Review Article

Molecular Syndromology

Mol Syndromol
DOI: 10.1159/000491004

Published online: July 13, 2018

Mouse Models of Syndromic Craniosynostosis

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Keywords
FGFR mutations · Mouse models

Abstract
Craniosynostosis is a common craniofacial birth defect. This review focusses on the advances that have been achieved through studying the pathogenesis of craniosynostosis using mouse models. Classic methods of gene targeting which generate individual gene knockout models have successfully identified numerous genes required for normal development of the skull bones and sutures. However, the study of syndromic craniosynostosis has largely benefited from the production of knockin models that precisely mimic human mutations. These have allowed the detailed investigation of downstream events at the cellular and molecular level following otherwise unpredictable gain-of-function effects. This has greatly enhanced our understanding of the pathogenesis of this disease and has the potential to translate into improvement of the clinical management of this condition in the future.

Craniosynostosis is a common feature of craniofacial birth defects, with a prevalence of 1:2,500 births [Cohen and Kreiborg, 1992]. It is characterised by premature fusion of calvarial bones and can occur along single or multiple cranial sutures. Around 30% of craniosynostosis occurs within a characterised craniofacial syndrome (syndromic craniosynostosis) with a genetic cause, whilst the majority of non-syndromic cases have a de novo cause (non-syndromic craniosynostosis) [Johnson and Wilkie, 2011]. The molecular basis for craniosynostosis is complex: for example, a genetic cause such as a dominant mutation within one of the fibroblast growth factor receptor ( ) 1, 2, and 3 genes are well known; yet, environmental factors, most notably intra-uterine head constraint, have also been hypothesised as among the predisposing factors to this condition [Muenke et al., 1994, 1997; Reardon et al., 1994; Johnson and Wilkie, 2011]. The phenotypic consequence of craniosynostosis is skull shape distortion with secondary sensory-neurological deficits through an increase of intracranial pressure [Derderian and Seaward, 2012]. Typically, mutations are responsible for the crouzonoid phenotype comprising of complex craniosynostosis, midfacial hypoplasia, strabismus, and brachycephaly [Johnson and Wilkie, 2011]. As a result of craniosynostosis, symptoms include optic atrophy, blindness, and hearing deficits [Derderian and Seaward, 2012]. There is currently no pharmacological treatment for craniosynostosis, with repeating surgical modalities as the primary option to accommodate normal brain growth by correcting skull dysmorphology and reducing intracranial pressure, a procedure known as craniectomy [Johnson and Wilkie, 2011]. Specifically,

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surgical interventions aim to re-open the suture (distraction osteogenesis) with calvarial remodelling [Park and Yoon, 2012].

Historically, a mutation in the *2* (Msh homeobox 2) gene was first to be associated with syndromic craniosynostosis, eliciting a clinical phenotype known as Boston type craniosynostosis [Jabs et al., 1993]. Mutations in genes encoding FGFRs were identified later and are perhaps the most common genes involved in syndromic craniosynostosis [Wilkie, 2005]. The most notable characteristics of craniofacial dysmorphology are often referred to as the crouzonoid phenotype, with coronal synostosis being the most common type of suture fusion [Wilkie and Morriss-Kay, 2001]. This usually results from autosomal dominant mutations that constitutively activate the FGF receptor and as such can be thought of as gain-of-function (GOF) mutations [Wilkie, 2005]. A generalisation is that the craniofacial spectrum elicited by FGFRs signalling misregulation will depend on the tissue specificity and precise allelic mutation within the receptor gene [Wilkie, 2005]. Allelic mutations affecting the ligand-binding domain (S252, P253, C278, and C342) account for 80% of all craniosynostosis cases. An interesting observation first mentioned by Wilkie [2005] is that identical substitutions across all FGFR paralogues are conserved at equivalent positions along the gene. For example, an amino acid change within the linker region of each receptor such as Proline 250 to Arginine (p.Pro250Arg) gives rise to either Pfeiffer (FGFR1), Apert (FGFR2), or Muenke (FGFR3) syndromes, with coronal synostosis being a common phenotype of all 3 [Wilkie, 2005]. FGFR1, 2, and 3 are all expressed along the edges of the calvarial bones with FGFR2 predominant in the osteogenic front [Iseki et al., 1999; Johnson et al., 2000]. However, the spatial localisation of the various splice forms is not well characterised due to their high sequence homology. Instead, it is mainly through isoform-specific knockouts in mouse models that their individual functions have been delineated (see below).

Undeniably, mouse models have offered a significant platform to study human disease progression, and generating models carrying specific knockin mutations can help to address questions concerning the phenotypic diversity caused by the various mutations identified in patients. Whilst a large body of research has focussed on the genomic landscape, the biochemical and transcriptomic consequences that influence cellular activity in vivo still remains to be fully elucidated. In order to advance translation to clinical practice, it will be critical to address the aberrant mechanisms that lead toward these craniofacial abnormalities. In this review, we will provide an overview of the currently available mouse models that have been associated with various forms of syndromic craniosynostosis (Table 1). Finally, we will draw conclusions about the n

1, mutating binding sites for FRS2 on ′ *1*, or preventing TRK autophosphorylation [Partanen et al., 1998]. Additionally, Partanen et al. [1998] have achieved isoform-specific knockout to exons 8 (IIIb) and 9 (IIIc) by inserting a stop codon into these exons. ′ *1* appears

mains largely normal. Thus, the lack of a craniofacial phenotype exhibited by FGFR1 LOF implies its role in craniofacial development is minor. However, the , *1* knockout mouse is embryonic lethal, suggesting the importance of the mesodermal isoform in early embryogenesis [Partanen et al., 1998].

FGFR2

FGFR2 is a positive regulator for osteoblast differentiation and manipulating this signalling pathway has consequences for osteoblastogenesis. It is well characterised that , *2*, the master regulator for osteoblast differentiation, is downstream of FGFR2 signalling [Miraoui et al., 2009]. Therefore, a substantial proportion of clinical syndromes and bone diseases have been related to signalling misregulation caused by this receptor. The role of FGFR2 was first characterised using knockout models. Several groups have generated , *2* knockout lines with similar phenotypes, yielding a series of gastrulation, placental and osteogenesis defects [Arman et al., 1998, 1999; Xu et al., 1998; Yu et al., 2003]. The first FGFR2 knockout was generated by targeted disruption to the kinase domain of the receptor, preventing autophosphorylation [Arman et al., 1998]. Other , *2* knockouts followed, by disrupting immunoglobulin loops along the receptor gene [Xu et al., 1998]. Xu et al. [1998] generated a knockout by removing exons encoding the IgIII loop responsible for ligand specificity. Despite homozygous lethality at E10.5, this study was the first to gain insights into the role of FGFR2 in limb development as these mutants fail to develop limb buds due to a loss of paracrine signalling that is responsible for tissue outgrowth [Xu et al., 1998]. It was later discovered from the , *2* / model that the IIIb isoform is critical for limb outgrowth as these mice have a complete loss of the appendicular skeleton [De Moerlooze et al., 2000; Revest et al., 2001]. FGF10 is a likely binding partner for FGFR2b as , *10* / mice exhibit striking similarities to , *2* / mice [Min et al., 1998; Sekine et al., 1999]. On the other hand, , *2* / mice illustrate that this isoform is required for normal craniofacial development as bi-coronal synostosis and underdevelopment of the auditory bulla were reported characteristics [Eswarakumar et al., 2002]. Others have also generated conditional , *2* knockouts to study tissue-specific effects: conditional knockout in the mesenchyme using *1* leads to defects in both axial and craniofacial skeleton [Yu et al., 2003]. Specifically, these mice have decreased bone density, truncated femurs owing to insufficient chondrocyte and osteoblast proliferation, brachycephaly, and dwarfism [Yu et al., 2003].

A large cohort of characterised craniofacial syndromes is commonly associated with *2* germline mutations [Wilkie, 2005]. GOF mutations in the *2* gene are characteristic of Apert, Crouzon, and Beare-Stevenson syndromes [Wilkie, 2005] and establish that FGFR2 signalling is a key player in craniofacial development. Crouzon syndrome is most commonly caused by a substitution mutation in *2* (FGFR2c-p.C342Y; at the DIII Ig loop) and is autosomal dominant [Reardon et al., 1994]. The substitution of a cysteine to a tyrosine residue results in the stabilisation of intermolecular disulphide bonds at the receptor extracellular domains, leading to constitutive activation [Eswarakumar et al., 2005]. The phenotypes associated with the IIIc isoform in Crouzon syndrome are mainly craniofacial, whilst the p.S252W mutation found in Apert syndrome is associated with additional limb phenotypes such as truncation and syndactyly, since the mutation affects both FGFR2 splice variants [Johnson and Wilkie, 2011]. Mouse models are available for the most common FGFR2 craniofacial syndromes: , *2* $^{+}$ (Crouzon), , *2* $^{+}$ (Crouzon), and , *2* ^{250} $^{+}$ (Apert) [Chen et al., 2003; Eswarakumar et al., 2004; Wang et al., 2005; Mai et al., 2010]. A common characteristic in these models are shortened midface, brachycephaly, and coronal suture obliteration, which mimicks the human disease phenotype. Interestingly, none of these models, including Apert mice, display a limb phenotype. On a cellular level, these mutations affect FGFR2 function by altering osteoblast proliferation, differentiation, and apoptosis in the suture. Eswaraskumar et al. [2004] reported around E13.5 an early increase in cellular activity at the osteogenic front that is responsible for suture obliteration in , *2* $^{+}$. Chen et al. [2003], however, reported increased apoptosis as being a key player for coronal synostosis development in a separate mouse model for Apert syndrome (, *2* ^{250} $^{+}$).

It is not well understood how a separate allelic mutation, also affecting the transmembrane domain of FGFR2, gives rise to Beare-Stevenson syndrome (, *2* $^{+}$) [Wang et al., 2012]. Similar to the C342Y mutation, FGFR2-Y394C stabilises intermolecular bonds of unpaired cysteine residues leading towards constitutive activation. However, despite showing craniofacial similarities, Beare-Stevenson patients have additional skin abnormalities including cutis gyrate (thickened scalp) and acanthosis nigricans (hyper pigmentation) [Wang et al., 2012]. A mouse model has been generated to study this mutation (, *2* $^{+}$), but the pathogenic origin of the cutaneous phenotype still remains unclear [Wang et al., 2012]. In addition to introducing GOF mutations, increasing gene

not yet available, perhaps owing to the potency of mutations affecting the kinase domain [Ornitz and Itoh, 2015]. There have been reports linking craniosynostosis to achondroplasia and thanatophoric dysplasia in the literature, but these links are not yet well established. For example, some patients with thanatophoric dysplasia exhibit a cloverleaf skull, suggestive of severe craniosynostosis [Tavormina et al., 1995], another mutation for achondroplasia (FGFR3-p.A391E) has been identified in Beare-Stevenson patients [Meyers et al., 1995] and similarly in the mouse, where an isolated study reports coronal synostosis in , 3 3 0 /+ [Lee et al., 2017].

The p.P250R mutation is the most common mutation identified in all 3 FGFR paralogues [Wilkie, 2005]. In FGFR3, this leads to Muenke syndrome, where unilateral or bilateral coronal synostosis is an apparent characteristic [Muenke et al., 1994]. The p.P250R mutation affects both FGFR3 isoforms and results in increased affinity for FGF ligands [Muenke et al., 1994; Wu et al., 2009]. A knockin of this mutation in the mouse recapitulated Muenke syndrome (, 3 2 /+) [Twigg et al., 2009]. However, the craniofacial phenotype was incompletely penetrant due to background differences between mouse strains. Despite the variability observed in the general craniofacial skeleton, it has been a useful mouse model for

this pathogenesis [Ting et al., 2009; Yen et al., 2010]. Both of these studies stress the importance of contact inhibition in controlling osteoblast differentiation in the mesenchyme, since conditional removal leads to mis-specification, and loss of positional information in specifying the suture boundary. Additionally, LOF mutations in

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available, *1* [Kiyozumi et al., 2006; Vissers et al., 2011]. Both models harbour interfrontal synostosis, with the latter mutant possessing a stronger phenotype [Vissers et al., 2011].

Conclusion and Future Directions

Mouse models have been critical to the study of human disease. Together with lineage tracing reporters, the phenotypes caused by knockin mutations have been critical to understand the pathways required for craniofacial development. Furthermore, mouse models provide a platform to test novel therapeutic strategies and management techniques [Perlyn et al., 2006; Wang et al., 2015; Maruyama et al., 2016; Rachwalski et al., this issue]. However, it is well understood in the murine research community that mouse models of human disease sometimes exhibit differences in phenotypic end points, which might simply be due to species differences. These can be the result of differing genetic redundancies and sensitivities. An example of this can be seen for a *23* mutation responsible for human Carpenter syndrome [Eggenschwiler et al., 2001; Jenkins et al., 2007]. LOF of the mammalian homologue of *23* in the mouse results in exencephaly and early embryonic lethality implicating dosage dependency of the phenotype [Eggenschwiler et al., 2001]. Other examples include the Ets domain-containing transcription factor ( ), where craniosynostosis is only observed in a mouse model harbouring a conditional allele ( ) reducing the expression level to about 30% in contrast to that of a heterozygote null allele (*1*

laser capture microdissection on several mouse models of craniosynostosis including Apert and Saethre-Choetzen syndromes.

Despite the plethora of mouse models generated for craniofacial syndromes, few studies have characterised the downstream signalling misregulation that must contribute to the phenotype. Further disruption of downstream effectors will undoubtedly help to delineate the complex relationship between signal transduction and gene expression. This could be achieved by generating mouse models specifically targeting those signalling intermediates. A comprehensive review of these mouse models has been listed in a recent paper by Dinsmore and Soriano [2018]. As growth factor signalling, such as FGF, is critical for craniofacial development, it would also be ideal to adopt mouse models that are designed to study cancer progression into birth defects research. This is because mouse models such as ^{6} $^{/+}$ or ^{12} $^{/+}$ have mutations that specifically disrupt multiple levels of a signalling cascade (i.e., MAPK/ERK) [Tuveson et al., 2004; Mercer et al., 2005]. Future studies will need to address the impact of the mutation on cascade activation at the level of the receptor in vivo. For example, it has been demonstrated by Miraoui et al. [2009] that the Apert mu-

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