HHS Public Access

Author manuscript

Neuron. Author manuscript; available in PMC 2020 February 27.

Published in final edited form as:

Neuron. 2016 May 04; 90(3): 427–431. doi:10.1016/j.neuron.2016.04.026.

C9ORF72-ALS/FTD: Transgenic Mice Make a Come-BAC

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Abstract

For five years, since the landmark discovery of the ***C9ORF72*** hexanucleotide repeat expansion in ALS/FTD, a transgenic mouse model has remained elusive. Now, two laboratories (Liu et al., 2016; Jiang et al., 2016) report the development of BAC transgenic mice that recapitulate features of the human disease.

Discovered in 2011, the GGGGCC hexanucleotide repeat expansion (HRE) in ***C9ORF72*** is now regarded as the most common genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (DeJesus-Hernandez et al., 2011; Renton et al., 2011). The HRE, located in the first intron, consists of 2–30 repeats in the general population and can range from hundreds to thousands of repeats in affected patients. Difficult to clone and prone to germline and somatic instability, these large expansions have presented a technical hurdle for the development of transgenic mouse models. In fact, the field has been largely advanced through studies of autopsy tissue and patient-derived iPS neurons, providing some evidence for pathophysiology, e.g., nucleocytoplasmic transport, and including the first candidate antisense oligonucleotide and small-molecule therapies (for a recent review, see Todd and Petrucelli, 2016). As powerful as the “true” human models are, having intact rodent models provides a multiplicity of additional benefits. Early attempts using a bacterial artificial chromosome (BAC) to express part (Peters et al., 2015) or all (O’Rourke et al., 2015) of the human C9 gene produced mice with characteristic molecular abnormalities (RNA foci, repeat-associated non-ATG [RAN] dipeptides) but no clinical or neuropathologic phenotype. Alternative approaches, using viral delivery of short HREs (Chew et al., 2015) or dipeptide repeat proteins (DPRs; Zhang et al., 2016), generated very provocative results, but with synthetic constructs and high levels of RNA and DPR overexpression. Now, two groups, Jiang et al. (2016) and Liu et al. (2016), report the development of BAC transgenic mice that use patient-derived gene constructs to recapitulate molecular, neuropathologic, and clinical features of C9-ALS/FTD. These mice will be valuable for studying disease mechanisms and testing therapeutics. Moreover, careful comparison between these models may yield additional insights into C9 pathogenesis.

The C9 protein is a DENN domain-containing protein that may play a role in endosomal trafficking. The issue of whether the C9 HRE causes disease by a loss or gain of function (or both) remains an open question. Numerous groups have reported that C9 mRNA expression

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is reduced in patient tissue (DeJesus-Her- nandez et al., 2011 and others), and C9 protein levels are also reduced in frontal cortex (Waite et al., 2014). Despite early reports of motor defects in invertebrate knockout models, conditional (Koppers et al., 2015) and germline (Atanasio et al., 2016; O'Rourke et al., 2016) ablation of the mouse homolog of C9 has been insufficient to cause neurodegeneration.

In this issue, Jiang et al. present findings from another germline knockout, which, as previously reported, develops dramatic hematopoietic abnormalities, with splenomegaly, lymphadenopathy, and premature death. Although 12-month-old null mice have mild deficits in social interaction and Rotarod performance, they do not show EMG abnormalities or spinal motor neuron loss. Brain pathology was not evaluated in the current study, but was absent in a similar model at 17 months (O'Rourke et al., 2016), suggesting these behavioral deficits may be more related to systemic illness than neurodegeneration. The heterozygous mice, more analogous to the human disease, show no pathological or behavioral phenotype. Interestingly, O'Rourke et al. did show an alteration in macrophage function and pathology, as reflected in a common aberrant transcriptome in the CNS of null mice and patient brain tissue, hinting that loss of C9 function could produce an altered neuro-inflammatory response. In aggregate, these rodent studies suggest that loss of function is unlikely to be a strong contributor to C9-ALS/FTD and that the gene may have a more profound role in hematopoietic function. However, no study to date evaluates the partial loss of C9 in the setting of the HRE.

Two C9 gain-of-function mechanisms, RNA toxicity and accumulation of RAN-translated DPRs, have received significant attention. Fluorescence in situ hybridization (FISH) reveals accumulation of sense (G_4C_2) and antisense (C_4G_2) RNA foci in patient tissue (DeJesus-Hernandez et al., 2011; Zu et al., 2013), and the C9 HRE sequesters RNA binding proteins, including regulators of nucleocytoplasmic trafficking and other fundamental processes (Donnelly et al., 2013). Antibodies to all six predicted products of C9 RAN translation (poly-GP, -GA, and -GR in the sense direction, and poly-GP, -PA, and -PR in the antisense direction) label inclusions in autopsy tissue (Zu et al., 2013). Genetic overexpression or exogenous delivery of DPRs causes toxicity in vitro and in vivo. However, the data conflict about which DPRs are most toxic. Since the precise mechanism of RAN translation in C9 is unknown, it has also been difficult thus far to experimentally separate the relative contributions of RNA gain of function versus RAN translation to C9-mediated neurodegeneration in model systems with human levels of expression.

Perhaps due to a high degree of variability, studies in autopsy tissue have failed to demonstrate a consistent effect of HRE length or C9 expression level on RNA foci or DPRs. The precise correlation between these molecular features and disease severity is also unclear, although patterns are starting to emerge in larger cohorts. Complicating matters are two recently generated BAC transgenic mice, both of which display RNA foci and DPRs, yet fail to develop a phenotype or neuropathology (O'Rourke et al., 2015; Peters et al., 2015). These studies raise the question of whether RNA foci and DPRs are insufficient to drive neurodegeneration (and may even be protective), or whether additional genetic or epigenetic factors are required to cause pathology.

To address these questions, Jiang et al. developed multiple BAC transgenic lines using a construct containing C9 exons 1–5 (with 140-kb 5' flanking sequence). Four lines were characterized, expressing 110 and 450 repeats (with increasing expression levels, designated 450A-C). As in previous studies, these mice did not show evidence of motor neuron disease. No deficits in weight, grip strength, or Rotarod performance were seen out to 18 months of age, and there were no deficits in resting EMG or myogenic motor-evoked potentials at 12 months. No motor neuron loss or gliosis was seen in the spinal cord or motor cortex.

However, a cognitive phenotype was detected. Lines 450B-C developed spatial learning and working memory deficits as well as increased anxiety. In 450B-C, but not 110 or 450A, mild loss of neurons was also seen in the hippocampus. No gliosis or TDP-43 mislocalization was observed, although increased phosphorylated TDP-43 was seen by western blot. RanGAP1 and Lamin B staining, recently implicated in C9 human brain, iPS neurons, and fly models, did not reveal nuclear pore pathology. Both sense and antisense RNA foci were detected, and there was a strong effect of C9 expression on foci density. Aggregates of sense but not antisense DPRs were detected by immunohistochemistry. Quantitative analysis by ELISA showed a strong correlation between repeat number, C9 expression, and poly-GP levels. With age, soluble poly-GP decreased and cellular aggregates increased. Remarkably, a single administration of antisense oligonucleotides led to a durable improvement in cognitive deficits, RNA foci, and DPRs.

By comparison, Liu et al. utilized a BAC construct containing the full C9 gene (exons 1–11, including 5' and 3' flanking sequences). Four lines of transgenic mice with HRE from 37 to 500 in length were characterized further. Beginning at 20 weeks, a subset of "acute" female mice from the highest repeat lines (500 and 500/32) developed sudden and rapidly progressive weight loss, inactivity, labored breathing, and death. Many of the remaining animals (500, 500/32, and 39/ 27) developed a milder, "slow progressive" phenotype including kyphosis, hindlimb clasping, anxiety, and intermittent seizures. Gait abnormalities were detected by 16 weeks in the 500 and 500/ 32 lines. Decreased grip strength and abnormal performance on the open field test (indicative of anxiety) were also seen.

Remarkably, these mice showed both peripheral and central neurodegeneration. "Acute" females showed denervation at the neuromuscular junction, neurogenic muscle atrophy, a mild shift in caliber of ventral root axons, ~40% loss of lumbar spinal motor neurons, and a 57% decrease in neurons of the motor cortex. In the brain, a 75% decrease in layer II/III cortical neurons, 65% loss of Purkinje cells, patchy hippocampal neuron loss, and reactive gliosis were seen. Cytoplasmic and nuclear TDP-43 aggregates were also found in acute animals. Slow progressive animals displayed more subtle pathology, with sprouting and axon swelling at the NMJ, mild shifts in ventral root axon caliber, ~30% loss of spinal motor neurons, and focal cortical and cerebellar neurodegeneration with hippocampal sparing.

Sense and antisense RNA foci were detected in 500 and 500/32 mice by 2 months of age (and never detected in 37 and 36/29). A novel observation was made that antisense but not sense foci correlated with the severity of neurodegeneration. RAN proteins were more challenging to detect in the mice than in patient tissue; however, using a newly generated antibody against insoluble GA, small inclusions were seen in symptomatic 500, 500/32, and

36/29 animals. Poly-GP aggregates were also detected in 500 and 500/32 lines. As reported by Jiang et al., an increasing burden of GA aggregates was seen with disease progression.

Taken together, these two studies represent a major advance in rodent modeling of C9-ALS/FTD. For the first time, mice expressing a human C9 transgene display a neurodegenerative phenotype. Moreover, these deficits appear to be reversible with antisense oligonucleotide treatment, paralleling effects seen previously in iPS neurons. Comparing the mouse models available to date (see Table 1), the precise combination of factors needed to generate a phenotype is still quite unclear; however, clues are emerging. As in humans, there is a size threshold above which the HRE becomes toxic. In mice with equivalent expression of 110 and 450 repeats (Jiang et al., 2016) or 37 and 500 repeats (Liu et al., 2016), only the mice with the higher repeat number develop symptoms. Whether differences in HRE size above this toxic threshold correlate with disease severity (i.e., whether there is length dependence among HREs ≥ 500) still remains to be demonstrated.

Superimposed on this is a major effect of the C9 expression level, which seems to govern both molecular and phenotypic severity. Jiang et al. characterized multiple lines of mice, all with 450 repeats, but with differing expression levels. Mice with low C9 expression, equal to endogenous mouse C9, never developed symptoms (450A), whereas mice expressing 3–4 times higher C9 levels (450B-C) showed a cognitive phenotype. There was also a clear influence of C9 expression on the burden of RNA foci and DPRs. Since the early BAC mice expressed C9 at ~1× (Peters et al., 2015) and ~2× (O'Rourke et al., 2015) endogenous mouse levels, the lack of phenotype in these mice may be explainable, at least in part, by low expression. Interestingly, Liu et al. showed that overexpression of even small HREs (36/29, ~3× endogenous) caused disease, highlighting a possible interaction between HRE size and expression level in determining disease risk.

Additional factors must be involved, since at every repeat size and expression level, the BAC mice reported by Liu et al. showed a more severe phenotype and neuropathological outcome–diffuse upper and lower motor neuron involvement (with patchy hippocampal loss)-compared to selective hippocampal pathology seen by Jiang et al. These differences are intriguing, given that some C9 patients develop a motor system disease while others develop a cognitive injury. The most obvious difference between studies is the background strain (FVB/NJ), particularly given the disparity between male and female mice. The background effect could be easily tested by crossing these mice onto a C57BL/6 background (or by crossing the other lines onto FVB/NJ). Another potential variable is the transgene construct and flanking sequences, as Liu et al. used the full-length gene with flanking sequences, while Jiang et al. used a truncated version. However, O'Rourke et al. used a similar full-length construct (with flanking sequences), and those animals failed to develop a phenotype. Given that the C9 HRE is in a noncoding region, the remainder of the C9 gene/protein may not be required for toxicity, supported by results from viral delivery of G4C2–66 or GA50, both of which lack other C9 gene elements and use an actin promoter (Chew et al., 2015; Zhang et al., 2016). Comparison between these animals, as well as the prior BAC lines, is needed to determine whether the full gene and flanking elements confer any relevant differences in temporal and spatial C9 expression.

Finally, these mice raise questions about the pathogenic role of RNA foci, DPRs, and other molecular features. There is already uncertainty in the field, given that RNA foci and DPRs do not necessarily accumulate in the most affected brain regions (highest in cerebellum, intermediate in cortex, and lower in the spinal cord). Adding to this, the first two BAC lines showed RNA foci early in life, at an even higher density than that reported in human studies, but failed to develop disease. Meanwhile, high-expressing 36/27 females developed a phenotype without detectable RNA foci (Liu et al., 2016). Similarly, both of the asymptomatic BAC lines expressed poly-GP, which in the mice generated by O'Rourke et al. was at levels comparable to human tissue by ELISA. However, no phenotype was seen. In the most severely affected "acute" mice, Liu et al. had difficulty even detecting DPR aggregates, using antibodies that readily detect such pathology in autopsy tissue. By comparison, median poly-GP levels in AAV-G_4C_2-66 mice are 7–70 times higher than those seen in human brain.

These discrepancies are challenging to interpret and are further complicated by differences in technique between labs. A systematic comparison of foci and DPRs across models may help clarify if any relationship exists with disease. TDP-43 pathology, on the other hand, characteristic of human C9-ALS/FTD, seems to correlate well with development of disease across all of the models. Nuclear pore pathology, a presumed fundamental pathway in C9, has not yet been as systematically evaluated, and emerging data suggest this could be an important correlate with neurodegeneration. Moving forward, these questions linking molecular pathology with disease manifestations will be critical to resolve, as they are central to our understanding of C9 pathogenesis and will directly impact therapy design and monitoring.

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Table 1.

Comparison of C9ORF72 Mouse Models

Study	Construct/Background	Repeats	Expression (versus mouse C9)	Phenotype	Pathology	RNA foci	RAN proteins	TDP-43 pathology	Nuclear pore pathology
BAC Transgenics									
O'Rourke et al., 2015	Full C9 gene (5′ 110 kb, 3′ 20 kb); C57BL/6	100–1,000	2× (protein)	N	N	Y	Y	N	ND
Peters et al., 2015	Exons 1–6 (5′ 140.5 kb); SJL/B6	500/300	1× (RNA)	N[a]	N[a]	Y	Y	N	ND
Jiang et al., 2016	Exons 1–5 (5′ 140 kb); C57BL/6	110, 450	110: 3–4× (RNA) 450A: 1× 450B: 3–4× 450C *het*: 3–4× 450C *hom*: 12×	Cognitive	CNS	Y	Y	N (although increased)	N (RanGAP1, Lamin B)
Liu et al., 2016	Full C9 gene (5′ 52 kb, 3′ 19.4 kb); FVB/NJ	37, 36/27, 500, 500/32	37: ≤ 1× (RNA) 36/29: 2–3× 500: ≤ 1× 500/32: 2×	Survival[b], Motor, Cognitive[c]	PNS, CNS	Y	Y (rare)	Y	ND
Viral Delivery									
Chew et al., 2015	AAV-G_4C_2-66 (actin promoter); C57BL/6	66	ND	Cognitive	CNS	Y	Y	Y	Y (RanGAP1, Pom121; unpublished data)
Zhang et al., 2016	AAV-GFP-GA_{50} (actin promoter); C57BL/6	N/A	N/A	Motor, Cognitive	CNS	N/A	Y (GA)	Y (rare)	Y (RanGAP1, Pom121)

Note: An inducible G_4C_2-80 mouse has also been developed (Hukema et al., 2014). Insufficient data were reported to fully compare with the above models. Y, yes; N, no; ND, not determined.

[a] Only male mice evaluated.

[b] females only.

[c] anxiety only.

Neuron. Author manuscript; available in PMC 2020 February 27.