signaling associated genes in response to elevated LPS plasma levels. A similar transcription pattern was obtained by culturing PBMCs from normal controls with LPS for a short time *in vitro*.

Similarly, Mougeot et al. found that peripheral blood lymphocytes (PBLs) display dysregulation of the ubiquitin/proteasome system (UPS) [63]. In particular, microarray analysis revealed upregulation of the ubiquitin-protein ligase E3- α -2 (UBR2) expression. UBR2 is known to act in synergy with UBR1 in a quality control mechanism for degradation of unfolded proteins. UBR2 upregulation correlated inversely with time since onset of disease and directly with the ALS functional rating scale (ALSF_{RS}-R), suggesting that UBR2 is increased early in the disease course and decreases as disease progresses. The authors confirmed with *in vitro* experiments that cultured PBMCs from ALS patients accumulated more ubiquitinated proteins than PBMCs from healthy controls in a serum-dependent manner, as expected from the transcription data.

Very recently, human samples have been used to interrogate micro RNA (miRNA) expression [64, 65]. Two studies successfully identified dysregulation of miRNAs in peripheral leukocytes [64] and monocytes [65] from SALS samples compared to controls. These miRNA are involved in pathways relevant to the CNS and, in particular, Butovsky et al. identified changes relevant for the inflammatory response, similarly to the expression pattern displayed by monocytes isolated from multiple sclerosis (MS) patients. These results suggest that miRNAs profiles found in the peripheral blood cells can be relevant to understand the pathogenesis of ALS and/or used as biomarkers of the disease.

4.2. Biomarkers in ALS

ALS is fatal rapidly progressive neurodegenerative disorder, characterized by the activation of an intricate network of pathways and still lacking an effective treatment beside Riluzole [1]. The diagnosis of ALS is still mainly based on clinical assessment of progression of symptoms, which results in a delay of about a year from symptom onset to diagnosis. Although the clinical course of disease can considerably vary from case to case, in the majority of cases death occurs within 2-5 years [66].

In this scenario, significant effort has been spent trying to identify molecules that could help classify different forms of ALS and lead to early diagnosis, as well as monitor disease progression.

The tissues mainly utilized for biomarker screening in the past five years have been peripheral blood mononuclear cells (PBMCs) and cerebrospinal fluid (CSF). PBMCs have already been shown to display some of the traits of the disease, such as increase in inflammatory

genes [65] and downregulation of Bcl-2 [67] and might, therefore, be used to investigate the disease during its progression as well as provide unique biomarkers.

Recently, Nardo et al. performed proteomic analysis of PBMCs isolated from 60 sporadic ALS patients and 30 healthy controls [68]. The authors identified and validated in a second cohort 14 protein biomarkers, that could discriminate between ALS patients and controls regardless of age and gender. Remarkably, of these 14 biomarkers, 5 were able to discriminate between ALS patients and individuals with other neuropathies and 3, among which TDP43, were markers of disease severity. Notably, these results are consistent with a CSF biomarker study reporting that TDP43 levels were increased in ALS patients [69]. The value of this result goes beyond the finding of a disease biomarker, as it supports the even more interesting hypothesis that TDP43 could be a common player in early disease in familial as well as sporadic ALS cases. This would confirm what is already suggested by the presence of TDP-43 positive aggregates in SALS biopsy samples.

Although there is still no consensus on valid biomarkers for ALS [70], proteome analysis has recently led to the identification of fetuin-A and transthyretin (TTR) as candidates to distinguish ALS patients with rapid versus slow disease progression. The upregulation of TTR and fetuin-A, involved in immune regulatory functions, could be associated with the inflammatory state of the CNS. At present, these markers were tested in two independent cohorts of 18 and 20 patients with a follow up of 2 years [71] and TTR had already been identified as a potential biomarker for ALS compared to controls in a previous study [72]. Although further validation is needed, these results are encouraging and would provide an invaluable tool to discriminate between patients with different disease progression rates. This would help clinician determine the timing for clinical intervention such as gastrostomy and non-invasive ventilation [73].

5. Stem cell technology for ALS research

Stem cells are defined as a population of cells that maintains the ability to self-renew and differentiate into several cell types of the adult body. In mammals, several tissues such as muscle, brain and bone marrow, harbor subtypes of stem cells that can give rise to a relatively small variety of different cell types. These adult stem cells are committed to certain cell lineages and do not produce cells from other tissue types under normal conditions. Unlike adult stem cells, embryonic stem cells that can be isolated from the inner cell mass of early stage embryos are pluripotent and can, therefore, still differentiate into virtually any cell type of the human body. While the collection of embryonic stem cells from mice is a widely accepted approach used for disease modeling, the use of human embryonic stem cells is controversial and rises severe ethical concerns. With the discovery that adult human fibroblasts can be reprogrammed to an embryonic stem cell like state, new hope arose for stem cell based approaches in human research. Induced pluripotent stem cells (iPS) are usually generated by the introduction of 2-5 defined pluripotency transcription factors into fibroblasts or other readily available differentiated cell types. These transcription factors

drastically alter gene expression in the target cells until some of them eventually become pluripotent and can then be isolated and amplified. Initially, the transcription factors were introduced by retroviral or lentiviral constructs leading to the integration of the transgene into the target genome. As the random integration of additional genes can disrupt/alter the expression of endogenous genes, more recent approaches rely on less invasive techniques such as transposons or RNA transfection [74]

The use of stem cell technologies in ALS research started in 2007 when mouse embryonic stem cells from the most prominent SOD1 model carrying the G93A mutation were established [75]. When differentiated into motor neurons, mild phenotypic differences between motor neurons expressing human wild type SOD1 or the G93A mutation could be observed. After several weeks in culture, SOD1 containing inclusions as well as the overall level of ubiquitinated proteins were more frequent in the motor neurons expressing the mutant human protein. In 2009, a human embryonic stem cell line was used to generate motor neurons that were then transfected with different SOD1 mutation containing constructs [76]. The researchers observed a reduction in neurite length and in line with the study of the mouse G93A embryonic stem cell derived motor neurons, reduced survival. However, in the human study, it is unclear whether the observed phenotype arises from the mutations or the increase of SOD1 abundance itself, as a control overexpressing wild type SOD1, was not generated.

Since the discovery of the iPS technology, huge efforts were put in the generation of patient specific iPS lines. Up to now, several hundred lines with various mutations have been generated and some are now becoming commercially available thereby getting accessible to a broad scientific community.

In 2008, Dimos et al reported the successful generation of motor neurons and glial cells from an ALS patient derived iPS line carrying a SOD1 mutation causing a mild disease phenotype [14]. Surprisingly, unlike the previous studies with mouse and human embryonic stem cells, no disease related phenotype was reported from these cells until now. This potential lack of phenotype could in part be explained by the patient's late onset and mild disease form. Another report from 2011, where motor neurons were generated from a patient harboring a VAPB mutation, did also not mention any phenotype despite reduced levels of VAPB. As the levels of this protein were already reduced in the fibroblasts used for the reprogramming, the lower levels in the resulting motor neurons could either be due to mutation induced expression or translational changes or could also be explained by an incomplete reprogramming of this genomic locus in the generated iPS lines [77]. Recently, the first report of an iPS line harboring a TDP-43 mutation was published [78]. The motor neurons generated from this line showed elevated TDP-43 levels, but no change in localization or signs of aggregate formation. In addition, motor neurons from both, control and TDP-43 mutant were phenotypically and functionally similar despite an elevated sensitivity to PI3K signaling inhibition and elevated cell death. These data suggest that the toxicity of this TDP-43 mutation might arise from its increased stability leading to a higher overall protein amount in the cell.

Despite the growing number of iPS lines from ALS patients available, no further reports of disease relevant phenotypes or major discoveries of disease mechanisms from the use of these cells were reported so far. In addition, observations from other neurological disorders show a similar trend: cells differentiated from embryonic stem cells or induced pluripotent stem cells often reflect only certain aspects of the disease and sometimes it takes several weeks before such differences can be recorded and/or the observed symptoms are very mild [79, 80]. The problems to reproduce patient phenotypes have ameliorated the initial excitement about this new method to model neurological diseases. More recently, it became clear that even cells differentiated from individual embryonic stem cell clones or iPS clones from the same patient can show substantial phenotypic differences, a phenomenon called clonal variation [81]. Similar observations were made in a study using iPS lines of ALS patients carrying TDP-43 mutations. After differentiation of individual iPS clones from the same patient into motor neurons, the levels of TDP-43 expression as well as aggregate formation and oxidative stress induced cell death showed substantial variation [82]. Considering the confusing reports, it has to be assumed that the reprogramming as well as the following differentiation mechanisms are not yet fully under control and more mechanistic research and standardization of the protocols will hopefully soon lead to more pronounced and reproducible results. Initial steps to improve reproducibility and differentiation are already under way. The main focus lies on the standardization of the initial characterization of the iPS clones prior to use as well as the improvement of differentiation protocols by addition of various small molecules and growth factors [83, 84].

While these approaches emerge, sporadic and familial forms of ALS can be modeled with cells isolated from human post mortem spinal cord or brain samples. A recent report demonstrated that post-mortem isolated neuronal progenitor cells from patients with sporadic or familial ALS, can be differentiated into astrocyte-like cells *in vitro*. Astrocytes from patients, but not from healthy controls conveyed toxicity to wild type mouse motor neurons in a co-culture [15]. This system provides a promising tool for testing of potential therapeutic approaches.

5.1. The promises and limitations of stem cells for therapeutic approaches

The disease modeling described in the upper section is in particular important for the huge proportion of ALS cases, where no causing mutation is known. Until the discovery of the iPS technology, this lack of knowledge made it impossible to model such cases *in vitro* in a cell based assay or with animal models, unless post-mortem cells could be collected. Now we can use skin fibroblasts or other readily available cell types from affected patients during different time points of disease progression prior to end stage. From these fibroblasts, various cell types that are known to play a crucial role in ALS can be generated and their behavior and interplay in a cell culture dish can be studied in-depth.

Despite emerging into an invaluable tool to study disease mechanisms, stem cells also hold a huge potential for the development of therapeutic approaches for ALS and many other neurodegenerative diseases. On the one hand, the generated cells can now be used in drug screenings to identify new target mechanisms or to assess potential new therapeutics. On

the other hand, the stem cells can be used for cell replacement approaches. For patients with known mutations, the cells can be genetically modified or corrected prior to reprogramming and differentiation. Such individualized strategies would allow the use of the patient's own cells for transplantation, thereby reducing the risk of graft rejection.

In ALS, a major future goal would be to produce and replace dying motor neurons. A proof of principle that motor neuron transplantation might become possible came from the observation that mouse embryonic stem cell derived motor neurons transplanted into the lumbar part of paralysed adult rats can actually survive and form functional neuromuscular junctions leading to phenotypical improvements [85]. However, in human ALS patients, the replacement of motor neurons might be more complicated due to the size differences, amount of cells needed and distance that the axon would have to grow out. In addition, it becomes more and more evident that ALS is a non-cell autonomous disease in which astrocytes, microglia and oligodendrocytes play a crucial role in modulating disease onset and progression [3, 15, 86]. In this context, a strategy approaching several cell types at a time might be more successful than bringing in healthy motor neurons alone into a heavily diseased environment. A promising candidate that can generate various cell types in the CNS and at the same time positively stimulate the neuronal environment by producing neuroprotective factors, are neuronal progenitor cells (also called neuronal stem cells). Several reports indicate that transplanted neuronal progenitor cells are able to differentiate into different cell types *in vivo* [87, 88]. Further, injection of NPCs has been shown to ameliorate disease progression in ALS rodent models even if most of the cells do not migrate or differentiate into other cell types in various neurodegenerative diseases [89]. However, it is not known how these cells would behave and survive when transplanted into a diseased environment.

One of the largest limitations for cell replacement strategies to date is the lack of efficiency as well as specificity during the amplification and differentiation step. The differentiation of ES or iPS cells into various different neurons or neuronal progenitor cells is guided by the application of different growth factors and small molecules. However, this process usually generates a mixed population containing many different cell types. The cell type of interest often represents only 30% or even less of the total population. Therefore, it might be difficult to generate enough cells for therapeutic applications.

When using ES or iPS lines to generate the cell type of interest, a further drawback is that a small portion of cells remains undifferentiated and immature, thereby representing a major risk factor for transplantation [90]. When neuronal progenitor cells derived from mouse iPS cells were injected into adult mouse brains, they formed tumors in up to 60% of the injected animals [88]. The use of more restricted cell types such as NPCs on the opposite, appears to be safer.

Finally, the generation and maintenance of a stable iPS lines from human adult cells is expensive and very time consuming [74]. If clinical applications are considered, a thorough characterization of several individual clones needs to be undertaken prior to use, making a widespread application of this approach today unlikely. Very recent reports indicate that fibroblasts can be directly differentiated into several types of neurons and even neuronal progenitor cells in a much faster and more efficient way than through iPSing [91]. It remains to

be evaluated to which extent these cells recapitulate neurodegenerative disease phenotypes, although a first report from fibroblast derived neurons from a familial Alzheimer's patient seem promising [92].

In summary, the technique of reprogramming holds great promises in terms of disease modeling and unraveling of underlying mechanisms of sporadic neurodegenerative diseases such as ALS. Despite the current confusion due to the various methods used to generate the lines, the observed clonal variations as well as the limited reflection of disease phenotypes, the field has advanced with tremendous speed if one considers that the first report about reprogramming of mouse fibroblasts was published only 6 years ago. With combined efforts and improved methods, a better understanding and control of the reprogramming mechanisms can be achieved, thereby facilitating the interpretation and usage of the generated cells.

6. Future trends in modeling ALS and discovering new therapies

The recent remarkable advancement in the cell biology field that adult fibroblasts can be reprogrammed to virtually originate all cell types have created a unique opportunity to model neurological disorders *in vitro*. iPS technology has already been applied to several neurodegenerative conditions, from Alzheimer's disease [93] to Down syndrome [94], as well schizophrenia [80], Rett syndrome [95] and ALS [14].

Although a large number of iPS cell lines from patients affected by various diseases have been made commercially available, it is still not clear how robustly these recapitulate the characteristics specific of each disease. Although the promises of iPS technology are to lead to high-throughput screenings to find new efficacious therapeutic targets, they are subject to some main limitations that have already been addressed in other sections of this book chapter. It is, therefore, of paramount importance that the properties of the differentiated cells are well characterized and it is verified that they are representative of the disease they are modeling.

However, some promising results have been obtained from a very recent study suggesting that iPS-derived motor neurons originated from patients carrying TDP-43 mutations display abnormalities typical of TDP-43 proteinopathy. These cells display elevated levels of soluble and detergent-resistant TDP-43 protein, decreased survival, and increased vulnerability to inhibition of phosphatidylinositol 3-kinase (PI3K) pathway [78] as well as shorter neurites and TDP-43 cytoplasmic aggregates [82]. These parameters can be used as readout for high-throughput drug screenings as well as short hairpin RNA (shRNA) library screenings. Indeed, Egawa and colleagues performed microarray analysis on iPS-derived motor neurons transduced with lentivirus expressing green fluorescent protein (GFP) under the control of the HB9 promoter. Based on the results obtained from gene expression analysis, the authors tested 4 drugs known to modulate transcription through histone modification and RNA splicing. Using the high content imaging analyzer InCell 6000, Egawa and colleagues found

that anacardic acid had protective effects against arsenite-induced motor neuron death and was able to decrease TDP-43 cytoplasmic aggregates as well as increase neurite length [82].

A different approach was taken in 2011 by Haidet-Phillips and colleagues [15], producing cells from patients without the use of viral vectors or induction of major epigenetic modifications. In this study, astrocytes were derived from NPCs isolated from ALS patients and it was observed that, regardless of their familial or sporadic origin, these cells were toxic to wild type murine motor neurons expressing GFP under HB9 promoter [15]. The authors found that SOD1 knockout via shRNA could rescue motor neurons at different extents depending on whether these were co-cultured on astrocytes from familial or sporadic cases. This study overcomes some of the major issues related to iPS cells and sets the premises for drug and shRNA screening to target pathways and single genes involved in astrocyte toxicity.

Concluding, it is clear that in the past five years the ALS field has seen a major change of scenario, where more tools are available to study more forms of FALS as well as the striking majority of SALS. As the recent genetic discoveries have highlighted the importance of previously unexplored pathways, i.e. RNA metabolism, also common targets linking sporadic and familial ALS have been identified, i.e. TDP-43 and SOD-1. Moreover, the advances in highthroughput screening technology with the advent of new gene profiling techniques, i.e. deep-sequencing, and high content imaging systems are bound to determine the beginning of a new era for ALS research.

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