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

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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 arteries⁵. 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 targeted in embryonic stem (ES) cells (Fig. 1a). 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 littersmates is shown in Fig. 1b.

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. 2a–h). The neural tubes of the mutants were often crooked (Figs 3i and 4h), 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. 2e–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. 2i,j). In addition, the amnion of mutant embryos appeared thickened and failed to completely enclose the body of the embryo (Fig. 2e,f), resulting in abnormal fusion of the amniotic and exocoelomic cavities. The chorion of mutant embryos also appeared abnormal, suggesting defects in chorician fusion (Fig. 2e,f).

![Fig. 1 Targeting 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 targeting vector used for homologous recombination is in the middle and the targeted allele is at the bottom. The targeting 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 targeting vector were 3.0 and 1.2 kb, respectively. The HSV-tk gene was linked to the 3' end of the targeting 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.](image-url)

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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. 2d). 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. 3a–c). β-gal was also localized to the cardiac crescent and lateral mesoderm (Fig. 3c). 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. 3d). β-gal expression was also detected in the umbilical and vitelline vessels, which are derived from extraembryonic mesoderm (Fig. 3f,h). At E11.5, β-gal expression persisted in the left ventricle, outflow tract (Fig. 3e,g,i) and placenta. β-gal expression was also detected in the mandibular component of the first branchial arch and sympathetic ganglia (Fig. 3e), reflecting the neural-crest contribution to these regions.

Staining of mutant embryos showed β-gal expression in the mesodermal layer of the visceral yolk sac (Fig. 3j,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–l, 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 d, sagittal sectioning through a E7.5 heterozygous embryo reveals staining in the trophoderm surrounding the ectoplacental cone, as well as in the amnion (am), chorion (ch), allantois (al), and the visceral yolk sac. At E7.75 (panel e), 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 (v) 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–n, 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. 3k–n). 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. 3k,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 folds8, Otx2 was expressed in the neural ectoderm of the head folds of mutant embryos (Fig. 4a). HNF3β, a marker for the notochord9, was also expressed in the correct pattern in mutant embryos (Fig. 4h), 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 Plt gene) was examined (Fig. 4i). We observed a dramatic reduction in expression of Plt in Hand1 mutants which is consistent with earlier findings of a study in which overexpression of Hand1 in Rcho-1 cells stimulated the PII promoter10.

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. 4b–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. 4f). MLC-2V is a ventricular-specific marker10; 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. 4g), whereas within the lateral mesoderm, Hand2 expression appeared to be unaffected. Expression of the cardiac markers MEF2C and Nx2.5 was unaffected in mutant embryos (data not shown).

In the chick, Hand1 and Hand2 are expressed homogeneously
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.\(^3,11\) 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 deficiency—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 targeting. A Hand1 genomic clone was isolated from a 129sv lambda library and partially characterized by restriction-mapping and DNA sequencing. To construct the targeting vector, a 3.0-kb BstEII 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 Sall-HindIII 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 targeting vector were confirmed by DNA sequencing.

The targeting vector was linearized with NotI, 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
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 cytochrome and covered. Whole-mount in situ hybridizations were performed with digoxigenin-labelled RNA run-off probes as described. Sources of cDNAs for making probes were as follows: Otx2 (ref. 8), Hnf3β, Napp1, MLC-1A, MLC-2A, MLC-2V, Hand2 (ref. 2), and Pli (ref. 16). After hybridization, probes were immuno-detected with alkaline phosphatase-conjugated antidigoxigenin antibody followed by colourization reactions.

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