Rare Diseases 2, e28688; 2014; © 2014 Landes Bioscience

Epistatic interactions between *Chd7* and *Fgf8* during cerebellar development

Implications for CHARGE syndrome

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Keywords: CHARGE syndrome, Chd7, Fgf8, cerebellum, embryo, mid-hindbrain, isthmus organizer, brain, development, mouse

Abbreviations: AVSD, Atrioventricular septal defects; FGF, Fibroblast Growth Factor; CHD7, Chromodomain Helicase DNA binding factor 7; Otx2, Orthodenticle homeobox 2; Gbx2, Gastrulation brain homeobox 2; Tbx1, T box transcription factor 1; Krox20 = Egr2, early growth response gene 2; CNS, Central Nervous System; MRI, Magnetic Resonance Imaging; ChIP-Seq, Chromatin Immunoprecipitation-sequencing; GCp, Granule cell precursor; r1, rhomobomere 1; r3, rhombomere 3; r5, rhombomere 5; IsO, Isthmus Organizer

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Submitted: 02/21/2014

Revised: 03/20/2014

Accepted: 03/27/2014

Published Online: 03/31/2014

Citation: Basson MA. Epistatic interactions between Chd7 and Fgf8 during cerebellar development: Implications for CHARGE syndrome. Rare Diseases 2014; 2:e28688; http://dx.doi.org/10.4161/rdis.28688

Addendum to: Yu T, Meiners LC, Danielsen K, Wong MT, Bowler T, Reinberg D, Scambler PJ, van Ravenswaaij-Arts CM, Basson MA. Deregulated FGF and homeotic gene expression underlies cerebellar vermis hypoplasia in CHARGE syndrome. Elife 2013; 2:e01305; PMID:24368733; http://dx.doi.org/10.7554/eLife.01305

CHARGE syndrome is a rare, autosomal dominant condition caused by mutations in the *CHD7* gene. Although central nervous system defects have been reported, the detailed description and analysis of these anomalies in CHARGE syndrome patients lag far behind the description of other, more easily observed defects. We recently described cerebellar abnormalities in CHARGE syndrome patients and used mouse models to identify the underlying causes. Our studies identified altered expression of the homeobox genes *Otx2* and *Gbx2* in the developing neural tube of *Chd7*$^{-/-}$ embryos. Furthermore, we showed that the expression of *Fgf8* is sensitive to *Chd7* gene dosage and demonstrated an epistatic relationship between these genes during cerebellar vermis development. These findings provided, for the first time, an example of cerebellar vermis hypoplasia in a human syndrome that can be linked to deregulated FGF signaling. I discuss some of these observations and their implications for CHARGE syndrome.

Epistasis in Human Disease

In the context of disease-susceptibility alleles, interactions between more than one risk allele that alters the incidence of disease to a significantly greater extent than expected from the simple addition of the individual allelic effects can be defined as epistatic interactions.[1] It is becoming increasingly clear that epistatic effects are of fundamental importance to understand the genetic basis of complex diseases that may be caused by the combination of many genetic risk factors, each with a relatively small contribution to disease susceptibility. In addition, conditions with relatively "simple" genetics but pleiotropic phenotypic effects, e.g., syndromes caused by autosomal dominant gene effects, might also be subject to strong epistatic effects. For example, 22q11del (DiGeorge) syndrome exhibits large clinical variability.[2] The existence of so-called "modifier" genes, i.e., protective or susceptibility alleles that alter the effect of the dominant disease-associated gene on disease incidence, has been postulated for many years. Studies in model organisms, typically making use of loss-of-function alleles, have been useful in identifying candidate genetic modifiers of disease.[2] However, it has proven rather difficult to identify modifier genes in the human population.

CHARGE Syndrome

CHARGE (coloboma of the eye, heart defects, atresia of the nasal choanae, retardation of growth and/or development, genital and/or urinary abnormalities, and ear abnormalities and deafness) syndrome is an autosomal dominant disorder with an estimated prevalence of 1 per 10 000.[3-5] Most patients (60–70%) have mutations in the *CHD7* (Chromodomain helicase DNA-binding protein 7) gene.[6-8] A CHARGE syndrome clinical diagnosis is normally made when three to four major abnormalities (coloboma, choanal atraesia,

ear defects, and cranial nerve dysfunction) are seen or two to three major and two to three minor criteria (e.g., genital hypoplasia, retarded growth, cardiovascular defects, orofacial cleft, or tracheo-esophageal fistula) are met.[9-11] However, CHARGE syndrome is characterized by high phenotypic variability, making diagnosis and the definition of key clinical characteristics difficult. Interestingly, *CHD7* mutations have also been reported in other conditions, most notably Kallmann syndrome and idiopathic hypogonadotropic hypogonadism with hearing loss[12,13] and velocardiofacial and/or DiGeorge syndrome.[14,15] Loss-of-function mutations in *Chd7* and *Tbx1* (a DiGeorge syndrome gene) interact during aortic arch development in mouse embryos, suggesting that the function of these genes intersect at some point.[15]

A key signaling pathway linked to *Tbx1* function is the FGF signaling pathway. *Tbx1* is required for normal *Fgf8* expression in the endoderm, and *Tbx1* and *Fgf8* loss-of-function alleles are in epistasis during pharyngeal development.[16] We therefore postulated that *Chd7* might also function upstream of the FGF signaling pathway. However, we did not detect a statistically significant interaction between *Chd7* and *Fgf8* loss-of-function alleles during aortic arch development.[15] There are several possible explanations for this result, including: (1) Our hypothesis is incorrect and *Chd7* haplo-insufficiency does not affect *Fgf8* gene expression or signaling and there is no epistatic relationship between these genes; (2) CHD7 can function upstream of FGF signaling, but these effects are highly context-dependent and not relevant during aortic arch development; or (3) *Chd7* and *Fgf8* interactions are modified, i.e., in epistasis with additional genetic factors and the genetic background used in this study masked any interactions. We turned to central nervous system (CNS) development in an attempt to resolve these questions.

CNS Defects are Prevalent in CHARGE Syndrome

CNS defects like arhinencephaly, hypoplasia of the cerebellum, and brainstem and cerebellar heterotopia are detected in 70–80% of CHARGE syndrome cases.[17-20] This incidence is in the same range as other more regularly reported clinical features of CHARGE syndrome: coloboma (82%), atraesia of the choanae (57%), ear abnormalities and deafness (95%), cranial nerve dysfunction (61%), heart defects (80%), retardation of growth/development (90%), and genito-urinary anomalies (60%). Thus, CNS defects are likely to represent a significant component of the clinical spectrum that typifies CHARGE syndrome. However, the exact scope and penetrance of CNS defects associated with CHARGE syndrome and *CHD7* deficiency remain to be fully defined.

Cerebellar Defects in CHARGE Syndrome

Until recently, reports on cerebellar anomalies in CHARGE syndrome were rare, with the most convincing evidence being described in pre-natal fetuses.[19,21,22] This has led to the erroneous assumption by some that cerebellar defects may not be a significant feature characteristic of CHARGE syndrome. However, there are two alternative explanations: (1) MRI examinations capable of detecting cerebellar defects in CHARGE syndrome are not routinely performed and (2) these defects are associated with the most severe cases of CHARGE syndrome that tend to be incompatible with life. Indeed, one study reports a correlation between patient survival and Atrioventricular septal defects (AVSD) with cerebellar and brainstem defects.[20]

We recently reported the first systematic analysis of cerebellar structure in MRI scans from a cohort of patients with *CHD7* mutations and diagnosed with CHARGE syndrome.[23] Approximately 50% of patients showed some cerebellar abnormality. These included cerebellar vermis hypoplasia (35%) and foliation defects (25%). The former defect was of particular interest, as our previous studies in mouse models have shown that inappropriate levels of FGF signaling in the embryonic isthmus organizer (IsO, a key signaling center located at the embryonic mid-hindbrain boundary) dramatically affected the size of the cerebellar vermis, with little effect on the size of the cerebellar hemispheres.[24] Thus, it appears that expansion of the vermis progenitor zone is critically dependent on high levels of FGF signals from the adjacent IsO. The identification of cerebellar vermis hypoplasia in CHARGE syndrome suggested a potential link between CHD7 and FGF signaling.

The examination of mouse models uncovered compelling evidence for epistatic interactions between *Chd7* and *Fgf8*. Whereas $Chd7^{+/-}$ and $Fgf8^{+/-}$ mouse embryos exhibited no discernable cerebellar defects, $Chd7^{+/-};Fgf8^{+/-}$ embryos presented with cerebellar vermis aplasia. This epistatic interaction between *Chd7* and *Fgf8* loss-of-function alleles identified a functionally important link between CHD7 and FGF signaling.

Having uncovered strong genetic evidence in support of a link between *Chd7* and *Fgf8*, we searched for a mechanistic explanation for these genetic observations. The most obvious prediction was that CHD7 was required for the normal expression of *Fgf8*, or other FGF signaling components. Indeed, we could show that *Fgf8* expression levels at the IsO directly correlated with *Chd7* genotype.[23] These findings placed CHD7 upstream of *Fgf8*.

These findings predict that hypomorphic mutations in the FGF signaling pathway will strongly enhance cerebellar vermis defects in CHARGE syndrome patients. It is of considerable interest to note that mutations in multiple genes in the FGF pathway have been reported in patients with Kallmann syndrome, consistent with a model whereby these gene mutations can interact epistatically to determine the incidence and perhaps severity of disease.[25] This raises the possibility that mutations or polymorphisms that affect the function and/or expression of FGF8 and other FGF signaling components might affect the penetrance and expressivity of cerebellar defects in CHARGE syndrome. Sequencing of the *FGF8* gene in a cohort of CHARGE syndrome patients with *CHD7* mutations has not identified any obviously damaging mutations in patients with or without cerbellar vermis defects

yet (personal communication, Conny van Ravenswaaij-Arts). Additional targeted or exome sequencing studies in CHARGE syndrome patients, with careful analyses of correlations between genetic features and particular phenotypes are likely to yield substantial insights into the etiology and genetic complexities of CHARGE syndrome.

Our identification of a link between CHD7 and FGF signaling at the IsO provides an explanation for the cerebellar vermis hypoplasia present in some CHARGE syndrome patients. Several patients had additional cerebellar defects, including abnormal foliation patterns.[23] Previous studies have also reported cellular heterotopia, the mis-localization of neurons in the cerebellum.[19,22] These observations imply additional roles for CHD7 in controlling postnatal cerebellar development, when granule cell precursor (GCp) proliferation in the external granule cell layer and subsequent migration of GCps into the cerebellar cortex drive cerebellar foliation.

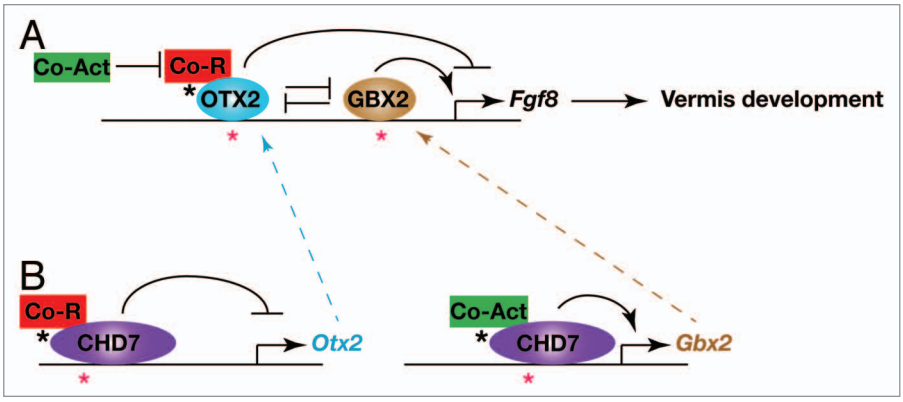

Figure 1. Gene regulatory interactions implicated in cerebellar vermis hypoplasia in CHARGE syndrome. The diagram depicts a model of the homeobox gene-regulatory network that impacts on *Fgf8* expression at the IsO. Possible regulatory mutations that might result in de-regulated *Fgf8* expression and cerebellar vermis hypoplasia are indicated by asterisks. (**A**) OTX2 represses *Fgf8* expression by interacting with transcriptional co-repressors, such as Groucho (Co-R, Tle4/Grg4), whereas GBX2 acts as a positive regulator of *Fgf8* expression, presumably through interacting with transcriptional co-activators (Co-Act). OTX2 and GBX2 cross-repress each other's expression. Mutations in OTX2 or GBX2 regulatory regions (red asterisks) or mutations that affect the ability of these factors to recruit or activate co-activators or repressors (black asterisk) might affect *Fgf8* expression levels and result in cerebellar vermis hypoplasia. (**B**) CHD7 functions as an *Otx2* repressor and GBX2 activator in the mid-hindbrain region, possibly also through interacting with additional chromatin remodelling and transcriptional regulators that contribute to repressive or activator activities. Mutations in *Otx2* or *Gbx2* regulatory elements to which CHD7 are recruited (red asterisks) or mutations affecting the activity or recruitment of additional co-activating or repressing factors (black asterisks) are predicted to affect *Otx2* or *Gbx2* expression, which in turn will affect *Fgf8* expression and cause cerebellar vermis abnormalities.

Identification of CHD7 as a Critical Regulator of Homeobox Gene Expression

CHD7 shares significant homology in amino acid sequence (44%) and domain organization with the *Drosophila* chromodomain factor kismet. The *kismet* gene was originally identified as a member of the Trithorax group of regulators and *kismet* mutant flies shows evidence of homeotic transformations and corresponding alterations in homeobox gene expression.[26] Given these similarities it is tempting to speculate that some of the developmental anomalies typical of *Chd7* deficiency might be caused by deregulated homeobox gene expression. Certainly, some evidence in support of this notion has been reported. For example, the expression of *Krox20*, a homeobox gene expressed in two specific segments of the embryonic hindbrain, rhombomere 3 (r3) and r5, is reduced in *Chd7*-deficient embryos.[23,27] Defects in r3 and r5 identity might be responsible for some of the cranial nerve paralysis observed in a high proportion of CHARGE syndrome patients, with the VIIth and VIIIth cranial nerves most commonly affected.[18]

The homeobox genes *Otx2* and *Gbx2* impart positional identity in the early anterior neural tube. The IsO forms at the sharp boundary between the anterior *Otx2* and posterior *Gbx2* expression domains that arises as a result of cross-repressive interactions between these transcription factors. Studies in various model organisms have accumulated a large body of evidence indicating that OTX2 functions as a potent repressor of *Fgf8* expression at the IsO.[28-30] Studies in the mouse have shown that transgenic *Otx2* misexpression could re-position the *Fgf8*-expressing IsO to more posterior positions in the embryo.[30] The intriguing links between CHD7/kismet and homeobox gene regulation led us to examine whether CHD7 depletion might affect *Fgf8* expression through changes in *Otx2* and *Gbx2* gene expression. We found that *Otx2* was de-repressed and *Gbx2* lost in the region normally fated to become r1. Intriguingly, we found that *Fgf8* expression was initialised at the correct position in *Chd7*$^{-/-}$ embryos and was therefore located in an *Otx2*-expressing region. Thus, rather than shifting the *Fgf8* expression domain as in transgenic *Otx2* misexpression experiments,[30] *Fgf8* expression was reduced, consistent with the role of OTX2 as a repressor of *Fgf8*. This finding has several important implications for understanding the potential genetic causes of cerebellar vermis hypoplasia in humans. Mutations that affect the activity of other endogenous repressors of *OTX2* and mutations in critical *OTX2* regulatory elements might be responsible for some cases of cerebellar vermis hypoplasia. This observation might also help explain the fact that mutations in the *FGF8* coding sequence have not as yet been described in patients with cerebellar vermis hypoplasia. Given the many critical developmental roles performed by FGF8 in development, mutations with potent enough effects on *FGF8* function are likely to be incompatible with life. However, one might predict that mutations in non-coding regions that specifically affect *FGF8* expression in the

IsO might predispose an individual to cerebellar vermis hypoplasia (**Fig. 1**).

Implications for Other CHARGE Syndrome Phenotypes

An obvious question that arises from our work on the cerebellum is whether the regulation of homeobox gene expression and FGF signaling by CHD7 is unique to this part of the embryo, or whether it might also explain other phenotypes associated with CHD7 deficiency. *Fgf8* and *Otx2* are involved in the development of several other organs and structures affected in CHARGE syndrome, including the eye, ear, olfactory placode and the forebrain. Intriguingly, *Otx2* expression is reduced in the otic vesicle and olfactory placode of *Chd7*$^{-/-}$ embryos, suggesting that CHD7 can regulate *Otx2* expression in other embryonic regions, but that the effect on *Otx2* expression is context-dependent.[31,32]

Concluding Remarks

Elucidating the developmental basis of CNS defects caused by CHD7 deficiency is important for understanding the etiology of cognitive impairments associated with CHARGE syndrome. In addition to its developmental roles, CHD7 functions as a key regulator of neural progenitor cell differentiation in the adult hippocampus (Feng et al.[33]). To what extent CHD7 deficiency in the hippocampus affects cognitive ability of adults with *CHD7* mutations remains to be determined.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

Acknowledgments

I acknowledge funding from the Wellcome Trust (091475) and the Medical Research Council (MR/K022377/1) who supported this work and thank Kieran Jones for comments on the manuscript.

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