Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1

Anthony B. Firulli[1], David G. McFadden[1], Qing Lin[1], Deepak Srivastava[1,3] & Eric N. Olson[1]

The basic helix-loop-helix (bHLH) transcription factors, Hand1 and Hand2 (refs 1,2), also called eHand/Hxt/Thing1 and dHand/Hed/Thing2 (refs 3,4), respectively, are expressed in the heart and certain neural-crest derivatives during embryogenesis. In addition, *Hand1* is expressed in extraembryonic membranes, whereas *Hand2* is expressed in the deciduum. Previous studies have demonstrated that *Hand2* is required for formation of the right ventricle of the heart and the aortic arch arteries[5]. We have generated a germline mutation in the mouse *Hand1* gene by replacing the first coding exon with a β-galactosidase reporter gene. Embryos homozygous for the *Hand1* null allele died between embryonic days 8.5 and 9.5 and exhibited yolk sac abnormalities due to a deficiency in extraembryonic mesoderm. Heart development was also perturbed and did not progress beyond the cardiac-looping stage. Our results demonstrate important roles for *Hand1* in extraembryonic mesodermal and heart development.

The mouse *Hand1* gene was targetted in embryonic stem (ES) cells (Fig. 1*a*). Homologous recombination resulted in deletion of the first exon of the gene, which encodes the bHLH region, and introduction of a *lacZ* reporter gene, encoding β-galactosidase (β-gal), at the position of the *Hand1* initiation codon. Mice heterozygous for the *Hand1* mutation were phenotypically normal and were intercrossed with C57/B6 and Swiss mice to obtain homozygous *Hand1* mutants. From these intercrosses, we did not detect homozygous mutant neonates, indicating that the homozygous mutation resulted in embryonic lethality. We genotyped embryos from timed pregnancies to determine the time of lethality. Prior to embryonic day (E) 8.0, homozygous mutants that appeared normal were detected at the predicted Mendelian frequency. At E8.5, mutant embryos began to show delayed growth, and by E10.5, they appeared necrotic and in the process of being resorbed. A Southern blot of yolk-sac DNA obtained from E9.5 embryo littermates is shown in Fig. 1*b*.

Embryos mutant in *Hand1* appeared to develop normally until the eight-somite stage, but failed to undergo turning. They also developed an ectoplacental cone, headfolds, a neural tube and notochord along the anterior-posterior axis, and between 9–14 somites before arresting in development (Fig. 2*a–h*). The neural tubes of the mutants were often crooked (Figs 3*l* and 4*h*), which may be a secondary consequence of nutrient-deficiency due to improper function of the yolk sac. Haematoxylin and eosin (H&E) staining of embryo sections revealed somites, headfolds, lateral mesoderm and blood islands in mutant embryos (Fig. 2*e–j*). The primary morphologic defects in mutant embryos appeared within the yolk sac and heart. Yolk sacs of mutant embryos had a rough, folded appearance in the area of contact with the ectoplacental cone and lacked defined vascularization. Transverse sections showed separation between the endodermal and mesodermal layers; the outer endodermal layer was extensively folded, suggesting its growth rate was not synchronized with the inner mesodermal layer (Fig. 2*i,j*). In addition, the amnion of mutant embryos appeared thickened and failed to completely enclose the body of the embryo (Fig. 2*e,f*), resulting in abnormal fusion of the amniotic and exocoelomic cavities. The chorion of mutant embryos also appeared abnormal, suggesting defects in chorionic fusion (Fig. 2*e,f*).

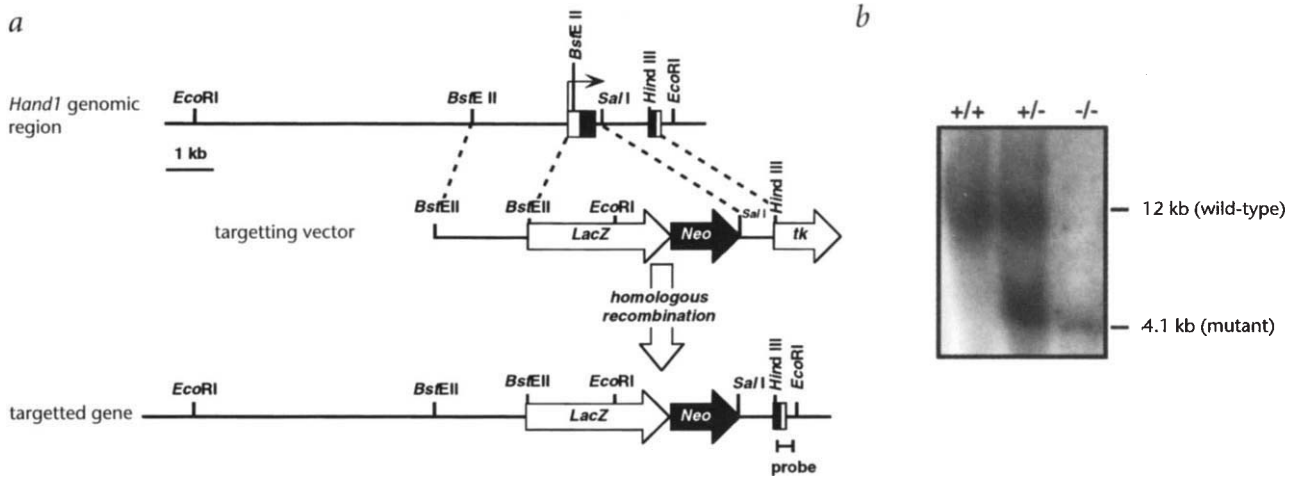

Fig. 1 Targetting of *Hand1*. ***a***, The mouse *Hand1* gene structure is shown at the top. Coding and non-coding regions of the two exons are indicated by black and white shading, respectively. The targetting vector used for homologous recombination is in the middle and the targetted allele is at the bottom. The targetting strategy introduced a *lacZ* reporter gene in the 5′ untranslated region of *Hand1*, followed by *PGK-neo*. The direction of *neo* transcription was the same as that of *Hand1*. The 5′ and 3′ arms of the targetting vector were 3.0 and 1.2 kb, respectively. The *HSV-tk* gene was linked to the 3′ end of the targetting vector to allow for negative selection in the presence of FIAU. Homologous recombination at the *Hand1* locus resulted in deletion of the entire first exon of the gene, as indicated, and the insertion of *lacZ* under control of the endogenous *Hand1* promoter. The position of the external probe used for Southern analysis is indicated. ***b***, Southern blot of yolk-sac DNA from E9.5 embryos.

Departments of [1]Molecular Biology and Oncology and [3]Pediatrics, The University of Texas Southwestern Medical Center at Dallas, 6000 Harry Hines Blvd., Dallas, Texas 75235-9148, USA. Correspondence should be addressed to E.N.O. e-mail: eolson@hamon.swmed.edu

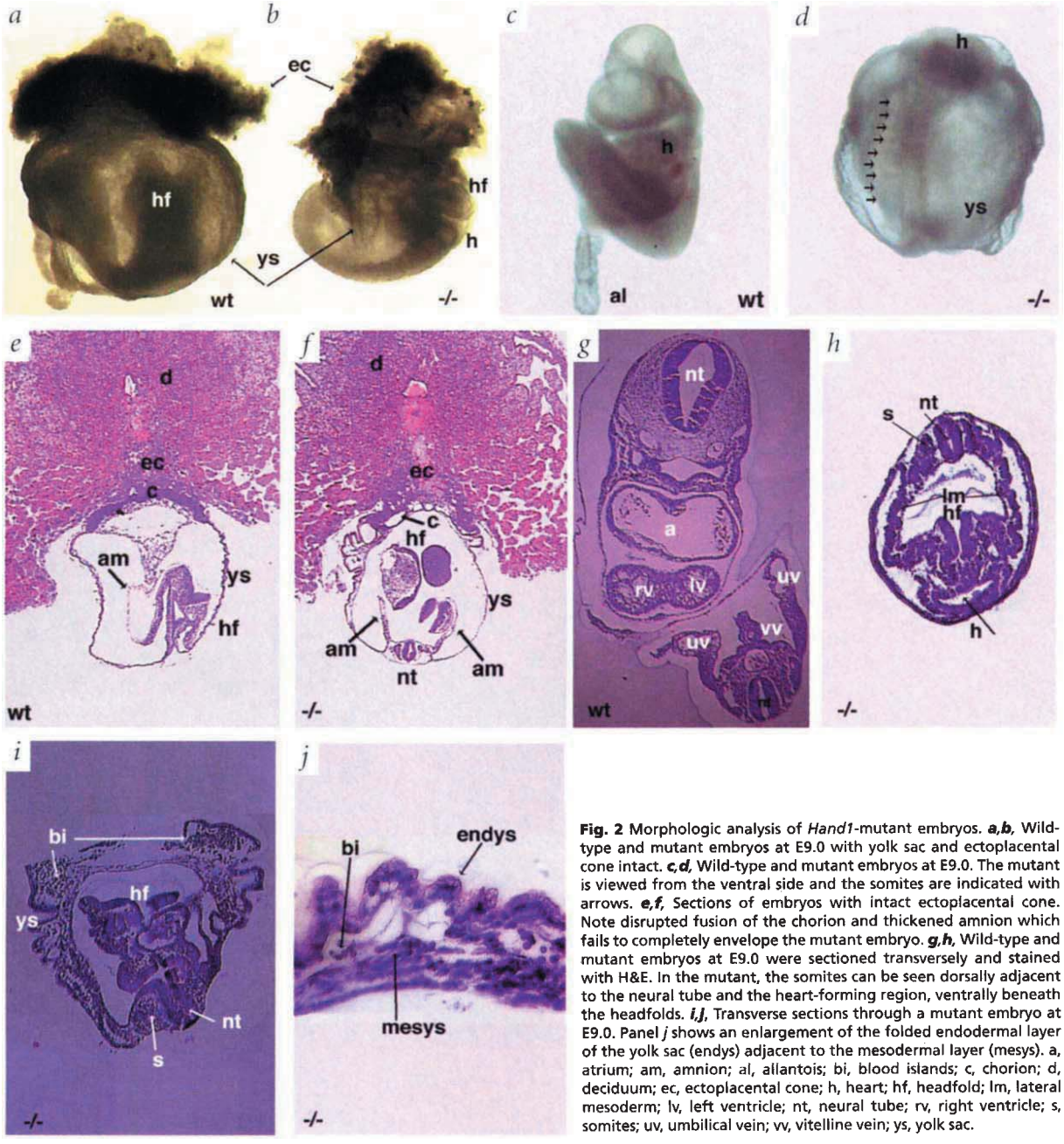

Fig. 2 Morphologic analysis of *Hand1*-mutant embryos. ***a,b,*** Wild-type and mutant embryos at E9.0 with yolk sac and ectoplacental cone intact. ***c,d,*** Wild-type and mutant embryos at E9.0. The mutant is viewed from the ventral side and the somites are indicated with arrows. ***e,f,*** Sections of embryos with intact ectoplacental cone. Note disrupted fusion of the chorion and thickened amnion which fails to completely envelope the mutant embryo. ***g,h,*** Wild-type and mutant embryos at E9.0 were sectioned transversely and stained with H&E. In the mutant, the somites can be seen dorsally adjacent to the neural tube and the heart-forming region, ventrally beneath the headfolds. ***i,j,*** Transverse sections through a mutant embryo at E9.0. Panel *j* shows an enlargement of the folded endodermal layer of the yolk sac (endys) adjacent to the mesodermal layer (mesys). a, atrium; am, amnion; al, allantois; bi, blood islands; c, chorion; d, deciduum; ec, ectoplacental cone; h, heart; hf, headfold; lm, lateral mesoderm; lv, left ventricle; nt, neural tube; rv, right ventricle; s, somites; uv, umbilical vein; vv, vitelline vein; ys, yolk sac.

The cardiogenic region of the mutants also failed to develop properly. Cardiogenic cells are normally specified in a region of the anterior lateral mesoderm known as the cardiac crescent at approximately E7.75 (the presomite stage; ref. 6). This region then elongates along the ventral midline to form the linear heart tube, which initiates contractions at E8.0 (the five- to seven-somite stage) and begins rightward looping soon thereafter. The heart tube formed in the *Hand1* mutant, but looping was abnormal, even though development continued for at least a day beyond the stage in which looping should have occurred, as evidenced by the number of somites (Fig. 2*d*). Contractions were occasionally observed in mutant hearts. Embryos from over 50 heterozygous *Hand1* intercrosses were examined between E8.0–10.0 and we detected little variability in the mutant phenotype.

In heterozygous embryos at E7.5, intense β-gal expression was detected from the mutant allele in the trophectoderm of the ectoplacental cone and extraembryonic mesodermal components of the chorion, amnion, allantois and visceral yolk sac (Fig. 3*a–c*). β-gal was also localized to the cardiac crescent and lateral mesoderm (Fig. 3*c*). There was strong staining in the lateral mesoderm along the entire length of the embryo between E8.0–9.0, as well as within the heart (not shown), and by E9.5, expression in the heart became localized to the left ventricular region and the outflow tract (Fig. 3*d*). β-gal expression was also detected in the umbilical and vitelline vessels, which are derived from extraembryonic mesoderm (Fig. 3*f,h*). At E11.5, β-gal expression persisted in the left ventricle, outflow tract (Fig. 3*e,g,i*) and placenta. β-gal expression was also detected in the mandibular component of the first branchial arch and sympathetic ganglia (Fig. 3*e*), reflecting the neural-crest contribution to these regions[7].

Staining of mutant embryos showed β-gal expression in the mesodermal layer of the visceral yolk sac (Fig. 3*j,m,o*) and indicated that the lateral mesodermal regions appear to be separated

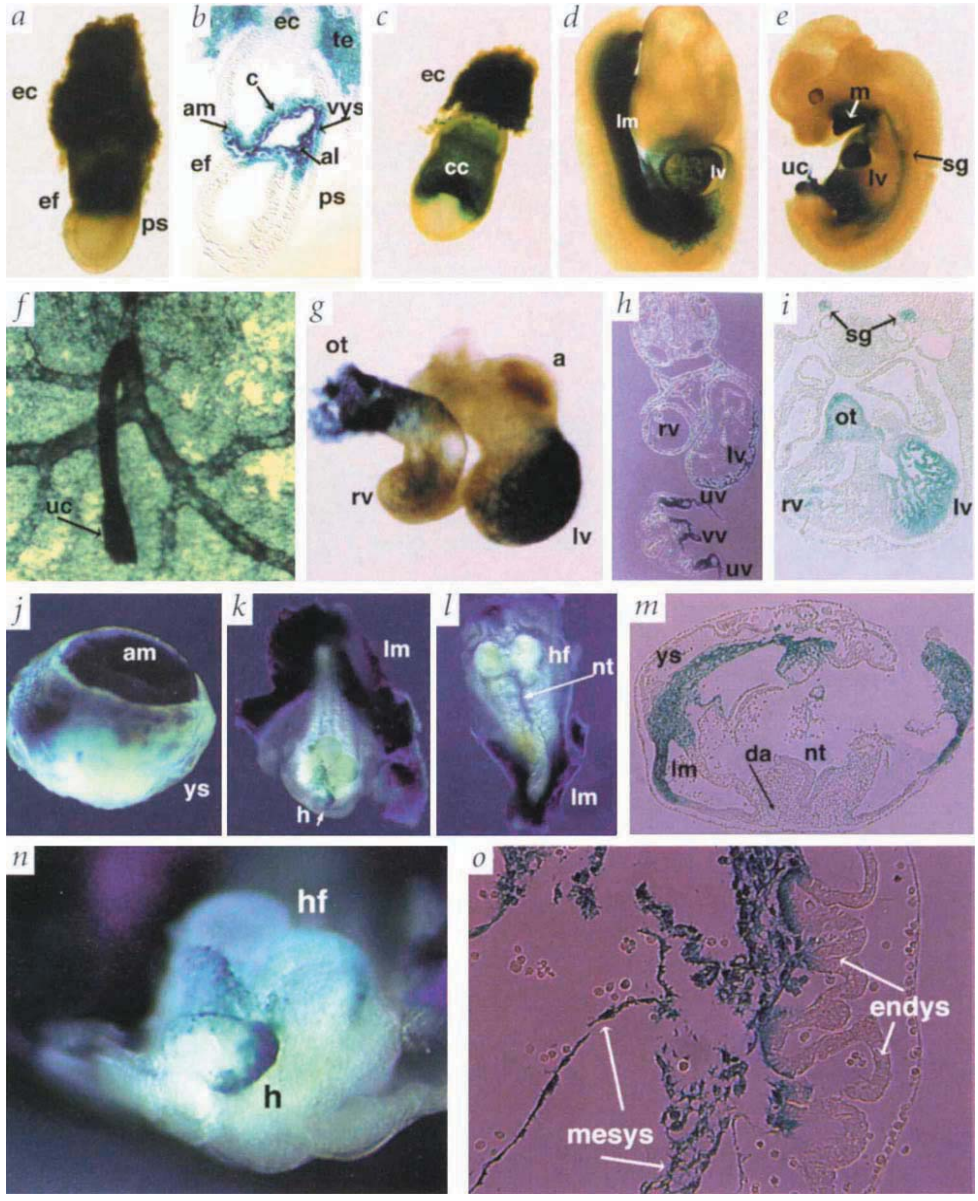

Fig. 3 Expression pattern of reporter gene integrated into the *Hand1* locus. In panels *a–i*, heterozygous embryos from intercrosses of *Hand1(+/-)* mice were stained for β-gal expression. Strong β-gal staining can be seen in the ectoplacental cone (ec) at E7.5 and E7.75 (***a,b,c***). In panel *b*, sagittal sectioning through a E7.5 heterozygous embryo reveals staining in the trophectoderm surrounding the ectoplacental cone, as well as in the amnion (am), chorion (c), allantois (al), and the visceral yolk sac. At E7.75 (panel *c*), expression of the reporter gene is observed in the cardiac crescent (cc). At E9.5, expression can be seen throughout the lateral mesoderm (lm), as well as within the left ventricle of the heart (lv; ***d***), in addition to the yolk-sac vasculature and the umbilical cord (***f***). A transverse section through a heterozygous embryo at E9.5 reveals expression in the left ventricle, as well as in the umbilical veins (uv) and vitelline vein (vv) at the caudal end of the embryo (***h***). At E11.5 (panel ***e***), analysis of the whole-mount heterozygous embryo reveals β-gal expression in the sympathetic ganglia (sg), the mandible (m), the umbilical cord (uc), and the left ventricle of the heart. In the heart at E11.5 (panel ***g***), expression is primarily observed in the left ventricle (lv) and outflow tract (ot) , but not in the right ventricle (rv) or atrium (a). A transverse section of the heart (panel ***i***), reveals expression in the left ventricle, the outflow tract, and the sympathetic ganglia. In panels *j–o*, homozygous mutant *Hand1-/-* embryos were analysed for β-gal expression. Panel ***j*** illustrates the yolk sac at E8.5. In a frontal view of an E9.0 mutant, the head folds (hf) are at the bottom and somites can be seen (panel ***k***). In the heart, expression appears to be enhanced in the left side of the cardiac region. The lateral mesoderm also shows strong β-gal staining. In a dorsal view of an E9.0 mutant (panel ***l***), the headfolds and body axis are indicated. A transverse section (panel ***m***) illustrates β-gal expression in mesodermal components of the yolk sac and outlying lateral mesoderm. In an expanded view of the heart-forming region (panel ***n***), the left-sidedness of β-gal expression in the heart is evident. Panel ***o*** shows high magnification of *Hand1-lacZ* expression in the yolk sac where endoderm of the yolk sac (endys) becomes separated from the mesodermal yolk sac (mesys) component.

from the body axis (Fig. 3*k–m*). Despite the fact that the heart did not develop beyond the heart-tube stage in the mutant, reporter-gene expression appeared to be enhanced on the left side of the cardiogenic region by E9.0 (Fig. 3*k,n*), which may reflect the onset of cardiac looping.

We examined the expression of a series of markers by whole-mount *in situ* hybridization to define potential molecular defects in *Hand1*-mutant embryos. The homeobox gene *Otx2* is normally expressed in the developing head folds[8]. *Otx2* was expressed in the neural ectoderm of the head folds of mutant embryos (Fig. 4*a*). HNF-3β, a marker for the notochord[9], was also expressed in the correct pattern in mutant embryos (Fig. 4*h*), indicating that the head and anterior-posterior axis were properly specified.

To examine the role of *Hand1* in trophoblast development, expression of the giant cell-specific marker placental lactogen I (encoded by the *Pll* gene) was examined (Fig. 4*i*). We observed a dramatic reduction in expression of *Pl1* in *Hand1* mutants which is consistent with earlier findings of a study in which overexpression of *Hand1* in Rcho-1 cells stimulated the *Pl1* promoter[3].

The cardiac markers, atrial natriuretic factor (ANF; encoded by *Nppa*) and myosin light chains MLC-1A, MLC-2A and MLC-2V were expressed within the cardiac crescent region of the mutant, indicating that cardiac commitment and differentiation can occur in the absence of *Hand1* (Fig. 4*b–f*). Defects in cardiac morphogenesis were revealed from the staining pattern of MLC-2A, which showed the failure of the posterior region of the forming heart tube to fuse (Fig. 4*f*). MLC-2V is a ventricular-specific marker[10]; its expression suggests that chamber specification occurred in the mutant. There appeared to be a slight reduction in *Hand2* expression within the heart-forming region (Fig. 4*g*), whereas within the lateral mesoderm, *Hand2* expression appeared to be unaffected. Expression of the cardiac markers MEF2C and Nkx2.5 was unaffected in mutant embryos (data not shown).

In the chick, *Hand1* and *Hand2* are expressed homogeneously

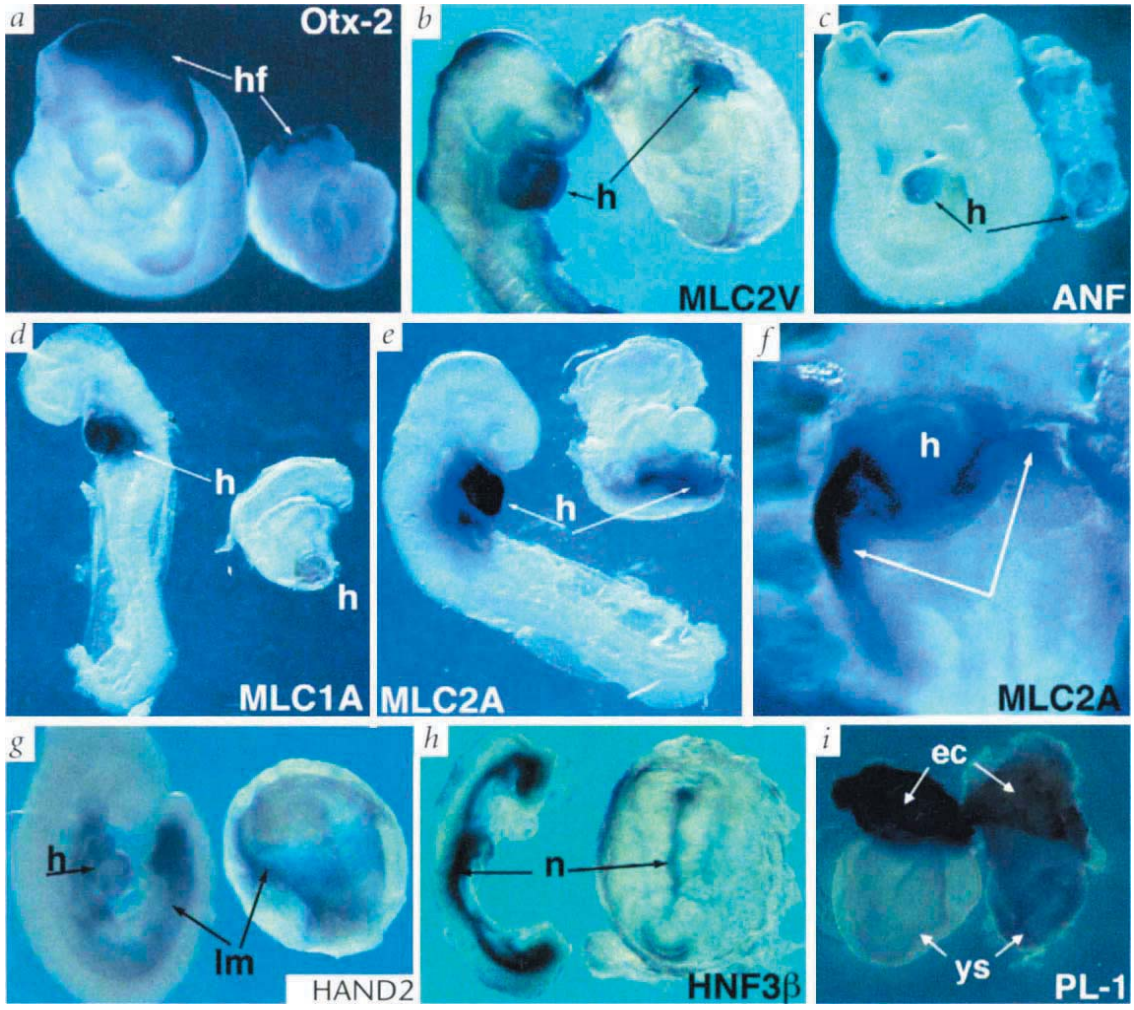

Fig. 4 Whole-mount *in situ* hybridization of wild-type and *Hand1*-mutant embryos. Wild-type and mutant embryos were stained by whole-mount *in situ* hybridization for the indicated markers. In each panel, the wild-type embryo is presented on the left and the mutant, on the right except in *f*, which shows a mutant only. ***a***, Head-fold (hf) expression of the head-specific marker, *Otx2*. ***b–e***, Expression of the cardiac markers: MLC-2V, ANF, MLC-1A, and MLC-2A (arrows) in wild-type and mutants. ***f***, Higher magnification of the mutant embryo in *e*, showing the incomplete fusion of the heart tube determined by the persistence of a cardiac crescent (arrows). ***g***, Expression of *Hand2* in the heart (h) and lateral mesoderm (lm) of wild-type and mutant embryos. ***h***, The notochord-specific marker HNF3β is expressed in a normal pattern in the notochords (n) of wild-type and embryos mutant in *Hand1*. ***i***, Expression of the trophoblast marker PL-I in the ectoplacental cones (ec) of wild-type and *Hand1*-null embryos. Head-staining of embryos in panel *c* is background.

throughout the developing heart and appear to play overlapping roles in cardiac looping[2]. During heart development in the mouse, Hand1 and Hand2 become restricted to the left and right ventricular regions, respectively, at the onset of looping and are the earliest chamber-restricted transcription factors identified to our knowledge[5,11]. This mutually-exclusive expression pattern may account for their apparent lack of redundancy in the developing heart of the mouse, as compared with the chick.

The probable cause of death of the *Hand1*-mutant embryos is nutrient defiency—probably due to the lack of *Pl1* expression in trophoblast giant cells. We have considered the possibility that the cardiac defects observed in *Hand1*-mutant embryos might arise secondarily to nutritional deficits due to abnormalities in the extraembryonic mesoderm. While we cannot formally rule this out, we believe that the cardiac defects are not solely due to extraembryonic abnormalities, because the cardiac defects appear highly specific, in contrast with other developmental events which occur normally in the absence of *Hand1*. As *Hand1*-mutant embryos developed at least 12 somites (approximately E8.5), the cardiogenic defects are unlikely to be a result of a general delay in growth. Unequivocal assessment of the cardiac functions of Hand1 will require cardiac-specific conditional gene modulation, *in vitro* embryo culture or tetraploid rescue.

Methods

Gene targetting. A *Hand1* genomic clone was isolated from a 129sv lambda library and partially characterized by restriction-mapping and DNA sequencing. To construct the targetting vector, a 3.0-kb *Bst*EII fragment extending upstream from the *Hand1* translational initiation codon was ligated to a β-galactosidase reporter gene. This *Hand1-lacZ* cassette was then cloned upstream of a neomycin-resistance gene linked to the phosphoglycerokinase (PGK) promoter. For the 3′ arm of genomic homology, we used a 1.2-kb *Sal*I-*Hin*dIII fragment that extended from within the first intron of the gene into the second exon. A thymidine-kinase gene under control of the herpes simplex virus promoter was linked downstream of the 3′ arm. All cloning junctions within the targetting vector were confirmed by DNA sequencing.

The targetting vector was linearized with *Not*I, prior to electroporation into AB2.2 129 ES cells, and plated onto G418-resistant STO feeder layers. Following positive-negative selection with G418 and FIAU, respectively, individual ES cell colonies were isolated and analysed by Southern blot for

homologous recombination at the *Hand1* locus, as previously described[12]. Homologous recombination was observed at a frequency of 1:30 in the 600 ES cell clones analysed. Two independent clones were injected into blastocysts obtained from C57/B6 mice, which were subsequently implanted into pseudopregnant Swiss foster female mice. Chimaeras that were obtained transmitted the *Hand1* mutation through the germline and the resulting offspring were intercrossed or were bred with Swiss mice to ultimately obtain *Hand1*-null embryos.

Genotyping. ES cell DNA, as well as tail genomic DNA, was analysed for the *Hand1* mutation by Southern-blot analysis[12]. Insertion of the *lacZ* and *neo* genes into the *Hand1* locus introduced an *Eco*RI site that could be used to distinguish the wild-type and targetted alleles, which yielded 12.0-kb and 4.1-kb fragments, respectively, following Southern analysis of *Eco*RI-digested DNA and hybridization with a labelled *Hin*dIII-*Bss*HII probe from the region 3′ of the targetted mutation.

***In situ* hybridization, β-gal staining and histology.** β-gal expression was detected in *Hand1* (+/–) and (–/–) embryos as described[13]. Briefly, embryos were dissected from the uterus and fixed in 2% paraformaldehyde-0.2% glutaraldehyde for up to 1 h, after which they were rinsed in PBS and incubated in PBS containing 5 mM $K_3Fe(CN)_6$, 5-mM $K_4Fe(CN)_6{:}3H_2O$, 2 mM $MgCl_2$ and 1 mg/ml X-gal overnight. Reactions were stopped by rinsing embryos with PBS, followed by further fixation in 4% paraformaldehyde.

Embryos that were prepared for sectioning were dehydrated in ethanol and embedded in paraffin. Transverse sections were cut at 10-μm intervals and dried on microscope slides. Paraffin was removed with xylene and sections were stained with H&E or simply treated with cytoseal and covered.

Whole-mount *in situ* hybridizations were performed with digoxygenin-labelled RNA run-off probes as described[14]. Sources of cDNAs for making probes were as follows: *Otx2* (ref. 8), HNF3β[9], *Nappa*[15], MLC-1A, MLC-2A, MLC-2V, *Hand2* (ref. 2), and *Pl1* (ref. 16). After hybridization, probes were immuno-detected with alkaline phosphatase-conjugated anti-digoxygenin antibody followed by colourization reactions.

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