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Hand1 regulates cardiomyocyte proliferation versus differentiation in the developing heart

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The precise origins of myocardial progenitors and their subsequent contribution to the developing heart has been an area of considerable activity within the field of cardiovascular biology. How these progenitors are regulated and what signals are responsible for their development are, however, much less well understood. Clearly, not only is there a need to identify factors that regulate the transition from proliferation of cardioblasts to differentiation of cardiac muscle, but it is also necessary to identify factors that maintain an adequate pool of undifferentiated myocyte precursors as a prerequisite to preventing organ hypoplasia and congenital heart disease. Here, we report how upregulation of the basic helix-loop-helix (bHLH) transcription factor Hand1, restricted exclusively to *Hand1*-expressing cells, brings about a significant extension of the heart tube and extraneous looping caused by the elevated proliferation of cardioblasts in the distal outflow tract. This activity is independent of the further recruitment of extracardiac cells from the secondary heart field and permissive for the continued differentiation of adjacent myocardium. Culture studies using embryonic stem (ES) cell-derived cardiomyocytes revealed that, in a *Hand1*-null background, there is significantly elevated cardiomyocyte differentiation, with an apparent default mesoderm pathway to a cardiomyocyte fate. However, Hand1 gain of function maintains proliferating precursors resulting in delayed and significantly reduced cardiomyocyte differentiation that is mediated by the prevention of cell-cycle exit, by G1 progression and by increased cell division. Thus, this work identifies Hand1 as a crucial cardiac regulatory protein that controls the balance between proliferation and differentiation in the developing heart, and fills a significant gap in our understanding of how the myocardium of the embryonic heart is established.

KEY WORDS: Heart, Hand1, Mouse, Tet-Off, Outflow tract, Cardiomyocyte, Proliferation, Differentiation

INTRODUCTION

Heart morphogenesis begins shortly after gastrulation with the specification of the cardiac precursors of the cardiac crescent in the anterior lateral mesoderm followed by the migration of these precursors to the ventral midline and their fusion to form the linear heart tube (Harvey, 2002). These cells comprise the so-called primary heart field that later contributes mainly to the left ventricle (Cai et al., 2003). Subsequent rapid elongation coincident with cardiac looping then occurs following the recruitment of cells to the growing poles of the heart (Kelly and Buckingham, 2002). At the arterial pole, the heart tube is extended by the addition of extracardiac cells that originate from progenitors in the pharyngeal mesoderm, dubbed the anterior (or secondary) heart field (AHF) (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). Cardiomyocytes arising from the AHF take on right ventricular and outflow tract (OFT) identities (Zaffran et al., 2004). More recently, the AHF itself has been further redefined as a subpopulation of a broader second lineage that not only contributes to the right ventricle and OFT at the arterial pole of the heart, but also to the atria and inflow regions at the venous pole (Meilhac et al., 2004). Inroads into deciphering transcriptional regulation in the cardiac lineages suggest a requirement for combinatorial activities of multiple transcription factors that have an impact on differentiation and deployment of myocardial precursor cells. However, factors that maintain a balance between proliferation and differentiation of different populations of myocardial precursors have not been identified.

Hand1 (previously known as eHAND, Hxt and Thing1), a member of the bHLH transcription factor family, is a candidate regulator of cardiac precursor cell fate. *Hand1* is expressed in distinct regions of the linear heart tube and, post-looping, becomes localised to both primary heart field and second lineage derivatives, specifically in the outer curvature of the presumptive left ventricle and the developing OFT, and also at lower levels in the outer curvature of the right ventricle. The role of Hand1 in heart development has previously been explored in mice lacking *Hand1*, but detailed analysis is confounded by early embryonic lethality resulting from extra-embryonic defects (Firulli et al., 1998; Riley et al., 1998). Rescue of the extra-embryonic defects by tetraploid aggregation experiments yielded limited insight into the precise cellular role of Hand1 in the heart because embryos were only partially rescued, with failed looping morphogenesis, defective chamber septation and impaired ventricular development (Riley et al., 1998).

Controversy surrounds the specific function of Hand1 in the heart in terms of whether it is involved in regulating looping morphogenesis as a primary role that feeds back directly onto ventricular development, or whether it is restricted to ventricular specification and maturation coincident with looping. Conditional targeting of *Hand1* has not resolved this issue, as the defects observed following heart-specific knockout of *Hand1* (McFadden et al., 2005) are less severe than would be predicted based on the reported phenotype of tetraploid-rescued conventional *Hand1*-null embryos (Riley et al., 1998). In the conditional targeting study by McFadden and colleagues (McFadden et al., 2005), *Hand1* cardiac knockouts under the control of a Nkx2.5 Cre driver formed hearts

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that clearly looped and had a degree of left-ventricular expansion. These observations are significantly milder than previously described for the tetraploid-rescued mutants, which are characterised by a complete failure in looping morphogenesis and aberrant ventricular differentiation (Riley et al., 1998). Putative limitations with respect to the Cre approach taken are probably caused by mosaicism of the Cre expression under the described Nkx2.5 promoter (McFadden et al., 2005) and/or a temporal delay in efficient Cre excision resulting in Hand1 being inactivated at the heart tube stage, excluding an effect on earlier cells expressing *Hand1* (Buckingham et al., 2005). Nevertheless, the conditional, heart-specific *Hand1* mutant embryos presented with left-ventricular hypoplasia, leading to the postulation that this specific anomaly could be the result of a proliferation defect in the primary heart field lineage (Buckingham et al., 2005). However, because of the early lethality of mouse embryos lacking *Hand1* and the limited insight gained from the conditional knockout approach, it was necessary to develop a model that would enable us to express *Hand1* in an appropriate spatially and temporally controlled manner, circumventing complications associated with extra-embryonic defects, which could then be used to assess its precise role during heart development. To achieve this, we engineered the tetracycline system into the endogenous *Hand1* locus. The Tet-Off transactivator (tTA) was targeted specifically by homologous recombination and thus placed under the control of the endogenous *Hand1* promoter and regulatory regions. The resulting mouse tTA knock-in strain (driver) was then crossed to multiple transgenic lines (responders) to regulate expression of a tet-responsive *Hand1* transgene. In light of the limitations of pre-existing loss-of-function mouse models, we sought to use the tet-system knock-in approach to induce overexpression of *Hand1*, restricted exclusively to *Hand1*-expressing regions, and to facilitate the analyses of early- and late-onset functions of Hand1 in the developing heart. At E9.5, overexpression of *Hand1* disrupted cardiac morphogenesis and brought about an extension of the heart tube and extraneous looping caused by the elevated proliferation of cardiac precursors in the distal OFT. In complimentary studies employing in vitro differentiated embryonic stem (ES) cells, the complete loss of *Hand1* resulted in elevated cardiomyocyte differentiation, whereas *Hand1* gain of function maintained cardiomyocyte proliferation, via G1 progression and delayed cell-cycle exit. The combined in vivo and in vitro studies, therefore, indicate a cell-autonomous role for Hand1 in regulating the balance between cardiomyocyte differentiation and proliferation during cardiac development.

MATERIALS AND METHODS

Generation of driver and responder mouse strains

For the driver targeting construct a 1 kb *Eco*RI-*Bam*HI fragment containing the tTA transactivator was excised from pTet-Off (Clontech) and cloned into pKS+. A 0.8 kb rabbit β-globin poly(A) sequence (van Ooyen et al., 1979) and neomycin-resistance cassette from pPNT (Tybulewicz et al., 1991) flanked by loxP sites were cloned downstream of the tTA. This construct was then subcloned into a *Sal*I-*Bgl*II digest of a *Hand1*-targeting vector described previously (Riley et al., 2000). For the responder transgenic construct, a full-length *Hand1* cDNA was cloned downstream of the tetracycline response element (TRE) in pTRE2 (Clontech) and a neomycin-resistance cassette was inserted downstream in the opposing orientation. The driver and responder mouse lines were generated by genOway (Lyon, France). The driver targeted line was established by homologous recombination in 129sv ES cells. Correctly targeted ES-cell clones were identified by Southern blot analysis, using a *Bgl*II digest of genomic DNA and a 5′ probe that is a 2 kb *Eco*RI/*Kpn*I fragment that hybridises to a 13 kb wild-type band and a 9 kb targeted mutant band. The responder transgenic strain was generated by 'Safe DNA Transgenesis' (http://www.genoway.com/). Positive ES-cell clones were again identified by Southern blot (see Fig. 1A-D). Transgene copy number was estimated by scanning densitometry using the Scion Image program (Scion Corporation; http://www.scioncorp.com/frames/fr_scion_products.htm). Positive clones were subsequently used for injection into C57BL/6-derived blastocysts for generation of chimeras using standard procedures, for both the targeted driver and transgenic responder lines.

Mouse maintenance and genotyping

Mice were maintained on a mixed background. Embryos were generated by mating heterozygous driver and responder mice. A total of 654 embryos were analysed and all were stage-matched to controls by somite count. The targeted driver allele was identified by a 690 bp product (S-5′-GGG-GTGGGGTGGGATTAGAT-3′ and AS-5′-AGAAGGGCCCAGGGAAG-ACT-3′). The presence of the responder transgene was detected by a 2.2 kb product (S-5′-GCGCCTGGCTACCAGTTACA-3′ and AS-5′-GGGGTG-GGGTGGGATTAGAT-3′).

ES cells and embryoid bodies

ES cells containing the transgenic responder construct were co-transfected with pBIG3r (Strathdee et al., 1999) containing the tTA under the control of a TK promoter and pTK-Hyg (Clontech). Positive clones stably expressing the tTA were isolated following hygromycin selection and presence of the tTA was determined by a 150 bp PCR product (S-5′-CAGCGCA-TTAGAGCTGCTTA-3′ and AS-5′-ATCTCAATGGCTAAGGCGTC-3′). RT-PCR was performed to confirm that the positive clones expressed both the tTA (primers as above) and the *Hand1* transgene, as detected by a 400 bp PCR product (S-5′-GATGGGACTGGAGAAGACCA-3′ and AS-5′-GAAGTCAGATGCTCAAGGGG-3′).

ES cells were cultured and differentiated in vitro to form embryoid bodies (EBs) derived from wild-type, control (transgenic responder construct alone), *Hand1*-overexpressing and *Hand1* heterozygous or null lines, as previously described (Riley et al., 2000; Smart et al., 2002). Single cardiomyocytes were isolated from adherent EB cultures at days 10 and 14, according to the protocol described previously (Maltsev et al., 1993).

RT-PCR

mRNA was isolated from whole embryos, cells and EBs using the Micro FastTrack 2.0 kit (Invitrogen), according to the manufacturer's instructions. The RNA was DNase treated before reverse transcription was performed using Superscript II RT (Invitrogen), according to the manufacturer's instructions. PCR was performed using standard conditions.

Optical projection tomography

Optical projection tomography (OPT) was performed essentially as described (Sharpe et al., 2002) on whole-mount embryos. Analysis and visualisation of OPT data was performed with Improvision Volocity 3.1.

Histology

Embryos were fixed, dehydrated and wax embedded as previously described (Moorman et al., 2001). They were then serially sectioned at 10-15 μm. In situ sections were counterstained with 0.5% Eosin, as described previously (Smart et al., 2002).

RNA in situ hybridisation

RNA in situ hybridisation on whole-mount embryos and sections was performed as previously described (Moorman et al., 2001; Smart et al., 2002), using riboprobes specific for *Hand1* (Cserjesi et al., 1995), atrial natriuretic factor (*Anf; Nppa* – Mouse Genome Informatics) and *Gata4* (Kuo et al., 1997), islet 1 (*Isl1*) and *Wnt11* (Cai et al., 2003), *Nkx2.5* (Lints et al., 1993), *Hand2* (Srivastava et al., 1997), and myocyte enhancer factor 2c (*Mef2c*) (Edmondson et al., 1994).

Immunofluorescence

Sections were dewaxed and rehydrated (Moorman et al., 2001), and stained with antibodies against MF20 (1/10; Developmental Studies Hybridoma), phospho-histone H3 (1/200; Upstate) and cleaved caspase 3 (1/100; Cell Signalling Technology). Sections were then counterstained with the nuclear marker bis-benzamide, as previously described (Hill and Riley, 2004; Riley et al., 2000).

Real-time PCR

Real-time qRT-PCR was carried out on RNA extracted from embryos at E9.5 and EBs following 14 days of differentiation. This was performed on an ABI 7000 Sequence Detector (Applied Biosystems) using SYBR Green (Absolute QPCR SYBR Green Mix, ABgene). Complementary DNA PCR primers were obtained from published sequences (Fijnvandraat et al., 2003a; Fijnvandraat et al., 2003b; Lekanne Deprez et al., 2002) or designed using Primer Express software (version 2.0, Applied Biosystems).

Western blot

EB lysates were prepared in RIPA lysis buffer following 14 days of differentiation. Western blot was performed by standard methods using antibodies against cyclin D2 (1/500; Abcam), Cdk4 (1/500; Santa Cruz) and Gapdh (1/1000; Chemicon). Scanning densitometry was performed as described above.

RESULTS

Generation of the *Hand1* overexpression model

The Tet-Off system was employed to investigate the effects of overexpressing *Hand1* in the developing heart, specifically in *Hand1*-expressing cells. Two independent mouse strains were generated: the driver (tTA) was targeted to the endogenous *Hand1* locus by homologous recombination in ES cells (Fig. 1A,B); and the responder (TRE2), was placed upstream of a full-length *Hand1* cDNA and randomly inserted, as a transgene, into ES cells (Fig. 1C). Correctly targeted and transgenic ES-cell clones were identified by Southern blotting and PCR (Fig. 1D,E). Southern blotting was performed to confirm germline transmission of both the driver and responder strains. Incorporation of a single tTA allele into the *Hand1* locus resulted in mice that were heterozygous for *Hand1* and thus exhibited no discernable phenotype in advance of driver/responder matings (Riley et al., 1998).

To determine whether the tTA was expressed in *Hand1*-expressing lineages in the driver strain, we performed RNA in situ hybridisation analysis to detect tTA transcripts in whole-mount embryos and in sections at E9.5. The results confirmed that tTA expression from a gross whole-mount perspective colocalises with *Hand1*-expressing regions, notably in the branchial arches, lateral plate mesoderm, distal OFT and left ventricular (LV) myocardium at E9.5 (Fig. 2A,B). In histological sections, there were some differences between Hand1 and tTA expression, reflecting a degree of mosaicism in the tTA driver, notably ectopic expression in the first branchial arch (Fig. 2E,F). As we have taken the approach of knocking-in the tTA driver into the endogenous *Hand1* locus and we know from previous studies that Hand1 is not required for its own expression (Riley et al., 2000), it is reasonable to assume that the tTA is subject to all of the appropriate temporal and spatial control as endogenous *Hand1*. Any variation, therefore, between *Hand1* and tTA expression should be minimal and inexplicable in light of our current understanding of the knock-in approach.

The fact that tTA expression, on the whole, recapitulated that of endogenous *Hand1* confirmed the knock-in approach to be suitable for driving *Hand1* overexpression exclusively in *Hand1*-expressing cells. In order to assess levels of *Hand1* expression, compound heterozygote embryos were generated by crossing the driver and responder strains. Compound heterozygotes expressed the transgenic *Hand1* cDNA by RT-PCR and northern blot (not shown), and, furthermore, had approximately twice as much *Hand1* expression, as measured by real-time quantitative PCR, than control embryos (Fig. 1F). The single founder animal (Fig. 1F) and subsequent F1 and F2 generations were used throughout the study. The transgene copy number for the original founder was four and no

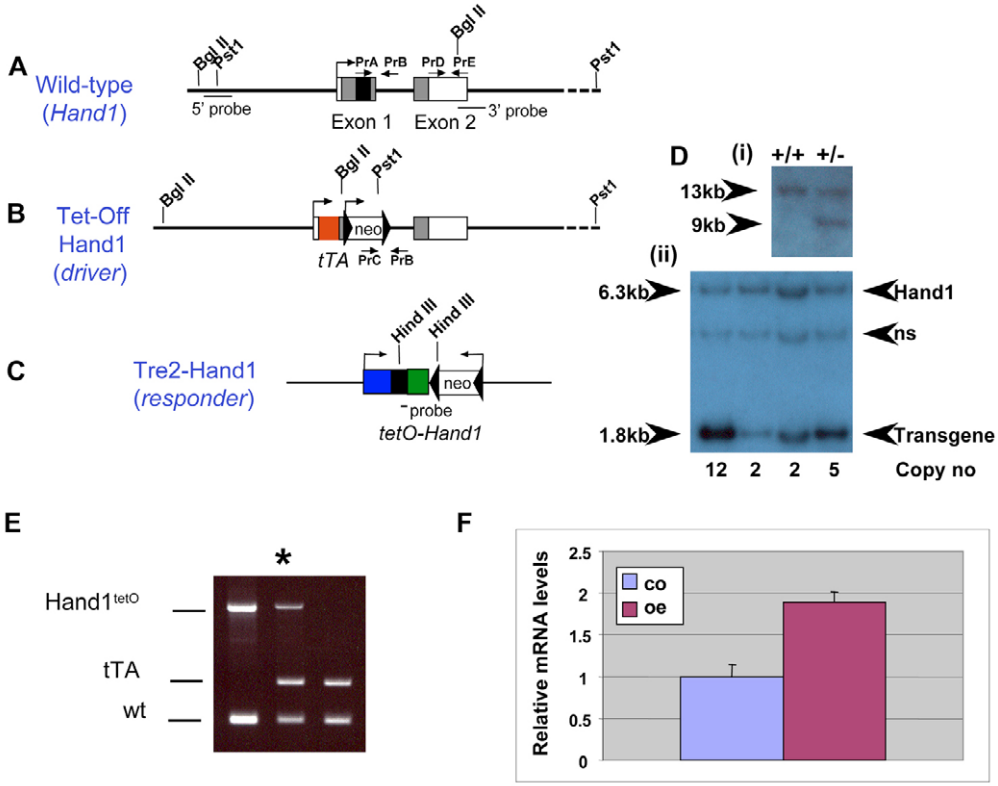

Fig. 1. Incorporating the Tet system into the *Hand1* locus to generate an overexpression model. (**A**) Wild-type *Hand1* consists of two exons (boxes: non-coding regions indicated in white, coding region is shaded, bHLH domain is shown in black) separated by a 1.5 kb intron. (**B**) The targeted *Hand1* locus contains the transactivator tTA (red) under the control of the endogenous *Hand1* promoter. (**C**) The tet-responsive Tre2 (blue)-Hand1 cDNA (black) upstream of a β-globin poly(A) (green) randomly integrated as a transgene. The primers PrA, PrB, PrC and PrD are as shown in A,B. (**D**) Southern blot analysis to confirm the targeting of the endogenous *Hand1* locus for the driver and varying copy number founder lines for the responder. (**i**) *Bgl*II digest with the 5′ probe illustrated in A. (**ii**) *Hind*III digest with the probe illustrated in C. (**E**) PCR genotyping of F1 following driver × responder matings (asterisk denotes compound driver/responder heterozygote). (**F**) Quantitative real-time PCR on RNA from compound heterozygote embryos at E9.5 reveals twice as much expression of *Hand1* compared with control littermates containing only driver or responder. Bars represent mean±s.e.m. of three real-time qRT-PCR experiments on single founder overexpression and control embryos. co, control; ns, non-specific; oe, overexpression.

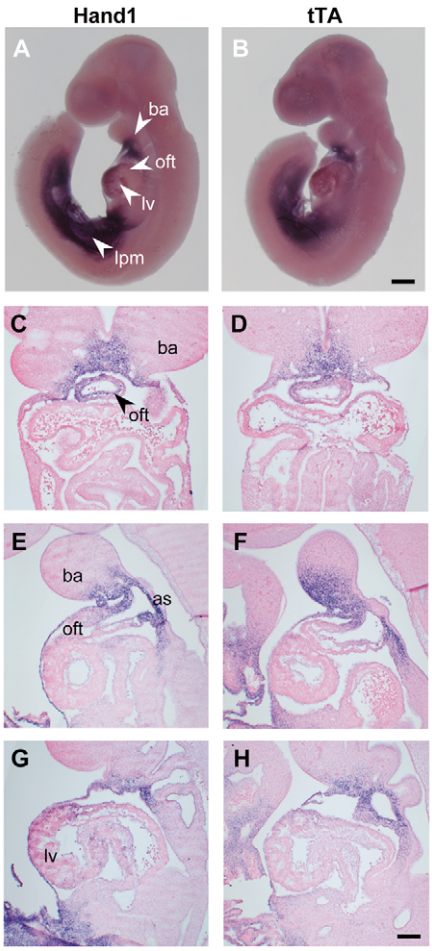

Fig. 2. The targeted tTA transactivator is expressed in an equivalent spatial pattern to endogenous *Hand1*. Whole-mount RNA in situ hybridisation analysis of *Hand1* (**A**) and tTA (**B**) in control embryos at E9.5. (**C**,**D**) Frontal sections through the distal OFT region. (**E-H**) Sagittal sections through the first branchial arch (E,F) and heart (G.H). as, aortic sac; ba, branchial arch; oft, outflow tract; lv, left ventricle. Scale bars: in B, 200 μm for A,B; in H, 50 μm for C-H.

correlation was observed between copy number and level of *Hand1* expression (data not shown). The *Hand1* responder transgene was not expressed in the absence of the tTA driver (data not shown), confirming that the system was not 'leaky'.

Overexpression of *Hand1* disrupts cardiac morphogenesis

The observation that *Hand1* overexpression in developing trophoblasts promotes terminal differentiation to a giant cell fate (Cross et al., 1995) and the fact that tetraploid-rescued *Hand1*-null embryos have hypoplastic hearts with downregulation of differentiation markers such as *Mlc2v* (*Myl2* – Mouse Genome Informatics) (Riley et al., 1998), suggest that *Hand1* overexpression in the developing heart may promote cardiomyocyte differentiation in vivo. In compound driver/responder heterozygotes we observed, as early as E8.0, expansion of the primary heart tube and extension at the earliest stages of looping compared to littermate controls (embryos containing either only the driver targeted allele or the responder transgene), suggesting hyperplasia and a putative expansion of cell number (Fig. 3). Extension of the primary heart tube at E8.0-E8.5 (Fig. 3A-L) was associated with a corresponding temporal and spatial overexpression of *Hand1*, as confirmed by in situ analysis (Fig. 3M-P). At E9.5, *Hand1*-overexpressing embryos revealed a distinct cardiac phenotype (Figs 4, 5). Hearts in which overexpression occurred clearly looped to the right, suggesting no apparent defect in the initiation and onset of looping. However, looping was abnormal, with a significant overextension of the heart tube. Most notably, the OFT was elongated distally and physically displaced from the inflow region (Fig. 4D). Coincident with the abnormal looping was defective LV development (Figs 4, 5). The *Hand1* overexpression embryos appeared morphologically normal in other lineages, and the phenotype could be reversed with the addition of the tet derivative doxycycline to pregnant mothers (data not shown), indicating that the embryonic defects observed were specific and indeed caused by overexpression of *Hand1*.

The outflow tract is elongated in *Hand1*-overexpressing embryos

In *Hand1*-overexpressing embryos, the distal OFT region was significantly extended and the heart tube displaced both ventrally away from the body wall and laterally to reveal extraneous rightward looping (Figs 4, 5). The abnormal looping of the heart is most probably a direct consequence of the OFT extension, as the heart tube might be expected to undergo additional turns/folds to become accommodated within a restricted pericardial region. In order to confirm the observation of OFT elongation, distal OFT length was measured in moderately (n=11) and severely (n=5) affected overexpression embryos and compared with transgene positive (n=9) controls (Fig. 5A-D). The distal OFT was defined as the region of the heart tube extending from the body wall to the apex of the so-called 'dog leg bend' (Anderson et al., 2003). In severely affected embryos, the OFT was found to be approximately twice as long as controls. In the moderately affected embryos, OFT length was found to be approximately 50% longer than in controls. Variation in the cardiac phenotype between *Hand1*-overexpressing embryos at mid-gestation stages (compare Fig. 4D-F with 4G-H) is most likely to be attributable to a degree of mosaicism at the level of tTA expression despite the knock-in approach (data not shown). However, the mosaicism fortuitously enabled us to assess a range of hypermorphic cardiac phenotypes, with the most severe case illustrated in Fig. 4G-I and Fig. 5E-H. Furthermore, hypermorphic embryos provided a valuable insight into changes in gene expression profiles in the heart, even when the anatomical defects observed were mild to modest (see below).

Abnormal ventricular development in severely affected *Hand1* overexpression embryos

In severely affected embryos, extraneous looping was accompanied by impaired LV differentiation and expansion leading to severe necrosis (Fig. 4G-I,M, Fig. 5H). In moderately affected embryos, the presumptive left ventricle was reduced in size but had otherwise undergone a degree of expansion and appeared to be developing normally (Fig. 4D-F,K). Sections through the hearts of the severely affected *Hand1*-overexpressing embryos revealed that the failure in ventricular expansion was associated with an almost complete absence of chamber lumen (Fig. 4M) caused by a combination of elevated myocyte density (control, 110±15 (n=6); overexpression, 185±12 (n=6); P<0.05) and cellular hypertrophy. The LV phenotype in the severely affected embryos may be a primary effect of *Hand1* overexpression in the LV myocardium itself, or could be a secondary effect caused by the impaired haemodynamics of the expanded OFT and extraneously looped heart tube.

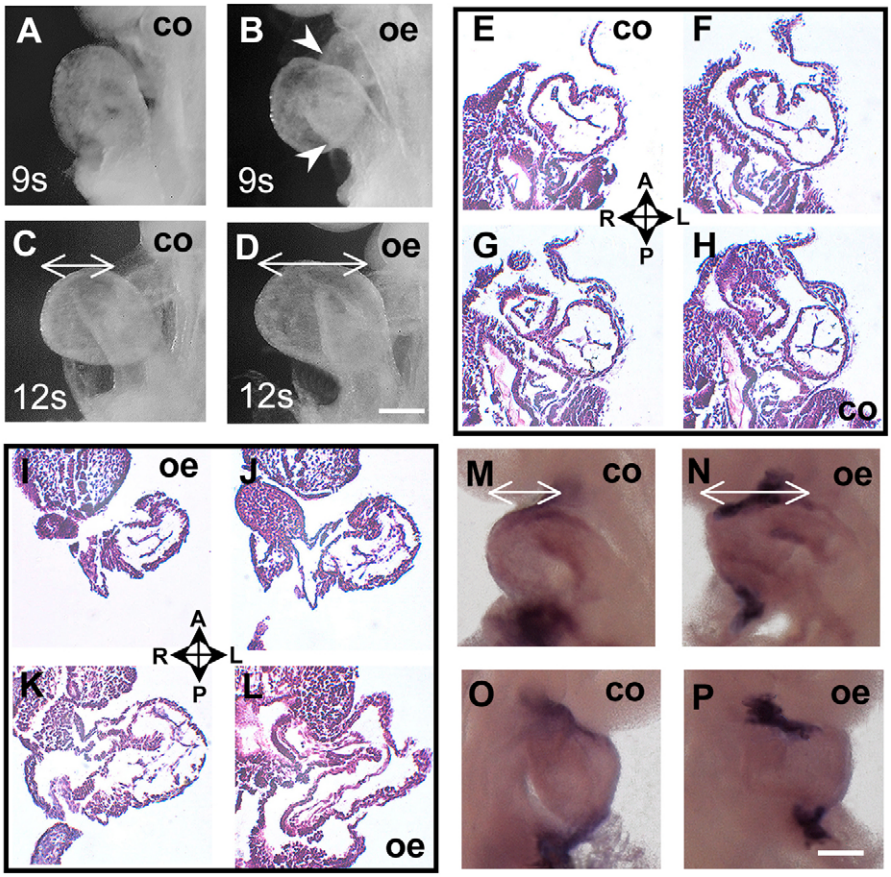

Fig. 3. Primary heart tube expansion in *Hand1*-overexpressing embryos at E8.0-8.5. Bright-field whole-mount left lateral views of hearts from somite-matched *Hand1*-overexpressing (oe) embryos and control (co) littermates dissected pre-embryonic turning (nine somites; **A**,**B**) and during turning (12 somites; **C**,**D**). Haematoxylin and Eosin stained frontal sections through the heart tube of a control (**E-H**) and overexpression embryo (**I-L**) at the 12-somite stage. Whole-mount in situ hybridisation for *Hand1* on control (**M**,**O**) and overexpression embryonic hearts (**N**,**P**). (A,B) The primary heart tube pre-turning is expanded (white arrowheads) and early stages of looping are accentuated in overexpressing embryos compared with control. Extension and exaggerated ventral and left lateral displacement of the heart tube can be seen as looping progresses during turning (depicted by white arrows; C,D; compare E-H with I-L). (M-P) Confirmation of appropriate spatial overexpression of *Hand1* in the mutant embryos at the 12-somite stage. co, control; oe, overexpression; s, somites. Anterior (A) – posterior (P) and left (L) – right (R) axes are indicated for E-L. Scale bar: in D,P, 50 μm for A-P.

In severely affected embryos, the extended OFT and impaired ventricular expansion was not compatible with survival beyond E10.5 (data not shown). Moderately and mildly affected embryos recover such that, at E12.5-14.5, heart development appears entirely normal (see Fig. S1 in the supplementary material) and these embryos survive to birth. Neither the mild nor moderate class of *Hand1*-overexpressing embryos revealed overtly abnormal cardiac morphogenesis. There appears to be a tolerance of variation in outflow-tract length, even amongst control somite-matched embryos (see Fig. 5D), which ensures normal development. The expansion of the OFT observed in the mildly and moderately affected mutants is presumably within this variance threshold such that the OFT is remodelled appropriately and LV maturation remains comparable with controls.

Altered cardiac gene expression in *Hand1* overexpression embryos

As *Hand1* expression in the second lineage is restricted to the aortic sac mesothelium (Fig. 2) and is excluded from key areas such as the distal pharyngeal mesoderm and the mesodermal core of the branchial arches (Kelly et al., 2001), we deemed it unlikely that the OFT expansion would be attributed to elevated migration, or elevated number, of extracardiac cells from the anterior heart field. To confirm this hypothesis, we investigated the contribution of the AHF in the *Hand1*-overexpressing embryos by examining the expression levels of: the LIM-domain-containing transcription factor *Isl1*, which is involved in the differentiation of AHF cells (Cai et al., 2003); *Mef2c*, which is believed to be a target of Isl1 (Dodou et al., 2004), and changes in which are thought to reflect defects in the formation of the AHF and its derivatives (Kelly and Buckingham, 2002); and *Hand2* (downstream of *Mef2c* in the same pathway) (Lin et al., 1997). In the case of all three markers – *Isl1* (Fig. 6A-F), *Mef2c* (Fig. 6G-L) and *Hand2* (data not shown) – there were no observable differences in expression between control and *Hand1*-overexpressing embryos, suggesting that the distal OFT expansion is not caused by an elevated deployment of AHF cells. This suggests that the effect of *Hand1* overexpression was autonomous to the distal OFT and may have arisen as a result of an inherent expansion in cardiac precursors in this region. To test this hypothesis, we looked at markers of cardiomyocyte differentiation by in situ hybridisation and quantitative real-time PCR. Atrial natriuretic factor (ANF) is a marker of differentiated myocardium in the atrial and ventricular chambers, but has previously been reported to be undetectable in the OFT (Habets et al., 2002). Not only was *Anf* (*Nppa* – Mouse Genome Informatics) downregulated in the trabeculated myocardium of the LV in modestly affected embryos (Fig. 7A-D), it was also downregulated in the OFT and restricted exclusively to the proximal OFT, suggesting alternate differentiation status along the proximal and distal axes (Fig. 7E,F). To assess the differentiation status specifically of the extended distal OFT in the overexpression embryos, we examined the levels of *Wnt11*, a marker of the myocardium of the OFT (Cai et al., 2003) implicated in the induction of cardiogenesis and in the cardiomyocyte differentiation program (Koyanagi et al., 2005; Pandur et al., 2002). *Wnt11* was expressed at high levels throughout the distal OFT in control embryos (Fig. 7G,I) but restricted to low-level expression in a subpopulation of cells in the myocardial wall of the OFT in overexpression embryos (Fig. 7H,J). This suggests a reduction in the level of myocardial differentiation coincident with distal OFT extension. Reduced myocardial differentiation in the primary heart

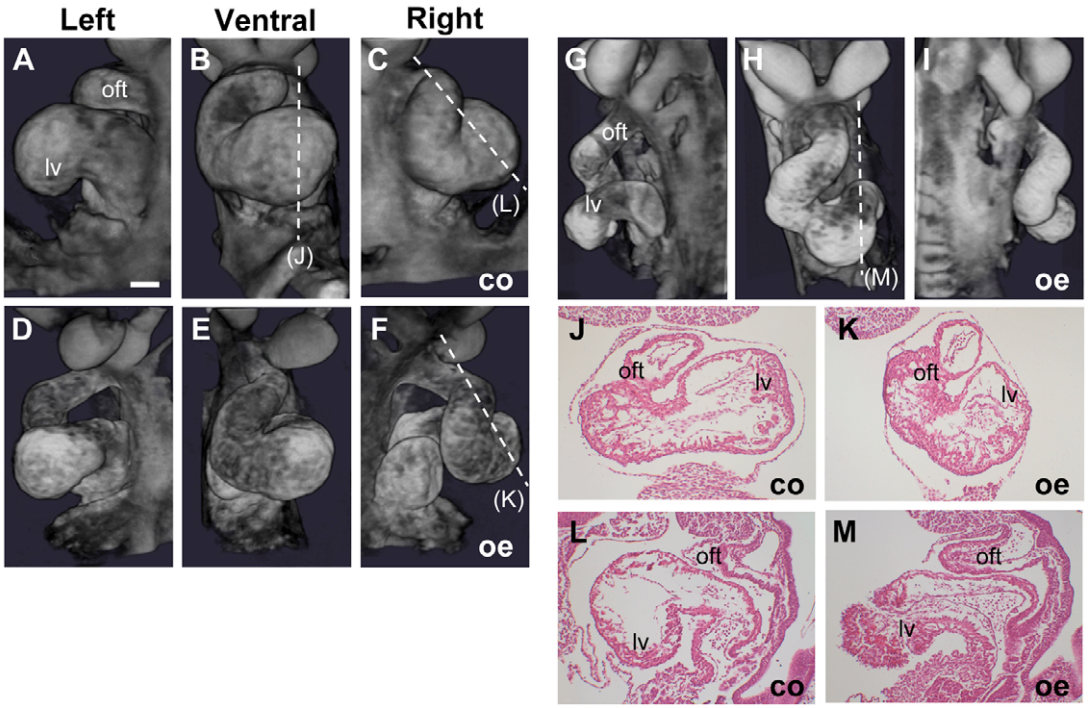

Fig. 4. *Hand1* overexpression promotes extension of the distal OFT, extraneous cardiac looping and impaired LV development. Rendered optical projection tomography of embryos at E9.5 (pericardium removed), viewed from left, ventral and right aspects, in control embryos (containing just the responder transgene; **A-C**), in *Hand1*-overexpressing embryos with a moderate phenotype (**D-F**) or in severe phenotype embryos (**G-I**). Frontal sections through the hearts of control (**J**) and moderate *Hand1*-overexpressing embryos (**K**) in the planes depicted in B and F, respectively. Sagittal sections through the hearts of control (**L**) and severe phenotype *Hand1*-overexpressing embryos (**M**) in the planes depicted in C and H, respectively. co, control; lv, left ventricle; oe, overexpression; oft, outflow tract. Scale bar: 100 μm in A.

tube and, notably, the LV was confirmed in the overexpression embryos by in situ hybridisation for the early cardiac markers *Nkx2.5* and *Gata4* (Fig. 8). Whereas robust expression for both markers was observed in the primary heart tubes of control embryos (Fig. 8A,C,E,G), *Nkx2.5* and *Gata4* were significantly downregulated throughout the heart, most notably in the left ventricle of the overexpression embryos (Fig. 8B,D,F,H).

Quantitative real-time PCR analyses for an array of cardiac markers were performed on RNA extracted from control versus *Hand1* overexpression embryos at E9.5. The results supported the downregulation of *Anf*, *Nkx2.5* and *Gata4* observed by in situ hybridisation and further revealed downregulation of chisel (*smpx* – Mouse Genome Informatics) as an additional early marker of cardiomyocyte differentiation (Fig. 8Q). By contrast, the expression of markers of gap junctions, such as *Cx40* (*Gja5* – Mouse Genome Informatics) and *Cx43* (*Gja1* – Mouse Genome Informatics), was unchanged, as was expression of the ventricle-specific homeobox gene *Irx4* (Fig. 8Q). Interestingly, neither expression of *Mef2c* nor *Hand2* was altered in the overexpression embryos (Fig. 8Q), consistent with the in situ hybridisation data (Fig. 6) and confirming that the effect of *Hand1* overexpression is independent of an enhanced recruitment of extracardiac cells from the AHF.

Elevated proliferation of cardiomyocytes in the distal outflow tract

As there appears to be reduced cardiomyocyte differentiation in the *Hand1* overexpression embryos, we determined whether there were altered levels of apoptosis or elevated cell proliferation by immunostaining with antibodies against cleaved caspase 3 (CC3) and phospho-histone H3 (PH3), respectively. We observed no difference in expression of CC3 between wild-type and overexpressing embryos, with very few cells throughout the developing heart undergoing apoptosis at this stage (data not shown). By contrast, there were significantly more proliferating cells located in the distal OFT region of overexpressing hearts compared with wild type, with large numbers of PH3 positive cells, as an indicator of cells undergoing mitosis, situated in the wall and lumen of the OFT [control, 125±10 (n=6); overexpression, 238±14 (n=6) P<0.01] (Fig. 9A-H). The observation of an over-proliferation of cardioblasts in the OFT of overexpression embryos is further supported by the downregulation of *Wnt11* described above (Fig. 7G-J).

Taken together, a lack of change in markers of the AHF, the downregulation of key genes associated with promoting cardiomyocyte differentiation and elevated levels of proliferation of cells in the distal OFT suggest that Hand1 does not affect deployment of cells into the OFT from the AHF, but rather that, once the extracardiac cells arrive in the OFT, they respond to Hand1 gain of function by continuing to proliferate as opposed to committing to a terminally differentiated cell fate.

Hand1 dosage influences cardiomyocyte differentiation

To confirm the in vivo findings and enable us to compare directly *Hand1* gain of function with a loss-of-function model, we characterised Hand1 activity in vitro using ES cells differentiated into EBs over a period of 14 days in floating culture. EB-derived cardiomyocytes represent a faithful model of the in vivo situation: not only do they exhibit characteristics of early chamber

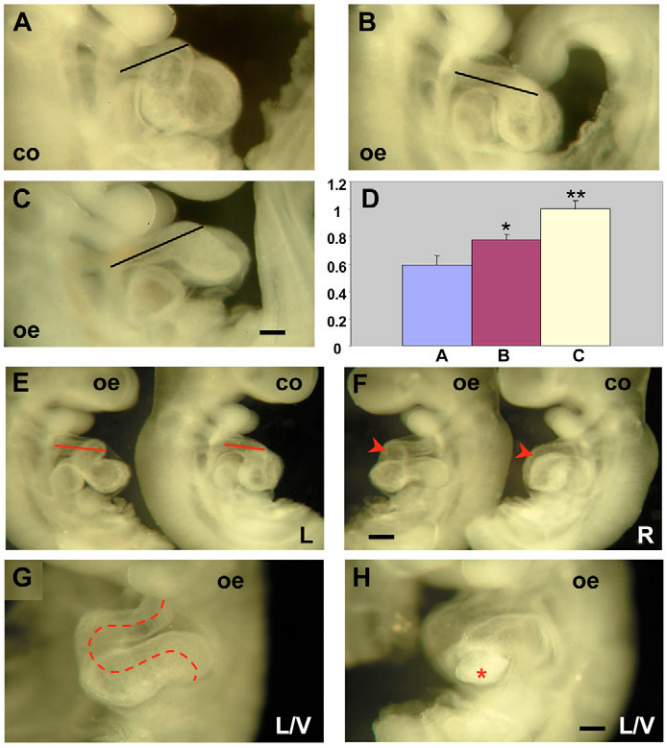

Fig. 5. *Hand1* overexpression promotes extension of the distal OFT, extraneous cardiac looping and impaired LV development. (**A-D**) *Hand1* overexpression in somite-stage matched embryos at E9.5 leads to moderate (B) and significant (C) extension of the distal OFT compared with control littermates (A); quantified in relative units (D), measurements are mean±s.e.m., values of n=9 (A), n=11 (B) and n=5 (C), based on distance depicted by black lines in A-C, *P<0.05, **P<0.01. The heart tube is extended (**E**) and displaced ventrally (**F**; red arrowheads) in overexpression embryos compared with controls. Looping is accentuated and highly convoluted (**G**) and, in severe cases, the presumptive LV (red asterisk) is small and necrotic (**H**). co, control; L, left; L/V, left-ventral; oe, overexpression; R, right. Scale bars: 100 μm in C,H; 200 μm in F.

myocardium but a substantial proportion are reminiscent of those observed in embryonic OFT (Fijnvandraat et al., 2003a; Fijnvandraat et al., 2003b). ES cells generated during the derivation of the responder mouse strain containing the *Tre2-Hand1* transgene responder construct were stably transfected with a tTA driver under the control of a TK promoter to generate a line that precociously overexpressed *Hand1* (see Fig. S2A in the supplementary material). *Hand1* expression is first detected in wild-type EBs at day 4 of differentiation (Smart et al., 2002). The *Hand1*-overexpressing line expressed the *Hand1* responder transgene precociously, prior to endogenous *Hand1* expression, between day 0 and 4 of differentiation (see Fig. S2B, inset i, in the supplementary material). Overexpression was maintained throughout differentiation up to day 14 (see Fig. S2B in the supplementary material), at which stage *Hand1* was expressed five- to sixfold higher in the *Hand1*-overexpressing cell line than in the control line (see Fig. S2B, inset ii, in the supplementary material).

The *Hand1*-overexpressing cells were then compared with either control or *Hand1*-null ES-cell lines (Riley et al., 2000) in terms of their inherent ability to form cardiomyocyte contractile foci and real-time expression of cardiac markers. *Hand1*-overexpressing EBs revealed a significant delay and reduction in the formation of foci compared with wild type, whereas *Hand1*-null EBs formed a much higher proportion of contractile foci, up to fivefold higher at day 10 of differentiation (Fig. 10A). To demonstrate that the beating foci observed were cardiomyocytes, and to confirm that the *Hand1*-overexpressing line was deficient in forming cardiomyocytes, cells were dispersed and isolated from EBs at day 14 and immunostained for sarcomeric myosin using the MF20 monoclonal antibody. Whereas cardiomyocytes associated with beating foci were readily identified in *Hand1*-null, wild-type and control cell lines (see Fig. S2C in the supplementary material), no cardiomyocytes were identified in the *Hand1*-overexpressing line, confirming the deficiency in cardiomyocyte differentiation in the gain-of-function line.

The levels of contractile foci were supported by the gene-expression profiles for each cell line (Fig. 10B). Commitment to a cardiac fate was evident in *Hand1*-overexpressing EBs from the corresponding expression of *Mef2c*, *Cx40* and *Irx4*, and *Mlc2v* as markers of chamber myocardium. Moreover, expression of *Irx4* and *Mlc2v* at levels comparable to control were indicative of progression towards ventricular-like differentiation (Fig. 10B). However, the significant reduction in the expression of *Nkx2.5*, *Gata4* and the pan-cardiac marker α-cardiac actin (Fig. 10B) suggests an overall reduction in cardiomyocyte differentiation. As the ventricular-specific markers (*Irx4* and *Mlc2v*) appeared normal, this suggests that there are no significant changes in EB-derived cardiomyocytes that resemble early chamber myocardium and that the reduced differentiation is possibly attributable to the OFT component of the EB-derived cardiomyocytes (Fijnvandraat et al., 2003a). This is consistent with the identification of independent populations of EB-derived cardiomyocytes, the gene expression profiles and electrophysiological activity of which are characteristic of either embryonic OFT or early chamber myocardium (Fijnvandraat et al., 2003a; Fijnvandraat et al., 2003b).

The observation of *Nkx2.5* inhibition following Hand1 gain of function suggests that, although *Nkx2.5* precedes *Hand1* expression in the developing heart in vivo and is thought to be higher up the transcriptional hierarchy (Biben and Harvey, 1997), Hand1 is able to feedback onto *Nkx2.5* and modulate not only its expression levels but subsequent induced myocardial differentiation. *Hand1*-null EBs, by contrast, revealed significantly elevated *Nkx2.5* levels; in fact, all of the cardiac genes examined (with the exception of *Cx40*) were highly upregulated in response to Hand1 loss of function (Fig. 10B). This is consistent with the observation of significantly increased contractile cardiomyocyte foci in *Hand1*-null EBs (Fig. 10A). An overall observation that *Hand1* overexpression affected a restricted cohort of the cardiac markers examined compared to loss of *Hand1* is almost certainly explained by the fact that the two parent ES-cell lines differed considerably in their profiles for the markers in question (data not shown). The differences in cardiomyocyte differentiation between the *Hand1*-null and *Hand1*-overexpressing cell lines indicate a non-redundant role for Hand1 in maintaining equilibrium between cardiomyocyte proliferation versus differentiation.

Cyclin D2 and its associated kinase, Cdk4, are downstream of Hand1

The altered levels and rates of differentiation in the *Hand1*-null and *Hand1*-overexpressing cell lines suggested that these lines may have associated alterations in cell-proliferation levels. Furthermore, as there is elevated proliferation in the OFT in *Hand1*-overexpressing embryos, accompanied by a decrease in differentiation, it follows that, in the event of *Hand1* overexpression, there may be a reduction in exit from the cell cycle, resulting in continued proliferation at the expense of differentiation.

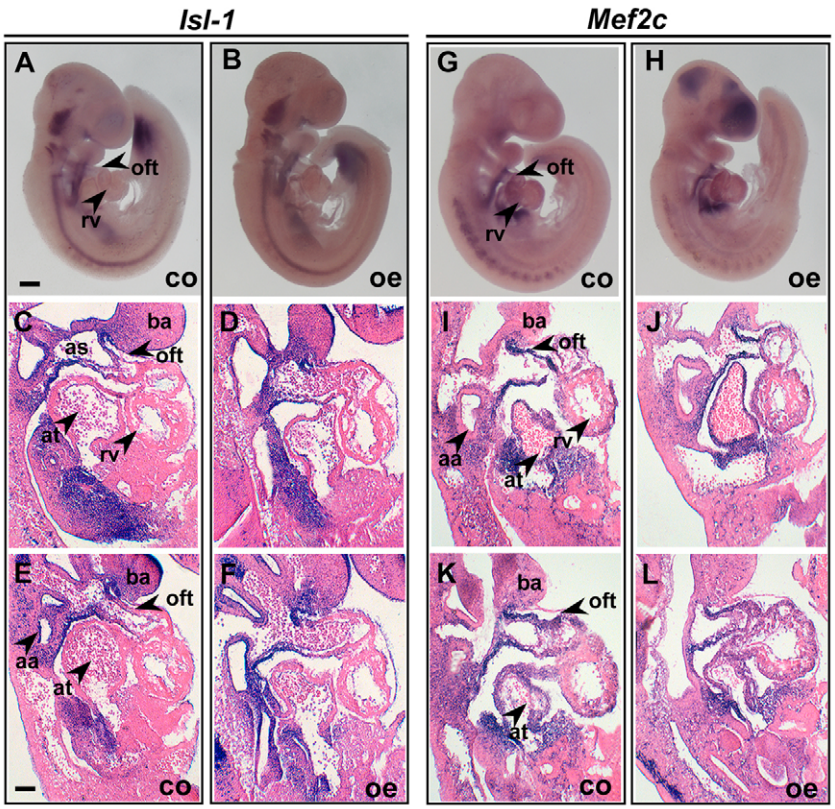

Fig. 6. Contribution of extracardiac cells from the AHF is unaffected in *Hand1*-overexpressing embryos. RNA in situ hybridisation analysis of *Isl1* and *Mef2c* in control embryos (**A**,**C**,**E**,**G**,**I**,**K**) and moderate phenotype *Hand1*-overexpressing embryos (**B**,**D**,**F**,**H**,**J**,**L**) at E9.5. Right lateral views of whole-mount embryos (A,B,G,H). Sagittal sections of the control (C,E,I,K) and *Hand1*-overexpressing hearts (D,F,J,L). There are no apparent differences in expression of the AHF marker *Isl-1* between control and overexpressing embryos (compare A,C,E with B,D,F) or in expression of markers of AHF derivatives, as indicated by *Mef2c* (compare G,I,K with H,J,L). aa, aortic arch(es); at, atrium; ba, branchial arch; co, control; oe, overexpression; oft, outflow tract; rv, right ventricle. Scale bars: 200 μm in A for A,B,G,H; 50 μm in E for C-F,I-L.

To provide further evidence for elevated levels of cell proliferation in a Hand1 gain-of-function background and mechanistic insight into how Hand1 might promote increased cell division, we examined levels of cyclin D2 and the associated cyclin-dependent kinase, Cdk4, in *Hand1*-overexpressing EB-derived cardiomyocytes. As cardiomyocytes develop, they lose the ability to proliferate and there appears to be a block in the gap phases of the cell cycle, most notably at G1, consistent with the terminal differentiation of these cells (Brooks et al., 1998). Progression through G1 is dependent upon the D-type cyclins and, although many different stimuli induce cell proliferation through different signalling pathways, activation of one or more of the D-type cyclins is necessary for most, if not all, of these pathways (Busk et al., 2005). In *Hand1*-overexpressing EBs, we observed significantly elevated protein levels of both cyclin D2 and Cdk4 compared with wild type; this was reciprocated by reduced levels for both proteins in EBs that were either heterozygous

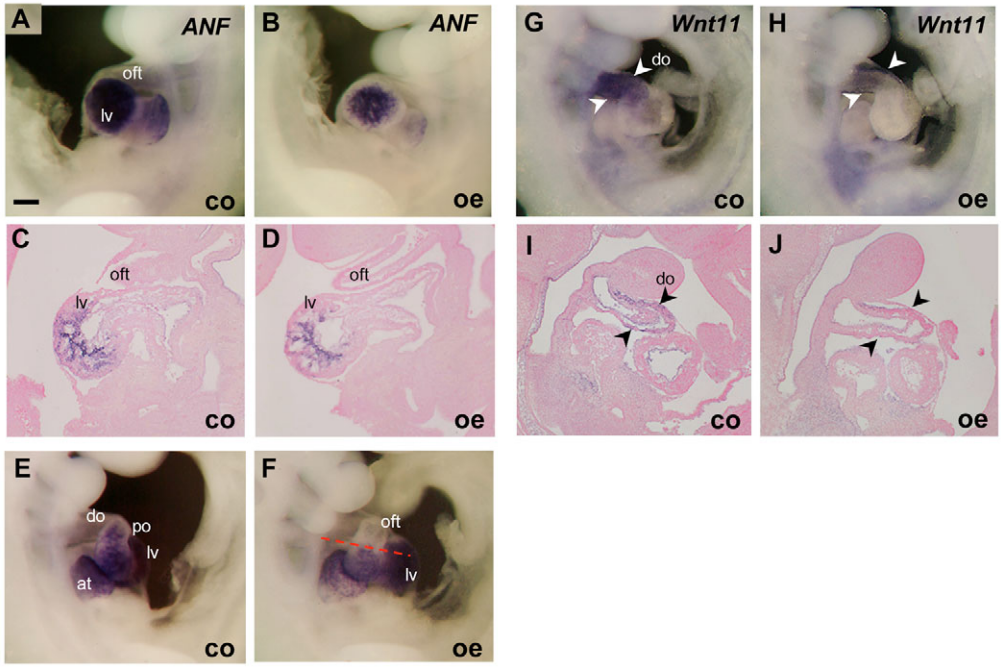

Fig. 7. Markers of cardiomyocyte differentiation are down regulated in *Hand1*-overexpressing embryos. RNA in situ hybridisation analysis of *Anf* and *Wnt11* in control embryos (**A**,**C**,**E**,**G**,**I**) and moderate phenotype *Hand1*-overexpressing embryos (**B**,**D**,**F**,**H**,**J**) at E9.5. Sagittal sections of the control (C,I) and *Hand1*-overexpressing hearts (D,J). Boundary of *Anf* expression in proximal outflow tract of overexpressing embryos is depicted by a broken red line in F. *Wnt11* in distal outflow tract (black and white arrowheads) is downregulated in overexpressing embryos (H,J) compared with control embryos (G,I). at, atrium; co, control; do, distal outflow tract; lv, left ventricle; oe, overexpression; oft, outflow tract; po, proximal outflow tract. Scale bar: 100 μm in A.

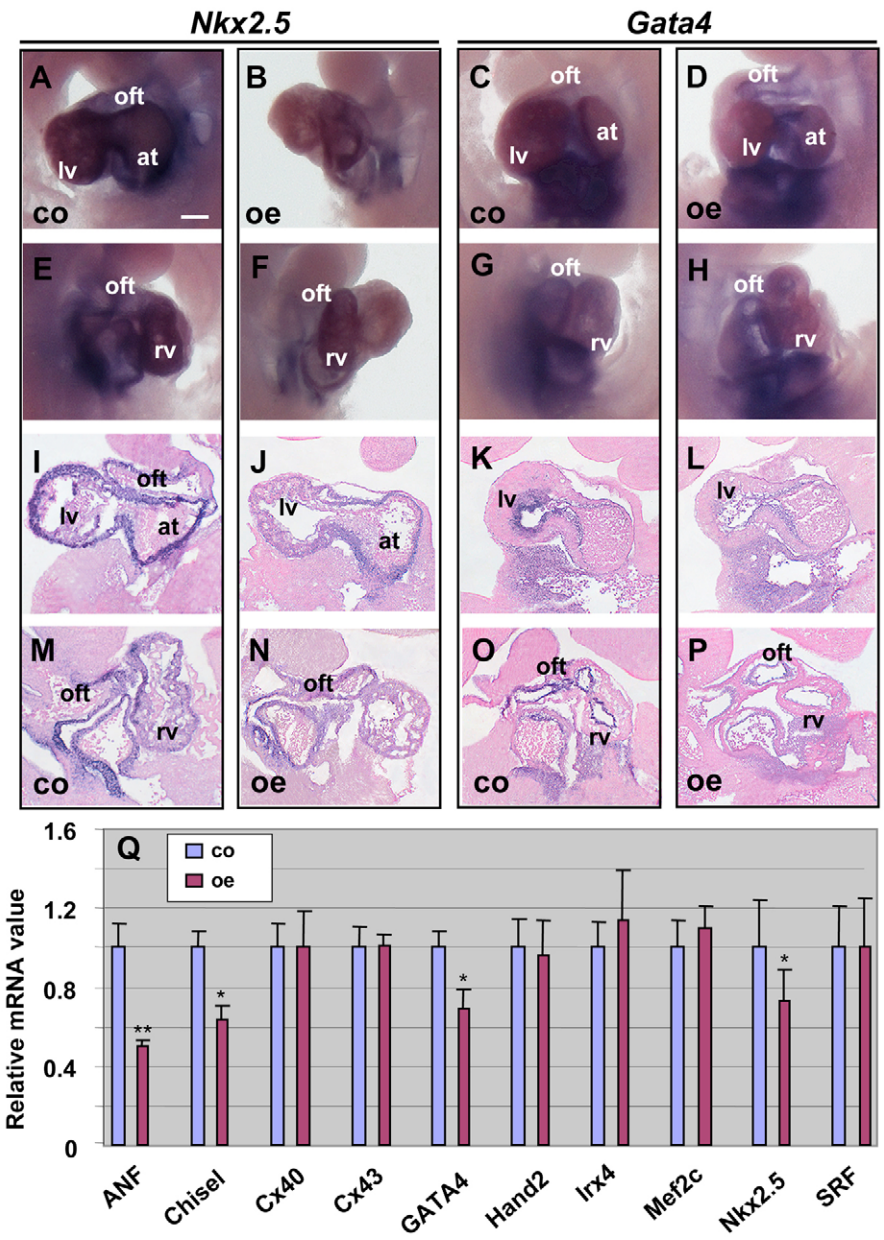

Fig. 8. Primary heart field markers are downregulated in *Hand1*-overexpressing embryos. RNA in situ hybridisation analysis of *Nkx2.5* and *Gata4* in control embryos (**A**,**C**,**E**,**G**,**I**,**K**,**M**,**O**) and moderate phenotype *Hand1*-overexpressing embryos (**B**,**D**,**F**,**H**,**J**,**L**,**N**,**P**) at E9.5. (I-P) Sagittal sections of the control (I,K,M,O) and *Hand1*-overexpressing (J,L,N,P) hearts. (**Q**) Quantitative real-time PCR analysis on RNA extracted from control or severe phenotype overexpression embryos for an array of cardiac markers. Means and standard deviation bars with 95% confidence limits are shown. **P*<0.05, ***P*<0.01. at, atrium; co, control; lv, left ventricle; oe, overexpression; oft, outflow tract; rv, right ventricle. Scale bar: 100 μm in A.

or homozygous mutant for *Hand1* (Fig. 10C,D). The differences in cyclin D2 and Cdk4 levels observed between Hand1 gain- and loss-of-function EBs appears to reflect the significant differences in numbers of differentiated cardiomyocytes/beating foci observed between the two populations (5% in the *Hand1*-overexpressing EBs versus 90% in the *Hand1*-null EBs) following 14 days of differentiation (Fig. 10A). Therefore, Hand1 activity influences cyclin D expression and associated CDK activity, both of which are hallmarks of cell-cycle entry and pivotal for continued cell proliferation.

DISCUSSION

We describe a previously unknown role for Hand1 as a significant cardiac transcription factor in regulating cardiomyocyte exit from the cell cycle to finely control the balance between cell proliferation and differentiation. The finding that Hand1 can promote proliferation in sub-regions of the developing heart while still allowing adjacent myocardial precursors to differentiate is novel and supports the idea that proliferation and differentiation are intrinsically linked and are not, as previously thought, mutually exclusive processes. Moreover, this study also suggests that Hand1 is able to promote either cell proliferation or differentiation in embryologically distinct lineages. Hand1 is both necessary and sufficient for the terminal differentiation of trophoblast stem cells to a giant cell fate (Cross et al., 1995; Hemberger et al., 2004), whereas, in the heart, overexpression results in expansion of the proliferative pool of undifferentiated cardiomyocyte precursors.

An important potential caveat to the findings of this study resides in the interpretation of the result of overexpression of *Hand1* as being either a gain-of-function or a dominant-negative effect, taking into account the reported role of Hand1 as part of heterodimeric complex (Firulli et al., 2000; Scott et al., 2000). Moreover, an additional consideration is whether, at high concentrations, specificity of Hand1 downstream effects may be lost resulting in an impact on non-endogenous Hand1 target genes. Although Hand1 functions as a heterodimer with E-factors in vitro, according to the classic bHLH

paradigm (Firulli et al., 2000; Scott et al., 2000), it can also function as a homodimer (Hill and Riley, 2004; Scott et al., 2000) and, indeed, a tethered Hand1 homodimer allele, generated by homologous recombination in ES cells, can rescue the Hand1-null phenotype (Hu et al., 2006). Therefore, a dominant-negative effect on heterodimer formation can be ruled out as almost certainly lacking functional significance in vivo. The specificity issue is one that can be levied at any loss- or gain-of-function model, as the effects can be wide ranging and are often indirect. In this case, the *Hand1* expression is actually only twofold above controls (Fig. 1). Therefore, we are working throughout with concentrations of Hand1 where significant non-specific effects are unlikely; this is borne out by restriction of the phenotype exclusively to areas of *Hand1* expression.

Hand1 overexpression, which is restricted to *Hand1*-expressing cells in the OFT, demonstrates how OFT myocardium following recruitment from the AHF is sensitised to Hand1, resulting in cell-cycle exit delay and maintenance of a proliferative myocardial progenitor population. *Hand1* is expressed neither in distal pharyngeal mesoderm nor in pharyngeal endoderm and, as such, is unlikely to be involved in regulating proliferation/differentiation of the AHF or second lineage. Our results demonstrate that the OFT extension and elongation of the heart tube in *Hand1*-overexpressing embryos occurs independently of second lineage deployment as the expression of *Isl1*, *Mef2c* and *Hand2* is unchanged in *Hand1*-overexpression embryos. The cells that migrate from the AHF into the embryonic heart are undifferentiated, and are thought to terminally differentiate into cardiomyocytes once they arrive at their destination in the developing heart (Waldo et al., 2001). It is, therefore, plausible that Hand1 crucially controls this differentiation process in the OFT myocardium, balancing levels of cell proliferation and differentiation to maintain appropriate OFT remodelling. In the event of *Hand1* overexpression in the OFT, the balance shifts towards continued proliferation, resulting in increased cell number, abnormal OFT development and subsequent aberrant morphogenesis of the heart. Interestingly, a shortening of the OFT was noted in the cardiac-specific knockout of *Hand1* (McFadden et al., 2005) and, although this was not investigated further, it has been suggested that the OFT defect may be secondary to an observed LV hypoplasia (Buckingham et al., 2005; McFadden et al., 2005). Our results indicate that Hand1 directly affects the development of the OFT and suggest that shortening of the OFT in cardiac-specific *Hand1*-null embryos may well be a primary defect of reduced cardiomyocyte proliferation.

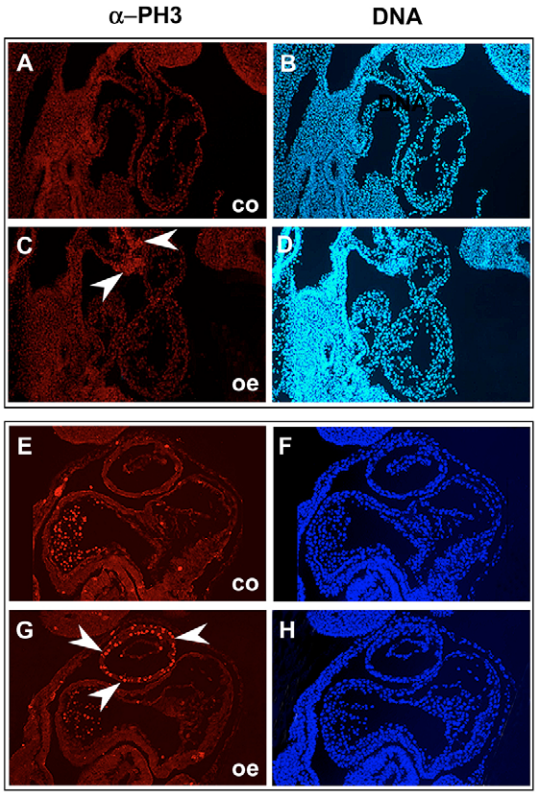

Fig. 9. Over-proliferation of cardiac precursors in the distal OFT of *Hand1*-overexpressing embryos. Sagittal (**A-D**) and frontal (**E-H**) sections through the distal OFT region of control or *Hand1*-overexpressing embryos at E9.5. Immunofluorescence with antibody specific to phospho-histone H3 (α-PH3) to mark proliferating cells (A,C,E,G). (B,D,F,H) Bis-benzamide counterstaining (DNA) of nuclei. White arrowheads in C and G indicate areas of proliferating cells. co, control; oe, over-expression.

Hand1 gain of function acts to promote continued proliferation of existing precursors in the distal OFT, enabling them to maintain an early phenotype whereby cardiomyocytes proliferate even as they begin to differentiate, and subsequently prevents further differentiation associated with cell-cycle exit. Conversely, in a *Hand1*-null background the heart tube fails to loop, the OFT is hypoplastic and the presumptive ventricle is thin walled (Riley et al., 1998). Moreover, *Hand1*-null ES cells differentiated in vitro give rise to a significantly higher proportion of beating foci compared with wild type and this is associated with a significant upregulation in markers of cardiomyocyte differentiation. Therefore, upstream regulation of *Hand1* levels in the expanding heart tube represents a switch between cardiomyocyte proliferation and differentiation during organogenesis, and Hand1 itself can regulate the progenitor pool in the developing OFT in a manner entirely distinct from signals intrinsic to and emanating from the AHF.

Overexpression of *Hand1* in the LV appears to promote further proliferation, thus preventing the outer curvature from expanding and ballooning ventrally as secondary myocardium to form the ventricular chamber. Secondary myocardium has a distinct molecular profile to that of the primary myocardium of the linear heart tube. Once this program is initiated, secondary myocardium is thought to balloon out from the outer curvature of the primary heart tube to form the four chambers of the heart (Christoffels et al., 2000). The downregulation of markers of secondary myocardium, such as *Anf* and chisel (Christoffels et al., 2000), in *Hand1*-overexpressing embryos may, therefore, explain the failure of the ventricle to balloon out, as differentiation into secondary myocardium is simply not initiated. In this case, Hand1 may be controlling the balance between differentiation and proliferation in the ventricular chamber such that, in the event of *Hand1* overexpression in the presumptive LV, as in the OFT, the balance shifts towards proliferation, resulting in an increased number of cells and reduced cardiomyocyte differentiation with the associated down-regulation of differentiation markers. Conversely, in tetraploid-rescued *Hand1*-null embryos, the thin myocardial wall of the LV in the absence of Hand1 can now be explained by a shift in balance towards decreased proliferation, rather than by secondary effects, as previously thought (Riley et al., 1998; Riley et al., 2000). The fact that the LV is a derivative of the primary heart field suggests that Hand1 uniquely impacts on derivatives of both the primary (LV) and secondary (OFT) lineages in a cell-autonomous manner. This is further supported by the extension of the heart tube observed in *Hand1*-overexpression embryos as early as E8.0, which suggests that elevated *Hand1* levels

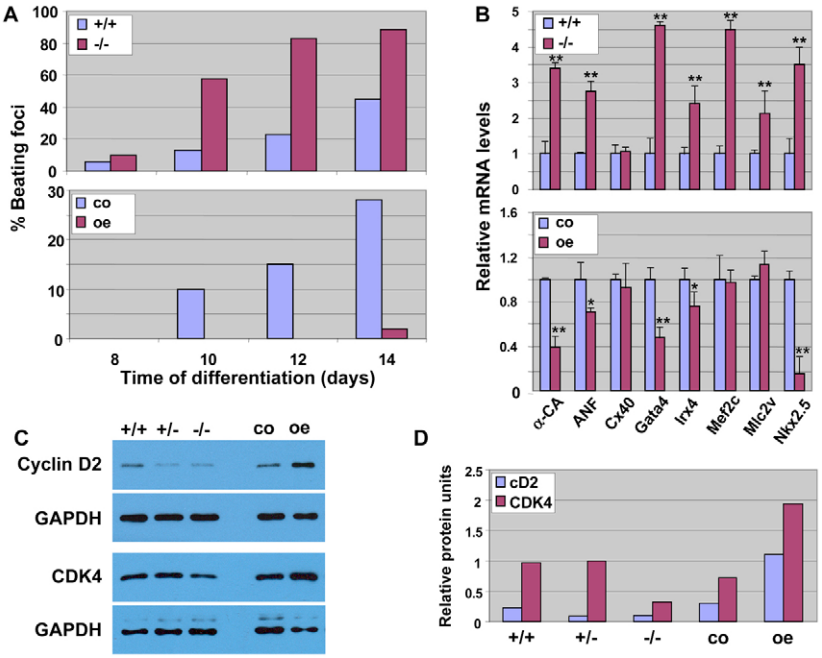

Fig. 10. Hand1 regulates cardiomyocyte proliferation versus differentiation in vitro via G1 cell-cycle progression or exit. (**A**) Myocardial beating in embryoid bodies (differentiated for up to 14 days) derived from wild-type (+/+), *Hand1*-null (–/–), control (responder transgene alone, co) and *Hand1*-overexpressing (oe) ES-cell lines. (**B**) Quantitative real-time PCR analysis on RNA extracted from embryoid bodies following 14 days of differentiation, for an array of cardiac markers. (**C**) Western blots of day-14 EBs using antibodies for cyclin D2, Cdk4 and Gapdh. (**D**) Quantification of protein levels from C normalised to Gapdh using scanning densitometry.

exert a proliferative effect on the cells arising from the primary heart field. It remains to be seen, however, whether the failure in the ballooning and lack of ventricular lumen is primarily linked to an over-proliferation of ventricular myocardium or is secondary to the elevated haemodynamic load and increased fluid pressure arising from the extended heart tube.

In *Hand1*-overexpressing EBs, reduced and delayed cardiomyocyte differentiation was associated with increased levels of cyclin D2 and Cdk4, which is indicative of sustained entry into, and progression through, the cell cycle and, consequently, of enhanced proliferation. The opposite was true in the *Hand1*-null EBs, where there appeared to be down-regulation of cyclin D2 and Cdk4, indicating premature cell cycle exit, lower levels of proliferation and enhanced differentiation of cardiomyocytes. Moreover, these observations suggest that cyclin D2 and Cdk4 are downstream of *Hand1*; although it remains to be elucidated whether Hand1 directly, or indirectly, regulates cyclin D2/Cdk4 expression.

The observation that *Hand1* lies upstream of cyclin D2 is important in light of recent studies implicating both genes in adult-onset cardiovascular disease. Adult transgenic mice expressing cyclin D2 in the myocardium show increased levels of basal cardiomyocyte cell-cycle activity (Pasumarthi and Field, 2002) and this increase in activity appears to result in a progressive reduction of infarct size and improved cardiac function (Rubart and Field, 2006). Additionally, it has been demonstrated that cyclin D2 represses hypertrophy by forcing cardiomyocytes through the cell cycle, resulting in cell proliferation. This suggests that cyclin D2 levels directly determine whether cells grow by hypertrophy or proliferation (Busk et al., 2005). *Hand1* has been shown to be downregulated in human ischemic and dilated cardiomyopathies (Natarajan et al., 2001), and also following the induction of cardiac hypertrophy in adult rodent hearts (Thattaliyath et al., 2002). Furthermore, a study on cardiac lineage protein 1 (*Clp1*; *Hexim1* – Mouse Genome Informatics)-null mice suggested that an observed downregulation of *Hand1* in the hearts of *Clp1* embryos directly promoted a foetal form of cardiac hypertrophy (Huang et al., 2004). The downregulation of *Hand1* during cardiac hypertrophy may, therefore, result in the downregulation of cyclin D2, which in turn is a likely contributing factor towards hypertrophic growth. This suggests that Hand1 and cyclin D2 are capable of modifying cardiac responses in the myocardium to external stimuli and that, collectively, they may play an important role during hypertrophic signalling.

Disruption in the balance of cell number during heart development, via over-proliferation and associated failure in differentiation, clearly leads to an inability to maintain appropriate morphogenesis. Conversely, failure to maintain an adequate pool of undifferentiated myocyte precursors results in organ hypoplasia, as is observed in mice lacking *Hand1*, and almost certainly contributes to the aetiology of congenital heart disease (CHD). OFT anomalies account for ~30% of all cases of CHD (Kelly and Buckingham, 2002). Reduced OFT length is associated with defective alignment with the atrioventricular junction and abnormal rotation of OFT myocardium, which in turn leads to abnormal ventricular septation and OFT anomalies such as double outlet right ventricle and persistent truncus arteriosus. Autonomous, Hand1-induced proliferation of cardiomyocyte precursors during heart-tube elongation provides insight into directing progenitor cells along a path towards integrated myocardium, which is of direct relevance to cell-based therapies for cardiovascular disease and cell-autonomous strategies for cardiac regenerative medicine.

Two potential approaches for deriving cell-based therapies are the transplantation of stem cells from an external source and the activation of resident stem cells within the heart. Clearly, for these to be viable options there is a need for further understanding of the precise molecular pathways involved in directing cardiomyocyte proliferation and differentiation, and how to generate greater numbers of functional cardiomyocytes. These studies collectively reveal that Hand1 is involved in maintaining the correct balance between proliferation and differentiation in the developing heart, suggesting that manipulation of Hand1 levels could potentially affect both of these avenues of therapeutic research.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/22/????/DC1

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