

# Ex vivo whole-embryo culture of caspase-8-deficient embryos normalize their aberrant phenotypes in the developing neural tube and heart

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## Abstract

**Caspase-8 plays the role of initiator in the caspase cascade and is a key molecule in death receptor-induced apoptotic pathways. To investigate the physiological roles of caspase-8 in vivo, we have generated caspase-8-deficient mice by gene targeting. The first signs of abnormality in homozygous mutant embryos were observed in extraembryonic tissue, the yolk sac. By embryonic day (E) 10.5, the yolk sac vasculature had begun to form inappropriately, and subsequently the mutant embryos displayed a variety of defects in the developing heart and neural tube. As a result, all mutant embryos died at E11.5. Importantly, homozygous mutant neural and heart defects were rescued by ex vivo whole-embryo culture during E10.5–E11.5, suggesting that these defects are most likely secondary to a lack of physiological caspase-8 activity. Taken together, these results suggest that caspase-8 is indispensable for embryonic development.**

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**Keywords:** caspase-8; apoptosis; hemorrhage; cardiac rupture; angiogenesis; whole-embryo culture

**Abbreviations:** ES, embryonic stem; FADD, Fas-associated death domain protein; MEF, mouse embryonic fibroblasts; PBS, phosphate-buffered saline; PECAM-1, platelet endothelial cell adhesion molecule-1; PFA, paraformaldehyde; RT-PCR, reverse-transcriptase and polymerase chain reaction; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling

## Introduction

Apoptosis, or programmed cell death, is the principal mechanism utilized by multicellular organisms to orchestrate tissue morphogenesis during development and maintain homeostasis in adulthood.<sup>1–3</sup> Apoptosis also occurs during defense processes and immune responses.<sup>4</sup> Studies on signal transduction mechanisms in apoptosis identified several molecules which effect and regulate this process. Of these molecules, caspases, a family of cysteine proteases, have been shown to act as the executioners of apoptosis.<sup>5</sup> In mammals, fourteen caspases have been identified, most of which are activated during apoptosis. Among these, caspase-8 (also known as FLICE/MACH/Mch5) is an initiator caspase with a long prodomain, termed a 'death effector domain' (DED), which activates downstream effector caspases (e.g. caspase-3, -6 and -7).<sup>6,7</sup> It has been shown that caspase-8 is essential for Fas-mediated apoptotic signaling.<sup>8,9</sup> Ligation of Fas results in the recruitment of caspase-8 via interaction with the adapter molecule FADD (Fas-associated death domain protein, also called MORT1). Recruitment of caspase-8 to the complex initiates autoprocessing of procaspase-8 to an active form which is released into the cytoplasm.<sup>10</sup> Activated caspase-8 cleaves and activates downstream caspases, thereby committing the cell to apoptosis. Caspase-8 also activates another apoptotic pathway, which, through processing of Bid, mediates release of cytochrome *c* from mitochondria and activation of caspase-9.<sup>11</sup> And, in addition to Fas, caspase-8 is involved in apoptotic signaling via other death receptors, such as tumor necrosis factor receptor type 1 (TNFR1), death receptor 3 (DR3) and TNF-related apoptosis-inducing ligand (TRAIL) receptors.<sup>12–15</sup> Clearly, caspase-8 is an important mediator for a wide range of apoptotic pathways.

During embryogenesis, apoptosis is utilized ubiquitously to eliminate unwanted or excess cells in the organism. Recently, the targeted disruption of several genes involved in apoptosis has been performed.<sup>16,17</sup> Among those analyzed, it has been shown that caspase-3, caspase-9 and Apaf-1 are involved in brain development during late embryogenesis.<sup>18–22</sup> Targeted disruption of mouse FADD results in developmental heart abnormalities and embryonic lethality.<sup>23,24</sup> Furthermore, caspase-8-deficient mice display impaired heart muscle development and accumulation of

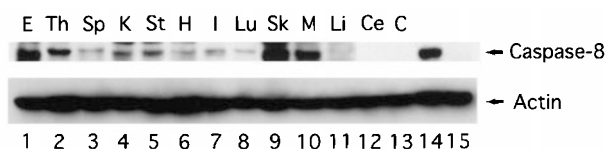
erythrocytes in the liver.<sup>12</sup> More recently, it has been reported that Casper (also called c-FLIP), which negatively regulates Fas-mediated apoptosis by interacting with FADD or/and caspase-8, is also involved in heart development during embryogenesis.<sup>25</sup> Taken together, evidence from FADD-, Casper- and caspase-8-deficient mice suggests that these molecules function in a Fas-independent manner during embryogenesis.

To investigate the biological function of caspase-8 *in vivo*, we generated mutant mice devoid of proteolytic function by targeted disruption of the protease domain using homologous recombination. Like the previous report on this knockout model,<sup>12</sup> we found that protease-deficient caspase-8 mutants die *in utero* and exhibit cardiac rupture. Moreover, we report the new finding of several additional embryonic and extraembryonic defects which arise during embryogenesis. We also report that our caspase-8 mutant embryos could be rescued by *ex vivo* whole-embryo culture, a finding which could help point to an explanation of the defects displayed by our mice. The present study suggests first the direct involvement of caspase-8 in early mouse embryonic development, particularly in organogenesis.

## Results

### Embryonic expression of caspase-8

In a previous study, we demonstrated that caspase-8 is expressed in adult tissues as well as in embryos at 9.5 days (E9.5) and 17 days (E17) of development by Northern blot analysis.<sup>26</sup> To confirm these findings, we further analyzed caspase-8 expression in embryos at the protein level (Figure 1). We prepared cell lysates from E8.5 embryos and from various tissues of E17 embryos and analyzed them by immunoblotting with an anti-mouse caspase-8 monoclonal antibody. As a positive control, we used cell lysates from JB-6 stable transfectants expressing mouse caspase-8. We detected a 55-kDa protein in JB-6 transfectants, but not in parental JB-6 cells lacking endogenous caspase-8 (Figure 1, lanes 14 and 15). Similarly, we observed a strong band of the same size in E8.5 embryos and in the thymus, skin and muscle of E17



**Figure 1** Immunoblot analysis of caspase-8 in mouse embryos. Approximately 70  $\mu$ g of cell lysates from E8.5 embryos (lane 1) and from various E17 embryonic tissues (lanes 2–13) were analyzed by SDS–PAGE followed by immunoblotting with anti-mouse caspase-8 antibody. Cell lysates from JB-6 transfectants expressing mouse caspase-8 and from parental JB-6 cells were also analyzed (lanes 14 and 15). Blots were also probed with anti-actin antibody as a standard control. Abbreviations: E, E8.5 embryo; Th, thymus; Sp, spleen; K, kidney; St, stomach; H, heart; I, intestine; Lu, lung; Sk, skin; M, muscle; Li, liver; Ce, cerebrum; C, cerebellum

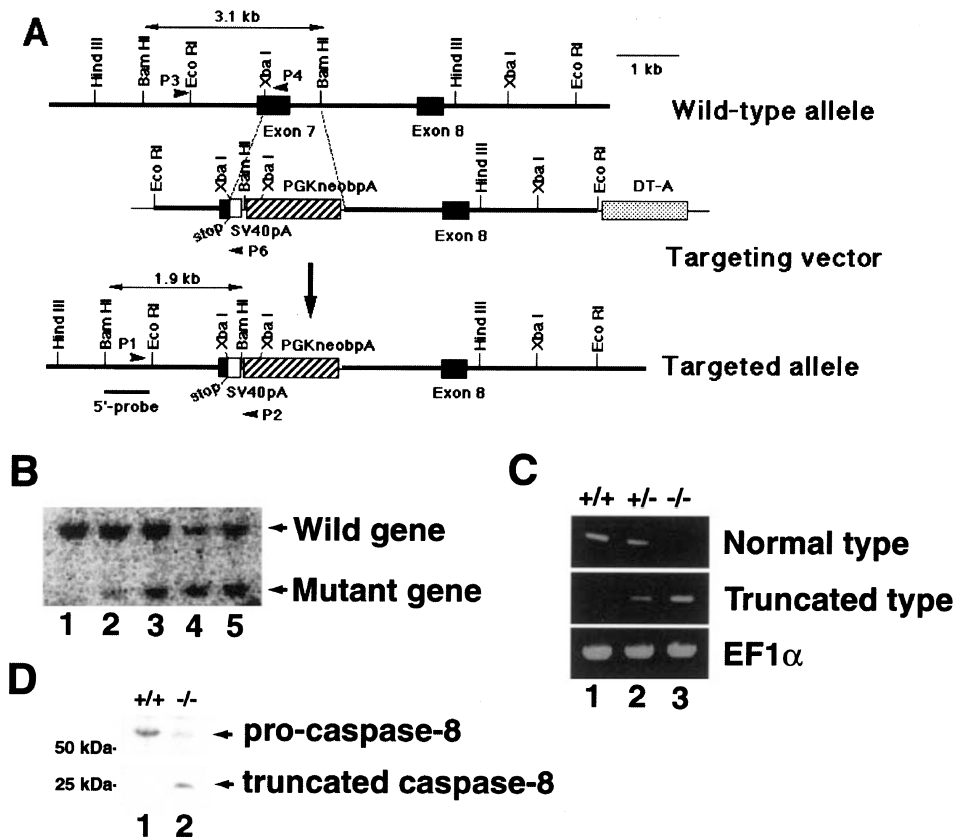
embryos (Figure 1, lanes 1, 2, 9 and 10). A faint band was observed in cell lysates from the spleen, kidney, stomach, heart, intestine and lung (lanes 3–8). Caspase-8 could not be detected in the liver and brain (Figure 1, lanes 11–13). Taken together, these data strongly suggest that caspase-8 is expressed in several tissues during mouse embryogenesis, at least between E8.5 and E17. To help determine the role played by caspase-8 during this period, we created caspase-8 knockout mice by targeted disruption.

### Targeted disruption of the mouse caspase-8 gene and generation of caspase-8 knockout mice

Caspase-10 (also known as FLICE2/Mch4), a caspase-8 homologue, plays a similar role to caspase-8 in Fas-mediated apoptotic signaling in humans,<sup>27</sup> and it was thought that caspase-10 might compensate for the lack of caspase-8 activity in caspase-8-null mice. We speculated that a truncated caspase-8 protein lacking the protease domain might act as a dominant-negative molecule and inhibit the function of caspase-10. To generate this caspase-8 mutant, we designed a targeting vector by which a stop codon and SV40 polyadenylation sequence would be inserted into exon 7 of the caspase-8 gene (Figure 2A). After transfection of the targeting vector and selection with G418, four homologous recombinant embryonic stem (ES) clones were isolated (Figure 2B), two of which were used to produce chimeric mice. Finally, we established independent lines of caspase-8 knockout mice from six chimerae and examined their progenies.

### Embryonic lethality of homozygous caspase-8-deficient mice

The established heterozygous mutants displayed normal phenotype and retained propagative activity. However, no homozygous mutants were observed among live pups genotyped from heterozygous mating, indicating that homozygous mutants are embryonic lethal. Therefore, we examined embryos of various stages (Table 1), and found that until E10.5, homozygous mutant embryos were detected in the expected Mendelian ratio (25%). However, all homozygous mutants obtained at later timepoints (E11.5 to E14.5) died accompanying with abnormal phenotypes. Most homozygous mutant embryos displayed signs of abdominal hemorrhage (described below in detail). This phenotype, observed in all of our mutant lines, was similar to that of caspase-8 null-mutant mice.<sup>12</sup> However, by reverse-transcriptase and polymerase chain reaction (RT–PCR) analysis of embryonic transcripts, we were able to detect truncated caspase-8 mRNA transcribed from the mutant allele (Figure 2C). Furthermore, by immunoblot analysis with an anti-caspase-8 antibody, a 55-kDa pro-caspase-8 was detected in wild-type mouse embryonic fibroblasts (MEFs), but not in homozygous mutant MEFs, whereas a peptide at 25 kDa corresponding to the DED domains of caspase-8 was observed in mutant MEFs (Figure 2D). These results suggest that our mice exhibit protease-deficient mutation, but not null-mutation, of the caspase-8 gene. We designate our homozygous mutant mice as *caspase-8*<sup>DED/DED</sup>.



**Figure 2** Targeted disruption of the mouse caspase-8 gene by homologous recombination. **(A)** Targeting strategy for homologous recombination. Exons of the caspase-8 gene are depicted as closed boxes. The SV40 polyA sequence, the PGKneobpA cassette and the diphtheria toxin A fragment gene (DT-A) cassette are indicated by open, hatched and dotted boxes, respectively. Primers (P1, P2, P3, P4 and P6) used for screening and RT-PCR are shown by arrowheads. A 5'-probe for Southern hybridization and DNA fragments expected after *Bam*HI digestion are indicated by a bold line and arrows, respectively. **(B)** Southern blot analysis of ES cell clones. Genomic DNAs isolated from parental cells (lane 1) and ES clones (lanes 2–5) were digested with *Bam*HI, resolved by electrophoresis and hybridized with a 5'-probe. The 3.1 kb and 1.9 kb DNA bands correspond to the wild-type and mutant genes, respectively. **(C)** RT-PCR analysis of caspase-8-deficient embryos. Total RNAs from E10.5 embryos obtained by crossing heterozygotic mice were amplified as described in Materials and Methods. PCR products derived from wild-type embryo (lane 1) and heterozygous (lane 2) and homozygous (lane 3) mutant embryos were analyzed by electrophoresis for detection of normal (top) and truncated (middle) caspase-8 transcripts. Primers for mouse EF1 $\alpha$  transcripts were used as a standard control (bottom). **(D)** Immunoblot analysis of MEFs. Cell lysates of wild-type (+/+) and homozygous caspase-8 mutant (-/-) MEF cells were analyzed by immunoblotting with anti-mouse caspase-8 monoclonal antibody specific to the DED domain of caspase-8. Size standards are shown on the left side

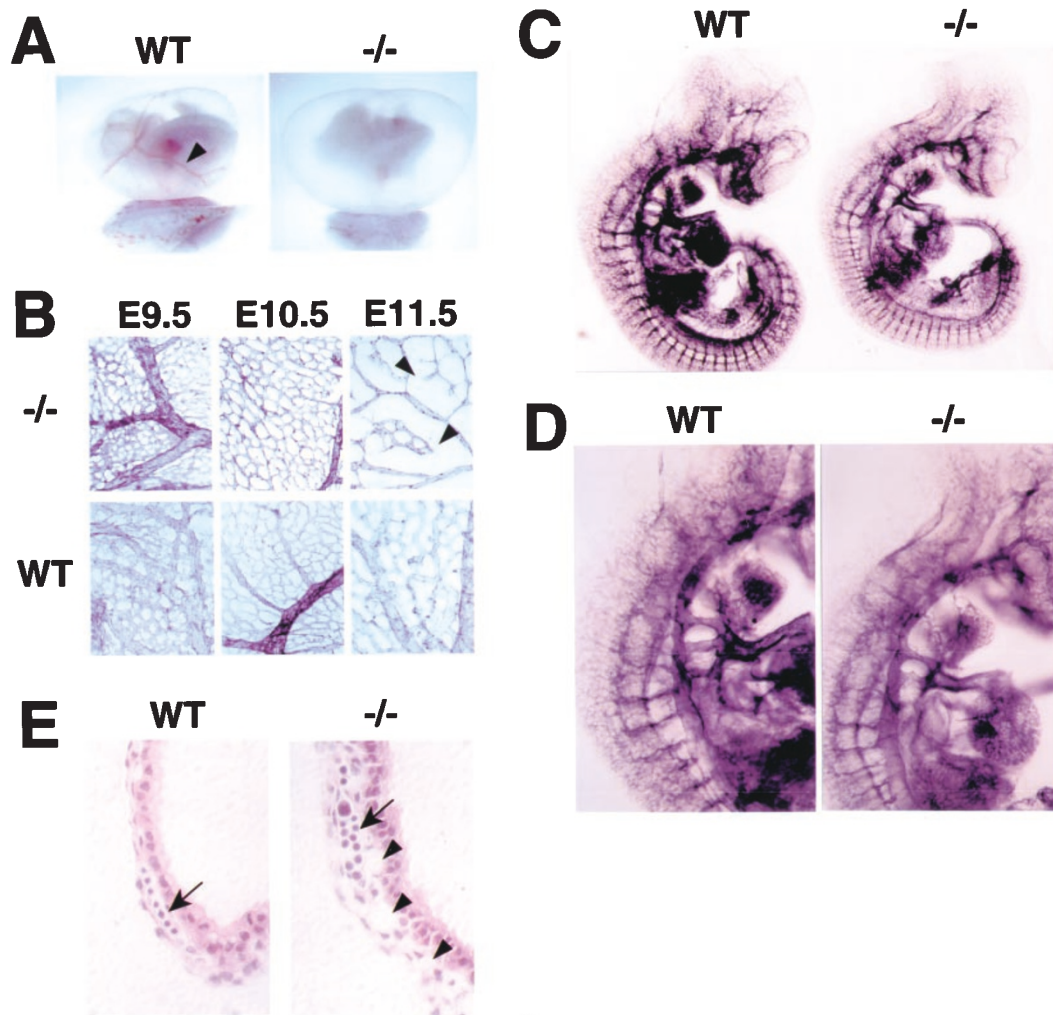
**Table 1** Genotyping of mice derived from caspase-8 heterozygous intercrosses

Age (days)	No. of genotypes (%)			No. of abnormal homozygotes <sup>a</sup> (%)
	+/+	+/-	-/-	
P21-28	22 (30)	51 (70)	0 (0)	
E14.5-	48 (36)	71 (53)	15 (11)	15/15 (100)
E12.5	18 (32)	31 (58)	6 (10)	6/6 (100)
E11.5	48 (27)	100 (60)	24 (13)	24/24 (100)
E10.5	72 (33)	87 (42)	54 (25)	3/54 (6)
E9.5	16 (34)	21 (46)	9 (20)	0/9 (0)
E8.5	6 (37)	8 (50)	2 (13)	0/2 (0)

The genotype of mice at indicated postnatal (P) or embryonic (E) days were determined by PCR analysis. <sup>a</sup>Morphology of the homozygous mutants were judged under the microscope.

### Extraembryonic defects in *caspase-8*<sup>DED/DED</sup> mutant embryos

Abnormal development of *caspase-8*<sup>DED/DED</sup> mutant mice was first observed in the extraembryonic tissue, the yolk sac. The yolk sac of the E11.5 *caspase-8*<sup>DED/DED</sup> mouse appears pale and displays underdeveloped vasculature (Figure 3A). To analyze these defects, we examined the yolk sac vascular endothelium using an antibody specific for platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) (Figure 3B). In wild-type yolk sacs, the vascular network was pruned and remodeled to form a branched and intricate tree-like network between E9.5 and E11.5. In contrast, *caspase-8*<sup>DED/DED</sup> yolk sacs still exhibited the honeycomb-like primary capillary plexus at E10.5. Eventually, regression of vessels was observed in the E11.5 mutant yolk sac. Upon histological



**Figure 3** Yolk sac defects in caspase-8-deficient embryos. **(A)** Morphology of yolk sacs from E11.5 wild-type (left) and *caspase-8*<sup>DED/DED</sup> (right) embryos. Large vitelline blood vessels (shown by an arrowhead) are clearly observed in the wild-type, but not the *caspase-8*<sup>DED/DED</sup> yolk sac. **(B)** Whole-mount immunohistochemistry of yolk sacs dissected from wild-type (bottom) and *caspase-8*<sup>DED/DED</sup> (top) embryos. Yolk sacs prepared at E9.5 (left), E10.5 (middle) and E11.5 (right) were immunostained with an anti-PECAM-1 antibody. Regressive vessels are indicated by arrowheads. **(C and D)** Whole-mount immunostaining of E10.5 wild-type (left) and *caspase-8*<sup>DED/DED</sup> (right) embryos. Embryos proper were immunostained with anti-PECAM-1 antibody as described in Materials and Methods. **(E)** Histological analysis of yolk sacs dissected from wild-type (left) and *caspase-8*<sup>DED/DED</sup> (right) embryos at E11.5. Yolk sac sections were stained with hematoxylin and eosin. A vessel containing blood cells and a vessel lacking blood cells are shown by an arrow and an arrowhead, respectively. Blood cells were detected in only a few vessels of each E11.5 mutant yolk sac. Abbreviations: WT, wild-type; *-/-*, *caspase-8*<sup>DED/DED</sup>

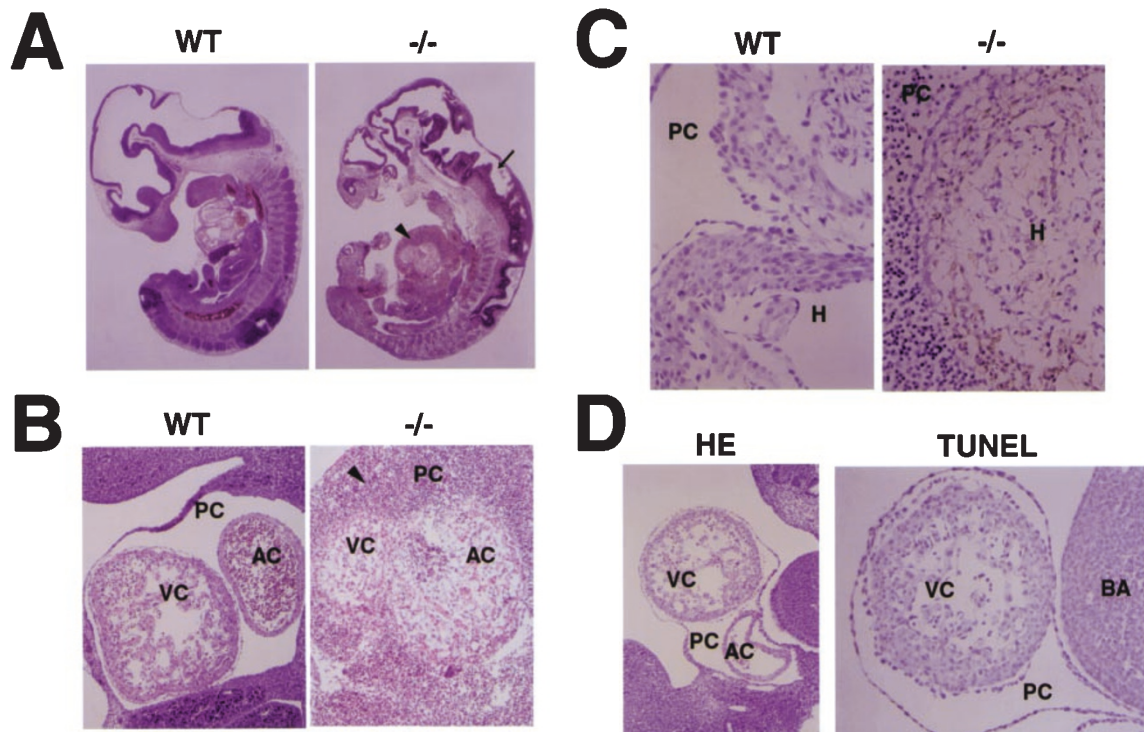
analysis of the vasculature, it was readily apparent that E11.5 mutant vessels contained far fewer blood cells than vessels of wild-type yolk sacs (Figure 3E). These results indicate that caspase-8 is necessary for normal maturation of the yolk sac primary capillary plexus into the more mature tree-like hierarchy of vessels, and probably for development of blood cells.

On the other hand, whole-mount immunostaining of E10.5 embryos with anti-PECAM-1 antibody revealed no differences between the vascular systems of wild-type and *caspase-8*<sup>DED/DED</sup> embryos proper (Figure 3C, D). A similar branched network of blood vessels was observed in both wild-type and mutant embryos. These results indicate that

caspase-8 activation is required for angiogenesis only in the extraembryonic yolk sac.

### Cardiac rupture in *caspase-8*<sup>DED/DED</sup> mutant embryos

To learn more about the defects in *caspase-8*<sup>DED/DED</sup> embryos associated with abdominal hemorrhage, detailed histological analyses were performed on E10.5–11.5 embryos. As shown in Figure 4A and B, we confirmed that the abdominal hemorrhage was the result of an efflux of blood cells into the pericardial cavity, and that the main muscular framework of the heart had undergone lysis. By terminal



**Figure 4** Histological analysis of heart tissue from caspase-8-deficient mice. (**A** and **B**) Hematoxylin and eosin (HE) staining of sagittal sections of E11.5 wild-type (left) and *caspase-8<sup>DED/DED</sup>* (right) embryos. Hemorrhage is shown by arrowheads in photographs of whole body (**A**) and pericardial cavity and heart (**B**). The arrow shows the neural tube, which developed irregularly, in the *caspase-8<sup>DED/DED</sup>* embryo. (**C**) TUNEL staining of hearts from E11.5 wild-type (left) and *caspase-8<sup>DED/DED</sup>* (right) embryos. TUNEL-positive cells (colored brown) are apoptotic. (**D**) HE and TUNEL staining of the E10.5 *caspase-8<sup>DED/DED</sup>* embryo. Sagittal sections were stained with hematoxylin and eosin (left) and analyzed by TUNEL staining (right). No TUNEL-positive cells were observed in the *caspase-8<sup>DED/DED</sup>* heart. Abbreviations: WT, wild-type;  $-/-$ , *caspase-8<sup>DED/DED</sup>*; H, heart; PC, pericardial cavity; AC, atrial chamber; VC, ventricular chamber; BA, branchial arch

deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) staining of *caspase-8<sup>DED/DED</sup>* E11.5 embryos, we found that the lytic change was due to apoptosis of cardiomyocytes (Figure 4C). Interestingly, neither cardiac rupture nor cardiomyocyte apoptosis was observed at E10.5 (Figure 4D). In addition, no apoptosis was observed in E11.5 *caspase-8<sup>DED/DED</sup>* embryos in tissues other than the heart (data not shown). Thus, it appears that this malformation event occurs during heart development between E10.5 and E11.5, subsequent to the appearance of yolk sac defects. We assume that cardiac rupture secondary to cardiomyocyte apoptosis is the major reason for embryonic lethality in caspase-8-deficient mice.

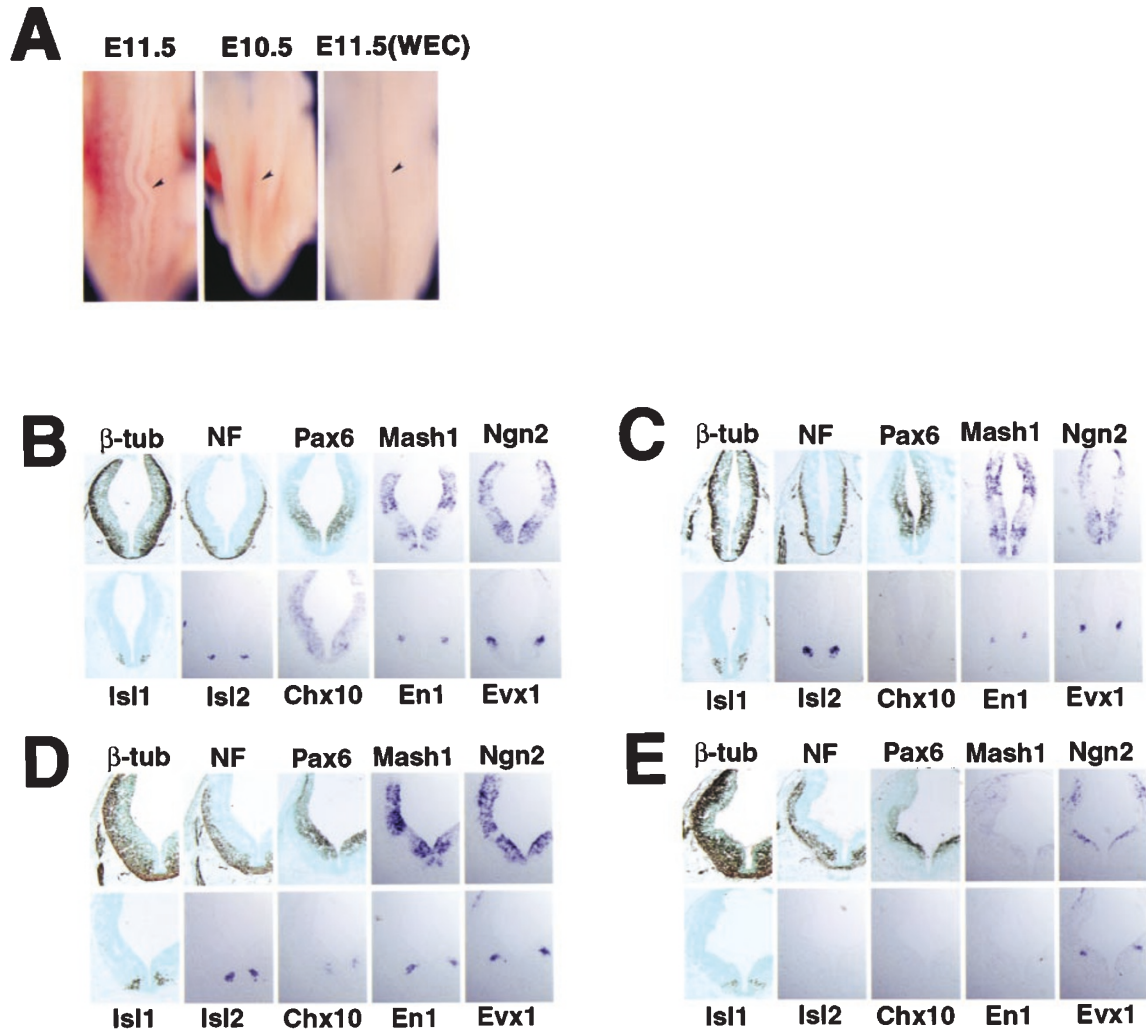
### Neural tube defects in *caspase-8<sup>DED/DED</sup>* mutant embryos

Another aberrant phenotype observed in the mutants was a defect in the neural tube. As shown in Figure 5A, the neural tubes of E11.5 *caspase-8<sup>DED/DED</sup>* embryos displayed a kink-like irregular pattern as compared with E10.5 embryos. These observations were confirmed by histological analysis (Figure 4). To further analyze the neural tube defect, we examined the expression of neurogenic and neuronal markers by *in situ* hybridization and immunohistochemical analyses (Figure

5B–E). Although *caspase-8<sup>DED/DED</sup>* and wild-type embryos exhibited similar expression patterns at E10.5, the former exhibited altered patterns by E11.5. The ventricular zone became narrower in the mutant, and expression of neurogenic cell markers in that region (Pax6, Mash1 and Neurogenin-2) was diminished relative to the wild-type. In addition, though some neuronal proteins ( $\beta$ -tubulin, neurofilament, and Islet-1) remained at wild-type levels, transcripts of other neuronal markers (Islet-2, Chox-10, En1, and Evx1) disappeared or were dramatically reduced in the mutant neural tube. Surprisingly, we were unable to detect massive apoptotic cells in the neural tube of mutant embryos (data not shown), in contrast with their prominence in the heart (Figure 4C). Based on these observations, we conclude that loss of function of caspase-8 affects neural tube development between E10.5 and E11.5.

### Rescue of *caspase-8<sup>DED/DED</sup>* mutant phenotypes in whole-embryo culture

It is difficult to distinguish whether the abnormal cardiomyocyte apoptosis is a direct result of caspase-8-deficiency or a secondary effect of cells lacking the physiological action of caspase-8. To understand the effect of the caspase-8-deficiency in the heart, we performed whole-embryo culture

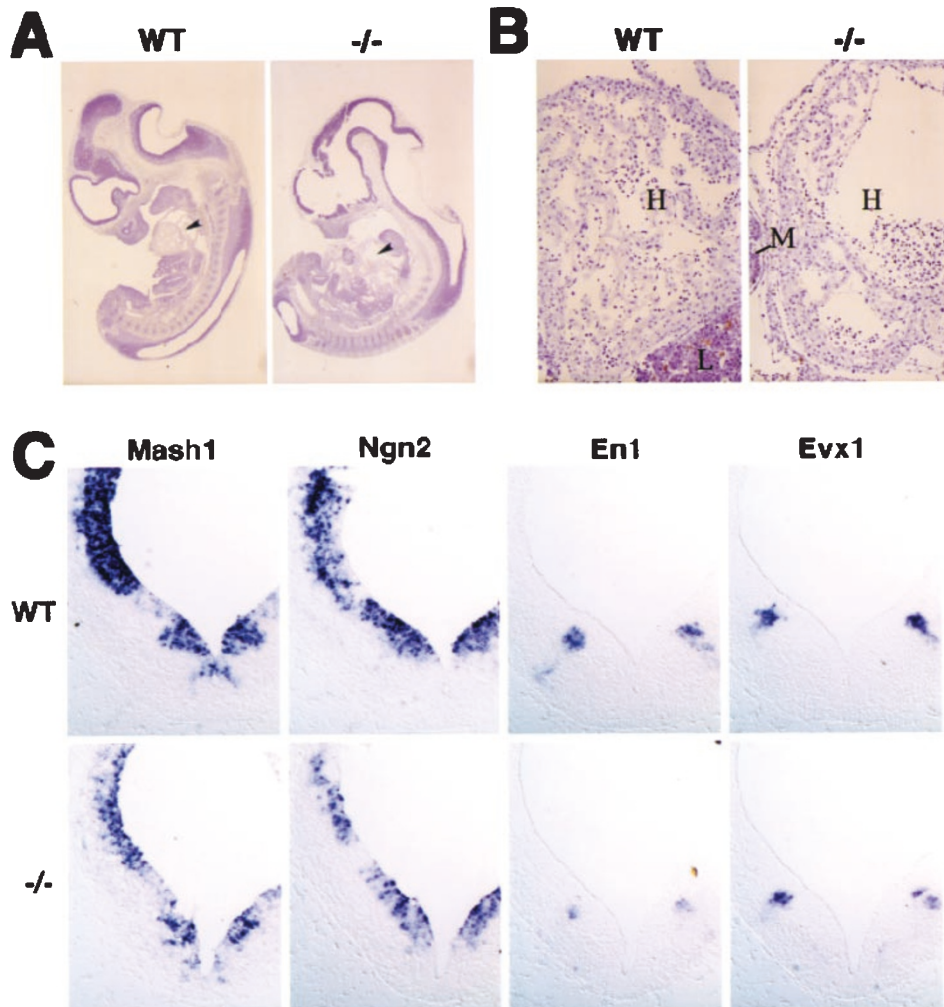


**Figure 5** Morphological and neuron-specific gene expression analyses of the caspase-8-deficient neural tube. **(A)** Morphology of the neural tubes in *caspase-8<sup>DED/DED</sup>* embryos. Neural tube kinks were observed in the embryo dissected at E11.5 (left), but not at E10.5 (middle) or in E11.5 embryo after whole-embryo culture (WEC) (right). Neural tubes are indicated by arrowheads. **(B–E)** Immunohistochemistry and *in situ* hybridization of neural tubes from wild-type **(B and D)** and *caspase-8<sup>DED/DED</sup>* **(C and E)** embryos. Transverse sections of hindbrain neural tube prepared at E10.5 **(B and C)** and E11.5 **(D and E)** were examined with antibodies against  $\beta$ -tubulin ( $\beta$ -tub), neurofilament (NF), Pax6 and Islet-1 (Isl1) and with RNA probes for Islet-2 (Isl2), Chox-10 (Chx10), En1, Evx1, Mash1 and Neurogenin-2 (Ngn2) as described in Materials and Methods

of *caspase-8<sup>DED/DED</sup>* mutant embryos and their littermates during E10.5–E11.5. It has been shown that this whole-embryo culture system mimics the *in vivo* situation during E8–E12.<sup>28</sup> If caspase-8-deficiency acts in a cell-autonomous manner in cardiomyocytes, the mutant phenotype would be reproducible by *ex vivo* culture. Unexpectedly, cardiac rupture was no longer observed in *caspase-8<sup>DED/DED</sup>* embryos after 24 hours cultivation, which grew as well as wild-type embryos from 34–38 somites to 45–50 somites comparable to E11.5 normal embryos *in vivo* (Figure 6A). Histological analysis of cultured *caspase-8<sup>DED/DED</sup>* embryos also showed normal heart development (Figure 6B). Myocardial trabeculation developed normally in *caspase-8<sup>DED/DED</sup>* hearts during culture. In addition, apoptotic myocytes were not observed in the heart by TUNEL staining, although a few apoptotic cells

were detected in other tissues including the midgut. These results indicate that caspase-8 is not directly involved in myocyte survival by itself, and we emphasize that the heart defect occurs as a secondary result.

Interestingly, whole-embryo culture of *caspase-8<sup>DED/DED</sup>* embryos also resulted in normalization of neural tube formation, as neural tube kinks disappeared upon whole-embryo culture (Figure 5A). Normal development of the neural tube of the *caspase-8<sup>DED/DED</sup>* embryo was confirmed by histological observation (Figure 6A). Furthermore, no differences were detectable in the expression patterns of neurogenic and neuronal markers between wild-type and *caspase-8<sup>DED/DED</sup>* embryos by *in situ* hybridization analysis (Figure 6C). Expression of both neurogenic (Mash1 and Neurogenin-2) and neuronal (En1 and Evx1) markers was



**Figure 6** Recovery of caspase-8-deficient embryos by *ex vivo* whole-embryo culture. (A) Histology of wild-type (left) and *caspase-8<sup>DED/DED</sup>* (right) embryos cultured during E10.5–E11.5. Sagittal sections of embryos (45–50 somites) grown up from E10.5 embryos (34–38 somites) during culture were stained with hematoxylin and eosin. The hearts are indicated by arrowheads. (B) TUNEL staining of hearts from wild-type (left) and *caspase-8<sup>DED/DED</sup>* (right) embryos after whole-embryo culture. TUNEL-positive cells (colored brown) were not detected in the heart, but were detected in other tissues such as liver and midgut of both wild-type and *caspase-8<sup>DED/DED</sup>* embryos. (C) *In situ* hybridization of neural tubes from wild-type (top) and *caspase-8<sup>DED/DED</sup>* (bottom) embryos after culture. Transverse sections of hindbrain tube were examined with RNA probes for Mash1, Neurogenin-2 (Ngn2), En1 and Evx1. Abbreviations: WT, wild-type; -/-, *caspase-8<sup>DED/DED</sup>*; H, heart; L, liver; M, midgut

detected in mutant as well as wild-type embryos. Thus, both major defects could be rescued by whole-embryo culture.

## Discussion

In this study, we generated caspase-8-deficient mice devoid of proteolytic function, but expressing the DED domains of caspase-8, by targeted disruption using homologous recombination. Homozygous *caspase-8<sup>DED/DED</sup>* mice display an inappropriate vascular system formed in the yolk sac and defects in the developing neural tube as well as heart and die at E11.5, clearly indicating for the first time that protease activity of caspase-8 is necessary for angiogenesis in the yolk sac and organogenesis during embryonic development.

Importantly, these mutant phenotypes can be rescued by *ex vivo* whole-embryo culture. No cardiac rupture or neural tube kinks occurred during culture (Figure 5A and 6), clearly indicating that these abnormalities may not all represent direct effects of caspase-8 deficiency. This evidence contradicts a previous report describing the direct involvement of caspase-8, its adapter FADD and the inhibitor molecule Casper in cell growth and survival of the developing heart.<sup>25</sup> In our current study, however, caspase-8 protease activity, which is required for induction of apoptosis in a variety of cells, was found to be absolutely necessary for tissue formation during embryonic development. Moreover, we found that homozygous caspase-8-deficient ES cells are able to differentiate into cardiomyocytes *in vitro*, suggesting no requirement of caspase-8 for

cell growth and differentiation (data not shown). One possible explanation for the aberrant phenotypes of the mutant mice is that a factor or factors released from cells which survive inappropriately due to failure of caspase-8-mediated apoptosis are responsible for the irregular development of the heart and neural tube as a secondary effect. During whole-embryo culture, these harmful factors could be diluted in the culture medium. Therefore, we hypothesize that caspase-8 and FADD transmit a death signal used to eliminate unwanted cells and that Casper antagonizes this signal in order to effect the survival of cells required for heart development. The whole-embryo culture system will be a valuable tool for assessing this hypothesis and understanding the physiological significance of caspase-8-mediated apoptosis during development, as well as the causal relations of defects observed in our protease-deficient caspase-8 mutant mice.

During development, the process of pruning is essential for the maturation of the vascular system. The term is used to describe the loss of excess endothelial cells generated during vasculogenesis, resulting in a tree-like pattern of vasculature typical of normal angiogenesis.<sup>29</sup> In this study, we have shown that this remodeling process is defective in the yolk sacs of caspase-8-deficient mice (Figure 3). Interestingly, this process was normal in the embryo proper. In addition, we confirmed by immunohistochemical analysis that vessels connecting the embryo proper and the yolk sac are normally developed and contain blood cells in *caspase-8<sup>DED/DED</sup>* embryos (data not shown). Therefore, it seems that caspase-8 is essential for extraembryonic vascularization. By RT-PCR analysis, we were able to detect caspase-8 transcripts in the yolk sacs of wild-type embryos isolated at E10.5 and E11.5 (data not shown). Furthermore, it has been shown that programmed capillary regression is accompanied by apoptosis.<sup>30</sup> These pieces of evidence suggest that pruning may be associated with caspase-dependent apoptosis and that caspase-8 is involved in this process.

Notch is an important molecule for controlling signal transduction and cell fate in vertebrate and invertebrate development.<sup>31</sup> Deficiency of Notch signaling influences organogenesis of many tissues. Interestingly, among the many defects resulting from disruption of the Notch signaling system, abnormalities of the heart, neural tube and yolk sac were observed which remarkably resemble those of our caspase-8-deficient mice.<sup>32–34</sup> This would suggest a potential connection between caspase-8 and the Notch signaling system. Further analysis, including an examination of the downstream molecules of the Notch signaling pathway in caspase-8-deficient mice, will be required in order to verify this potential connection.

We have demonstrated that caspase-8 is essential during mid-stage mouse embryonic development. However, the fact that caspase-8 was clearly observed in the thymus, skin and muscle of the wild-type E17 embryo (Figure 1) suggests that it may play an equally important role during the later stages of embryogenesis. Recent studies have shown that Fas transcripts are detectable in the developing thymus of E16.5 embryo,<sup>35</sup> and that mouse thymocytes undergo apoptosis when treated with an agonistic anti-Fas

antibody.<sup>36</sup> In addition, deficiency of FADD resulted in abnormal development of thymocytes in the mutant mice.<sup>24</sup> Because the activities of FADD, Fas and caspase-8 are so closely intertwined, and because caspase-8 protein is present in the E17 thymus, it is reasonable, based on these findings, to predict an important functional role for caspase-8 in thymic development.

To understand the role of caspase-8 *in vivo*, we and another group have generated caspase-8-deficient mice and shown that caspase-8 is involved in embryonic development. However, knowledge from these knockout mice is not sufficient to fully determine the physiological role of caspase-8 in embryogenesis. Consequently, these studies raise new questions, including how caspase-8 is activated during development. As Fas-, TNFR1-, DR3-DR6- and TRAIL-deficient embryos are viable,<sup>37–42</sup> caspase-8 activation must occur in a manner independent of death receptors. Furthermore, it has been shown that both caspase-3- and Bid-deficient embryos displayed normal development of the heart, neural tube and yolk sac.<sup>18,43</sup> As these molecules are both caspase-8-specific substrates, it seems reasonable to predict the existence of another target molecule recognized by caspase-8 during embryogenesis. Clearly, the caspase-8-mediated signaling pathway, as it functions during embryonic development, requires further elucidation.

## Materials and Methods

### Construction of the targeting vector and establishment of caspase-8 knockout mice

A targeting vector was constructed for generating a truncated caspase-8 protein through introduction of a stop codon at exon 7 of the mouse *caspase-8* gene.<sup>26</sup> In brief, a 1.2-kb *EcoRI*–*XbaI* fragment of the 5' homologous region was ligated with the stop sequence, the polyadenylation signal derived from SV40 virus and a PGK-neo resistance gene expression cassette (PGKneobpA).<sup>44</sup> Then, the 5-kb *BamHI*–*EcoRI* fragment from the 3' homologous region containing exon 8 and the MC1-diphtheria toxin A fragment gene expression cassette (DT-A)<sup>45</sup> were fused at the 3' end of the vector. The targeting vector was linearized and introduced into R1 ES cells<sup>46</sup> by electroporation. After screening by G418 selection at 180 µg/ml for 7–10 days, four recombinants were identified from 500 ES clones by PCR analysis and confirmed by Southern blot analysis with a 5'-probe (see Figure 2).

Chimeric mice were produced from two independent *caspase-8<sup>DED/+</sup>* ES clones by the aggregation method<sup>46</sup> using C57BL/6 8-cell-stage eggs and subsequent implantation into CD1 foster mothers.<sup>47</sup> The resulting chimeric males were mated with C57BL/6 females (SLC, Japan). Germline transmission of the mutant allele was verified by PCR and Southern blot analyses of tail DNA from offspring of C57BL/6 genetic background. The genotypes of embryos obtained by cross-mating the heterozygous *caspase-8<sup>DED/+</sup>* mice were also confirmed by PCR and Southern blot analyses.

### Southern blot analysis

Genomic DNAs isolated from ES clones were digested with the restriction enzyme *BamHI*, electrophoresed through a 0.7% agarose



gel and transferred to nylon membranes (Hybond-N, Amersham Biosciences). Hybridization was performed as previously described<sup>48</sup> using [<sup>32</sup>P]-labeled DNA probes made with a Ready-to-Go DNA labeling kit (Amersham Biosciences).

### PCR and RT-PCR analyses

PCR amplification for screening of ES clones and for genotyping of embryonic fibroblasts, embryos and mice was performed with two pairs of primers: primer P1 (5'-CCGTATGTCTGAAGACAGCTACTGT-3' from intron 6) and primer P2 (5'-CTCTATGGCTTCTGAGGCG-GAAAG-3' from PGKneobpA) for detection of the targeted allele, and primer P3 (5'-ATGAGATCCTGTGCCTTCTCCCG-3' from intron 6) and primer P4 (5'-GTCACCGTGGGATAGGATACAGCA-3' from exon 7) for the normal allele. PCR was carried out as follows: initial denaturation at 96°C for 3 min following by 40 cycles of 94°C for 1 min, 67°C for 2 min, 72°C for 3 min and extension at 72°C for 10 min.

For RT-PCR analysis, total RNAs (3 µg) isolated from E10.5 embryos using Isogen (Nippongene, Japan) were used for first-strand cDNA synthesis with an oligo-dT primer on Ready-To-Go RT-PCR beads (Amersham Biosciences), according to the manufacturer's recommendations. First-strand cDNAs were amplified with three sets of primers: primers P4 and P5 (5'-ATGGCGGAAGTGTGACTCG-3' on exon 5) for detection of normal caspase-8 transcripts, primers P5 and P6 (5'-ATGTTTCAGGTTCCAGGGGAGGTG-3' in the SV40 polyA region) for truncated caspase-8 transcripts, and primers P7 (5'-CCTGATTGTTGCTGCTGGTGG-3') and P8 (5'-GTCACGAA-CAGCAAAGCGACCAAG-3') for mouse EF1 $\alpha$  transcripts as a standard control. PCR was performed under the conditions described above and PCR products were analyzed by 1% agarose gel electrophoresis.

### Establishment of stable transfectants and embryonic fibroblasts

To establish transfectants stably-expressing mouse caspase-8, we used JB-6 cells, a human T lymphoma Jurkat cell variant that is caspase-8-deficient.<sup>49</sup> We transfected mouse caspase-8 cDNA with the neo resistance gene into this variant and selected them in 1.5 mg/ml of G418. An established JB-6 stable clone and parental JB-6 cells were maintained in RPMI-1640 with 10% fetal calf serum. MEF cells were established from E10.5 embryos and their genotype was determined by PCR. These cells were maintained in D-MEM with 10% fetal calf serum.

### Immunoblot analysis

For preparation of cell lysates, stable transfectants expressing mouse caspase-8 and MEF cells were lysed in lysis buffer (150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1% EDTA, a cocktail of protease inhibitors and 20 mM Tris-HCl pH 7.5). Similarly, cell lysates from E8.5 embryos and from several E17 embryonic tissues were prepared after homogenization in the lysis buffer. These lysates were then analyzed by SDS-PAGE followed by immunoblotting with anti-mouse caspase-8 and anti-actin (Chemicon) monoclonal antibodies. Prior to immunoblotting, the membrane transferred with cell lysates of MEF cells was immersed in phosphate-buffered saline (PBS) containing 3% formaldehyde for 10 min. Then, immunoblot analysis was performed as described previously.<sup>50</sup> An anti-mouse caspase-8 monoclonal antibody was generated against full-length of mouse caspase-8 produced in *E. coli* and recognizes the DED domain of caspase-8 (Kazama *et al.*, unpublished data).

### Ex vivo whole-embryo culture

For whole-embryo culture, embryos (34–38 somites) were dissected at E10.5 from pregnant mice after heterozygous mating. Cultivation of embryos was carried out at 37°C for 24 h in rat serum containing 2 mg/ml glucose as described.<sup>28</sup> After culture, embryos (45–50 somites) were fixed in 4% paraformaldehyde (PFA) in PBS overnight for histology, immunohistochemistry and *in situ* hybridization. Genomic DNAs for genotyping were prepared from yolk sacs after culture.

### In situ hybridization

Embryos were dissected at E10.5 and E11.5 and after whole-embryo culture, and fixed with 4% PFA in PBS overnight. Frozen sections were cut on a cryostat and hybridization was performed as previously described.<sup>51</sup> RNA probes for mouse *Islet-2* (a gift from Dr. S Pfaff), *Chox-10* (a gift from Dr. R MacInnes), *En1*, *Evx1* (gifts from Dr. M Goulding), *Mash1* and *Neurogenin-2* (gifts from Dr. F Guilmot) were prepared for hybridization.

### Histological analysis and TUNEL staining

For histological analysis, embryos and yolk sacs were dissected at E10.5 and E11.5 and after whole-embryo culture, fixed in neutral formalin solution (pH 7.4), embedded in paraffin, and cut into 3.5 µm sections for hematoxylin and eosin staining. For *in situ* demonstration of apoptosis, TUNEL staining was performed according to the manufacturer's instructions (Wako, Japan). After labeling, samples were counter-stained with hematoxylin.

### Immunohistochemical analysis

Frozen sections from E10.5 and E11.5 embryos were immunostained essentially as described<sup>51</sup> using anti- $\beta$ -tubulin antibody (Sigma), anti-neurofilament antibody (2H3, Developmental Biology Hybridoma Bank), anti-Pax6 antibody<sup>52</sup> and anti-*Islet-1* antibody (40.2D6, Developmental Biology Hybridoma Bank). Detection of immunoreactivity was performed using an ABC kit (Vector Laboratories).

### Whole-mount immunohistochemistry

Whole-mount immunohistochemistry was performed as previously described.<sup>53</sup> Briefly, embryos dissected at E10.5 and yolk sacs prepared at E9.5, E10.5 and E11.5 were fixed in PBS containing 2% PFA for 2 h at 4°C, immersed in cold PBS overnight and treated with PBS containing 0.15% H<sub>2</sub>O<sub>2</sub> and 0.1% NaN<sub>3</sub> at 4°C for 1 h. After washing with PBS, embryos and yolk sacs were preincubated in blocking solution (PBS-MBT) containing 2% milk, 0.2% bovine serum albumin, 0.6% goat serum and 0.1% Triton X-100 at 4°C for 1 h. Then, the samples were incubated with a rat monoclonal anti-PECAM-1 antibody (BD-PharMingen) in PBS-MBT at 4°C overnight and washed five times, 1 h each, with PBS-MT containing 2% milk and 0.1% Triton X-100 at room temperature. Subsequently, they were incubated with horseradish peroxidase-conjugated goat anti-rat IgG antibody (Cappel) in PBS-MBT at 4°C overnight, washed five times with PBS-MT, and developed with PBS containing 0.1% Triton X-100, 0.03% 3,3'-diaminobenzidine and 0.08% NiCl. After washing with PBS, embryos were destained with a mixture of benzyl alcohol and benzyl benzoate.

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