observed within the base population (Fig. 4A). Genome-wide fitness variation was estimated to be so large in our experimental assay that mutations with a selection coefficient as large $s = 0.5$ would commonly be trapped in the living dead. This indicates that the proportion of the mutational spectrum whose fixation is promoted by recombination is large. In general, the advantage to recombination demonstrated by these experiments will be manifest in any genome, or genomic subunit, when there is sufficient fitness variation relative to the strength of natural selection, i.e., when a nontrivial proportion of the mutation spectrum will be trapped in the living dead.

Although we did not explicitly examine the accumulation of new harmful mutations, our results reinforce a previous experiment's results (*34*) that demonstrated an accelerated accumulation of harmful mutations in nonrecombining genomes. In general, deleterious mutations fix only when the selection coefficient is weak relative to stochastic noise generated by binomial sampling error and background selection (i.e., $|s| < 1/N_e$) (*22*, *23*). In the absence of recombination a mildly deleterious mutation need only fix by chance within the progenitor tail ($N_p \ll N$) before it will ultimately fix population-wide due to recurrent selective sweeps of the fittest genomes (*13*, *17*). Accordingly, the smaller effective size of a nonrecombining population will cause a wider portion of the spectrum of deleterious mutations to accumulate.

The substantial costs associated with sexual recombination are well established (*1*), making its prevalence in nature an evolutionary enigma. Our results experimentally verify a counteracting advantage of recombining compared to clonal lineages: reduced accumulation of harmful mutations and increased accumulation of beneficial mutations. The magnitude of this benefit will accrue over geological time and promote the superior persistence of recombining lineages at both the level of species within communities (clonal versus sexual species) and genes within chromosomes (nonrecombining Y-linked versus recombining X-linked genes).

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42. The standardized fitness of $w^+ = (\overline{W}_{w^+} - \overline{W})/\overline{W}$, where $\overline{W}_{w^+}$ is the fitness of a $w^+$ allele averaged over all of its genetic backgrounds, and $\overline{W}$ is mean fitness. This value is related to gene frequency change by, $\Delta p_{w^+} = p_{w^+}(\overline{W}_{w^+} - \overline{W})/\overline{W}$.
43. These autosomes were derived from the base population (depicted as open rectangles). The X-clone generator stock was continuously backcrossed to the LHM base population to maintain genetic diversity of its autosomes.
44. Twice as many Y-clone generator females were used to propagate each generation (160 compared to 80 X-clone generator females) because they produced 50% fewer offspring (due to aneuploid gametes associated with the autosomal translocation). To retain high levels of sexual selection, the 160 Y clone generator females were combined with the males in two groups of 80 virgin females each. The first group of females was combined with the males, and then 4 hours later the second group was added. The 80 virgin females in the synthetic X treatment were combined with the males as a single group. Each generation the total population of flies was evenly distributed among five vials (28.5 mm by 95 mm), with 20 males and 32 females per vial in the synthetic Y treatment, and 20 males and 16 females per vial in the synthetic X treatment. Each vial had a 10-cm extension sleeve attached to its top to provide additional space for pupating juveniles and for courtship among adults.
45. An excess of eggs was laid in the culture vials that were used to propagate the each generation. These were culled to achieve a density matching that to which the base population had adapted for over 200 generations. In generation 1, the vials were not culled sufficiently. At elevated density the $w^+$ red-eyed flies have a fitness advantage over and beyond that experimentally produced by artificial selection. Accordingly, the first generation was not included in statistical analyses, but the significance of all tests remain unchanged with or without the inclusion of this generation.


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# Liver Organogenesis Promoted by Endothelial Cells Prior to Vascular Function


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**The embryonic role of endothelial cells and nascent vessels in promoting organogenesis, prior to vascular function, is unclear. We find that early endothelial cells in mouse embryos surround newly specified hepatic endoderm and delimit the mesenchymal domain into which the liver bud grows. In *flk-1* mutant embryos, which lack endothelial cells, hepatic specification occurs, but liver morphogenesis fails prior to mesenchyme invasion. We developed an embryo tissue explant system that permits liver bud vasculogenesis and show that in the absence of endothelial cells, or when the latter are inhibited, there is a selective defect in hepatic outgrowth. We conclude that vasculogenic endothelial cells and nascent vessels are critical for the earliest stages of organogenesis, prior to blood vessel function.**


The early stages of visceral organ development serve as a model for changes in cells and tissues that occur in various biological contexts. During the embryonic specification of tissues such as the liver, endodermal epithelial cells receive stimuli from mesodermal cells that cause changes in gene expression and cell division. The endodermal cells differentiate and proliferate within an epithelium, and then begin to move into the surrounding connective tissue. Finally, the cells form a new domain of condensed tissue mass that becomes vascularized. These transitions, which are common to organogensis, tissue regeneration, and tumor growth, require the careful orchestration of signals between epithelial cells, mesenchymal

cells, and endothelial cells. During these processes, endothelial cell precursors, or angioblasts, differentiate and form primitive tubules (vasculogenesis), or primitive tubules branch from preexisting vessels (angiogenesis) (*1*, *2*). Presently, there is an extensive literature on the signals that induce vasculogenesis and angiogenesis (*1*–*3*), but little is known about the ability of endothelial cells to promote organ development, apart from vascular function.

The liver is an excellent model to investigate this issue because the liver vasculature is necessary for the tissue's early hematopoietic function. Hepatic cells are induced within the embryonic endoderm by day 8.5 of mouse gestation (E8.5) (*4*) and hematopoietic cell invasion of the nascent liver occurs by E10 (*5*), so rapid tissue growth and vascular development are essential.

An early role for endothelial cells in liver organogenesis was suggested by studies of the septum transversum. This mesoderm-derived tissue domain surrounds the embryonic midgut endoderm and elicits a secondary morphogenetic induction within the nascent liver bud (*6*–*8*). The septum transversum domain has been suggested to contain vasculogenic endothelial cells (*9*, *10*). However, due to a lack of early endothelial cell markers and genetic mutations that eliminate endothelial cell lineages, previous studies did not rigorously assess the potential contribution of angioblasts or early endothelial cells to the morphogenetic induction of the liver or other endoderm-derived organs. In the present study, we use such tools and a liver bud culture system to assess the role of endothelial cells in the morphogenetic phase of organ development.

**Endothelial-endoderm interactions prior to liver bud emergence.** At embryonic day 8.5 to 9.0 (E8.5 to 9.0), newly specified hepatic cells begin to multiply within the endodermal epithelium (Fig. 1, A to C, "h.e."), and at E9.5, they migrate into the surrounding septum transversum mesenchyme (Fig. 1D, s.t.m., red circled domain; boxed region, enlarged in Fig. 1E, contains the emerging liver bud). Occasionally at E9.5, and clearly at E10.5, dark staining hematopoietic cells within developing sinusoids can be seen near or in the liver region (Fig. 1E, lower left arrow; Fig. 1F, arrowheads). To determine when angioblasts or endothelial cells begin to be associated with the liver bud,

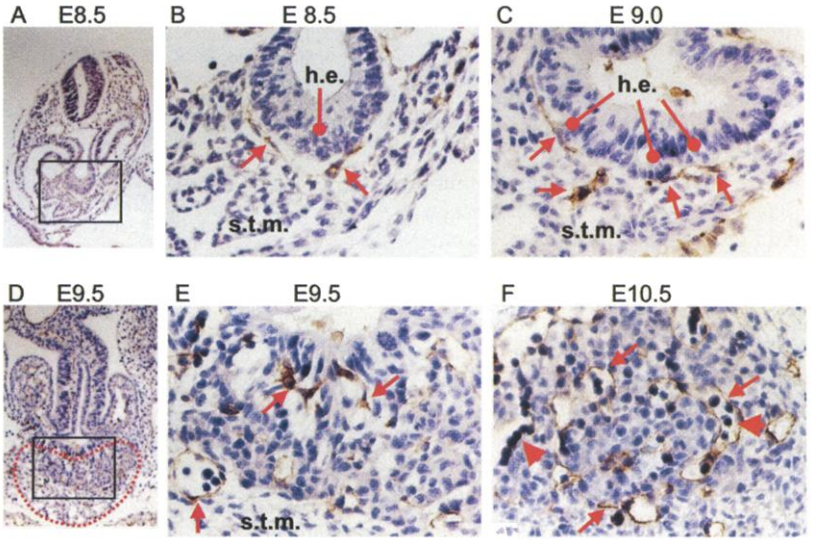


**Fig. 1.** Interactions between endothelial cells and hepatic endoderm before liver bud emergence. Transverse sections of mouse embryos at E8.5 (**A** and **B**), E9.0 (**C**), E9.5 (**D** and **E**), and E10.5 (**F**). Vascular endothelial cells were detected as brown-stained cells with anti-PECAM (arrows; arrowheads denote hematopoietic cells). h.e., hepatic endoderm; s.t.m., septum transversum mesenchyme. Red circled area in (D) denotes the s.t.m. Boxed areas in (A) and (D) are magnified in (B) and (E) and encompass the liver bud. Original magnification, ×100 (A and D); ×400 (B, C, E, and F). The field in (D) appears smaller than in (A) because the embryo is larger.

we stained embryo sections with an antibody against platelet endothelial cell adhesion molecule (anti-PECAM, CD31), a definitive marker for embryonic endothelial cells (*11*, *12*). As a second marker, we performed β-galactosidase (β-Gal) staining of heterozygous mice in which the LacZ gene had been recombined into the *flk-1* locus, resulting in LacZ expression like the native *flk-1* gene (*13*). Flk-1 (VEGFR-2) is a cell surface receptor for vascular endothelial growth factor (VEGF) and is highly expressed in embryonic angioblasts and endothelial cells (*14*–*17*). Although *flk-1* homozygotes are embryonic lethal, heterozygotes are normal (*13*). As expected, PECAM- and *flk-1*$^{LacZ}$–positive cells lined the nascent sinusoids that contain the hematopoietic cells at E9.5 to E10.5 (Figs. 1, E and F, and 2F). Notably, the nascent sinusoids at E9.5 were irregular and consisted mostly of primitive vascular structures lined by endothelial cells that only occasionally had been invaded by hematopoietic cells.

However, angioblasts or endothelial cells were detected as early as E8.5 to E9.0, as a loose necklace of cells interceding between the thickening hepatic epithelium and the septum transversum mesenchyme (Figs. 1B and 2B, arrows). At E9.0, angioblasts or endothelial cells predominated where segments of the hepatic endoderm begin to break into the mesenchyme (Fig. 1C, arrows). During these E8.5 to E9.0 stages, the endothelial cells were separated from one another, and we did not detect closed vascular structures or local hematopoietic cells. At E9.5, the hepatic cells migrated into the domain of septum transversum mesenchyme where endothelial cells begin to form vascular structures, but not beyond (Figs. 1, D and E, and 2, E and F). We conclude that prior to blood vessel formation and function, angioblasts or endothelial cells physically interact with nascent hepatic cells. This interaction precedes liver bud emergence and persists from the hepatic endoderm stage (E8.5) (Fig. 1B) through the formation of the liver proper (E10.5) (Fig. 1F).

**Defect in liver bud emergence in the absence of endothelial cells.** To test the hypothesis that endothelial cells promote hepatic morphogenesis, we crossed *flk-1* heterozygous mice to generate homozygous null embryos. Such embryos form early angioblasts, but not mature endothelial cells or blood vessels, and the mutant angioblasts are defective in migrating to their normal sites of vascular development (*13*). In *flk-1*$^{-/-}$ embryos at E9.0, no angioblasts or endothelial cells were evident around the hepatic endoderm or in the septum transversum mesenchyme (Fig. 2D). However, in such embryos, the hepatic endoderm begins to thicken normally (Fig. 2, B and D) and reverse transcriptase–polymerase chain reaction analysis showed that liver genes such as *albumin*, *transthyretin*, and *Hex* are all induced (*12*), reflecting that the initial hepatic induction takes place (*18*).

Shalaby *et al.* (*13*) previously observed that the homozygous mutant *flk-1* allele causes embryonic lethality by E10.5. As expected, *flk-1*$^{-/-}$ embryos exhibited a relative lack of β-Gal–positive cells (Fig. 2, C and G). Although *flk-1* homozygous embryos exhibited retarded growth, compared with heterozygous embryos (Fig. 2, E and G), they develop similar numbers of somites (*13*). Furthermore, the

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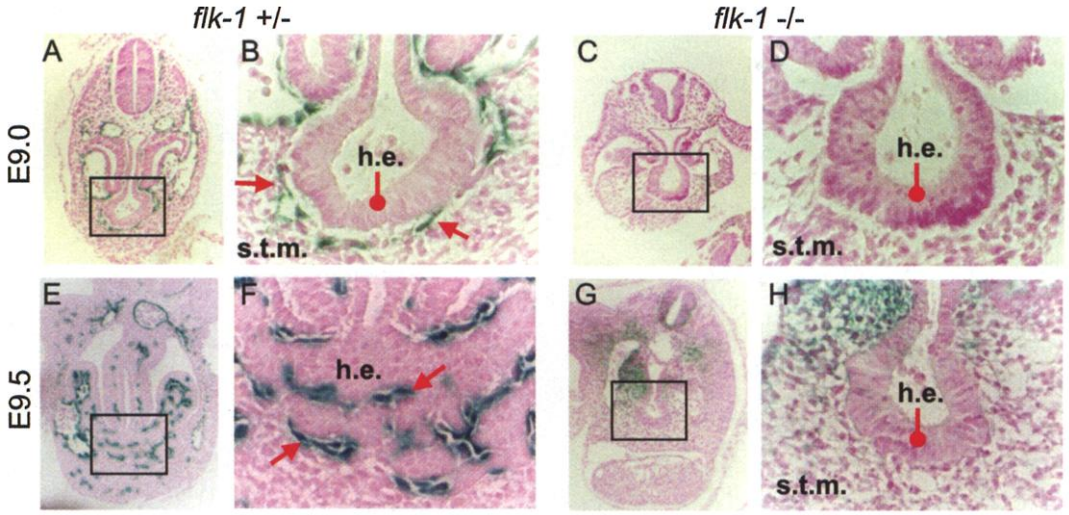


**Fig. 2.** (**A** to **H**) Lack of liver bud emergence in *flk-1* homozygous mutant embryos. Transverse sections of mouse embryos stained for β-Gal to visualize expression of the *flk-1*$^{LacZ}$ allele. Bluish-green cells are positive (arrows). The lack of growth of hepatic endoderm (h.e.) from E9.0 to E9.5 in the *flk-1*$^{-/-}$ embryos, compared with the extensive growth and vascular network formation in *flk-1*$^{+/-}$ embryos, is apparent.

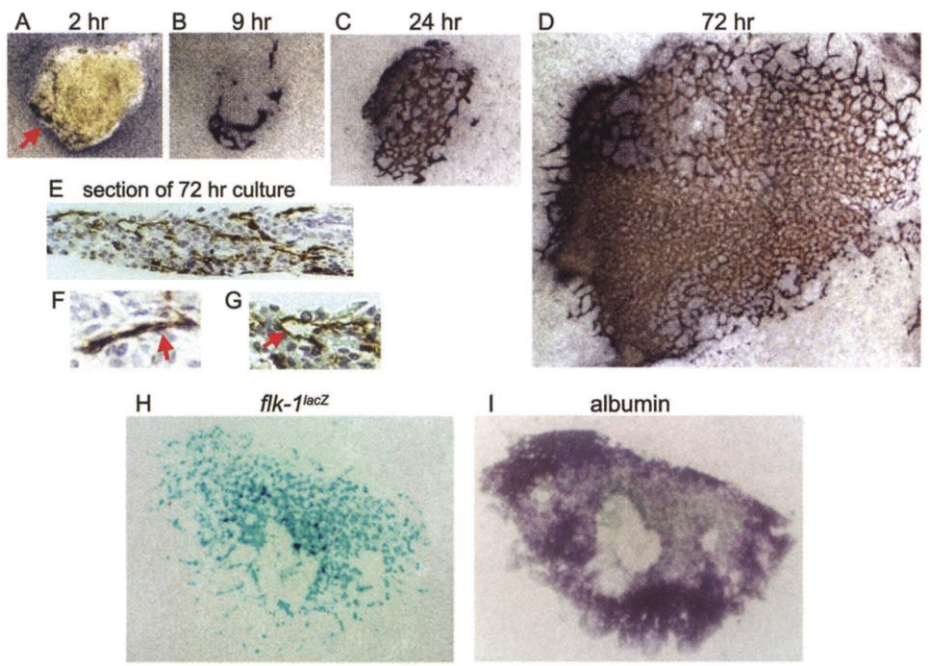


**Fig. 3.** Liver bud culture system permissive for vascular development in vitro. (**A** to **D**) Liver buds from wild-type embryos were cultured for various times and stained with anti-PECAM. Magnification, ×100; (D) is a composite of four images. The arrow in (A) denotes PECAM-positive cells, but note the absence of vascular network structures that begin to develop in vitro by 9 hours (B). (**E**) Section of a 72-hour culture; (**F** and **G**) magnified areas. (**H**) A 72-hour *flk-1*$^{LacZ}$ heterozygous explant stained for β-Gal; the blue network indicates vascular areas. (**I**) In situ hybridization of the explant in (H), revealing albumin mRNA expression in the vascular area.

*flk-1*$^{-/-}$ embryos still exhibited growth between the E9.0 and E9.5 stages; enhanced closure of the neural tube, narrowing of the gut, and increased area of the septum transversum mesenchyme cells were apparent (Fig. 2, C and G). Yet, despite these changes in embryonic growth from E9.0 to E9.5, the size of the hepatic endoderm in the *flk-1*$^{-/-}$ embryos remained essentially constant (compare Fig. 2, D and H). Furthermore, in the *flk-1*$^{-/-}$ embryos at E9.5, there were no hepatic cells proliferating into the surrounding septum transversum mesenchyme (Fig. 2H). By contrast, in *flk-1* heterozygous and wild-type embryos at E9.5, there was extensive hepatic cell invasion (Figs. 1E and 2F). We conclude that the presence of angioblasts and/or early endothelial cells is critical for liver bud outgrowth, prior to the formation of a functioning local vasculature and prior hepatic recruitment of hematopoietic cells.

**Liver vascular development in vitro.** The impaired hepatic morphogenesis in the *flk-1*$^{-/-}$ embryos may have been due to secondary effects of the embryos' overall impaired growth and vitality, or to effects of the *flk-1* mutation on the hematopoietic system (*13*). To determine whether endothelial cells within and around the liver bud promote hepatic growth in isolation of the rest of the embryo, we developed an embryo tissue explant system that supports liver vasculogenesis. We found that cultivating E8.5 to E9.5 liver bud tissue at the air-gas interface on a Transwell filter membrane, with Matrigel in the culture medium, permits extensive endothelial cell growth and vascular morphogenesis (*19*).

Figure 3A shows an explant from an E9.5 (~25 somite) embryo that was fixed after 2 hours in culture and stained for PECAM (*14*). Isolated PECAM-positive cells and cell clusters were evident (Fig. 3A, arrow), but no obvious vascular network had formed. After 9 hours in culture, the explants flattened out on the membrane, becoming difficult to see; yet PECAM staining revealed the beginning of a vascular network (Fig. 3B). By 24 hours, a web of vessel-like structures was prominent throughout the explants (Fig. 3C), and by 3 days, a considerable extent of vascular development and overall growth had occurred [Fig. 3D; same magnification as (A) to (C)]. In addition, a thin layer of fibroblastic cells typically grew beyond the thick, vascularized tissue domain; these cells are probably derived from the septum transversum mesenchyme (Fig. 4B). Transverse sections of the 72-hour explants revealed the thickness of the main tissue mass and the presence of vascular structures throughout (Fig. 3E), including small, lumenized vessels (Fig. 3F, arrow) and larger, vascular structures that were lined by PECAM-positive cells (Fig. 3G, arrow), analogous to those seen in liver buds in embryos (Fig. 1F). Explants from *flk-1*$^{LacZ}$ heterozygous embryos also exhibited a web of vascular structures upon staining for β-Gal, reflecting *flk-1* gene expression (Fig. 3H). In situ hybridization (*12*) on such explants showed that most of the *flk-1*$^{LacZ}$–positive tissue, i.e., the vascularized region, encompassed the domain of hepatic cells, as seen by the expression of serum albumin mRNA (Fig. 3I). Importantly, these data precisely mimic the in vivo observation of endothelial cells delimiting the domain

of the septum transversum into which the hepatic cells proliferate (Figs. 1E and 2F). We conclude that the new culture conditions were permissive for de novo vascular development of liver tissue, in the absence of potential influences outside the liver bud domain.

**Impaired hepatic endoderm outgrowth in *flk-1* mutant explants.** We made use of several approaches with the explant system to investigate the necessity of endothelial cells to promote hepatic morphogenesis. In the first, we compared the growth of wild-type, *flk-1* heterozygous, and *flk-1* homozygous mutant liver bud tissue cultivated in vitro. Explant cultures were established and allowed to grow for 72 hours, during which time they were photographed and subjected to in situ hybridization for serum albumin mRNA to monitor the growth of the hepatic domain. Explants cultured for 1 hour remained on top of the membrane and resulted in refraction of the light in the surrounding medium (Fig. 4A, whitish domain around the explant circled in red). By 72 hours, whitish fibroblastic cells surrounded the primary, thick masses of cells (Fig. 4B), and most of the latter cells expressed albumin mRNA (Fig. 4C). We quantitated the cellular area of the explants and determined that, for wild-type and heterozygous *flk-1*, the total area spanned by the tissue explants, including the fibroblastic cells, increased about 15-fold over the 72-hour period (Fig. 4H) (*12*).

Although *flk-1* homozygous mutant explants were usually smaller at the outset (Fig. 4D), they too exhibited about a 15-fold increase in overall cellular area during the 72-hour growth period (Fig. 4, E and H). However, the primary, thick tissue mass of the *flk-1* homozygous explants usually remained small, and most of the growth was exhibited by the fibroblastic cells. To quantitate the growth of the hepatic cells, we compared the area of albumin mRNA-positive cells to the total cell area for wild-type, heterozygous, and homozygous *flk-1* explants (*12*). Whereas comparable growth was observed in the hepatic domain of wild-type and heterozygous explants, to about 20% of the total cell area, the hepatic domain of homozygous *flk-1* explants grew to only about 5% of the total cell area (Fig. 4G). The difference in growth of hepatic cells was statistically significant ($P = 0.0025$; paired Student's $t$ test). We conclude that the lack of endothelial cells in the *flk-1* explants specifically affects the outgrowth of the hepatic endoderm, while not affecting the growth of the surrounding fibroblast cells or the initial expression of early liver genes in the endoderm. We further note that hepatic outgrowth, induced by endothelial cells in the wild-type and heterozygous explants, occurs in the absence of a functioning vasculature and in isolation from the rest of the embryo.

**Ongoing presence of endothelial cells needed for hepatic outgrowth.** As a second approach to investigate whether endothelial cells are required for hepatic morphogenesis, we sought to inhibit the growth and development of endothelial cells in liver bud explants from wild-type embryos. This would allow us to determine whether endothelial cells are needed continuously or whether they provide an initial stimulatory signal that is maintained by hepatic or septum transversum mesenchyme cells. To this end, we used NK4, a new angiogenesis inhibitor that potently suppresses tumor growth and metastasis (*20*). NK4 contains four "kringle" domains from hepatocyte growth factor (HGF) (*21*). Although the mechanism by which NK4 inhibits angiogenesis is unknown, NK4 has structural similarity to angiostatin, an angiogenesis inhibitor composed of kringle domains from an internal fragment of plasminogen (*22*).

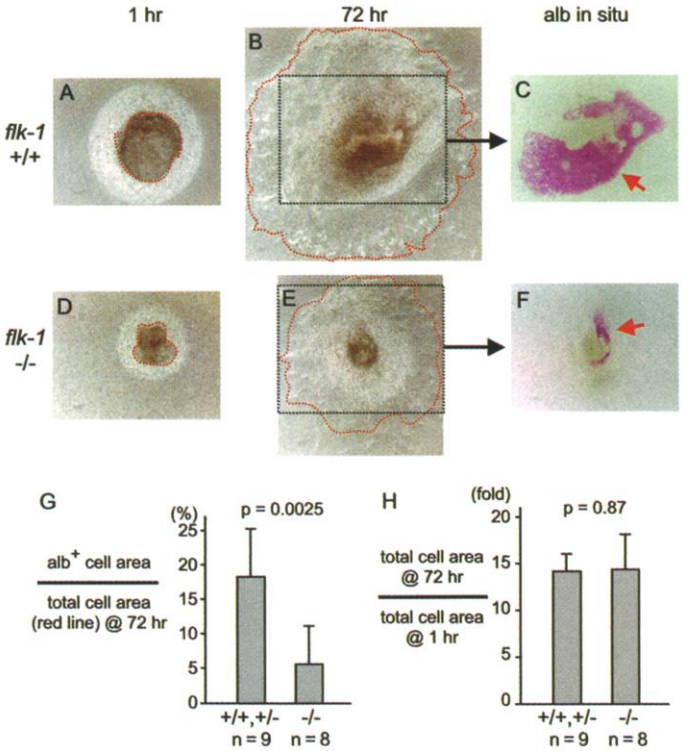


**Fig. 4.** Impaired hepatic outgrowth in *flk-1* homozygous mutant explants. (**A** to **F**) Embryonic explants of the designated genotypes at different times of cell culture. The total cell area is circled in red. The whitish ring around the cell mass in (A) and (D) is due to the light refraction; the whitish area just within the red circles in (B) and (E) reflects the presence of fibroblastic cells. Boxed areas in (B) and (E) are shown in (C) and (F), respectively, as the results of in situ hybridization with an albumin antisense probe and detected with FastRed. Arrows denote albumin mRNA-positive regions. (**G**) Albumin-positive cell area relative to the total cell area at 72 hours. A paired Student's $t$ test shows that the data are significantly different ($p = 0.0025$). (**H**) Total cell area at 72 hours relative to the total cell area at 1 hour. Paired Student's $t$ test shows no significant difference.

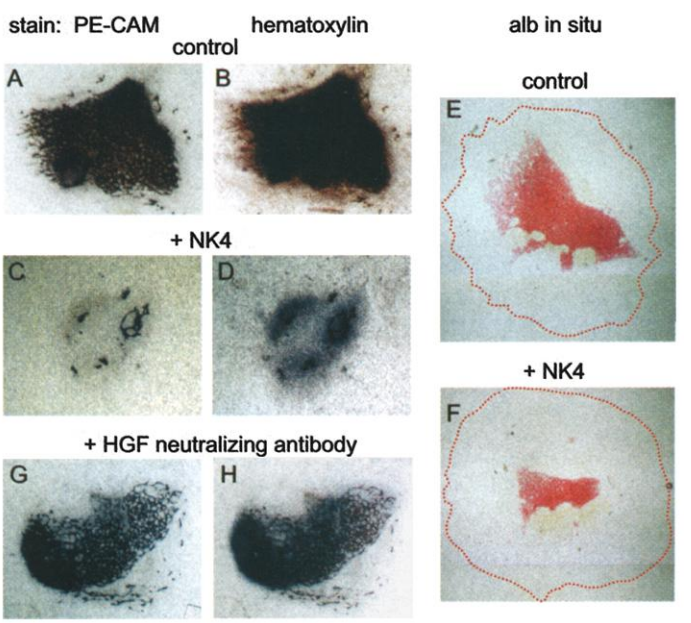


**Fig. 5.** Inhibition of vascular development in explants inhibits hepatic growth. The liver buds of E9.5 embryos were cultured for 72 hours in the presence of 500 nM bovine serum albumin (**A** and **B**), 500 nM NK4 (**C** and **D**), or 30 μg/ml anti-HGF immunoglobulin G (**G** and **H**). The liver bud cultures were stained for PECAM (A, C, and G) and then with hematoxylin (B, D, and H) to visualize the presence of nonendothelial cells. In (**E**) and (**F**), fibroblastic cell areas were outlined in red and albumin-positive cells were detected with FastRed.

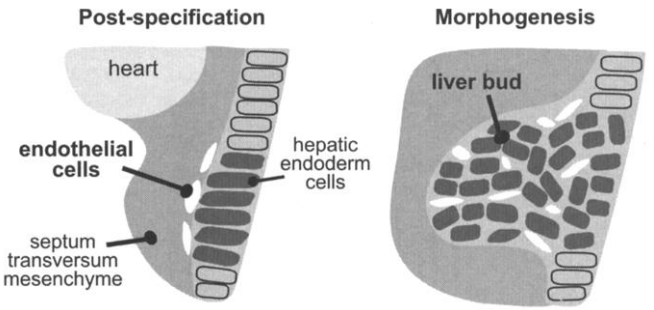


**Fig. 6.** Morphogenetic role of early endothelial cells in liver organogenesis. (Left) Shortly after hepatic specification, early endothelial cells intercede between the hepatic endoderm cells and the septum transversum mesenchyme. The source of these early endothelial cells is not known. (Right) The early endothelial cells promote the morphogenetic phase of organogenesis, where the hepatogenic cells multiply and migrate into the septum transversum mesenchyme to develop the liver bud.

Liver buds isolated from E9.5 embryos were cultured in the presence or absence of NK4, subjected to PECAM staining to detect endothelial networks, and then stained with hematoxylin to visualize the primary cell mass (*12*). In contrast to the extensive vascular network seen in the control cultures (Fig. 5A), vascular network formation was strongly inhibited in all explants treated with 500 nM NK4 (Fig. 5C). In such cultures, the growth of the primary tissue mass was also greatly inhibited (Fig. 5, B and D), as was the fraction of albumin-positive cells relative to fibroblasts (Fig. 5, E and F). To test the possibility that NK4 affects hepatic outgrowth by inhibiting HGF signaling, we cultured liver buds with an HGF-neutralizing antibody, using the same concentrations and preparations that inhibited HGF in Transwell cultures of lung explants (*23*). No inhibition of vascular development or of tissue growth was observed (Fig. 5, G and H; $n = 3$). Importantly, the lack of effect of an HGF inhibitor on the initial phase of hepatic endoderm outgrowth in vitro is consistent with gene-disruption studies showing that HGF signaling is critical after, but not during, the initial emergence of the liver bud in embryos (*24*). Taking these findings together with those from the *flk-1* mutant explants and embryos, we conclude that endothelial cells and/or nascent vascular structures, prior to the formation of functioning vessels, are needed continuously to promote early hepatic morphogenesis.

**Role of endothelial cells in organogenesis and other tissue transitions.** We found that early endothelial cells and nascent vascular structures interact with newly specified hepatic endoderm, prior to liver bud emergence, and promote hepatic morphogenesis (Fig. 6). Although the most extensive hepatic outgrowth in vitro occurs after endothelial cells have assembled into vascular structures (Fig. 3, A and D), morphogenesis of the liver bud in *flk-1* mutant embryos arrests at the stage prior to liver vascular formation (Figs. 1, B and C, and 2, B, D, and H). Thus, both endothelial cells and nascent vascular structures may provide an organogenic stimulus, clearly preceding the function of blood vessels in providing oxygenation, metabolites, or blood cells to the liver region. Liver bud emergence requires the transitions from a columnar epithelium to cells dividing more rapidly and migrating through the mesenchyme; we find that endothelial cells and nascent vessels promote early events in these transitions, comparable to the time of function of the earliest known liver transcription factors, *Hex* (*25*) and *Prox1* (*26*).

Signaling from endothelial cells recruits mesenchymal cells to nascent vessel walls (*27*, *28*) and promotes myocyte differentiation within the developing heart (*29*). These examples relate to the development of the cardiovascular system itself, rather than to tissues served by the vasculature, and it remains to be determined whether different signals are used in each context. We also found angioblasts or early endothelial cells associating with emerging buds of embryonic lung and pancreas, and the nascent glandular portion of the stomach, suggesting a general role in gut tissue organogenesis (*12*).

The cell interactions that we have found to occur during organogenesis may be recapitulated in adult tissues. For example, during liver regeneration, signaling between endothelial cells and hepatocytes may be critical to establish a sinusoidal liver architecture (*30*–*32*). During tumorigenesis, vascular development is a major influence on tumor growth (*22*, *33*), and vascular cells or structures, independent of the blood supply, may be influential. We therefore suggest that understanding how endothelial cells promote early organogenesis may impact our understanding of these other processes. In addition, the new culture techniques and the role of endothelial cells discovered here should facilitate future efforts to reconstitute organ systems in vitro, for cell differentiation studies and drug testing, and in vivo, for therapeutic purposes.

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