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ASD-relevant Animal Models of the Foxp Family of Transcription Factors

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Abstract

Autism is a neurodevelopmental disorder with a multifaceted association between genes and the environment. Currently, in the majority of patients, the etiology of autism is not known and coupled with increasing prevalence rates, along with the high degree of heritability of autism, the development of animal models is crucial for studying and developing therapies for autism. A key characteristic of autism is marked abnormalities in the acquisition and use of language. Thus, to understand and ultimately treat autism is an especially difficult task because no animal produces language, as it is defined in humans. In this review, we will discuss the *FOXP* family of genes, which are a group of transcription factors that have been linked to both autism, as well as language in humans. Due to the association of language/communication and the Foxp family of transcription factors, animal models with targeted disruptions of Foxp functioning are powerful tools for understanding the developmental signaling pathways that may be vulnerable in autism.

Keywords: FOXP2; FOXP1; Autism; Genetics; Rodent vocalization

Introduction

The etiology of autism, or the more broadly defined Autism Spectrum Disorders (ASD), is multifaceted and is likely due to a combination of genetic and environmental interactions [1,2]. Genetically modified animal models are amenable for studying these interactions, and for testing biochemical therapeutics in a high-throughput manner. Nevertheless, it is imperative for any proposed animal model of autism to recapitulate the basic fundamental core features of the disorder, as those observed in humans and for these "symptoms" to be reversed by the same drugs used to treat patients [3]. This definition of a valid animal model is complicated in the development of ASD animal models because disrupted communication is a core feature of ASD, and language is perceived to be a human-specific feature. Thus, here we present animal models for genes implicated in language, *Foxp2* and *Foxp1*, and discuss their relevancy to understanding the pathophysiology of ASD.

FOXP Family

The FOXP family of transcription factors consists of three genes expressed in the brain: *FOXP1, FOXP2*, and *FOXP4*. Of these three genes, more is known about FOXP2 due to the identification of a mutation in *FOXP2* over a decade ago, in a large intergenerational family termed the KE family [4]. Affected members of the KE family were found to have a mutation in the DNA-binding domain of *FOXP2,* which resulted in these individuals exhibiting verbal dyspraxia, a below average IQ, and syntactic impairments in language [5,6]. Subsequent studies have shown that this heterozygous mutation of an arginine to histadine (R553H) in the forkhead DNA binding domain of *FOXP2,* prevents the FOXP2 protein from binding to DNA [7], and thus likely inhibits its ability to directly regulate transcriptional events. Additional types of mutations including a premature stop mutation, leading to protein truncation have been identified in other individuals and families with similar speech and language phenotypes (for a detailed review of these mutations [8,9]). *FOXP2* has a highly conserved amino acid sequence, as well as a conserved distribution in brain regions that are involved in vocal communication, such as the neocortex, basal ganglia and cerebellum [8]. This conservation across species in areas of the brain implicated in diseases such as ASD [10], makes the study of *FOXP2* animal models relevant to understanding the normal neurodevelopmental mechanisms of these brain regions and circuits. In humans, *FOXP2* has had accelerated divergence from a common ancestor sequence with chimpanzees, as evidenced by two human-specific amino acids in its sequence [11]. This rapid change in the molecular evolution of *FOXP2* occurred around the same time as the emergence of language in the human lineage, and these changes have also been shown to be important for a unique transcriptional program for the human form of *FOXP2* [12]. *FOXP2* transcriptionally regulates genes involved in neuronal development, neurite outgrowth, dendritic branching, and axonal morphology [12-14]. Therefore, the evidence for FOXP2 involvement in language, its expression pattern in the brain, and its transcriptional regulation of important neurodevelopmental genes has led to the hypothesis that *FOXP2* may have a role in neurodevelopmental disorders, such as ASD.

Several studies have investigated whether genetic variations in *FOXP2* itself are associated with ASD; however, since there have been both positive and negative results reported in the literature, these studies are inconclusive [15-23]. In addition, recent genome-wide surveys of Copy Number Variation (CNV) in ASD samples have not found variation in *FOXP2* associated with the disorder [24-26]. Nevertheless, strong support for *FOXP2* playing a role in ASD can be found in its regulation of downstream signaling pathways. For example, candidate gene approaches have found that *FOXP2* regulates the expression of *CNTNAP2, MET*, and *SRPX2*, all ASD related genes [27-29]. In addition, genome-wide DNA binding and gene expression studies have identified numerous target genes of *FOXP2* in either human or mouse tissue that are associated with ASD [14,30-32]. Together, these studies suggest that signaling pathways downstream of *FOXP2* regulation of gene expression are particularly vulnerable in ASD.

FOXP1 is highly homologous to *FOXP2*, and is also expressed

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in the developing human brain, in both neocortex and basal ganglia [33]. For a more detailed review on expression patterns of *FOXP* family members, see [32,34]. The conservation of functional domains and overlapping expression in certain brain regions among *FOXP* family members suggests the potential for functional redundancy, and/or synergistic activities. In fact, *FOXP1, FOXP2*, and *FOXP4* not only form homodimers, but they also have the capacity to form heterodimers with each other, to regulate transcription [35]. Thus, through this heterodimerization mechanism, both *FOXP1* and *FOXP4* may participate in *FOXP2*-mediated signaling pathways, important for speech and language. Unlike *FOXP2*, a more direct association between *FOXP1* and ASD has been discovered recently. Several studies have uncovered mutations, deletions, or copy number variations of *FOXP1* in individuals with ASD, or other neurodevelopmental disorders such as intellectual disability [36-42]. In addition, some affected individuals with *FOXP1* mutations have abnormally enlarged ventricles, as assessed by MRI [37]. The discovery of increased brain volume in patients with a *FOXP1* mutation is consistent with previous reports of ASD patients [43-45]. Similar to *FOXP2*, *FOXP1* can also repress expression of *CNTNAP2*, suggesting convergent signaling pathways of ASD genes by FOXP family members [39]. Moreover, these results again highlight a potential mechanism for *FOXP2* participation in signaling pathways vulnerable in ASD, through its heterodimerization with *FOXP1*. Additional studies of *FOXP1* targets will be required to fully assess the involvement of *FOXP1*-regulated signaling pathways in ASD. Taken together, these data support a role for both *FOXP1* and *FOXP2* in the regulation of signaling pathways involved in ASD.

Rodent Ultrasonic Vocalizations

The most challenging aspect of ASD biology to model in rodent systems is the disruption to language and communication. However, it has been well established that rodents communicate through the use of Ultrasonic Vocalizations (USVs) [46,47], and numerous studies have identified alterations in USVs in mice, with genetic modifications in genes associated with ASD [48-52]. USVs are produced by many species of rodents in the context of social encounters, and function as a short-distance communication method since USVs attenuate rapidly in space [53,54]. Moreover, many predators have limited hearing capacity for ultrasonic tones, thus providing rodents an ideal method for communicating with intended receivers, while minimizing the threat of predation [46,54]. USVs emerge early in infancy in many rodent species, (i.e. within the first few days of life for mice and rats) [47,55]. Pup distress USVs are triggered by aversive events, such as maternal separation and hypothermia [56,57]. These maternal separation calls are the most widely used method for eliciting and analyzing vocalizations in young rodents [58-60]. Maternal separation calls are effective stimuli because these infant vocalizations readily elicit maternal retrieval behavior in many strains of rats and mice [61-64]. The most parsimonious interpretation of these data is that pups' ultrasonic calls communicate an aversive emotional state to their mothers, resulting in pup retrieval. Changes in USVs may thus, alter the behavioral feedback circuit between pup and mother leading to potential changes in other behavioral responses during development, such as anxiety-related behaviors [65,66]. Pups cease producing separation-induced distress calls around the time when their eyes and ear canals open at approximately 14 days of age [67], further suggesting that these particular vocalizations are not intended for non-maternal conspecifics [55]. The study of pup USVs, within the context of ASD is therefore, not only important for understanding mechanisms of vocalizations, but also how alterations in biobehavior feedback at an early age may impact later development.

Animal Models and the Foxp Family

Since disrupted communication ability is a core feature of ASD, and this particular facet of ASD is particularly challenging to address in animal models. The association between language/communication and the Foxp family of transcription factors makes animal models with disruption to Foxp functioning, potentially informative for understanding the molecular biology of this phenotype in ASD, as well as vocalizations in general. A number of *Foxp2* mutant mice have been developed, and the study of these animals have provided insight into the contribution of *Foxp2* to vocalizations and other behaviours, that may be relevant to the study of ASD, such as learning and memory (see table 1 for a complete summary of these lines).

Shu et al. [68] generated the first *Foxp2* Knockout (KO) mouse. Shu et al. [68] found that homozygous loss of *Foxp2* (*Foxp2*$^{-/-}$) in mice leads to severe motor defects, and the mice typically die by postnatal day 21. The subsequent generation of an additional line of *Foxp2*$^{-/-}$ mice, using a conditional null allele approach has supported this finding [69]. In addition, the *Foxp2*$^{-/-}$ mice also have reduced postnatal weight gains, although the underlying etiology of this is unknown, and could be tied to non-nervous system requirements for *Foxp2* function [68-70]. Moreover, heterozygous loss of *Foxp2* (*Foxp2*$^{-/+}$) also leads to a reduction in postnatal weight gain in one strain of mice [68], but unlike the *Foxp2*$^{-/-}$ mice, the *Foxp2*$^{-/+}$ mice are viable [68,69]. In addition to the development of *Foxp2* null allele mice, other *Foxp2* mutants have been developed. Mice have been engineered with a specific mutation in *Foxp2*, analogous to the mutation in the affected members of the KE, mice family (R552H), or with a nonsense mutation in *Foxp2* analogous to the mutation in an additional family with disruptions in speech and language (S321X) [9,70]. Finally, mice with a "humanized" form of *Foxp2* have been generated by substituting the exon that yields the two human-unique amino acids of *FOXP2* into the orthologous exon of the mouse *Foxp2* gene [71]. The resultant *Foxp2*$^{hum/hum}$ mice are viable, and do not have any growth issues.

Anatomically, major developmental abnormalities in the cerebellum have been reported in both homozygous null and point mutation *Foxp2* mutants. These mice were found to have atypical cerebellar growth, e.g. nonconforming alignment of Purkinje cells and incomplete migration of granular cells [68-70,72]. In addition, one report has found heterozygous null *Foxp2* mutants to have slight differences in cerebellar development, as compared to wild type mice [68], while the other studies have not reported differences in heterozygotes [69,70]. Alterations in the cerebellum are relevant to the study of ASD, as there is increasing evidence that cerebellar deficits are associated with ASD [10]. Moreover, *Foxp2*$^{R552H/+}$ mice were found to have impaired Long-term Depression (LTD) in the striatum, but a more rapid induction of LTD in the cerebellum [70]. These same mice were also tested using *in vivo* multielectrode recordings and displayed negative modulation of striatal neuron firing rates, which is in contrast to the positive modulation exhibited by wild type mice [73]. Additionally, these *Foxp2*$^{R552H/+}$ mice also demonstrate abnormally high continuous striatal activity [73]. This pattern of activation in the striatum is irregular, because the majority of neurons in the striatum typically show low in vivo firing rates [74]. In contrast to the impaired LTD of *Foxp2*$^{R552H/+}$ mice, *Foxp2*$^{hum/hum}$ mice have increased LTD in the striatum [71]. These changes in excitation/inhibition, and/or plasticity may also be relevant to many of the biological changes ascertained in patients with ASD [2].

In addition to anatomical abnormalities, homozygous *Foxp2* mutants have discernible motor deficiencies as measured by righting reflex, negative geotaxis, and rotarod testing [68-70,72]. In contrast,

Gene	Reference	Mutation	USV	Other behavior	Anatomy
Foxp2	**Shu (2005)**	**Null Allele**	**PN 6; PN 10**		
		***Foxp2*$^{-/-}$**	1) Reduced total number 2) Duration, frequency, bandwidth comparable to WT	1) Severe motor defects 2) Die by PN 21	1) Atypical cerebellar growth 2) Reduced postnatal weight gain
		***Foxp2*$^{2-/+}$**	1) Reduced total number duration, peak frequency, bandwidth comparable to WT	1) Similar to WT on Morris water maze	1) Minor changes in cerebellum 2) Reduced postnatal weight gain
	French (2007)	**Null Allele cKO**			
		***Foxp2*$^{-/-}$**		1) Severe motor defects 2) Die by PN 21	1) Atypical cerebellar growth 2) Reduced postnatal weight gain
		***Foxp2*$^{-/+}$**		1) No gross motor defects	1) Normal cerebellar growth 2) Normal postnatal weight gain
	Fujita (2008)	**KE family mutation**	**PN 8**		
		***Foxp2*$^{R552H/R552H}$**	1) Reduced total number and produced mainly "clicks"	1) Severe motor defects 2) Die by PN 21	1) Atypical cerebellar growth 2) Reduced postnatal weight gain
		***Foxp2*$^{R552H/+}$**	1) Decrease in total number 2) Able to produce a variety of calls but with reduced duration	1) Slight motor defects	1) Normal postnatal weight gain
	Groszer (2008)	**KE family mutation**	**PN4**		
		***Foxp2*$^{R552H/R552H}$**	1) No reduction in total number	1) Severe motor defects 2) Die by PN 21	1) Atypical cerebellar growth 2) Reduced postnatal weight gain
		***Foxp2*$^{R552H/+}$**	1) No differences	1) No gross motor defects 2) Slight reduction in motor skill learning	1) Impaired LTD in striatum 2) Climbing fiber and parallel fiber input on to Purkinje cells were normal but faster induction of LTD 3) Normal postnatal weight gain 4) Normal cerebellar growth
	Enard (2009)	**Humanized Foxp2**	**PN 4, PN 7, PN 10, PN 13**		
		***Foxp2*$^{hum/hum}$**	1) No differences in total number of calls or duration 2) Lower start frequency and lower min and max frequency	1) No gross motor defects 2) Reduced exploratory activity in a novel environment	1) Reduction in dopamine brain that express Foxp2 2) Longer neurite outgrowth 3) Stronger LTD 4) Changes in gene expression patterns in the striatum
		***Foxp2*$^{WT/KO}$**		1) Slightly impaired motor learning on the rotarod	1) Increase in dopamine concentrations in all regions of brain that express Foxp2 2) Increases in serotonin levels in nucleus accumbens 3) Shorter neurite outgrowth 4) Gene expression showed opposite patterns than observed in Foxp2hum/hum
	Gaub (2010); French (2011); Kurt (2012)	**KE family mutation**	**PN 4**		
		***Foxp2*$^{R552H/R552H}$**	1) No reduction in total number 2) More low acoustic USVs with similar duration as WT		1) Reduced postnatal weight gain
		***Foxp2*$^{R552H/+}$**	1) No reduction in total number and similar duration as WT	1) Learned auditory-motor association task slowly but reached WT levels	1) Normal postnatal weight gain 2) Striatal neurons showed more negative modulation than the characteristic positive modulation 3) Higher than normal ongoing firing rates for medium spiny neurons
		***Foxp2*$^{S321X/S321X}$**	1) Small reduction in total number 2) More low acoustic USVs similar duration, and fewer frequency jumps as WT		1) Reduced postnatal weight gain
		***Foxp2*$^{S321X/+}$**	1) No reduction in total number 2) More high acoustic USVs but longer duration than WT	1) Did not learn auditory motor association task	1) Normal postnatal weight gain
Foxp1	**Rousso (2008)**	**Null Allele**			
		***Foxp1*$^{-/-}$**			1) Foxp1 involved in motor neuron formation in spinal cord
	Surmeli (2011)	**Motor neuron cKO**			
		Olig2: Cre; Foxp1flox/flox			1) Foxp1 involved in sensory-motor connectivity of neurons in spinal cord
Foxp4	**Rousso (2012)**	**Null Allele**			
		***Foxp4*$^{-/-}$**			1) Increased neural tube defects, lack of lateral ventricles, and holoprosencephaly

PN=postnatal day; WT= wild type; cKO=conditional knockout.

Table 1: Summary of Foxp family mouse models including data for USVs, other behaviors, and anatomy.

Foxp2$^{-/+}$ mutants, *Foxp2*$^{R552H/+}$, and *Foxp2*$^{hum/hum}$ mice, either did not exhibit motor deficits, or displayed minor deficits in motor skill acquisition [68,70,72]. A recent study examining the *Foxp2*$^{R552H/+}$, and *Foxp2*$^{S321X/+}$ mice has found that these two different point mutations affect the ability of these mice to learn how to associate auditory stimuli with a motor output behavior [75]. In this study, two different acoustic tones were played. The mice were conditioned to jump a hurdle separating two chambers, in response to one tone, but abstain from jumping the hurdle, in association with a different tone. *Foxp2*$^{R552H/+}$ mice learned the task, but at a slower rate than wild type mice, whereas *Foxp2*$^{S321X/+}$ mice exhibited a slow, flat learning curve that never reached wild type levels [75]. These data suggest an important distinctive behavioral outcome, as a consequence of two different human-based mutations of the *Foxp2* gene on learning behavior. These studies also highlight the involvement of *Foxp2* in brain circuitry, underlying different types of learning that may be vulnerable in neurodevelopmental disorders, such as ASD.

Examination of USVs in *Foxp2* mouse models has found that both heterozygous and homozygous *Foxp2* mutant mice show differences in the total number of vocalizations emitted, compared to wild type litter mates [68,72]. In contrast, Groszer et al. [70] report finding no reduction in the total number of USVs emitted by *Foxp2* mutants, compared to controls [76]. An analysis of the acoustic properties of the vocalizations produced by both homozygous and heterozygous *Foxp2* mutants yielded no differences in the call duration, frequency, or bandwidth [68,70,72]. Interestingly, studies using humanized *Foxp2* mice have found that the vocalizations of these mice have a lower frequency onset, and lower minimum and maximum frequency means [71]. An extension of previous work by Gaub et al. [76] found *Foxp2*$^{R552H/R552H}$ mice produced: 1) the same total number of USVs and 2) these USVs were the same duration as the USVs produced by wild type mice, with the only distinction being that the USVs were produced at a lower frequency. In contrast, *Foxp2*$^{S321X/S321X}$ mice produced fewer total numbers of USVs, and those USVs were produced at a lower frequency, with no significant difference in the duration of the calls, as compared to wild type mice. Moreover, *Foxp2*$^{R552H/+}$ and *Foxp2*$^{S321X/+}$ mice showed no difference in the total number and duration of the USVs, as compared to wild type mice, however, these two mutant lines tended to produce USVs, at a higher frequency than those calls produced by *Foxp2*$^{R552H/R552H}$ and *Foxp2*$^{S321X/S321X}$ mice [76]. Because much of the neural circuitry underlying USVs still remains unknown, it is unclear which aspects of *Foxp2* function and expression are driving this altered behavior. Moreover, it is currently believed that mouse vocalizations are innate, and are not learned in a manner that is similar to language in humans, although there is some recent evidence that adult vocalizations in the male mouse may have features reminiscent of vocal learning [77]. Thus, it is plausible that maternal separation USVs only recapitulate motor aspects of language, and may not serve as appropriate models for the cognitive aspects of language. However, as discussed above, there are other social and communicative aspects of USVs that occur between pup and mother, making them an appropriate model for the study of autistic-like behaviors in rodents. In addition, the gene expression studies that have been carried out in both *Foxp2* knockout mice [14] and *Foxp2* humanized mice [71], have identified several ASD genes as potential *Foxp2* targets in these animal models. Therefore, it is possible that evolutionary conserved *Foxp2* targets are important for the coordination of motor movements responsible for both USVs and language.

FoxP2 is also expressed in zebra finch brain, in areas comparable to the mammalian expression pattern [33]. In addition, *FoxP2* expression changes during song acquisition, pointing to an important role for *FoxP2* in learned song, which is in contrast to its potential role in inherent USVs in mice [33,78-80]. Furthermore, reduction of *FoxP2* in the zebra finch brain leads to a decrease in spine formation, as well as a disruption to song learning [13,81]. Changes in neurite outgrowth have also been found in mouse models of *Foxp2*, [14,71], thereby suggesting a conserved cellular function for *FoxP2*, that may have a functional impact on vocalization circuitry. Together, these data from rodent and songbird emphasize a critical role for *FoxP2* in the motor circuitry underlying vocalizations. What these data do not address is the function of human *FOXP2* in the cognitive aspects of language. However, gene expression studies comparing human and chimpanzee *FOXP2* support the idea that human *FOXP2* has a unique transcriptional program that may be contributing to higher cognition and language [12]. Further studies of *Foxp2* animal models, such as conditional knockout of *Foxp2* in specific brain regions will facilitate understanding the role of *Foxp2* in brain development. Such experiments, together with gene expression studies in human and non-human primate cells and tissues, should assist in parsing out the conserved function of *FOXP2* in vocalizations, as well as potential human-specific functions of *FOXP2* in language signaling pathways.

Recent work in genetically modified rodents has also implicated *Foxp1* in Central Nervous System (CNS) motor function. Homozygous *Foxp1* knockout mice are embryonic lethal at E14.5 due to defects in the cardiovascular system [82], making the study of the role of *Foxp1* in behavior impossible to ascertain in these mice. However, examination of the developing spinal cord in these mice has shown that Foxp1 is required for motor neuron specification [83,84]. In addition, a conditional knockout of Foxp1 in motor neurons leads to profound impairments in limb coordination during motor movements [85]. Additional mice with selective reduction of *Foxp1* in other areas of the CNS should be informative as to a potential role for *Foxp1* in USVs, social behavior, or repetitive behaviors. Furthermore, the targets of *Foxp1* in the developing mammalian brain have yet to be determined, and should provide insight into whether *Foxp1* also transcriptionally regulates signaling pathways containing ASD genes similar to *Foxp2*. Thus, animal models of both *Foxp2* and Foxp1 that recapitulate key aspects of the behaviors affected in humans with ASD (i.e., vocalizations and complex social behaviors) have the potential for modeling key neurodevelopmental phenotypes, for which functional interventions and therapeutics can be tested.

Future Directions

Future studies utilizing whole genome sequencing in even larger patient cohorts than has been conducted for exome sequencing should more thoroughly address the contribution of both *FOXP2* and *FOXP1* in ASD. Since, there is a greater appreciation that the genetic architecture of ASD in most cases is due to polygenic contributions, rather than dominant single gene mutations [2], it will be even more important to understand the function and the gene targets of these transcription factors in the developing brain. In addition, the high homology, ability to heterodimerize, and overlapping expression patterns of *FOXP4* to the other FOXP family members in the brain, make *FOXP4* another prime candidate for study in animal models. However, since homozygous loss of *Foxp4* is embryonic lethal due to cardiovascular and neural tube deficits [86,87], CNS-specific conditional alleles of *Foxp4* will be warranted.

Since the development of reliable animal models is a critical step towards understanding and ultimately treating ASD, this task has been particularly daunting due to the inherently human-specific cognitive

nature of language, that is characteristically disrupted in ASD. As such, the use of animal models for understanding ASD has some inherent limitations. However, the majority of signaling pathways and circuitry used in language most certainly has been built upon existing pathways, and circuitry utilized for vocalizations and working memory in other species. Thus, understanding these neurobiological features using animal models can provide important insights into developmental mechanisms at risk in ASD. In addition, animal models other than mice may provide novel insight. For example, rat pups have more complex vocalizations, including a phenomenon termed "maternal potentiation", that may be useful in modeling ASD-like behaviors [65]. Since genetically modified rats (and potentially other organisms, such as non-human primates) can be rapidly generated using new technologies, such as zinc finger nucleases [88], both USVs and other cognitive behaviors may be better suited for study in other species, such as the rat. Due to the relationship of the FOXP family of transcription factors with vocalizations, language and cognitive diseases, these genes have the potential to bring unique insight into the pathophysiology of ASD. Although the uncovering of genes important for ASD and other neurodevelopmental disorders is still ongoing, the brain-expressed FOXP family members are rare examples of transcription factors important for brain development, language, and ASD.

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