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Human pluripotent stem cell models of Fragile X Syndrome

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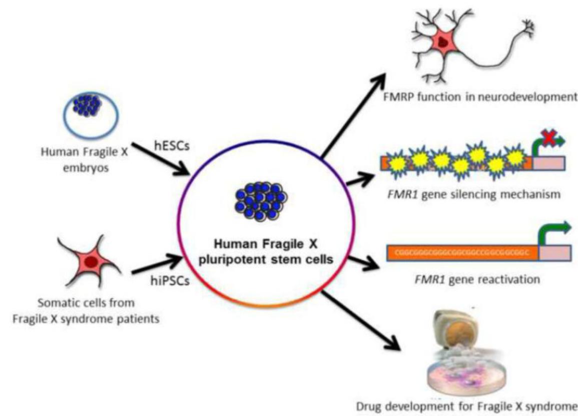
Graphical abstract



I. Introduction

Fragile X syndrome (FXS) is the most common inherited cause of intellectual disability with a prevalence of 1 in 5000 (Coffee et al., 2009). Characteristics include learning deficits and IQ between 20 and 60 as well as hyperactivity, attention deficit disorder, and autistic-like behavior (Hagerman and Hagerman, 2002). At least 25% of individuals with FXS meet the diagnostic criteria for autism (Cohen et al., 1991; Fisch et al., 1986; Hagerman et al., 2005; Hatton et al., 2006; Kaufmann et al., 2004; Lathe, 2009; Reiss et al., 1986). Individuals with FXS also have an increased incidence of seizures and reduced motor coordination. FXS features are not limited to the nervous system and include connective tissue dysplasia, facial dysmorphia, hyperextensible joints, mitral valve prolapse and macro-orchidism.

FXS is caused by a mutation in a single gene, the Fragile X Mental Retardation Gene 1 (*FMR1*), resulting in lack of the Fragile X Mental Retardation Protein (FMRP) (Pieretti et al., 1991; Verkerk et al., 1991). As in many neuropsychiatric disorders, animal models have been useful in studying characteristics and potential mechanisms underlying FXS. FXS

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models have shown that FMRP is an RNA binding protein that binds to specific mRNAs to control their location and protein translation (Eberhart et al., 1996). This function implies that FMRP plays a crucial role in neuronal development, function and synaptic plasticity (Liu-Yesucevitz et al., 2011; Sidorov et al., 2013). The absence of FMRP results in increased protein synthesis, leading to enhanced signaling in a number of intracellular pathways, including the mTOR, mGLuR5, ERK, Gsk3*β*, PI3K, and insulin pathways. Studies in animal models demonstrate that FMRP also plays a crucial role in neuronal development. FMRP deficiency leads to fate switch of neural precursor cells from neuron to glia lineages and increased death of immature neurons, leading to reduced neuronal production (Guo et al., 2011; Guo et al., 2012b; Luo et al., 2010). FMRP-deficient neurons also exhibit impaired morphological development of neuronal dendrites and spines. Data from mouse models has informed our understanding of FXS and several drug trials have been instituted in FXS patients as a direct result of these studies.

Yet, there are several critical reasons why it is necessary to use human cells to define underlying mechanisms that lead to FXS characteristics, particularly those affecting the nervous system. First, the epigenetic silencing of the *FMR1* gene that causes FXS occurs only in human. The causal mutation in FXS is a trinucleotide CGG repeat expansion. When the mutational expansion of the CGG repeats exceeds 200 in humans, it leads to methylation of the repeats and the *FMR1* promoter, chromatin condensation, and a loss of FMRP expression. Mice engineered to mimic the human mutation in the *FMR1* gene do not show methylation and silencing characteristic of the gene in humans (Brouwer et al., 2007). These results indicate that epigenetic mechanisms in human and mice are different and preclude the ability to study epigenetic mechanisms of *FMR1* silencing in mouse models of FXS.

Differences between the formation and structure of the brain in mice and humans also present challenges to understanding the mechanisms of abnormal brain development and function in FXS. The formation of the brain is prolonged in humans, taking months compared to weeks in mice. More importantly, the human brain is more reliant on the role of interneurons and astrocytes and so FXS mouse models may not adequately reveal differences in these particular systems. For example, human interneuron development occurs over a protracted period of time and integrates unique mechanisms to generate more numerous and more elaborate interneurons (Hansen et al., 2013; Hansen et al., 2010; LaMonica et al., 2012; Lui et al., 2011; Marin, 2013; Tyson and Anderson, 2013). Thus, it is important to study the cause and consequences of *FMR1* silencing in neural development and function in the human context.

II. FXS is caused by a human specific mutation

II. A. Epigenetic regulation of gene expression

Epigenetic mechanisms, mediated by DNA methylation, histone modification, and noncoding RNAs are known to play significant roles in regulating stem cells and development as well as adult neuroplasticity (Jobe et al., 2012).

II. A. 1. DNA methylation—DNA methylation is catalyzed by methyl transferases including *de novo* Dnmt3a and Dnmt3b that add methyl groups onto unmethylated DNA and

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Dnmt1 that recognizes hemi-methylated DNA and maintains DNA methylation. A majority of genomic DNA methylation, particularly in the brain, is at cytosine residues in the context of CpG dinucleotide (mCG), with additional methylation at non-CpG sites (mCA, mCT, mCC or collectively called mCH). Genome-wide DNA methylation studies have demonstrated a drastic increase in DNA methylation levels in neurons during postnatal development that coincides with neuronal maturation, suggesting a critical role for DNA methylation during neuronal development. Active DNA demethylation involves multi-step chemical reactions by several groups of proteins and through the production of 5-hydroxymethylation of cytosine (5hmC) (Piccolo and Fisher, 2014).

II. A. 2. Histone modification—Chromatin comprised of nucleosome repeats of 147 base pairs (bp) of DNA sequence wrapped around two copies each of histone proteins, H2A, H2B, H3 and H4 can exist in either highly condensed heterochromatin associated with gene silencing or loosely packed euchromatin associated with gene expression. The amino (N)-terminal tails of core histones are subject to a variety of covalent modifications including acetylation, methylation, ubiquitination, phosphorylation, ribosylation, SUMOylation, etc. The combination of these histone modifications are called "the histone code" and binding of modified histones to specific genomic regions control the activation or repression of the associated genes (Bernstein et al., 2007). Some histone modifications like acetylation of lysine 9 and 14 (Ace-H3) and di- or tri-methylation of lysine 4 (H3K4) are signatures of actively expressed chromatin and referred to as “active histone marks”. Other marks, such as di- or tri-methylation of lysine 9 (H3K9) or lysine 27 (H3K27) on histone H3 are associated with silent chromatin domains and are referred to as “repressive histone marks”. These histone modifications are catalyzed by enzymes such as histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyl transferases (HMTs), and histone demethylases (HdMTs).

II. A. 3. Non-coding RNAs—A large portion of the genome is transcribed into non-protein-coding RNA called noncoding RNA (ncRNA). Among them, the best studied are small ncRNAs including microRNAs. Recently, the involvement of long ncRNAs (lncRNAs) is increasingly recognized as an important aspect of regulation (Jobe et al., 2012).

All three epigenetic mechanisms are, to some extent, involved in *FMR1* gene expression and shutdown. DNA methylation of the expanded CGGs in the *FMR1* gene is the major, if not only, cause of *FMR1* gene inactivation in FXS. Associated with this striking DNA methylation change, histone marks in the *FMR1* gene locus also shift from an active to a repressive state. Several lncRNAs within the *FMR1* gene locus also undergo inactivation, but it is unclear how much they contribute to FXS and whether they are involved in the gene inactivation process. As detailed below, extensive efforts have been devoted to understanding their roles in *FMR1* gene silencing and activation so as to better understand the underlying cause of FXS.

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II. B. *FMR1* Gene Silencing

The causal mutation in FXS is a trinucleotide CGG repeat expansion in the *FMR1* gene. Expansion of the CGG repeats over 200 leads to *FMR1* silencing and is thought to occur about 11 weeks of gestation *in vivo* (Willemsen et al., 2002).

II. B. 1. CGG repeats and *FMR1* silencing—CGG trinucleotide repeats are normally present in the *FMR1* gene of all humans. Population genetics studies have shown that the modal number of CGG repeats is 30, with significant numbers of individuals with repeats both below and above this number (Mailick et al., 2014). In most people with the number of near modal CGG repeat numbers, the CGG repeat usually remains stable through generations. However, due to reasons that are not understood, the CGG repeats sometimes expand through the germ line leading to CGG repeat lengths between 55 to 200, termed pre-mutation. Once considered unaffected, individuals with a pre-mutation *FMR1* gene have more recently been shown to exhibit some mild to moderate pathological presentations termed Fragile X-associated disorders, even though *FMR1* gene transcription is not reduced (Lozano et al., 2014). These disorders include premature ovarian insufficiency (POI) and Fragile X-associated tremor/ataxia syndrome (FXTAS). Mutational expansion of the CGG repeats beyond 200 triggers methylation of the repeats and the *FMR1* promoter, chromatin condensation, and a loss of transcription, resulting in FXS. The mechanism that prompts *FMR1* gene inactivation remains unclear and is an active field of study.

The importance of *FMR1* methylation in gene silencing is illustrated by the existence of individuals who are mosaic for *FMR1* gene methylation. These FXS "methylation mosaics" have an absence of methylation in a subpopulation of cells, reduced methylation in different cells, or absence of methylation in one allele in females (Stoger et al., 2011). As described in Section V, rare males with *FMR1* full-length CGG expansion mutations show no or only mild symptoms because their CGG and *FMR1* gene are unmethylated (Hagerman et al., 1994; Loesch et al., 1993; Loesch et al., 2004; Loesch et al., 2012).

II. B. 2. Genetic and epigenetic signatures of *FMR1*—Extensive studies have compared the genetic and epigenetic signatures of active and repressed *FMR1* genes (Figure 1). DNA footprinting studies have identified four footprints in the *FMR1* gene promoter that correspond to the consensus binding site of four transcription factors, α-PAL/NRF1, Sp1, H4TF1/Sp1-like, and c-myc. These footprints are found in *FMR1* in several different cell types derived from normal individuals but absent in *FMR1* of cells derived from FXS individuals (Drouin et al., 1997; Schwemmle, 1999; Schwemmle et al., 1997). Therefore *FMR1* gene repression is correlated with the absence of transcription factor binding. Drouin et al., also made an interesting observation that the same footprint sites are present in both *FMR1* gene and the gene of huRNP-A2, a ribonucleoprotein. The authors predicted that FMRP might have similar a RNA transport function as huRNA-A2. This function for FMRP was later confirmed by FMRP regulation of activity-dependent RNA transport during neuronal dendritic spine development (Dictenberg et al., 2008).

Using "3C" chromosome conformation analysis of a 170 kb locus encompassing the human *FMR1* gene, Gheldof et al., discovered a significant difference in chromosome conformation

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between *FMR1*-expressing versus non-expressing cells. The *FMR1* gene promoter is at the center of a 50 kb chromosome domain exhibiting less interactions among each other in *FMR1*-expressing cells compared to in FXS cells (Gheldof et al., 2006). These results suggest that silencing of the *FMR1* gene is associated with broader changes at the chromosome level than previously anticipated.

Several studies have compared epigenetic signatures of active versus repressed *FMR1* promoters (Figure 1). Chromatin immunoprecipitation studies have shown that in normal cells with *FMR1* gene transcription, the 5' region of *FMR1* gene is associated with acetylated H3 and H4 that are associated with actively transcribed genes (Coffee et al., 1999). However in FXS cells, there is a significantly reduced level of acetylated H3 or H4 associated of the *FMR1* gene. The levels of acetylated H3 on the *FMR1* promoter are inversely correlated with repeat sizes (Coffee et al., 2002). In addition, H3K4 methylation is decreased in FXS cells whereas H3K9 methylation is increased, consistent with the inactive status of *FMR1* gene in FXS cells. These changes in histone modifications are restricted to *FMR1* promoter regions (Gheldof et al., 2006). Furthermore, the silencing of *FMR1* gene in human embryonic stem cells is associated with loss of active chromatin markers including H3K4me2 and gain of H3K9me3 (Avitzour et al., 2014). However, studies of full mutation males with an unmethylated *FMR1* promoter show increase in deacetylated H3 and H4 and methylated H3K9 (Pietrobono et al., 2005; Tabolacci et al., 2008b) suggesting that histone modification might be independent of DNA methylation in *FMR1* gene shutdown. The seemingly contradictory messages from these studies suggest increasingly complex mechanisms regulate *FMR1* gene expression. To that point, FMRP has been shown to interact with microRNAs (Liu et al., 2014) and the human *FMR1* gene locus encodes several lncRNAs (Pastori et al., 2014; Peschansky et al., 2015). Further other genes such as *FAM 11A* (Shaw et al., 2002) and several long noncoding RNAs such as antisense *FMR1* (AS*FMR1*) (Ladd et al., 2007) and FMR6 (Pastori et al., 2014) in the locus are also methylated and silenced in addition to *FMR1*.

II. B. 3. Potential mechanisms of *FMR1* silencing—Several potential mechanisms underlying *FMR1* gene inactivation have been proposed and investigated.

A DNA methylation boundary was discovered upstream of the *FMR1* gene at about 685 to 800 nucleotides from the CGG repeats (Naumann et al., 2009) and several nuclear proteins, including insulator protein CTCF, bind to this region. Binding of CTCF to this region is lost in FXS cells with *FMR1* silenced (Ladd et al., 2007). A later report (Lanni et al., 2013) confirmed that CTCF binding is needed to prevent *FMR1* gene silencing, but not to prevent general DNA methylation. The authors speculate that CTCF may function by modulating chromosome conformation. Another study using human embryonic stem cells suggests that changes in histone markers precede DNA methylation changes (Eiges et al., 2007). Further, CGG containing *FMR1* mRNA was found to inhibit its own expression. Demethylation of the *FMR1* promoter leads to increased repressive chromatin marker H3K27 methylation binding to the promoter that is dependent on the presence of mutant mRNA (Kumari and Usdin, 2014). Recently, *FMR1* silencing was shown to be mediated by *FMR1* mRNA containing long CGG repeats (Colak et al., 2014). Therefore, several mechanisms may be at play in silencing *FMR1* expression.

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Recapitulation of the *FMR1* silencing mutation has been unsuccessful in animal models, particularly mouse, because epigenetic silencing does not occur in the same way. Therefore, it is necessary to use human cells to define the mechanisms of *FMR1* silencing.

III. Human pluripotent stem cells as a model to study FXS

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and particularly induced PSCs (hiPSCs), offer a model system to reveal cellular and molecular events underlying normal and abnormal neural development. hESCs are isolated from preimplantation embryos and retain the two characteristics unique to stem cells: self-renewal and pluripotency (Thomson et al., 1998). hiPSCs are reprogrammed from somatic cells by forced expression of stem cell genes and have the characteristics of hESCs (Takahashi et al., 2007; Yu et al., 2007). Patient-derived hiPSCs provide a paradigm to understand neurological disease pathogenesis, including FXS, in the human genetic background.

While providing an unparalleled tool for the study of early human brain development, there are multiple factors in hPSC studies that introduce variability and affect the ability to compare data from multiple studies. Variability can be introduced through patient differences, iPSC reprogramming methods, and neuronal differentiation paradigms. Many of these problems can be overcome by using cells from enough different individuals to enable statistically meaningful results. Alternatively, either engineered or spontaneously-generated isogenic cell lines can provide a more practical alternative to limit genetic diversity.

III. A. Embryonic Stem Cells

FXS hESCs were first isolated and reported from preimplantation embryos carrying the FXS mutation by Verlinsky in 2005 (Verlinsky et al., 2005). The heritability of FXS enables the identification of affected embryos through the use of preimplantation genetic diagnosis during the *in vitro* fertilization process. hESCs can be isolated from the inner cell mass (ICM) of these embryos for research purposes (Ben-Yosef et al., 2008; Kuliev et al., 2005; Pickering et al., 2003; Stephenson et al., 2009). Since this initial report, other FXS hESCs have been published (Eiges et al., 2007; Gerhardt et al., 2014). Yet only a very few FXS hESC lines are approved and listed on the NIH Human Embryonic Stem Cell Registry, thereby hindering the use of these cells by NIH-funded researchers.

Eiges et al., provided the first detailed characterization of a single human FXS hESC line (Eiges et al., 2007). The data confirmed that the cells derived from a FXS preimplantation embryo met the criteria for hESCs and that the cells retained the full length mutation of the *FMR1* gene. Surprisingly, however, the *FMR1* gene was unmethylated and expressed in these cells. Epigenetic silencing did not occur until these cells were differentiated and thus provided a new paradigm in which to study the mechanisms of *FMR1* silencing (Figure 2). More recent evidence from multiple FXS hESC lines, however, suggests that the epigenetic silencing in FXS hESCs may occur more easily than initially observed (Avitzour et al., 2014). These results also suggest that the initial embryonic ICM cells may have different methylation of the *FMR1* gene at the time of stem cell derivation. Therefore, epigenetic silencing can occur in the undifferentiated state. As more human FXS hESCs are reported and characterized, the prevalence of this phenomenon will be revealed.

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III. B. Induced pluripotent stem cells

The ability to generate hiPSCs from somatic cells of FXS individuals has enabled the generation of FXS hiPSC lines from patient fibroblasts (Bar-Nur et al., 2012; Brick et al., 2014; de Esch et al., 2014; Doers et al., 2014; Halevy et al., 2015; Kaufmann et al., 2015). Without exception, the methylated, silenced *FMR1* mutation in the patient fibroblasts is retained through the reprogramming process (Urbach et al., 2010). Reprogramming also causes a rare unmethylated full mutation in patient somatic cells to be silenced (de Esch et al., 2014). Therefore, it is not yet possible to isolate full mutation FXS hiPSCs that have the unsilenced gene (Figure 2). These cells are useful to test mechanisms of epigenetic reactivation. These cells are also valuable for studying the effect of *FMR1* loss as cells differentiate from the undifferentiated state.

IV. Neural differentiation of FXS hPSCs

Most of what we know about the role of FMRP in neural development comes from FXS mouse models. FMRP is an RNA binding protein that binds to specific mRNAs to control the location and protein translation of these mRNAs (Eberhart et al., 1996). FMRP plays a crucial role in neuronal development, function and synaptic plasticity (Liu-Yesucevitz et al., 2011; Sidorov et al., 2013). How the loss of FMRP manifests in the human nervous system is unknown. Although higher density but immature long and thin neuronal dendritic spines are consistently found in FXS postmortem brains (Hinton et al., 1991; Irwin et al., 2001; Wisniewski et al., 1991), the underlying mechanisms of this phenotype is not well defined. Mouse models have revealed that neural cells that lack FMRP exhibit neurogenesis and neuronal maturation deficits (Li and Zhao, 2014). Further, altered synaptic plasticity has been established in the FXS mouse model and is thought to be due to the lack of FMRP's role as a negative regulator of translation (Liu-Yesucevitz et al., 2011). It is important to define whether human FXS neural cells have similar deficits so that therapeutics to affect neural development and function in FXS can be more intelligently designed.

IV. A. Neural differentiation from hPSCs

To take advantage of the power of hPSCs to model human brain development and to define the steps that go awry in FXS, it is critical to differentiate hPSCs into the specific neural subtypes that are affected in FXS (Kim et al., 2014a). This likely includes all neural subtypes, but hPSC neural research in general is focused on cortical excitatory neurons and astrocytes, with emerging work on interneurons and other glial subtypes. The differentiation of neural cells from hPSCs was pioneered by Su-Chun Zhang beginning with the initial report of neuron differentiation from hESCs (Zhang et al., 2001). The Zhang lab and others developed core methods for the generation of dorsal forebrain derived (cortical-like) neurons (Chambers et al., 2009; Eiraku et al., 2008; Pankratz et al., 2007; Watanabe et al., 2005). These methods are effective for hiPSCs as well although there is variable efficiency between different hiPSC lines that can affect the interpretation of results (Hu et al., 2010). Neurons generated through these and similar methods neurons have characteristics that correspond primarily to excitatory projection neurons in the cortex (Hansen et al., 2011; Mariani et al., 2012; Shi et al., 2012). Therefore they are of value in the study of FXS, where dendritic spines of excitatory neurons are altered. Other neuronal subtypes as well as astrocytes

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(Emdad et al., 2012; Krencik et al., 2011; Ruiz et al., 2010; Shaltouki et al., 2013) and oligodendrocytes (Czepiel et al., 2011; Hu et al., 2009; Liu et al., 2011; Pouya et al., 2011; Sharp et al., 2011) can be differentiated from hPSCs. More recently, the directed differentiation of interneurons from hPSCs has been accomplished (Kim et al., 2014b; Liu et al., 2013; Maroof et al., 2013; Nicholas et al., 2013), thus providing the ability to generate a range of human neural subtypes for disease modeling.

FMRP is expressed in all neural cells (Bakker et al., 2000; Devys et al., 1993; Willemsen et al., 2004), so the characterization of many cells would be valuable to better understand FXS. While the focus of FXS neurobiology has been on excitatory neurons, emerging evidence suggests that inhibitory neurons are also dysfunctional in FXS (Cea-Del Rio and Huntsman, 2014). Astrocytes express FMRP and evidence from mouse suggests that the lack of FMRP in astrocytes may be detrimental (Jacobs et al., 2012; Jacobs et al., 2010; Pacey and Doering, 2007). Recent human neuroimaging studies suggest that individuals with FXS have white matter defects that may link development and function of oligodendrocytes to FXS neuropathology (Green et al., 2015; Villalon-Reina et al., 2013)

IV. B. Neural differentiation from FXS hPSCs

Despite the establishment of FXS hESCs and hiPSCs, there is relatively limited data on the development and phenotypic characterization of human FXS neurons derived from PSCs and no reports of glial cells specifically differentiated from FXS hPSCs.

The most comprehensive description of the neural development from FXS PSCs comes from hESCs by Dalit Ben-Yosef's lab, the first to characterize FXS hESC-derived neural cells (Telias et al., 2013). The authors differentiated three FXS hESC lines into neurons and assessed neuronal development and function. The differentiation paradigm was fairly standard, although the subtype of neurons generated was neither defined nor optimized and may have been a ventrally derived neuronal subtype given the addition of sonic hedgehog (SHH), a ventralizing morphogen. The study showed that the FXS hESCs expressed less *FMR1* as they differentiated, consistent with the previous report (Eiges et al., 2007). FXS hESCs, when differentiated, had reduced expression of neural induction genes and a delay in neurogenesis, although neurons could be generated. The neurons had deficits in neuronal maturation as evidenced by decreased neuronal gene expression, increased progenitor gene expression and immature electrophysiological properties. The authors conclude from their study that FXS hESCs can successfully differentiate into neurons *in vitro* and *FMR1* silencing mimics that during embryogenesis.

Only limited studies to date have addressed the neuronal development of human neurons derived from FXS iPSCs (Doers et al., 2014; Halevy et al., 2015; Sheridan et al., 2011). Similar to results from FXS hESCs, dorsal forebrain neurons can be generated from FXS hiPSCs, although progenitor characteristics were not well-defined in any study. The gene expression patterns of hiPSC-derived FXS neurons suggest defects in neuronal differentiation (Sheridan et al., 2011) and maturation (Halevy et al., 2015) similar to what have been shown in mouse models (Guo et al., 2011; Guo et al., 2012a; Guo et al., 2015). We have found that hiPSC-derived FXS neurons exhibit defective neurite outgrowth (Doers et al., 2014). A recent study using a novel micro-raft culture method, showed that FXS

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hiPSC-derived neurons exhibit reduced pre-synaptic vesicle recycling (Niedringhaus et al., 2015), recapitulating what has been shown in mouse models (Deng et al., 2011).

Therefore, the limited studies using human FXS hESCs and hiPSCs suggest that FMRP is important for neural precursor differentiation and neuronal maturation. Further experiments are acutely needed on more human hPSCs lines to delineate: 1) specific phenotypes in neural progenitors (e.g. cell cycle abnormalities, cell death); 2) phenotypes in differentiation (e.g. developmental delay, fate switch); 3) phenotypes affecting synaptic development and synaptic plasticity; and 4) electrophysiological properties of mature human FXS neurons. The achievement of these goals will enable critical studies to dissect the mechanisms of FMRP's actions in human cortical neurons.

V. Reactivation of the *FMR1* gene in hPSCs

V. A. Rationale for *FMR1* gene restoration as a potential therapy

Since the coding sequence of the silenced *FMR1* gene is normal, a possible therapeutic strategy is to restore the transcription of *FMR1* in FXS (Figure 1). In fact, there are now several reports of males with *FMR1* full-length CGG expansion mutations who show no or mild symptoms because their *FMR1* genes are unmethylated (Hagerman et al., 1994; Loesch et al., 1993; Loesch et al., 2004; Loesch et al., 2012; Pietrobono et al., 2005; Smeets et al., 1995; Tabolacci et al., 2008b). Therefore an unmethylated *FMR1* gene carries out normal functions resulting in near normal intelligence instead of intellectual disability associated with FXS.

The mouse genetics studies shed more hope of this notion. We have discovered that restoring *FMR1* in adult-born new neurons using inducible genetics restore several adult neurogenesis-dependent learning and memory in mice (Guo et al., 2011). These data suggest that the neuronal developmental deficits seen in FMRP-deficient neurons might be reversible.

V. B. *FMR1* gene restoration strategies

The identification of effective methods to reactivate the *FMR1* gene and restore FMRP expression has been extremely challenging. A number of studies have shown that treatment of human FXS lymphoblastoid cell lines with a DNA methyltransferase (DNMT) inhibitor 5-azacytidine (5azaC) or 5-azadeoxycytidine (5azadC) results in partial reactivation of the *FMR1* gene and FMRP expression. Upon treatment, the *FMR1* promoter becomes passively unmethylated through cell division (Chiurazzi et al., 1998; Pietrobono et al., 2002). Importantly, the increase in *FMR1* mRNA production is associated with increased active chromatin marker binding and decreased repressive chromatin marker binding to the *FMR1* promoter (Brendel et al., 2013; Kumari and Usdin, 2014; Pietrobono et al., 2002; Tabolacci et al., 2008a) (Figure 1). In contrast, methotrexate, a folate antagonist that acts by inhibiting dihydrofolate reductase (DHFR) and has some DNA methylation inhibition activity does not reduce DNA methylation in the *FMR1* promoter. DHFR does lead to some mRNA but not protein expression (Brendel et al., 2013). These results suggest that reversing DNA methylation of CGG repeats might be a promising method for gene restoration therapy.

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Chemicals affecting histone modification have also been explored for *FMR1* reactivation. Most studies have so far focused on Class I, II, and IV HDAC inhibitors include butyrate and trichostatin A (TSA). Using human FXS lymphoblastoid cell lines, Chiurazzi et al., have shown that treatment with HDAC inhibitors, phenylbutyrate, sodium butyrate and TSA, leads to moderately increased *FMR1* gene transcription, yet less than compared to the effect of 5azaC or 5-azadC (Chiurazzi et al., 1999). However other studies show no *FMR1* transcription after TSA treatment (Coffee et al., 2002; Coffee et al., 1999) or VPA treatment (Tabolacci et al., 2008a). Interestingly, combined treatment with 5azaC and HDAC inhibitor leads to 2–5 fold higher reactivation compared to 5azaC treatment alone (Chiurazzi et al., 1999). Recently, more effective reactivation, comparable to that by 5azaC, has been achieved by using splitomicin (SPT), an inhibitor of SIRT1 a class III HDAC (Biacsi et al., 2008; Kumari and Usdin, 2014). Knockdown of SIRT1 in either lymphoblastoid cell lines or FXS patient-derived fibroblasts leads to increased deacetylation of H4K16 and increased *FMR1* gene transcription without significantly affecting DNA methylation. This study suggests that inhibition of certain key HDACs may be able to reactivate the *FMR1* gene without altering DNA methylation.

The limited success of using known epigenetic reagents to reactivate *FMR1* genes has prompted studies to explore novel chemical reagents and molecules to reactivate the *FMR1* gene. A major challenge is to establish a screening method that can effectively and efficiently report *FMR1* gene expression. Several reports of screening technologies have been recently published. In one study, FXS hiPSCs were differentiated into neural progenitor cells (NPCs), immunostained with an FMRP antibody, and analyzed by high content imaging for FMRP levels (Kaufmann et al., 2015). Using this system 50,000 compounds covering epigenetic targets and known FMRP regulated pathways were screened and several compounds (identity not revealed) that induced weak reactivation were identified (Kaufmann et al., 2015). In another screen, dual FMRP antibodies were used to establish a time-resolved fluorescence resonance energy transfer (TR-FRET) dual antibody assay to increase specificity. Human iPSC-derived NPCs were used to screen ~5000 compounds including a FDA-approved drug library. Six hits were identified that enhanced *FMR1* gene transcription modestly, although no significant FMRP was detected (Kumari et al., 2015). Interestingly, one of the identified compounds is SB216763 that we have previously found to rescue learning deficits in *FMR1*-null mice through enhancing Wnt signaling (Guo et al., 2012a). Yet, none of the compounds identified so far can reactivate *FMR1* expression to near normal levels, necessitating new and better strategies.

The discovery and rapid development of gene editing technology have opened new avenue for gene correction-based therapies. The Zinc Finger Protease (ZFN) and Transcription activator-like effector nucleases (TALENs) methods, though promising, were difficult to use and had relatively low specificity (Hsu et al., 2014). Nevertheless, proof of concept experiments have shown that TALEN can be used to correct AT-rich repeats in FATS a common fragile site in mice (Ma et al., 2014). The newest CRISPR/Cas9-based gene editing method is significantly easier to use and exhibits much higher specificity (Zhang et al., 2014). Recently, Park et al used CRISPR/Cas9 to delete the CGG repeats in the silenced *FMR1* gene in FXS hiPSCs and demonstrated activation of *FMR1* gene (Park et al., 2015). Although the efficiency of this deletion is extremely low and the deletion is not restricted to

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the CGG repeat, the results of this work suggest that deletion of silenced CGG repeat might be a promising gene reactivation strategy for FXS. In addition to deletion of CGG, Cas9 can also be used to deliver a transcriptional activator (e.g.VP64) to specific silenced genes to reactivate them (Scott et al., 2014). With the fast advancement in gene editing and gene therapy, reactivation of *FMR1* using genetic methods may become feasible in the near future.

VI. Future Directions

Although much work has been done using FXS mouse models, how FMRP regulates human neurogenesis and neuronal development remains unclear. In addition, the *FMR1* gene inactivation and reactivation studies have not been successfully translated *in vivo*. These hurdles are due to several reasons. First and foremost, the most-widely used mouse model of FXS has a knockout of the *FMR1* gene rather than a repeat expansion in the gene, rendering it useless for studies of reactivation. Additionally, mice engineered with expanded repeats in the *FMR1* gene do not show methylation and silencing characteristic of the gene in humans (Brouwer et al., 2007; Ludwig et al., 2014). Further, some of the demethylating agents can be toxic and need to be targeted to the brain where FMRP expression is most relevant. Lastly, DNMT inhibitors inhibit DNA methylation of newly synthesized DNA during cell division; therefore their effect on post-mitotic neurons is unclear. Therefore an *in vivo* model that recapitulates the pathology of human FXS neurodevelopment will be necessary for testing new therapeutic reactivation strategies. In addition, gene-specific reactivation of *FMR1* using novel gene editing methods may provide good alternatives for chemical-based reactivation. However delivery of these genetic reagents to human brains presents major hurdles clinically. Lastly, the questions remain whether certain pathways and functions can be restored after the critical period. Future research in these areas will shed light on both basic mechanisms and therapeutic potentials for FXS.

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Highlights

- Fragile X syndrome (FXS) is characterized by intellectual disability and autism.
- Human specific epigenetic silencing of the FMR1 gene causes FXS.
- Human pluripotent stem cells are a model to study human neural development in FXS.
- Human FXS pluripotent stem cells provide insight into FMR1 epigenetic silencing.

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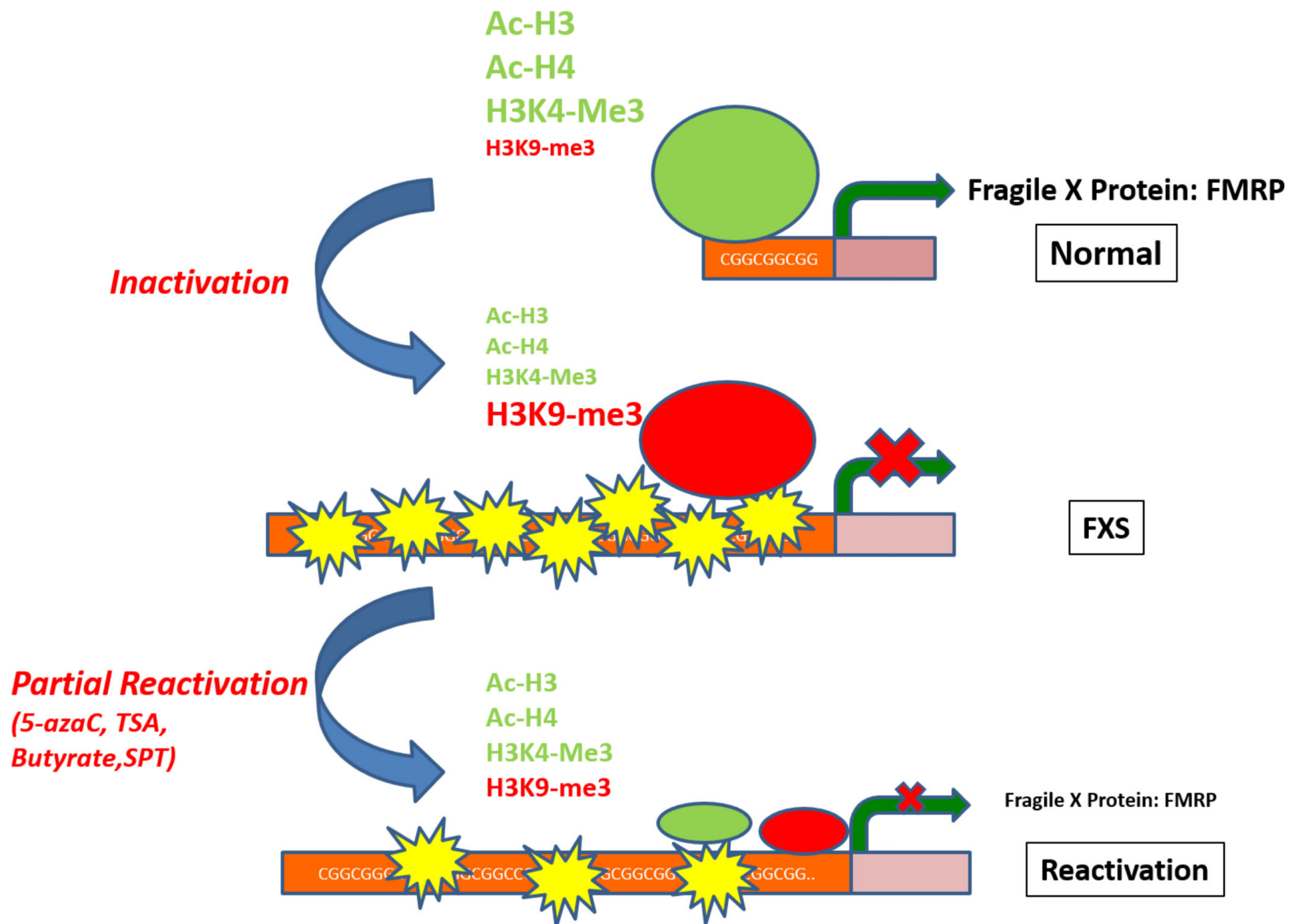

Figure 1. Epigenetic profiles of the human *FMR1* gene

The *FMR1* gene promoter in normal cells is unmethylated and is enriched with active chromatin markers such as acetylated Histone H3 (Ac-H3), acetylated Histone H4 (Ac-H4), trimethylated H3 at lysine 4 (H3K4-me3) but low in inhibitory chromatin markers such as trimethylated histone H3 at lysine 9 (H3K9-me3). The *FMR1* gene promoter in cells from FXS patients is methylated and is enriched with inactive chromatin marker such as H3K9-me3 but low in active chromatin markers. Treatment with DNA methyltransferase inhibitors such as 5azaC or HDAC inhibitors such as trichostatin A (TSA), butyrate or splitomicin (SPT) can partially change the histone signature and reactivate the *FMR1* gene.

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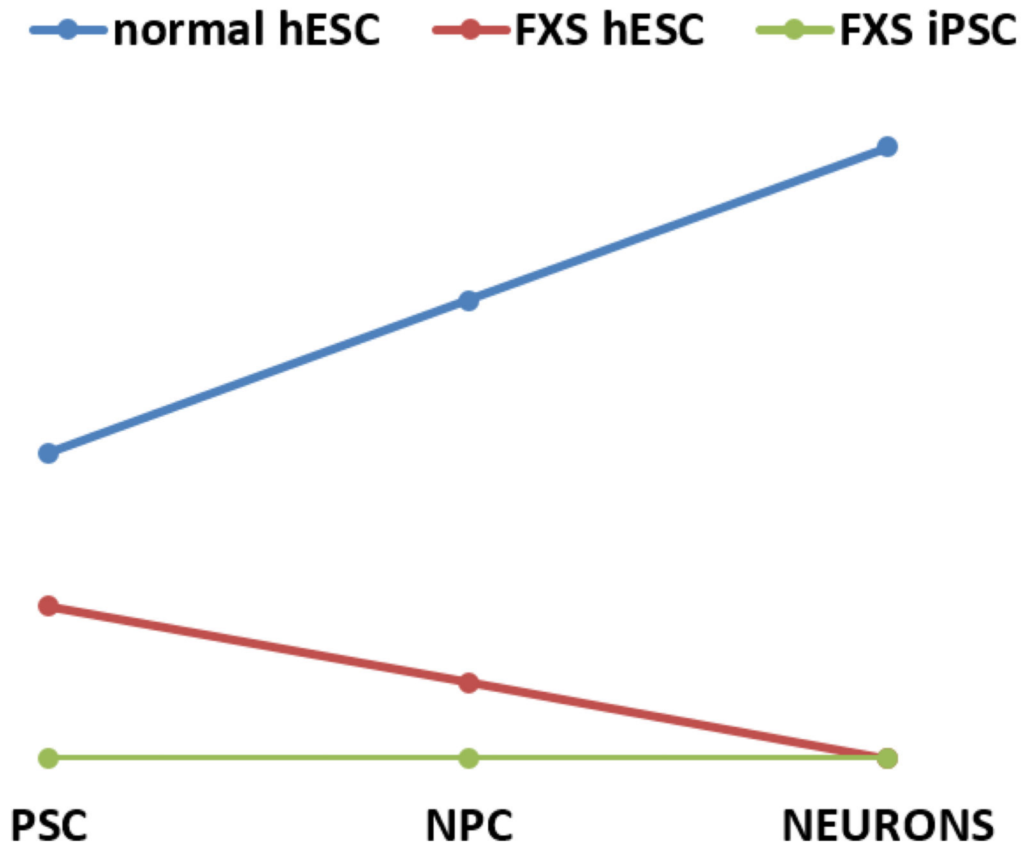

Figure 2. Neuronal differentiation-dependent *FMR1* gene inactivation

Graph depicts the *FMR1* gene expression in hPSCs during neuronal differentiation. The *FMR1* gene is expressed in normal hESCs and its expression levels increase during neuronal differentiation (blue line). *FMR1* is expressed in FXS hESCs but at lower levels than normal due to partial inactivation and is gradually silenced during differentiation (red line). The *FMR1* gene is silenced in FXS hiPSCs and throughout neural differentiation. (PSC=pluripotent stem cells, NPC=neural progenitor cells).