CHAPTER 6

DISCUSSION

In the present study an inducible transgenic Tet-on system was taken advantage to express a transgene which causes FXTAS disease, the expanded premutation CGG. The transgene was bound to a GFP reporter to help the visualization of transgene expression. The induction of the transgene was under control of dox, which activates the transgene through the activation of the tetracycline-responsive element (30). Furthermore, by using specific driver promoters with the reverse-tetracycline transactivator, the expression of transgene can be focused to specific areas. In this study several driver promoters were used, the driver promoter hnRNP-rtTA which expresses the transgene ubiquitously in all tissues (62), GFA2-rtTA which is restricted in astrocytes and Bergmann glia (32), and PrP-rtTA which expresses in all cell types in the brain except for the Purkinje cells (31).

Expression of expanded premutation CGG as RNA was proven to be toxic and lethal when it was expressed in all tissues as shown in Tet-on-99CGG-eGFP/hnRNP-rtTA mice after dox treatment. Our Tet-on-99CGG-eGFP/GFA2-rtTA transgenic mice did not show any expression of the transgene. It does not mean the system is not feasible; it might be due to either an absence or a very low of expression because of an unknown mechanism. A failure in generating Tet-on-11CGG-eGFP line has happened since it exhibited loss of expression after several

breedings. In the transgenic line Tet-on-99CGG-eGFP/PrP-rtTA, the expression of transgene was in a mosaic pattern, especially in hippocampus, cerebellum, striatum, as well as in the kidney which was considered as a leakage. The expression of expanded premutation CGG transcript in Tet-on-99CGG-eGFP/PrP-rtTA mice produced the same neuropathological effect as seen in FXTAS, the ubiquitin-positive intranuclear inclusions. The ubiquitin-positive inclusions were found in the area where 99CGG-GFP expression was observed. The ubiquitin-positive inclusions were detected after 12 weeks of trangene expression by dox in drinking treatment. The number increased when dox induction was lengthened till 16 weeks, while expression of the transgene for only 2 and 4 weeks did not seem to be enough to create the ubiquitin-positive inclusions.

6.1. Leakage of Tet-on-99CGG-eGFP/PrP-rtTA

In Tet-on-99CGG-eGFP/PrP-rtTA mice, there was leakage in the kidney. This finding was similar with another previous study using PrP driver promoter which documented leakages in kidney and heart (31). It seems leakage in kidney occurs commonly in transgene using PrP driver promoter. We are convinced that the leakage occurs in the driver promoter PrP-rtTA, not in the Tet promoter since we did not find GFP expression in the bigenic mice Tet-on-99CGG-eGFP/PrP-rtTA without dox treatment as well as in the monogenic mice Tet-on-99CGG-eGFP with/without dox. It is important to concern about the leakage since the expression of expanded RNA is considered to be toxic and can cause death. As we know from Tet-on-99CGG-eGFP/hnRNP-rtTA mice, that they died after 5 days of

toxic RNA expression in many tissues. It was fortunate to have these PrP mice to look normal even after 16 weeks of expanded RNA leakage expression in the kidney. This leakage can be a clue that the Tet-on-99CGG-eGFP/hnRNP-rtTA mice died were not due to the expression in the kidney. Expression in other vital tissues such as liver, heart and intestine might cause the death in these mice, or the combination of toxic RNA expression in several tissues could also be possible as the cause of the death. It might be necessary to conduct an apoptosis assay on several tissues of Tet-on-99CGG-eGFP/hnRNP-rtTA mice to get closer to the answer of the cause of the death.

6.2. Toxic RNA and inclusions

Bigenic mice Tet-on-99CGG-eGFP/PrP-rtTA exhibited the presence of ubiquitin-positive intranuclear inclusion after 12 weeks toxic RNA expression. The number of inclusions increased with longer dox induction, 16 weeks. It differs with the CGG KI mice which start to show the presence of inclusion at the age of 30 weeks. Another transgenic mouse model of FXTAS in which the expression is restricted in Purkinje cells, displays the ubiquitin-positive inclusion at the age of 8 weeks (61). Studies using *drosophila melanogaster* flies reported that high expression of 60CGG RNA can induce neurodegenerative phenotype similarly with lower expression of 90CGG, indicating either CGG length or expression level determine the toxicity (60).

The inclusions might not only contain ubiquitin, but also other proteins as well as the expanded RNA as seen in inclusions of other mouse models. In the CGG KI mice and L7/Pcp2 transgenic mouse, the inclusions contain 20S proteasome, Hsp40, and Rad23B (29). Recently Sam68 was also detected in the inclusion of CGG KI mice (51). Those proteins can also be present in our inducible transgenic mice. Further analysis is required to reveal the contents of the inclusions. It is very likely that the contents of inclusions in every mouse model of FXTAS are similar. But there will be many differences in the compositions of inclusions when we compare the murine inclusions with human FXTAS inclusions. It has been shown in CGG KI mice which do not contain Pur-α, hnRNP A2/B1, α-β crystalline, and lamin A/C within the inclusions (52), while they are found in human FXTAS inclusions. The difference in composition could be caused by the difference of the disease progression between those two species, or it is possible that random sequestration to the proteins takes place.

The presence of ubiquitin, 20S core complex, and Hsp40 in the inclusions indicates the role of proteasome degradation pathway in the cause of tremor/ataxia syndrome. Ubiquitin proteasome pathway is also affected in other trinucleotide repeat and ataxia disorders, such as Huntington's disease, SCA (spinocerebellar ataxia) type 1, SCA type 3, SCA type 7 and oculopharyngeal muscular dystrophy (63-68). Studies of polyglutamine disorders showed that the disruption of the ubiquitin-proteasome degradation pathway were able to cause dysregulation of important genes, leading to neuronal cell death (69, 70). It has been shown that Sam68 was also sequestered within the inclusions in human and mouse FXTAS

(51). The trapped Sam68 in the inclusions causes the depletion of Sam68 in cells expressing expanded premutation CGG. Sam68 itself plays a role in mRNA metabolism such as alternative splicing regulation, nuclear export, somatodendritic transport, polyadenylation and translation (71). In FXTAS patients, alteration of Sam68-regulated SMN2 splicing was found; however the correlation between the splicing alteration and FXTAS outcome remains unclear (51).

6.3. Reversibility study

Inducible transgenic mice can be used to answer some questions regarding to FXTAS disease. One question comes to reveal whether cessation of toxic expanded premutation CGG RNA expression can ameliorate the condition of FXTAS patients. Using inducible transgenic mice, studies on SCA type 1 have answered the same question since this disease is also developed by a gain of function mechanism as well as the presence of inclusions. After cessation of transgene expression, mutant Ataxin-1, many disease outcomes such as the histological and behavioral were recovered (72). The inducible transgenic system is a powerful technique to answer the reversibility question. This system allows us to freely either induce or stop the target gene expression. Thus this system is suitable for reversibility study in FXTAS. The neurobehavioral and histological pathology will be evaluated upon the cessation of the toxic expanded premutation CGG RNA. Once it is proven to be reversible, then an effort to interfere the RNA expression will be established to reduce the toxicity of elevated Fmr1 RNA by

therapeutic compounds such as antisense DNA oligomers (ASO) and inhibitory RNAs (RNAi).

The histological pathology hallmark for FXTAS is the ubiquitin-positive intranuclear inclusion (14, 19). This hallmark would be used as the reversibility parameter. However ubiquitin are not the only marker that can be used for the reversibility parameter since other proteins might also be revealed to be present in the inclusions. The reversibility is expected to result in the disappearance of proteins as well as the expanded RNA aggregates within the inclusions. Immunohistochemistry using antibody against the possible protein sequestered in the inclusions might complete the reversibility assessment. FISH technique might also be useful to examine the disappearance of RNA aggregates after toxic RNA cessation. This study is now in the step where ubiquitin inclusions have succeeded to be produced, and knowing the timing of inducement for ubiquitin formation. The next step is to stop the expression by cessation of dox treatment, and wait for some time to remove the expression, and then observe whether the ubiqutin inclusion would also disappear. Study using cell expressing expanded premutation CGG has given a clue that a drug treatment can disperse the aggregates in the inclusions (51).

6.4. Astrocytes and glutamate excitotoxicity involvement for disease progression

Astrocytes are important for glutamate uptake, which eliminates glutamate from synapses. A decrease in astrocytes can cause toxic accumulation of

extracellular glutamate. Furthermore, glial cells are essential for the development and maintenance of synaptic networks by releasing trophic factors (73). Excess of glutamate resulting glutamate toxicity is suspected to play a role in pathophysiology of neurodegenerative diseases, such as dementia, Parkinson's, Alzheimer's, and Huntington's diseases, and amyotrophic lateral sclerosis (73, 74). The abnormality of astrocytic glutamate transporters causes increased toxic of glutamate in amyotrophic lateral sclerosis (75). SCA7 transgenic mice which express toxic mutant ataxin-7 in Bergmann glia show a marked impairment in GLAST-mediated glutamate transport, and resulting in neurological phenotype (76). Other cerebellar disorders such as SCA5 and Huntington's disease also reveal the abnormalities of glutamate transporters (77, 78).

Observation of the FXTAS human brain showed the presence of ubiquitin-positive inclusions in both neurons and astrocytes. The astrocytes demonstrated hypertrophic changes which were abnormal in sub-cortical white matter (79). In KI mice which is a very good model for FXTAS, there is no or lack of ubiquitin-positive inclusions as well as morphological abnormalities in astrocytes, while humans FXTAS do demonstrate them. Although these mice showed FXTAS neurological features such as ubiquitin-positive inclusions in neurons, deficits in motor, learning, and memory; these mice showed lack of significant degenerative neuropathology compared to human. The neurological outcomes are relatively mild compared with the human situation, and some neurodegenerative features seen in human such as Purkinje cell drop-out, ubiquitin-positive inclusions in astrocytes, significant neurodegenerative phenotype, are absent in these mice (29).

The absence of ubiquitin-positive inclusions in astrocytes of the KI mice is likely because of the lack expression of *Fmr1* in the astrocytes. This absence could indicate the importance of astrocytes involvement in FXTAS pathology. Exposure of toxic RNA in astrocytes which possibly cause reduced astrocytic glutamate uptake resulting in glutamate toxicity might be important for producing severe FXTAS outcome.

Glutamate toxicity is proposed to be able to produce severe outcome of neurological disease particularly FXTAS because glutamate toxicity might produce the excitotoxicity effect to Purkinje cells which can lead to non-cell autonomous Purkinje cell dropout. It has been shown that expression of toxic mutant ataxin-7 using driver promoter GFA2 which expressed only in the Bergmann glia of mice can cause an impairment of glutamate uptake through a reduction in GLAST expression, leading to Purkinje cells degeneration (76). In human FXTAS, the ubiquitin-positive inclusions were not found in Purkinje cells; however they displayed Purkinje cell loss/dropout (14). A non-cell-autonomous mechanism is considered to be the possible cause of this finding. This mechanism might also underlie the Purkinje cell dropout in transgenic SCA7 mice with PrP driver promoter. Expression of mutant ataxin-7 in the neuronal and non-neuronal cells throughout the brain except for the Purkinje cells, leads to neurodegeneration of Purkinje cells in the transgenic SCA7 mice (76).

Purkinje cells itself are important for motor movement coordination. One study showed that L7/Pcp2 FXTAS transgenic mice, which expressed toxic expanded premutation CGG only in Purkinje cells, developed ubiquitin-positive

intranuclear inclusions in the Purkinje cells, Purkinje cell dropout and behavioral anomalies such as a decline in neuromotor learning abilities (61). The Purkinje cell dropout was not present in CGG KI mice (29). It is likely the Purkinje cell dropout is necessary to produce a more severe form of neuropathology in FXTAS. The absence of inclusions in astrocytes and Bergmann glia in CGG KI mice, and the phenomenon of non-cell-autonomous Purkinje cell degeneration, suggest that neurotoxicity in astrocytes and Bergmann glia might be crucial to generate excitotoxicity to the Purkinje cells which drives to Purkinje cells degeneration, hence exhibiting severe outcome of FXTAS.

The phenomenon as mentioned above drives to a question whether ubiquitin-positive inclusions in both neurons and astrocytes (as seen in human) are necessary to produce complete FXTAS pathology. Using inducible transgenic mice with PrP-rtTA driver promoter, a clue regarding to this question is getting closer. It is possible because the transgenic mice with PrP-rtTA promoter will express the toxic RNA in neuron and astrocytes in the brain except for the Purkinje cells (31). TheTet-on-99CGG-eGFP/PrP-rtTA mice have shown the restriction of the transgene expression in the brain, although a leakage in kidney was seen. The expression was observed mainly in hippocampus, cerebellum and striatum. The expression of expanded RNA transgene for 12 weeks in these mice was able to produce ubiquitin-positive intranuclear inclusions in the areas where transgene expression was found. These mice will be used for further studies to assess the clinical and neuropathological correlate to FXTAS. We hypothesize the

clinical and neuropathological of this mouse model will mimic the human situation, showing severe form of FXTAS.

Another question regarding to the role of astrocytes is whether expression of toxic RNA only in astrocytes and Bergmann glia excluding the neurons is sufficient to produce FXTAS disease. Mouse model with Tet-on-99CGG-eGFP/GFA2-rtTA actually is a good model to answer the question because these transgenic mice can restrict the expanded RNA expression only in astrocytes and Bergmann glia. This produces the possibility to study the role of astrocytes and Bergmann glia in the disease progression. It was hypothesized that expression of the expanded RNA only in astrocytes and Bergmann glia is sufficient to cause the FXTAS outcome. Unfortunately the Tet-on-99CGG-eGFP/GFA2-rtTA mice which showed expression in the organotypical slices, did not exhibit expression *in vivo* due to an absence or a very low expression of the transgene. To this transgenic line, thing necessary to do is conducting a new microinjection to establish some new founders.