Dual functions of α4β1 integrin in epicardial development: initial migration and long-term attachment

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The epicardium of the mammalian heart arises from progenitor cells outside the developing heart. The epicardial progenitor (EPP) cells migrate onto the heart through a cyst-mediated mechanism in which the progenitors are released from the tissue of origin as cysts; the cysts float in the fluid of the pericardial cavity and attach to the naked myocardial surface of the heart, and cells in the cysts then migrate out to form an epithelial sheet. In this paper, we show that the gene encoding the α4 subunit of α4β1 integrin (α4β1) is essential for this migratory process. We have generated a knockin mutation in mice replacing the *α4 integrin* gene with the *lacZ* reporter gene, placing *lacZ* under the control of the *α4 integrin* promoter. We show that in homozygous mutant embryos, the migration of EPP progenitor cells is impaired due to inefficient budding of the cysts and a failure of the cells in the cysts to migrate on the heart. This study provides direct genetic evidence for essential roles for α4β1 integrin–mediated cell adhesion in the migration of progenitor cells to form the epicardium, in addition to a previous finding that α4β1 is essential for maintaining the epicardium (Yang, J.T., H. Rayburn, and R.O. Hynes. 1995. *Development.* 121:549–560).

Introduction

Cell migration is critically important during embryonic development. One interesting migratory event occurs during the formation of the epicardium of the heart. The heart of vertebrate animals is comprised of three cell layers: the outer epicardium, the myocardium, and the inner endocardium. A developing heart initially consists of only the myocardium and the endocardium. Later in development, progenitor cells outside the heart migrate onto the heart to form the epicardium (for review see Manner et al., 2001). The newly formed epicardium provides nearly all of the cellular elements of the subepicardial and intermyocardial connective tissues, and of the coronary vasculature. The epicardial progenitor (EPP)* cells originate from the pericardial surface of the septum transversum, called the proepicardial serosa (PS), in close proximity to the sinus venosus and the dorsal wall of the developing ventricle. In the PS, the EPP cells form bleb-like villi. The progenitor cells reach the heart by two cellular mechanisms. In the first mechanism, the villi form a transient tissue bridge that contacts the dorsal surface of the develop-

ing ventricle directly; once cells attach to the heart they spread as a continuous epithelial sheet. This mechanism predominates in avian embryos (Manner, 1992). In the second mechanism, EPP cells bud out from the villi as aggregates or cysts that float freely in the pericardial cavity; as the cysts contact the myocardial surface of the heart, they attach to the myocardium, and the cells spread from the cysts to form isolated patches of epicardial sheets, subsequently coalescing to form a coherent epicardium. This cyst-mediated mechanism predominates in dogfish and mammalian embryos (Viragh and Challice, 1981; Komiyama et al., 1987). In both mechanisms, the progenitor cells undergo active cell movement as they spread on the surface of the heart, as evidenced by the formation of ruffles and pseudopods on cells at the advancing edge of the spreading epicardial cells (Ho and Shimada, 1978).

The molecular mechanism by which the EPP cells adhere to and migrate on the surface of the heart is not known. We have shown previously that the gene encoding the α4 subunit of α4β1 integrin (α4β1), a cell adhesion receptor, is expressed in the EPP cells as they migrate onto the surface of the heart, suggesting that α4β1 integrin may play an important role in this migratory event (Pinco et al., 2001). α4β1 binds to fibronectin (Wayner et al., 1989; Guan and Hynes, 1990), a major component of the extracellular matrix, and VCAM-1 (Osborn et al., 1989; Elices et al., 1990), a mem-

Figure 1. **Generation of *lacZ* knockin mice.** (A) Restriction maps of wild-type *α4 integrin* locus, targeting vector, and targeted allele after recombination at the *α4 integrin* locus. The first and second coding exons of *α4 integrin* are represented as closed boxes. The *lacZ* cDNA is indicated by a hatched box. Arrows mark the transcription start site for the *α4 integrin* promoter. SA, splice acceptor; IRES, encephalomyocarditis virus internal ribosome entry site; pA, simian virus 40 polyadenylation sequence; Neo, pGK-neomycin resistance cassette; TK, pGK-thymidine kinase cassette. The bars under the restriction map of the targeting vector indicate the sizes of the flanking sequences homologous to the wild-type *α4 integrin* locus. The bars above the restriction map of the wild-type allele and below that of the targeted allele indicate the sizes of restriction fragments hybridizing to an external probe (heavy line). (B) Southern blot analysis of mice that are wild-type or heterozygous for the targeted allele. The 6- and 7-kb fragments were derived from the wild-type and targeted alleles, respectively. (C) PCR analysis of yolk sacs from wild-type embryos and those that were heterozygous or homozygous for the knockin allele. KI, knockin allele; WT, wild-type allele.
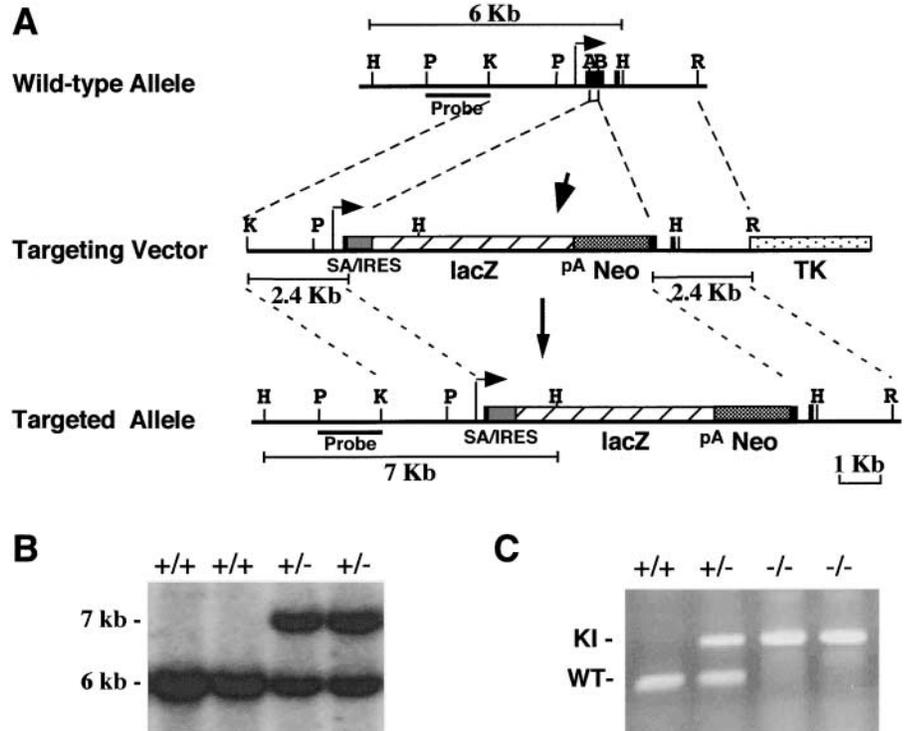
ber of the immunoglobulin superfamily. At late embryonic stages, α4β1 is localized to the epicardium, VCAM-1 to the myocardium, and fibronectin to the subepicardial space between the epicardium and the myocardium (Kwee et al., 1995; Yang et al., 1995). In *α4 integrin*– and *VCAM-1*–deficient embryos at embryonic day (E)11.5 the epicardium is missing (Kwee et al., 1995; Yang et al., 1995), although the epicardium in the α4-null embryos at E10–11 is normal (Yang et al., 1995). This result shows that the binding between α4β1 and VCAM-1 is essential for the maintenance of the epicardium, but α4β1 appears not to be essential for the formation of the epicardium.

In this paper, we report on our studies during which we generated a mouse in which the *lacZ* gene is knocked in at the *α4 integrin* locus, replacing the endogenous *α4 integrin* gene with the *lacZ* gene under the control of the *α4 integrin* promoter. This mouse line carries a null mutation of *α4 integrin* but has a genetic background different from the previous *α4 integrin* knockout mouse line. Our analysis of embryos that are homozygous for the knockin allele shows that α4β1 is essential for the EPP cells to migrate onto the surface of the heart to form the epicardium.

Results

Disruption of *α4 integrin* gene by knocking in *lacZ* at the *α4 integrin* locus

To introduce a marker for the cells that normally express the *α4 integrin* gene, we generated a knockin mouse in which *α4 integrin* was disrupted by replacing 91 bp of the first cod-ing exon with a *lacZ* reporter, placing *lacZ* under the control of the endogenous *α4 integrin* promoter. This replacement removed 22 of 39 residues of the signal peptide and 8 residues from the NH$_2$ terminus of the mature α4 integrin protein, leaving the 5′ untranslated region of the *α4* gene intact (Fig. 1 A). This knockin allele, *α4KI-lacZ*, resulted in the same detrimental effects as the previously generated *α4 integrin* knockout allele (Yang et al., 1995), which we refer to here as *α4KO*. As observed with the conventional knockout mutation, the knockin mutation resulted in two embryonic defects. 50% of the homozygous mutant embryos had a defect in chorio–allantois fusion and died at ∼E11; another 50% of the homozygous mutant embryos did not have this defect, but had a cardiac defect with hemorrhage and died at ∼E12 (unpublished data).

To determine if the *lacZ* gene was expressed under the control of the *α4 integrin* promoter, heterozygous *α4KI-lacZ* embryos at E8.5 and 9.5 were analyzed by X-gal staining for β-galactosidase activity. The *lacZ* expression pattern was compared with that of *α4 integrin* mRNA in wild-type embryos at the same stages. As judged by in situ hybridization, *α4 integrin* mRNA was expressed in the chorion (Fig. 2 A) and cranial neural crest cells (Fig. 2 C) at E8.5 (Pinco et al., 2001). At E9.5, *α4 integrin* mRNA was expressed in the PS and in migrating EPP cells (Fig. 2 E) (Pinco et al., 2001). The knocked-in *lacZ* was also expressed in the cells of the chorion, cranial neural crest, PS, and the migrating EPP cells (Fig. 2, B, D, and F). Thus, the *α4KI-lacZ* embryos expressed *lacZ* in a pattern consistent with the endogenous *α4 integrin* transcript.
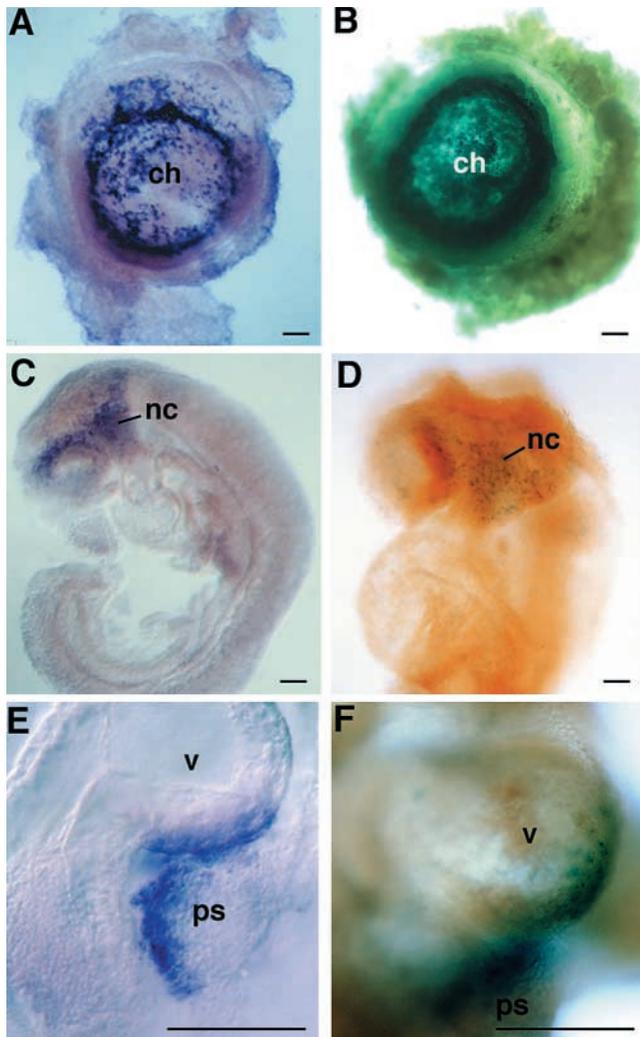
Figure 2. ***LacZ* is expressed under the control of the endogenous α*4 integrin* promoter in α*4KI-lacZ* embryos.** Heterozygous α*4KI-lacZ* embryos at E8.5–9.5 were stained with X-gal. Note that the patterns of expression of *lacZ* in these embryos (B, D, and F), as judged by X-gal staining, were identical to those of α*4* mRNA in wild-type embryos (A, C, and E). Both α*4 integrin* mRNA and X-gal were expressed in the chorion (A and B), cranial neural crest cells (C and D), and the PS and the EPP cells that are migrating onto the heart (E and F). ch, chorion; nc, neural crest cells; v, ventricle. Bars, 0.1 mm.

The epicardium fails to form in homozygous α*4KI-lacZ* embryos

Because the knocked-in *lacZ* was expressed in the PS and the EPP cells that are migrating onto the heart, *lacZ* provided a useful marker for these cells. We examined β-galactosidase activity by X-gal staining in E9.25 (22–24 somites) and 9.5 (26–28 somites) embryos homozygous (α4-null) or heterozygous (α4-positive) for the α*4KI-lacZ* allele. At E9.25, blue cells were seen in the PS area in both α4-positive and -null embryos (Fig. 3, A and B). Many blue cells were found on the heart in α4-positive embryos (Fig. 3 A), but only a few blue clumps were found on the heart in α4-null embryos (Fig. 3 B). At E9.5, the α4-positive embryos had fewer blue EPP cells in the PS area and more blue cells covering the heart (Fig. 3 C). In contrast, the α4-null embryos had significantly more blue cells in

the PS area, but hardly any blue cells on the heart except a few blue clumps (Fig. 3 D). This phenotype had 100% penetrance (n = 9).

To confirm that the blue cells covering the heart in α4-positive embryos were indeed the epicardial cells, we sectioned the X-gal–stained embryos in paraffin. The histological analysis showed that the blue cells on the hearts of α4-positive embryos formed a single layer of epicardial cells, which had a morphology distinct from the myocardial cells (Fig. 4, A, C, and E). On the hearts of α4-null embryos, this epicardial cell layer was absent; the few blue clumps were cell aggregates that failed to spread into an epithelial sheet (Fig. 4, B, D, and F). In α4-null embryos at E10.5 (see Fig. 6) and 11.5 (unpublished data), the epicardium was also missing, indicating that this epicardial defect was not due to a developmental delay. We conclude that the epicardium failed to form in homozygous α*4KI-lacZ* embryos.

EPP cells fail to migrate on the heart in the absence of α4 integrin

Lineage tracing studies have demonstrated that the epicardial cells originate from the PS (Manner, 1999). EM studies by others have shown that, in mouse embryos, EPP cells migrate onto the heart through a cyst-mediated mechanism (Viragh and Challice, 1981; Komiyama et al., 1987). The EPP cells bud out from the PS as cysts, which float freely in the fluid of the pericardial cavity and then attach to the surface of the heart near the PS or some distance away from the PS; once attached to the surface of the heart, the cells in the cysts proliferate and migrate out of the cysts as continuous epithelial processes. The cysts bud out from the PS at E9.25–9.5. In α4-positive embryos at this stage, some budded cysts were seen near the PS area that were *lacZ*-positive (Fig. 4 E, arrow); a majority of the cysts had attached to the heart, which were seen as *lacZ*-positive patches (Fig. 3 A, arrows). Whole-mount examination of the patches at a higher magnification showed that each patch is a cyst with outspreading processes (Fig. 3 E). Histological sections of these embryos showed that the blue patches of cells on the hearts were indeed epicardial cells (Fig. 4, A and C). In α4-null embryos at this stage, a majority of the cysts failed to reach the heart. Many *lacZ*-positive cysts were found in the peritoneal cavity (Fig. 4 F). These cysts must be mislocalized EPP cysts, as, in α4-positive embryos, no *lacZ*-positive cysts were found in areas other than the PS and the heart (Fig. 4 E). In the α4-null embryos, a few cysts were able to reach the heart, which were the blue clumps mentioned earlier (Fig. 3, B and D, arrows). These cysts formed close contact with the myocardium as shown by histological studies (Fig. 4, B and D), but the EPP cells in the cysts failed to migrate out (Fig. 3 F). At later stages, the cysts were often seen as large cell aggregates (Fig. 3, G and H), indicating that the EPP cells in the cysts proliferated but failed to spread into an epithelial sheet. These results indicate that, in the absence of α4β1, the EPP cells can adhere to the surface of the heart and proliferate, but cannot migrate, suggesting that α4β1 is essential for the migration of EPP cells on the surface of the heart.

Figure 3. **Whole mounts of X-gal–stained α4KI-lacZ embryos between E9.25 and 9.75.** E9.25 (A, B, E, and F), 9.5 (C and D), or 9.75 (G and H) embryos heterozygous (A, C, and E, α4-positive) or homozygous (B, D, F, G, and H, α4-null) for the α4KI-lacZ allele were stained with X-gal. Note that blue EPP cells were present on the heart of α4-positive embryos (A and C) but were largely absent from the heart of α4-null embryos (B and D). In α4-positive embryos, the EPP cells reached the heart by a cyst-mediated mechanism; the cysts released by the PS attached to the heart and the EPP cells in the cysts migrated out as processes (E, white arrowheads). In α4-null embryos, a few cysts attached to the heart (B and D, arrows) but the EPP cells failed to migrate out from the cysts (F–H). Black arrows, cysts that had attached to the heart. a, atrium; v, ventricle. Bars: (A–D) 0.5 mm; (E–H) 0.01 mm.
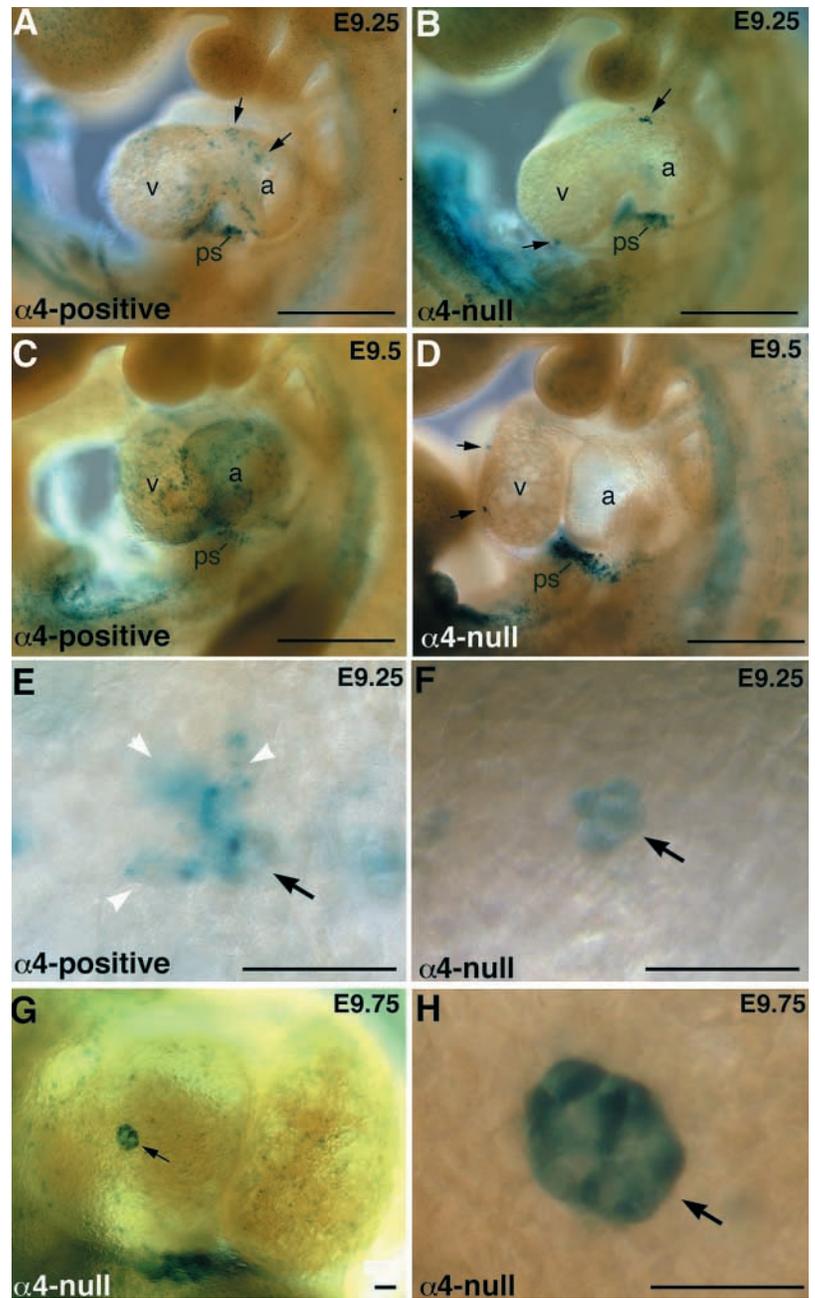
Formation of proepicardial cysts was less efficient in the absence of α4 integrin

As mentioned earlier, α4-null embryos at E9.5 had significantly more *lacZ*-positive cells in the PS area than the α4-positive embryos at the same stage (Fig. 3, C and D), suggesting that the cysts may bud less efficiently in α4-null embryos. To determine if this was the case, we counted the number of blue patches on the hearts of α4-positive embryos. We found that, at E9.25, the embryos had ∼30 cyst-derived patches on the heart. At E9.5, the number of patches doubled by estimation. In α4-null embryos at E9.25–9.5, far fewer cysts (an average of 3 cysts, *n* = 9) were present on the heart (Fig. 3, B and D, arrows). At E9.5, many cysts (an average of 10 cysts, *n* =5) were found in the peritoneal cavity (Fig. 4 F). Thus, the total number

of cysts in an α4-null embryo was <20 on average, much lower than that (i.e., the EPP patches on the heart) found in α4-positive embryos. Apparently, a majority of EPP cells failed to form cysts and remained in the PS. To determine if the cyst formation was defective in the α4-null embryos, we examined the PS area of these embryos more closely. We found that the PS in the α4-null embryos not only had many more *lacZ*-positive cells than those in the α4-positive embryos, but its morphology was also different from that in the α4-positive embryos. The PS of α4-positive embryos had a cauliflower-like shape (Fig. 5, A and C) and was an aggregate of hollow vesicles that were lined by *lacZ*-positive cells, many of them appeared to have already been released from the PS as cysts (Fig. 5 C, arrowheads). In contrast, the PS of the α4-null embryos had larger villi protrusions with budding cysts (Fig. 5, B and D); the villi
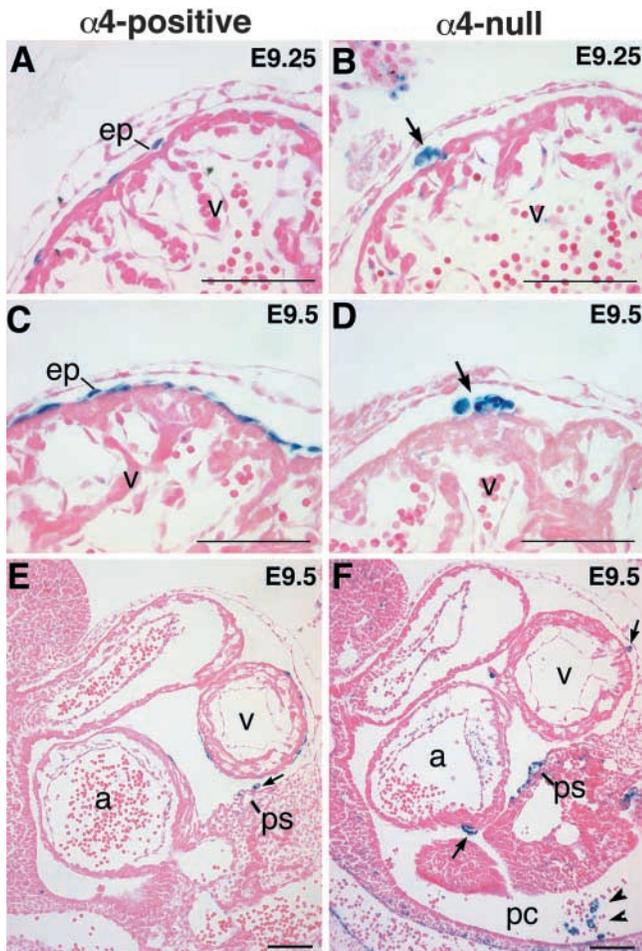
Figure 4. Histological analysis of EPP cells in *α4KI-lacZ* embryos at E9.25 and 9.5. E9.25 (A and B) or 9.5 (C–F) embryos were stained with X-gal and sectioned in paraffin. Note that in the heterozygous *α4KI-lacZ* embryos (A, C, and E, α4-positive) cysts bud out from the PS (E, arrow) and the blue EPP cells formed a single epicardial layer (A and C). In the homozygous *α4KI-lacZ* embryos (B, D and F, α4-null) many blue cysts failed to reach the heart and were found in the peritoneal cavity (F, arrowheads); some cysts attached to the heart (arrows) but they failed to spread into a single cell layer (B and D). a, atrium; v, ventricle; ep, epicardium; pc, peritoneal cavity. Bars, 0.1 mm.

were hollow and were lined by *lacZ*-positive cells, but they did not have the vesicle-like morphology seen in α4-positive embryos (Fig. 5 D). We counted the number of newly released vesicle-like cysts at the PS area of α4-positive and α4-null embryos in each of the histological sections that had the PS. The average number of newly released cysts found on the sections of α4-positive and α4-null embryos was ∼5 and 2, respectively. We conclude that budding of the cysts is less efficient in the α4-null embryos and α4β1 integrin is involved in this process.

Dual functions of α4β1 are revealed by the α4-null mutation on different genetic background

A mouse line carrying a knockout mutation of *α4 integrin* gene was previously generated (Yang et al., 1995). Histological analysis on embryos homozygous for this knockout allele (here we refer to this allele as *α4KO* to distinguish it from the *α4KI-lacZ* allele) shows that the epicardium is present at

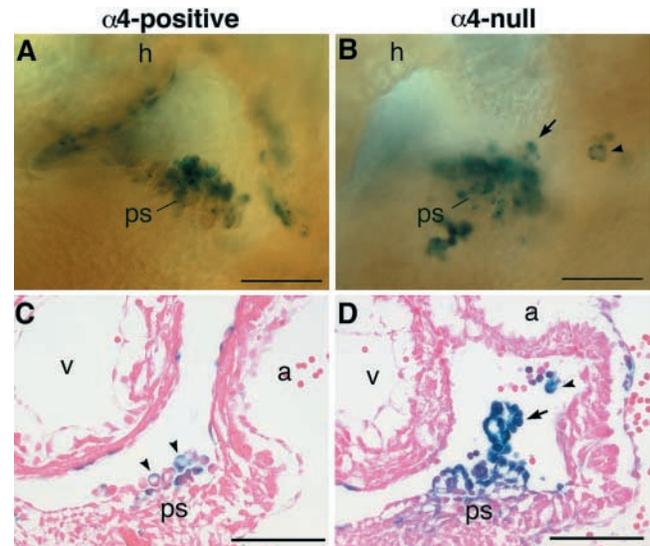**Figure 5. Whole-mount and histological analysis of PS in *α4KI-lacZ* embryos at E9.25.** E9.25 embryos that were heterozygous (A and C, α4-positive) or homozygous (B and D, α4-null) for *α4KI-lacZ* were stained with X-gal and photographed whole-mount (A and B) or sectioned in paraffin (C and D). Note that the morphology of the PS in α4-positive and α4-null embryos were different. Arrowheads, vesicle-like cell aggregates; arrows, villi with budding cysts. h, heart; a, atrium; v, ventricle. Bars, 0.1 mm.

E10.5 but absent at E11.5. At E10.5, these embryos formed a single epicardial layer surrounding both the primitive atrium and ventricle with a morphology identical to that in wild-type embryos at the same stage (Yang et al., 1995). In contrast, histological analysis on E10.5 embryos that were homozygous for the *α4KI-lacZ* allele showed no trace of an epicardial layer in the hearts (Fig. 6, B and E) with 100% penetrance (*n* = 10). To determine if the higher dosage of β-galactosidase in the homozygous *α4KI-lacZ* embryos had any dominant effect, we examined E10.5 embryos that were compound heterozygous for *α4KO* and *α4KI-lacZ*, which were α4-null but had only one copy of *lacZ*. The embryos (*n* = 6) also failed to form the epicardium (Fig. 6, C and F). Therefore, the more severe epicardial defect did not result from the expression of *lacZ*. It is likely that the more severe defect displayed by the *α4KI-lacZ* mice is due to variations in the genetic background. The mouse line carrying the *α4KI-lacZ* allele has a mixed genetic background of C57BL/6NCI and 129SvEvTac, whereas the line carrying the *α4KO* allele has a mixed genetic background of C57BL/6J and 129SvPas. It has been documented that significant genetic variability exists between the 129SvEvTac and 129SvPas lines (Simpson et al., 1997). We conclude that α4β1 has two independent functions in the development of the epicardium: migration of EPP cells to form the epicardium and maintenance of the epicardium once it is formed.

Localization of VCAM-1 and fibronectin in embryonic heart during epicardial development α4β1 has two ligands, VCAM-1 and fibronectin

It has been shown that at E11.5 α4β1 is localized to the epicardium, VCAM-1 to the myocardium and fibronectin to the subepicardial space between the epicardium and the

Figure 6. **Histological analysis of hearts in E10.5 embryos.** Mouse embryos at E10.5 were stained with X-gal and sectioned in paraffin. The genotypes of the embryo sections were: (A and D) α4KI-lacZ/+; (B and E) α4KI-lacZ/α4KI-lacZ; and (C and F) α4KI-lacZ/α4KO. Note that in all embryos, the knocked-in lacZ was expressed in the endocardial cushions and the PS. Embryos homozygous for α4KI-lacZ (B and E) or compound heterozygous for α4KI-lacZ and α4KO (C and F) did not form the epicardium. The arrow in C points to a cyst that attached to the heart but failed to spread. e, endocardial cushion; ep, epicardium; v, ventricle. Bars, 0.1 mm.
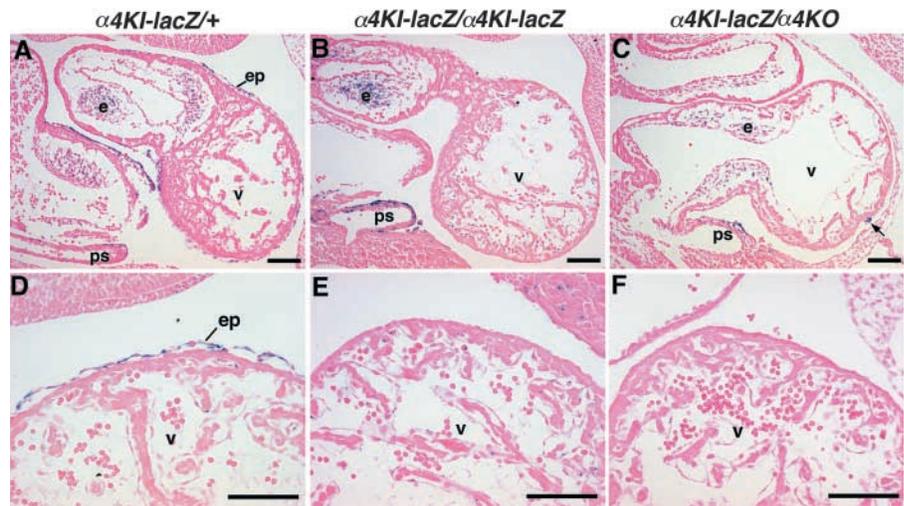
myocardium (Kwee et al., 1995; Yang et al., 1995). Here we examined the expression of VCAM-1 and fibronectin in the heart at E9.25 when EPP cells begin to migrate. We found that fibronectin was localized peripherally to the EPP cells that are budding out from the PS and to the myocardial cells (Fig. 7, A and C); VCAM-1 was localized in the myocardium (Fig. 7, B and D). Therefore, α4β1 may interact with fibronectin during the cyst-budding event and may interact with fibronectin and/or VCAM-1 as the EPP cells migrate out of the cysts that attached to the heart.

In vitro migration of PS explant cells required α4β1 integrin

To determine if the binding of α4β1 to VCAM-1 and fibronectin is essential for the migration of EPP cells, we performed a modified Boyden chamber assay on PS explant tissues. The PS is a loose tissue which can be readily dissected out. When the isolated PS explants were stained whole-mount with X-gal, the cells in the explants were blue, indi-

cating that the explants contained mostly EPP cells (unpublished data). In a Boyden chamber, a membrane with 8-μm pores separates the chamber into two compartments. In our study, the membrane was coated on both sides with VCAM-1 or fibronectin and the bottom compartment was filled with the same substrate. As a control, the membranes were not coated with any substrates. PS explants were placed in the top compartments and cell migration through pores was examined by nuclear staining of the cells at the bottom of the membrane. The results are summarized in Table I.

When the membranes were not coated with any substrates, 2 out of 18 α4-positive PS explants adhered to the membranes and had some cells migrating through the pores. Thus, the membrane or the PS explants can facilitate a low level of nonspecific background adherence and migration. When the membranes were coated with VCAM-1, all α4-positive explants (n = 14) adhered to the membranes and had cells migrating through the pores (Fig. 8, A and B). In contrast, seven out of eight α4-null explants failed to adhere

Figure 7. **Fibronectin and VCAM-1 localization by immunostaining.** Embryo sections were stained with an anti-fibronectin (A and C) or an anti–VCAM-1 antibody (B and D). C and D are higher magnifications of A and B, respectively, showing the PS region. Note that fibronectin is localized in the periphery of cysts that are budding out (arrowhead) and in the myocardium. VCAM-1 is localized in the myocardium. a, atrium; v, ventricle; my, myocardium. Bars, 0.1 mm.
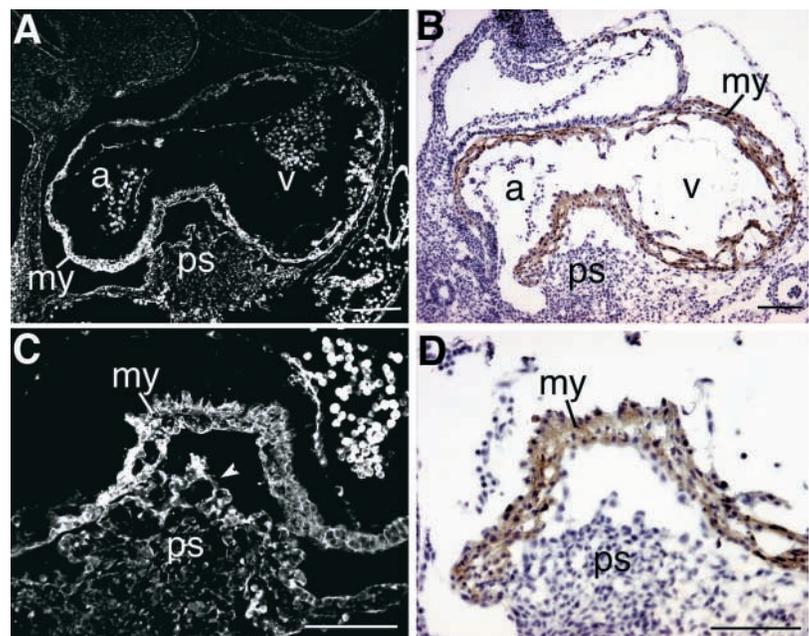
Table I. **Adhesion and migration of PS explants in vitro**

Substrate	Genotype	Total number of samples	Number of samples adhered[a]	Number of samples migrated[b]
No substrate	α4-positive	18	2 (11%)	2 (11%)
VCAM-1	α4-positive	14	14 (100%)	14 (100%)
	α4-null	8	1 (12.5%)	1 (12.5%)
Fibronectin	α4-positive	12	12 (100%)	12 (100%)
	α4-null	7	7 (100%)	0 (0%)

[a]Samples that showed nuclear staining on the top side of the membrane.
[b]Samples that showed nuclear staining on the bottom side of the membrane.

to VCAM-1–coated membranes. We conclude that α4β1 is required for VCAM-1–dependent adhesion of the PS explant. The one α4-null sample that adhered also had cells migrating through the pores. With only one sample, we could not draw any conclusions about the requirement for α4β1 in VCAM-1–dependent migration of the PS explant. Nevertheless, our results showed that, in the presence of α4β1, the PS explants were able to migrate on VCAM-1.

All α4-positive ($n = 12$) and α4-null ($n = 7$) explants adhered to fibronectin-coated membranes; however, only the α4-positive ($n = 12$) and not the α4-null ($n = 7$) explant cells migrated through the pores (Fig. 8, C–F). This result indicates that α4β1 is required for fibronectin-dependent migration of PS explant cells in vitro. However, explant cells can adhere to fibronectin in the absence of α4β1.

Discussion

α4 integrin is a marker for EPP cells

In this paper, we analyzed a knockin mouse in which the α4 integrin gene was replaced with lacZ. By comparing the lacZ expression pattern with α4 integrin mRNA in wild-type embryos, we showed that the knocked-in lacZ is faithfully expressed under the control of the α4 integrin promoter. Both α4 integrin mRNA and the knocked-in lacZ are expressed in the PS and the newly formed epicardium, making α4 integrin/knocked-in lacZ a marker for the EPP cells. Because there are no existing markers for the EPP cells, we carried out histological analyses to confirm that the cells expressing α4 integrin/knocked-in lacZ were indeed the progenitor cells that gave rise to the epicardium. We showed that the lacZ-positive cells in the PS of α4KI-lacZ embryos form the cauliflower-shaped tissue that has been shown by lineage tracing studies to give rise to the epicardium (Manner, 1999). It has been well established that in mammals, the EPP cells migrate onto the heart predominantly through a cyst-mediated process (Viragh and Challice, 1981; Komiyama et al., 1987). In heterozygous α4KI-lacZ mice, we observed lacZ-positive cysts that were released from the PS, and that landed and spread on the heart in a manner identical to the previously described cyst-mediated process. Finally, by histological analysis, we showed that the lacZ-positive cells
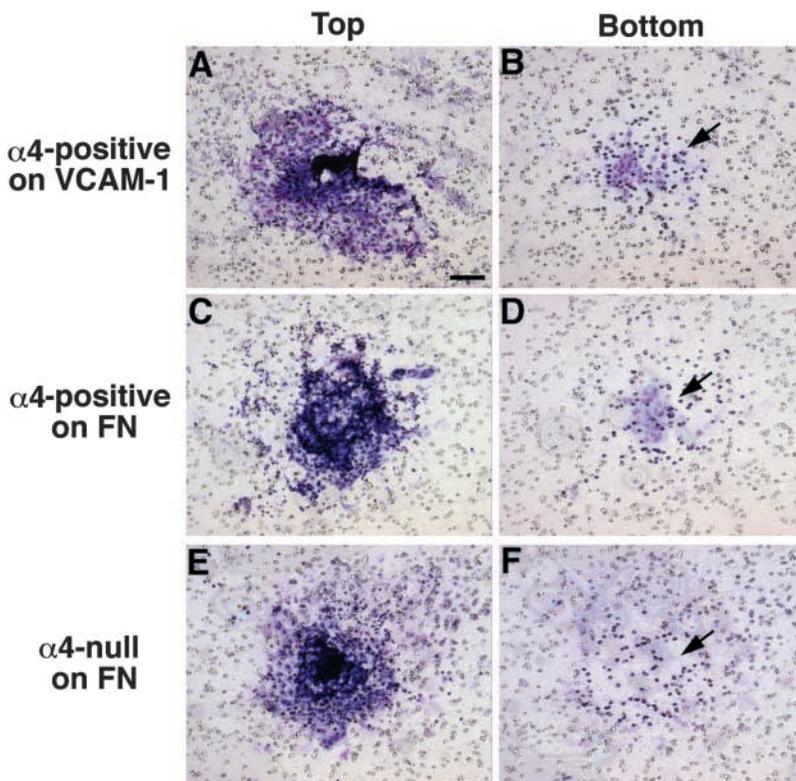

Figure 8. **Adhesion and migration of PS explant cells in Boyden chambers.** PS explants from α4-positive (A–D) or -null (E–F) embryos were placed on membranes coated with VCAM-1 (A and B) or fibronectin (C–F). After overnight incubation, each explant was photographed (A, C, and E) and removed from the top side; the bottom side was then photographed (B, D, and F). Note the nuclei of explant cells that adhered to VCAM-1 and fibronectin (A, C, and E) and migrated through pores (B and D, arrows). α4-null explant cells failed to migrate through pores (F, arrow). FN, fibronectin. Bar, 0.1 mm.

from the cysts that spread on the heart are epicardial cells. We conclude that *α4 integrin*/knocked-in *lacZ* is expressed in the EPP cells and in the newly formed epicardium and can be used as a marker for these cells. *α4 integrin* is also expressed in neural crest cells (Kil et al., 1998; Pinco et al., 2001). However, there is no evidence for any contribution of neural crest cells to the epicardial cell lineage. Cardiac neural crest cells give rise to the outflow tract of the heart and may also contribute to the endocardial cushions. Both tissues express *α4 integrin*, but they are anatomically distinct from the epicardium. Thus, the expression of *α4 integrin* in these tissues does not prevent the use of *α4 integrin* as a marker for the epicardial cell lineage.

α4β1 is essential for the migration of EPP cells on the myocardial surface of the heart

The *lacZ* marker in the *α4KI-lacZ* mouse allowed us to study the role of α4β1 in a unique cyst-mediated migratory event during the formation of the epicardium. We found that, in the absence of α4β1, the proepicardial cysts budded from the PS less efficiently; once released, a majority of the cysts failed to reach the heart and were found in the peritoneal cavity instead. A few cysts did reach and attach to the heart, but the EPP cells in the cysts failed to migrate out to form an epithelial sheet. Thus, α4β1 is involved in multiple events during the cyst-mediated migration of EPP cells to form the epicardium.

EM studies have shown that, as the EPP cells spread on the surface of the heart, the advancing edge of the cells forms ruffles and pseudopods and actively moves (Ho and Shimada, 1978). Active cell spreading movement could be regulated by differential cell–cell and cell–substratum adhesion (Ryan et al., 2001). In the case of the EPP cells spreading on the myocardium, the adhesive strength of the EPP cells to the myocardium (substratum) may depend on the binding of α4β1 on the surface of EPP cells to VCAM-1 on the surface of the myocardial cells. In the absence of α4β1, the EPP cells may become less adhesive to the myocardium and adhesion among the EPP cells may become the predominant force, preventing the cells from migrating out. Integrin-mediated signaling can regulate cadherin expression in skeletal muscle (Huttenlocher et al., 1998). Cross talk between integrin- and cadherin-mediated pathways may also occur in the migration of EPP cells. Alternatively, α4β1 may play a direct role in regulating cell motility. The latter idea is supported by studies showing that the cytoplasmic tail of α4 promotes cell motility (Kassner et al., 1995), and by our in vitro Boyden chamber assay showing that the α4-null PS explant cells have a defect in fibronectin-dependent cell motility. However, the two mechanisms may not be mutually exclusive.

α4β1 has two major ligands, VCAM-1 and fibronectin. Both of them are localized to the myocardium; fibronectin is also localized to the PS. To determine the role of these ligands in the adhesion and migration of the EPP cells, we tested the ability of PS explant cells to adhere and migrate on these ligands. Our data show that α4β1 is required for PS explant cells to adhere to VCAM-1. VCAM-1 is essential for the attachment of mature epicardial cells to the myocardium (Kwee et al., 1995). It is likely that interactions between α4β1 and VCAM-1 also play an important role in the attachment of proepicardial cysts to the myocardium. In contrast to VCAM-1, we show that fibronectin can facilitate adhesion of PS explants in the absence of α4β1. This fibronectin-dependent adhesion may be mediated by another fibronectin receptor, such as α5β1, which is also expressed in EPP cells (unpublished data). Such an integrin(s) may also facilitate the attachment of the α4-null proepicardial cysts to the myocardial surface in vivo. Although all α4-null PS explants adhered to fibronectin, none had cells migrating on fibronectin. This result indicates that α4β1 is required for the PS explant cells to migrate on fibronectin. Because fibronectin is localized to the myocardium and to the periphery of budding proepicardial cysts, it is likely that the interaction between α4β1 and fibronectin plays key roles in the migration and morphogenetic movement of EPP cells on the heart and in the PS. Because VCAM-1 is localized in the myocardium, and the α4-positive PS explant cells can migrate on VCAM-1, VCAM-1 may also play an important role in the α4β1-dependent migration of EPP cells in vivo.

In the homozygous *α4KI-lacZ* embryos, only a few cysts attached to the heart; the majority of them landed in the peritoneal cavity. This defect could be due to a failure of the cysts to attach to the myocardium or to navigate onto the heart. It has been thought that the proepicardial cysts emigrate to the heart by a free-floating mechanism (Komiyama et al., 1987). Our data has opened a possibility that this migration may not be a random event, but may be regulated by factors secreted by the myocardium. Because fibronectin is localized on the myocardium, it may also be released by the myocardium into the fluid in the pericardial cavity, which may provide chemotactic activities for attracting the cysts. Alternatively, the cysts may simply be less adhesive to the heart in the absence of α4β1.

Another role of α4β1 during epicardial development is in the formation of the proepicardial cysts. The mechanism of cyst formation is not known, although the vesicle-like morphology of the PS and the epicardial fate of the cysts has been well established based on lineage tracing and EM studies (Manner et al., 2001). This event apparently involves movement of cells in the PS to form vesicles that are lined by a single layer of epithelial cells, a morphogenetic event that is reminiscent of vasculogenesis where progenitor cells coalesce into a epithelium that encloses a lumen. In the absence of α4 integrin, the EPP cells form larger villi and the cysts bud less efficiently. It is possible that the vesicle/cyst formation requires precise adhesion of the EPP cells with their surrounding extracellular matrix, which could be mediated by the binding between α4β1 and fibronectin, as fibronectin is localized at the periphery of the budding cysts in the PS.

Phenotypic variation between *α4KO* and *α4 KI-lacZ* mice is due to difference in their genetic backgrounds

An *α4 integrin* knockout mouse (*α4KO*) was generated previously, which has a different genetic background and shows a less severe epicardial defect compared to the *α4KI-lacZ* mouse. In homozygous *α4KO* embryos, the epicardium forms initially but is lost at a later stage (Yang et al., 1995). When the embryos are compound heterozygous for the *α4KI-lacZ* and *α4KO* alleles, they have the same EPP defect

as the homozygous *α4KI-lacZ* embryos. The results from the compound heterozygotes show that (a) the EPP defect is not due to the knocked-in *lacZ*; and (b) the embryos have the EPP defect as long as α4β1 is absent and the embryos carry a partial genetic background of the *α4KI-lacZ* strain. The phenotypic difference between the *α4KI-lacZ* and *α4KO* strains might be explained by an involvement of more than one pair of cell adhesion molecules. In the *α4KO* strain, another pair of cell adhesion molecules may compensate for the function of α4β1 in epicardial formation, but not for the function of this integrin in maintaining the epicardium. However, in the *α4KI-lacZ* strain the other pair of cell adhesion molecules might not be able to rescue the function of α4β1 in epicardial formation due to some dominant modifier(s). Because the homozygous (α4-null) and heterozygous (α4-positive) *α4KI-lacZ* embryos have the same genetic background, yet only the homozygotes have the EPP defect, α4β1 must play a major role in EPP formation. Thus, the EPP defects of our *α4KI-lacZ* mice reveal essential functions of α4β1 in the formation of the epicardium in addition to the previously shown function of this integrin in maintaining the epicardium. The new in vivo functions of α4β1 uncovered in this study may provide important insight into the role of α4β1 in cell migration and morphogenesis.

Materials and methods

Generation of knockin mice

The *α4 integrin* genomic locus was targeted by homologous recombination using a 5.7-kb Exon 1–containing genomic fragment (Yang et al., 1995), which was ligated into Bluescript SK (Stratagene). A 91-bp Ava I/Bsm I fragment in the first exon of the *α4 integrin* gene was replaced with a DNA insert containing a splice acceptor (Friedrich and Soriano, 1991), an encephalomyocarditis virus internal ribosome entry site (Ghattas et al., 1991), the *lacZ* cDNA, a simian virus 40 polyadenylation sequence (Friedrich and Soriano, 1991), and a pGK-neo cassette (McBurney et al., 1991). This insertion leaves 2.4 kb of the genomic DNA on both sides of the insert. A PGK-tk cassette was inserted at the 3′ end of the genomic fragment (Fig. A). The linearized plasmid was electroporated into MC3 embryonic stem (ES) cells, generated and provided by the Johns Hopkins University School of Medicine (Baltimore, MD) transgenic facility. The cells were selected with 300 μg/ml G418 and 2 μM gancyclovir. Homologous recombination was detected in 25% of the drug-resistant ES cell colonies by Southern blot analysis using an external 5′ probe (Fig. 1, A and B). The targeted clones were injected into C57BL/6NCI blastocysts to generate chimeras. F1 founders were generated by crossing chimeric males to C57BL/6NCI females. For routine genotyping, PCR analysis was performed using internal primers (Fig. 1 C) as follows: wild-type, 5′-AGCCTGAAGCATC-CCTGG-3′ 5′-GGTCCAGGTTGTAGGAGTG-3′; targeted, 5′-TGACCGCT-TCCTCGTG-3′ 5′-GAGTAGCCAAACAGCGTGC-3′. The *α4KO* mouse line was provided by Richard Hynes (MIT, Cambridge, MA).

Visualization of β-galactosidase in whole-mount and histological sections of mouse embryos

Whole mount X-gal staining was performed as described (Hogan et al., 1994). Briefly, embryos were fixed in 0.2% glutaraldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer, pH 7.3, 5 mM EGTA and 2 mM MgCl₂. The fixed embryos were washed three times in 0.1 M phosphate buffer, pH 7.3, containing 0.02% Nonidet-P40 and 2 mM MgCl₂. The embryos were then stained overnight at 37°C with 1 mg/ml X-gal in wash buffer with 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide. The embryos were photographed using Axioskop Microscope (Carl Zeiss) in conjunction with a Photometrics Coolsnap fx CCD camera controlled by IPLab-spectrum software (BioVision Technologies, Inc.).

For histological studies, the X-gal–stained embryos were postfixed in 4% paraformaldehyde overnight at 4°C. The fixed embryos were dehydrated through an ethanol series, cleared in xylene and embedded in paraffin. Sections (8 μm) were counterstained with eosin.

VCAM-1 and fibronectin immunohistochemistry

Frozen sections were used for immunostaining with an antibody against VCAM-1 (M/K-2; American Type Culture Collection). The immunostaining procedure was described previously (Yang et al., 1995). Briefly, the frozen sections were fixed in acetone, washed two times in PBS containing 0.1% BSA, blocked with blocking buffer (0.1% BSA and 10% normal goat serum in PBS), and incubated with the anti–VCAM-1 antibody (diluted in blocking buffer). The sections were then washed in PBS, incubated with biotinylated secondary antibody, washed again in PBS, incubated with 0.3% H₂O₂ in methanol, washed in PBS, and incubated with ABC reagent mix (Vector Laboratories). The sections were then incubated with DAB/H₂O₂ substrate, counterstained with hematoxylin, dehydrated in ethanol and xylene and mounted.

Paraffin sections were used for immunostaining with an antiserum against fibronectin, provided by Richard Hynes (MIT, Cambridge, MA). The immunostaining procedure was as described (George et al., 1993). Briefly, paraffin sections were dewaxed in xylene and dehydrated through a graded ethanol series into H₂O. The sections were blocked with 10% fibronectin-depleted serum, 0.05% Tween-20, and 0.02% azide in PBS. The same blocking buffer was used to dilute the primary and secondary antibodies. After blocking, the sections were incubated with the primary antibody, washed in PBS, incubated with rhodamine-conjugated secondary antibody, washed three times in PBS, and mounted.

Modified Boyden chamber assay

A modified Boyden chamber assay was carried out in Micro Chemotaxis Chambers (Neuro Probe, Inc.) as described (McCarthy et al., 1983). 10 μg/ml mouse plasma fibronectin (Life Technologies) or soluble VCAM-1, provided by Roy Lobb (Biogene, Cambridge, MA) in DME were added to the bottom compartments of the chambers. Membranes (8-μm pore size) were overlaid onto the wells, the chambers assembled and the same substrate was added to the top compartments. The chambers were incubated at 37°C for 2 h to coat the membrane with the substrate on both sides. The substrate in the top compartment was then removed and PS explant was placed in the top compartments along with DME. The chambers were incubated at 37°C overnight. The membranes were then removed, fixed in methanol for 10 min, stained with Giemsa (Sigma-Aldrich), and rinsed with water. After photographing the PS explants on the membrane, the top side of the membrane was wiped with a Q-tip to remove all of the cells that remained on the top side. The bottom side of the membrane was then photographed. The appearance of Giemsa-stained nuclei on the top and bottom sides was examined. The nuclear staining on the top side indicates cell adhesion, and that on the bottom side indicates migration through pores.

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