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High content analysis in amyotrophic lateral sclerosis

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a devastating disease characterized by the progressive loss of motor neurons. Neurons, astrocytes, oligodendrocytes and microglial cells all undergo pathological modifications in the onset and progression of ALS. A number of genes involved in the etiopathology of the disease have been identified, but a complete understanding of the molecular mechanisms of ALS has yet to be determined. Currently, people affected by ALS have a life expectancy of only two to five years from diagnosis. The search for a treatment has been slow and mostly unsuccessful, leaving patients in desperate need of better therapies. Until recently, most pre-clinical studies utilized the available ALS animal models. In the past years, the development of new protocols for isolation of patient cells and differentiation into relevant cell types has provided new tools to model ALS, potentially more relevant to the disease itself as they directly come from patients. The use of stem cells is showing promise to facilitate ALS research by expanding our understanding of the disease and help to identify potential new therapeutic targets and therapies to help patients. Advancements in high content analysis (HCA) have the power to contribute to move ALS research forward by combining automated image acquisition along with digital image analysis. With modern HCA machines it is possible, in a period of just a few hours, to observe changes in morphology and survival of cells, under the stimulation of hundreds, if not thousands of drugs and compounds. In this article, we will summarize the major molecular and cellular hallmarks of ALS, describe the advancements provided by the *in vitro* models developed in the last few years, and review the studies that have applied HCA to the ALS field to date.

Contents

1. Review

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease affecting predominantly motor neurons (MN), with an incidence of 3.9 individuals for every 100,000 people in the U.S. (Center for Disease Control and Prevention – https://wwwn.cdc.gov/als/ALSReportsNew.aspx). Hallmarks of the disease are progressive muscular weakness and dystrophy, caused by loss of upper MNs in the motor cortex and lower MNs in the brainstem and spinal cord (SC). ALS typically progresses rapidly and results in patient death within three to five years after diagnosis; the cause of death for the majority of patients is respiratory failure (Rowland and Shneider, 2001). Inherited cases of

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the disease, referred to as familial ALS (fALS), account for 5–10% of the patient population; non-inherited cases, as well as those of unknown origin are called sporadic ALS (sALS) and affect the remaining population. Both sALS and fALS have a very similar clinical profile, suggesting common disease mechanisms (Robberecht and Philips, 2013). Our current understanding of ALS is that the disease is a multifactorial disorder with a cumulative pathology in which more than one cellular mechanism is disrupted, causing a series of detrimental events contributing to MN death. Some of the events characterizing ALS are: the aggregation and misfolding of proteins, high levels of oxidative stress, excitotoxicity, deficient axonal transport, mitochondrial dysfunctions and inflammation (Bruijn et al., 2004). The neurodegeneration occurring in ALS has also been described as a non-cell autonomous occurrence (Ilieva et al., 2009), meaning that the MN death is a consequence of a pathological state of other cell types, such as microglia (Beers et al., 2006a; Dennys et al., 2015; Frakes et al., 2014), astrocytes (Di Giorgio et al., 2007; Haidet-Phillips et al., 2011; Meyer et al., 2014; Serio et al., 2013; Nagai et al., 2007; Yamanaka et al., 2008; Song et al., 2016) and oligodendrocytes (Philips et al., 2013; Ferraiuolo et al., 2016) that either spread their toxicity or simply provide less support compared to a non-pathological condition.

The last 20 years of research have resulted in remarkable advancements in the identification of many genetic factors behind the onset and the progression of the disease. Mutations responsible for the onset of ALS have been identified in various genes; the copper-zinc superoxide dismutase 1 (SOD1) gene was the first to be described in 1995 (Rosen et al., 1993), followed by many others including the transactivation response DNA-binding protein 43 (TDP43) (Sreedharan et al., 2008) and the FUS RNA binding protein (Kabashi et al., 2008). One of the latest to be identified is an expansion in hexanucleotide repeat GGGCC of the chromosome 9 open reading frame 72 (C9ORF72) (DeJesus-Hernandez et al., 2011), which account for 50% of familial cases. Other genes have been associated with familial or sporadic ALS such as Optineurin (OPTN) (Maruyama et al., 2010), Sequestosome 1 (SQSTM1) (Fecto et al., 2011), Angiogenin (ANG) (Greenway et al., 2006), Survival of motor neuron 1 (SMN1) (Burghes & Beattie, 2009) and NEK1 (Brenner et al., 2016), although with a minor epidemiological incidence (Zufiría et al., 2016) (for a complete list see https://ghr.nlm.nih.gov/condition/amyotrophic-lateral-sclerosis/show/Related+Gene(s)). These genes code for proteins involved in many unrelated cellular processes confirming the complexity of the disease.

The identification of genetic causes of ALS has allowed the development of *in vivo* and *in vitro* models that recapitulate the biochemical and molecular events occurring in the pathology. More than 20 transgenic mouse models of SOD1 are currently available, expressing either the human wild type (WT) protein or different mutated forms; transgenic mouse strains carrying the human WT or mutated TDP43 and FUS proteins have also been established (McGoldrick et al., 2013). More recently, the C9orf72 Bacterial Artificial Chromosome (BAC) transgenic mouse also became available (O'Rourke et al., 2015; Peters et al., 2015; Liu et al., 2016). These models have different advantages and reflect the disease in various ways; however, SOD1 mice remain the most used in research. For example, studies conducted on SOD1 mice have revealed that a toxic gain of function rather than a loss in protein function is responsible for the ALS pathophysiology; $SOD1^{-/-}$ mice showed no difference in neurological or morphological levels when compared to littermate controls (Reaume et al., 1996). Reaume and colleagues have shown that $SOD1^{-/-}$ mice, compared to their littermate controls, show only an increased vulnerability to stress in MNs, while the cell number or functionality was not impaired. Conversely, mice overexpressing mutated forms of the SOD1 protein, such as the $SOD1^{G93A}$ (Gurney et al., 1994), $SOD1^{G37R}$ (Wong et al., 1995) and the $SOD1^{G85R}$ (Brujin et al., 1997) have shown a selective death of MNs and a progressive fatal paralysis, despite little to no change in SOD1 enzyme activity or, for some mutations, even an increase thereof (Cleveland and Rothstein, 2001). Mutant SOD1 protein forms intracellular aggregates, detected in multiple cell types including MNs and astrocytes (Bruijn et al., 1998) and cause a series of detrimental cellular events with elevation of reactive oxygen species (ROS) production (Xu et al., 2005), endoplasmic reticulum (ER) stress (Tobisawa et al., 2003), inhibition of proteosomal degradation (Urushitani et al., 2002), mitochondrial dysfunctions (Vande Velde et al., 2008) among the most cited. Additionally, studies with ALS animal models also support the hypothesis that non-cell autonomous mechanisms are responsible for MN death in ALS; indeed, when mutated SOD1 was expressed exclusively in motor neurons, animals showed no sign of ALS (Pramatarova et al., 2001; Lino et al., 2002) or only mild signs of motor neuropathy (Jaarsma et al., 2008), suggesting that maybe other type of cells are involved in the pathology. Animal models are a crucial resource in the investigation of disease mechanisms and to test potential treatments; however, their use entails several limitations, as they cannot truly replicate the human disease. In animals, the development of ALS symptoms is dependent on the expression of the transgene as well as the gender and the genetic background of the animal. Transgenic animals are only modeling the disease associated with their specific genome alteration; for example, if we consider the $SOD1^{G93A}$ mouse, which is one of the most frequently used ALS and mutant SOD1 model, it would represent only familial cases of ALS, thus less than 2% of total cases. In addition, some findings could be limited to the population carrying the specific mutation at the glycine 93, which could significantly narrow the patient population benefiting from the treatment. Moreover, animal models typically cannot be used for high throughput studies, which is a great limitation for large-scale therapeutic investigations.

Studying molecular mechanisms of the disease as well as testing new drugs or gene-based therapies directly in a wider and human scenario may provide more reliable information on therapeutic approaches that can be more successfully translated to the clinic. For this purpose, researchers have developed methods to isolate cells from postmortem human samples that can be cultured *in vitro* and expanded for a limited time (Palmer et al., 2001). In the context of ALS, neural progenitor cells (NPCs) from the lumbar spinal cord of ALS post-mortem patients, once differentiated into astrocytes, were shown to dramatically affect MN survival compared to control astrocytes (Haidet-Phillips et al., 2011; Re et al., 2014). Moreover, reduction of SOD1 levels in astrocytes from sporadic ALS patients that do not carry a mutation in this gene, lead to substantial increase in survival of co-cultured motor neurons. Using a similar *in vitro* model Re and colleagues, further confirmed that in sporadic cases of ALS, SOD1 toxicity in MNs alone is not responsible for the MN death (Re et al., 2014). A limitation of post-mortem isolated cell based models is that they represent only very late stages of the disease and they are limited in their potential for expansion to large high content analyses due to limited growth. Additional advancements have come from the advent of reprogramming methods which are based on the de-differentiation of somatic cells, into stem cell-like progenitor cells or induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006; Yamanaka et al., 2007). Unlike cells obtained from post-mortem samples, fibroblasts can be obtained directly from living patients, and can be used to model the disease at various stages of progression, rather than being limited to the end stage. Once fibroblasts are converted to progenitors or iPSCs, they provide an unlimited source of material thanks to their high proliferative capacity (Yamanaka et al., 2007) and can be differentiated into many cell types including neuronal cells (Yamanaka et al., 2007; Crompton et al., 2013; Karumbayaram et al., 2009). Several human iPSC lines carrying various ALS mutations are now publicly available and have been successfully differentiated into MNs or glial cells (Dimos et al., 2008; Hester et al., 2011; Amoroso et al., 2013; Bilican et al., 2012). The first iPSC line that had been generated was from an 82 year-old female ALS patient carrying a mutation in the SOD1 gene (Dimos et al., 2008); derived iPSCs were successfully differentiated to MNs and characterized. Subsequently, a series of other studies derived iPSC lines from both familial and sporadic cases (Bilican et al., 2012; Sareen et al., 2013a; Burkhardt et al., 2013) of ALS. Although the use of iPSC lines for ALS studies has revealed important