Short Communication

Neural tube closure depends on expression of Grainyhead-like 3 in multiple tissues

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ARTICLE INFO

Keywords:
Neural tube defects
grainyhead
spina bifida
curly tail
mouse embryo

ABSTRACT

Failure of neural tube closure leads to neural tube defects (NTDs), common congenital abnormalities in humans. Among the genes whose loss of function causes NTDs in mice, Grainyhead-like3 (Grhl3) is essential for spinal neural tube closure, with null mutants exhibiting fully penetrant spina bifida. During spinal neurulation Grhl3 is initially expressed in the surface (non-neural) ectoderm, subsequently in the neuroepithelial component of the neural folds and at the node-streak border, and finally in the hindgut endoderm. Here, we show that endoderm-specific knockout of Grhl3 causes late-arising spinal NTDs, preceded by increased neural curvature of the caudal region which was shown previously to suppress closure of the spinal neural folds. This finding supports the hypothesis that diminished Grhl3 expression in the hindgut is the cause of spinal NTDs in the curly tail, carrying a hypomorphic Grhl3 allele. Complete loss of Grhl3 function produces a more severe phenotype in which closure fails earlier in neurulation, before the stage of onset of expression in the hindgut of wild-type embryos. This implicates additional tissues and NTD mechanisms in Grhl3 null embryos. Conditional knockout of Grhl3 in the neural plate and node-streak border has minimal effect on closure, suggesting that abnormal function of surface ectoderm, where Grhl3 transcripts are first detected, is primarily responsible for early failure of spinal neurulation in Grhl3 null embryos.

1. Introduction

In higher vertebrates neural tube closure is achieved through coordinated shaping and bending of the neural plate to form bilateral neural folds which adhere in the midline (Greene and Copp, 2014; Nikolopoulou et al., 2017). Closure propagates between initial sites of adhesion to seal the intervening open regions, termed neuropores. Failure to complete closure results in severe birth defects, termed neural tube defects (NTDs), which include spina bifida and anencephaly (Copp et al., 2013). The closure process is not solely dependent on intrinsic properties of the neuroepithelium (Pai et al., 2012; Ray and Niswander, 2012). The leading edges of the neural folds at the closure site comprise neuroepithelium with overlying surface (non-neural) ectoderm and intervening extracellular matrix. Notably, initial contact appears to be mediated by surface ectoderm cells at the boundary with the neuroepithelium at both cranial and spinal levels (Ray and Niswander, 2016; Rolo et al., 2016).

Among the genes required for neural tube closure, members of the grainyhead-like family of transcription factors (Gustavsson et al., 2008; Ting et al., 2003a) appear essential at both cranial and spinal levels of the body axis. For example, mice lacking expression of Grhl3 (previously Get1 or Som) exhibit fully penetrant spina bifida, with a low frequency of exencephaly, the developmental forerunner of anencephaly (Ting et al., 2003a, 2003b; Yu et al., 2006).

At later stages of development and post-natally, Grhl3 has key functions in epithelia, being required for differentiation of the epidermis during late-fetal development. Knockouts display defective barrier formation, as well as impaired wound healing (Gordon et al., 2014; Hopkin et al., 2012; Ting et al., 2005; Yu et al., 2006). The function of Grhl3 in epidermal differentiation parallels the requirement for Drosophila grainyhead (grh) in regulating cuticle development (Uv et al., 1997). Although attention has focussed on the role of Grhl3 in surface ectoderm and skin, expression is also present in epithelia of the
gastrointestinal tract, bladder and lung (Kudryavtseva et al., 2003). Cdhl1 (encoding E-cadherin), a marker of surface ectoderm and epithelia, is a direct target of Grhl3 in mouse mammary gland cells (Aloai et al., 2015).

At neurulation stages, Grhl3 is expressed from the earliest stages in the surface ectoderm, in keeping with its later function in epidermal differentiation. However, it is also expressed in other sites including transiently in the spinal neuroepithelium and in the hindgut endoderm (Gustavsson et al., 2007; Gustavsson et al., 2008; Ting et al., 2003a, 2003b). The identification of Grhl3 expression in several tissues during neurulation raises questions over which expression site(s) is essential for neural tube closure. For example, it has been proposed that Grhl3 contributes to delineation of the border between surface ectoderm and the neuroepithelium (Kimura-Yoshida et al., 2015).

A requirement for Grhl3 function in the hindgut endoderm was suggested by analysis of the curly tail (ct) mouse strain, which carries a hypomorphic allele of Grhl3 and exhibits partially penetrant spinal NTDs (Gustavsson et al., 2007; Van Straaten and Copp, 2001). Neural tube closure is apparently unaffected in approximately 50% of homozygous ct/ct embryos, while affected embryos can be recognised by the presence of an enlarged posterior neuropore (PNP) at embryonic day 10.5 (E10.5; Copp, 1985). This is indicative of subsequent failure or delay of closure of the spinal neural tube, which lead to spina bifida (approximately 15% of embryos) or a tail flexion defect (approximately 40%), respectively. Failure of PNP closure in ct/ct embryos correlates with reduced cellular proliferation rate in the hindgut endoderm and notochord (Copp et al., 1988a). In combination with ‘normal’ proliferation in the neuroepithelium, this causes a growth imbalance between dorsal and ventral tissues leading to increased ventral curvature of the caudal region of the embryo. Increased curvature mechanically opposes closure, as demonstrated in both mouse and chick embryos (Van Straaten et al., 1993); indeed, splitting of the caudal region allows PNP closure to progress normally in cultured ct/ct embryos (Brook et al., 1991). Moreover, normalisation of the growth imbalance, either through suppression (Copp et al., 1988b) or stimulation of proliferation (Cogram et al., 2004; Leung et al., 2013), prevents spinal NTDs in the ct mutant.

Grhl3 expression is diminished in ct/ct embryos and their spinal NTDs can be prevented by Bac transgene-mediated expression of Grhl3 (Gustavsson et al., 2007). In transgenic embryos, Grhl3 expression was increased in each of the endogenous sites of expression, therefore leaving open the question of whether Grhl3 expression is required in tissues other than the hindgut for neural tube closure. Moreover, the frequency of NTDs in the ct/ct strain is strongly influenced by genetic modifiers, raising the possibility that one of these is responsible for the hindgut phenotype (Lettis et al., 1995; Neumann et al., 1994; Van Straaten and Copp, 2001; de Castro et al., 2012). These considerations led us to investigate the tissue-specific requirements for Grhl3 function in neural tube closure, in order to ask whether expression is needed in more than one tissue, possibly at different developmental stages.

2. Materials and Methods

2.1. Mice

A conditional (floxed) allele of Grhl3 (designated Grhl3fi/+; ) has been described (Yu et al., 2006). These mice were crossed to β-actin-Cre mice to generate heterogeneous null, Grhl3fi/-, mice used in subsequent experimental matings. Tissue-specific cre-driver lines were Sox17-2A-iCre (Engert et al., 2009), Nkx1-2-Cre (Albors et al. http://dx.doi.org/10.1101/045872) and Grhl3-Cre (Camerer et al., 2010). Cre lines were visualised by crosses to mice carrying the Gr(Rosa)26SorcreIRESERTFPScas reporter allele (Srinivas et al., 2001). Mice were genotyped by PCR of genomic DNA, as described (Camerer et al., 2010; Gustavsson et al., 2007; Yu et al., 2006).

Animal studies were carried out under regulations of the Animals (Scientific Procedures) Act 1986 of the UK Government, and in accordance with guidance issued by the Medical Research Council, UK in Responsibility in the Use of Animals for Medical Research (July 1993). Litters were generated by timed matings in which mice were paired overnight and the day of finding a copulation plug was designated embryonic day 0.5 (E0.5). YFP expression in whole mount embryos was visualised by direct fluorescence. Embryos for in situ hybridisation were rinsed in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) in PBS at 4 °C overnight.

2.2. Morphological measurements

The PNP length of embryos at E8.5 to E10.5 was measured using an eye-piece graticule, from the tip of the tail bud to the rostral limit of the open neural folds. For measurements of ventral curvature, the caudal region of E9.5-10.5 embryos was isolated from the body, photographed and curvature of the caudal region measured as described (Brook et al., 1991; Brouns et al., 2011).

Whole mount in situ hybridisation was performed as previously reported (de Castro et al., 2012; Gustavsson et al., 2007), following which embryos were embedded in albumin-gelatine and 40 μm sections obtained on a vibratome. Images were processed using Photoshop (Version 6.0) for cropping and figures were prepared using Adobe Illustrator software.

2.3. Quantitative real time RT-PCR (qRT-PCR)

RNA was isolated from the caudal region of E8.5 (10-14ss, cut at somite 10), E9.5 (15-16ss, cut at somite 12) and E10.5 (26-31ss, cut at somite 14) embryos. Total RNA was isolated using TRIzol Reagent (Gibco) followed by DNase treatment (DNA-free, Ambion). Primers for qRT-PCR were designed to give a product of 150–250 bp (sequences available on request) and optimised for 60°C annealing temperature. RNA extraction, cDNA synthesis and quantitative RT-PCR were performed using glyceraldehyde-3-phosphate dehydrogenase (Gapdh) for normalisation, as previously (Gustavsson et al., 2007; Brouns et al., 2011). qRT-PCR used iTAQ Universal SYBR Green Supermix assay (Bio-Rad) on a CFX96 system (Bio-Rad) with analysis using Bio-Rad CFX Manager software. Individual experiments were combined and analysed using Sigma Stat software (ANOVA or t-test).

3. Results

3.1. Excess body curvature in Grhl3 null embryos

Genetic mapping and transgenic rescue provide evidence that the major genetic cause of NTDs in ct/ct embryos is a hypomorphic allele of Grhl3 (here denoted Grhl3fi). In this model, the causative cellular mechanism is thought to be diminished proliferation in the hindgut and consequent excess curvature of the caudal region that opposes closure (see above; Van Straaten and Copp, 2001). However, it remained formally possible that reduced proliferation in the hindgut was a result of modifier gene action with NTDs resulting from summation with a deleterious effect of insufficient Grhl3 expression in another tissue. We investigated this question in embryos carrying combinations of null or ‘floxed’ alleles of Grhl3 (Yu et al., 2006). We first examined litters from intercross of Grhl3fi/- mice. At late E10.5, the caudal region of Grhl3fi/- embryos showed obvious excess curvature (Fig. 1A-D) and the posterior neuropore (PNP) remained extensively open (Fig. 1B, D), whereas closure is complete in all wild-type embryos at this stage. Earlier on E10.5, when PNP closure is still incomplete in wild-type and Grhl3fi/- embryos (Fig. 1E, F), the latter already showed a significantly increased ventral curvature of the caudal region (Fig. 1G).

These observations are consistent with the hypothesis that diminished Grhl3 expression can affect axial curvature, as observed in affected ct
embryos at the same stage (Brook et al., 1991). Hence, increased curvature in ct/ct embryos is unlikely to be due solely to genetic modifiers.

3.2. Loss of Grhl3 expression in the gut endoderm is sufficient to cause spinal NTDs

Next, we directly tested the requirement for Grhl3 expression in the hindgut by conditional deletion of Grhl3 in the endoderm. A ‘floxed’ allele of Grhl3, designated Grhl3<sup>fl/fl</sup> (Yu et al., 2006) was recombined by Sox17-2A-ICre, which is active in the hindgut from E8.5 but not in other Grhl3 expression sites (Engert et al., 2009). Litters from matings of Sox17<sup>2A-ICre</sup>; Grhl3<sup>fl/fl</sup> with Grhl3<sup>fl/fl</sup> mice (dams included either parental genotype) were analysed at E11.5-15.5 (Fig. 2A-C). NTDs were not observed among Cre-negative Grhl3<sup>fl/fl</sup> or Grhl3<sup>+/+</sup> embryos (both genotypes are effectively heterozygous), whereas spina bifida occurred in 60% (6/10 embryos) and tail flexion defects in 70% (7/10) of Sox17<sup>2A-ICre</sup>; Grhl3<sup>fl/fl</sup> embryos. We conclude that loss of Grhl3 expression in the gut endoderm is sufficient to cause NTDs.

In order to confirm that gut-conditional NTDs result from failure of PNP closure, rather than later re-opening of a closed neural tube, additional litters were collected at E10-10.5. Measurements were made of PNP length (Fig. 2D), an enlarged PNP being indicative of ensuing delay or failure of closure (Copp, 1985). The mean PNP length of Sox17<sup>2A-ICre</sup>; Grhl3<sup>fl/fl</sup> embryos became abnormally enlarged from the 24–27 somite stage (Fig. 2D; Fig. S2), consistent with failure of the normal onset of Grhl3 expression in the hindgut endoderm from this stage (Fig. 3A).

3.3. Grhl3 is required in multiple tissues during neural tube closure

All Grhl3<sup>fl/fl</sup> fetuses generated in the experimental intercross of Sox17<sup>2A-ICre</sup>; Grhl3<sup>fl/fl</sup> with Grhl3<sup>fl/fl</sup> developed spina bifida (Fig. 2B) and the extent of the open spinal lesion appeared greater than in hindgut-conditional mutants (examples shown in Fig. 2C). Notably, PNP length was already significantly enlarged in Grhl3<sup>fl/fl</sup> embryos at 20–23 somites, in contrast to hindgut-conditional embryos whose PNP length had not yet become different from wild-type and heterozygous embryos (Fig. 2D). Hence, failure of PNP closure occurs at an earlier stage in the constitutive null embryos than in hindgut-conditional. To investigate this further, additional experimental litters were generated by intercrossing Grhl3 heterozygotes. PNP length was enlarged from as early as the 8–11 somite stage (at E8.5) in Grhl3<sup>fl/fl</sup> embryos (Fig. 3B). This suggests that spinal neurulation becomes defective immediately after initiation of closure which typically occurs at the 5–7 somite stage. Failure of the initiation event (Closure 1) itself leads to craniorachischisis, which was not observed in Grhl3<sup>fl/fl</sup> embryos, here or in previous studies (Ting et al., 2003a; Yu et al., 2006). Hence, the entire of spinal closure, post-Closure 1, requires Grhl3 function.

We demonstrated a requirement for Grhl3 in the hindgut during later spinal neurulation, but Grhl3 is not expressed in the hindgut at E8.5-9, when closure first fails (Fig. 3A). Therefore, Grhl3 function in another embryonic tissue must underlie this early-stage closure defect. In embryos with 8–11 somites, the surface ectoderm is the only tissue at the axial level of the ‘zippering point’ which expresses Grhl3 (Fig. 3A), suggesting a vital role for Grhl3 function in this tissue for progression of closure. A putative role for the surface ectoderm in NTDs is consistent with the finding that initial contact of the neural fold tips in the spinal region is mediated by surface ectoderm cells at the border with the neural plate (Rolo et al., 2016). We analysed selected genes that function in epidermal differentiation in the caudal region of embryos at E9, shortly after onset of epidermal specification of the surface ectoderm. By qRT-PCR, Trp63 (encoding Tap63) (Koster et al., 2007), a key transcriptional regulator of epidermal specification, and its target Tpfap2c (Koster et al., 2007), were significantly down-regulated in Grhl3 null embryos (Table S1), suggesting a possible delay in specification of the surface ectoderm. By E10.5, the basal keratin Krt5 was also down-regulated in Grhl3 null embryos (Table S2),
although Krt8 and Krt18 (expressed in uncommitted surface ectoderm) were not significantly altered at E10.5.

Although Grhl3 is only expressed in the surface ectoderm at the PNP closure site at E8.5, mRNA is also detected in the posterior part of the embryo corresponding to the node-streak border and caudo-lateral epiblast, where neuro-mesodermal progenitors (NMPs) are located (Henrique et al., 2015)(Fig. 3A). Later, at E9.5, mRNA is also detected in the neural plate of the PNP as well as in the hindgut endoderm even later, at E10-10.5 (Gustavsson et al., 2007)(Fig. 3A). Expression in the surface ectoderm and neural plate at E9.5 was confirmed using Grhl3cre/+ embryos, in which β-galactosidase is expressed from the Grhl3 locus (Fig. S1A-C), consistent with previous studies of the Grhl3cre allele (Camerer et al., 2010). Moreover, use of Grhl3cre to recombine R62R-YFP for lineage tracing revealed expression in all surface ectoderm cells and in a mosaic pattern within the neuroepithelium and paraxial mesoderm (Fig. S1D-H)(Rolo et al., 2016), consis-
Fig. 3. Grhl3 is required for spinal neurulation prior to expression in neural plate and/or hindgut. (A) Grhl3 exhibits a dynamic expression pattern during neurulation. Expression is evident in the surface ectoderm on the outside of the spinal neural folds, and at E8.5 in the caudal lateral epiblast (white arrowhead) as well as some cells in the neuroepithelium (black arrowhead). Expression is detected in the neuroepithelium (np) at E9.5 and hindgut at E10.5. Dashed white lines in i-iii indicate level of sections in i-iii; scale bars represent 0.5 mm in i-iii and 0.1 mm in i-iii. PNP length delineated by dashed black lines in Aii. (B) Analysis at E8.5-10.5 shows that the mean PNP length of Grhl3 null embryos (n = 78) is significantly enlarged from the 8–11 somite stage onwards compared with Grhl3 heterozygous (Grhl3 fl/fl; n = 152) and wild-type (n = 100) littermates (# significantly different compared with other genotypes; p < 0.001, ANOVA; n = 10–47 embryos at each somite interval except 2–5 embryos at 28–31 somite stage). (C-F) Expression of Sox2 (C-D) and Nkx1-2 (E-F) does not differ in E9.0 Grhl3fl/fl embryos (D, F) from other genotypes (C, E). Transverse sections (C–F; at level of white dashed lines in C–F) show expression of both genes in the open neural folds (D). Hindgut is outlined by black dashed lines in E–F. Scale bars represent 0.5 mm in D–F; and 0.1 mm in C and C–F.

In order to further investigate the tissue-specific requirement for Grhl3 expression, we performed additional experimental matings (Nkx1-2Cre+/++;Grhl3f/f x Sox17Cre+/++;Grhl3f/f) to generate embryos in which the ‘floxed’ Grhl3 allele was recombined in both hindgut endoderm and neural plate (Nkx1-2Cre+/++;Sox17Cre+/++;Grhl3f/f) but not surface ectoderm. In this cross, PNP closure was still incomplete at late E10.5 (32 or more somites) among 4/5 Nkx1-2Cre+/++;Grhl3f/f and 2/3 Sox17Cre+/++;Grhl3f/f embryos, compared with only 1/6 cre-negative Grhl3f/f controls (Fig. 3C). As in the previous cross, the PNP length of embryos expressing Sox17Cre+/++;Grhl3f/f was significantly enlarged from the 24–27 somite stage onwards. The additional presence of Nkx1-2Cre+/++;Grhl3f/f did not lead to a further increase in mean PNP length (Fig. 3C). There was a significant increase in ventral curvature of the caudal region of Sox17Cre+/++;Grhl3f/f and Sox17Cre+/++;Grhl3f/f embryos compared with Grhl3f/f controls (Fig. 3D). In contrast, ventral curvature of Nkx1-2Cre+/++;Grhl3f/f embryos was comparable to controls (Fig. 3D). These findings support the hypothesis that it is the loss of Grhl3 expression in the hindgut that is primarily responsible for excess curvature and failure of PNP closure.

Together, these data suggest that Grhl3 expression in the hindgut, and to a much lesser extent in the neuroepithelium, is required at the later stages of spinal closure at E10.5-10.5, whereas spinal neural tube closure first requires Grhl3 function in the surface ectoderm, from an early stage after the initial closure event at E8.5.

4. Discussion

Spinal neurulation appears to be highly dependent on Grhl3 with diminished expression, as in the curly tail (ct/ct) strain (Gustavsson et al., 2007), complete loss in Grhl3f/f (Yu et al., 2006) or tissue-specific loss in the hindgut (this study) all causing failure of PNP
closure, leading to spina bifida. During neural tube closure, Grhl3 is expressed not only in the surface ectoderm, but also at additional sites (neuroepithelium, gut endoderm and node-streak border/caudo-lateral epiblast). Our findings demonstrate a requirement for Grhl3 expression in at least two tissues, including the hindgut. Previous studies suggested that NTDs in curly tail embryos result from defective hindgut cell proliferation that disrupts closure via a biomechanical effect of excess axial curvature on neural fold elevation and apposition (Van Straaten and Copp, 2001). The neuroepithelium of the PNP is tightly attached to the underlying notochord and hindgut by extracellular matrix (O’Shea, 1987), so that defective growth of the hindgut serves to biomechanically deform the overlying neural plate. Splinting of the caudal region to avoid development of body axis curvature rescues PNP closure in otherwise untreated curly tail embryos (Brook et al., 1991), further indicating the importance of this pathogenic mechanism for spinal NTDs. In line with this hypothesis, rescue of PNP closure by inositol (Cogram et al., 2004), nucleotides (Leung et al., 2013) or Grhl3-BAC transgenesis (Gustavsson et al., 2008) is associated with stimulation of proliferation in the hindgut. Moreover, polymorphic variants of lamin B1 that differentially affect NTD frequency in curly tail embryos show concordant differences in cell proliferation (de Castro et al., 2012).

In the present study, we obtained strong supporting evidence for Grhl3 involvement in the hypothesised mechanism of curly tail spinal NTDs, in particular from the finding of low spinal NTDs in gut-specific conditional Sox17cre; Grhl3f/− mutant embryos. This observation provides an independent demonstration of the requirement for Grhl3 expression in the hindgut, during completion of PNP closure. Additional evidence includes the relatively late stage at which PNP closure first becomes abnormal in both curly tail and Sox17cre; Grhl3f/− mutant embryos (E10), which correlates with the stage of onset of Grhl3 expression in the hindgut during wild-type development.

Grhl3 is presumably required, therefore, to promote a normal rate of cell proliferation in the embryonic hindgut, at least at neurulation stages. However, studies in other systems implicate Grhl3 as a negative influence on cell proliferation. For example, in developing skin, Grhl3 mutations cause hyper-proliferation (Ting et al., 2005), while in normal tongue papillae, loss of Grhl3 results in enhanced cell proliferation (Adhikari et al., 2017). Hence, the specific requirement for Grhl3 in hindgut cell proliferation remains to be determined.
In addition to the relatively late requirement for Grhl3 expression in the embryonic hindgut, stage-dependent analysis shows there is also an earlier requirement for Grhl3 in spinal neurulation. Null embryos develop severe NTDs owing to early onset of abnormal PNP closure, from E8.5, more than a day before this is observed in endoderm-specific mutants. While closure initiation (Closure 1) occurs successfully, the whole of the subsequent spinal closure fails in Grhl3−/− embryos, generating extensive spina bifida. Several lines of evidence suggest a vital role of Grhl3 in the surface ectoderm at this early stage (8–11 somites) when closure starts to be delayed. First, Grhl3 expression can only be detected in the surface ectoderm at this stage. Expression in the neuroepithelium does not begin until 12 or more hours later, and hindgut expression has an even later onset. Second, conditional inactivation of Grhl3 specifically in the neuroepithelium using Nkx1-2cre is not detrimental to neural tube closure, arguing against a key role for Grhl3 in the neuroepithelium. Third, genetic markers of surface ectoderm establishment and differentiation are reduced in expression in the caudal region of Grhl3−/− embryos at the stage when neural tube closure defects first become apparent. Hence, Grhl3 expression in the surface ectoderm appears essential for the early stages of spinal neural tube closure in the mouse embryo.

A priority for future work is to conduct a conditional gene targeting approach to specifically eliminate Grhl3 function from the early surface ectoderm, in order to directly test its hypothesised role in early spinal neurulation. This approach was not possible in the present study, owing to the lack of a suitable cre-driver for early surface ectoderm. While Keratin14-cre is frequently used to eliminate gene function in the epidermis (Jonkers et al., 2001), we found that significant cre-mediated recombination occurs only following completion of spinal neurulation (data not shown), making K14-cre unsuitable for this study. The only other commonly used cre-driver for the surface ectoderm are required to enable its developmental roles to be fully evaluated.

In conclusion, Grhl3 null embryos are subject to two successive closure-preventing insults: an initial surface ectoderm-mediated defect that hampers closure during early spinal neurulation, and a subsequent inhibitory influence imposed by caudal curvature resulting from lack of Grhl3 expression in the hindgut. The latter causes low spina bifida in homozygotes for the Grhl3fl/fl allele, and renews the earlier closure delay in Grhl3−/− null embryos. In terms of clinical relevance, this study provides proof-of-principle for a multi-hit model of birth defects resulting from a single key gene with distinct, critical functions in sequentially developing embryonic tissues.

Acknowledgments

We thank Kate Storey for sharing unpublished reagents. We thank Gabriel Galea and Evangelia Nikolopoulos for helpful discussions.

Funding

The project was funded by the Medical Research Council (G0802163, J003794 to NG, AC) and Wellcome Trust (087525 to AC, NG). NG and AC are supported by Great Ormond Street Hospital Children’s Charity. Research was supported by the NIHR Great Ormond Street Hospital Biomedical Research Centre.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.01.016.

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