Title : Effect of *Esr1* gene polymorphisms on parental behavior in female mice

Name: Mallarapu, Lalithadevi

Affiliation: Mouse Genomics Resource Laboratory (MGRL), Department of Genetics, School of Life Science

Introduction:

Parental care is critical for offspring's survival. Across mammalian species, females exhibit extremely diverse parental care (maternal behaviors) to ensure the mental and physical well-being of the offspring. Across species, a wide range of maternal behaviors have been observed, generating questions about how animals recognize their offspring and how brain circuits drive and control maternal display of affection. Previously, several studies shown that endocrine changes during pregnancy and child birth are associated with enhanced maternal care and establishment of new neural circuits. Changes in hormones throughout the pregnancy influence serotonergic and dopaminergic circuits in particular, modify the hormonal receptor levels in brain and thus neural activity. Most importantly, the increase in hormonal estrogen levels in later phase of pregnancy enhances the induction of maternal care. However, studies on rodents revealed that though virgin mice avoid or attack the pups initially, after a short period of sensitization with foster pups, female mice show nearly spontaneous maternal care and rats take several days of pup exposure sessions to accept the pups. These reports summarize that even though the maternal receptivity depends on several factors, upon pup stimulus, formation of neural connections and the expression followed by activation of specific proteins in defined neuronal subregions is critical.

ESR1 gene function is very well studied in humans. Several studies reported a long list of *ESR1* gene sequence variants -including mutations and polymorphisms correlated with various behavioral disorders in humans. In humans, polymorphisms in *ESR1* gene reported to cause anxiety, depression, anti-social behavior and harsh maternal parenting. Most of the reported polymorphisms are located in noncoding regions of the genome and do not alter the amino acid sequence, hence the molecular mechanisms in changing the behavior were not well established. Unless we establish the molecular

mechanism underlying the change in behavior, it is impossible to find the possible therapies/ drugs to treat behavioral disorders.

Method:

In the present study, *Esr1* gene sequence of 18 mice strains (http://www.informatics.jax.org/marker/MGI:1352467) was compared to check whether there is any variation in the gene sequence. Specific primers were designed to identify the polymorphism in the coding region of interest. I used one of the wild derived mice strains MSM/Ms (MSM; found in Mishima, Japan) and commonly used laboratory strain C57BL/6J (B6) in this study. These mice strains are already reported to have a difference in their behavior, where MSM shows higher aggression and social behavior when compared to B6. The coding exon 1 sequence of *Esr1* gene of MSM was compared with B6 by performing the sequence analysis. A 9bp (GCC; codes for alanine; AAA) coding polymorphism was identified in B6. This 9bp is present in the long stretch of GCC (alanine) repeat region and this kind of trinucleotide repeats are common in human and rodent genome. The importance of presence of alanine repeats in ESR1 are not studied yet., however in a more general way, these repeats are known to be causative for several congenital diseases with neurocognitive phenotypes in humans. It is therefore of general interest to explore the significance of this 9bp polymorphism in B6 genome. In the present study, I aimed to explore the importance of 9bp presence in B6 *Esr1* gene in the context to maternal behavior.

Results and Discussion:

In this study, I screened the entire coding exons for *Esr1* gene polymorphism identification. Though there are more than 20 SNPs present among the various mouse strains, only 3 polymorphisms resulted in change in the amino acid sequence. Here, I focussed on polymorphism rs245996394 (“in-frame” deletion) in B6 mice, which is located in the GCC repeat (coding region) on the N-terminal domain of ESR1 protein and also there was a difference in GCC repeat length between wild and laboratory mouse strains.

Deletion of GCC repeat (9bp) in B6 resulted in significant reduction of protein levels

As it was established that polyalanine expansions cause protein mis-folding, Alpha-fold prediction of protein structure of three genotypes, showed that dimer form of *Esr1*$^{\Delta 9/\Delta 9}$ protein is more similar to *Esr1*$^{+/+}$ compared to *Esr1*$^{\Delta 9/+}$ and the deletion effected the amino acid composition in β-sheet formation (data not shown). As structural prediction data is completely based on sequence modelling and I could not confirm those predictions with any *in vitro* assays, this data could not provide the actual confirmation about change in protein structure. However, it provided the basic idea that heterozygous deletion of 9bp (3 alanine's) effected the protein conformation and interactions. Since structural data was not strong enough address the changes in ESR1, I checked the protein levels through western blotting. A prominent reduction of hypothalamic ESR1 levels was observed in *Esr1*$^{\Delta 9/+}$ females where *Esr1*$^{\Delta 9/\Delta 9}$ maintained similar protein levels when compared to *Esr1*$^{+/+}$. The significant reduction in ESR1 levels was only observed at 65kD monomer length (the canonical protein). The other protein transcript which expresses at 46kD (ESR1 protein isoform without AF1 domain containing polyalanine repeat) in ESR1 protein did not show any difference in protein expression among the three genotypes. These results suggests that though the decrease in polyalanine length have caused reduction in protein levels, the deletion did not affect all the ESR1 protein isoforms. The lower protein levels can also be possibly addressed with ESR1 protein instability as it was reported earlier polyalanine repeats may lead to protein misfolding and subsequent loss of function. If polyalanine repeats have a role in ESR1 gene, the question here is why the homozygous deletion of 9bp does not show any effect on the females like heterozygous deletion. Compared with other transcription factor genes, where polyalanine expansions resulted in lower protein levels, ESR1 may function differently because of its dimeric nature. Hence, there is a possibility that similar polyalanine length in homozygotic alleles might be favourable condition than different allelic length in heterozygotes. At present based on the given results it is very difficult to address this phenomenon.

Higher Esr1 mRNA expression

The earlier finding of reduced ESR1 levels in hypothalamus led me to check whether 9bp deletion affected its receptor expression. I observed the higher *Esr1* mRNA levels upon

heterozygous deletion. Earlier reports showed that, *ESR1* polymorphism rs9340799 determines the degree of cancer susceptibility by influencing the gene expression *via* transcriptional regulation. In addition, another report showed that polymorphisms might alter the transcription levels of the *Esr1*, but the underlying mechanism remained unclear. There is a possibility that 9bp deletion, affected the transcriptional regulation in heterozygotes which might have resulted in higher mRNA levels.

The higher *Esr1* and lower *Esr2* mRNA expression in hypothalamus shows that both the receptors balance the estrogen functions by compensating for each other. However, there was no change in *Esr1* mRNA expression in ovary between three groups and also *Esr1*$^{\Delta 9/+}$ females maintained healthy estrus cycle despite lower Estradiol levels in proestrus. These results indicate that the effect of 9bp deletion might be tissue specific or some other signalling pathway is involved. As ESR1 functions through different signalling pathway and so far, the present study can only speculate that polymorphisms in ESR1 might affect its signalling pathway which in turn decides the fate of events in brain.

Heterozygous deletion of 9bp in B6 genetic background affects pup retrieval behavior

Hypothalamic MPOA is considered as main driving region for maternal behavior. When MPOA ESR1 positive cells were optogenetically inactivated, females were unable to retrieve the pups. These observations showed that heterozygous deletion of 9 bp led to abnormal parental behavior in virgin females. In the elaborated maternal behaviors, it was observed that initially all females hesitated to approach the pups, though in later sessions they were quick in approaching and sniffing the pups. To approach 1st and 2nd pups' females took almost similar time irrespective of the genotype. However, in case of 3rd pup approach, *Esr1*$^{\Delta 9/+}$ females took longer time in approaching. Immunostaining results showed lower ESR1 expressing cells in MPOA in *Esr1*$^{\Delta 9/+}$ females. Lower expression can relate to delayed pup retrieval and higher offspring mortality in heterozygote females. I also checked for expression of neuronal excitation marker protein cFOS and found that this protein expression was low in *Esr1*$^{\Delta 9/+}$ females. Low expression can be explained because these females maintained less pup contact. These results indicate that heterozygous deletion of 9bp in B6 background is not favourable for females to perform the normal retrieving behavior. The reduced tendency to retrieve pups in heterozygote

females can be correlated with the previous report on human where SNPs in *ESR1* gene resulted in harsh maternal parenting. In addition, human studies showed that, women with SNPs in *ESR1* gene showed various behavioral outcomes like psychiatric disorders, dementia, cognitive impairment, sudden changes in mood and depression. However, for the same SNP, behavioral outcomes of each study differs from one another. For example, *ESR1* PvuII polymorphism is associated with increased Alzheimer's risk in Caucasian populations, but not in Asian populations. These variations are particularly relevant in populations consisting of different ethnicities. Overall, all these findings implicated that *Esr1* gene polymorphisms alter mRNA and protein expression levels in brain, which might have altered the estrogen signalling pathways resulting in behavioral disorders. Though present study only focussed on maternal behavior, it might be an indication that studying the polymorphisms in mice will open the more ways to understand the molecular mechanisms for change in behaviors in humans.

Conclusion:

In conclusion, my study identified that mice from different genetic background, i.e., laboratory strain and wild derived strains differ in *Esr1* gene structure. I found a repeat number polymorphism of GCC in ESR1 coding region between B6 and MSM. This polymorphism i.e., deletion of 9bp in B6- *Esr1* gene resulted in abnormal maternal behavior. Briefly, our findings include; (i) increased pup mortality in $Esr1^{+/\Delta 9}$ (heterozygous) females, $Esr1^{+/\Delta 9}$ females showed reluctance in pup retrieval (took more time), (iii) reduced expression of ESR1 and cFOS (immediate early gene) in the MPOA of $Esr1^{+/\Delta 9}$ mice after maternal behavior implicating the reluctance in pup retrieval, (iv) higher *Esr1* mRNA expression and lower protein levels in hypothalamus of $Esr1^{+/\Delta 9}$ females, indicating the effect of polymorphism on regulation at either transcription or translation level. Altogether, this study provides a glance of how a *Esr1* genetic polymorphism has affected maternal behavior.