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ORIGINAL INVESTIGATION

Examination of *FGFRL1* as a candidate gene for diaphragmatic defects at chromosome 4p16.3 shows that *Fgfrl1* null mice have reduced expression of *Tpm3*, sarcomere genes and *Lrtm1* in the diaphragm

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Abstract *Fgfrl1* (also known as *Fgfr5;* OMIM 605830) homozygous null mice have thin, amuscular diaphragms and die at birth because of diaphragm hypoplasia. *FGFRL1* is located at 4p16.3, and this chromosome region can be deleted in patients with congenital diaphragmatic hernia (CDH). We examined *FGFRL1* as a candidate gene for the diaphragmatic defects associated with 4p16.3 deletions and re-sequenced this gene in 54 patients with CDH. We confirmed six known coding single nucleotide polymorphisms (SNPs): c.209G > A (p.Pro20Pro), c.977G > A (p.Pro276Pro), c.1040T > C (p.Asp297Asp), c.1234C > A (p.Pro362Gln), c.1420G > T (p.Arg424Leu), and c.1540C > T (p.Pro464Leu), but we did not identify any gene mutations. We genotyped additional CDH patients for four of these six SNPs, including the three non-synonymous SNPs, to make a total of 200 chromosomes, and found that the allele frequency for the four SNPs, did not differ significantly between patients and normal controls ($p \geq 0.05$). We then used Affymetrix Genechip® Mouse Gene 1.0 ST arrays and found eight genes with significantly reduced expression levels in the diaphragms of *Fgfrl1* homozygous null mice when compared with wildtype mice—*Tpm3*, *Fgfrl1* ($p = 0.004$), *Myl2*, *Lrtm1*, *Myh4*, *Myl3*, *Myh7* and *Hephl1*. Lrtm1 is closely related to Slit3, a protein associated with herniation of the central tendon of the diaphragm in mice. The Slit proteins are known to regulate axon branching and cell migration, and inhibition of *Slit3* reduces cell motility and decreases the expression of *Rac* and *Cdc42*, two genes that are essential for myoblast fusion. Further studies to determine if *Lrtm1* has a similar function to *Slit3* and if reduced *Fgfrl1* expression can cause diaphragm hypoplasia through a mechanism involving decreased myoblast motility and/or myoblast fusion, seem indicated.

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Introduction

In recent years, array comparative genomic hybridization has been used to map chromosome regions that contain genes required for normal diaphragm formation (Slavotinek et al. 2006; Kantarci et al. 2006; Scott et al. 2007). These

array studies and previous cytogenetic case reports have shown that monosomy for distal chromosome 4p16 can be associated with congenital diaphragmatic hernia (CDH; for review of chromosome aberrations at 4p16 and CDH, see Holder et al. 2007). Deletions of 4p16 are also associated with Wolf–Hirschhorn syndrome (WHS), and there are several reports of WHS patients who have had terminal deletions of chromosome 4p and CDH (Tachdjian et al. 1992; Howe et al. 1996; Sergi et al. 1998; van Dooren et al. 2004; Van Buggenhout et al. 2004; Casaccia et al. 2006; Slavotinek et al. 2006; Table 1). The smallest chromosome 4p16.3 deletion associated with a diaphragmatic defect was 2.4 Mb in size and extends from chromosome 4pter to probe D4S43 (Casaccia et al. 2006). The recurrent deletions in WHS patients with CDH establish the 4p16.3 terminal region as highly likely to contain a gene that is involved in diaphragm development, with the critical region being the terminal 2.4 Mb of 4p16.3 as described above (Casaccia et al. 2006). However, CDH is not invariably associated with WHS and terminal 4p16.3 deletions, and there are reports of WHS patients with deletions encompassing *FGFRL1* in whom CDH was not noted (Engbers et al. 2009).

We used Endeavour and TOPPgene gene prioritization software to examine the terminal 2.4 Mb of chromosome 4p16.3 for candidate genes for CDH. Although the results returned from both programs were not entirely in agreement, *Fgfrl1* (also known as *Fgfr5;* OMIM 605830; Wiedemann and Trueb 2000; Kim et al. 2001; Sleeman et al. 2001) returned as a possible candidate gene from the two separate programs (Table 2). *Fgfrl1* has seven exons, of which the last six are coding exons. Early in murine development at E10.5, *Fgfrl1* is present in the brain, cranial placodes, pharyngeal arches, somites and heart (Catela et al. 2009). At E15–E17, *Fgfrl1* is expressed throughout the diaphragm and the intercostal and tongue muscles (Trueb and Taeschler 2006). The gene is also highly expressed in nasal, rib and tracheal cartilage and in the intermediate cartilage from bone primordia (Trueb et al. 2003; Trueb and Taeschler 2006). Although the exact function of Fgfrl1 is unknown, the protein forms constitutive dimers and promotes cell adhesion mediated by heparan sulfate, a glycosaminoglycan of the extracellular matrix (Rieckmann et al. 2008). Fgfrl1 also binds Fgf2, a stimulator of muscle cell proliferation, although it does so with a lower binding affinity than Fgfr2 (Sleeman et al. 2001).

Table 1 Summary of diaphragmatic defects in patients with monosomy for chromosome 4p16

Patient	Clinical diagnosis	Karyotype	Mapping; estimated size	Diaphragm defect	Pulmonary hypoplasia	Reference
Case A	WHS	Not done	Not done	CDH, type not described	L lung	Lazjuk et al. (1980)
Case B	WHS	Not done	Not done	CDH, type not described	L lung	Lazjuk et al. (1980)
Case 5	WHS	46,XY,del(4)(p16)	Not done	CDH, L-sided	Bilateral	Tachdjian et al. (1992)
–	WHS	46,XX, der(4) t(4;13)(p16;q32) ish der(4)t(4;13)(p16;q32) (WHS-)	Not done	CDH, type not described	Not mentioned	Tapper et al. (2002)
–	WHS	46,XY,del(4)(p16)	Not done	CDH, type not described	Not mentioned	Howe et al. (1996)
–	Dandy-Walker malformation; facial cleft	46,XX,del(4)(p16)	Not done	CDH, type not described	Not mentioned	Howe et al. (1996)
–	WHS	46,XX,del(4)(pter → 13)	Not done	CDH, L-sided	Bilateral	Sergi et al. (1998)
–	WHS	46,XY.ish del(4)(p16.1)	RP274B16 (deleted) RP11-173B23 (not deleted)	CDH, L-sided Bochdalek	Bilateral	Van Dooren et al. (2004)
Patient 1	WHS	46,XX,del(4)(p16.3)	RP513G18 (deleted); RP11-489M13 (not deleted); 3.81 Mb	CDH, L-sided	Not mentioned	Van Buggenhout et al. (2004)
–	WHS	46,XX,del(4)(p15.2)	Not done	CDH		Basgul et al. (2006)
–	WHS	46,XX ish del(4)(p16.3)	D4S43 to telomere; 2.4 Mb	CDH, L-sided	Not mentioned	Casaccia et al. (2006)
–	WHS	46,XY,del(4)(p16)	RP11-97H19 (deleted); RP11-101J14 (not deleted); 8 Mb	CDH, L-sided	Bilateral	Slavotinek et al. (2006)

WHS Wolf–Hirschhorn syndrome, *CDH* congenital diaphragmatic hernia, *L* left, *L-sided* left-sided

Table 2 Gene prioritization rankings for diaphragmatic hernia using Endeavour and TOPPgene software programs

Ranking	Endeavour[a]	TOPPgene[b]	Overall *p* value (TOPPgene only)[b]
1	*FGFRL1*	*SPON2*	0.008
2	*FGFR3*	*FGFR3*	0.020
3	*IDUA*	*FGFRL1*	0.193
4	*WHSC1*	*TACC3*	0.244
5	*CTBP1*	*WHSC1*	0.291
6	*MAEA*	*RNF212*	0.652
7	*SPON2*	*KIAA1530*	0.658
8	*MXD4*	*TMEM175*	0.701

Only the top eight ranked genes are shown

[a] Endeavour (http://homes.esat.kuleuven.be/~bioiuser/endeavour/endeavour.php)

[b] TOPPgene prioritization software (http://toppgene.cchmc.org/)

Fgfrl1 homozygous null mice have been created by removing the first two exons of *Fgfrl1*, and the mutant mice were found to have thin and amuscular diaphragms (Baertschi et al. 2007). These mice did not have diaphragmatic hernias, but at E18.5, the liver was pushed tightly against the dorsal and costal regions of the diaphragm to form a small protrusion and the dorsal and costal ends of the diaphragms had a thickness of 60% of the wildtype diaphragms (Baertschi et al. 2007). The muscle hypotrophy was specific to the diaphragm and the remaining muscles in the *Fgfrl1* null mice were normal (Baertschi et al. 2007). A second mouse model of *Fgfrl1* loss of function was also made by deleting exons 3–7 of *Fgfrl1* (Catela et al. 2009). This mutant was described as having the same diaphragm defects as previously reported (Baertschi et al. 2007; Catela et al. 2009), reduced growth, skeletal anomalies resembling those found in the WHS phenotype in humans, a reduction in laryngeal cartilage size, cardiac defects with faulty septation of the heart, anemia and maldevelopment of the yolk sac (Catela et al. 2009). In both mouse models of loss of *Fgfrl1* function, heterozygotes were normal (Catela et al. 2009). In *Danio rerio*, depletion of the *Fgfrl1a* and *Fgfrlb* genes caused malformations of the lower jaw and inhibited the development of cartilage formed by the branchial arches (Hall et al. 2006). *FGFRL1* has therefore been implicated in the pathogenesis of the craniofacial anomalies found in WHS (Catela et al. 2009; Engbers et al. 2009).

We asked if *FGFRL1* was implicated in the pathogenesis of CDH associated with 4p16.3 deletions and WHS. We re-sequenced *FGFRL1* in patients with CDH and normal controls and performed expression arrays on cDNA obtained from the diaphragms of homozygous null and wildtype mice. The following paper describes our results.

Materials and methods

Gene prioritization software

We used Endeavour (http://homes.esat.kuleuven.be/~bioiuser/endeavour/endeavour.php) and TOPPgene prioritization software (http://toppgene.cchmc.org/). For training or comparison genes, we provided the genes previously determined to be mutated in human syndromes with CDH, including *WT1*, *GPC3*, *CHD7*, *NIPBL*, *EFNB1*, *LRP2*, *DLL3*, *FBN1*, *CHRNG*, *STRA6* and *HCCS* (Entezami et al. 1998; Bulman et al. 2000; Li et al. 2001; Revencu et al. 2004; Vissers et al. 2004; Vasudevan et al. 2006; Wimplinger et al. 2006; Kantarci et al. 2007; Pasutto et al. 2007; Antonius et al. 2008), and genes known to be associated with diaphragmatic defects in null mice, including *Fog2*, *Slit3*, *Robo1* and *Lox* (Hornstra et al. 2003; Yuan et al. 2003; Ackerman et al. 2005). We used all of the Ref Seq genes from chromosome 4, nucleotides 1–2,406,426 (numbering according to hg 18; UCSC Genome Browser, http://genome.ucsc.edu) as the candidate genes, using the location of D4S43 to provide the outer boundary of the test region (Casaccia et al. 2006; UCSC genome browser http://genome.ucsc.edu/). Although the results of both programs were not entirely in agreement, both programs ranked *Fgfrl1* highly, and the finding of diaphragmatic defects in mice that were homozygous null for Fgfrl1 lead to our selection of this gene for initial study.

Patients

DNA samples were obtained from patients with diaphragmatic defects using two protocols approved by the Committee for Human Subjects Research (CHR) at the University of California, San Francisco (UCSF; CHR numbers H41842-22157-07 and H41842-26613-05). We used 54 DNA samples from patients with isolated diaphragmatic hernias (the ethnic mix of this group was 48 Caucasian, 33 Hispanic, 7 African American, 6 Asian and 6% unknown or mixed) and 55 samples from patients with diaphragmatic hernia and additional anomalies (the ethnic mix of this group was 44 Hispanic, 36 Caucasian, 9 Asian, 7 African American and 4% other/unknown). None of the patients with additional anomalies had been diagnosed with an underlying genetic syndrome as an explanation for their diaphragmatic defect. Some of these patient samples were derived from dried neonatal blood specimens obtained by the California Birth Defects Monitoring Program as part of a larger genetic investigation of risk factors of birth defects. Use of the human specimens for the purposes of the main study and the current sub-study was approved by the California Committee for the Protection of Human Subjects. The control samples comprised 109 samples from patients

of Caucasian ethnicity without known diaphragmatic defects or skeletal anomalies (Rieckmann et al. 2008).

Genomic sequencing

We sequenced the exons and the exon–intron boundaries of coding region for the *FGFRL1* gene in an initial group of 54 patients with isolated CDH. For selected single nucleotide polymorphisms (SNPs) (p. Pro20Pro, p.Pro362Gln, p. Arg424Leu, p. Pro464Leu), we genotyped additional patients who had CDH and additional anomalies to make a total of 200 genotyped chromosomes. We examined 80–100 bp of the 5′ untranslated region upstream from the start codon of *FGFRL1*, but the promoter was not studied. We obtained data on the frequency of *FGFRL1* SNPs in controls by sequencing 109 control patients as described above and by examining the published SNP frequency in public databases (dbSNP; http://www.ncbi.nlm.nih.gov/sites/entrez). We used a two-tailed Fisher's exact test to compare the allele frequencies for the *FGFRL1* SNPs between CDH patients and controls. We used PolyPhen (http://genetics.bwh.harvard.edu/pph/) to predict the functional effect of non-synonymous SNPs in *FGFRL1*.

cDNA array hybridization

We used GeneChip® Mouse Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA, USA) to hybridize three independently obtained cDNA samples from *Fgfrl1* null mice and *Fgfrl1* wildtype mice from three separate experiments. These gene chips contain 750,000, unique 25-mer oligonucleotide probes that interrogate 28,853 murine genes with an average of 27 probes across the full length of each gene (http://www.affymetrix.com/products_services/arrays/specific/mousegene_1_st.affx). All of the probes map to exons that are well supported as coding regions of known genes.

RNA samples were obtained from murine fetuses at E18.5 and genotyped to establish sex and *Fgfrl1* status. RNA was treated with DNAse (Invitrogen, Carlsbad, CA, USA) and cDNA samples were amplified, hybridized, washed and stained according to the manufacturer's specifications (GeneChip WT cDNA synthesis and amplification kit, Applied Biosystems, Carlsbad, CA, USA) by the J. David Gladstone Institutes Genomics Core Facility at UCSF (http://www.gladstone.ucsf.edu/gladstone/php/?sitename=genomicscore). Negative controls were performed by replacing RNA with RNase-free water.

Gene expression analysis

Basic processing, quality control and normalization of the six Affymetrix Mouse Gene 1.0 ST chips were done at the Swiss Institute for Bioinformatics in Lausanne using Affymetrix Power Tools (http://www.affymetrix.com/partners_programs/programs/developer/tools/powertools.affx). The processed chips were found to be of good quality since in each case the probe set relative log expression mean was between 0.1 and 0.23, and because no clear outlier was observed (Fig. 1). Differentially expressed genes were identified utilizing linear models (limma) and Benjamini–Hochberg correction for multiple testing. Only genes with adjusted p values of less than 0.1 were further investigated.

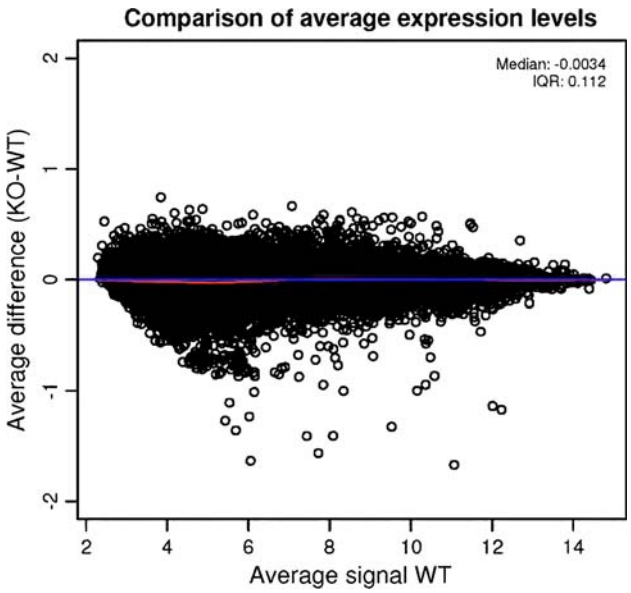

Fig. 1 Comparison of gene expression levels in wildtype diaphragm at E18.5 (*X*-axis) compared to knock-out—wildtype diaphragm gene expression (*Y*-axis) from three independent experiments. There are few significant outliers over a range of genes with low expression to high expression

Reverse transcription-polymerase chain reaction (RT-PCR) with SybrGreen

We used SYBRGreen and RT-PCR to quantify *Fgfrl1* and *Myh4* expression from the diaphragm of wildtype and knockout mice. cDNA from *Ffgrl1* null and wildtype mice was obtained from total RNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Primer pairs were *Myh4* forward primer: CGAAGGCGGAGCTACGGT, *Myh4* reverse primer: GGCCATGTCCTCAATCTTGTC; *Fgfrl1* forward primer: ACAAGGCCGGTGCCATCAAC; *Fgfrl1* reverse primer TGGAACGAGTCCGCTGGATT. PCR was conducted in triplicate with 20 mL reaction volumes of water, PerfeCTa SYBR Green SuperMix Reaction Mixes (Quanta Biosciences Inc., Gaithersburg, MD, USA) with Rox as a passive reference, 5 ng cDNA, and 0.9–0.15 mM each primer depending on optimization of the

primer set. Water and SYBR Green Supermix were aliquoted into individual tubes, one for each cDNA sample. cDNA was then added to the aliquoted Supermix and water. The combined Supermix, water and cDNA was then aliquoted into a 384-well plate. The primers and water were mixed together and added to the Supermix, water, and cDNA in the 384-well plate. PCR was conducted on the ABI 7900HT (Applied Biosystems) using the following cycle parameters: 1 cycle of 95° for 3 min, 40 cycles of 95° for 15 s, 60° for 1 min, and 1 cycle of 95° for 15 s, 60° for 15 s, 95° for 15 s. Analysis was carried out using the SDS software (version 2.3) supplied with the ABI 7900HT to determine the Ct values of each reaction. A dissociation curve was run after amplification to ensure the amplification of a single product. Ct values were determined for three test and three reference reactions in each sample, averaged, and subtracted to obtain the ΔCt [ΔCt = Ct (test locus) − Ct (control locus)]. PCR efficiencies were measured for all custom assays and were greater than or equal to 90%. Therefore, relative fold difference was calculated for each primer/probe combination as $2^{-\Delta Ct} \times 100$. *Gapdh* was used as a control.

Taqman probe analysis

cDNA from *Ffgrl1* null and wildtype mice was obtained from total RNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). The expression of *Tpm3* (Taqman probe Mm00445589_m1; Applied Biosystems), *Myl3* (Taqman probe Mm00803032_m1), *Lrtm1* (Taqman probe Mm00620949_m1), *Myh7* (TaqMan probe Mm00600555_m1) and *Myl2* (TaqMan probe Mm00440384_m1) were run according to protocol of the manufacturer using an ABI Prism 7500 sequence detection system (Applied Biosystems). All reactions were run in sets of four identical reactions. *Hprt* expression (TaqMan probe Mm03024075_m1) did not differ significantly in expression between *Fgfrl1* null and wildtype mice, and was used as a control (data not shown). Quantification analysis was performed as above using the ΔΔCt method using *Hprt* as a control.

Results

Gene prioritization software

Using all of the available Endeavour databases and the training gene set listed above and Endeavour prioritization software, *FGFRL1* returned as the gene with the highest prioritization score from the 43 genes and transcripts contained within the 2.4-Mb interval on chromosome 4p16.3 (Table 2). Using TOPPgene, *FGFRL1* ranked third with a *p* value of 0.193, behind *SPON2* ($p = 0.008$) and *FGFR3* ($p = 0.02$) (Table 2). However, a mouse model of loss of *Spon2* (also known as *mindin*) function showed no evidence of pulmonary or diaphragmatic defects, although the gene is expressed in fetal and adult lungs (He et al. 2004). Mice with *Fgfr3* loss of function have had severe kyphosis, long tails, curved femurs and loss of the inner pillar cells of the ear, consistent with the function of *Fgfr3* as an inhibitor of chondrocyte proliferation, but the *Fgfr3* null mice have not had CDH or diaphragmatic abnormalities (Colvin et al. 1996; Deng et al. 1996). After considering the available data from mouse models of loss of gene function and both gene prioritization programs, we selected *Fgfrl1* for our initial studies.

Genomic sequencing

The SNPs identified in *FGFRL1* in patients with CDH and controls have been shown in Table 3. The allele frequencies for all of the SNPs were not significantly different from those expected with Hardy–Weinberg equilibrium in either patients or controls (data not shown). Our results confirmed six known coding SNPs in *FGFRL1*: c.209G > A (p.Pro20Pro), c.977G > A (p.P276P), c.1040T > C (p.Asp297Asp), c.1234C > A (p.Pro362Gln), c.1420G > T (p.Arg424Leu), and c.1540C > T (p.Pro464Leu). We did not identify any novel SNP or mutations in *FGFRL1* in either patients or controls. For c.209G > A, a synonymous SNP, the allele frequency in CDH patients was of borderline statistical significance ($p = 0.048$) when compared to the allele frequency in control patients, but the allele frequencies in the CDH patients did not differ significantly from the allele frequencies found in craniosynostosis patients ($p = 0.49$). The remainder of the allele frequencies for the coding SNPs did not differ significantly between patients with CDH and normal controls (Table 3). However, it is interesting that one patient with WHS, CDH and a 4p16.3 deletion including *FGFRL1* (Slavotinek et al. 2006) was also sequenced and was found to be hemizygous for the minor allele for three of the six SNPs (p. Pro20Pro, p. Pro362Gln, and p.Pro464Leu) on the remaining 4p16.3 allele. Although the first of these SNPs is synonymous, the second SNP returned a position-specific independent counts (PSIC) score difference of 1.854 with the interpretation of 'possibly damaging' by PolyPhen and the third SNP returned a PSIC score of 1.831 with the same interpretation of 'possibly damaging'. It is therefore feasible that these SNPs resulted in reduced protein function on the intact allele of this patient, thus predisposing to diaphragmatic hypoplasia because of a further reduction in *FGFRL1* expression.

Table 3 Coding and non-coding sequence alterations in *FGFRL1* in patients with diaphragmatic hernia

FGFRL1 Exon	Nucleotide	Amino acid	dbSNP	Allele frequency in diaphragmatic hernia patients (number of chromosomes)	Allele frequency in control patients (number of chromosomes)	Allele frequency in craniosynostosis patients from dbSNP (number of chromosomes)
2	c.209G > A	p.Pro20Pro	rs4647942	*G* = 0.58 (202)	*G* = 0.709 (86)	*G* = 0.525 (40)
				A = 0.42	*A* = 0.291	*A* = 0.475
5	c.727G > T	p.Arg193Leu	rs4647937	*G* = 1.0 (108)	*G* = 1 (84)	*G* = 1.0 (40)
				T = 0	*T* = 0	*T* = 0
6	c.977G > A	p.Pro276Pro	rs58679007	*G* = 0.99 (100)	*G* = 1 (84)	Not available
				A = 0.01	*A* = 0	
6	c.1040T > C	p.Asp297Asp	rs4647946	*T* = 0.98 (180)	*T* = 0.99 (218)	*T* = 0.95 (40)
				C = 0.02	*C* = 0.01	*C* = 0.05
7	c.1234C > A	p.Pro362Gln	rs4647930	*C* = 0.79 (200)	*C* = 0.725 (218)	*C* = 0.725 (40)
				A = 0.21	*A* = 0.275	*A* = 0.275
7	c.1420G > T	p.Arg424Leu	rs4647931	*G* = 0.96 (200)	*G* = 0.97 (216)	Not available
				T = 0.04	*T* = 0.03	
7	c.1540C > T	p.Pro464Leu	rs4647932	*C* = 0.92 (200)	*C* = 0.93 (216)	*C* = 0.925 (40)
				T = 0.08	*T* = 0.07	*T* = 0.075
FGFRL1 Intron	Nucleotide	Amino acid	dbSNP	Allele frequency in diaphragmatic hernia patients (*n* number of chromosomes)	Allele frequency in control patients (*n* number of chromosomes)	Allele frequency in craniosynostosis patients from dbSNP (*n* number of chromosomes)
IVS3	+16G > A	–	–	*G* = 0.95 (104)	*G* = 0.92 (176)	Not available
				A = 0.05	*A* = 0.08	
IVS4	+10G > C	–	rs4647933	*G* = 0.99 (92)	*G* = 0.99 (172)	*G* = 0.95 (40)
				C = 0.01	*C* = 0.01	*C* = 0.05
IVS6	+47G > A	–	rs4647944	*G* = 0.93 (108)	*G* = 0.94 (218)	*T* = 0.925 (40)
				A = 0.07	*A* = 0.06	*A* = 0.075
IVS6	+76G > T	–	–	*G* = 0.98 (102)	*G* = 0.995 (218)	Not available
				T = 0.02	*T* = 0.005	

dbSNP http://www.ncbi.nlm.nih.gov/sites/entrez

cDNA array hybridization

We found only eight genes with significantly reduced expression levels ($p < 0.05$) in *Fgfrl1* homozygous null mice compared to wildtype mice (see Table 4 for Summary and Supplementary data for results of 200 genes). These genes were *Tpm3*, *Fgfrl1* (as expected; $p = 0.004$), *Myl2*, *Lrtm1*, *Myh4*, *Myl3*, *Myh7* and *Hephl1*. We confirmed a reduction in the expression of *Tpm3*, *Myl3*, *Lrtm1*, *Myh7* and *Myl2* in homozygous null mice when compared with wildtype controls using Taqman probes and in *Fgfrl1* and *Myh4* using SYBR Green and RT-PCR (Table 5). We were unable to verify the reduction in *Hephl1* expression, as we could not reliably amplify this gene using SYBR Green and there was no commercially available Taqman probe. There were no genes that were significantly overexpressed in the *Fgfrl1* homozygous null mice. *Fog2* (*Zfpm2*) and *Slit3,* two genes known to cause diaphragm hypoplasia in mice (Yuan et al. 2003; Ackerman et al. 2005), did not show significantly altered expression in the diaphragm of mutant mice compared to wildtype controls, with both genes having p values of 0.49 (Supplementary data).

Discussion

The fibroblast growth factor receptors (FGFRs) share a canonical structure comprising an extracellular region with three immunoglobulin-like (Ig-like) domains, a stretch of acidic residues between the first and second Ig-like domains termed the acidic box, a transmembrane helix, and an intracellular domain with tyrosine kinase activity (Mohammadi et al. 2005). Binding of FGFs and heparan

Table 4 Comparative gene expression between *FGFRL1* null mice and wildtype littermates

Gene	Array *p* value	Array expression level in *FGFRL1* null mice relative to wildtype mice	Function	Disease associated with loss of function	Reference(s)
Tpm3	0.0015	−2.34	Skeletal muscle contraction	AD, AR Nemaline myopathy	Danhaive et al. (2007), Lehtokari et al. (2008), Wada et al. (1996)
Fgfrl1	0.0038	−2.0	Cell adhesion	Diaphragm hypoplasia	Baertschi et al. (2007), Catela et al. (2009)
Myl2	0.0100	−3.34	Cardiac and smooth muscle contraction	Cardiomyopathy	Flavigny et al. (1998), Richard et al. (2003)
Lrtm1	0.0117	−1.73	Unknown	Candidate gene for Zimmerman-Laband syndrome	Kim et al. (2007)
Myh4	0.0216	−1.89	Skeletal muscle contraction	None known	Soussi-Yanicostas et al. (1993)
Myl3	0.0216	−2.65	Skeletal muscle contraction	Cardiomyopathy	Richard et al. (2003), Fokstuen et al. (2008), Møller et al. (2009)
Myh7	0.0216	−2.27	Skeletal muscle contraction	Cardiomyopathy	García-Castro et al. (2003), Richard et al. (2003), Meredith et al. (2004), Hershberger et al. (2008), Møller et al. (2009)
Hephl1	0.0478	−1.05	Unknown	None known	Nil

AD autosomal dominant, *AR* autosomal recessive

Table 5 Results for RT-PCR using SYBR-Green and Taqman probes for genes with reduced expression in *Fgfrl1* null mice

Gene	Ct (avg.)	ΔCt = avg. Ct (gene) − avg. Ct *Gapdh*	Relative % expression normalized to *Gapdh* (Sybr) = 2^-dCt*100	Relative expression normalized to knock-out 2^ΔΔCt
Fgfrl1 knock-out	33.93	9.65	0.12	1.0
Fgfrl1 wildtype	28.27	4.7	3.85	30.89
Lrtm1 knock-out	37.59	13.3	0.01	1.0
Lrtm1 wildtype	33.14	9.57	0.13	13.31
Myh4 knock-out	38.19	13.9	0.01	1.0
Myh4 wildtype	32.05	8.48	0.28	42.93
Gene	Ct (avg.)	ΔCt = avg. Ct (gene) − avg. Ct *Hprt*	Relative % expression normalized to *Hprt* (Taqman) = 2^-dCt*100	Relative expression normalized to knock-out 2^ΔΔCt
Myl2 knock-out	32.3	−1.35	255.03	1.0
Myl2 wildtype	26.99	−5.69	5156.15	20.22
Myh7 knock-out	29.06	−4.59	2402.89	1.0
Myh7 wildtype	25.41	−7.29	15414.77	6.42
Tpm3 knock-out	33.02	−3.28	973.60	1.0
Tpm3 wildtype	28.42	−7.37	16582.21	17.03
Myl3 knock-out	35.19	−1.11	215.90	1.0
Myl3 wildtype	30.78	−5.01	3224.98	14.94

RT-PCR reverse transcription polymerase chain reaction, *avg.* average, *Ct* cycle threshold

sulfate to the FGFRs induces dimerization (Mohammadi et al. 2005). An active complex of two FGFs, two FGFRs and one or two heparan sulfate chains results in trans-phosphorylation of specific tyrosine kinase residues in the intracellular domain of the FGFR, recruiting adapter proteins and enabling downstream signaling by diverse pathways including the PLCγ pathway, P-I-3 Kinase-AKT/PKB pathway and the Ras-MAP kinase pathway (Dailey et al. 2005).

Fgfrl1 is the most diverged of the Fgfrs (Wiedemann and Trueb 2000; Kim et al. 2001). Although it is structurally related to the other Fgfrs, with the NH_2-terminal of the protein having three Ig-like domains, six cysteines, an acidic box and one transmembrane domain, Fgfrl1 lacks both the histidine–alanine–valine motif that is critical for binding other FGFR molecules and the tyrosine kinase domain that is required for intracellular signaling (Wiedemann and Trueb 2001; Kim et al. 2001; Sleeman et al. 2001). Fgfrl1 is increased at sites of cell–cell contact and the protein is hypothesized to promote cell adhesion (Rieckmann et al. 2008). Fgfrl1 can therefore be considered similar to a nectin, a class of Ig-like, transmembrane cell adhesion molecule required for contact inhibition of cell movement and proliferation (Takai et al. 2008). Finally, there is some evidence that Fgfrl1 may act as a decoy receptor to coordinate the distribution of free FGF ligand and inhibit FGF signaling, as Fgfrl1 can inhibit the FGF-stimulated growth of MG63 cells (Trueb et al. 2003) and inhibit the FGF-stimulated activity of an FGF-inducible responsive element in a luciferase experiment (Rieckmann et al. 2009).

Our cDNA arrays examined comparative gene expression from the diaphragms of sex-matched, *Fgfrl1* homozygous null mice and wildtype littermates at E18.5. The murine diaphragm forms between E11.5 and E13.5, (Leinwand et al. 2002), but E18.5 was chosen because of the ease of diaphragmatic dissection at this later stage of gestation. We also believe that an examination of this time period is still useful, as at least some genes that have been implicated in diaphragm development (for example, *Slit3* and *Fog2*), are expressed at this later stage of development in addition to earlier time periods. The expression of *Fgfrl1* was reduced in the *Fgfrl1* homozygous null mice compared to wildtype littermates (Table 4; $p = 0.004$), validating the array methodology. Only seven genes besides *Fgfrl1* showed significant decreases in expression level in the mutant diaphragms when compared with wildtype littermates: *Tpm3*, *Myl2*, *Lrtm1*, *Myh4*, *Myl3*, *Myh7* and *Hephl1*. Tropomyosin-3 (*Tpm3*; OMIM 191030) showed the greatest reduction in expression in the *Fgfrl1* mutant mice (Table 4). This gene encodes a component of the thin filaments of the sarcomere, and loss of function mutations in *TPM3* have been reported in patients with nemaline myopathy and congenital fiber type disproportion (Laing et al. 1995; Clarke et al. 2008; Lehtokari et al. 2008). Interestingly, diaphragm hypoplasia is a recognized complication of severe nemaline myopathy (Wada et al. 1996; Danhaive et al. 2007). However, in patients with *TPM3* loss of function mutations, the diameter of the type I slow fibers is reduced, as *TPM3* encodes the slow isoform of skeletal muscle α-tropomyosin (Lehtokari et al. 2008). As the *Fgfrl1* null mice had normal muscle fiber histology and the ratio of fiber subtypes was unaltered when compared with normal littermates (Baertschi et al. 2007), this suggests that the reduction in *Tpm3* expression in the *Fgfrl1* null mice was a secondary phenomenon, attributable either to the deficiency of *Fgfrl1* and/or to the muscular hypoplasia of the diaphragm.

The expression arrays also demonstrated reduced expression for four other genes that encode sarcomeric proteins: Myosin, light chain-2 (*Myl2*; OMIM 160781), Myosin, heavy chain-4 (*Myh4*; OMIM 160742), Myosin, light chain-3 (*Myl3*; OMIM 160790), and Myosin, heavy chain-7 (*Myh7*, OMIM 160760). *Myl3* and *Myh7* both encode slow-twitch, type I muscle isoforms (Jandreski et al. 1987), whereas *Myl2* regulates myosin ATPase activity in smooth muscle. Heterozygous, missense *Myl2* mutations have been described in hypertrophic cardiomyopathy (Poetter et al. 1996; Flavigny et al. 1998; Richard et al. 2003). Mutations in *Myh7* have also been described in hypertrophic and dilated cardiomyopathies (García-Castro et al. 2003; Richard et al. 2003; Hershberger et al. 2008; Møller et al. (2009) and have been estimated to account for up to 40–50% of hypertrophic cardiomyopathies. *Myh7* mutations have also been reported in myosin storage myopathy and Laing myopathy (Meredith et al. 2004), but diaphragm weakness is not a common component of these myopathies. *Myl3* mutations are rare in dilated and hypertrophic cardiomyopathies (Richard et al. 2003; Fokstuen et al. 2008; Møller et al. (2009), whereas mutations in *Myh4* have not been reported in cardiac disease. We were not able to find any link between mutations in these genes and diaphragmatic defects.

Increased expression of *Tpm3* and *Myh7*, both components of the slow-twitch fibers that predominate in the diaphragm, has previously been noted in wildtype diaphragms compared to wildtype hindlimbs in mice (Porter et al. 2004). As these genes are expressed in the muscular component of the developing diaphragm, it is plausible that the differences in expression seen for the sarcomere genes could be related to the muscular hypoplasia of the diaphragm per se, independent from *Fgfrl1* expression, or the differences in expression could be related the muscular hypoplasia of the diaphragm in addition to the absence of *Fgfrl1* expression. For example, the reduced expression of *Tpm3* and the other sarcomere genes in the *Fgfrl1* null diaphragms may reflect reduced myoblast adhesion and secondary diaphragm thinning due to the postulated role of *Fgfrl1* in cell adhesion. However, at a minimum, our results demonstrate that the expression of these genes is important for the late stages of murine diaphragm development in utero.

Little is known regarding the functions of *Lrtm1* and *Hephl1*, the remaining two differentially expressed genes from our array studies. Leucine-rich transmembrane protein 1 (*Lrtm1*) has three coding exons that produce a 356 amino

acid membrane protein of unknown function (Kim et al. 2007). Transcripts have been isolated from many cDNAs from the pineal gland, lung and eye (Unigene; http://www.ncbi.nlm.nih.gov/unigene), and there does not seem to be an obvious relationship with muscle hypoplasia. A BlastP analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that Lrtm1 shows strong homology to Slit3 (1e-19) and Slit2 (4e-16), suggesting that Lrtm1 and Slit3 could share a similar function. This is interesting, as *Slit3* homozygous null mice have diaphragmatic hernias resulting from a failure of the septum transversum, or central tendon, of the diaphragm, to separate from the liver (Yuan et al. 2003). Slit3 was localized to the plasma membrane and mitochondria of a macrophage cell line, RAW264.7, and inhibition of *Slit3* reduced cell motility and the activity of two small GTPases known to be involved in actin cytoskeleton organization, *Cdc42* and *Rac* (Begum et al. 2004; Tanno et al. 2007). Rac1 and Cdc42 have been shown to be essential for myoblast fusion in the diaphragm in conditional mutant mice (Vasyutina et al. 2009). Further studies are needed to investigate whether *Lrtm1* is also involved in cell motility or myoblast fusion in the diaphragm and to define its relationship with *Fgfrl1*. The Slit proteins have also been shown be important in axon guidance and cell migration (Ma and Tessier-Lavigne 2007). The protein similarity between Lrtm1 and Slit3 is intriguing, as it suggests that there may be more than one gene with a role in cell migration that is important in diaphragm development, perhaps acting by providing guidance cues for muscle cell or muscle nerve cell migration, if Lrtm1 does indeed share a similar function to Slit3.

The last gene, *Hephl1,* has greatest similarity to *Hephaestin* (*Heph*; OMIM 300167), a gene encoding a transmembrane protein with ferroxidase activity that can function as an iron transporter in intestinal cells (Vulpe et al. 1999; Griffiths et al. 2005). *Heph* is mutated in sex-linked anemia (sla) mice that have a microcytic, hypochromic anemia with deficient export of iron from enterocytes (Petrak and Vyoral 2005), but Heph shows no known link with human disease and we were unable to find any connection between Heph and diaphragm development.

We chose to re-sequence *FGFRL1* in patients with CDH because of reports of two independent mouse models of loss of *Fgfrl1* gene function that had muscular hypoplasia of distinct, distal regions of the diaphragm (Baertschi et al. 2007; Catela et al. 2009) and the finding of *FGFRL1* as a candidate gene for CDH associated with 4p deletions. Our re-sequencing studies did not identify any new sequence alterations in *FGFRL1* in CDH patients, nor did they demonstrate a significant difference in the frequency of SNPs between control and patient population groups (Table 3). Despite the relatively small number of genotyped patients, our interpretation of the results is that the CDH patients studied were representative of this population and that further sequencing would be unlikely to uncover any significant differences from normal controls in terms of the allele frequencies for these SNPs in *FGFRL1*. In addition, several of the SNPs have relatively high heterozygosity scores (for example, see p.Pro20Pro and p.Pro362Gln; Table 3), thus making biological significance possibly less likely due to the relatively common frequency of carriers. However, one CDH patient who had a 4p16.3 deletion that included *FGFRL1* was hemizygous for the minor allele for three of the six SNPs, raising the possibility that the CDH in this patient may have been related to reduced *FGFRL1* expression caused by his deletion and these SNPs, or by the deletion of *FGFRL1* and at least one other gene at 4p16.3.

Sequencing of *FGFRL1* has previously been performed in 55 patients with congenital skeletal malformations comprising 22 patients with non-syndromic craniosynostosis and 19 with short stature (Rieckmann et al. 2008). In a female with a clinical diagnosis of Antley–Bixler syndrome (craniosynostosis, radio-ulnar synostosis and genital anomalies), a frameshift mutation in exon 6 of *FGFRL1* caused by an insertion of four nucleotides was identified (Rieckmann et al. 2008). The mutant and wildtype protein both localized to the plasma membrane, but the mutant protein lacked a sorting signal and was not removed from the plasma membrane as quickly, enabling interactions with FGF ligands of a longer duration and thus suggesting a gain of function, in contrast to Fgfrl1 wildtype protein, which was removed from the plasma membrane more quickly (Rieckmann et al. 2008). This patient was also re-sequenced for P450 oxidoreductase mutations, a gene known to cause Antley–Bixler syndrome, and a missense mutation, p. Ala287Pro, and a novel mutation at the 3′ splice junction of exon 8 changing the donor sequence AGgtacca into AGatacca (Rieckmann et al. 2008) were identified. There are no other published re-sequencing studies of the *FGFRL1* gene. Our failure to detect mutations or significant polymorphisms in *FGFRL1* in CDH patients could be construed as further evidence that diaphragm hypoplasia can be a pathologically and genetically distinct process from CDH.

Conclusion

We studied *FGFRL1* as a candidate gene for the diaphragmatic defects found with monosomy for 4p16.3 and in WHS. Re-sequencing of this gene showed no novel mutations or SNPs that were significantly associated with CDH. However, our cDNA expression arrays showed a significant decrease in the expression level of *Lrtm1* and several sarcomere genes in E18.5 diaphragms from *Fgfrl1* homozygous null mice when compared with wildtype littermates. Lrtm1

shows similarity with Slit3, a gene involved in cell motility that has previously been implicated in hernias involving the central tendon of the murine diaphragm, and that can alter the expression of two genes, Rac and Cdc42, that are essential for myoblast fusion. It remains to be seen whether *Lrtm1* has a similar function to *Slit3*, and if *Fgfrl1* therefore might cause diaphragm hypoplasia through a mechanism involving cell motility and/or myoblast fusion.

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