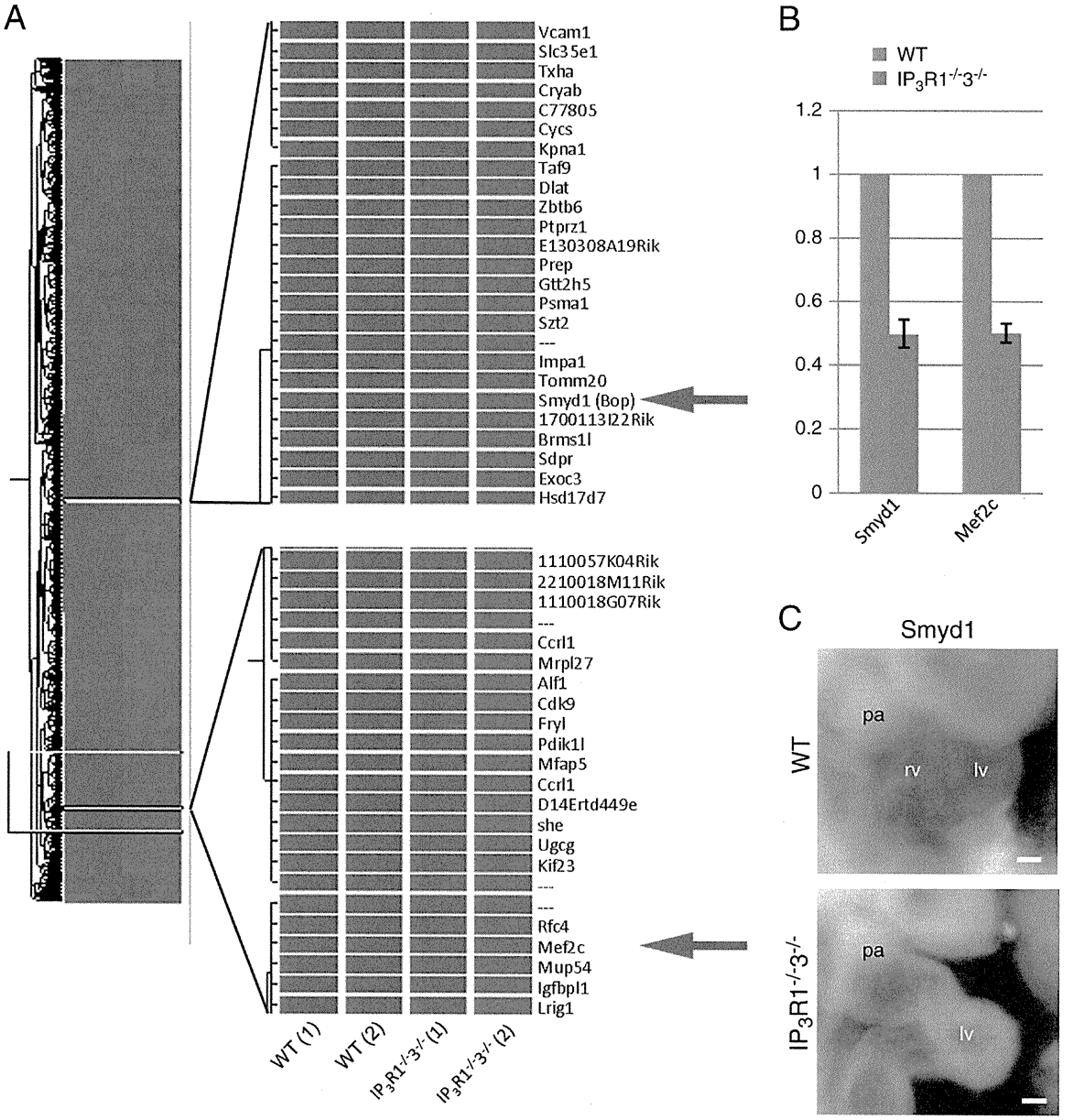

Fig. 5. Downregulation of the Mef2c–Smyd1 pathway in $IP_3R1^{-/-}3^{-/-}$ hearts. (A) A heat map was generated from results of the microarray by GeneSpring (Agilent, ver. 7.3.1) to compare fold change patterns of the genes expressed in wildtype (WT) and $IP_3R1^{-/-}3^{-/-}$ hearts. Results of two independent experiments, (1) and (2), for WT and $IP_3R1^{-/-}3^{-/-}$ hearts are shown. Red and green colors indicate relatively high and low levels of expression, respectively. (B) qRT-PCR for *Smyd1* and *Mef2c* expression were shown. Error bars indicate standard errors (n = 3). (C) Expression of mouse Symd1 is shown in the right lateral views of wildtype and $IP_3R1^{-/-}3^{-/-}$ embryo at E9.25. lv, left ventricle; pa, pharyngeal arch; rv, right ventricle. The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE28186).

These data suggest that IP_3R-mediated Ca^{2+} signaling dependent on a redundant role of IP_3R1 and IP_3R3 may involve, at least in part, the Mef2C–Smyd1 pathway during the development of the SHF.

4. Discussion

4.1. A redundant role of IP_3R1 and IP_3R3 is essential for development of the SHF

This study has demonstrated that IP_3R1 and IP_3R3 are redundantly essential for the development of the SHF that gives rise to the cardiac outflow tract and the right ventricle. $IP_3R1^{-/-}IP_3R3^{-/-}$ embryos show normal development of the linear heart tube and initiation of the rightward looping by E8.5, but have hypoplasia of the outflow tract and the primitive right ventricle just one day later. Our molecular analyses revealed that there is overlapping expression of IP_3R1 and IP_3R3 in the SHF at E8.5–9.5, which is consistent with the cardiac phenotype of $IP_3R1^{-/-}IP_3R3^{-/-}$ mutant embryos. The expression of molecular markers of the SHF, *Isl1*, *Bmp4* and *Hand2*, is reduced in $IP_3R1^{-/-}IP_3R3^{-/-}$ mutants, but expression of *Tbx1* and *Fgf8* along with *Nkx2.5*, *Mlc2a*, *Mlc2v*, *Tbx5* and *Anf* is not altered. This suggests that a specific defect occurs in a subpopulation of the SHF rather than as a global defect. Our data indicate that the hypoplasia of the outflow tract and the primitive right ventricle in $IP_3R1^{-/-}IP_3R3^{-/-}$ embryos occurs mainly as a result of enhanced apoptosis of mesodermal cells in the SHF. Therefore, Ca^{2+} signaling redundantly mediated by IP_3R1 and IP_3R3 may function in the survival of SHF cells. Recent advances in developmental biology have elucidated numerous signaling pathways that control SHF development, including Bmp, Fgf, Hedgehog and Wnt signaling [23]. Our data is the first demonstration that IP_3R-mediated intracellular Ca^{2+} signaling plays a role in the development of the SHF and its derivatives.

Although both IP_3R1 and IP_3R3 are expressed throughout the developing heart, the phenotypes associated with null mutations in both genes are largely confined to the SHF and its derivatives. This anatomic restriction of cardiac defects probably reflects a specific redundant role of IP_3R subtypes in the Ca^{2+} signaling that operates inside and outside the SHF. Expression of both IP_3R1 and IP_3R3 is relatively broad, but their expression overlaps in the SHF, and null mutations in both genes resulted in enhanced apoptosis in the SHF of splanchnic mesoderm. Recently, Liang et al. showed that IP_3R3 negatively regulated apoptosis during mouse embryonic stem cell differentiation [24]. Intriguingly, downregulation of IP_3R3 appeared to affect mesodermal and mesoendodermal differentiation, but not ectodermal differentiation, suggesting a specific function of IP_3R3 in the development of the mesoderm, consistent with our results. Alternatively, the SHF may be susceptible to altered gene expression during heart development around E9.0–9.5. Further studies using conditional gene inactivation would provide some clues on this issue.

4.2. A possible molecular mechanism underlying IP_3R-mediated Ca^{2+} signaling in the SHF

Although the molecular mechanisms underlying IP_3R-mediated Ca^{2+} signaling in the SHF are not fully understood, our microarray analysis has demonstrated that several genes related to cardiac development and Ca^{2+} signaling, including *Smyd1* and *Mef2c*, are significantly downregulated in $IP_3R1^{-/-}IP_3R3^{-/-}$ mutant hearts. *Smyd1* encodes a muscle-restricted transcriptional repressor and putative histone methyltransferase. Intriguingly, mice lacking the *Smyd1* gene, or the *Mef2c* gene that directly regulates *Smyd1*, die from abnormalities in the formation of the right ventricle and outflow tract [25], similar to the $IP_3R1^{-/-}IP_3R3^{-/-}$ mutant embryos. Furthermore, our histologic analysis of the $IP_3R1^{-/-}IP_3R3^{-/-}$ embryos appeared to show an expansion of the extracellular matrix similar to the *Smyd1* mutant (Fig. 1L). Recently, Mef2c has been shown to play an important role in development of the SHF as a direct downstream effector of Isl1 [26]. Together with these findings, our microarray and qRT-PCR analyses raise the possibility that IP_3R may play a role in regulation of the transcriptional cascade in development of the SHF. A recent study suggested that both the translocation of Mef2c into the nucleus as well as Mef2c transcriptional activation are compromised in the absence of the endoplasmic reticulum-localized Ca^{2+}-binding protein, calreticulin [27]. Cells deficient in calreticulin have impaired IP_3R-dependent Ca^{2+} release from the endoplasmic reticulum [28], and calreticulin-deficient mice display embryonic lethality with impaired cardiac development [29]. We speculate that developmental defects in the $IP_3R1^{-/-}IP_3R3^{-/-}$ mutant heart may result, at least in part, from the impairment of calreticulin-dependent Mef2c activation.

The mutant embryos for either the *Smyd1* or the *Mef2c* genes have a more severe phenotype than the $IP_3R1^{-/-}IP_3R3^{-/-}$ mutant embryos. This may be as a result of a partial downregulation of Mef2c–Smyd1 signaling in $IP_3R1^{-/-}IP_3R3^{-/-}$ mutant embryos and suggests that IP_3R-mediated Ca^{2+} signaling pathway is not the sole regulator of the Mef2c–Smyd1 transcriptional cascade in the SHF. To date, it has been shown that Isl1 and a forkhead transcription factor, Foxh1, directly activate transcription of Mef2c in the SHF [26].

4.3. Clinical implications of altered signaling events in the SHF

Congenital heart defects (CHDs) in human occur in nearly 1% of all live births and are the major cause of infant mortality and morbidity, yet the underlying genetic etiology of over 80% of CHDs remains unknown. Direct or indirect perturbation of the SHF cell deployment leads to a spectrum of outflow tract defects, including persistent truncus arteriosus, double outlet right ventricle and tetralogy of Fallot that account for approximately 30% of CHD. Cells derived from the SHF may also contribute to later growth of the heart since there are a small number of multipotent *Isl1*-positive cells in the early postnatal heart [30]. Numerous signaling molecules have been identified that regulate cells in the SHF and constitute part of the signaling environment to maintain these cells in a progenitor state and/or control their differentiation into the cardiac outflow tract [23]. Here, we show that IP_3R1 and IP_3R3 are essential for development of the SHF, although their precise roles remain to be determined. Further investigation of the signaling molecules involved in the development of the SHF would provide a new insight into both the etiology of CHD and the application of stem cells for the repair of outflow tract defects.

5. Conclusions

Our results revealed that IP_3R1 and 3 are genetically redundant and essential for the cardiovascular development. Redundant roles of IP_3R1 and 3 may be implicated in the regulation of cell death and the Mef2c–Smyd1 transcriptional cascade during the development of the SHF.

Disclosures

None declared.

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RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART A

The Impact of Cardiac Surgery in Patients with Trisomy 18 and Trisomy 13 in Japan

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Congenital heart defects (CHD) are very common in patients with trisomy 18 (T18) and trisomy 13 (T13). The surgical indication of CHD remains controversial since the natural history of these trisomies is documented to be poor. To investigate the outcome of CHD in patients with T18 and T13, we collected and evaluated clinical data from 134 patients with T18 and 27 patients with T13 through nationwide network of Japanese Society of Pediatric Cardiology and Cardiac Surgery. In patients with T18, 23 (17%) of 134 were alive at this survey. One hundred twenty-six (94%) of 134 patients had CHDs. The most common CHD was ventricular septal defect (VSD, 59%). Sixty-five (52%) of 126 patients with CHD developed pulmonary hypertension (PH). Thirty-two (25%) of 126 patients with CHD underwent cardiac surgery and 18 patients (56%) have survived beyond postoperative period. While palliative surgery was performed in most patients, six cases (19%) underwent intracardiac repair for VSD. Operated patients survived longer than those who did not have surgery ($P < 0.01$). In patients with T13, 5 (19%) of 27 patients were alive during study period. Twenty-three (85%) of 27 patients had CHD and 13 (57%) of 27 patients had PH. Atrial septal defect was the most common form of CHD (22%). Cardiac surgery was done in 6 (26%) of 23 patients. In this study, approximately a quarter of patients underwent surgery for CHD in both trisomies. Cardiac surgery may improve survival in selected patients with T18.

Key words: trisomy 18; trisomy 13; cardiac surgery

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INTRODUCTION

Trisomy 18 (T18) and trisomy 13 (T13) represent common and important chromosomal disorders with multiple congenital anomalies. The incidence of T18 is estimated to 1/3,600–8,500, and that of T13 is estimated to be 1/10,000–20,000 [Carey, 2010]. Congenital heart defects (CHD) are present in about 80% of children with T13 and 90% of children with T18 [Carey, 2010]. Large systemic to pulmonary shunt lesions are common defects, and polyvalvular disease and mitral atresia are characteristic,

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especially in T18. Progressive pulmonary vascular obstructive change was observed in autopsy cases of T18 as early as 2-month old [Van Praagh et al., 1989].

These trisomies are also associated with multiple extracardiac defects, severe growth failure and developmental delay and only 5–10% survived beyond the first year [Rasmussen et al., 2003; Jones, 2006; Carey, 2010]. In a population-based study from the Northern region of England, the most common mode of death in patients with T18 and T13 was reported to be central apnea [Wyllie et al., 1994; Embleton et al., 1996]. The authors stated that cardiac surgery could not be justified in patients with T18 and those with T13. Even if survival occurs beyond the first year of life, patients with T18 and T13 frequently have marked developmental delay and respiratory and/or feeding problems that require intensive daily care. Therefore, surgical treatment for CHD in these patients has been limited even though their heart defects were expected to be successfully repaired from a view point of hemodynamics.

Recently, Kosho et al. [2006] showed improved survival of patients with T18 under intensive care including respiratory support, cardiac inotropic agents, and gastrointestinal surgery. Although they did not perform cardiac surgery in any of those patients, 25% of patients with T18 survived 1 year. Moreover, Graham et al. [2004] evaluated the outcome of cardiac surgery in 35 patients with T18 and T13, through data from the Pediatric Cardiac Consortium (48 centers in the United States, Canada, and Europe). They showed that 91% discharged alive and the patients without an extended preoperative ventilator requirement did not require prolonged mechanical ventilation after surgery [Graham et al., 2004]. Kaneko et al. [2008] compared outcomes of three distinct periods with different management: the first period, both pharmacological ductal intervention and cardiac surgery withheld; the second period, only pharmacological ductal intervention offered; the third period, both strategies available. The median survival time was significantly longer in the third (243 days), than the first (7 days) and the second (24 days). Kaneko et al. [2009] subsequently reported the outcome of 17 patients with T18 who underwent cardiac surgery including 11 having palliative surgery without cardiopulmonary bypass, 4 having palliative surgery followed by intracardiac repair (ICR), and 3 having primary ICR. Their survival time ranged from 12 to 1,384 days (median, 324 days), and 14 (82%) of them were discharged home with improved symptoms. However, it remains unclear whether cardiac surgery could affect their natural history and improve their lifespan because of lack of the large-scale follow-up study.

In order to accumulate further clinical evidence and determine the operative outcome of CHD in patients with T18 and T13, we performed a nationwide survey and analyzed clinical data, especially cardiac surgery and the outcome in these trisomies.

PATIENTS AND METHODS

Between July, 2005 and March, 2008, questionnaires made by the Committee for Genetics and Epidemiology of Cardiovascular Diseases in the Japanese Society of Pediatric Cardiology and Cardiac Surgery were sent to affiliated hospitals that belonged to this nationwide network. The questionnaires included outcome at survey, genotype, birth weight, gestational age, parental age at birth, family history, cardiac phenotype, coexistence of pulmonary hypertension (PH), type of cardiac surgery, age at surgery, operative outcome, and extracardiac phenotype of patients with T18 and T13 diagnosed and followed at those hospitals. One hundred thirty-four patients with T18 and 27 patients with T13 were registered from 15 of 98 (15%) hospitals with neonatal intensive care units and analyzed in this surveillance. No patient was excluded in spite of incomplete answers.

Group comparison for mean difference was analyzed by Student's *t*-test or Mann–Whitney *U*-test. Associations between survival and various anomalies were estimated by Fisher's exact probability test. Logrank test was used to detect the difference of survival rate using Kaplan–Meier method between operated and non-operated patients. A *P*-value less than 5% was defined as statistically significant. These statistical analyses were performed using Statcel (OMS, Tokorozawa, Saitama).

RESULTS

General information of patients analyzed in this study is shown in Table I. Female patients were predominant in those with T18 (female to male ratio: 1.9) consistent with previous reports [Jones, 2006]. The results of karyotyping were reported in 41 patients with T18 and 12 patients with T13. Extracardiac anomalies were shown in Table II. Cerebellar hypoplasia and gastrointestinal defects including esophageal atresia and hepatoblastoma were characteristic anomalies in patients with T18.

TABLE I. General Information in Patients Analyzed in This Study

	Trisomy 18	Trisomy 13
Number	134 (M 46, F 86, UK 2)	27 (M 11, F 15, UK 1)
Median age at survey (range)	4.8 months (0 day–19.9 years)	3 months (0 day–9.0 years)
Median birth weight (range)	1,692 g (650–2,698 g)	1,976 g (664–2,620 g)
Median gestational age (range)	37 weeks (28–42 weeks)	36 weeks (26–40 weeks)
Median maternal age at birth (range)	34 years (21–45 years)	32 years (24–44 years)
Median paternal age at birth (range)	36 years (21–50 years)	38 years (25–50 years)

M, male; F, female; UK, unknown.
Unknown sex: 2 (1.5%) of 134 in trisomy 18 and 1 (3.7%) of 27 in trisomy 13.
Unknown genotype: 93 (69%) of 134 in trisomy 18 and 15 (56%) in trisomy 13.

TABLE II. Extracardiac Anomalies in Patients With Trisomy 18 and Trisomy 13

Trisomy 18 (n = 134)	Trisomy 13 (n = 27)
Overlapping finger 111 (83%)	Cleft palate/lip 15 (56%)
Cerebellular hypoplasia 79 (59%)	Polydactyly 10 (37%)
Esophageal atresia 13(10%)	Umbilical hernia 4 (15%)
Hepatoblastoma 3 (2%)	

TABLE III. Cause of Death in Patients With Trisomy 18 and Trisomy 13

	Trisomy 18 (n = 97)	Trisomy 13 (n = 16)
Respiratory failure	38 (39%)	4 (25%)
Heart failure	25 (26%)	5 (31%)
Arrhythmia	12 (12%)	
Sudden death	15 (15%)	3 (19%)
Unknown	7 (7%)	4 (25%)

Overall Outcome of Patients With Trisomy 18

Twenty-three patients (17%) of 134 analyzed patients with T18 were alive at this survey (5 males and 18 females). Median age of surviving patients was 2.4 years ranging from 1 month to 19.9 years old. Three (13%) of these 23 patients had mosaicism of T18 and their ages were 3 years and 9 months, 16 years, and 19 years. One hundred five patients (78%) of 134 patients with T18 died at the median age of 3.6 months (40 males and 65 females) and 2 of them (1.9%) who died at 24 days and a year and 11 months old age showed mosaicism of T18. Outcomes of the remaining 6 patients were not reported. The vast majority of patients expired within 12 months as shown in Figure 1A. Although about 25% (survival rate 0.25) of patients with T18 survived the first year, the survival rate remained as high as 0.14 after 1 year, suggesting the relatively better survival in the subsequent years than in the first year (Fig. 1A). The median birth weight of patients who survived was significantly higher than expired patients with T18 (1,923 g in surviving patients vs. 1,701 g in expired patients, $P = 0.032$). Median gestational age of patients who survived was significantly longer than expired patients (39 weeks in survived patients vs. 37 weeks in expired patients, $P < 0.01$). The cause of the death was reported in 97 deceased patients (Table III). Thirty-eight patients (39%) of them were reported to die from respiratory failure, including 10 patients diagnosed with central apnea. Twenty-seven (71%) of the 38 patients died during the neonatal period. Cardiac involvement caused death in 25 patients from heart failure (26%) and 12 patients from arrhythmia (12%). Sudden death was also observed in 15 patients (15%). It is a remarkable finding that 6 of 15 patients died suddenly after successful cardiac surgery. Gastrointestinal defects did not significantly affect survival of T18 compared to those without gastrointestinal defects in this survey ($P = 0.37$).

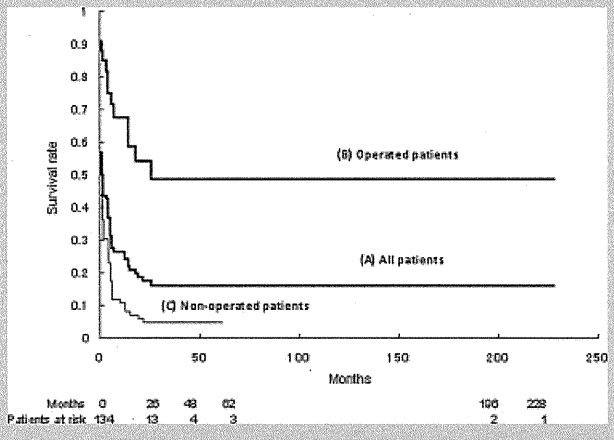

FIG. 1. The Kaplan–Meier survival curve of patients with trisomy 18. The survival rate of trisomy 18 remarkably declined after 26 months (A). Patients with cardiac surgery (B) survived significantly longer than those without cardiac surgery (C, $P < 0.01$).

Congenital Heart Defects in Patients With Trisomy 18

One hundred twenty-six (94%) of 134 patients with T18 had CHD. Details of CHD in T18 were shown in Figure 2A. Ventricular septal defect (VSD) was the most common form of CHD up to 75 patients (59%) with T18 and 68% of those with VSD had other pulmonary-systemic shunts, such as atrial septal defect (ASD) and patent ductus arteriosus (PDA; Fig. 2B). Double outlet right ventricle (DORV) was the second most common CHD found in 14 (11%) of 126 patients and 73% of them did not have pulmonary stenosis resulting in excessive pulmonary flow. Three cases of mitral atresia with DORV (21%) were reported in 14 patients with DORV and T18 (Fig. 2C), which appeared to be characteristic CHD in T18 reported as previous autopsies [Van Praagh et al., 1989].

Sixty-five patients (52%) of 126 patients with CHD in T18 suffered from PH due to pulmonary-systemic shunt lesion. Valvular disease was identified 58 (46%) of 126 patients with T18, including 33 multiple valvular lesions. Fourteen patients showed both atrioventricular and semilunar valvular defects.

Cardiac Surgery in Patients With Trisomy 18

Cardiac surgery was performed in 32 (25%) of 126 CHD patients with T18. Postoperative survival was observed in 18 (56%) of 32 operative cases at this survey. Postoperative periods of these survivors were ranged from 2 to 216 months. The remaining 14 patients died after surgery and 2 of them died within 1 month after surgery. The median age at surgery was 1.8 months of age ranging from 1 day to 18.6 months of age. Fourteen (61%) of 23 patients with palliation and 2 (40%) of 5 patients with intracardiac surgery were alive at this survey (Table IV). Two patients were received two-staged surgery (palliative repair followed by ICR) and one of them survived. The complex heart lesions such as hypoplastic left heart (HLH), single ventricle, and persistent

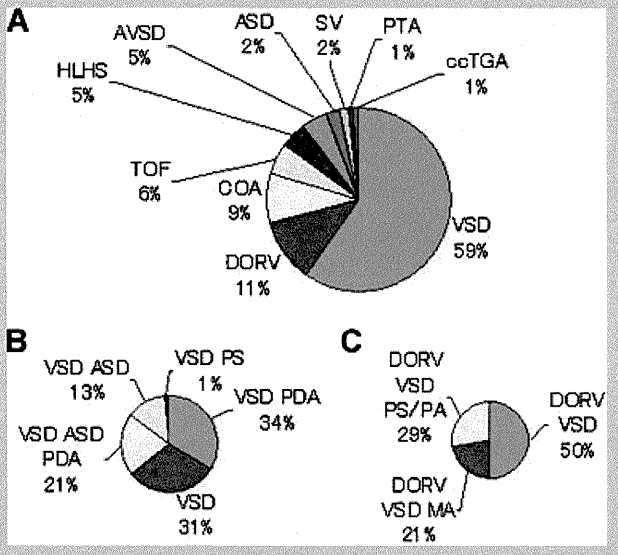

FIG. 2. Details of congenital heart defects (A), ventricular septal defect (B), and double outlet right ventricle (C) in patients with trisomy 18. A: VSD is the most common congenital heart defect in trisomy 18. B: VSD is frequently accompanied with other left to right shunt lesions, such as ASD and PDA in trisomy 18. C: DORV with mitral atresia is characteristic in trisomy 18. VSD, ventricular septal defect; DORV, double outlet right ventricle; COA, coarctation of aorta; TOF, tetralogy of Fallot; HLH, hypoplastic left heart; AVSD, atrioventricular septal defect; ASD, atrial septal defect; SV, single ventricle; PTA, persistent truncus arteriosus; ccTGA, congenitally corrected transposition of great arteries; PDA, patent ductus arteriosus; PS, pulmonary stenosis; MA, mitral atresia; PS/PA, pulmonary stenosis/pulmonary atresia.

truncus arteriosus (PTA), and congenitally corrected transposition of great arteries were not operated in this study. Thirty (93%) of 32 operated patients manifested PH before surgery, which was improved after cardiac surgery in 17 patients of them (57%).

TABLE IV. Details of Congenital Heart Defects and Operative Procedures in Trisomy 18

CHD	Operation	Survival	Procedure (number)
VSD	13	7	PAB (10) ICR (1) UK (2)
VSD PDA	8	4	PAB + DL (7) ICR (1)
VSD PDA ASD	2	1	UK (2)
VSD PAPVC	2	1	ICR (1) ICR + PVR (1)
COA VSD	4	2	PAB + COR (3) ICR (1)
DORV VSD PS	1	1	CS (1)
DORV VSD MA COA AS	1	1	PAB (1)
TOF	1	1	BTS (1)
Total	32	18	

CHD, congenital heart defects; VSD, ventricular septal defect; PDA, patent ductus arteriosus; ASD, atrial septal defect; PAPVC, partial anomalous pulmonary venous connection; COA, coarctation of aorta; DORV, double outlet right ventricle; PS, pulmonary stenosis; MA, mitral atresia; AS, aortic stenosis; PAB, pulmonary artery banding; ICR, intracardiac repair; UK, unknown; DL, ductus ligation; PVR, pulmonary vein repair; COR, coarctation of aorta repair; CS, central shunt; BTS, Blalock-Taussig shunt.

Kaplan–Meier survival curves were illustrated for operated and non-operated patients with T18 (Fig. 1B,C). The survival estimate of those who were operated (Fig. 1B) was significantly higher than of those who were not operated (Fig. 1C) in this T18 cohort ($P < 0.01$). Although the vast majority of patients with T18 were not operated in this study, the longest observational period of non-operated patients was 62 months, which was much shorter than that of operated patients.

Overall Outcome of Patients With Trisomy 13

Five (19%) of 27 patients with T13 were alive at this survey (4 males and 1 female). The complex heart defects such as HLH and PTA were not operated. The median age of surviving patients was 3.5 years of age ranging from 0 day to 5.1 years old. Sixteen (59%) of 27 patients were deceased (3 males, 12 females, and 1 unknown gender) and the median age of death was 1 month. Mosaicism of T13 was reported in 1 surviving patient (20%) and 1 expired patient (6.3%). Congenital anomalies except heart were listed in Table II. Kaplan–Meier survived curve was illustrated in Figure 3 showing that the vast majority of them were dead within the first year. The cause of death of patients with T13 included heart failure (31%), respiratory failure (25%), and sudden death (19%; Table III).

Congenital Heart Defects and Cardiac Surgery in Patients With Trisomy 13

Twenty-three (85%) of 27 patients with T13 had CHD. ASD was the most common CHD found in 5 cases (22%) followed by VSD in 4 cases (17%), and DORV in 3 cases (13%) as shown in Figure 4. Two of 3 patients with DORV were accompanied with pulmonary atresia. Thirteen (57%) of 23 CHD were accompanied by PH.

Six (26%) of 23 patients received cardiac surgery and half of them (50%) were alive at the time of this survey. Age at surgery was distributed between 4 days and 9.5 years. Three (50%) of the 6 operated patients had PH, which improved after surgery. Six surgical procedures included three palliations (50%), along

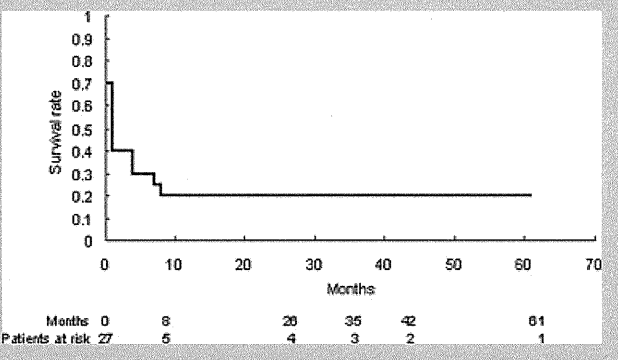

FIG. 3. The Kaplan–Meier survival curve in patients with trisomy 13. The survival rate of trisomy 13 remarkably declined after 8 months.

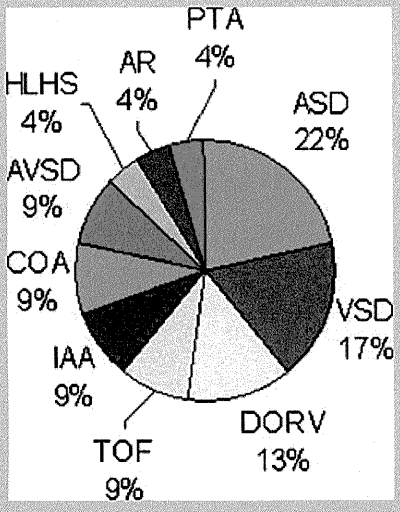

FIG. 4. Details of congenital heart defects in patients with trisomy 13. ASD was the most common congenital heart defect in patients with trisomy 13. Most of DORV accompanied with excess pulmonary blood flow. ASD, atrial septal defect; VSD, ventricular septal defect; DORV, double outlet right ventricle; TOF, tetralogy of Fallot; IAA, interruption of aortic arch; COA, coarctation of aorta; AVSD, atrioventricular septal defect; AR, aortic regurgitation; PTA, persistent truncus arteriosus.

with 2 (67%) surviving and 2 ICR (33%) with 1 (50%) surviving (Table V).

DISCUSSION

In order to delineate the current situation of cardiac surgery in patients with T18 and T13, we performed the nationwide survey through the network of Japanese Pediatric Cardiology and Cardiac Surgery. In our study, 94% of T18 and 85% of T13 had CHD. The vast majority of CHD had left to right shunt lesions and excessive pulmonary blood flow, which probably resulted in PH that was observed in 52% of patients with T18 and in 57% of those with T13, respectively. Twenty-six percent of patients with both trisomies received surgery for CHD and only 2 of 32 patients died in perioperative period in T18. It is noteworthy that operated patients survived significantly longer than non-operated patients in patients with T18 (Fig. 1B,C), although it is possible that operated patients had simple CHD and/or less extracardiac problems. As for patients with T13, our data collection was not detailed enough to analyze the surgical impact on their survival. In our limited data, operative survival rate of T13 seemed not inferior to that of T18, suggesting that cardiac surgery may be tolerated in at least some patients with T13. Recently, there is a growing tendency to take cardiac surgery into consideration in both trisomies according to clinical status despite the poor life expectancy [Carey, 2005, 2010; Jones, 2006]. This trend has probably been growing in the background of patients' support groups, general respect for parental autonomy, and accumulation of clinical evidence such as long-term survivors and effectiveness of intensive treatment [Graham et al., 2004; Kosho et al., 2006; Kaneko et al., 2008, 2009]. Our nationwide survey supports this trend providing information from relatively large numbers of patients with T18 and T13.

TABLE V. Details of Congenital Heart Defects and Operative Procedures in Trisomy 13

CHD	Operation	Survival	Procedure
VSD ASD PDA	1	1	PAB + DL (1)
ASD PDA	1	0	UK (1)
AVSD PDA	1	1	ICR (1)
COA VSD PDA	1	1	PAB + DL (1)
IAA APW VSD	1	0	ICR (1)
TOF	1	0	BTS (1)
Total	6	3	

CHD, congenital heart defects; VSD, ventricular septal defect; ASD, atrial septal defect; PDA, patent ductus arteriosus; PAB, pulmonary artery banding; DL, ductus ligation; UK, unknown; AVSD, atrioventricular septal defect; ICR, intracardiac repair; COA, coarctation of aorta; IAA, interruption of aortic arch; APW, aortopulmonary window; TOF, tetralogy of Fallot; BTS, Blalock-Taussig shunt.

With regard to surgical procedures in patients with T18 and T13, it is debatable whether an intracardiac repair using cardiopulmonary bypass or a palliative repair should be employed for operative survival. In our study, about 20–30% of patients received ICRs and half of them survived postoperatively in both trisomies, although majority of patients underwent palliative repairs (Tables IV and V). Kaneko et al. [2009] reported better postoperative survival with palliative repairs than primary ICRs among 17 patients with T18 who had life-threatening extracardiac problems. On the other hand, Van Dyke and Allen [1990] reported a T18 patient with VSD and PDA who survived for more than 5 years after ICR. Graham et al. [2004] also reported that 32 (91%) of 35 patients with T18 and T13 who underwent cardiac surgery were discharged from hospitals alive. Their primary surgical procedures included 18 ICRs, 13 palliative repairs as well as 4 PDA ligations, suggesting that palliative surgery such as pulmonary artery banding (PAB) might not be always indicated as a primary repair to alleviate the risk of surgery [Graham et al., 2004]. This series included four patients with VSD who underwent ICRs after PAB. Teraguchi et al. [1998] also described two patients with T18 and large VSD who survived after the ICR following PAB. It should be also noted that all studies described above excluded intracardiac surgery for complex type of CHD, such as HLHS.

Our study was based on questionnaires about CHD and extracardiac anomalies and had limitation for the assessment of clinical severity of each patient. We could not recruit all patients with T18 and T13 because of partial response (15% of affiliated hospitals) to our survey. We might have missed patients who died earlier, resulting in increased survival or surgical success rate. The vast majority of patients died by 26 months in T18 and 8 months in T13, reflecting the short life expectancy. On the other hand, a small number of patients at risk may overestimate the survival rate of both trisomies as no death seemed to occur thereafter (Figs. 1 and 3). Hence, this study may be defined as a multi-institutional observation with some bias rather than as a population-based analysis.

Because no well-defined criteria for cardiac surgery are available for patients with T18/T13, the indication of operation was completely dependent on each physician's decision, and we did not know how physicians struggled and finally decided to operate on patients with T18 and T13. Although our study suggested that operated patients survived longer than non-operated patients with T18, general postoperative survival of simple CHD including VSD, ASD, and PDA should reach more than 99% in nonsyndromic patients, whereas it declined as low as 50% in both trisomies in this study. Even though cardiac surgery successfully corrected abnormal hemodynamics, non-heart-related death could be encountered in T18 and T13 (Table III). Respiratory failure including central apnea may not be avoided in these trisomies regardless of outcomes of cardiac surgery. Unexpected or sudden death was also reported, which might be related to apnea. On the other hand, it was evident that some patients with T18 and T13 could survive and discharge hospital [Carey, 2010]. Technical difficulties in performing surgical treatment of relatively simple CHD in patients with T18/T13 are being overcome and cardiac surgery could be considered in those without severe extracardiac problem.

In this study, although we analyzed the impact of cardiac surgery on survival, we could not evaluate the impact of cardiac surgery on indices concerning quality of life such as hospital discharge rate, rate of home mechanical ventilation, rate of home oxygen therapy, rate of oral feeding without nasal tube. It is important to evaluate how well the patients become after cardiac surgery in future studies.

CONCLUSION

Cardiac surgery for CHD was performed in a quarter of patients with T18 and T13 and resulted in better life outcome, especially in T18, in the nationwide survey in Japan. The indications of cardiac surgery for these patients need to be considered electively from the individual patient's status along with continuous support for decision-making by the parents. It is still not clear whether cardiac surgery improves the long-term prognosis of patients with T18 and T13; however, cardiac surgery may be indicated for patients with relatively simple CHD and/or extracardiac complications. We believe that the opinions of parents of patients should be strongly respected in determining whether such surgery is performed. Physicians should provide information to the family sufficient to enable optimal decision-making. Pediatric cardiologists as well as clinical geneticists need not only to perform counseling and respect parent's decision, but also address the opinions as professionals based on medical experience, knowledge, and ethics.

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Hand2 function in second heart field progenitors is essential for cardiogenesis

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ABSTRACT

Cardiogenesis involves the contributions of multiple progenitor pools, including mesoderm-derived cardiac progenitors known as the first and second heart fields. Disruption of genetic pathways regulating individual subsets of cardiac progenitors likely underlies many forms of human cardiac malformations. *Hand2* is a member of the basic helix loop helix (bHLH) family of transcription factors and is expressed in numerous cell lineages that contribute to the developing heart. However, the early embryonic lethality of *Hand2*-null mice has precluded lineage-specific study of its function in myocardial progenitors. Here, we generated and used a floxed allele of *Hand2* to ablate its expression in specific cardiac cell populations at defined developmental points. We found that *Hand2* expression within the mesoderm-derived second heart field progenitors was required for their survival and deletion in this domain recapitulated the complete *Hand2*-null phenotype. Loss of *Hand2* at later stages of development and in restricted domains of the second heart field revealed a spectrum of cardiac anomalies resembling forms of human congenital heart disease. Molecular analyses of *Hand2* mutant cells revealed several genes by which *Hand2* may influence expansion of the cardiac progenitors. These findings demonstrate that *Hand2* is essential for survival of second heart field progenitors and that the graded loss of *Hand2* function in this cardiac progenitor pool can cause a spectrum of congenital heart malformation.

Introduction

Congenital heart defects (CHDs) represent the most common form of human birth defects and occur in nearly 1% of live births (Hoffman and Kaplan, 2002). The recognition that individual pools of cardiac progenitors contribute to specific regions of the heart suggests that some CHDs may be due to disruption of genetic pathways that control migration, survival, expansion or differentiation of distinct populations of cells that contribute to the heart. Because of the dynamic nature of early embryonic development, it is also likely that the developmental requirement for critical genes within progenitors occurs during specific developmental windows.

Cell lineage analyses have demonstrated that the heart develops from multiple sources of cells (reviewed in Buckingham et al., 2005; Olson, 2006; Srivastava, 2006). Two progenitor cell populations, the first heart field (FHF) and second heart field (SHF) are derived from the lateral plate and splanchnic mesoderm, respectively. The third lineage is derived from cardiac neural crest (CNC) cells. In mice, the FHF forms the crescent shaped heart primordium at embryonic day (E) 7.5. At E8.0, these cells fuse at the ventral midline to form the primary heart tube and later contribute to most of the left ventricle (Buckingham et al., 2005). Meanwhile, the SHF cells, initially medial and caudal to the FHF, migrate through the pharyngeal mesoderm into the heart tube from both the anterior and posterior poles as the heart tube breaks symmetry and bends to the right. Molecularly distinct subsets of the SHF cells contribute to the outflow tract myocardium, right ventricle and atria. By E10.5, CNC cells migrate from their birthplace along the dorsal aspect of the neural folds into the outflow tract to ultimately septate the outflow into two distinct vessels, where they also differentiate into vascular smooth muscle cells.

Hand2, also known as *dHAND*, is a member of the basic helix-loop-helix (bHLH) family of transcription factors. *Hand2* is expressed in the heart, limb bud, and numerous neural crest derivatives during embryogenesis (Srivastava et al., 1995; Srivastava et al., 1997). In the heart, *Hand2* is expressed throughout the entire primary heart

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tube during early embryonic stages, with dominant expression in the right ventricle and outflow tract as the heart tube loops. *Hand2*-null (*Hand2*$^{-/-}$) mice show severe hypoplasia of the right ventricle and growth retardation from E9.5, with death by E10.5 (Srivastava et al., 1997; Yamagishi et al., 2001).

The severe phenotype and early embryonic lethality of *Hand2*$^{-/-}$ mice have precluded the determination of the precise roles of *Hand2* within the individual cell types where it is expressed. The tissue-specific deletion of *Hand2* in neural crest cells and in the limb bud has revealed essential roles in each tissue (Galli et al., 2010; Morikawa and Cserjesi, 2008). To determine the function of *Hand2* in specific subsets of myocardial progenitors that contribute to the heart as well as the cellular mechanisms underlying the severe hypoplasia of the right ventricle in *Hand2*$^{-/-}$ mice, we established and examined conditional knockout alleles of *Hand2* in specific SHF domains.

Materials and methods

Gene targeting and genotyping

To generate a conditional allele of *Hand2, loxP* sites were placed flanking the two introns of *Hand2*. For selection purposes, a neomycin cassette, flanked by two *frt* sites, was also placed upstream of the first exon. Homologous recombination and deletion of the Neo cassette by Flip-*frt* recombination created the *Hand2*^{loxp} allele (Fig. 1A). Genotyping was accomplished by digesting DNA with BamHI and ClaI and Southern analysis with a 5′ ^{32}P-radiolabeled probe as described (Srivastava et al., 1997). This process produced a 4.5-kb band representing the *Hand2*^{loxp} allele, a 5.9-kb band representing the null allele, and a 7.5-kb band representing the wild type (Fig. 1B). *Cre* driver (male) and *Hand2*$^{+/-}$ (female) mice were mated to yield *Cre:Hand2*$^{+/-}$ mice (male), which were mated with *Hand2*$^{loxp/loxp}$ mice to generate *Cre:Hand2*$^{loxp/-}$ conditional knockout mice.

Generating conditional knockout mice

Hand2^{loxp} mice were mated with four different *Cre* driver lines: *Tbx1Cre* (Maeda et al., 2006), *Mef2cCre* (Dodou et al., 2004), and *Islet1Cre* (Cai et al., 2003), which excise the floxed gene in distinct second heart field domains; and *Nkx2.5Cre* (McFadden et al., 2005), which is active in both the right and left ventricles. Each *Cre* line was crossed with *Hand2*$^{+/-}$ mice to obtain *Cre:Hand2*$^{+/-}$ males. These mice were crossed with *Hand2*$^{loxp/loxp}$, *Hand2*$^{loxp/+}$, *and Hand2*$^{loxp/+}$; *ROSA*^{LacZ} females. All mouse lines were of mixed C57BL6/129SVEJ background. We collected the resulting embryos between E8.5 and E18.5. A summary of cardiac cell types affected by each Cre line as previously published using reporter lines is provided below:

E9.5		Islet1Cre	Mef2cCre	Nkx2.5Cre	Tbx1Cre
Outflow tract	Myocardium	++	++	−	++
	Endocardium	+	++	−	+
Right ventricle	Myocardium	++	++	++	Partial
	Endocardium	+	++	+	Partial
Left ventricle	Myocardium	Partial	Partial	++	−
	Endocardium	−	−	+	−
Atrium	Right	Partial	−	−	−
	Left	−	−	−	−

Histology

The embryos from timed matings were harvested and fixed overnight in 4% paraformaldehyde/PBS. After fixation, embryos were rinsed in PBS, dehydrated overnight in 70% ethanol, and embedded in paraffin wax. Histological sections were cut and stained with hematoxylin and eosin or used for other analyses, such as TUNEL and cell-proliferation assays.

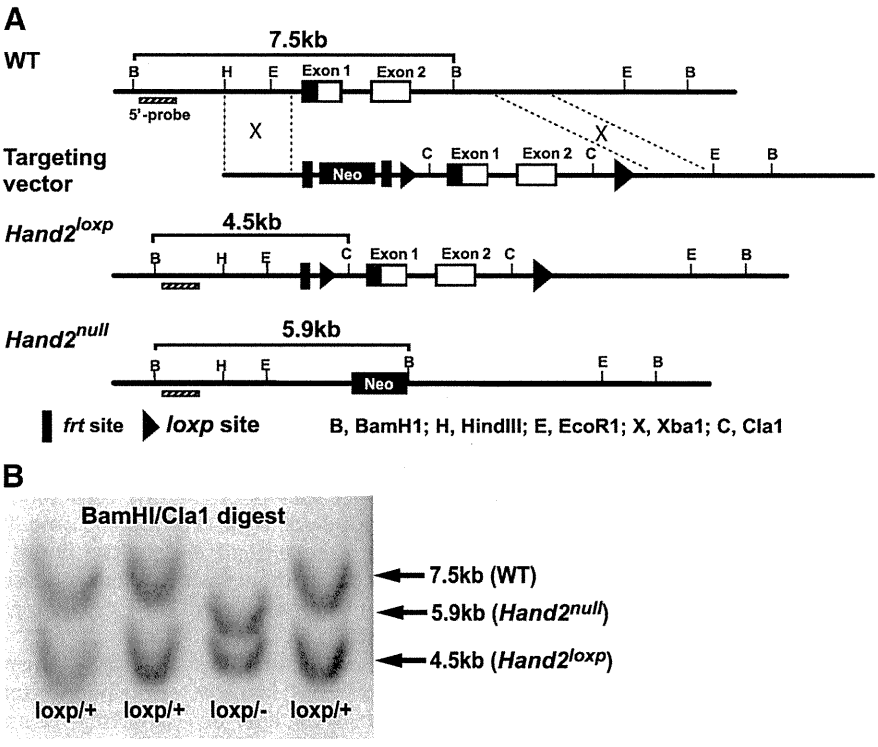


Fig. 1. Strategy for tissue-specific inactivation of *Hand2* using *Cre-loxp* system. A) Targeting strategy for generating the conditional allele of *Hand2*. In the targeting vector, a *Neo* resistance gene cassette was flanked by two *frt* sites, and two loxp sites were inserted surrounding the entire *Hand2* gene. After targeting, F1 mice were crossed with *flp* transgenic mice to remove the *Neo* cassette, resulting in the *Hand2*^{loxp} allele. We also used the previously published *Hand2*-null allele for this study. B) Genotyping was performed by Southern analysis with *BamHI* and *Cla1* digested genomic DNA. B, *BamHI*; H, *HindIII*; E, *EcoRI*; X, *XbaI*; C, *ClaI*. WT, wild type.

LacZ staining of embryos

The embryos from timed matings were harvested and prefixed for 2 h in 4% paraformaldehyde, 0.25% glutaraldehyde/PBS at 4 °C. Beta-galactosidase staining was performed as described (Yamagishi et al., 2003).

TUNEL assay and cell-proliferation assay

TUNEL staining and proliferation assays were performed on transverse sections from E9.0 embryos embedded in paraffin wax. The Cell Death Detection Kit (Roche) was used for TUNEL assays. Proliferation assays used primary anti-phosphohistone H3 antibody (rabbit, diluted 1:200 in 1% BSA/PBS), biotin-labeled goat anti-rabbit IgG, and FITC-strept-avidin. A statistical analysis was performed using the Student's *t*-test. A P value of 0.05 or less was considered significant.

Messenger RNA expression array analysis

E9.0 mouse hearts from wild-type, *Hand2*$^{-/-}$, or *Nkx2.5Cre:Hand2*$^{loxp/-}$ mice were harvested using Trizol reagent (Invitrogen) for total RNA isolation. The total RNA (50 ng) was labeled and hybridized to a mouse mRNA expression microarray (Affymetrix) for analysis. Gene expression values were obtained from Affymetrix CEL files with the GC-RMA package from Bioconductor.

Quantitative RT-PCR

Total RNA was isolated from E9.0 hearts with Trizol reagent (Invitrogen) and 20 ng was reverse transcribed using the Multi Reverse Transcriptase by Random Primer Labeling method (Roche). Quantitative RT-PCR (qRT-PCR) was performed using an ABI7900 with Taqman primer sets for selected cardiac developmental genes, and results were normalized to the expression of *Gapdh*. Statistical analysis was performed using the Student's *t*-test. The results shown are representative of more than three independent experiments.

Results

Generation of Hand2 floxed allele

To determine the function of *Hand2* in specific domains, we generated a conditional knockout allele of *Hand2* (Fig. 1A). Homologous recombination in mouse embryonic stem (ES) cells resulted in ES cells containing *loxp* sites flanking the *Hand2* gene and *frt* sites surrounding the *Neomycin* (*Neo*) resistance cassette 5′ of the first exon. Successful site-specific heterozygous recombination was confirmed by Southern analysis (Fig. 1B). Two distinct targeted ES cell lines were injected into blastocysts and the resulting high percentage chimeras were bred for the germline transmission of the targeted allele. Heterozygous mice were intercrossed with mice that ubiquitously expressed *Flp*-recombinase to remove the *Neo* gene (*Hand2*$^{loxp/+}$). Like *Hand2*$^{+/-}$ mice, *Hand2*$^{loxp/+}$ mice were phenotypically normal and fertile. The intercross of *Hand2*$^{loxp/+}$ mice generated *Hand2*$^{loxp/loxp}$ mice, and mice with this genotype approximated Mendelian inheritance at birth, survived to adulthood, and bred normally. The deletion of *Hand2* from the *Hand2*$^{loxp/+}$ or *Hand2*$^{loxp/loxp}$ conditional alleles depends on the expression and activity of Cre-recombinase. Several domain-specific *Cre* driver mice that express Cre-recombinase in domains of interest were mated with *Hand2*$^{+/-}$ mice and further crossed with *Hand2*$^{loxp/+}$ or *Hand2*$^{loxp/loxp}$ mice to obtain mouse embryos with domain-specific ablation of *Hand2* gene function.

Hand2 function is required in early SHF cardiac progenitor cells

We ablated *Hand2* with the *Nkx2.5*-enhancer-driven *Cre* transgenic mouse that results in Cre-recombinase activity in the right and left ventricular chambers after E8.5, but not in the earlier cardiac progenitors prior to their differentiation into cardiac cells (McFadden et al., 2005). *Nkx2.5Cre:Hand2*$^{loxp/-}$ mice formed both right and left ventricles (Fig. 2 B,F), although they were slightly smaller than those of the wild-type embryos at E9.5 (Fig. 2 A,E) and the mutant mice died by E12.5. Thus, the deletion of *Hand2* after initial specification of cardiac progenitors appeared to affect the expansion of cardiomyocytes in the embryonic heart, but to a much lesser degree than observed in the complete *Hand2*-null state.

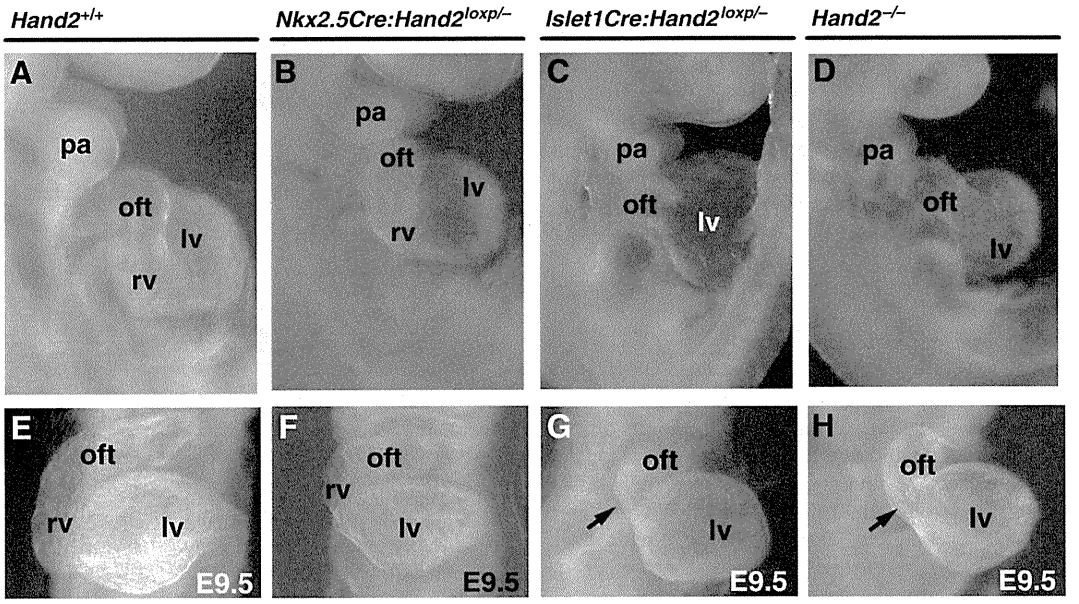


Fig. 2. *Hand2* deletion in the SHF mimics cardiac defect of the *Hand2*$^{-/-}$ germline mutant. Right lateral views (A–D) and frontal views (E–H) of the heart of embryos at E9.5. In *Nkx2.5Cre:Hand2*$^{loxp/-}$ embryos, the right ventricular (rv) segment was formed, although it was slightly smaller than that of wild type. *Islet1Cre:Hand2*$^{loxp/-}$ embryos demonstrated severe hypoplasia of the rv similar to *Hand2*$^{-/-}$ hearts (arrows in G and H). pa, pharyngeal arch; oft, outflow tract; lv, left ventricle.

Hand2 expression is high in the early cardiac progenitors of the FHF and SHF and begins to decline by E9.5 (Srivastava et al., 1995, 1997), suggesting that it might have earlier functions in cardiac progenitor cells. To investigate this, we used the *Islet1Cre* mouse that expresses Cre-recombinase in all of the early SHF cells, prior to the onset of sarcomeric gene expression (Cai et al., 2003). *Islet1Cre:Hand2*$^{loxp/-}$ embryonic hearts died by E10.5 with severe hypoplasia of the right ventricle and a shortened outflow tract (Fig. 2 C,G and 3 A–D), phenocopying complete *Hand2*$^{-/-}$ hearts (Fig. 2 D,H). These results suggest that *Hand2* expression and function in the early SHF progenitors are essential for their contribution to the developing heart and that the *Hand2*$^{-/-}$ cardiac defect (Srivastava et al., 1997) largely reflects its function in the SHF.

Hand2 is involved in survival of SHF progenitors of the pharyngeal mesoderm

To investigate the cause for right ventricular hypoplasia in the *Islet1Cre:Hand2*$^{loxp/-}$ embryos, we performed lineage analyses of the SHF cardiac progenitor cells by crossing this mouse into the *Rosa26*^{lacZ} background (Zambrowicz et al., 1997). In this system, the Cre-mediated recombination of the *Rosa26*^{lacZ} allele resulted in β-galactosidase (β-gal) production from the recombined, constitutively expressed *Rosa26* locus. Thus, β-gal staining (blue) marked derivatives of the SHF that once expressed *Islet1*. At E9.5, blue stain was detected in the pharyngeal region and outflow tract of *Islet1Cre:Hand2*$^{loxp/+}$; *Rosa26*^{LacZ} embryos, indicating that the SHF cardiac progenitor cells were migrating, as expected, from the pharyngeal mesoderm into the outflow tract (Fig. 3E). In contrast, fewer *LacZ*-positive cells were found in *Islet1Cre:Hand2*$^{loxp/-}$; *Rosa26*^{LacZ} embryos at E9.5 (Fig. 3F). The reduction was especially pronounced in the pharyngeal region of these embryos, suggesting that the decrease in progenitor cell number occurred before their migration into the outflow tract.

To determine if the decreased number of progenitor cells from the SHF resulted from abnormal cell death or decreased proliferation, we performed TUNEL and proliferation assays on the embryos collected at E9.0. More cell death was detected in the pharyngeal mesoderm of both *Islet1Cre:Hand2*$^{loxp/-}$ and *Hand2*$^{-/-}$ mutants than in the wild type embryos (Fig. 3G), while the number of TUNEL positive cells in the outflow tract was only significantly greater in the *Hand2*$^{-/-}$ embryos (Fig. 3H), likely reflecting the influence of *Hand2* in neural crest derivatives in the outflow tract as previously reported (Thomas et al., 1998). Immunohistochemical analyses using anti-Ph3 antibody revealed no significant difference in cell proliferation among the wild-type, *Islet1Cre:Hand2*$^{loxp/-}$ and *Hand2*$^{-/-}$ embryos (data not shown). These results suggest that *Hand2* is required within the SHF progenitor cells to ensure their survival and that the severe right ventricular hypoplasia that occurs from the loss of *Hand2* is a result of enhanced apoptosis of the SHF cells within the pharyngeal mesoderm before they contribute to the heart.

Loss of Hand2 in subsets of SHF progenitors causes a spectrum of right heart lesions

The discovery of distinct subpopulations of SHF progenitors raises the possibility that some forms of CHD involving the outflow tract or right ventricle are caused by the loss of some or all SHF cells during cardiogenesis. Having demonstrated that *Hand2* was essential for the survival of SHF progenitors, we investigated whether the loss of *Hand2* in smaller subsets of SHF could result in cardiac anomalies more similar to CHDs in humans. Transgenic mice with Cre-recombinase under the control of the SHF-specific enhancer of *Mef2c* (*Mef2cCre*) express Cre-recombinase in a distinct subset of the SHF-derived cells that is narrower than that of the *Islet1Cre* driver mice, encompassing the outflow tract and right ventricle, including the ventricular septum (Dodou et al., 2004). The *Mef2cCre:Hand2*$^{loxp/-}$ embryos had no gross growth retardation until E12.5, but died around E13.5. By morphological analyses, the right ventricle was smaller in the *Mef2cCre:Hand2*$^{loxp/-}$ mutant embryos than in the wild type

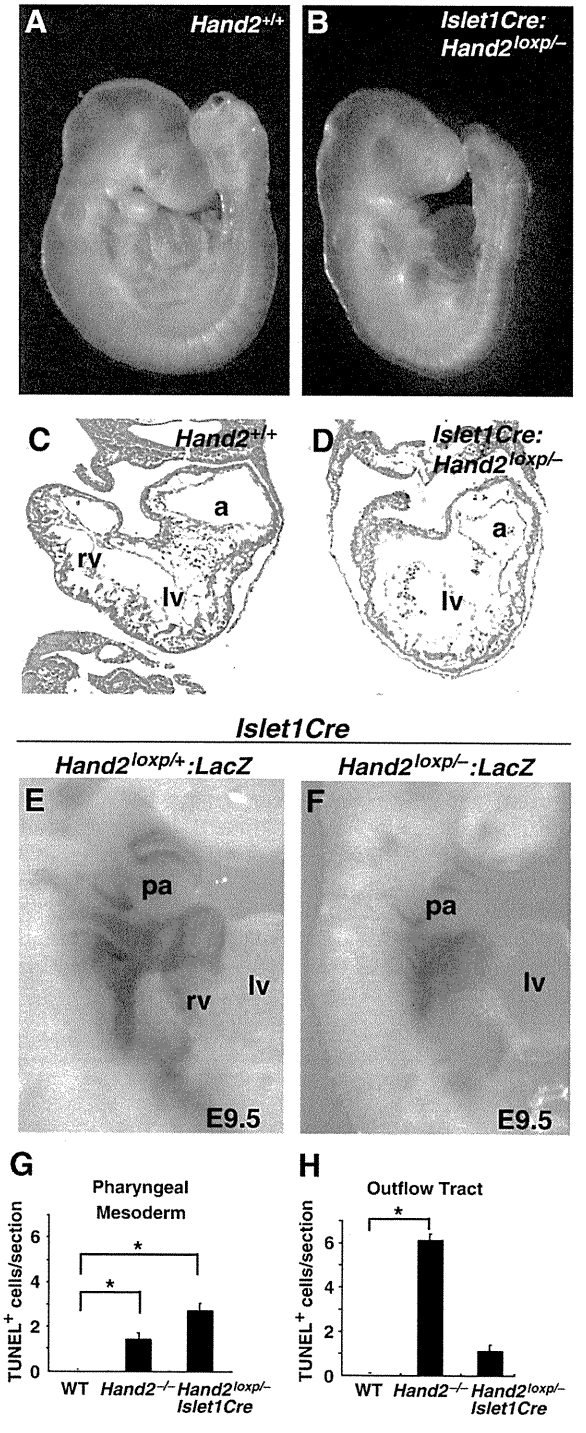


Fig. 3. *Hand2* deletion in the SHF results in reduced number of cardiac progenitor cells in the pharyngeal region. Right lateral views of wild-type (A) or *Islet1Cre:Hand2*$^{loxp/-}$ (B) embryos at E9.5. Growth retardation of mutant embryos was noted. Transverse section from wild-type (C) or *Islet1Cre:Hand2*$^{loxp/-}$ (D) embryos at E9.5 demonstrated severe hypoplasia of right ventricle (rv) and a thin walled left ventricle (lv) in mutants, similar to *Hand2*-nulls. Compared with *Islet1Cre:Hand2*$^{loxp/+}$; *Rosa26*^{LacZ} embryo (E), LacZ staining was reduced in *Islet1Cre:Hand2*$^{loxp/-}$; *Rosa26*^{LacZ} (F), indicating a reduced number of cardiac progenitor cells in the SHF of this mutant. Results of TUNEL assay on wild type, *Hand2*$^{-/-}$, and *Islet1Cre:Hand2*$^{loxp/-}$ E9.0 embryos in the pharyngeal mesoderm (G) and the outflow tract (H) are shown. Excessive cell death was observed in the pharyngeal mesoderm of *Islet1Cre:Hand2*$^{loxp/-}$ and *Hand2*$^{-/-}$ embryos, and in the outflow tract of *Hand2*$^{-/-}$ embryos. pa, pharyngeal arch; a, atrium. *p = 0.05.

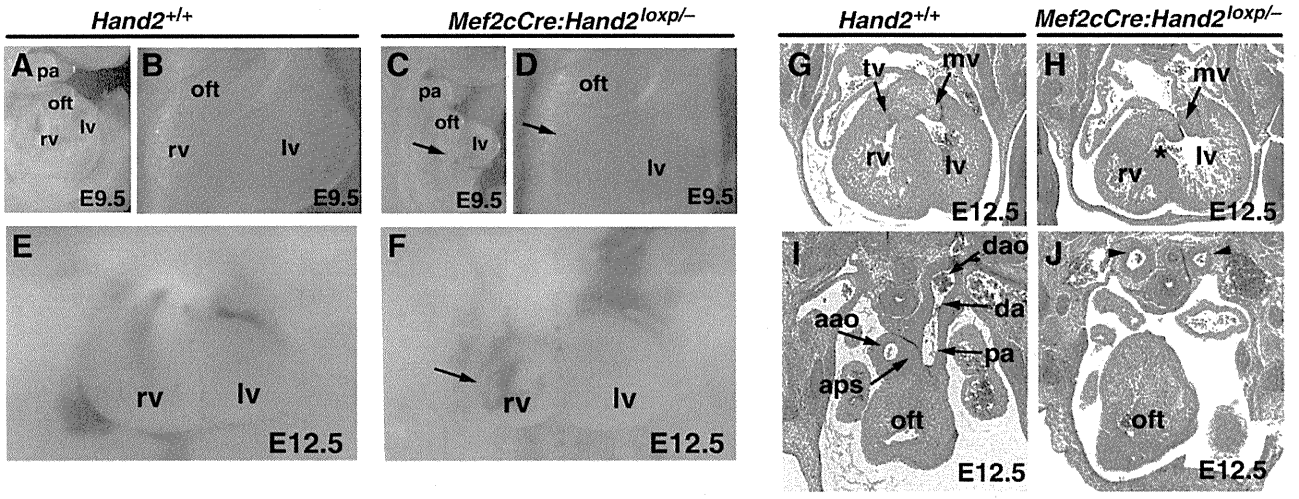


Fig. 4. Hypoplastic right ventricle with tricuspid atresia in *Mef2cCre:Hand2* conditional knockout mice. Wild-type (A, B) and *Mef2cCre:Hand2*$^{loxp/-}$ hearts (C, D) at E9.5 are shown in right lateral (A, C) and frontal views (B, D). Wild-type (E) and *Mef2cCre:Hand2*$^{loxp/-}$ hearts (F) at E12.5 are shown in frontal views. A small and thin right ventricle (rv) was observed in *Mef2cCre:Hand2*$^{loxp/-}$ (arrows in C, D, F). Transverse sections of ventricular level (G, H) or outflow tract (oft) level (I, J) in hearts of wild-type (G, I) or *Mef2cCre:Hand2*$^{loxp/-}$ (H, J) embryos. *Mef2cCre:Hand2*$^{loxp/-}$ hearts showed a small rv, interventricular septal defect (asterisk in H) and no evidence of a tricuspid valve (tv). While wild type demonstrated appropriate asymmetric remodeling for primitive aortic arches into ascending aorta (aao), main pulmonary artery (pa), ductus arteriosus (da), and descending aortic arch (dao) (I), *Mef2cCre:Hand2*$^{loxp/-}$ mutants displayed bilateral descending aortas (arrowheads in J) with no evidence of outflow septation. lv, left ventricle; mv, mitral valve; aps, aortico pulmonary septum.

embryos at E9.5 (Fig. 4 A–D), but larger than the *Hand2*$^{-/-}$ or *Islet1Cre:Hand2*$^{loxp/-}$ right ventricles. Analyses of these mutants at E12.5 revealed a hypoplastic right ventricle with relatively thin myocardium (Fig. 4 E–H). The *Mef2cCre:Hand2*$^{loxp/-}$ mutant embryos lacked the anlage of a tricuspid valve between the right atrium and ventricle in any level of section, and displayed ventricular septal defects (Fig. 4H). This anatomy is reminiscent of a type of human CHD known as tricuspid atresia. At this stage, the distal outflow tract was divided into two vessels in the wild type (Fig. 4I), but an immature truncus arteriosus was noted in *Mef2cCre:Hand2*$^{loxp/-}$ mutants (Fig. 4J), probably as a result of the delayed remodeling of the outflow tract and aortic arches.

We previously showed that the transcription factor *Tbx1* is expressed in cells whose descendants contribute mainly to the distal right ventricular outflow tract and pulmonary artery (Yamagishi et al., 2003; Maeda et al., 2006). We used an enhancer of *Tbx1* driving *Cre-recombinase* (*Tbx1-Cre*) to delete *Hand2* in this narrower subset of the SHF-derived cells (Maeda et al., 2006). The analyses of *Tbx1Cre:Hand2*$^{loxp/-}$ mutants at E10.5 revealed a shortened outflow tract, while the right ventricle appeared to be developing normally (Fig. 5 A–D). By E13.5, the *Tbx1Cre:Hand2*$^{loxp/-}$ embryos had smaller right ventricles and outflow tracts than the wild-type mice (Fig. 5 E,F) and died by E15.5.

We used the *Rosa26*^{LacZ} mouse to lineage trace the consequence of *Hand2* deletion in *Tbx1*-expressing cells. *LacZ* staining of E12.5 *Tbx1Cre:Hand2*$^{loxp/-}$; *Rosa26*^{LacZ} mutant embryos highlighted the narrowing of the outflow tract and hypoplasia of the outlet, or subpulmonary conus, of the right ventricle compared to *Tbx1Cre:Hand2*$^{loxp/+}$; *Rosa*^{LacZ} littermates (Fig. 5 G–J). The transverse sections of embryos at E14.5 further demonstrated that the right ventricular cavity of *Tbx1Cre:Hand2*$^{loxp/-}$ mutants was smaller than the wild type, although the right ventricular muscle wall thickness was comparable (Fig. 5 K,L). The decreased chamber volume was likely secondary to hypoplasia of the infundibular components, as the pulmonary valve appeared normal (Fig. 5 M,N). Notably, the relationships of the great vessels to the ventricular chambers and the ventricular septum were normal in all of the mutant embryos. Hypoplasia of the infundibular components (outlet portion) of the right ventricle is often observed in many forms of human CHD and may reflect loss of the distal SHF progenitors.

Analyses of Hand2-dependent cardiac gene expression

To elucidate the gene expression alterations that may underlie the severe right ventricular hypoplasia in *Hand2*$^{-/-}$ mice and *Islet1Cre:Hand2*$^{loxp/-}$ mice, we performed an mRNA expression array analysis that compared the E9.0 wild-type, *Hand2*$^{-/-}$, and *Nkx2.5Cre:Hand2*$^{loxp/-}$ embryonic hearts. Since severe right ventricular hypoplasia was not demonstrated in the *Nkx2.5Cre:Hand2*$^{loxp/-}$ animals, we hypothesized that genes dysregulated in the *Hand2*$^{-/-}$ hearts, but not in the *Nkx2.5Cre:Hand2*$^{loxp/-}$ hearts, may be implicated in the defects specifically resulting from ablation of *Hand2* in the SHF.

We selected several key regulators of cardiac development that appeared to be dysregulated by microarray (Fig. 6A) and performed qRT-PCR analyses to validate the array results. Interestingly, expression levels of *Gata4*, *Has2* and *Bmp5* were significantly downregulated in the *Hand2*$^{-/-}$ hearts, but not in the *Nkx2.5Cre:Hand2*$^{loxp/-}$ hearts, by array (Fig. 6B) and qRT-PCR (Fig. 6 C–E). Decreased levels of *Has2* in the *Hand2*$^{-/-}$ hearts may reflect the smaller right ventricle since *Has2* is more enriched in this domain; however, the downregulation of *Gata4* and *Bmp5*, among other dysregulated genes, may contribute to the severe right ventricle hypoplasia observed in *Hand2*$^{-/-}$ or *Islet1Cre:Hand2*$^{loxp/-}$ mice. The downregulation of *Hand2* was also confirmed in both *Hand2*$^{-/-}$ and *Nkx2.5Cre:Hand2*$^{loxp/-}$ hearts by qRT-PCR (Fig. 6F) and other genes dysregulated in both settings such as Klf4 and Klf7 may contribute to the expansion defect.

Discussion

We sought to dissect the roles of *Hand2* in the various cardiac progenitor cells where it is normally expressed and to gain an understanding of the process by which *Hand2* controls heart development. We created a floxed *Hand2* allele, which we used to delete *Hand2* in specific domains. Through the analyses of these mutant mice, we found that *Hand2* was required in the SHF progenitors for their early survival. Temporal- or spatially-restricted ablation of *Hand2* function in the SHF revealed its necessity for the normal development of the outflow tract, tricuspid valve, and ventricular septum. Interestingly, the abnormalities seen in mice with conditional ablation of *Hand2* in discrete sub-domains shared some resemblance to several human congenital heart malformations. The gene expression analyses of these

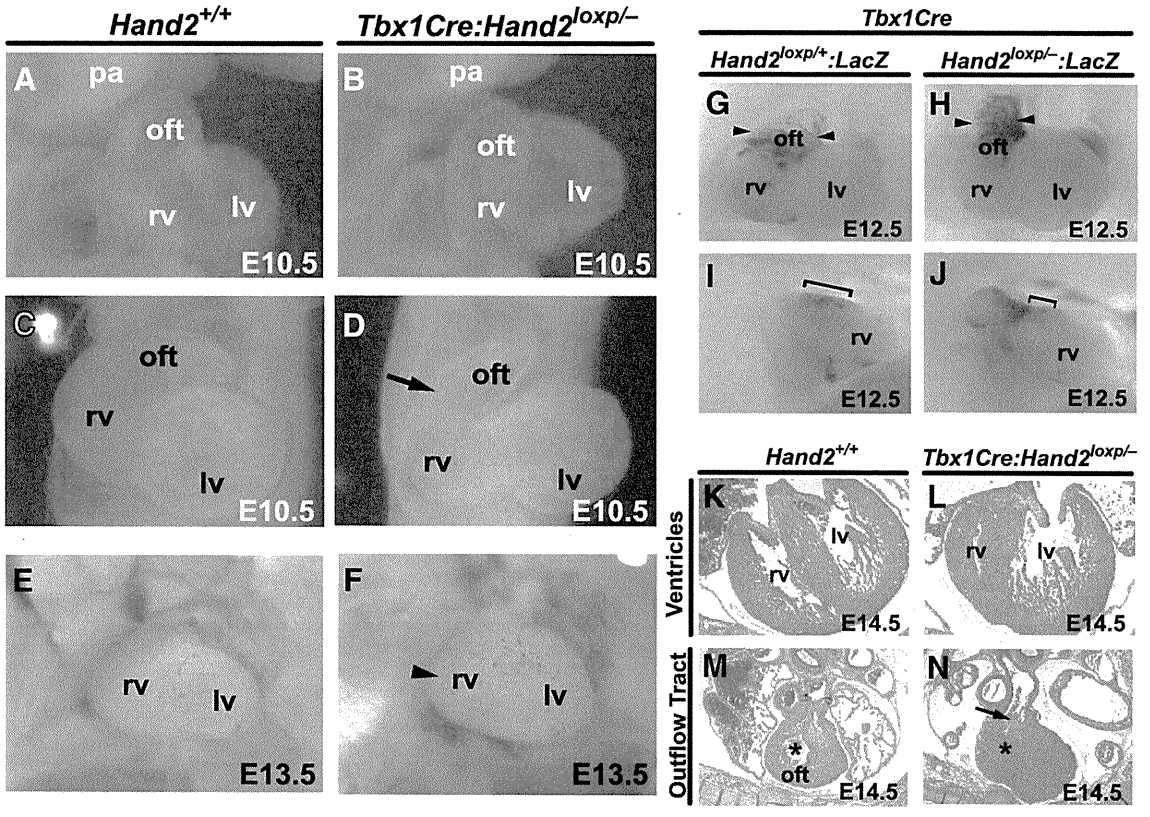


Fig. 5. Subpulmonary conus defect in *Tbx1Cre:Hand2* conditional knockout mice. Right lateral views (A, B) and frontal views (C, D) of the heart at E10.5 and frontal views at E13.5 (E, F) of wild type (A, C, E) and *Tbx1Cre:Hand2*$^{loxp/-}$ embryos (B, D, F). The outflow tract (oft) was shorter in the *Tbx1Cre:Hand2*$^{loxp/-}$ (arrow in D) than in the wild type. Note the small right ventricle (rv) in *Tbx1Cre:Hand2*$^{loxp/-}$ at E13.5 (arrowhead in F). Frontal (G, H) and right lateral (I, J) views of *Tbx1Cre:Hand2*$^{loxp/+}$: *Rosa*^{LacZ} (G, I) and *Tbx1Cre:Hand2*$^{loxp/-}$: *Rosa*^{LacZ} (H, J) embryos with *lacZ* staining at E12.5. Narrowing (between arrowheads in G, H) and shortening of the oft (brackets in I, J) could be seen in *Tbx1Cre:Hand2*$^{loxp/-}$: *Rosa*^{LacZ}. Transverse section at ventricular level (K, L) or outflow tract level (M, N) of wild type (K, M) or mutant (L, N) at E14.5. Small rv in *Tbx1Cre:Hand2*$^{loxp/-}$ compared to wild type (K). Wild type embryos had a right ventricular oft lumen (asterisk in M), whereas *Tbx1Cre:Hand2*$^{loxp/-}$ had no evidence of the lumen (asterisk in N) below the pulmonary valve (arrow in N) at any level of section, suggesting the absence of infundibular components. pa, pharyngeal arch; lv, left ventricle.

mutant mice revealed potential effectors of *Hand2* in right ventricular and general myocardial development.

Hand2 is essential in the SHF for right ventricular development

In this study, we found that the loss of *Hand2* in the broadest SHF domain phenocopies the ventricular defect observed in global *Hand2* knockout, while its ablation in a subset of SHF and FHF is not sufficient to phenocopy the *Hand2*$^{-/-}$ mutant hearts. We conclude that the SHF is the critical region of *Hand2* expression required for proper development of the right ventricle and that this requirement is prior to the migration of the SHF cells into the heart. Intriguingly, the loss of *Hand2* in the SHF led to enhanced apoptosis of cardiac progenitor cells in the pharyngeal mesoderm before they participated in the process of cardiac development, resulting in severe hypoplasia of the right ventricle. Thus, it is likely that *Hand2* is essential for the survival of undifferentiated cardiac progenitor cells in the SHF. The deletion of *Hand2* after cardiac differentiation had begun using the ventricular-specific *Nkx2.5-Cre* transgenic mouse resulted in a later defect of ventricular expansion, similar to that observed upon the deletion of *Hand2* in the domain of the myocardial sarcomeric gene, *cardiac Troponin T* (Morikawa and Cserjesi, 2008).

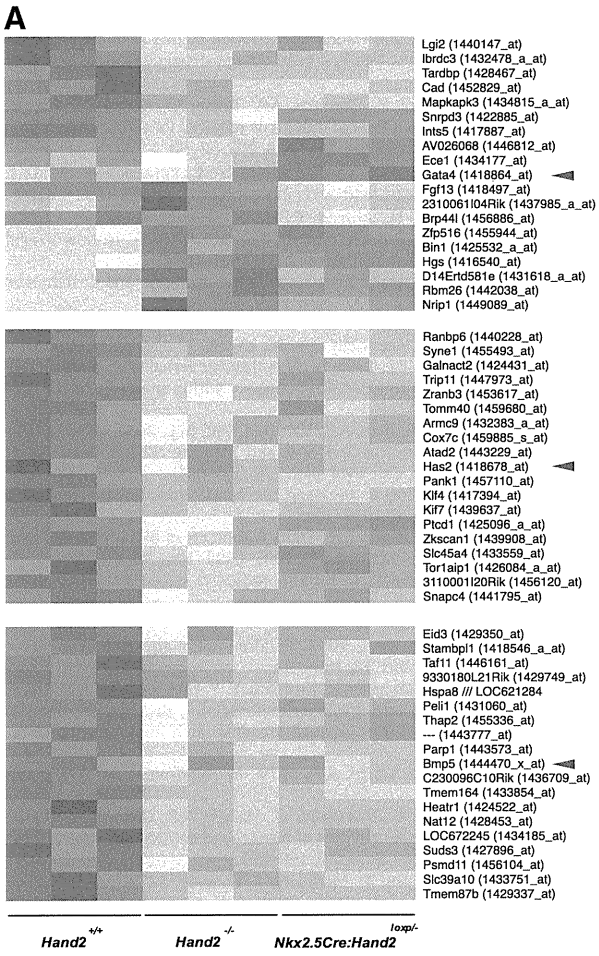
Similarity between Hand2 conditional knockout hearts and human CHDs

Deleting *Hand2* in a subset of the SHF cells resulted in various degrees of hypoplastic right heart syndrome that correlated with the breadth of the SHF cell population that was targeted. For example, the *Tbx1Cre:Hand2*$^{loxp/-}$ phenotype was similar to severe right ventricular outflow tract obstruction that retards right ventricular chamber growth. In contrast, *Mef2cCre:Hand2*$^{loxp/-}$ mice developed small, thin-walled right ventricles and tricuspid valve atresia, likely reflecting the broader deletion of *Hand2* in cells that contribute to most of the right ventricular chamber.

While homozygous deletion of *Hand2* has not been described in humans, several reports indicate that the *Hand2* heterozygous deletion is part of 4q syndrome, which involves developmental craniofacial, musculoskeletal, and cardiac defects. 4q33, the *Hand2* locus (Natarajan et al., 2001), has been identified as the critical region of 4q syndrome (Towns et al., 1979; Yu et al., 1981), and CHDs were diagnosed in half of the 4q syndrome patients. The defects included pulmonary valve stenosis or atresia, patent ductus arteriosus, tetralogy of Fallot, tricuspid valve atresia, and coarctation of the aorta. (Keeling et al., 2001; Strahle and Middlemiss, 2007). Some of these may reflect *Hand2*'s function in the neural crest, as neural crest-specific ablation of *Hand2* causes similar loss of neural crest derivatives in the heart (Morikawa and Cserjesi, 2008) and the deletion of the pharyngeal arch-specific *Hand2* enhancer causes craniofacial defects (Yanagisawa et al., 2003). The overlapping phenotypes of *Hand2* SHF ablation in mice and 4q syndrome in humans suggests a potential role for *Hand2* in human CHDs, and points to the relevance of abnormal SHF progenitor cell development to human heart disease.

Putative downstream targets of Hand2 implicated in ventricular development

In an effort to examine gene expression defects in the SHF of *Hand2*$^{-/-}$ mice, we found that the expression of the zinc finger



B

Gene	Mouse Genotype		
	Hand2$^{+/+}$	*Hand2*$^{-/-}$	*Nkx2.5Cre:Hand2*$^{loxp/-}$
Gata4	1.000	0.398	0.791
Has2	1.000	0.315	0.787
Bmp5	1.000	0.208	0.849

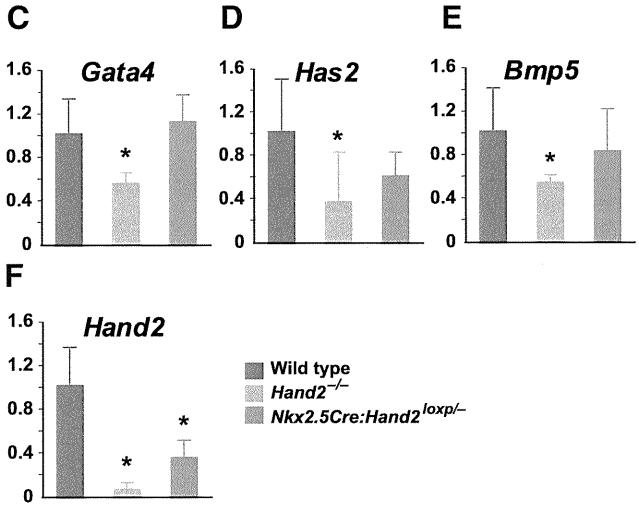


Fig. 6. mRNA microarray analysis of *Hand2* mutant hearts. (A) Microarray studies comparing gene expression profiles in wild-type versus *Hand2*$^{-/-}$ and *Nkx2.5Cre:Hand2*$^{loxp/-}$ mouse embryonic hearts. Heat map represents a portion of the genes downregulated in *Hand2*$^{-/-}$ and *Nkx2.5Cre:Hand2*$^{loxp/-}$, highlighting Gata4, Has2, and Bmp5 (arrowheads). Red-yellow gradient shows decrease in heat map value. (B) Results from mRNA expression microarray using wild-type, *Hand2*$^{-/-}$ and *Nkx2.5Cre:Hand2*$^{loxp/-}$ mouse embryonic hearts of selected cardiac development genes. Each number is a ratio of expression level compared with wild-type set to 1.0. (C–F) qPCR analyses were performed to validate the results of the microarray analysis for *Gata4* (C), *Has2* (D), *Bmp5* (E), and *Hand2* (F). Asterisks denote significant changes in gene expression ($p<.05$) compared with wild type.

transcription factor, *Gata4*, was reduced in the *Hand2*$^{-/-}$ hearts, but not in *Nkx2.5Cre:Hand2*$^{loxp/-}$. Interestingly, *Gata4* expression was also unchanged in mice containing a specific mutation in the *Hand2* DNA-binding domain, and these mice lived to a similar stage as the the *Nkx2.5Cre:Hand2*$^{loxp/-}$ mice (Liu et al., 2009). *Gata4* is normally expressed in the SHF and directly regulates *Hand2* during right ventricular development (McFadden et al., 2000), and *Gata4* conditional knockout mice demonstrate severe hypoplasia of the right ventricle with reduced expression of *Hand2* (Zeisberg et al., 2005). Thus, there may be a reinforcing loop between *Hand2* and *Gata4* that amplifies the expression of both genes early during SHF and subsequent right ventricular development.

In addition to *Gata4*, the expression of *Has2* and *Bmp5* was reduced in the *Hand2*$^{-/-}$ hearts compared to the wild type and *Nkx2.5Cre:Hand2*$^{loxp/-}$. *Has2* encodes hyaluronan synthase-2, which is essential for hyaluronic acid secretion into the extracellular matrix between the early endocardium and myocardium, known as the cardiac jelly (Camenisch et al., 2000; Mjaatvedt et al., 1998). The cardiac jelly mediates reciprocal signaling between the endocardium and myocardium that results in myocardial cell proliferation and maturation. Reduced expression of Has2 is associated with hypoplastic right ventricle (Mjaatvedt et al., 1998) and may also contribute to the abnormalities in *Hand2*$^{-/-}$ or *Islet1Cre:Hand2*$^{loxp/-}$ mice. However, it is also possible that the decrease in Has2 and other genes uniquely expressed in the right ventricle simply reflects the lack of right ventricular cells in the developing heart.

The bone morphogenic protein (BMP) family of signaling molecules mediates the simultaneous activation of Smad and Wnt signal transduction in the cardiogenic pathway, including induction of the earliest markers of cardiac differentiation, such as *Gata4*, *Nkx2.5*, and *Mef2c* (Pal and Khanna, 2006). In chick embryos, *Bmp5* is expressed in the outflow tract myocardium and may play a role in the process of the recruitment of cardiomyocytes to the outflow tract (Somi et al., 2004). Indeed, the loss of Bmp5 and Bmp7 resulted in outflow tract defects (Solloway and Robertson, 1999), suggesting that the reduced *Bmp5*-mediated signaling could contribute to the decreased numbers of SHF progenitors observed upon the reduction of *Hand2* dosage. It remains to be determined whether *Gata4*, *Has2* or *Bmp5* is directly or indirectly regulated by *Hand2*, but the regulation of Gata4 appears to be DNA binding-independent (Liu et al., 2009).

Conclusions

By establishing a conditional knockout allele of *Hand2* utilizing a variety of unique Cre-driver mouse lines, we have revealed some tissue-specific roles of the *Hand2* gene during the development of early cardiac progenitors. More importantly, the loss of *Hand2* in specific sub-domains of the SHF supports the idea that some forms of CHD might be a result of insufficient cells derived from discrete pools of progenitor cells. Our identification of genes that are specifically dysregulated when early SHF cells lack *Hand2* and are unaffected in the later knockout offers an opportunity to focus on pathways

uniquely important for the survival and expansion of cells populating the right ventricle. Further investigation of the function of these factors may contribute to the identification of the underlying causes of an array of CHDs affecting the human population.

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The Antiproliferative Action of Progesterone in Uterine Epithelium Is Mediated by Hand2

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fungi establish an intracellular life style and turned these rhizobia from free-living bacteria into nitrogen-fixing endosymbionts. However, although the endomycorrhizal symbiosis is widespread in the plant kingdom only very few plant lineages, namely legumes and *Parasponia*, have recruited this mechanism for the rhizobial nodule symbiosis. Studies on the constraints underlying this evolutionary event in *Parasponia* can provide insight into whether and how this nitrogen-fixing symbiosis can be transferred to other nonlegumes.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S16
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The Antiproliferative Action of Progesterone in Uterine Epithelium Is Mediated by Hand2

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During pregnancy, progesterone inhibits the growth-promoting actions of estrogen in the uterus. However, the mechanism for this is not clear. The attenuation of estrogen-mediated proliferation of the uterine epithelium by progesterone is a prerequisite for successful implantation. Our study reveals that progesterone-induced expression of the basic helix-loop-helix transcription factor Hand2 in the uterine stroma suppresses the production of several fibroblast growth factors (FGFs) that act as paracrine mediators of mitogenic effects of estrogen on the epithelium. In mouse uteri lacking Hand2, continued induction of these FGFs in the stroma maintains epithelial proliferation and stimulates estrogen-induced pathways, resulting in impaired implantation. Thus, Hand2 is a critical regulator of the uterine stromal-epithelial communication that directs proper steroid regulation conducive for the establishment of pregnancy.

A sequential and timely interplay of the steroid hormones 17β-estradiol (E) and progesterone (P) regulates critical uterine functions during the reproductive cycle and pregnancy (*1*–*3*). Whereas E drives uterine epithelial proliferation in cycling females, P counteracts E-induced endometrial hyperplasia. In mice, preovulatory ovarian E stimulates uterine epithelial growth and proliferation on days 1 and 2 of pregnancy (*1*). However, starting on day 3, P produced by the corpora lutea terminates E-mediated epithelial proliferation. In response to P, epithelial cells exit from the cell cycle and enter a differentiation pathway to acquire the receptive state that supports embryo implantation on day 4 of pregnancy (*4*–*6*). To identify the P-regulated pathways that underlie the implantation process, we had previously examined alterations in mouse uterine mRNA expression profiles in the periimplantation period in response to RU-486 (mifepristone), a well-characterized progesterone receptor (PR) antagonist (*7*). Our results identified *Hand2*, a critical regulator of morphogenesis in a variety of tissues (*8*, *9*), as a potential PR-regulated gene. Real-time polymerase chain reaction (PCR) confirmed that the expression of Hand2 mRNA was greatly reduced in the uteri of RU-486–treated mice (*10*) (fig. S1A). The expression of Hand2 protein, localized exclusively in the uterine stroma, was also abolished after RU-486 treatment (fig. S1B), which indicated that PR controls Hand2 expression in the mouse uterus during early pregnancy.

To further confirm P regulation of *Hand2*, ovaries were removed from nonpregnant mice, and then these animals were injected with either vehicle or P. We observed intense nuclear expression of Hand2 protein in uterine stromal cells after P treatment. Similar treatment of PR-null females showed no induction of Hand2 protein (Fig. 1A). These results established that P induces Hand2 expression in the uterine stroma. Consistent with its regulation by P, Hand2 expression was observed in the stromal cells underlying the luminal epithelium on days 3 and 4 of pregnancy (Fig. 1B).

To investigate the function of *Hand2* in the uterus, we created a conditional knockout of this gene in the adult uterine tissue. Crossing of mice harboring the "floxed" *Hand2* gene ($Hand2^{f/f}$) with PR-Cre mice (in which Cre recombinase was inserted into the PR gene) generated $Hand2^{d/d}$ mice in which the *Hand2* gene is deleted selectively in cells expressing PR. As shown in fig. S2, *Hand2* expression was successfully abrogated in uteri of $Hand2^{d/d}$ mice. A breeding study demonstrated that $Hand2^{d/d}$ females are infertile (table S1). An analysis of the ovulation and fertilization in $Hand2^{f/f}$ and $Hand2^{d/d}$ females revealed no significant difference in either the number or the morphology of the embryos recovered from their uteri (fig. S3, A and B). The serum levels of P and E were comparable in $Hand2^{f/f}$ and $Hand2^{d/d}$ females on day 4 of pregnancy, which indicated normal ovarian function (fig. S3, C and D).

We next examined embryo attachment to the uterine epithelium by using the blue dye assay, which assesses increased vascular permeability at implantation sites. $Hand2^{f/f}$ mice displayed distinct blue bands, indicative of implantation sites on day 5 of pregnancy (fig. S4). In contrast, none of the $Hand2^{d/d}$ females showed any sign of implantation. Implanted embryos with decidual swellings were also absent in $Hand2^{d/d}$ uteri on days 6 and 7 of pregnancy. Histological analysis of $Hand2^{f/f}$ females on day 5 of pregnancy

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showed, as expected, a close contact of embryonic trophectoderm with uterine luminal epithelium (Fig. 1C, a and b). In contrast, in *Hand2*$^{d/d}$ uteri, blastocysts remained unattached in the lumen (Fig. 1C, c and d). These results suggested that, in the absence of Hand2 expression in the stroma, the luminal epithelium fails to acquire competency for embryo implantation.

In mice, the window of uterine receptivity coincides with the P-mediated down-regulation of ER activity in uterine luminal epithelium (*5*, *6*). The levels of PR and estrogen receptor α (ERα) proteins in the luminal epithelium or stroma of *Hand2*$^{d/d}$ uteri were comparable to those of *Hand2*$^{f/f}$ controls (fig. S5). An examination of the phosphorylation of ERα at serine 118, indicative of its transcriptionally active state (*11*), revealed a sharp reduction of this modification in the luminal epithelial cells of *Hand2*$^{f/f}$ uteri on days 3 and 4 of pregnancy (fig. S6, a to d). In contrast, an increase in ERα phosphorylation was evident on these days in luminal epithelium of *Hand2*$^{d/d}$ uteri (fig. S6, e to h).

Consistent with this increase in ER transcriptional activity, expression of mRNAs corresponding to mucin 1 (*Muc-1*) and lactoferrin (*Lf*), well-characterized E-responsive genes in uterine epithelium (*12*), was significantly elevated in the *Hand2*-null uterus on day 4 of pregnancy (Fig. 2A). In contrast, the expression of *Ihh* (*13*), *Alox15* (*7*), and *Irg1* (*7*), which are known P-responsive genes in uterine epithelium, remained unaltered in *Hand2*$^{d/d}$ uteri (fig. S7). In addition, the mRNA levels of *Hoxa10*, a P-regulated stromal factor (*3*), and *Nr2f2*, a downstream target of *Ihh* in the uterine stroma (*13*), were unaffected in the uteri of *Hand2*$^{d/d}$ mice. However, the expression of leukemia inhibitory factor (*Lif*), a glandular factor that regulates uterine receptivity (*2*), was significantly reduced in *Hand2*$^{d/d}$ uteri (fig. S8).

Down-regulation of Muc-1 in the luminal epithelium is indicative of a receptive uterus (*12*). In contrast, persistent Muc-1 expression impairs acquisition of uterine receptivity and embryo implantation. On days 4 and 5 of gestation, a marked reduction in Muc-1 level was seen in uterine epithelia of *Hand2*$^{f/f}$ mice, consistent with the attainment of receptive status (fig. S9). However, Muc-1 expression persisted in uteri of *Hand2*$^{d/d}$ mice at the time of implantation. Thus, elevated epithelial ER signaling led to increased expression of Muc-1 and corresponded to disrupted uterine receptivity and implantation failure in *Hand2*$^{d/d}$ mice.

In normal pregnant uteri, the receptive state is also marked by a cessation in epithelial cell proliferation before implantation (*1*, *2*). As expected, in *Hand2*$^{f/f}$ mice, Ki-67, a cell proliferation marker, was undetectable in the uterine epithelium as it attains receptive status on day 4 of pregnancy (Fig. 2B, a). *Hand2*$^{d/d}$ uteri, on the other hand, exhibited robust Ki-67 expression in the luminal epithelium, which indicated sustained epithelial cell proliferation in the absence of *Hand2* (Fig. 2B, b).

The persistent proliferative state of uterine epithelium in the *Hand2*$^{d/d}$ mice raised the possibility that stromal expression of Hand2 mediates P action that opposes E-mediated epithelial proliferation. Administration of E to ovariectomized *Hand2*$^{f/f}$ and *Hand2*$^{d/d}$ mice led to robust uterine epithelial proliferation (Fig. 2C, a and b). Treatment with P alone induced proliferation exclusively in the uterine stromal cells of both genotypes (Fig. 2C, c and d). In *Hand2*$^{f/f}$ mice pretreated with P, administration of E showed no proliferative activity in the epithelium, which suggested a complete blockade of E-dependent proliferation by P. Under similar treatment conditions, *Hand2*$^{d/d}$ uteri exhibited a marked E-induced epithelial proliferation, indicating an absence of antiproliferative effects of P on the uterine epithelium (Fig. 2C, e and f). These results established Hand2 as a critical mediator of the actions of P in the stroma that inhibit E-dependent epithelial proliferation.

To identify downstream target(s) of Hand2 in the uterus, we performed gene expression profiling of uterine stromal cells isolated from *Hand2*$^{f/f}$ and *Hand2*$^{d/d}$ mice on day 4 of pregnancy. This study revealed elevated expression of mRNAs corresponding to several members of the fibroblast growth factor family (FGFs)—namely, *Fgf1*, *Fgf2*, *Fgf9*, and *Fgf18*—in uterine stroma of *Hand2*$^{d/d}$ mice. Real-time PCR confirmed the induction of *Fgf1*, *Fgf9*, *Fgf2*, and *Fgf18* mRNAs in uterine stromal cells of *Hand2*$^{d/d}$ mice (Fig. 3A). The expression of *Hbegf* mRNA, encoding the heparin-binding epidermal growth factor (HB-EGF), was also increased in the mutant uteri (fig. S10). In contrast, the expression of mRNAs of several other growth factor genes was either unaffected or slightly reduced in the *Hand2*-null uteri (fig. S10). We also observed that the uterine expression of *Fgf2*, *Fgf9*, and *Fgf18* progressively declined with the rise of *Hand2* expression as pregnancy advanced from day 1 to day 4 (fig. S11).

FGFs exert their paracrine responses through the cell surface FGF receptors (FGFRs) and a

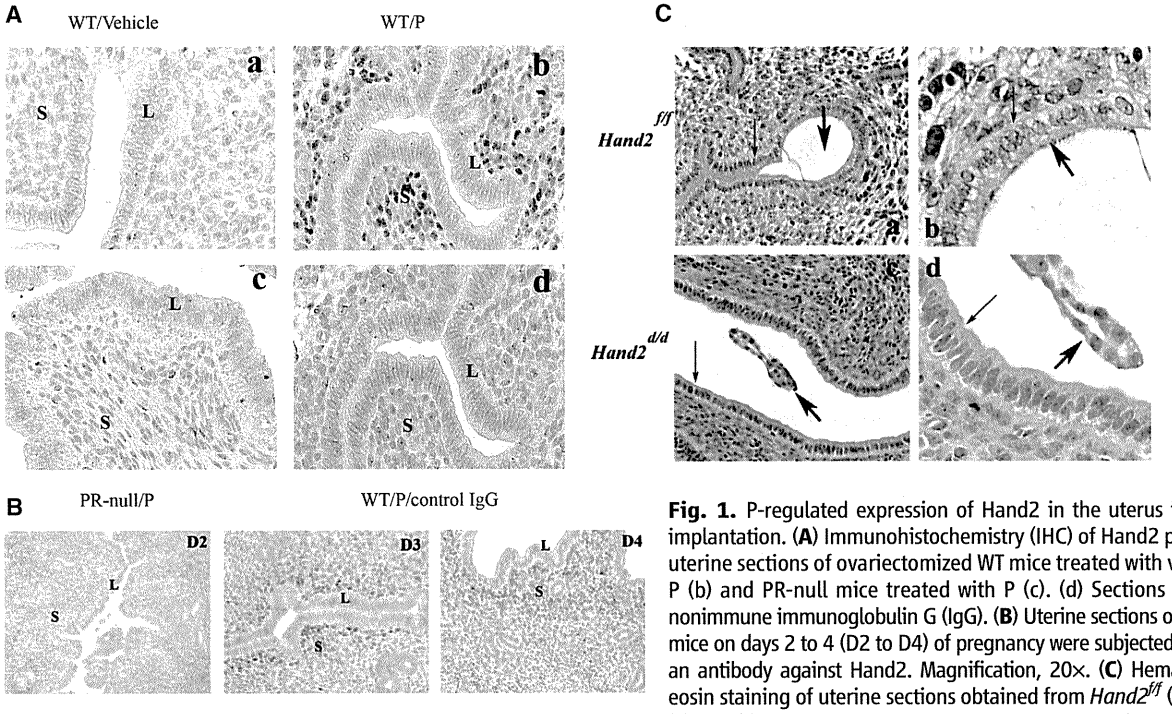

Fig. 1. P-regulated expression of Hand2 in the uterus is critical for implantation. (**A**) Immunohistochemistry (IHC) of Hand2 protein in the uterine sections of ovariectomized WT mice treated with vehicle (a) or P (b) and PR-null mice treated with P (c). (d) Sections treated with nonimmune immunoglobulin G (IgG). (**B**) Uterine sections obtained from mice on days 2 to 4 (D2 to D4) of pregnancy were subjected to IHC using an antibody against Hand2. Magnification, 20×. (**C**) Hematoxylin-and-eosin staining of uterine sections obtained from *Hand2*$^{f/f}$ (a and b) and *Hand2*$^{d/d}$ (c and d) mice on day 5 (n = 6) of pregnancy. (b) and (d) Magnified images of (a) and (c), respectively. Wide and narrow arrows point to embryo and luminal epithelium. L and S represent luminal epithelium and stroma, respectively.