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REVIEW

# Modeling diseases of noncoding unstable repeat expansions using mutant pluripotent stem cells

Shira Yanovsky-Dagan, Hagar Mor-Shaked, Rachel Eiges

Shira Yanovsky-Dagan, Hagar Mor-Shaked, Rachel Eiges, Stem Cell Research Laboratory, Medical Genetics Institute, Shaare Zedek Medical Center affiliated with the Hebrew University School of Medicine, Jerusalem 91031, Israel

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Correspondence to: Rachel Eiges, PhD, Stem Cell Research Laboratory, Medical Genetics Institute, Shaare Zedek Medical Center affiliated with the Hebrew University School of Medicine, 12 Shmu'el Bait Street, PO Box 3235, Jerusalem 91031, Israel. rachela@szmc.org.il
Telephone: +972-2-6555129
Fax: +972-2-6666135

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## Abstract

Pathogenic mutations involving DNA repeat expansions are responsible for over 20 different neuronal and neuromuscular diseases. All result from expanded tracts of repetitive DNA sequences (mostly microsatellites) that become unstable beyond a critical length when transmitted across generations. Nearly all are inherited as autosomal dominant conditions and are typically associated with anticipation. Pathologic unstable repeat expansions can be classified according to their length, repeat sequence, gene location and underlying pathologic mechanisms. This review summarizes the current contribution of mutant pluripotent stem cells (diseased human embryonic stem cells and patient-derived induced pluripotent stem cells) to the research of unstable repeat pathologies by focusing on particularly large unstable noncoding expansions. Among this class of disorders are Fragile X syndrome and Fragile X-associated tremor/ataxia syndrome, myotonic dystrophy type 1 and myotonic dystrophy type 2, Friedreich ataxia and C9 related amyotrophic lateral sclerosis and/or frontotemporal dementia, Facioscapulohumeral Muscular Dystrophy and potentially more. Common features that are typical to this subclass of conditions are RNA toxic gain-of-function, epigenetic loss-of-function, toxic repeat-associated non-ATG translation and somatic instability. For each mechanism we summarize the currently available stem cell based models, highlight how they contributed to better understanding of the related mechanism, and discuss how they may be utilized in future investigations.

**Key words:** Unstable repeat associated disorders; Human embryonic stem cells; Patient-derived induced pluripotent stem cells; Disease modeling; Epigenetics; repeat-associated non-ATG translation; RNA toxicity; Repeat somatic instability



**Core tip:** This review summarizes the current contribution of mutant pluripotent stem cells (diseased HESCs and patient-derived induced pluripotent stem cells) to the research of unstable repeat pathologies by focusing on particularly large unstable noncoding expansions. It demonstrates their importance as an unlimited cell source for generating rarely available impaired cells in culture, and as a model system for

exploring the mechanisms that are involved with this class of mutations. For each mechanism we describe the currently available stem cell based models, highlight how they contributed to better understanding of the related mechanism, and discuss how they may be utilized in future investigations.

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## INTRODUCTION

Unstable repeat expansions, termed also dynamic mutations, are the cause of over 20 different neurodevelopmental, neurodegenerative and neuromuscular heritable conditions that result from a change in the number of repeat units in coding genes when ordered in tandem (for comprehensive review see[1]). Repeat instabilities change across generations, and may vary between cells within affected individuals. Nearly all are inherited as autosomal dominant conditions and are typically associated with anticipation, meaning that the disease symptoms tend to be more severe and occur earlier in age with successive generations. Most unstable repeat associated disorders are caused by the addition of repeat units (mostly tri-nucleotides) that, above a certain threshold, lead to misregulation of genes and/or to their RNA/protein products. Disease associated unstable repeat expansions can be classified into two groups according to the length and gene location of the expansion. One class of disorders results from small repeat copy changes (few to tens of units) that usually reside in exon coding regions of genes. Among them are the ploy glutamine CAG expansion disorders like Huntington disease, Huntington's disease like 2 (HDL2), Dentatorubral-pallidoluysian atrophy (DRPLA), Spinal and bulbar muscular atrophy (SBMA) and spinocerebellar ataxia (SCA) 1, 2, 3, 6, 7 and 17, which lead to protein gain-of-function alterations. A second class of unstable repeat expansion disorders results from large expansions, ranging from hundreds to thousands of copies. Large pathogenic repeat expansions are typically located in noncoding regions including promoters, introns and untranslated regions (UTRs) of genes[2]. Among disorders in this class are Fragile X syndrome (FXS) and [Fragile X-associated tremor/ataxia syndrome (FXTAS); both FXS and FXTAS are caused by large expansion of a CGG repeat in the 5'-UTR of the *FMR1* gene[3-5]], [myotonic dystrophy type 1 (DM1); caused by a CTG expansion in the 3' UTR of the *DMPK* gene[6-8]] and type 2 (DM2; caused by a CCTG expansion in intron 1 of the *ZNF9* gene[9]), [Friedreich ataxia (FRDA); caused by a GAA expansion in intron 1 of the *Frataxin* gene[10]], C9 related [amyotrophic lateral sclerosis and/or frontotemporal dementia (ALS-FTD); caused by a GGGGCC[11] expansion in intron 1 of the *C9orf72* gene[12]], and [Facioscapulohumeral Muscular Dystrophy (FSHD); caused by a contraction of the D4Z4 macrosatellite repeat in sub-telomeres of chromosome 4q35].

The outcome of the expansion mutation may be different depending on its gene location and length. Unlike small expansions, which commonly result in alterations in protein function, large noncoding expansions introduce further complexity because they can lead to either loss-of-function, RNA gain-of-function, toxic protein gain-of-function, or to a combination of all these pathogenic mechanisms in unison[13]. In addition and in contrast to small expansions, large noncoding expansions frequently coincide with marked changes in repeat tract length between and within tissues of affected individuals[8,14,15]. This phenomenon, termed somatic repeat instability, results in mosaicism for expansion size and occasionally correlates with disease age of onset and severity. Modeling dynamic mutations, specifically large expansions, in mice can be particularly challenging due to the difficulty in artificially inducing and stably maintaining very large repeat expansions (especially CG-rich) *in vitro* and *in vivo*. In addition, despite the similarities between mouse and human, there are still major differences between the two species, leading to dissimilarities in biochemical pathways and phenotypes. These crucial discrepancies emphasize the need for complement model systems that will reproducibly copy the underlying mechanisms and clinical phenotypes in human.

Mutant human pluripotent stem cells, embryonic [human embryonic stem cells (HESCs)] or artificially induced [induced pluripotent stem cells (iPS cells)], may provide a great opportunity to study unstable repeat expansions by complementing existing models[16]. They are expected to be especially useful in the study of heritable conditions where the animal model fails to fully recapitulate the phenotype of the disease in human, or when disease relevant cell cultures from patients are unavailable. The many advantages to the utilization of these cells for biomedical research include the fact that they are human derived, untransformed cells with an unlimited self-renewing capacity. In addition, they have the potential to differentiate into a wide range of cell types in culture, and recapitulate early human embryo development while they differentiate *in vitro*. Moreover, as mutant pluripotent stem cells can be derived directly from genetically affected embryos that are obtained through preimplantation genetic diagnosis (PGD) procedures (HESC)[17], or by reprogramming somatic cells obtained from patients (iPS)[18], they can reproduce disease cellular phenotypes as they occur *in vivo* without the need to artificially intervene with their genome through genetic manipulation. In addition, they are human derived and potentially be used to generate large amounts of impaired disease relevant cells in culture. This is particularly beneficial in the

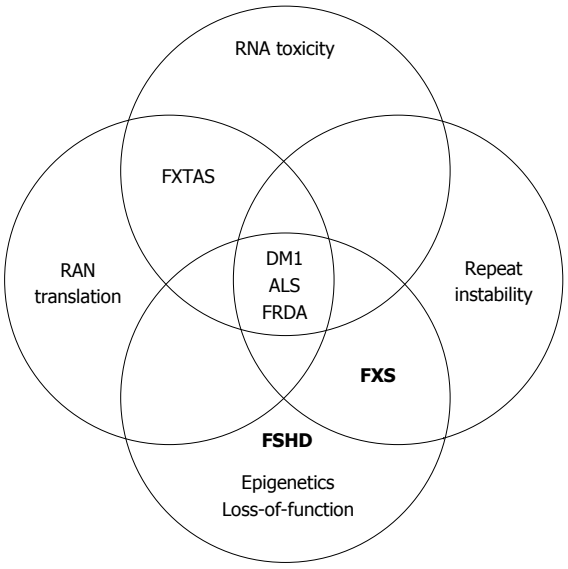


**Figure 1 Modeling pathogeneic noncoding repeat expansions by the use of mutant pluripotent stem cells.** Unstable noncoding repeat expansions have several features in common. They typically display somatic instability, and can lead to either epigenetic loss-of-function, toxic RNA gain-of-function, toxic RAN translation, or to a combination of all these pathogenic mechanisms. Future research might discover more similarities in the pathogenesis of these and additional repeat associated disorders. Disease modeling through the use of mutant HESCs and iPS cells is anticipated to be particularly useful considering their potential to generate impaired cells, and their ability to recapitulate developmentally regulated events upon differentiation. Indeed, using this model system, new mechanistic insights have thus far been gained for diseases marked in bold. FSHD: Facioscapulohumeral Muscular Dystrophy; FXS: Fragile X syndrome; ALS: Amyotrophic lateral sclerosis; FRDA: Friedreich ataxia; FXTAS: Fragile X-associated tremor/ataxia syndrome; DM1: Myotonic dystrophy type 1.

case of unstable repeat pathologies, where studies are frequently limited to postmortem brain samples or to unsuitable cell types obtained from patients such as peripheral blood cells or skin fibroblasts. Furthermore, as these cells can recapitulate early stage embryo development, they may be particularly valuable in modeling disease associated mechanisms that are developmentally regulated such as those that are elicited by differentiation. In terms of applied research, mutant pluripotent stem cells provide a powerful cell culture based system for gene correction. For instance, they may facilitate the efficient induction of irreversible changes in DNA that will correct the disease causing mutation by shortening the repeat tract through genome editing or other gene manipulation approaches[19]. They can also provide a platform for drug screening and development, conditioned by the accessibility of efficient differentiation protocols and the availability of reliable biomarkers.

In this review we summarize the current contribution of mutant pluripotent stem cells to the research of unstable repeat pathologies by focusing on common mechanisms that are associated with large unstable noncoding expansions (Figure 1). A complete survey of the data regarding the use of mutant pluripotent stem cells for modeling phenotypes of coding unstable repeat expansions, and the use of these cells as a platform for gene therapy, are beyond the scope of this review and can be found elsewhere[20-22]. The discussion here will thus be limited to only pathological noncoding repeat expansions. For each mechanism we highlight the currently available pluripotent stem cell models by describing their exclusive utility for investigation of the mechanism, how they contributed to better understanding of the related mechanism, and we raise potential routes for future investigation.

## TOXIC RNA GAIN-OF-FUNCTION

Over the past decade it has become evident that RNAs containing abnormally large microsatellite repeat expansions underlie the pathogenesis of at least 9 dominantly inherited human diseases. These are mostly neurological and neuromuscular diseases, including DM1[23], DM2[24], FXTAS[25], C9/ALS-FTD[26], SCA3[27], SCA8[28], SCA10[29], SCA31[30] and HDL2[31]. These diseases arise when the repeat-containing RNA disrupts the function of specific RNA binding proteins (RBPs) in *trans*. The paradigm for such toxic RNA gain-of-function pathology is DM1, where a CUG repeat expansion located in the 3'-UTR of the *DMPK* gene interferes with the cellular function of specific CUG-binding splice-regulating proteins termed MBNL1 and CUG-BP1 (CLEF protein family)[32,33]. The altered function of these splice regulators hinders the normal processing of transcripts encoding over twenty different muscle genes, resulting in inappropriate expression of embryonic rather than adult splice variants in adult disease manifesting tissues[34,35]. The general hallmarks of unstable repeat disorders with RNA-mediated toxicity are the accumulation of repeat-containing RNAs in intra-nuclear inclusions along with the sequestration of common RBPs (mainly proteins involved with RNA metabolism) and alternative splicing misregulation[36]. Co-localization of the large ribonucleoprotein (RNP) aggregates with ubiquitin and proteasome subunits is a common feature[37]. These striking parallels have led to the identification of the most common (currently known) mechanisms underlying noncoding unstable microsatellite disorders. This subclass of disorders is caused not due to the loss of function of the mutant transcripts and haploinsufficiency, but rather from the loss of function of RBPs that aggregate with the lengthy RNAs, and possibly from the toxic effect of the aggregates themselves. As the RNA seeds accumulation of the proteins it interacts with, it alters their biological function by changing their abundance and/or localization in the cell. Furthermore, since most of these expansions are bi-directionally transcribed, multiple repeat-containing toxic RNAs seem to be involved[38-40]. The susceptibility of neurons to this process is a common feature, most likely related to the ageing course of this specific cell type. Considering the reversibility of the process[41-44], it may be possible to develop effective therapeutic strategies to ameliorate

the symptoms of these diseases.

Taking advantage of two DM1 affected pluripotent stem cell lines derived from embryos identified through PGD, Denis *et al*[45] produced impaired early progenitor neural cells in culture. To validate the pathological relevance of their differentiated cells, they show that DM1-neural stem cells (NSCs) and derived neurons harbored intranuclear RNP foci, contain the mutant RNA and co-localize with the splice factor MBNL1. Interestingly, functional characterization of DM1 HESC-derived neurons revealed reduced proliferative capacity and increased autophagy linked to the mTOR signaling pathway. In addition, the researchers were able to demonstrate that the loss of function of MBNL1 in wild type (WT) HESCs results in alteration of the mTOR signaling pathway, whereas gain-of-function experiments rescued the phenotype. Collectively, their findings provided a mechanism by which the DM1 mutation might affect a major signaling pathway, and highlighted the pertinence of using pluripotent stem cells to study neuronal defects in general. A complement model system to support the formation of intranuclear RNA foci by *in vitro* derived neurons was developed in iPS cells, validating the potential of pluripotent stem cells to offer an unlimited cell resource for CNS mechanistic studies[46].

A different disease for which pluripotent stem cells have been employed to further understand the toxic RNA-mediated mechanism underlying unstable repeat expansions is in C9/ALS-FTD. Amyotrophic lateral sclerosis (ALS) and/or frontotemporal dementia (FTD) is an autosomal dominant neurodegenerative disorder characterized by adult onset of one or both of these conditions in an affected individual[47-49]. The leading cause for ALS-FTD is a $G_4C_2$ repeat expansion in the first intron of the *C9orf72* gene, between noncoding exons 1a and 1b. Expansion of $G_4C_2$ repeat tract beyond a certain threshold, results in RNA gain-of-function resulting from bi-directional transcription[40,50]. Its low incidence (1:100000) and mainly sporadic occurrence (90%), late onset, and accelerated progression, have thus far facilitated the preparation of iPS cells, but not HESC lines. By establishing multiple iPS clones from skin biopsies of patients' fibroblasts with over 1000 $G_4C_2$ repeats, researchers were able to generate motor neurons in culture that recapitulate key neuropathological features of ALS-FTD including RNA foci reminiscent of those observed in postmortem brain autopsies from patients[51]. Interestingly, the RNA foci containing $G_4C_2$ repeats were present in all iPSC-derived human neurons as well as in undifferentiated iPSCs and primary fibroblasts. This suggests that these foci are not restricted to a specific cell type or developmental stage, as one would predict if functionally significant. Moreover, transcription of the repeat containing C9orf72 transcripts, led to accumulation of $G_4C_2$ repeat-containing RNA foci selectively in various C9-ALS iPSC-derived neural cell types[52]. It was also shown that the pathologic RNA foci co-localize with the major known RBPs like hnRNPA1, Pur-$\alpha$[52] and ADARB2[53], suggesting that they may be able to disrupt RNA metabolism. Furthermore, it was found that the *in vitro* derived C9-ALS motor neurons present altered expression of genes involved in membrane excitability, and demonstrated a diminished capacity to fire continuous spikes upon depolarization compared to a WT motor neuron control[52]. By targeting the RNA foci through antisense oligonucleotide (ASO), either through knockdown (KD) of the full length C9orf72 transcripts or by differentially targeting the repeat containing mRNA isoforms (V1 and V3), researchers were able to suppress RNA foci formation and reverse gene expression defects in C9-ALS neuronal cells[52,53], as well as, rescue to some extent disease phenotype (Glutamate toxicity[53]). These data show that patient-derived motor neurons can be used to elucidate the pathogenic role of the toxic RNA in C9/ALS-FTD. More generally, they neatly illustrate the power of mutant pluripotent cells as a translational platform for therapeutic development.

Many questions regarding this repeat-associated mechanism remain to be addressed. For example, what dictates the cell-type specificity of this process? How is it age related? What is the protein composition of the aggregates in each disorder and, what is the nature of the inclusions? Are they toxic or do they simply represent clearance of toxic RNA? In addition, although animal models have provided firm evidence for RNA gain-of-function as a major contributing mechanism[54,55], clinical and pathophysiological differences between human and available animals (mouse and Drosophila) limit their usefulness in delineating specific mechanisms underlying these toxic RNA-mediated pathologies[56-61]. Therefore, a complement human-based model system is needed to enable molecular characterization of the onset and course of this class of conditions.

## REPEAT-MEDIATED EPIGENETIC MODIFICATIONS

Among the disease associated expansions there are several, counting FXS, FRDA, DM1, and C9/ALS-FTD, that results from particularly large noncoding expansions (hundreds to thousands of copies) that reside within CpG islands (CGIs). CGIs typically remains free of DNA methylation and are rich in histone modifications associated with transcriptionally active chromatin. Yet, expansion of repeat copy number beyond a certain threshold leads to local acquirement of abnormal CpG methylation in addition to loss of active histone modifications and concomitant gain of repressive histone modifications that are typical to densely packed chromatin (like H3K4 demethylation and H3K9 and H3K27 tri-methylation, respectively). These aberrant epigenetic modifications can change local gene transcription. Their effect may vary from one locus to another, depending on the location of

the expansion in the gene. While in some genes it abolishes the activity of a promoter (as in FXS and C9orf72)[62,63] or an enhancer (as in DM1)[64], in other genes it interferes with RNA transcription elongation (as in FRDA)[65] or termination (as in FSHD[66]). Furthermore, even when hypermethylation leads to the loss of promoter activity, the outcome may be very different. For example, while in FXS transcriptional silencing results in FMRP protein deficiency (loss-of-protein function)[67], in C9/ALS-FTD epigenetic silencing may potentially lead to increased use of an alternative promoter (RNA gain-of-function)[39]. In addition, in some disorders hypermethylation seems to be acquired in a developmentally regulated process (FXS)[17] whereas in others it is more of a tissue or an age-dependent phenomenon (DM1)[68]. Therefore, it is essential to first understand when, where and how hypermethylation affects local gene expression, in order to examine whether and by what mechanism this contributes to disease pathogenesis. More generally, by unraveling how repeat expansions lead to the loss of CGI identity, we may gain new insights on how CpG islands are normally protected from *de novo* methylation throughout the genome.

The availability of mutant pluripotent stem cell lines carrying pathologic repeat expansions may provide a great model system to address the questions of when, where and how hypermethylation is aberrantly acquired. In particular, such cell lines can be utilized to better determine the timing, intervene with the mechanism, and monitor the effect of epigenetic modifications. In addition, they may provide an exceptional opportunity to rescue the mutant phenotype by resetting the undesired epigenetic modifications that are coupled with the expansion through cell reprogramming.

To date, pluripotent stem cell-based studies related to the epigenetic aspects of unstable repeat expansions have been mainly limited to FXS. In FXS, when the CGGs reach the full mutation range, they lead to the spread of abnormal CpG methylation and repressive histone modifications at the 5' regulatory region, resulting in epigenetic gene silencing and consequently to FMRP protein deficiency in patients[62]. Initially, when the first FXS XY HESC line was established, it was found to express FMR1 at comparable levels with WT HESCs and to be completely FMR1 unmethylated[17]. These findings validated a commonly accepted view that FMR1 epigenetic silencing is a developmentally regulated process that is triggered by differentiation. Moreover, it encouraged investigators to propose that since epigenetic FMR1 inactivation is differentiation-dependent (rather than being maternally transmitted in the gamete), it may be possible to rescue the cellular phenotype of FXS neurons simply by removing the epigenetic marks that are erroneously gained, through cell de-differentiation in culture. However, as more FXS HESC lines were available, it became apparent that FMR1 hypermethylation commonly occurs in the undifferentiated state. Taking advantage of a large set of FXS HESCs (9 cell lines, including the former), Avitzour and colleagues found that FMR1 hypermethylation frequently occurs in these cell lines (six of nine lines, ranging from 24% to 65%)[69]. This suggests that the wide variability in methylation levels between and within the different FXS HESC lines reflects a widespread event within mutant FMR1 embryos, where methylation state is initially set. It should be interesting to determine whether FXS preimplantation embryos are already FMR1 methylated, and whether methylation at this stage is incomplete. Mechanistically, the researchers tried to address the question of how the CGG expansion at the FMR1 locus leads to *de novo* methylation of the CpG island at the 5'-UTR of FMR1[69]. They aimed to associate FMR1 hypermethylation with CTCF binding next to the repeats, taking in to consideration the well-known role of CTCF as an insulator binding protein that counteracts heterochromatin spreading[70,71] and taking in to account previous reports demonstrating CTCF occupancy next to the CGG repeats at the FMR1 locus in differentiated cells. However, since no enrichment for CTCF could be detected, in both WT and affected HESC lines, this study ruled out the possibility that hypermethylation may be attributed by the binding loss of CTCF, as formerly suggested[38,72]. Employing two other XY FXS HESC lines, Colak and colleagues uncovered an mRNA-mediated mechanism that drives epigenetic FMR1 inactivation in a way that relies on neuronal differentiation[73]. In this study the researchers showed that the CGG-lengthy FMR1 mRNA hybridizes to the complementary CGG-repeat portion of the *FMR1* gene to form an RNA:DNA duplex. Upon differentiation the RNA:DNA hybrid elicits gene silencing on the protein and mRNA levels. According to their study, disrupting the interaction of the mRNA with the CGG-repeat portion of the *FMR1* gene at a critical time point during cell differentiation prevents promoter silencing. It remains to be determined whether silencing in undifferentiated cells is achieved by the same mechanism or by distinct one, and whether the loss of FMR1 mRNA and protein in the FXS HESC-derived neurons involves DNA hypermethylation.

In line with this study, it has been proposed that it may be possible to reverse the adverse effect of the modifications and correct the mutant phenotype by reprogramming terminally differentiated FXS cells. However, somatic cell reprogramming of FXS patients' fibroblasts by numerous independent studies failed to de-methylate and reactivate the gene simply by de-differentiation[74], suggesting that once established, methylation is irreversible and/or is acquired prior to blastocyst formation[75-77]. In line with this view, it should be feasible to obtain FXS-iPS clones that are FMR1 active from somatic cells with a full mutation provided that they carry an unmethylated full expansion at least in some of their cells. On the other hand, perhaps reprogramming may wrongly lead to hypermethylation of this mutation prone site, regardless of its initial

epigenetic state. Indeed, reprogramming fibroblasts from an unusual asymptomatic subject, bearing a full mutation that is entirely unmethylated, resulted in complete silencing, regardless of the reprogramming method used[78]. Maybe the high levels of DNMT3b, that are induced as part of the reprogramming process[79], led to this unexpected result. This suggests that FXS iPS cells, and potentially additional iPS models for epigenetically regulated disorders, may not serve as an ideal model for better understanding the mechanism by which hypermethylation is induced. Reprogramming of somatic cells from additional subjects with hypo-methylated expansions will be required in order to establish whether this is a widespread event that is irrelevant to the disease. Regardless, it should be possible to use these disease affected iPS cells to explore the consequence of FMRP deficiency or reverse this effect in *in vitro* differentiated neurons, a disease relevant cell type. Indeed, re-activation of FMR1 by the treatment with the de-methylating agent 5-azaC has already been successfully practiced in neuron derived FXS-iPS cells[80].

Apart from FXS HESCs, there is indirect evidence to suggest that hypermethylation is recapitulated in patient derived iPS cells for other unstable repeat related loci. One example is C9/ALS-FTD iPS cells that carry a $G_4C_2$ expansion at the *C9orf72* gene. In the C9orf72 the $G_4C_2$ repetitive sequence is flanked by two CpG islands (CGIs) that span from the promoter sequence into intron 1 of *C9orf72*. Like many CGIs in the genome, this region typically remains unmethylated and is transcriptionally active. Yet, expansion to more than 90 repeats leads to the formation of one large CGI exclusively in patients, and to local acquirement of abnormal CpG methylation upstream[11]. Hypermethylation is coupled with the local gain of repressive histone modifications, and is responsible for the inactivation of variant 2 of *C9orf72* that, unlike repeat-containing variants 1 and 3, initiates transcription from exon 1b and harbors the $G_4C_2$ sequence in its promoter[26]. Overall, C9/ALS-FTD iPS cells demonstrate altered gene expression. While some reports demonstrate general reduction in the transcription of all variants, others show differential expression favoring variants 1 and 3 over variant 2[26,52,81]. All together, this suggests that hypermethylation in fact exist in these cells. Nevertheless, the contribution of epigenetic gene silencing to disease pathogenesis is not fully clear. There is conflicting evidence regarding the correlation between hypermethylation and type of pathology (ALS *vs* FTD), age of onset or severity[11,82,83]. Experimental evidence in animal models demonstrates inconsistent results[84,85]. On the other hand, hypermethylation was suggested to have a protective role from the accumulation of pathogenic RNA/protein foci contributed by repeat-containing mRNA variants 1 and 3[86]. These conflicting studies warrant further investigation regarding the contribution of DNA methylation to ALS-FTD pathogenesis, and may be considerably advanced by the use of *in vitro* derived neurons from C9/ALS-FTD pluripotent cells.

Another elegant example for the power of pluripotent stem cells in resolving the contribution of epigenetics to disease pathogenesis is in FSHD. FSHD is caused by a contraction of a macrosatellite repeat (D4Z4 repeat, 3.3 kb unit) located in the sub-telomeres of chromosome 4q35. Non-affected individuals carry 11-100 repeat units, while FSHD patients have less than 10 repeats, but must have at least one unit to manifest the disease[87-89]. Interestingly, the loss of DNA methylation and reduced H3K9me3 that coincide with repeat contraction have been found to be causally related to the up-regulation of a full transcript from the *DUX4* gene, a transcript which is exclusive to the most distal (telomeric) unit of the D4Z4 repeat containing region[66,80-93]. Expression of the full length DUX4 transcript, which is exclusive to FSHD myotubes, is responsible for the induction of nuclear foci, impaired myogenesis and ultimately to cell death by apoptosis[94]. Immediately downstream to the most-telomeric unit there is a polymorphic site that acts as an alternative polyadenylation signal that controls the length and stability of the DUX4 transcript. While normal alleles produce short transcripts and truncated proteins, contracted FSHD alleles utilize a cryptic splice donor to form a full length toxic protein. As alternative polyadenylation seemed to be a tissue-specific process, it was speculated to be a developmentally regulated event that is mediated by the loss of repressive epigenetic modifications due to D4Z4 repeat contraction. By comparing WT and FSHD iPS cell lines, Snider and colleagues[94] found that both cell types equally express full length DUX4, independent of repeat number. However, upon differentiation, full length expression was switched off and converted back to short transcripts in WT controls while full length expression persisted in the diseased cells. Importantly, the researchers were able to correlate the switch in splice site usage with the enhancement of repressive chromatin modifications (H3K9me3) in the WT differentiated cells. Thus, it appears that H3K9me3 modification (and most likely CpG methylation) mediates the splicing for the truncated protein upon differentiation, and that the contraction of the D4Z4 repeats abolishes this developmentally regulated process. It remains to be determined how D4Z4 contraction interferes with the normal induction of heterochromatin. Nevertheless, the use of FSHD iPS in this particular study, neatly illustrates the power of mutant pluripotent stem cells in resolving the molecular mechanisms contributing to disease pathogenesis.

## RAN TRANSLATION

During the past years it has become apparent that a third potential mechanism may act in the context of pathogenic repeat expansions. A novel form of

translation was found by Ranum's group, which results from translation in the absence of an initiating AUG codon next to disease causing unstable microsatellite repeats[95]. They found that the CAG repeat tract at the DMPK (DM1) and ATXN8 (SCA8) loci undergo noncanonical translation in disease relevant tissues of patients, resulting in the production of homopolymeric proteins in all three frames (CAG, AGC, and GCA). This newly discovered phenomenon was termed RAN translation, abbreviated for Repeat Associated Non-ATG translation.

To date, RAN translation has been shown to occur across four different types of repeat motifs: CAG, CTG, CGG and GGGGCC, in a growing number of conditions, including DM1, SCA8[95], FXTAS[96] and C9/ALS-FTD[39,40,50,97,98]. Among its effects, RAN translation leads to ubiquitin-positive intracellular protein inclusions, which may be toxic to cells[96]. Furthermore, researchers have shown that when repeats are bi-directionally transcribed to form sense and anti-sense transcripts, six potential homopolymeric proteins can be produced[48] as each transcript can be RAN translated in three different reading frames. Importantly, they provided evidence that long repeat tracts can express multiple RAN-translated products, simultaneously, in a single cell and suggested that RAN translation may contribute to disease pathogenesis by a protein gain-of-function mechanism[95]. In addition, as longer expansions are more liable to the accumulation of RAN translated products, it was speculated to be involved with disease anticipation[99]. Nonetheless, evidence for a role of RAN translation, if any, in disease pathogenesis still awaits further exploration.

Thus far, RAN translation has been documented in pluripotent stem cells only for C9/ALS-FTD. Indeed, postmitotic neurons derived from C9/ALS-FTD iPS cells have already been established, and were shown to present disease-relevant phenotypes that include RAN translation products in the form of cytoplasmic TDP-43 negative/p62 positive protein inclusions[51]. Immunofluorescent staining of the C9/ALS-FTD iPSNs (induced pluripotent stem cells derived neurons) revealed cytoplasmic poly-(Gly-Pro) inclusions similar to those observed in C9orf72 expansion carriers postmortem affected brains[53]. In addition, as the appearance of RAN peptide foci was coupled with increased sensitivity to cellular stress induced by autophagy inhibitors[51,53], it raised the possibility that this mechanism may contribute (directly or indirectly) to impaired autophagy function in ALS-FTD and possibly additional conditions associated with RAN translation[53].

There is by now enough evidence to indicate that mutant differentiated iPS cells can authentically recapitulate the late onset and tissue-specific cellular phenotypes that are typically seen in human C9orf72 postmortem brain samples. It should be interesting to explore whether similar cellular phenotypes can be reproduced in iPS cells derived from younger, yet asymptomatic, C9 mutation carriers. In addition, it would be useful to establish additional C9/ALS-FTD iPS cells from patients who carry small, intermediate and large expansions to correlate inclusions with repeat tract size, C9orf72 transcription and disease severity. Such associations are not yet fully understood. Nevertheless, it should be kept in mind that mitigation of RNA foci and RBP aggregation by antisense oligonucleotide (ASO) treatment in affected iPSNs had no effect on the formation of RAN-translation inclusions[53]. This would suggest that the detected homopolymeric inclusions may not have a major role in disease pathology. However, as only one type of di-peptide repeat was monitored [poly-(Gly-Pro)], it may be possible that in fact the other RAN peptides (such as the ones that are produced by antisense transcription) are crucial for the manifestation of the abovementioned cellular phenotypes. In any case, even if the RAN-translated products observed in iPS derived neurons will be proved to be irrelevant to disease pathology, they may be useful as a biomarker, providing an opportunity to develop a platform to test therapeutic agents for their effect on brain features of the disease. Compounds that will reverse the formation of the inclusions in the diseased cells are expected to offer a starting point for therapeutic development.

Many questions regarding RAN translation arise. For example, what is the clinical significance of RNA translation? Does RAN translation occur across all unstable microsatellite disorders? Is it age dependent? When and what determines its tissue specificity? In addition, it is still not yet clear which RNA structures and flanking sequences are required to facilitate RAN translation? Intrastrand RNA repeat associated hairpins and G-quadruplex structures have been suggested as candidates for RAN translation involvement[95,99-101]. When more stem cell based disease models (HESC and/or iPS) will become available, it is anticipated that human pluripotent stem cells will be important in clarifying at least some of these fundamental questions.

## REPEAT INSTABILITY

Repeat instability is a form of dynamic mutation that results from a change in the number of repeat units when ordered in tandem. Unlike static mutations, which involve permanent change in the DNA, repeat instabilities change across generations, and may vary between and within tissues of affected individuals (for comprehensive reviews see[102,103]). We refer to germ-line instability when the change in repeat number occurs during transmission from parent to offspring, and somatic instability when the change takes place in the somatic cells of the patient. Germ-line instability, which is frequently biased towards maternal or paternal transmissions, is typically associated with anticipation (reviewed in[103]). This is because the longer the repeat tract the more likely it will undergo expansion

in the next generation. As a result, disease symptoms worsen from one generation to the next. However, in some pathologies repeat instability extends beyond the germ-line, leading to a high degree of variation in repeat tract length in affected individuals[14,104,105]. Unlike in germ-line repeat instability, the contribution of somatic instability to disease pathogenesis is not at all times clear. In some conditions there is an obvious correlation between expansion size and cell dysfunction[106,107], whereas in others this association in not noticeable[108,109]. Nevertheless, it is imperative to understand the molecular details of this mutation-associated phenomenon, considering that in some cases expansion size is clinically significant and may be used as a prognostic marker[110].

It is important to note that not all expansions are alike in range and location. While expansions residing in coding regions are usually restricted in length (tens of copies) and limited to germ-line mutations, noncoding expansions are frequently large (hundreds-thousands of copies) and typically coincide with somatic instability. Furthermore, in some disorders somatic instability can be restricted to early embryonic development or to highly replicating cells, whereas in others it may be tissue-specific or age-dependent, and may be limited to particular post-mitotic cell types. Therefore, while the type of mutation is common to all of these heritable and devastating conditions, the marked variation in the pattern and distribution of expansion size among them may stem from assorted mechanisms.

It is generally accepted that repeat instability results from local disruption of DNA replication, repair or recombination (for comprehensive review see[111]). Regardless of the mechanism, all proposed models for instability are based on the formation of unusual structures in the DNA (like hairpins and G-quadruplexes) that are eventually resolved by the addition (expansions) or deletion (contractions) of repetitive units[112-120]. It remains to be determined when and how these changes occur. In that sense, expansion carrying pluripotent stem cells provide an exceptional opportunity to gain more insights on the mechanisms that are involved with repeat instability. This is because they recapitulate early molecular events as they happen *in vivo*. They can be induced to differentiate into a wide range of cell types, allowing the monitoring of repeat instability in diseased relevant cells that are otherwise inaccessible for research like heart muscle cells or post-mitotic neurons. In addition, there is no evidence for further repeat amplification beyond the range observed in somatic cell of patients. Thus far, only a limited number of reports dealing with repeat instability in pluripotent cells have been published. All are descriptive in nature. For example, by following CTG expansion size at the *DMPK* gene of DM1-affected HESC lines, it was possible to demonstrate a change in repeat tract heterogeneity along with time in culture[121,122]. Furthermore, by inducing the cells to differentiate *in vitro*, instability was lost[122], apparently contradicting observations *in vivo*[123,124]. Nevertheless, it should be emphasized that tract-length heterogeneity within cell lines does not necessarily indicate that instability in fact exists, but rather proves that instability has occurred sometime before or during cell line growth in culture. This equally holds for CGG expansions at the FMR1 locus, where repeat length heterogeneity is clearly observed in a large cohort of FXS HESC lines[17,69]. Interestingly, in this case variations seem to be inversely correlated with CpG hypermethylation, a cis element that was formerly identified to constrain instability in FXS-affected fibroblasts[125]. Indeed, CGG somatic instability has been shown to be largely restricted to early fetal development and to cease with epigenetic gene silencing of FMR1[15]. In addition, as these cell lines are widely heterogeneous for expansion size and FMR1 hypermethylation, it may indicate that differentiation by itself is irrelevant to the repression of repeat instability in FXS, as formerly suggested[17]. It will be essential to generate single cell lineages from those FXS HESCs and monitor them for CGG repeat instability during growth *in vitro*, to unambiguously show that instability is, in fact, active in these cell cultures. Alternatively, it may be particularly useful to use patient iPS cells for these types of studies, considering the clonal origin of iPS cells. Two relevant examples for this application are C9/ALS-FTD iPS cells, where $G_4C_2$ tract size heterogeneity is evident both in undifferentiated and in *in vitro*-derived neurons[51], and the GAA expansion at the frataxin gene in FRDA[126,127]. On the other hand, the iPS based complimentary system should be cautiously tested before being adopted, as reprogramming may incorrectly enhance instability at repetitive loci as reported in FRDA iPS cells[128].

Apart from monitoring repeat instability along with time or as a consequence of reprogramming/differentiation in culture, diseased pluripotent stem cells (HESCs or iPS cells) may provide an unusual opportunity to gain new insights into the mechanisms underlying this mutational event. For example, it was shown that when the mismatch repair proteins, including MSH2 and MSH6, are downregulated upon differentiation repeat instability is dramatically enhanced in DM1 (CTG), HD (CAG)[122] and FRDA (GAA)[126,128] pluripotent stem cells, suggesting that repeat instability is assigned to difficulties in DNA repair at the these particular loci. This is in contrast to CGG somatic instability in FXS, where perturbations in DNA replication are assumed to be the underlying mechanism. In line with this perception is the study of Gerhardt and colleagues[129], who employed the DNA combing methodology to associate CGG expansion with alterations in origin of replication (ORI) usage and rate of progression in undifferentiated FXS HESCs. Using a pair of FXS HESC lines, these researchers reported a difference in ORI usage ex-

clusive to undifferentiated FXS cells, proposing a switch in replication direction relative to the CGG repeats. According to their study, unlike in WT HESCs, replication proceeds predominantly from a downstream ORI in FXS HESCs, resulted in the replication of the CGGs by lagging strand synthesis (rather than by the leading strand) which favors expansions to occur. It remains to be determined whether the change in ORI usage in FXS HESCs is a cause or effect of the expansion, and whether it is mechanistically related to CGG somatic instability. The availability of a large cohort of FXS HESC lines that are diverse in their epigenetic state and CGG instability should help to address the aforementioned questions.

Lastly, it should mentioned that while all of the aforementioned studies relate to the issue of somatic instability using mutant pluripotent stem cells, it should be most attractive to utilize the same systems in order to recapitulate germ-line instability and better understand why such disease associated repetitive sequences become increasingly unstable during germ-line transmission. This can be accomplished once efficient protocols for inducing HESC/iPS to differentiate into primordial germ cells and fully matured gametes will be available. Indeed, differentiation of ES cells into fully matured oocytes[130] and the generation of viable offspring from these cells[131] have already been accomplished in mice, paving the way for such studies to be carried out in human cells in the near future.

## CONCLUDING REMARKS

HESCs are undifferentiated pluripotent cell lines with the potential for unrestricted self-renewal as well as the ability to differentiate into a wide range of cell types[132-134]. They are established from *in vitro* fertilization (IVF)-derived embryos, and can be obtained from genetically affected embryos following PGD procedures. The derivation of HESCs directly from diseased embryos provides a powerful tool for disease modeling[135,136] by affording the exploration of disease relevant tissues[137,138] and/or developmental stages[139] that are otherwise inaccessibly for research. The main limitation of this approach is that it relies on the availability of genetically affected embryos, a valuable resource that is accessible to only a small number of stem cell deriving centers worldwide. In such cases, a complement model system may be employed which relies on the derivation of patient-derived iPS cells; ES-like cells that are established by somatic cell reprogramming of patients' cells in culture[140]. The primary advantage of this alternative system is that it facilitates the establishment of pluripotent cell lines that share many features with HESCs and harbor the disease causing mutation/s without the need for affected embryos. As such, and although artificially induced, it has become the most common alternative for modeling human diseases in culture by many researchers[16,141-146]. Nevertheless, iPS cells can be useful only if the disease is not embryonic lethal, and if complete erasure of the epigenetic modifications that are normally gained with cell differentiation do not interfere with investigation of disease pathogenesis. It should be mentioned that both HESC and iPS cells have been reported to carry typical chromosomal aberrations resulting from culture adaptation[147,148]. For this reason it is very important to perform routine characterization for chromosomal abnormalities in HESC and iPS cells lines.

The main benefits of both mutant HESC- and iPS-based model systems include the fact that they: (1) are human derived; (2) naturally carry disease causing mutations; (3) have the potential to differentiate into a wide range of cell types; (4) can self-renew without limitation while maintaining their undifferentiated and pluripotent potential; and (5) they mimic, to some extent, early embryo developmental stages as they spontaneously differentiate *in vitro* and *in vivo*. For all these reasons, they have become a commonly used model system for studying various disease associated mechanisms, particularly in cases where animal models do not authentically reproduce the affected human phenotype, or when the disease relevant cell types are unavailable for research.

In the context of disease causing unstable noncoding repeat expansions, which account for approximately half a dozen heritable conditions, the availability of mutant pluripotent stem cells is of particular importance. This is because most of these diseases are neuron-associated, restricting human-based research to a limited number of postmortem brain samples or to the use of disease irrelevant cell types. In addition, since noncoding expansions are hard to clone, it has been extremely difficult to artificially produce them in culture or to generate appropriate animal models[149-153]. In general, pluripotent stem cells seem to reproduce many of the pathological features that are observed in noncoding repeat expansion disorder patient cells[52,53,127,154-157]. Some of these features include nuclear RNA-protein foci, di-peptide cytoplasmic inclusions and hypermethylation. Therefore, stem cells are useful for basic research studies related to the different mechanisms that are involved with this group of disorders. By enlarging the number and repeat expansion heterogeneity of mutant stem cells cohort, we may gain new insights related to the underling mechanisms that are associated with these pathologies early during embryo development. Once efficient differentiation protocols will be established, it will also be possible to generate large amounts of cells from affected tissues in culture. Studying cell types that are particularly vulnerable in these diseases will improve our understanding regarding the tissue specificity seen in patients. Moreover, such cells can be utilized for drug screening and development studies. Stem cells provide a convenient platform to genetically intervene with the molecular defect that is inherently found in the cells. Considering the recent advancements in the

**Table 1 Modeling unstable noncoding repeat expansions repeat pathologies using pluripotent stem cells**

| Disease | Molecular mechanism | Ref. |
|---|---|---|
| DM1 | Toxic RNA gain-of-function | [45,46] |
| C9/ALS-FTD | | [47-49,51-53] |
| FXS | Reaped-mediated epigenetic | [17,62,69,71-76,78] |
| C9/ALS-FTD | modifications | [52] |
| FSHD | | [92] |
| C9/ALS-FTD | RAN translation | [51,53] |
| FXS | Repeat instability | [17,69,127] |
| C9/ALS-FTD | | [51] |
| FDRA | | [124-126] |
| DM1 | | [119,120] |

DM1: Myotonic dystrophy 1; C9/ALS-FTD: C9 related amyotrophic lateral sclerosis and/or frontotemporal dementia; FXS: Fragile X-syndrome; FSHD: Facioscapulohumeral muscular dystrophy; FDRA: Friedreich ataxia.

field of artificially engineered nucleases (particularly the CRISPR/Cas9 system), it may be possible in the near future to insert, replace or remove repetitive DNA sequences from the genome of HESCs/iPS cells in a fairly uncomplicated procedure. This may hold a great promise for the development of new therapeutic approaches and hopefully will allow the ease, or even cure, pathologies resulting from unstable repeat expansions.

At present, mutant pluripotent stem cell lines are available for FXS, FXTAS, DM1, FRDA, C9/ALS-FTD, FSHD, and FRDA (Table 1). Most of these cell lines have been utilized for generating disease relevant cell types in order to validate the feasibility of the stem cell model system. In a few cases, where disease pathology is developmentally regulated, they have been particularly informative. For example, in FSHD stem cell lines facilitated the discovery of alternative polyadenylation signals responsible for the generation of toxic protein in a differentiation-dependent fashion[94]. In the case of FXS, an mRNA-mediated mechanism that drives epigenetic *FMR1* gene silencing in affected neurons was uncovered in stem cells as well[73]. In other cases, particular cellular phenotypes in differentiated stem cell-derived cultures have been monitored by effect of therapeutic agents or by defining proteins that are associated with pathological protein inclusions, as reported in C9/ALS iPS-derived neuronal cells[52,53].

As a note of caution, it should be understood that the development of efficient stem cell differentiation protocols is not that straightforward. In particular, it can be quite challenging to use stem cells for the derivation of homogeneous cell populations that are functionally impaired. Moreover, molecular phenotypes in mutant pluripotent cells must be validated to see if they reproduce molecular events that are observed in diseased individuals. For example, despite all the benefits of iPS cells, investigators are becoming increasingly more aware of epigenetic instability in these cell types[158-161] as many iPSs wrongly acquire hypermethylation as a consequence of reprogramming[78,162-164]. Accordingly, a comparison between mutant HESCs and patient-derived iPS cells will be essential in future investigations of the epigenetic aspects of repeat expansion diseases especially because many mechanistic questions still remain to be addressed in this area. Unanswered questions in the field include what molecular events lead to hypermethylation of repeat expansions? What pathogenic role do nuclear riboprotein or cytoplasmic di-pepetide inclusions have, and what are the proteins that are sequestered into these structures? What are the mechanisms that are responsible for germ-line and somatic repeat instability, and why does somatic instability differ according to patient age and by tissue-specificity among differing genomic loci? Given proper experimental conditions, the answers to these riveting questions may be waiting to be uncovered by stem cell-based model systems.

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