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Heart development in fibronectin-null mice is governed by a genetic modifier on chromosome four

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Abstract

Absence of the fibronectin (*FN*) gene leads to early embryonic lethality in both 129S4 and C57BL/6J strains due to severe cardiovascular defects. However, heart development is arrested at different stages in these embryos depending on the genetic background. In the majority of 129S4 *FN-null* embryos, heart progenitors remain at their anterior bilateral positions and fail to fuse at the midline to form a heart tube. However, on the C57BL/6J genetic background, cardiac development progresses further and results in a centrally positioned and looped heart. To find factor(s) involved in embryonic heart formation and governing the extent of heart development in *FN-null* embryos in 129S4 and C57BL/6J strains, we performed genetic mapping and haplotype analyses. These analyses lead to identification of a significant linkage to a 1-Mbp interval on chromosome four. Microarray analysis and sequencing identified 21 genes in this region, including five that are differentially expressed between the strains, as potential modifiers. Since none of these genes was previously known to play a role in heart development, one or more of them is likely to be a novel modifier affecting cardiac development. Identification of the modifier would significantly enhance our understanding of the molecular underpinning of heart development and disease.

Keywords: Fibronectin; Heart development; Genetic modifier; Mapping

1. Introduction

The heart is the first organ to form in the developing vertebrate embryo and the survival of the embryo and the well-being of the adult are critically dependent on the integrity of the developmental program guiding heart formation (Srivastava, 2006). One of the earliest events during cardiogenesis is the coalescence of the bilateral cardiac primordia toward the midline to form a single heart tube (Buckingham et al., 2005) – the failure of this to happen leads to *cardia bifida* and lethality.

Genetic analyses in zebrafish and mice have uncovered a number of genes and processes required for the formation of a single heart tube from the paired cardiac primordia. First, cardiac mesoderm is derived from the early wave of cells migrating through the primitive streak, and the deletion of genes that affect this process (*Mesp1* and *Nap1*) gives rise to *cardia bifida* (Rakeman and Anderson, 2006; Saga et al., 1999). Second, endoderm provides an essential signal for the migration of the cardiac lateral plate mesoderm toward the midline, and genes required for endoderm specification (*cas, bon, oep*) (Alexander et al., 1999; Kikuchi et al., 2000; Schier et al., 1997) or morphogenesis (*fau, GATA4, Hrs, Furin, Foxp4*) (Komada and Soriano,

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1999; Kuo et al., 1997; Li et al., 2004; Reiter et al., 1999; Roebroek et al., 1998) are required for the formation of a central heart tube. Hypoxia-regulated processes requiring HIF1α are also involved. Finally, genes important for cell migration (*paxillin, fibronectin, S1P receptor, NAP1*) (George et al., 1993; Hagel et al., 2002; Kupperman et al., 2000; Rakeman and Anderson, 2006), cell polarity (e.g. *fibronectin*, *NAP1*) (Rakeman and Anderson, 2006; Trinh and Stainier, 2004), extracellular matrix synthesis (*mtx1*) (Sakaguchi et al., 2006), or protein prenylation (*hmgcr1b*) (D'Amico et al., 2007) are essential for the formation of a centrally located heart tube.

Interestingly, the genetic makeup of laboratory mouse strains affects the phenotypes of mice carrying various knockout alleles. The most dramatic variations, from embryonic lethality in one strain to survival past birth in a different strain, are seen in mice carrying deletions in genes encoding an Rb family member p130 or EGF receptor (LeCouter et al., 1998; Threadgill et al., 1995). Heart function is also affected by strain-specific genetic variation. For example, adult wild-type mice of C57BL/6J strain possess the "heart of an athlete" compared with the hearts of A/J mice (Hoit et al., 2002). Genetic differences between strains also affect the degree of cardiac defects in some knockout mice. A deletion in the *HIF1α* gene results in *cardia bifida* in 32% of the embryos on a mixed 129X1-Swiss background while a central looped heart develops in all embryos from a mixed 129Sv/J × C57BL/6J strain (Compernolle et al., 2003; Iyer et al., 1998).

FN-null embryos have *cardia bifida* in the 129S4 strain of mice but develop a central looped heart in the C57BL/6J strain (George et al., 1997). Interestingly, the point mutant *natter* in zebrafish, which introduces a translational stop codon at the beginning of the *FN*1 message and abrogates the expression of fibronectin, also shows a strain-dependent *cardia bifida* phenotype (Trinh and Stainier, 2004). These phenomena suggest that there exist genetic factors whose identity and importance for heart development could be uncovered by performing genetic mapping analysis. Identification of new genes involved in early cardiogenesis may also facilitate our understanding of congenital heart defects. So far, mutations in a number of genes essential for embryonic heart development (e.g. *GATA4, Notch1, Nkx2.5, Tbx1, Tbx5*) have been found to underlie familial cases of congenital heart disease (Garg et al., 2003, 2005; Li et al., 1997; Merscher et al., 2001; Schott et al., 1998).

To identify new genes involved in heart development, we decided to map factor(s) governing heart formation in the *FN-null* embryos derived from F2 intercrosses between 129S4 and C57BL/6J strains. We used high-throughput genotyping of single-nucleotide polymorphisms (SNPs) and a dense SNP haplotype map of 129S4 and C57BL/6J strains (Wade and Daly, 2005) to identify a 1-Mbp interval on mouse chromosome four containing a modifier of cardiogenesis in *FN-null* embryos. Furthermore, by performing microarray analysis on wild-type, heterozygous, and *FN-null* embryos, we identified 21 candidate genes expressed in this interval, five of which were differentially expressed between 129S4 and C57BL/6J strains. This is the first example, to our knowledge, of mapping a quantitative trait locus of an embryonic lethal phenotype.

2. Results and discussion

2.1. Phenotypic analysis of FN-null embryos

All *FN-null* embryos from the C57BL/6J background developed a centrally located and looped heart by e9.3 (Fig. 1c and f). However, in 72% of 129S4 *FN-null* embryos, heart development stopped following the migration of cardiac primordia to their anterior–lateral positions (Fig. 1a and d). In the remaining 129S4 *FN-null* embryos, the heart primordia migrated and fused to form a central heart ball (Fig. 1b and e); however, none of the 129S4 *FN-null* embryos developed a looped heart tube (Table S1). Analysis of 62 *FN-null* embryos from F1 crosses between 129S4 and C57BL/6J, showed that, similar to 129S4 embryos, the largest group among the *FN-null* F1 embryos (37 out of 62) had bilateral cardiac primordia.

2.2. Genetic mapping

To perform genetic mapping analyses, we collected two independent sets of *FN-null* embryos from F2 intercrosses (there were 175 and 165 embryos in each set) and classified them into three groups according to their heart phenotypes (Fig. 1). Group 1 comprised embryos with unfused bilateral heart primordia, group 2 included embryos with a small- or medium-sized heart in the shape of a ball, and group 3 comprised embryos that developed a looped heart. Both sets of F2 embryos had similar distributions of phenotypes (Table S2). Each embryo was then genotyped using a genome-wide panel of SNP markers spaced about 20 cM apart on each chromosome. Each set of embryos was genotyped with a different set of genetic markers. These genome-wide scans indicated a potential linkage to the distal arm of chromosome four (Fig. 2a). We performed linkage analysis on the first and second sets of F2 embryos independently, and observed linkage to chromosome four in similar locations in each case (Fig. S1). An additional panel of SNP markers placed on chromosome four allowed us to narrow down the position of the modifier to a 5-Mbp interval (Fig. 2a and b). Other genomic regions did not show significant linkage (Fig. 2a). Furthermore, we did not detect genetic interactions between the locus on chromosome four and other chromosomal loci with LOD scores above 2.5 (Table S3).

We reasoned that the genetic modifier on chromosome four could affect either of two processes: migration of the lateral primordia to the midline or subsequent morphogenesis of the heart tube. In an attempt to distinguish between these two possibilities, we performed linkage analysis using different combinations of phenotypic groups. For example,

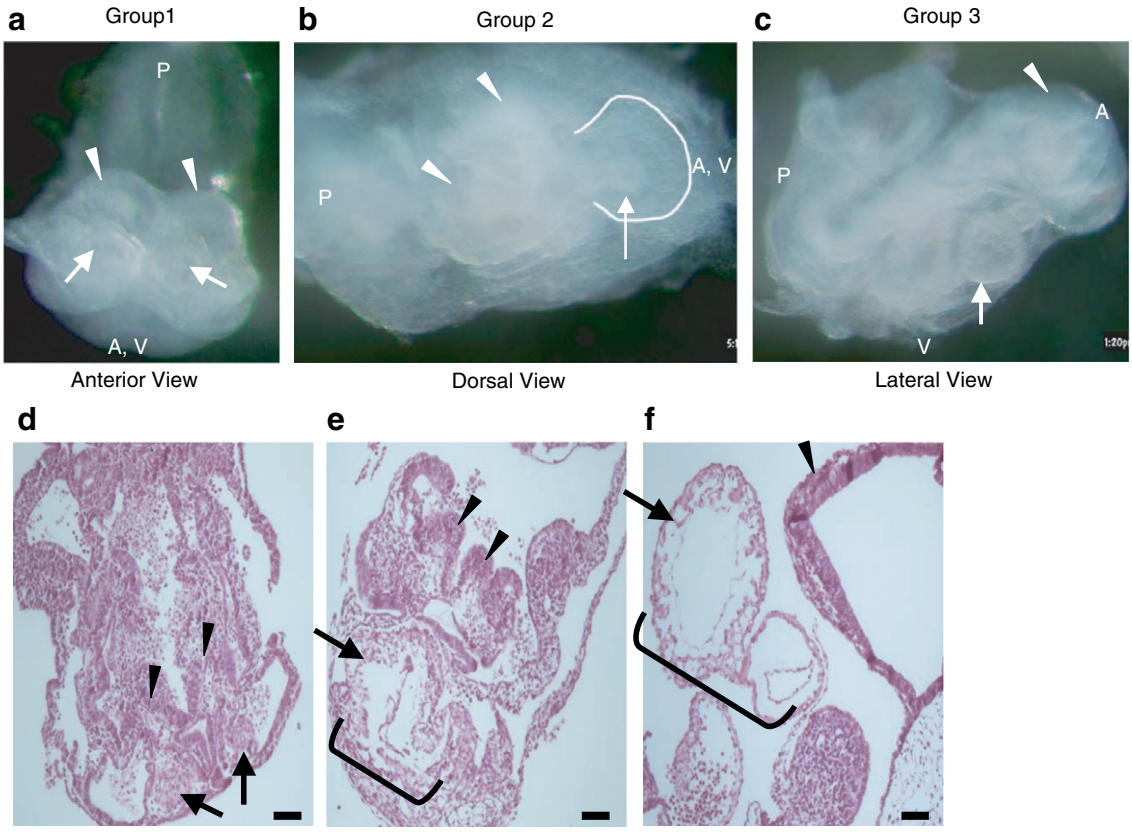

Fig. 1. Phenotypes of *FN-null* embryos. (a and d) A whole-mount view and a transverse section through an embryo representative of the majority of *FN-null* embryos from the 129S4 strain and of F2 embryos belonging to group 1. Arrows point to the two cardiac primordia, arrowheads point to the head folds. Anterior (A) and ventral (V) are at the bottom and posterior (P) is at the top. (b and e) A whole-mount view and a transverse section through a representative F2 embryo from group 2. Arrow points to the single heart ball outlined in B and encompassed by the bracket (e). Arrowheads point to headfolds. Anterior is to the right in (b) and at the bottom in (e). (c and f) A whole-mount view and a sagittal section through an embryo representative of *FN-null* embryos from C57BL/6J strain and F2 embryos belonging to group 3. Anterior is to the right in (c) and at the top right corner in (f). Arrow points to the central heart which is also delineated by a bracket in (f). Arrowhead points to the head. Scale bars in (d, e and f) are 65 μm.

to determine whether the modifier on chromosome four affected the progression from the bilateral cardiac primordia stage to a central heart, we analyzed embryos from group 1 against embryos from combined groups 2 and 3. While our analysis showed that the peak on chromosome four was mainly due to the genetic differences between groups 1 and 3 (Table S4), we obtained the highest LOD score, 5.5, when the three phenotypic classes were combined into two groups, one group contained embryos with unfused lateral heart primordia (group 1), while the other group consisted of embryos in which fusion of the primordia occurred (groups 2 and 3). This suggested that we were mapping a modifier affecting the event of coalescence of the two heart primordia into a single heart tube, a migration process that ordinarily happens during normal heart development (Buckingham et al., 2005).

The peak on chromosome four spanned five megabases (Mbp) (Fig. 2b) between 134 and 140 Mbp (2004 genome assembly). The LOD score of 5.5 corresponds to a pointwise (two-sided) p value of 2.5×10^{-6} and a genome-wide significance level of 0.003 (0.003 is the number of times that a linkage of LOD 5.5 could have been found fortuitously). These values comprise significant evidence (Lander and Kruglyak, 1995) that this region of chromosome four is linked to a gene(s) affecting heart development in *FN-null* embryos, and we term this locus modifier influencing single heart assembly (*Misha-1*).

The highest predicted LOD score (5.5) within the peak is 2 cM away from the closest genotyped marker, w4_13753, at 138 Mbp (Fig. 2b). Those embryos having two C57BL/6J alleles at the w4_13753 locus are approximately twofold more likely to develop a centrally located heart than to arrest at the bilateral primordia stage, and embryos homozygous or heterozygous for the 129S4 allele at w4_13735 have a 50/50 chance either to develop a central heart or to arrest at the bilateral primordia stage (Table S5). This suggests that, while a locus within the 5-Mbp region on chromosome four contains the major modifier of *FN-null* phenotype, there exist other quantitative trait loci, which contribute to strain-specific differences in heart development of *FN-null* mice.

While our experiments suggested that the locus on chromosome four contains one or more modifiers affecting the coalescence of the bilateral primordia into a single heart tube, the heart phenotype is not the only difference between these two groups, another notable

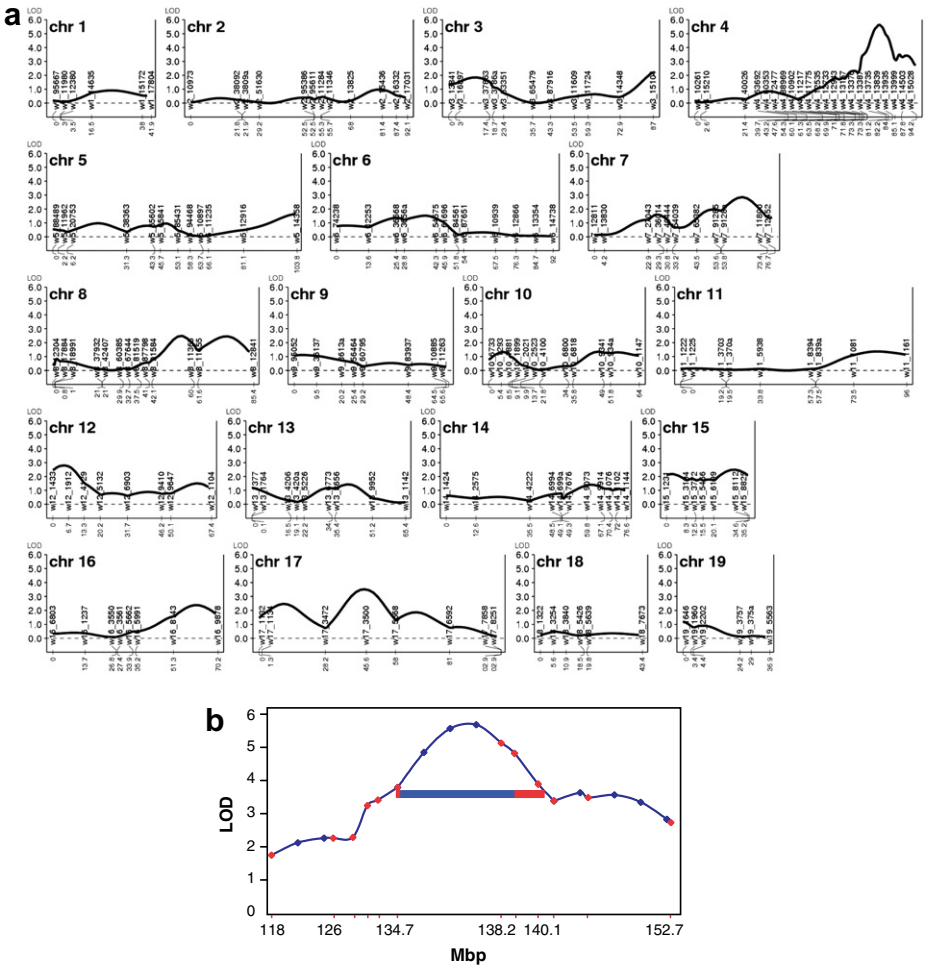

Fig. 2. A genetic modifier of heart development in *FN-null* mice is linked to the distal arm of chromosome four. (a) Genomescan of 341 F2 samples identified a linkage peak on chromosome four. For each chromosome, positions of the SNP markers in cM are plotted on the *x*-axis and LOD scores are plotted on the *y*-axis. Penetrance scan is shown, representing analysis of embryos from group 1 against embryos from combined groups two and three. Peaks on chromosomes eight and seventeen are not reproducible between the two sets of embryos and probably result from combining two different sets of markers used for the genome-wide scan. Chromosome four was genotyped with a single set of markers for all of the 341 embryo samples. (b) Linkage peak and haplotype map of the region on chromosome four. Embryos were pooled into two phenotypic groups (group 1 vs. groups 2 and 3) and analyzed using penetrance scan. Red marks and red points on the graph indicate positions of genotyped SNPs. Blue points on the curve show the points calculated by the MapmakerQTL. Blue bar – haplotype block shared between 129X1, 129S1, 129S4 and C57BL/6J strains based on the genotyping of 298 SNPs in this region (see Section 3). Marker w4_13753 at 138.2 Mb is the only polymorphism detected in this region. Red bars – regions containing SNPs between the 129S4 and C57BL/6J strains.

difference is the presence of vessels in embryos with at least a central heart ball, in contrast to those embryos without a heart (George et al., 1997). Therefore it is possible that the modifier locus affects an earlier stage in embryogenesis, correlating with the extent of the heart development. In order to understand the role of this locus in embryogenesis and heart formation, we need to identify the gene within it that acts as modifier.

2.3. Further analyses of the region

To narrow down the region containing the candidate modifier(s), we determined those areas within the 5-Mbp interval that contain polymorphisms between 129S4 and C57BL/6J strains, using a mouse SNP chip (Wade and Daly, 2005). This experiment showed that none of the 298 genotyped markers located between 134.8 and 139.1 Mbp was polymorphic between 129S4 and C57BL/6J strains (http://www.broad.mit.edu/~mjdaly/mousehapmap).

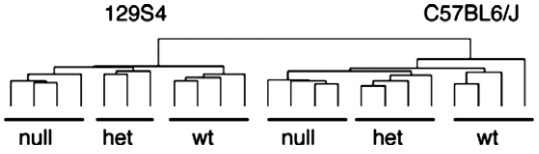

Fig. 3. Gene expression analysis of wild-type, heterozygous and *FN-null* embryos from 129S4 and C57BL/6J strains. Hierarchical unsupervised clustering of gene expression profiles distinguishes embryos from 129S4 and C57BL/6J strains and they cluster according to the number of wild-type *FN* alleles and according to genetic background.

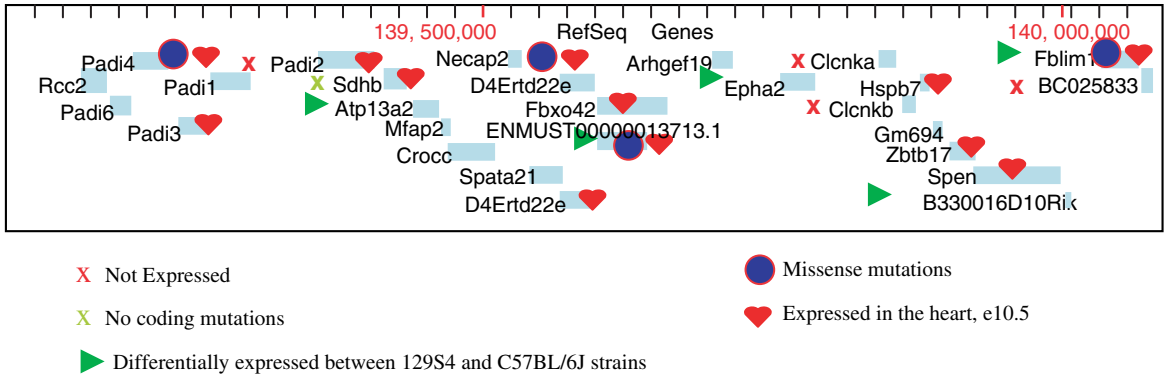

Fig. 4. Candidate modifier genes. There are 26 genes annotated in this 1-Mbp region of chromosome four. Twenty-five of them are annotated by RefSeq and are shown in the figure; the expression of one additional gene, ENMUST00000013713.1, is detected by the Affymetrix probe 1459714 at 139.67 Mbp. Genes expressed in the hearts of e10.5 embryos were found by mining the Entrez GEO database. Missense mutations were found by using the Perlegen database and by direct sequencing of cDNAs (see Section 3 and Table 1). Genes not expressed in the region were determined using Affymetrix absence and presence calls.

Table 1
Candidate modifier genes – expression and polymorphisms

	Affymetrix probe No.	Gene name/symbol	Expressed at e8.0	Differentially expressed	Expressed in the heart at e10.5	Missense mutations
1	1426897_at	RCC2	Yes	No	No	NS
2	1437084_at	PADi6	Yes	No	No	NS
3	1422760_at	PADi4	Yes	No	Yes	A573V
4	1419767_at	PADi3	Yes	No	Yes	NS
5	1419323_at	PADi1	Yes	No	No	NS
6	1418252_at	PADi2	No	No	Yes	NS
7	1418005_at	Sdhb*	Yes	No	Yes	None*
8	1428340_at	ATP13a2	Yes	Up in 129S4	Yes	NS
9	1417359_at	Mfap2	Yes	No	Yes	NS
10	1427338_at	Rootletin	Yes	No	No	T289A, E360G, V811M, S845N
11	1418961_at	NECAP2	Yes	No	Yes	I104L
12	1455718_at	Spata21	Yes	No	No	NS
13	1434482_at	D4Ertd22e	Yes	No	Yes	NS
14	1453382_at	Fbxo42	Yes	No	Yes	NS
15	1459714_at	ENMUST 00000013713.1*	Yes	Up in C57BL/6J	Yes	L40I, C106R, L168I, A107E*
16	1437629_at	ArhGEF19*	Yes		No	G492S*
17	1421151_at	EphA2	Yes	Up in C57BL/6J	No	NS
18	1457076_at	Gm693	Yes	No	No	NS
19	1455677_at	Clcnka	No	No	No	NS
20	1450340_at	Clcnkb	No	No	No	A380V, I431V, A554T, V571M, L637P, S667F
21	1421290_at	Hspb7	Yes	No	Yes	NS
22	1416224_at	Zbtb17	Yes	No	Yes	NS
23	1420397_at	Spen	Yes	No	Yes	NS
24	1455492_at	B330016D10Rik	Yes	Up in C57BL/6J	No	NS
25	1418569_at	Fblim1*	Yes	Up in C57BL/6J	Yes	I159R*
26	1451528_at	BC025833	No	No	No	NS

There are 26 genes annotated in the 1-Mbp region of chromosome four. Microarray analysis showed that 22 of these genes are expressed in e8.0 embryos and five of these genes are differentially expressed between the strains. Genes expressed in the hearts of e10.5 embryos were found by browsing Entrez GEO database. Missense mutations (C57BL/6J –> 129S4) were found by mining the Perlegen database and by direct sequencing. NS, not sequenced; these genes did not contain coding SNPs in the Perlegen database. Asterisks denote genes whose cDNAs were sequenced at 8× coverage from 129S4 and C57BL/6J strains.

Clearly this is a conserved haplotype block shared between the two strains. We resequenced several putative polymorphisms in this region – only one, w4_13753, was a true polymorphism and is the only known polymorphic marker in this region. We initially hoped that w4_13753 might help identify the modifier. However, there are no known or predicted genes in the near vicinity of w4_13753. The closest gene to the left of this marker

(TIR2) is 60 kb away and the closest gene on the right (Pax7) is 25 kb away. Neither gene is differentially expressed between 129S4 and C57BL/6J strains. While we cannot absolutely rule out the possibility that a rare polymorphism in this region could contribute to the modifier effect, this analysis suggests that the modifier(s) is more likely located within the 100 kbp on the left and/or 1 Mbp on the right sides of the peak, regions that do differ between strains (shown in red in Fig. 2b). We hypothesize that it is most likely that the potential modifier lies in the 1-Mbp region since it is closer to the two genotyped markers (at 138.2 and 139.1 Mbp) with the highest LOD scores (Fig. 2b).

The potential modifier must be a gene expressed in the embryos we analyzed. It could be either a differentially expressed gene, whose levels depend on the genetic strain or on the number of wild-type *FN*-alleles, or it could be a gene carrying a missense mutation. Since the levels of FN protein depend on the number of functional *FN* alleles (George et al., 1993), we determined gene expression profiles of *FN-null*, *FN*-heterozygous, and wild-type embryos at e8.0 of development, from both 129S4 and C57BL/6J strains by performing microarray analyses. Our analyses indicated that the expression profiles were segregated according to the strain and the genotype of the embryos (Fig. 3) and that 22 out of 26 annotated genes in the 1-Mbp region were expressed (Fig. 4 and Table 1). Among the expressed genes, five were differentially expressed between 129S4 and C57BL/6J strains. We did not find any genes in this interval that were differentially regulated by FN. Limited sequence analysis of the candidate genes (see Section 3) together with data from Perlegen (http://mouse.perlegen.com) showed that at least seven of the genes in the region contained missense mutations (Fig. 4 and Table 1), and that one gene, *Sdhb*, a potential tumor suppressor known to regulate HIF1α (Gottlieb and Tomlinson, 2005), does not have any coding SNPs, leaving 21 candidate modifier genes.

A potential modifier could be one of the genes differentially expressed between strains, such as *Fblim1*. *Fblim1* is a presumptive transcription factor that shuttles between the nucleus and the cytoplasm in response to Ca^{2+} and binds to Nkx2.5 (Akazawa et al., 2004), a transcription factor involved in heart development. This protein is interesting, because in the cytoplasm, it associates with actin stress fibers (Takafuta et al., 2003), appearance of which is modulated by the cellular adhesion to extracellular matrix proteins including fibronectin. Fblim1 contains three LIM domains and harbors a SNP between 129S4 and C57BL/6J strains located N-terminal to the first LIM domain leading to a missense mutation of Ile 159 (C57BL/6J) to Arg (129S4). A second interesting differentially expressed gene is the previously unidentified ENMUST00000013713.1. This gene has a GTPase domain, which harbors four SNPs between 129S4 and C57BL/6J strains leading to missense mutations in this domain (Table 1).

Alternatively, the modifier may not be differentially expressed between the strains but could have altered function due to a mutation. One such gene could be *ArhGEF19*, a novel gene with a predicted guanidine nucleotide exchange factor (GEF) domain and harboring Gly 492 (C57BL/6J) to Ser (129S4) mutation in this domain.

To identify the candidate modifier gene, we have tried to modify the levels of differentially expressed genes directly in 129S4 or C57BL/6J *FN-null* embryos, and used *FN-null* ES cells from 129S4 or C57BL/6J strains to generate tetraploid chimeras. These chimeras result from injecting diploid ES cells into tetraploid blastocysts, giving rise to embryos that are mainly derived from the ES cells and to extraembryonic tissues, derived from the tetraploid blastocysts. Even though fibronectin is a secreted protein, we hoped that its incorporation into the insoluble extracellular matrix of extraembryonic tissues would not lead to its distribution within the embryo. However this was not the case – the tetraploid chimeras made with *FN-null* ES cells of either strain, contained fibronectin within the embryo and developed hearts (data not shown). This was possibly due to the FN protein derived from rare wild-type tetraploid cells contributing to the embryo (Eakin et al., 2005) or, perhaps more likely, from diffusion of FN from the tetraploid extraembryonic tissues. While this approach was not useful to identify the modifier of *FN-null* phenotype, it would be useful for identifying a modifier of an embryonic lethal phenotype of a cell-autonomous gene such as integrin α5 (Yang et al., 1993).

Several approaches are available to identify the modifier of heart development in *FN-null* embryos from among the candidate genes. One could use RNAi to decrease the levels of differentially expressed genes: *ATP13a2* in 129S4 $FN^{+/-}$ ES cells or *Fblim1, ENMUST00000013713.1, Epha2*, and *B330016D10Rik* in C57BL/6J $FN^{+/-}$ ES cells. These modified ES cells would then be used to generate transgenic mice. Alternatively, $FN^{+/-}$ mice carrying BACs spanning the 1-Mbp region of chromosome four could be generated. However, these experiments are beyond the scope of the present paper.

2.4. Conclusion

It has been known for a long time that knockouts of some genes produce variable phenotypes depending on the mouse strain. This is interesting and could potentially allow identification of new and/or unexpected players in a particular process. We took advantage of the difference in heart formation in *FN-null* embryos from 129S4 and C57BL/6J strains with the aim of identifying new factors involved in heart development. Using a high-throughput SNP genotyping technology, which requires only nanogram quantities of genetic material, together with the recently available haplotype map of mouse strains and genome-wide expression analysis, we have narrowed the search to 21 primary candidate genes and outlined strategies that should be useful in identifying the candidate modifier.

3. Experimental procedures

3.1. Embryo samples

The *FN-null* allele was generated in 129S4 ES cells (George et al., 1993). 129S4 *FN-null* embryos were directly derived from the germline transmission of these cells. *FN-null* allele in the C57BL/6J strain was obtained by backcrossing *FN*-heterozygous mice from 129S4 strain to C57BL/6J mice for thirteen generations. *FN-null* embryos were isolated from *FN*-heterozygous crosses on the morning of embryonic day (e) 9.3 *post coitum* (p.c.), photographed and assigned a grade of one through three, depending on the heart phenotype. $FN^{+/-}$ mice and embryos of either strain are phenotypically normal. DNA was prepared using the DNeasy Qiagen kit according to the manufacturer's instructions and stored at −80 °C. Embryos were genotyped by PCR to confirm the homozygosity of the *FN-null* allele (George et al., 1993), and the presence of the Y chromosome using primers 5′-GCGCCCCAT GAATGCATTTAT G-3′ and 5′-CCCTCCGATGAGGCTG-3′ to detect the SRY gene.

3.2. Sample genotyping

Samples were genotyped to detect single-nucleotide polymorphisms (SNPs) (http://www.broad.mit.edu/~mjdaly/mousehapmap database) using the Sequenom MassArray (San Diego, CA, USA) mass spectrometry system as described previously (Petryshen et al., 2005). The data were analyzed using Mapmaker and MapmakerQTL (Kruglyak and Lander, 1995) using penetrance scan (Hamilton et al., 1997). Linkage to chromosome one was analyzed starting at 95 Mbp since all C57BL/6J *FN-null* embryos possess a significant amount of 129S4 genome carried over with the *FN-null* allele (*fibronectin* is situated at 70 mb on chromosome one).

3.3. Genotyping polymorphisms between 129S4 and C57BL/6J strains

DNA from 129S4 and C57BL/6J strains was hybridized to the mouse SNP chips as described (Wade and Daly, 2005). The results are available at http://www.broad.mit.edu/~mjdaly/mousehapmap web site. All genomic coordinates are according to the 2004 assembly of UCSC Genome Browser (http://genome.ucsc.edu).

3.4. Gene expression profiling

Total RNA was collected from six-somite wild-type, heterozygous, and *FN-null* embryos from 129S4 and C57BL/6J strains in the morning of the ninth day p.c. and purified using Trizol (Invitrogen). All wild-type and heterozygous embryos had crescent-shaped hearts, *FN-null* embryos from 129S4 strain had *cardia bifida* and those from C57BL/6J strain had hearts in the shape of a ball. *FN-null* embryos do not develop somites and thus we collected these embryos at the same day and time of development as the wild-type embryos. We obtained about 1 μg of total RNA from each embryo and used 100 ng to amplify and label mRNA according to the Affymetrix instructions (www.affymetrix.com). The labeled mRNA was hybridized to the Affymetrix 430 2.0 arrays. Data were analyzed using S-plus and GenePattern software packages.

3.5. Sequence analysis

cDNA was generated from total RNA isolated from 129S4 and C57BL/6J embryos at day e8.0. Guided by the sequence of the C57BL/6J mouse genome, we designed primers to amplify and sequence cDNAs encoding a few candidate modifier genes: *ArhGEF19, SdhB, ENMUST0000001 3713.1,* and *Fblim1*. *ENMUST00000013713.1* and *Fblim1* are two of the five genes differentially expressed between 129S4 and C57BL/6J strains, corresponding to Affymetrix probe Nos. 1459714 and 1418569. We determined that the probe set 1459714 assayed the expression of *ENMUST00000013713.1* since we amplified the cDNA corresponding to this gene using probe sequences from this probe set as reverse primers and primers lying in the predicted exons of *ENMUST00000013713.1* as forward primers. These cDNAs were isolated and sequenced from each of the two parental strains (results given in Table 1).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2007.05.004.

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