Human TDP-43 and FUS selectively affect motor neuron maturation and survival in a murine cell model of ALS by non-cell-autonomous mechanisms

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Abstract
TAR DNA-binding protein 43 (TDP-43) and fused in sarcoma (FUS) were recently found to cause familial and sporadic amyotrophic lateral sclerosis (ALS). The mechanisms by which mutations within these genes cause ALS are not understood. We established murine embryonic stem cell (ESC)-based cell models that stably express the human wild-type (WT) and various ALS causing mutations of TDP-43 (A315T) and FUS (R514S, R521C and P525L). We investigated their effect on pan-neuron as well as motor neuron degeneration. Finally, non-cell-autonomous mediated neurodegeneration by muscle cells was investigated. Expression of mutant hTDP-43, but not wild-type TDP-43, as well as wild-type and mutant hFUS proteins induced neuronal degeneration with partial selectivity for motor neurons. Motor neuron loss was accompanied by abnormal neurite morphology and length. In chimeric coculture experiments with control motor neurons and mutant muscle cells (as their major target cells), we detected that mutant hTDP-43 A315T as well as wild-type and hFUS P525L expression only in muscle cells is sufficient to exert degenerative effects on control motor neurons. In conclusion, our data indicate that a selective vulnerability of motor neurons expressing the pathogenic ALS-causing genes TDP-43 and FUS, is, at least in part, mediated through non-cell-autonomous mechanisms.

Key words: Amyotrophic lateral sclerosis, TAR DNA binding protein 43, fused in sarcoma, translocated in sarcoma, non-cell-autonomous neurodegeneration, neuropathology, models, neuromuscular disease

Introduction
Amyotrophic lateral sclerosis is the most frequent type of motor neuron disease affecting 2–4 per 100,000 people per annum worldwide. Survival time is approximately 1–5 years after symptom onset (1–4). In ALS, motor neurons are predominately affected, with cells undergoing degeneration and death. In 1993, mutations in the human SOD1 gene encoding for the superoxide dismutase-1 were first reported to be causative for familial ALS (FALS) (5). Cell and animal models expressing mutant hSOD1 in glial cells leading to progressive degeneration of motor neurons suggest non-cell-autonomous disease mechanisms (6–9).

In 2006 and 2009, mutations in the genes TDP-43 and FUS were identified to cause FALS as well as sporadic ALS. The molecular pathophysiology of motor neuron degeneration caused by mutant TDP-43 and FUS genes remains still largely enigmatic, but is suggested to be distinct from those of mutant SOD1 (10,11). Since both TDP-43 and FUS are RNA-binding proteins, mechanisms involving RNA dysmetabolism were thought to mediate motor neuron degeneration (12). TDP-43 is known to bind β-actin mRNA and human low molecular weight neurofilament mRNA (13,14) and thus to regulate neurite morphology, thereby implicating neuronal autonomous mechanisms in TDP-43 and FUS-mediated motor neuron degeneration (15). Non-cell-autonomous pathological effects caused by astrocytes could not be observed in an induced pluripotent stem cell (iPSC) model of mutant hTDP-43 (16).

The investigation of pathophysiological mechanisms in motor neuron degeneration in ALS became increasingly investigated through the use of cell models. Currently, multiple cell models exist, including non-neuronal ones such as HeLa cells, HEK cells and other non-neuronal cell types (17–19), Drosophila models (20,21), murine neural cell models (8,22), embryonic stem cell (ESC) models generated from transgenic mice (6), a human embryonic stem cell model (7), iPSC-based cell models
generated from patients (23–25), and transgenic mice and rat models (26–30). According to these cell models, murine mESC are uniquely suitable for the analysis of motor neuron survival, function and pathology due to their unlimited proliferation and differentiation potential and their substantially shorter generation and motor neuronal differentiation time compared to human ESC or iPSC models. Moreover, these cell models are particularly suitable to differentiate between cell-autonomous and non-cell-autonomous mechanisms by the respective coculture approaches. We thus generated mESC-based in vitro cell models on the basis of mutant hTDP-43 or hFUS genes to dissect the pathophysiological events in mutant TDP-43 and FUS-mediated motor neuron degeneration.

Materials and methods

Embryonic stem cells

Murine ESCs harbouring a bacterial artificial chromosome (BAC)-based eGFP reporter construct under control of the Hb9 promoter (Hb9::GFP) were kindly provided by Jared Sterneckert (24).

Plasmids and PCR

Expression constructs containing the human cDNA of wild-type TDP-43, missense mutant TDP-43 A315T, wild-type FUS, missense mutants FUS R514S, FUS R521C or FUS P525L were kindly provided by Robert H. Baloh (TDP-43) and Daisuke Ito (FUS). The cDNAs were cut out via KpnI/ApaI (Fermentas) restriction, digested and ligated into the pDsRed-Monomer-C1 expression vector (Clonetech Laboratories, Inc.). Correct constructs were amplified in E. coli, purified by anion-exchange (Plasmid Kit, Qiagen) restriction, digested and ligated into the pDsRed-Monomer-C1 expression vector (Clonetech Laboratories, Inc.). Correct constructs were amplified in E. coli, purified by anion-exchange (Plasmid Kit, Qiagen) restriction, digested and ligated into the pDsRed-Monomer-C1 expression vector (Clonetech Laboratories, Inc.). Correct constructs were amplified in E. coli, purified by anion-exchange (Plasmid Kit, Qiagen) restriction, digested and ligated into the pDsRed-Monomer-C1 expression vector (Clonetech Laboratories, Inc.). Correct constructs were amplified in E. coli, purified by anion-exchange (Plasmid Kit, Qiagen) restriction, digested and ligated into the pDsRed-Monomer-C1 expression vector (Clonetech Laboratories, Inc.). Correct constructs were amplified in E. coli, purified by anion-exchange (Plasmid Kit, Qiagen) restriction, digested and ligated into the pDsRed-Monomer-C1 expression vector (Clonetech Laboratories, Inc.). Correct constructs were amplified in E. coli, purified by anion-exchange (Plasmid Kit, Qiagen) restriction, digested and ligated into the pDsRed-Monomer-C1 expression vector (Clonetech Laboratories, Inc.). Correct constructs were amplified in E. coli, purified by anion-exchange (Plasmid Kit, Qiagen) restriction, digested and ligated into the pDsRed-Monomer-C1 expression vector (Clonetech Laboratories, Inc.). Correct constructs were amplified in E. coli, purified by anion-exchange (Plasmid Kit, Qiagen) restriction, digested and ligated into the pDsRed-Monomer-C1 expression vector (Clonetech Laboratories, Inc.).

Stable transfection

Murine Hb9::GFP ESCs were transfected with linearized plasmids by electroporation using the Amaxa™Mouse ES cell Nucleofector™Kit (Lonza). Stable integration of the plasmid into the murine genome was proven by PCR analysis and immunofluorescence imaging (Figure 1B, C).

Motor neuron differentiation

For long-term experiments, non-transfected mESCs (non-transgenic control, nt-control) and the stably transfected mESCs were differentiated into the neural lineage by embryoid body (EB) formation (see Figure 1A for a scheme of the experimental design).

Muscle cell differentiation

Induction of muscle differentiation for coculture analysis was achieved by EB formation of mESCs using the hanging drop technique.

Motor neuron/muscle cell cocultures (chimeric cultures)

For a defined coculture with motor neurons, non-transfected mESCs were differentiated into motor neurons and sorted for Hb9::GFP fluorescence as single cell suspension and were plated on maturated muscle cells and cultivated for 24 h in medium.

For details of the experimental set-up please refer to the Supplementary material online to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1055275.

Results

Generation of hTDP-43 and hFUS transgenic mESC lines

Murine embryonic stem cells (Hb9::GFP mESCs) were used to generate stable cell lines expressing the human variants of wild-type and mutant TDP-43 and FUS. TDP-43 and FUS cell lines were separately generated and stable colonies were selected by G418 treatment. Stable insertion of the constructs was confirmed by PCR analysis with specific primers detecting the fusion site of the transgenic fusion protein. All of the generated mESC lines showed a specific DNA band representing either a part of the neomycine resistance gene or a part of the appropriate transgenic fusion protein (Figure 1B). Transgene-positive lines were differentiated and analysed for hTDP-43 and hFUS fusion protein expression by fluorescence imaging. To distinguish plasmid-derived exogenous proteins from endogenous wild-type TDP-43 and FUS the transgenes were tagged with DsRed-Monomer C1 fluorescent protein (named ‘DsRed’). As expected, hTDP-43 and hFUS transgenic proteins were mainly localized in the nucleus while the DsRed fluorescence protein alone (vector control) was present throughout the whole cell (Figure 1C), thereby proving that the physiological protein distribution was not impaired by the fusion to the DsRed. Quantification of the total amount of transgenic cells in differentiated cultures resulted in 12.2 ± 1.9% transgene-positive cells of the total cell number without variation between cell lines (one-way ANOVA, F = 2.021, p = 0.0715).

Expression of ALS causing genes affects neuron survival

We used ESCs from mice expressing green fluorescent protein (GFP) downstream from the motor neuron specific Hb9 promoter to label motor neurons. Differentiation of these murine ESCs into motor neurons was induced by embryoid body
formation for five days and followed by neuron maturation in monolayer cultures that were analysed after an additional 5–14 days. The number of motor neurons and other neuron types were determined by immunofluorescence imaging. ESC lines transfected with the empty vector showed similar results compared to the non-transgenic (nt)-control ESC line with no significant difference in the number of Tuj1+
neurons and GFP+ motor neurons (Supplementary Figure 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1055275). The presence of differentiated motor neurons and other neuronal subtypes was directly affected by the expression of mutant hTDP-43, but not wild-type hTDP-43 protein: after five days of maturation, hTDP-43 A315T expressing neural cultures lost all GFP-expressing motor neurons, whereas wild-type hTDP-43 expressing cultures behaved similarly to non-transgenic control cultures (nt-control) (Figure 2A, Supplementary Table 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1055275). At the same time-point, the amount of Map2+ neurons in hTDP-43 A315T expressing cultures was significantly reduced (Figure 2A, Supplementary Tables 2/3 to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1055275). Analysing the amount of GFP+ motor neurons after 24 h of neuron maturation, we found approximately 31% GFP+ motor neurons under control conditions, whereas wild-type and mutant hTDP-43 expressing cultures harboured 65–70% fewer GFP+ motor neurons compared to control conditions (vector control, 29%; wild-type hTDP-43, 9%; hTDP-43 A315T, 11.5%).

After 14 days of maturation/aging, we observed a dramatic loss of 1.4% Tuj1+ and 1.1% Map2+ neurons in hTDP-43 A315T neuronal monolayer cultures compared to nt-control conditions (Figure 2A, Supplementary Tables 2/3 to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1055275). The survival of Map2+ neurons was significantly higher in both nt-control and wild-type hTDP-43 conditions compared to hTDP-43 A315T cultures. Already by day 5 of maturation, analyses of GFP+ to Map2+ neuron numbers revealed a selective loss of GFP+ motor neurons in the cultures expressing the hTDP-43 A315T system (Figure 2A, right panel, Supplementary Table 4 to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1055275). Quantification revealed no difference in the number of GFAP+ astrocytes (nt-control, 2.1 ± 0.5%; wild-type hTDP-43, 5.6 ± 0.7%; hTDP-43 A315T, 1.9 ± 0.1%) (Supplementary Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1055275).

In contrast, analysis of cultures expressing hFUS revealed no reduction of GFP+ motor neurons after five days in wild-type and mutant hFUS culture conditions, whereas after 14 days of maturation/aging, GFP+ motor neuron numbers were significantly lost in cultures expressing wild-type and mutant hFUS (Figure 2B, Supplementary Table 5 to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1055275). Tuj1+ and Map2+ neurons were in significantly reduced amounts in wild-type and mutant hFUS cultures as well (Figure 2B, Supplementary Tables 6/7 to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1055275). The most striking difference between TDP-43 and FUS cultures was that wild-type hFUS expression led to complete loss of motor neurons when analysed at day 14 of maturation/aging, suggesting that expression of wild-type hFUS has the same effect as expression of mutant hTDP-43 or mutant hFUS proteins (Figure 2A, B). The reduced ratio between the numbers of GFP+ and Map2+ neurons in cells expressing wild-type and mutant hFUS showed a selective vulnerability of GFP+ motor neurons only at day 14 (Figure 2B, right panel; Supplementary Table 8 to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1055275).

Quantification of the total amount of transgenic cells in differentiated cultures showed no differences between cell lines over time (two-way ANOVA, F = 0.1297, p = 0.8787), indicating an additional effect of neuronal maturation/aging on neurodegeneration seen in our cultures. These data suggest that the expression of hTDP-43 A315T and wild-type and mutant hFUS proteins induce neuronal degeneration with partial selectivity for GFP+ motor neurons in differentiated and maturated/aged transgenic murine ESCs cultures.

Protein aggregation and cytoplasmic mislocalization by expression of hTDP-43 A315T

The formation of aggregates or inclusions and protein mislocalization of pathogenic TDP-43 and FUS are hallmarks of ALS (31–33). We therefore investigated the presence of aggregate formation and mislocalization of the transgenic proteins. Notably, only hTDP-43 A315T, but not cultures expressing wild-type hTDP-43 or wild-type or mutant hFUS, exhibited aggregation formation or abundance of the respective protein in the cytoplasm (Figure 3). Interestingly, cytoplasmic mislocalization of hTDP-43 A315T was only found in cells of mesodermal origin (Figure 3A). We could detect only rarely cytoplasmic localization of hTDP-43 by wild-type hTDP-43 expression (Supplementary Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1055275). Neither hTDP-43 nor hFUS protein aggregates nor any cytoplasmic localization could be visualized in neurons (Figure 3B, Supplementary Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1055275).

Non-cell-autonomous effects cause motor neuron degeneration in vitro

We observed initial hints for non-cell-autonomous effects in cultures expressing hTDP-43 but not hFUS. Even though the transgene was not expressed by neurons themselves, there was obvious neuronal
Figure 2. Quantification of neuronal cell types in differentiated non-transgenic and hTDP-43 and hFUS transgenic mESC lines in monolayer cultures. (A) In vitro fluorescent GFP\(^+\) motor neurons and immunocytochemical detected Map2\(^+\) and Tuj1\(^+\) neurons were counted in hTDP-43 transgenic cultures after five and 14 days of maturation in monolayer cultures. Ratios of the amounts of GFP\(^+\) motor neurons and total Map2\(^+\) neurons were calculated to demonstrate selective vulnerability of motor neurons (bw panel). (B) In vitro fluorescent GFP\(^+\) motor neurons and immunocytochemical detected Map2\(^+\) and Tuj1\(^+\) neurons were counted in wild-type and mutant hFUS transgenic cultures after five and 14 days of maturation in monolayer cultures. Ratios of cell amounts of GFP\(^+\) motor neurons and total Map2\(^+\) neurons are shown. Data represent mean values ± s.e.m. of at least three independent experiments. Statistical results of two-way-ANOVA are displayed in Supplementary Tables 1–8\(^*\), \(*p < 0.05\), \(* * p < 0.01\), \(* * * p < 0.001\) (Bonferroni-adjusted post hoc two-sided t-test).
Figure 3. Protein mislocalization and neuronal degeneration of neurally differentiated non-transgenic and hTDP-43- and hFUS transgenic mESCs. Representative images are depicted. (A) Determination of abnormal cytoplasmic localization of transgenes by antibody enhanced in vitro fluorescence signal in GFP$^+$ neurons and muscle cells ($\alpha$-SMA$^+$ and Desmin$^+$ cells). The expression of DsRed fluorescent protein alone showed normal nuclear and somatic localization. Exclusively, the expression of hTDP-43 A315T mt protein resulted in cytoplasmic mislocalization and aggregation formation that was only found in $\alpha$-SMA$^+$ cells but not in neurons or astrocytes (see Supplementary Figure 1) (B) Visualization of GFP$^+$ motor neurons and Tuj1 stained neurons growing close to transgenic cells resulted in remarkably different neuron morphology after 14 days of maturation in monolayer cultures. Neurons in control cell lines, DsRed fluorescent protein expressing and wild-type hFUS and hFUS P525L expressing cultures showed normal neurite morphology and neuron shape whereas neurons in wild-type hTDP-43 and even more pronounced in hTDP-43 A315T expressing cultures revealed an abnormal cell body shape and neurite morphology. There were no GFP$^+$ motor neurons left in hTDP-43 A315T, wild-type hFUS and hFUS P525L expressing cultures. The DsRed fluorescent protein was enhanced by rabbit-anti-DsRed antibody detection. Cell nuclei were counter-stained with Hoechst. Scale bar, 50 and 100 μm. → visualize the lacking expression of transgenes in neurons and the neuron morphology differences.
degeneration in both cultures expressing wild-type and mutant hTDP-43 (Figure 3B) with a pronounced phenotype in the hTDP-43 A315T line. These changes were characterized by neuron and selective motor neuron death (Figure 2) as well as shortened and fragmented neurites (Figure 3). None of these ALS hallmarks could be observed in other neuroectodermal cell types, e.g. astrocytes (Supplementary Figure 1B to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1055275). On the other hand, the remaining neurons in cultures of wild-type and mutant hFUS looked healthy (Figure 3, Supplementary Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1055275).

To prove whether non-cell-autonomous effects are sufficient to evoke motor neuron degeneration, we generated transgenic muscle cells as the major connective cell type of motor neurons by overexpressing wild-type and mutant hTDP-43 or hFUS proteins and cocultured them with healthy (nt-control) GFP$^+$ motor neurons (Figure 4A, representative images). Quantitative analysis of murine ESC muscle cell differentiation resulted in a total amount of 48.7% (nt-control) and 74.2% (transgenic cell lines) of myosin$^+$, SMA$^+$ and desmin$^+$ muscle cells of which 18.8 ± 5.6% of the cells showed positive transgene expression by a DsRed fluorescence signal (data not shown). The amount of GFP$^+$ motor neurons collected via FACS immediately before plating yielded approximately 20%. Within the remaining

Figure 4. Chimeric cultures of healthy motor neurons cocultivated on top of non-transgenic and diseased (transgenic) muscle cell cultures. (A) Immunocytochemical representation of the muscle/motor neuron coculture system (depicted are examples of detected different transgene-expressing muscle types). (B) Relative quantification of the number of remaining non-transgenic motor neurons, their total neurite length, longest neurite length (suggestive for the axon) and branching points were quantified. Non-transgenic motor neurons cultured on top of non-transgenic-, DsRed fluorescent protein (vector-control)-, wild-type hTDP-43-, hTDP-43 A315T-, wild-type hFUS- and hFUS P525L expressing muscle cells were analysed. All data are depicted relative to the non-transgenic motor neurons growing on the control muscle cells. Total neurite lengths of motor neurons were significantly reduced if cocultured on hTDP-43 A315T expressing muscle cells ($p = 0.0002$; two-sided unpaired $t$-test). The expression of both wild-type hFUS and hFUS P525L proteins in muscle cells significantly affected the total neurite length (wild-type hFUS: $p = 0.025$, FUS P525L: $p = 0.001$; two-sided unpaired $t$-test). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. 

Non-cell-autonomous motor neuron degeneration in ALS
motor neurons, the neurite lengths and the branching points of GFP+ motor neurons were analysed by immunofluorescence imaging after 24 h in coculture. The number of motor neurons was not altered by transgene expression (Figure 4B). Cocultures of nt-control and vector control muscle cells with healthy motor neurons revealed no differences in neurite lengths and number of branching points. In contrast, motor neurons growing on top of transgenic hTDP-43 A315T and hFUS muscle cells showed a significantly reduced total neurite length compared to the nt-control. Strikingly, wild-type hFUS expression also led to a significant reduction in the total neurite length.

In summary, we showed that hTDP-43 A315T as well as both wild-type and hFUS P525L expressed in muscle cells clearly exert a non-cell-autonomous degenerative effect on nt-control motor neurons (Figure 4B).

Discussion

Here we report the generation of a murine ESC-based cell model of ALS by expression of wild-type and mutant hTDP-43 and hFUS genes. Investigating heterogeneous neuro-ectodermal cultures, we found increased neurodegeneration with partial selectivity for motor neurons by expression of hTDP-43 A315T as well as wild-type and mutant hFUS. Furthermore, we identified degenerative non-cell-autonomous effects of motor neurons caused by muscle cells expressing hTDP-43 A315T or wild-type and hFUSP525L. Interestingly, wild-type TDP-43 did not induce a comparable pathology.

Analysing murine hTDP-43 animal models revealed contrasting results, including the hypothesis of both loss-of-function and gain-of-function pathology (34). However, a common feature is that mutant hTDP-43 (A315T or M337V) leads to premature death and motor neuron degeneration. It is, however, still unclear whether overexpression of wild-type hTDP-43 also causes motor neuron disease. Using both Thy-1 promoter- and prion protein promoter (PrP)-driven overexpression of wild-type hTDP-43 was reported to cause a phenotype with many features of motor neuron disease (28,29,35). Another study using expression of wild-type hTDP-43 and Q331K or M337V mutants, however, reported only tremor in wild-type animals while the variant hTDP-43 strains caused significant age-dependent motor deficits. Interestingly, these mice did not die prematurely, but showed rather a stable phenotype after the initial motor impairment. Finally, in a rat model of hTDP43 overexpression, only rats expressing mutant hTDP43 developed symptoms of motor neuron disease (36). In line with that, we detected degeneration of motor neurons only in the mutant TDP-43 A315T line. In older mice representing a more advanced form of the disease, neurons other than motor neurons also were affected by degeneration (36), perfectly fitting our data showing decreased Map2+ neurons next to the complete loss of motor neurons in aged cultures. Importantly, and in contrast to the human neuropathology, many of the murine TDP-43 models showed neurodegeneration in the absence of TDP-43 aggregation and with normal nuclear TDP-43 expression (27,30,37,38). This might explain why we could hardly detect cytoplasmic TDP-43 mislocalization and aggregation in our murine cell model.

Since FUS was discovered to cause ALS more recently than was TDP-43, there are currently few murine TDP-43 models. Mitchell et al. reported a progressive motor neuron degeneration in mice when overexpressing wild-type hFUS (39), while Huang et al. reported that only mutated FUS caused motor neuron disease in a rat model. The different reports showed the appearance of aggregated cytoplasmic FUS in different amounts depending upon the severity of the underlying mutation (39,40). This is different from our cell model in which we did not find FUS aggregation. One reason might be that within a monolayer cell model a toxic gain of function could become relevant much earlier and, thus, the respective motor neurons already degenerated before FUS accumulation become obvious. Similar to the mouse models, we detected a complete loss of motor neurons in the wild-type and mutant hFUS lines, suggesting that FUS effects are distinct from those of TDP-43.

For both mutations we show a stable expression of the transgenes over time; thus, there is no direct correlation of transgene accumulation and neurodegeneration. The progressive decline in motor and other neurons in our aging cultures may be due to different reasons, including cumulative toxicity in such an artificial system or cellular aging. Stem cell-derived motor neurons are very young neurons compared to neurons from adult animals/humans. This means that a stepwise acquisition of neuronal function implicates an increase in their vulnerability to many different cues. Importantly, the appearance of pathophysiological phenotypes in long-term cultures supports the hypothesis of a degenerative disease rather than a developmental disorder.

Our study has two major limitations. First, our differentiation protocol led to less than 20% motor neurons. Even though we had approximately 20% motor neurons in young cultures with progressive degeneration in aged cultures, we cannot completely rule out that this is partly due to a maturation defect by the transgenes. However, even though the yield of motor neurons was limited, we were able to show a robust motor neuron disease phenotype. Secondly, we only rarely observed transgene expression in motor neurons themselves, whereas other neuron types expressed it more obviously. However, we clearly saw a selective vulnerability of motor neurons due to transgene expression and we could detect a progressive decline in motor neuron numbers in
long-term cultures (mimicking cellular aging) that was much higher in cells expressing the transgene. This suggests that motor neurons expressing the transgenes died early in motor neuron development and, thus, were not detectable at later stages. This would be in line with data from a TDP-43 rat model claiming that embryonic expression of mutated hTPD-43 is lethal (36). Additionally, Sephton et al. published that TDP-43 is developmentally regulated and highly expressed during embryonic development and decreases in levels in postnatal development (14). Expression of TDP-43 and FUS showed a remarkable reduction in adulthood in peripheral tissues but not in motor neurons (41). Thus, future studies should use inducible expression systems to overcome this early toxicity.

Even though the transgenes were barely observable in motor neurons themselves, the motor neurons underwent degeneration in cultures expressing hTDP-43 A315T as well as in wild-type and mutant hFUS proteins, leading us to the assumption of non-cell-autonomous effects by transgenic cells growing in the immediate microenvironment of motor neurons. The majority of these cells were of muscle origin. Coculture experiments confirmed a non-cell-autonomous effect on healthy motor neurons by transgene-expressing muscle cells, the major connective cells of motor neurons. Consistently, a recent report showed that the expression of pathogenic hSOD1 in muscle cells alone causes motor neuron degeneration (42). For SOD1-ALS, similar results were additionally found to be mediated by glial cells in human ESC-based and murine ALS models (6–9). Recently, Grad et al. demonstrated that non-cell-autonomous SOD1 can be taken up by neighbouring cells and, thereby, propagate SOD1 misfolding (43). Re et al. found that non-cell-autonomous toxicity by human primary SALS and FALS astrocytes selectively contributes to the death of human motor neurons. The process is named necroptosis (44).

Whereas non-cell-autonomous effects of SOD1 are well studied, there exist only preliminary data on TDP-43 and no data on FUS for such effects. Pathological TDP-43 A315T expression in astrocytes did not result in a pathological effect on motor neurons (38). Correspondingly, the study of Serio et al. could not find any non-cell-autonomous effects in a human iPSC model using cocultures with mutant hTDP-43 astrocytes (16). These differences among SOD1 and TDP-43 non-cell-autonomous effects generated by different cell types that interact with motor neurons (glial cells and muscles) evoke mechanistic differences in the pathologies of SOD1 and TDP-43.

Recently, there have been discussions regarding the role of muscle cells in motor neuron disease (MND), and it has been suggested that muscle in MND is not simply the victim of, but rather a major contributor to, the disease. Selective expression of mutated genes was shown to be causal for the MND phenotype and for survival in an SOD1 ALS mouse model (45) as well as in a Kennedy’s disease mouse model (46). Furthermore, peripheral depletion of polyQ-AR in a mouse model of Kennedy’s disease significantly improved survival and motor neuron degeneration (47). Significant neuropathological changes are seen in skeletal muscle of sporadic ALS patients (48). Currently there are no data regarding any pathological non-cell-autonomous effects of TDP-43 or FUS expression in muscle cells. We thus present novel initial data on the existence of non-cell-autonomous causative factors for motor neuron degeneration of both wild-type and hFUS P525L and hTDP-43 A315T induced by cocultured muscle cells.

Conclusions
Here we show selective motor neuron degeneration in hTDP-43 A315T as well as in wild-type and mutant hFUS using in vitro models of ALS. In long-term cultures we also observed non-specific neuronal loss, not just in motor neurons, resembling spreading of cortical neuron pathology also seen in ALS (49) and frontotemporal lobar degeneration. Finally, using a coculture system we observed non-cell-autonomous effects mediating motor neuron degeneration by hTDP-43 A315T and both wild-type and mutant hFUS protein expression in muscle cells, as the major connective cell type of motor neurons.

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Supplementary material available online

Supplementary material, Figures 1 and 2 and Tables 1–8 to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1055275.