

Chapter 1

Modeling Fragile X Syndrome in Human Pluripotent Cells

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Background

Fragile X Syndrome (FXS) was first identified as a distinct form of inherited intellectual disability in 1969 by Lubs, who described the physical constriction on the long arm of the X chromosome in lymphocytes isolated from FXS patients [1, 2]. This constriction was referred to as a fragile site, giving the syndrome its name. Apart from the cytogenic phenotype, FXS is recognized by mild to severe intellectual disability, with some patients displaying autistic behavior. This neurological symptom is accompanied by developmental delay, susceptibility to seizures, and dense and immature dendritic spines in affected neurons. Other, non-neurological symptoms include macroorchidism and distinct facial features such as a long narrow face with prominent ears, joint laxity and flat feet [3].

Genetic Variance Underling the Syndrome

It is now known that FXS is the leading cause of inherited intellectual disability in males and is the major monogenic cause of autism, affecting approximately one in every 4,000 boys and one in 8,000 girls worldwide [4–7]. In 1991, the syndrome was found to be caused by a single gene on the X chromosome, exhibiting length variation in patients [8]. The gene was named Fragile X mental retardation

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gene 1 (*FMRI*) [8] and its position correlated precisely to the location in which the physical constriction was first found [9]. The gene's variation in length was found to stem from a CGG repeat located at the 5' untranslated region (UTR) of *FMRI*. This CGG expansion at the 5' UTR of the gene leads to CpG methylation of the region, which spreads to the *FMRI* promoter [10]. This methylation is accompanied by hypoacetylation of histones and heterochromatinization. These epigenetic changes result in the silencing of *FMRI* and therefore the Fragile X mental retardation protein (FMRP) is not produced [11, 12].

In a healthy population, CGG repeats vary from 6 to 54 repeats [13]. If the number of repeats is between 55 and 200, the individual is referred to as a carrier with a premutation allele [13]. Premutation alleles do not cause FXS but they do cause other distinct disorders and are prone to major expansion of the repeats, especially during the female meiosis [13]. When the CGG repeat length exceeds 200, the allele is considered as having a full mutation and results in FXS [13]. Due to the location of the affected gene, FXS has almost complete penetrance in males while only 50 % of females show FXS symptoms. In some cases, deletions or missense mutations in the *FMRI* gene can also cause FXS. In other rare cases, males with a full mutation allele show no symptoms of the syndrome, implying that the epigenetic changes at the expansion region are the cause of the disease and not the CGG repeats themselves [14, 15].

FMRP Function

FMRP is a highly conserved protein found in animals such as fruit flies, mice, and humans [1, 8]. It is expressed in most mammalian tissues but is most abundant in the brain and testes [16]. Most of its functions are known from studies of brain tissues, as the neural phenotype has the greatest effect on the patient's quality of life. In the brain, FMRP is found primarily in neurons [16], where it plays an important role in synaptic plasticity. It is an RNA-binding protein that can selectively bind as much as 4 % of the mRNA in the mammalian brain [1, 17]. As FMRP is also present in the nucleus and can move to the cytoplasm, it may act as a cargo protein [18], shuttling to the periphery many of its target mRNAs, known to localize at dendrites [19]. Much work has been done on the interaction of FMRP with its target transcripts and it is now known that FMRP regulates translation by acting as a translational repressor. It does so by associating with polyribosomes [20–22] and inhibiting translation initiation [23], or by stalling ribosomes during translation elongation [22]. Several works have also shown that FMRP interacts with the microRNA (miRNA) pathway as both the *Drosophila melanogaster* ortholog (*dFMRI*) and the mammalian FMRP associate with components of the RNA-induced silencing complex (RISC) and several miRNAs [1, 6, 24–27].

Animal Models

Since *FMRI* is highly conserved across species, much work has been done on animal models (Fig. 1.1a). Null mutations of *dFmr1* produce flies with abnormal neuronal architecture and synaptic function [28–30], impairment of long-term memory [31], and reduced courtship interest [32]. The advantage of the fruit fly model is the ability to manipulate gene expression in different tissues using a variety of techniques, and the robust ability to study changes in behavior.

The mouse ortholog of *FMRI*, *Fmr1*, is also located on the X chromosome and shares 97 % homology in the amino acid sequence with the human gene [33]. KO mice exhibit many of the phenotypes seen in FXS patients. Like in affected humans, dendrites of KO mice are denser and have immature spines [34]. Other FXS characteristics such as learning and memory disability, increased susceptibility to seizures, and macroorchidism are also seen in KO mice [35].

These animal models are critical for our understanding of the various functions of *FMRI*, its importance in different tissues and cell types, and the conserved pathways underlying FXS in which it plays a part. However, there is no animal model

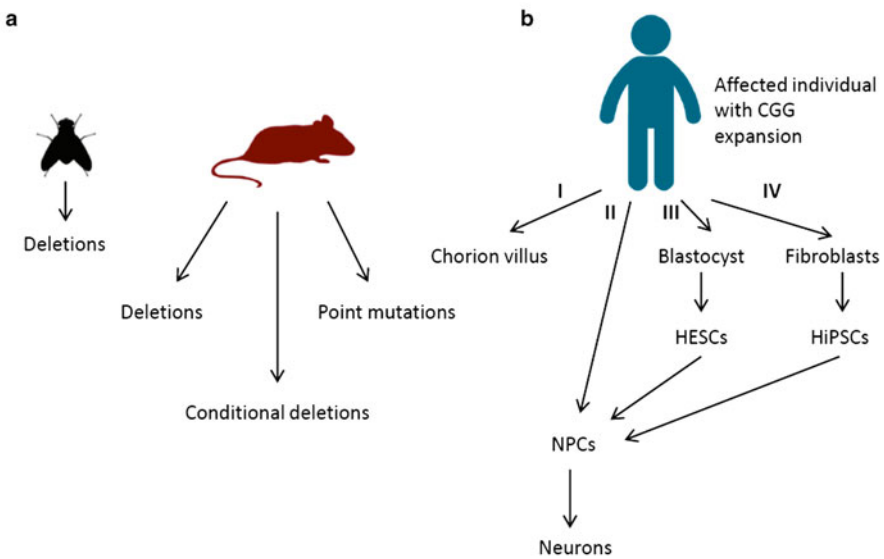


Fig. 1.1 Modeling fragile X syndrome in animals and humans. (a) To model the syndrome in animals, null mutations are created either by deletions or point mutations in flies or mice. (b) Different tissues from affected individuals are used to study the syndrome in humans. (I) Chorion villus samples are taken from the affected fetus. (II) Neural progenitor cells (NPCs) are taken from postmortem individuals. (III) Human embryonic stem cells (HESCs) are created from affected blastocysts. (IV) Human-induced pluripotent stem cells (HiPSCs) are generated by reprogramming of somatic cells (fibroblasts). The pluripotent cells can be differentiated into NPCs and then further differentiated into neurons

for the study of the CGG repeat expansion, nor the epigenetic silencing which causes the syndrome in humans. Even genetically engineered mice, with an extended number of CGG repeats, do not undergo methylation of the promoter and thus the gene remains active [36]. Understanding the mechanism and the timing of *FMRI* silencing during development is a key step in the search for treatment. Moreover, as it appears that *FMRI* plays a crucial role in differentiation and maturation of neurons [7, 37], it is of great importance to study these processes in humans as to better understand the role of FMRP in brain development.

Modeling FXS in Human Embryonic Stem Cells

A few studies on FXS were performed on human tissue samples, including chorion villus samples and tissues of affected fetuses [10, 38] (Fig. 1.1b). However, these models give only a snapshot of the syndrome status. To study the neural phenotype, neural progenitor cells (NPCs) were derived from a fetal FXS human brain [37] (Fig. 1.1b). These NPCs were used to generate neurons, which displayed fewer and shorter neuritis with smaller cell body and volume. Studies such as these are extremely valuable in terms of data yet access to human tissues is restricted and hard to come by and so comparison between different affected individuals is also scarce. Therefore, in order to better understand the different aspects of the disease such as CGG expansion, timing of *FMRI* silencing, and the function of *FMRI* in different tissues and cell types, all of which occur at different points in time during development and differentiation, a new model was needed. In 2007, human embryonic stem cells (ESCs) from FXS affected embryos (FXS-ESCs) were derived [39] and enabled the study of all of the above issues (Figs. 1.1b and 1.2). In one study [39], FXS-derived ES cell line was established from a blastocyst obtained from a preimplantation genetic diagnosis (PGD). The embryo was a male who inherited a full mutation allele from a carrier mother. Interestingly, although they carried the full mutation, FXS-ESCs showed both *FMRI* mRNA expression and the presence of FMRP. This finding showed that the transcriptional silencing of *FMRI* is a developmentally regulated process. To further understand the mechanism controlling the silencing of *FMRI*, FXS-ESCs were differentiated by injecting them into immunodeficient mice. The injected cells produced teratomas which were dissociated and analyzed. Analysis of *FMRI* mRNA levels in the teratomas showed a clear reduction in transcript levels, indicating that *FMRI* inactivation is indeed dependent upon differentiation. Methylation status and histone modifications were also analyzed and showed that while methylation of the *FMRI* promoter does increase early in the differentiation process, it does so in a very modest way. In contrast, histone modifications such as H3-tail acetylation and H3K9 methylation, which correlate with positive and negative regulation of transcription, respectively, show marked differences upon differentiation. H3-tail acetylation falls dramatically upon FXS-ESCs differentiation, whereas H3K9 methylation increases. The importance of this study, apart from the generation of a new model system, is a clear indication that *FMRI* is

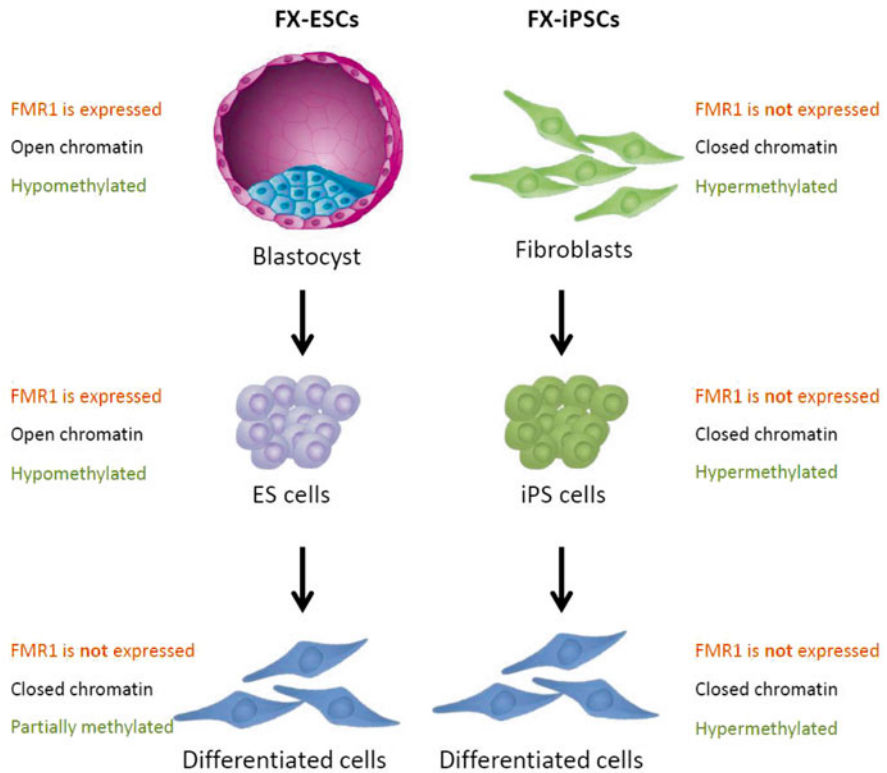


Fig. 1.2 Comparison of embryonic and induced pluripotent stem cell-derived models for fragile X syndrome

silenced in FXS embryos only during development and that the inactivation is initiated by chromatin modifications prior to DNA methylation.

In further studies, additional FXS-ESCs lines were derived from spare IVF-derived embryos diagnosed by PGD [40, 41]. In one of them, the association between early neural differentiation and the silencing of *FMR1* was examined, by producing active neurons from FXS-ESCs which were able to create neuronal networks [41]. This study showed that differentiation into neurospheres induced a steady increase in *FMR1* expression in control lines while neural differentiation of the FXS-ESCs lines did not. This difference was accompanied by aberrant expression of several early neural genes in FXS-ESCs-derived neurons, while other neural genes and pluripotency genes behaved in a normal manner. This observation further asserts that *FMR1* plays an important role in early stages of neurogenesis. Further analysis indicated that FXS-ESCs neurite formation and elongation did not show significant differences from control lines. FXS-ESCs were further differentiated to produce mature neurons, which lacked FMRP, displaying full silencing of *FMR1* during the 50 days of the differentiation process. Neurogenic potential of the

FXS-ESCs was lower compared to control and had a bias towards glial lineage. This may be linked to the aberrant neural gene expression seen at the early stages of the differentiation process. FXS-ESCs derived neurons were also analyzed for functional qualities. Functional analysis demonstrated that while FXS-ESCs can differentiate into viable neurons with passive properties similar to control cells, they hardly developed functional synaptic connections.

Although human stem cells derived from embryos hold great potential as a tool for understanding the basic mechanism of the disease, there are limitations to this model. First, in many countries, the generation of human stem cells from discarded IVF embryos presents an ethical challenge. Second, FXS is represented by profound variability in patients, ranging from the varying length of the repeats, through the methylation levels, and to the neurological phenotype itself. The degree of intellectual impairment also varies between different individuals, as only about 30 % of full mutation carriers display autistic behavior [42, 43]. Additionally, some carriers of the full mutation allele do not display any of the syndrome's phenotypes [14, 15]. As this variability is not inherited from the parents and is detected only after PGD analysis, the probability of acquiring numerous human embryonic stem cells displaying the entire spectrum of genetic and epigenetic differences is quite small and may span over many years.

Modeling FXS in Human-Induced Pluripotent Stem Cells

Recent advances in induced pluripotent stem cells (iPSCs) technology may resolve all of the aforementioned obstacles. Nowadays, creating iPSCs from somatic cells of any given individual is easy and reproducible. Recently, iPSCs from FXS patients (FXS-iPSCs) have been derived [44–46] (Fig. 1.2). Although iPSCs resemble human embryonic stem cells in most aspects, FXS-iPSCs differ in one critical manner. Despite successful reprogramming, it seems that the *FMRI* gene is resistant to the process and remains silent, maintaining both methylation status and repressive histone modifications [44] (Fig. 1.2). In this study [44], 11 FXS-iPSCs clones were derived from two different tissues of four affected male patients. These clones were then compared to multiple cell types including FXS-ESCs and wild-type iPSCs. The *FMRI* gene was found to be silent in all of FXS-iPSCs in contrast to all control cells. Comparison of DNA methylation revealed that the FXS-iPSCs retained the high methylation levels seen in their fibroblasts of origin. Further analysis of histone modifications in both FXS-iPSCs and FXS-ESCs and wild type cells revealed that the FXS-iPSCs are enriched for repressive chromatin markers in contrast to the control cells which are enriched for transcriptionally active chromatin markers. Thus, while the FXS model in human ESCs demonstrated the temporal silencing of *FMRI*, in FXS-iPSCs, *FMRI* was already inactive in the undifferentiated state (Fig. 1.2). FXS-iPSCs are still an important model as they are very useful for the analysis of derived neurons lacking the expression of *FMRI*. Another issue that must be addressed when creating iPSCs from individuals carrying the full mutation

allele is the stability or instability of the CGG repeats. It was observed that during reprogramming, or during the cells expansion following the reprogramming process, the CGG repeats can become unstable and either decrease or increase in length compared to the fibroblasts of origin. This change in repeat length may also lead to changes in the methylation status of the promoter and reactivation of the gene [45]. In the same study [45], two iPSCs subclones derived from the same FXS mosaic donor, one with a permutation allele and the other with a full mutation allele, were tested for their ability to differentiate into neurons. Although both subclones differentiated into neurons, the clone harboring the full mutation allele produced much shorter and fewer dendrites, despite the common genetic background.

Drug Screening in FXS-iPS Cells

Although FXS-iPSCs may not always be a suitable model to study the silencing mechanism of the syndrome, they constitute a unique model system for the study of the disease phenotype in diverse subpopulations of FXS-affected individuals, and for drug screening. A study aimed to evaluate the reactivation of *FMRI* in FXS-iPSCs and their neuronal derivatives through epigenetic modulation drugs showed not only that reactivation is possible but also uncovered additional layers of epigenetic control on *FMRI* [47]. The drug of choice in this study was a demethylating agent called 5-azacytidine (5-azaC), an FDA-approved drug previously shown to reactivate *FMRI* in immortalized FXS lymphoblastoid cell lines [11]. The 5-azaC concentrations used in the above study [47] were in accordance with physiological levels found in plasma of cancer patients treated with the drug. As a result of 5-azaC treatment on FXS-iPSCs, *FMRI* was robustly reactivated with levels of expression ranging between 15 and 45 % of control cells. The ability of 5-azaC to restore *FMRI* expression was further tested on neurons derived from FXS-iPSCs. Like the results observed in FXS-iPSCs, their neuronal derivatives also reactivated *FMRI*. In both the FXS-iPSCs and the derived neurons, reactivation occurred at the translational level, as detected by the presence of FMRP. Expression of the gene persisted at similar levels even after drug withdrawal. In addition to re-expression, epigenetic modifications such as DNA methylation, histone methylation, and acetylation of the *FMRI* locus were also examined after 5-azaC treatment. While DNA methylation of the promoter in untreated cells was very high, the treated iPSCs and neurons showed a marked reduction in DNA methylation. Histone modifications indicative of transcriptionally active genes revealed almost complete restoration of both modifications analyzed compared to the control iPSCs, which express *FMRI*. However, the histone modification indicative of repressed chromatin was not affected by 5-azaC. This observation may explain why expression levels of *FMRI* remained lower than those of the control cells and suggests the existence of additional layers of epigenetic control on the *FMRI* gene. While 5-azaC has a global effect on methylation and is not an ideal drug when it comes to treating infants or children, this study showed the potential in using iPSCs to produce relevant cell

types for drug screening in order to not only reactivate *FMR1* but also broaden our understanding of the epigenetic regulation controlling and underlying the disease.

Due to their availability, iPSCs can now be used to study associated syndromes for which human ESCs are not available, such as Fragile X-associated “Tremor/Ataxia Syndrome” (FXTAS). FXTAS is a neurodegenerative disorder affecting carriers of the permutation allele. Production of iPSCs from affected FXTAS individuals revealed that the permutation allele is enough to impair synaptic density, neurite length, and the overall function of iPSCs-derived affected neurons [48].

Concluding Remarks

Because of the variability in FXS patients, the potential embodied in human ESCs and FXS-iPSCs is vast. Future experiments will try to address open questions concerning FXS such as the basic molecular mechanisms underlying the syndrome and the cause of the CGG expansion during the female meiosis. The main advantage of using iPSCs to study FXS lies in the availability and the ease of generating pluripotent cells and their derivatives from a wide range of affected individuals and carriers. FXS-iPSCs and their derivatives will enable us to broaden our search for new drugs; these will either reactivate *FMR1* itself or its key downstream targets which display aberrant activity in affected cells. In using FXS-iPSCs, there are neither ethical concerns nor obtainable restrictions, allowing us to study all aspects of the syndrome, screen for drugs, examine response variation in the population and better tailor the treatment to each case and individual.

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