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Embryonic mesodermal defects in α_5 integrin-deficient mice

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SUMMARY

A loss of function mutation of the murine α_5 integrin gene generated by gene targeting in embryonic stem cells is a recessive embryonic lethal. The mutant embryos start to show observable defects by day 9 of gestation and die around day 10-11. The α_5-null embryos have pronounced defects in posterior trunk and yolk sac mesodermal structures, suggesting a role for $\alpha_5\beta_1$ integrin in mesoderm formation, movement or function. However, the embryos progress significantly further than embryos null for fibronectin, for which $\alpha_5\beta_1$ integrin is a receptor, suggesting the involvement of other fibronectin receptors. In vitro studies on cells derived from the α_5-null embryos confirm that the $\alpha_5\beta_1$ integrin is not expressed on mutant cells and show that the mutant cells are able to assemble fibronectin matrix, form focal contacts, and migrate on fibronectin despite the complete absence of the $\alpha_5\beta_1$ fibronectin receptor integrin. All these functions have previously been thought to involve or require $\alpha_5\beta_1$. The results presented show that these cellular functions involving fibronectin can proceed using other receptors.

Key words: α_5 integrin, fibronectin, embryonic stem cell

INTRODUCTION

Adhesive interactions between cells and their surrounding extracellular matrix (ECM) play critical roles in development and in a variety of physiological and pathological processes (Hynes and Lander, 1992; Adams and Watt, 1993). Cell-ECM interactions are mediated primarily by a family of cell surface receptors called integrins (Hynes, 1992). Integrins are heterodimeric transmembrane glycoproteins providing links between extracellular matrix and intracellular cytoskeleton, and may be involved in signal transduction processes. The ligands for integrins include a number of ECM proteins such as fibronectin (FN), laminin, collagens and vitronectin. Even though each integrin has a characteristic ligand specificity, many integrins have multiple ECM ligands and each ECM protein has multiple integrin receptors.

$\alpha_5\beta_1$ integrin is a FN receptor, which binds to the RGD region of FN (Pytela et al., 1985). Studies using various cell culture systems have suggested that $\alpha_5\beta_1$ is involved in many cellular processes including cell proliferation and oncogenic transformation (Plantefaber and Hynes, 1989; Giancotti and Ruoslahti, 1990; Schreiner et al., 1991), assembly of FN-rich extracellular matrices (Roman et al., 1989; Akiyama et al., 1989; Fogerty et al., 1990; Giancotti and Ruoslahti, 1990), cell migration (Akiyama et al., 1989; Schreiner et al., 1989; Giancotti and Ruoslahti, 1990; Bauer et al., 1992), regulation of gene expression (Werb et al., 1989), wound healing (Guo et al., 1991), and T cell activation (Shimizu and Shaw, 1991).

It has also been suggested that $\alpha_5\beta_1$ may play important roles during embryogenesis and differentiation. $\alpha_5\beta_1$ is expressed at high levels in early *Xenopus* embryos (Whittaker and DeSimone, 1993) and is widely expressed in chicken embryos, whereas its expression in adult tissues is more limited (Muschler and Horwitz, 1991). α_5 is expressed by invading human cytotrophoblast cells (Damsky et al., 1992), in developing heart and lung (Roman et al., 1991; Roman and McDonald, 1992) and in developing and regenerating peripheral nerves (Lefcort et al., 1992). As nerves develop, the levels of $\alpha_5\beta_1$ and its ligand, FN, fall. This pattern of early expression followed by down-regulation is characteristic of this integrin in a variety of systems including keratinocytes (Adams and Watt, 1990) and erythroid precursors (Patel and Lodish, 1986; Rosemblatt et al., 1991). The regulated expression of $\alpha_5\beta_1$ integrin suggests that it may be performing one or more of the functions revealed by the in vitro analyses but that proposition has been difficult to test in intact embryos. Furthermore, there exist multiple integrins which can act as receptors for FN (Hynes, 1992) raising the possibility of functional redundancy.

In this paper, we report our studies on the in vivo functions of the α_5 integrin subunit. By gene targeting in mouse embryonic stem (ES) cells, we have generated a null mutation of α_5. We report that this mutation is a recessive embryonic lethal, and that the α_5-null embryos display severe posterior and extraembryonic mesodermal defects. We also examined cultured α_5-null embryonic cells to study the role of $\alpha_5\beta_1$ in FN matrix assembly, focal contact formation and cell migration. While confirming the importance of $\alpha_5\beta_1$ integrin in embryogenesis, the results demon-

strate that many cellular processes in which this integrin has been implicated can proceed in its absence.

MATERIALS AND METHODS

Construction of the targeting vector

A 860 bp 5′ fragment of mouse α_5 integrin cDNA (D. J. G. Rees and R. O. Hynes, unpublished) was used to isolate a genomic clone from a library derived from a D3 mouse ES cell line (provided by D. Gray and R. Jaenisch, Whitehead Institute, M.I.T.). Restriction mapping, Southern blot analysis and DNA sequencing were used to locate the first coding exon of the α_5 integrin gene and a 6.9 kb fragment containing this exon was ligated into Bluescript SK (Stratagene). A PGK-neo-poly(A) cassette (McBurney et al., 1991) isolated from plasmid pKJ1 by digestion with *Eco*RI and *Hin*dIII was inserted between two *Eag*I sites within the 6.9 kb fragment; the 5′ *Eag*I site lies in the intron upstream from the initiation codon, and the second *Eag*I site lies within the first coding exon. This insertion results in replacement of 330 bp of the α_5 integrin gene, leaving 850 bp of the α_5 integrin locus (the short arm) 5′ and 5.7 kb (the long arm) 3′ of the neomycin-resistance gene. A PGK-tk cassette (Rudnicki, et al., 1992) was inserted at the end of the short arm of the construct (see Fig. 1A).

Growth, transfection and selection of targeted ES clones

Methods for growth and transfection of ES cells were described previously (George et al., 1993). The D3 line of ES cells (Doetschman et al., 1985, a generous gift from Dr Janet Rossant, Mt. Sinai Hospital, Toronto, Ontario, Canada) was cultured on irradiated fibroblast feeders in ES cell medium, which contains DMEM (high glucose) with 26 mM Hepes, pH 7.5, 14 mM sodium bicarbonate, 15% fetal bovine serum (Hazleton), 1× non-essential amino acids (Gibco) and 0.1 mM β-mercaptoethanol, supplemented with 1000 units/ml LIF (ESGRO from GIBCO-BRL).

4×10^7 ES cells, our passage 6, in 1.6 ml cold Hepes-buffered saline (25 mM Hepes, 134 mM NaCl, 5 mM KCl and 0.7 mM Na_2PO_4, pH 7.1) plus 40 μg linearized targeting vector DNA were electroporated at 240 volts and 500 micro-Farads in a Gene Pulser (Biorad) and plated on twelve 10-cm plates containing G418-resistant mouse embryonic fibroblast feeder cells for 26 hours before initiation of selection. Two plates were selected with G418 alone to determine transfection frequency. All other plates were selected with 200 μg/ml active G418 (Gibco) and 2 μM gancyclovir (Syntex Corp.). After 8 days of selection, 288 double-resistant colonies were picked and expanded on feeder cells. Half the cells from each colony were frozen in 10% DMSO in fetal bovine serum and half were lysed for DNA extraction.

DNA extraction and Southern blot analysis

ES cells were washed with PBS and lysed (150 mM NaCl, 20 mM Tris, pH 7.5, 5 mM EDTA. 5% SDS and 0.25 mg/ml proteinase K) for 30 minutes at 37°C and lysates were stored at −20°C. DNA was prepared by phenol extraction and ethanol precipitation and redissolved in 50 μl of 10 mM Tris (pH 7.5) and 0.1 mM EDTA. The same procedure was used for extracting DNA from yolk sacs and embryos, except that lysis was overnight. The method of Laird et al. (1991) was used to extract DNA from tail biopsies. Southern blot analyses were carried out as described elsewhere (Church and Gilbert, 1984), using a 2.2 kb *Kpn*I/*Nco*I fragment 5′ of the targeting vector as probe (Fig. 1A).

Generation of germline chimeras

Embryo manipulations were carried out as described (Bradley, 1987). ES cells were prepared and injected into C57Bl/6J blastocysts as described by George et al. (1993). Chimeric progeny were identified by coat color and chimeric males were bred to C57Bl/6J females. Germline transmission of the mutant allele was detected by Southern blot analysis of tail DNA.

Culture of embryonic cells

To culture embryonic cells, the heads and hearts of E8.5-9.5 embryos were removed and the remainder washed twice in PBS and twice in ES medium and dissociated with sterile tweezers. The dissociated tissues in 1 ml of ES medium were plated in a well of a 24-well plate precoated with 0.1% gelatin. After 7 days in culture, cells were either used for surface iodination, or subcultured to obtain a more homogeneous cell culture. Cells were trypsinized and half the cell suspension transferred to an uncoated well containing 1 ml of ES medium. After 1 hour at 37°C, the medium was removed, and the cells were washed to remove non-adherent cells. Fresh medium was added to the plate, which at this point contained a relatively homogeneous population of fibroblastic cells. If cells were incubated more than 1 hour before replacing the medium, a clustered cell type remained in addition to the fibroblastic cells. After 5-7 days culture, cells were either used for experiments or subcultured by trypsinization. The genotype of each embryonic cell culture was tested by Southern blot analysis.

Surface iodination and immunoprecipitation

Cell surface labeling with ^{125}I was performed by the lactoperoxidase-glucose oxidase method (Hynes, 1973). Cells were labelled with 100 μCi of Na [^{125}I] (Du Pont, NEN) per well, washed and lysed with 800 μl cold lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5 mM $CaCl_2$, 0.5% NP-40). Lysates were preabsorbed with 100 μl of protein A Sepharose (Pharmacia LKB) for 1 hour, the Sepharose beads removed by centrifugation and the resulting supernatants used for immunoprecipitation.

For immunoprecipitation, 200 μl of cell lysate, 10 μl of 5% NP-40 and 90 μl of 10 mg/ml BSA (Boehringer Mannheim) in lysis buffer were mixed. 5 μl of antiserum against α_5 (160, Hynes et al.,1989), β_1 (363, Marcantonio and Hynes, 1988) or α_5 preimmune serum were then added to the tube. After incubation for 1 hour, 30 μl of protein A-Sepharose beads (a 1:1 slurry preabsorbed with 10 mg/ml BSA in lysis buffer) were added. After 1 hour at 4°C, the beads were sedimented and washed 4× with cold lysis buffer, followed by boiling in 40 μl non-reducing SDS sample buffer. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis on 7% resolving gels with 4% stacking gels (Laemmli, 1970).

Immunofluorescence

For examining FN matrix assembly, cells were cultured in ES medium for 2 days on coverslips coated with 0.5% gelatin and stained with an antiserum against rat plasma FN (61.1, prepared as described by Mautner and Hynes, 1977). For examining focal contacts, cells were cultured overnight in 1% fetal bovine serum on coverslips coated with 10 μg/ml rat plasma FN and stained with antibodies against vinculin (Sigma), talin (Sigma), β_1 (363, Marcantonio and Hynes, 1988) and α_5 (160, Hynes et al.,1989). Embryonic sections were stained with anti-FN serum, as described (George et al., 1993).

Cell migration assay

Cell migration was carried out in Micro Chemotaxis Chambers (Neuro Probe Inc.) as described by McCarthy et al. (1983). Rat plasma FN was diluted in DME and 35 μl were dispensed into blind well portions of the microchamber. Membranes (8-μm pore size, Neuro Probe Inc.) were overlaid onto the wells, the chambers assembled and 50 μl of cell suspension (3×10^5 cells/ml in serum-free DME) were added to each top well of the assembly. Chambers were incubated for 4 hours at 37°C, the membranes were removed and fixed in methanol for 5-7 minutes, stained with Giemsa

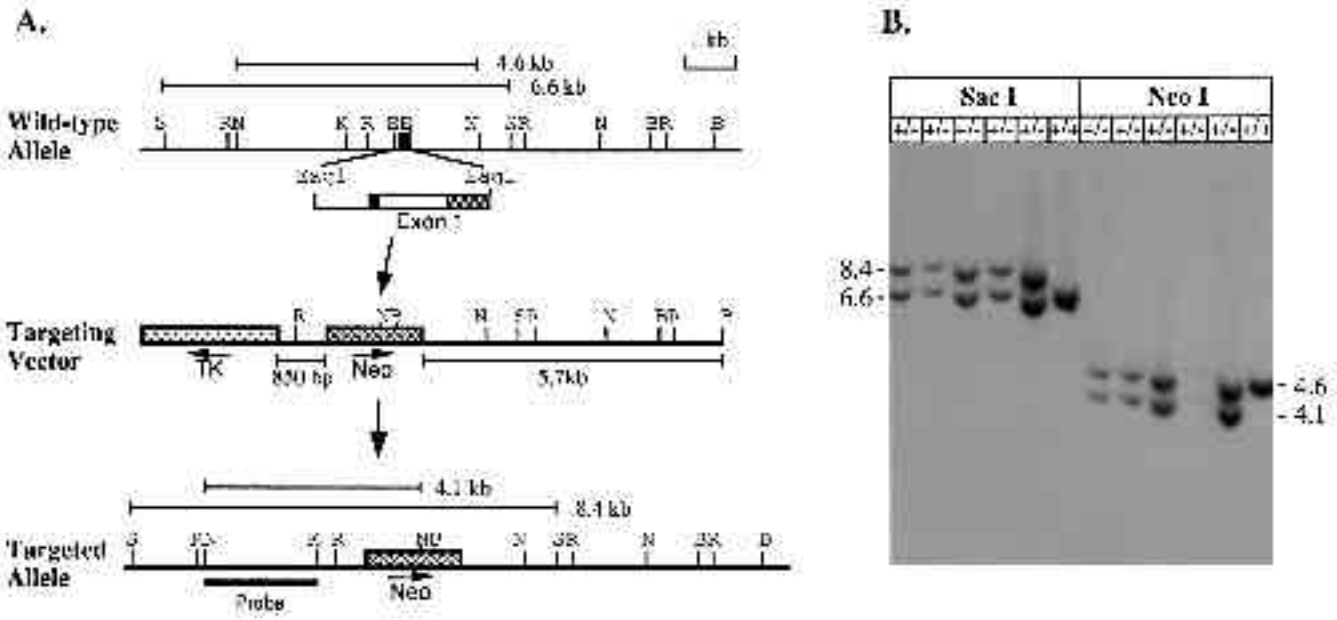

Fig. 1. Disruption of the first coding exon of the α_5 integrin gene in ES cells. (A) Targeting vector and recombination at the α_5 integrin locus. Restriction maps of the wild-type allele, the targeting vector and the targeted allele of the α_5 integrin gene are shown. An *Eag*I fragment containing most of the first coding exon is enlarged to illustrate the structure of the exon: black box, 5′ untranslated sequence; open box, signal sequence; shaded box, sequence encoding the mature protein. The PGK-neomycin resistance cassette and PGK-thymidine kinase cassette are designated as Neo and TK, respectively; arrows show the orientations of the genes. The bars under the restriction map of the targeting vector indicate the sizes of the flanking sequences homologous to the wild-type α_5 integrin locus. The bars on top of the restriction maps of wild-type and targeted alleles indicate the sizes of restriction fragments hybridizing to the probe used for Southern blot analysis (heavy line). Restriction sites: B, *Bam*HI; E, *Eag*I; K, *Kpn*I; N, *Nco*I; R, *Eco*RI; S, *Sac*I. (B) Southern blot analysis of targeted ES cells. The 8.4 kb and 4.1 kb fragments were derived from the wild-type allele; the 6.6 kb and 4.6 kb fragments were derived from the targeted allele (see A).

(Sigma), rinsed with water and air dried. Cells that had migrated through the membrane were counted; 15 non-overlapping high power fields (200×) per well, and the data were analyzed using the *t*-test in Statworks (Cricket Software, Inc).

PCR analysis of genotypes of embryos in paraffin sections

Embryonic tissue scraped from several sections was pooled in 30 μl double-distilled H_2O, incubated with 10 ng proteinase K (55°C for 20 minutes, 95°C for 10 minutes) and divided in half for separate PCR analyses with primers specific for the wild-type and targeted alleles. Maternal decidual tissue was used for positive controls. PCR (polymerase chain reaction) conditions were as described by the manufacturer of Taq polymerase (Perkin-Elmer Cetus) except that primers were annealed for 2 minutes at 55°C and 40 amplification cycles were performed. Oligonucleotide primers were as follows.

Wild-type allele: 5′-CGTTGAGTCATTCGCCTCT-3′;
5′-CTACCGCGTCTAGGTTGAAGC-3′.
Targeted allele: 5′-GACAATCGGCTGCTCTGA-3′;
5′-GCAGGCATCGCCATG-3′.

The amplified PCR product was separated by electrophoresis on a 1% agarose gel, and analyzed by Southern hybridization (Church and Gilbert, 1984).

Morphological and histological analysis

Embryos were dissected as described by Cockroft (1990). Whole-mount embryos were examined and photographed (Ektachrome160T film) with a dissecting microscope (Nikon). For histology, embryos were fixed in Carnoy's solution (60% ethanol, 30% chloroform and 10% glacial acetic acid) for 45 minutes when dissected or for 6 hours when in deciduo, dehydrated through an ethanol series, cleared in xylene and embedded in paraffin. Sections (8 μm) were stained with Harris hematoxylin and eosin, examined and photographed (Tri-X film, Eastman Kodak) with an Axiophot microscope (Carl Zeiss).

RESULTS

Disruption of the α_5 integrin gene in ES cells by homologous recombination

A genomic DNA fragment was isolated containing the first coding exon of the α_5 integrin gene, encoding the 5′ untranslated sequence, the signal peptide (43 amino acids) and 32 amino acids of the mature protein, and was used to construct a targeting vector (Fig. 1A) for disrupting the α_5 integrin gene in ES cells. A 330 bp *Eag*I fragment containing most of the first coding exon was replaced with a neomycin-resistance cassette for positive selection (see Materials and Methods). Since this replacement removes the translational initiator, ATG, and the sequence encoding the entire signal peptide, a complete disruption of the gene should result upon homologous recombination between the targeting vector and the chromosome. In order to select against cells with random insertions (negative selection), a herpes simplex virus thymidine kinase gene was also added to the targeting vector (Mansour et al., 1988).

The targeting vector was transfected into D3 ES cells and cultured in double selection medium containing G418 and gancyclovir. Resistant colonies were picked, expanded and

screened by Southern blot analysis (Fig. 1A,B). After screening 288 clones, 7 independent positive clones were obtained. Considering a 4-fold enrichment from the double selection, the targeting frequency was 1/164. Fig. 1 shows that the sizes of the targeted bands were as predicted from the restriction map. The targeted restriction fragments also hybridized to a probe derived from the neomycin-resistance cassette, and no other neomycin-positive bands were detected (data not shown), indicating that the mutated gene fragment was inserted as expected, and that no random insertions occurred.

The α_5 integrin mutation is a recessive embryonic lethal

Three independent targeted ES clones were injected into C57Bl/6J blastocysts, and the blastocysts transferred to pseudopregnant females. All three lines generated offspring with 30 to 100% chimerism of coat color, and chimeric animals from all three transmitted the mutation through the germline. Mice heterozygous for the mutation were identified by Southern blot analysis of tail DNA. Heterozygous mice did not display any obvious abnormalities; they were of normal size and fecundity and, up to one year of age, have not demonstrated any obvious defects.

To obtain mice homozygous for the α_5 mutation, the heterozygous mice were intercrossed. Among 120 liveborn progeny, there were 38 homozygous wild-type and 82 heterozygotes, but no homozygous mutant offspring. Thus, the α_5 mutation is a recessive embryonic lethal mutation. The genotypes of embryos derived from the intercrosses were determined by Southern blot analysis using DNA extracted either from the yolk sacs or from the embryos themselves. The results are summarized in Table 1. At days 8.5 to 10.5 of gestation, deformed embryos were observed and Southern blot analysis showed that all these were homozygous α_5 mutants. The genotypes of embryos dissected at days 8.5 and 9.5 of gestation showed a fairly good Mendelian distribution. At day 10.5 of gestation, on the other hand, the numbers of homozygous mutant embryos were reduced and most showed obvious deterioration. Of a total of 56 embryos dissected at days 11.5 and 12.5, no homozygous mutants were found. We conclude that the α_5 mutants die around days 10 to 11 of gestation.

Table 1. Genotypes of progeny from intercrosses beween heterozygous parents

		Genotype		
Stage	Total	+/+	+/−	−/−
E8.5	68	18	31	19*
E9.5	124	35	62	27*
E10.5	70	22	37	11*
E11.5	41	20	20	0
E12.5	15	4	11	0
E16.5	6	4	2	0
P28	120	38	82	0

*The embryos were abnormal.

Homozygous mutant embryos do not express α_5 integrin at the cell surface

To verify that we have indeed generated an α_5 integrin null mutation, we cultured cells from day 8.5 embryos (see Materials and Methods) obtained from heterozygous intercrosses and examined them for the presence of α_5 integrins on the surfaces by surface iodination and immunoprecipitation using an antiserum against α_5 integrin; preimmune serum and anti-β_1 integrin antiserum were used as controls. The genotypes of these embryos were determined by Southern blot analysis of DNA extracted from their yolk sacs (Fig. 2A). While both α_5 and β_1 integrins were immunoprecipitated from wild-type and heterozygous embryonic cells (Fig. 2B, embryos 1, 3 and 5), only β_1, but not α_5, was precipitated from the homozygous mutant cells (Fig. 2B, embryos 2 and 4). These results show that α_5 integrin is absent from the surfaces of cells derived from homozygous mutant embryos and that we have generated a true α_5-null mutation.

Homozygous α_5-null embryos have posterior defects

The phenotypes of α_5-null homozygous mutant embryos at days 8-10.5 of gestation were studied by examining whole-mount embryos. At days 8-8.5, most of the mutant embryos had similar numbers of somites as their wild-type littermates (4-13 pairs). However, the anteroposterior axes of the mutants were slightly shorter (Fig. 3 A-F). At day 8, the curvatures of the mutant embryos differed from the wild-type

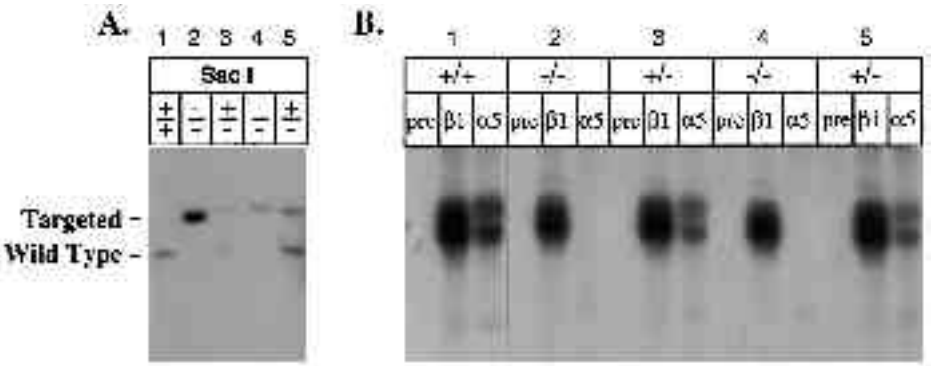

Fig. 2. Expression of α_5 integrin on the surfaces of cells derived from wild-type and mutant embryos. (A) Southern blot analysis of DNA extracted from the yolk sac of each embryo and digested with *Sac*I. The probe for the Southern blot analysis is shown in Fig. 1A. (B) Immunoprecipitations of surface-labeled embryonic cells. Cells cultured from the embryos were surface-labeled with ^{125}I, lysed and immunoprecipitated using antisera against α_5 or β_1 or preimmune serum, as described in Materials and Methods. A number of integrin α subunits associating with β_1 were coprecipitated when β_1-specific antiserum was used. The upper major band in the gel represents a number of integrin α subunits that comigrated on the SDS-gel; the lower major band represents β_1. However, β_1 was coprecipitated with α_5 when α_5-specific antiserum was used. Notice that α_5-specific antiserum failed to precipitate any proteins from homozygous mutant cells. pre, preimmune serum; β1, β_1-specific antiserum; α5, α_5-specific antiserum.

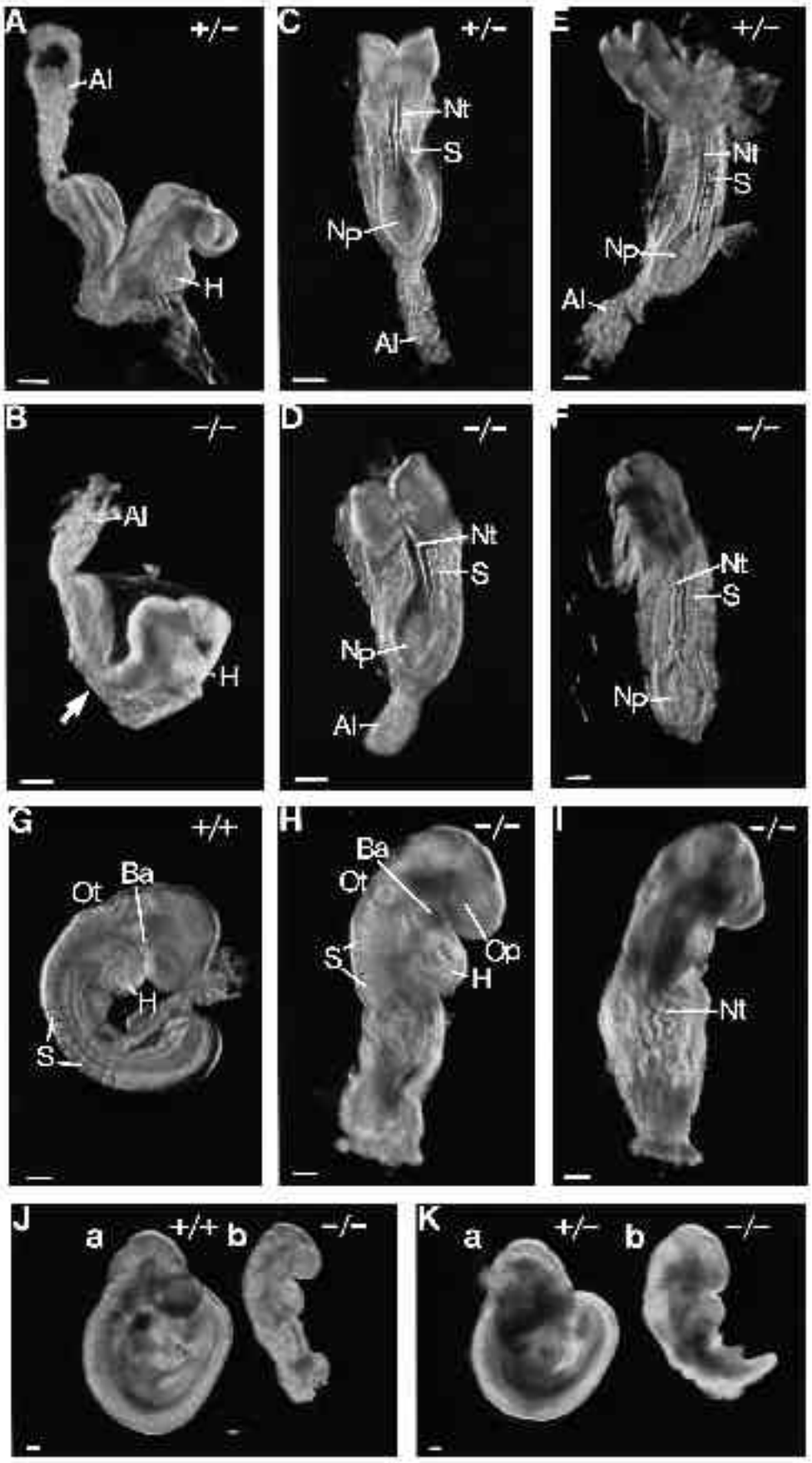

Fig. 3. Whole-mounts of wild-type, heterozygous and α_5-null embryos at days 8 to 10.5 of gestation (E8-E10.5). (A) Side view of an E8 wild-type embryo. The embryo has not turned. (B) Side view of an E8 mutant embryo. The embryo is similar to the wild-type embryo in A, except that the curvature of the mutant embryo is slightly different (marked by an arrow). (C) Dorsal view of the E8 wild-type embryo in A. The embryo has 4 pairs of somites. (D) Dorsal view of the E8 mutant embryo in B. The embryo also has 4 pairs of somites, but its anteroposterior axis is slightly shorter than that of the wild-type embryo in C and the neural folds are atypical in shape. (E) Dorsal view of an E8.5 wild-type embryo. The embryo has 13 pairs of somites. (F) Dorsal view of an E8.5 mutant embryo. The embryo has 7 pairs of somites. The allantois of the embryo was present but was removed during dissection. Notice the distortion of the neural tube. (G) Side view of an E9 wild-type embryo. The embryo has turned to a fetal position, with about 15 pairs of somites. (H) Side view of an E9.5 mutant embryo. The posterior trunk region of the embryo is deformed. Although delayed, the anterior region of the embryo is relatively normal, similar to that of the E9 wild-type embryo in G, with 7 pairs of somites in the anterior region. Note the presence of otic and optic anlagen. (I) Dorsal view of the mutant embryo in H. Notice the absence of somites and kinked neural tube in the deformed posterior region of the embryo. (J) Wild-type (a) and a mutant (b) embryo from the same litter (E9.5). Notice that the mutant embryo is delayed. (K) An E9.5 wild-type embryo (a); an E10.5 mutant embryo (b). Notice that the mutant embryo lacks the posterior trunk, while its anterior part is similar to that of the E9.5 wild-type embryo. Abbreviations: Al, allantois; Ba, branchial arch; H, heart; S, somites; Np, neural pore; Nt, neural tube; Ot, otic pit; Op, optic vesicle. The bar at the lower-left corner of each embryo corresponds to 0.2 mm.

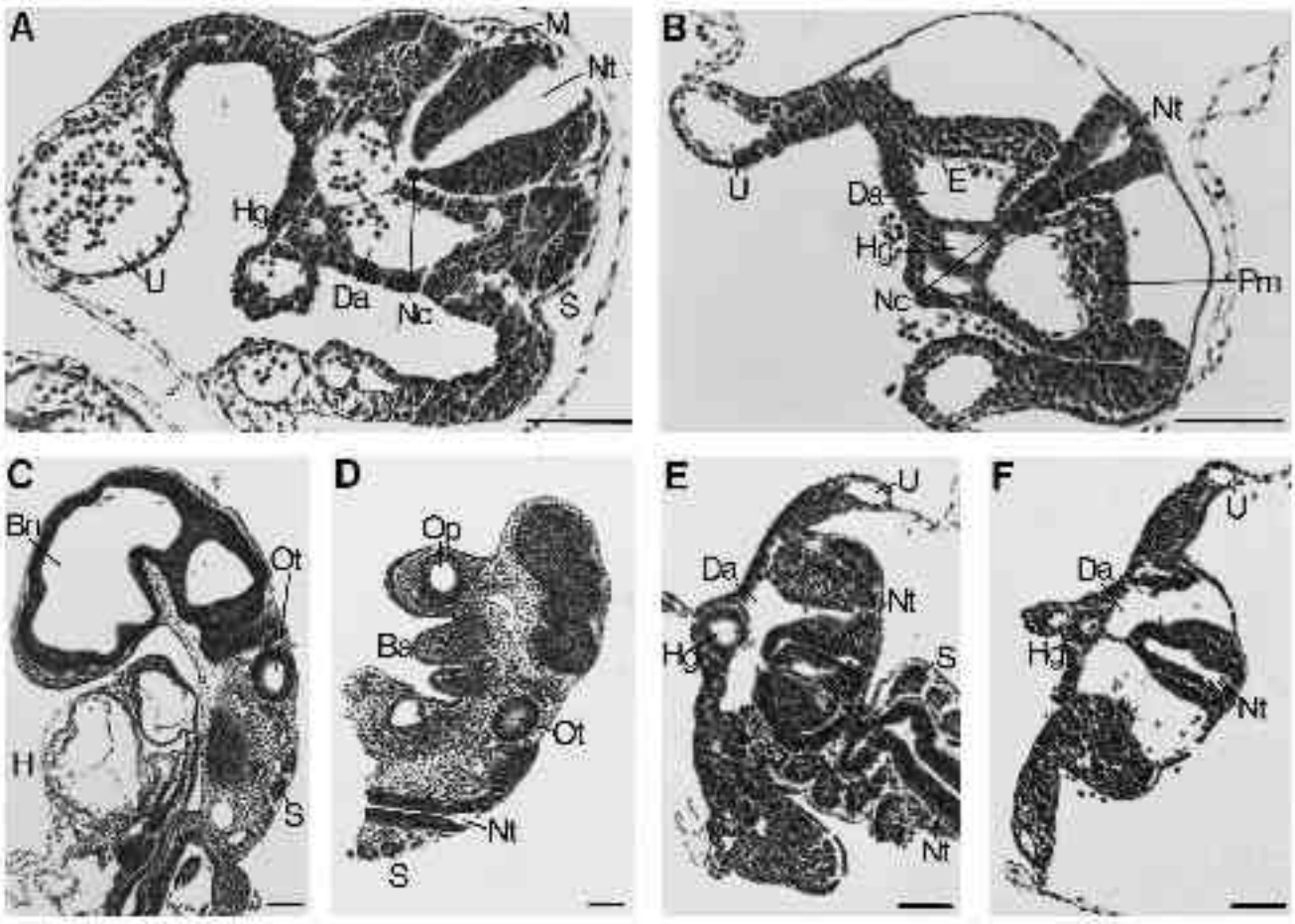

Fig. 4. Histological analysis of wild-type and α_5-null embryos at day 9.5 of gestation. (A) A transverse section of the posterior trunk region of a wild-type embryo. The neural tube is flanked by somites, which are derived from paraxial mesoderm. On the dorsal side of the somites are mesenchymal cells, which are either mesoderm-derived, or neural crest cells. (B) A transverse section of the posterior trunk region of a mutant embryo. Notice the empty space flanking the neural tube as a result of the deficit in paraxial mesoderm and mesenchymal cells. Also notice the leakage of primitive blood cells into the abnormal space, which is more prominent in other sections (e.g. see F). (C-F) Nonadjacent, serial sections of a mutant embryo. The anterior of the mutant is fairly normal (C and D), whereas the posterior shows neural tube and mesodermal defects (E and F). Notice the kinked neural tube in E and defective paraxial mesoderm and dorsal aorta in F. Abbreviations: Ba, branchial arch; Bn, brain; Da, dorsal aorta; E, endothelium; H, heart; Hg, hindgut; M, mesenchymal cells; Nc, notochord; Nt, neural tube; Ot, otic pit; Op, optic vesicle; Pm, paraxial mesoderm; S, somites; U, umbilical vein. The bar at the lower-right corner of each picture corresponds to 0.1 mm.

(Fig. 3A,B). At day 8.5, the mutants showed clear distortions of their neural tubes (Fig. 3E,F). At day 9.5, the mutant embryos were significantly delayed and were equivalent in size to a normal day 9 embryo (Fig. 3G,H,J). By day 9, wild-type embryos had turned into the fetal position, and their posterior trunks had extended in a C shape (Fig. 3G). In contrast, day 9.5 mutant embryos were only partially turned, and their posterior trunks were truncated, with a length only about one third that of a normal day 9 embryo (Fig. 3H). At this stage, the truncated posterior trunk regions of the mutants had pronounced defects. The neural tubes were very kinked and somites were absent (Fig. 3I). However, the anterior regions of mutant embryos, including the heads, branchial arches and hearts appeared grossly normal. The neural tubes and somites (7-10 pairs) appeared normal anteriorly (Fig. 3H,I). By day 10.5, the sizes of the mutant embryos were equivalent to those of normal day 9.5 embryos. The heads and hearts of the mutants appeared to develop relatively normally, but the posterior regions failed to develop (Fig. 3K).

The posterior trunk regions of α_5-null mutants have neural tube and mesodermal defects

To study the phenotype of α_5-null mutants further, histological sections of day 9.5 embryos were examined (Fig. 4). Fig. 4A,B shows transverse sections of a wild-type and a homozygous mutant embryo in the posterior trunk region. Typically, transverse sections of the deformed posterior trunk regions of homozygous mutant embryos showed absence of somites and reduced mesoderm in the region flanking the neural tube, leaving empty spaces in these areas. In some cases, somite-like structures appeared to form, but were localized away from the neural tube, lateral in the sections (Fig. 4E,F). Mesenchymal cells, normally present on the dorsal side of the somites, were also missing. The dorsal aortae were not fully closed, resulting in leakage of

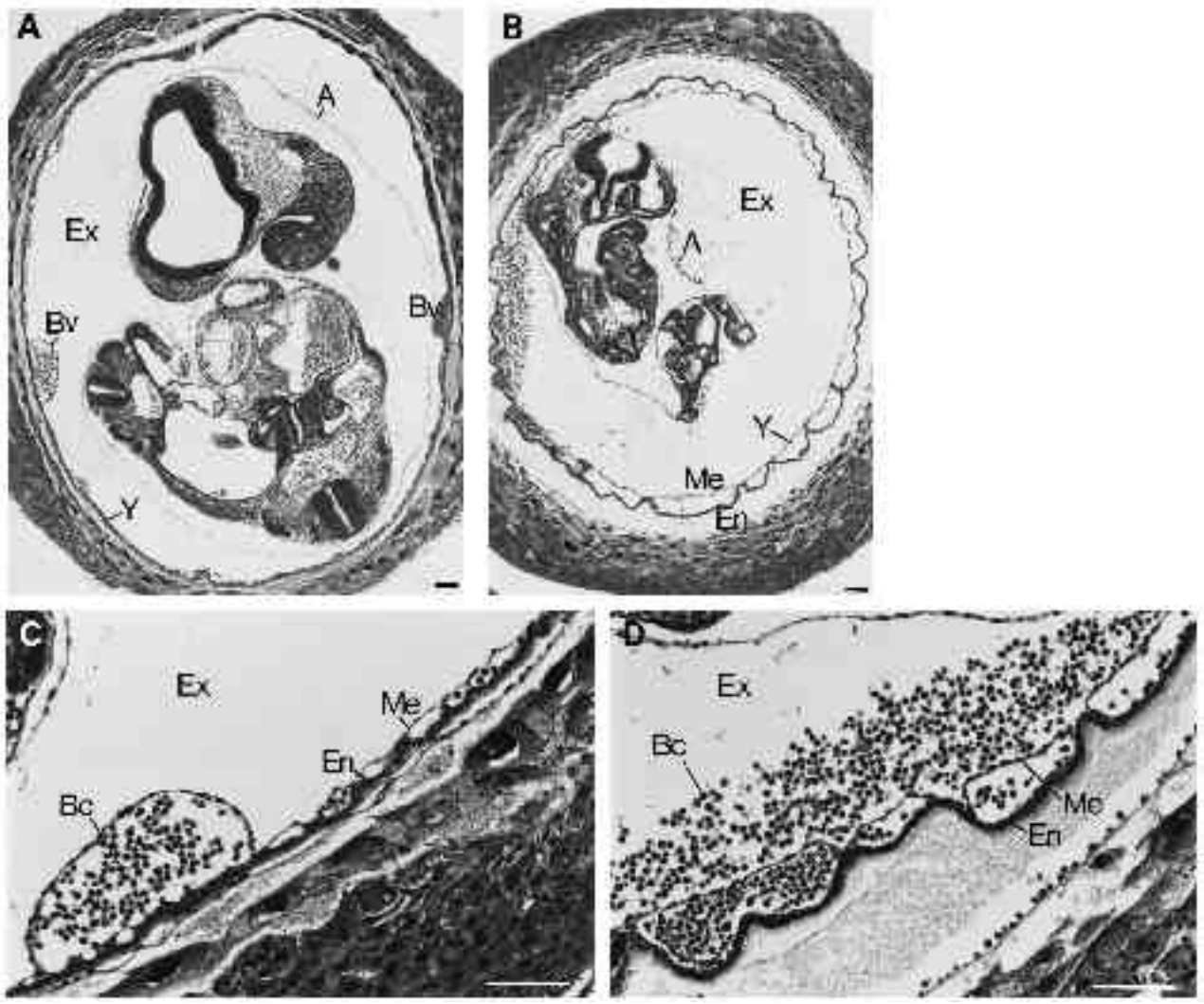

Fig. 5. Histological analysis of extraembryonic tissues of wild-type and α_5-null embryos. (A) A transverse section of an E9.5 wild-type embryo. The blood islands have fused to form blood vessels. (B) A transverse section of a mutant embryo at day 9.5 of gestation. The blood vessels are not appropriately formed. The extraembryonic endoderm and mesoderm in the yolk sac are separated. Notice that the surface area of the mesoderm appears to be less than that of the endoderm and the yolk sac as a whole appears retracted toward the embryo. Also notice the leakage of primitive blood cells from extraembryonic mesoderm into the exocoelomic space. (C) The section in A at higher magnification. Notice that the primitive blood cells are contained within the blood vessels. (D) The section in B at higher magnification. Note that blood cells are also present in the exocoelomic space. Abbreviations: A, amnion; Bv, blood vessel; En, extraembryonic endoderm; Me, extraembryonic mesoderm; Ex, exocoelomic space; Bc, primitive blood cells; Y, yolk sac. The bar at the lower-right corner of each picture corresponds to 0.1 mm.

blood cells into the abnormal mesoderm-deficient space, even though endothelium appeared to form along the wall of the dorsal aorta in most cases. The lateral structures such as umbilical vein were indistinguishable from those of wild-type embryos. Notochord appeared to form in mutant embryos, although it was not clear whether the notochords were normal. Serial sections of a mutant embryo are shown in Fig. 4C-F. The first two sections are sagittal and parasagittal sections of the anterior region of the mutant embryo, which show relatively normal anterior structures including brain, heart, branchial arches, neural tube and somites. The following two sections show the deformed posterior region of the embryo, in which distortion of the neural tube and the deficit of mesodermal structures flanking the neural tube are apparent. No distinct boundary was apparent between the relatively normal and deformed regions in the mutant embryos, and the defects became more severe towards the deformed posterior trunk region.

Homozygous α_5-null embryos have vascular and extraembryonic defects

At day 8 of normal gestation, blood islands begin to form within the extraembryonic mesoderm of the yolk sac, where primitive blood cells are formed. At day 9.5 of gestation, blood islands have fused to form blood vessels in the yolk sacs of wild-type embryos. In contrast, sections of day 9.5 mutant embryos (Fig. 5) showed that the blood vessels were not appropriately formed, and large numbers of blood cells

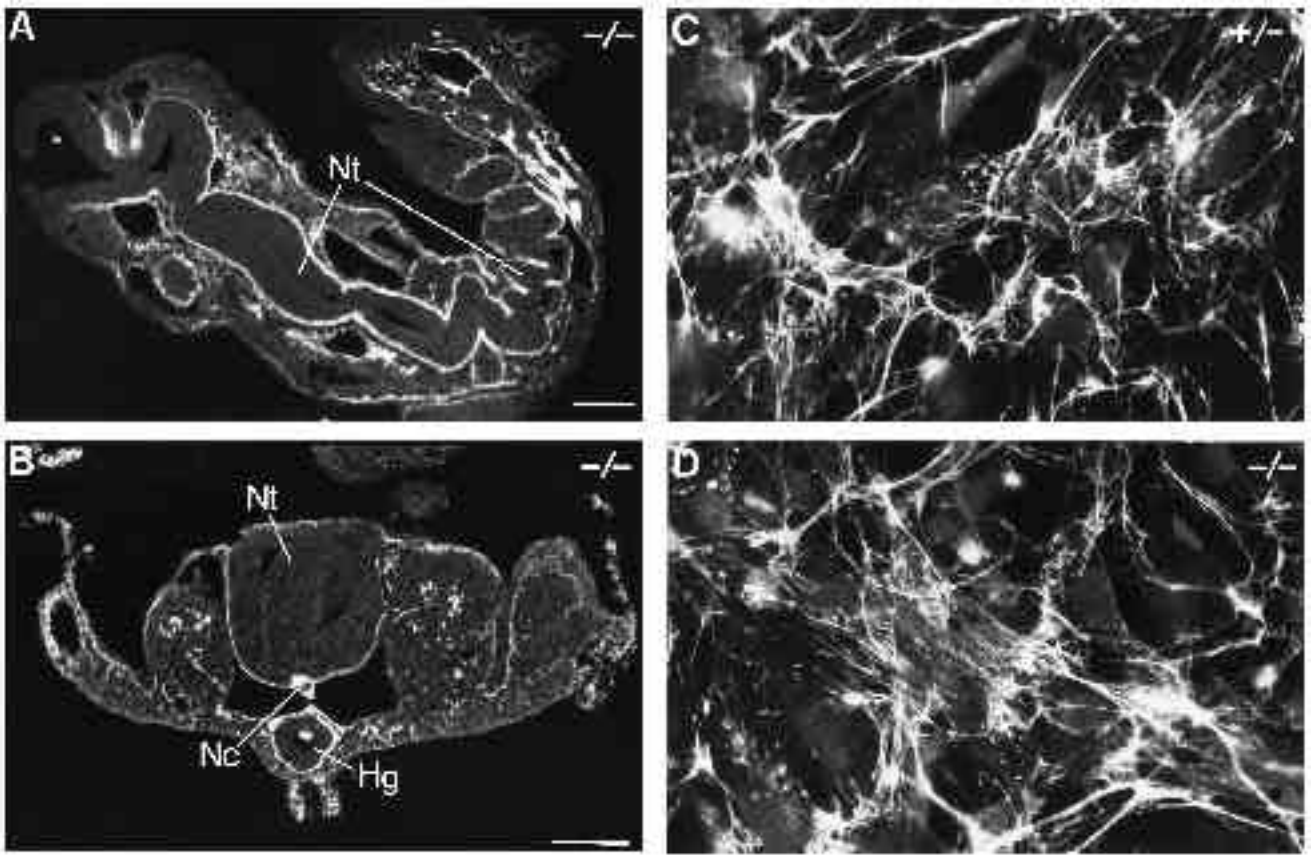

Fig. 6. Immunofluorescence showing expression of FN and assembly of FN matrix by wild-type and α_5-null embryos. (A,B) Immunofluorescence of longitudinal (A) and transverse (B) sections of E9.5 mutant embryos using an antiserum against FN. Note the distribution of FN in basement membranes around neural tube, notochord and hindgut and in the mesoderm flanking the neural tube. A clearly shows the kinky neural tube characteristic of α_5-null mutant embryos. Abbreviations: Nc, notochord; Nt, neural tube; Hg, hindgut. The bar at the lower-right corner of each picture corresponds to 0.1 mm. (C,D) Immunofluorescence of cells derived from wild-type (C) and mutant (D) embryos using antiserum against FN. Notice that the FN matrix assembled by the mutant cells is similar to that formed by the wild-type cells.

had leaked into the exocoelomic space. The extraembryonic mesoderm was separated from the extraembryonic endoderm as a result of defective cell-matrix adhesion or a deficit of mesoderm. It appeared that the overall surface area of the mesodermal layer was less than that of the endoderm, perhaps leading to splitting apart of the two layers. The blood appeared to be diffused rather than contained within discrete blood vessels. Although primitive blood cells were able to enter the embryonic circulation, fewer blood cells were present in the heart and blood vessels of the embryos (Fig. 4). These observations are consistent with there being leakage of blood cells from the vessels of the mutant embryos, suggesting that there were vascular defects both in the embryo and in the extraembryonic vasculature, which may be the cause of developmental arrest of the α_5-null embryos beyond day 9.5 of gestation.

Cells derived from α_5-null embryos are able to assemble FN matrix

Since in vitro data have suggested that $\alpha_5\beta_1$ integrin may be important for FN matrix assembly, we examined FN matrix formation in α_5-null mutant embryos. Immunofluorescence staining of embryonic sections showed that FN was expressed in the α_5-null mutant embryos, and the pattern of FN-rich matrix was indistinguishable from that of wild-type embryos (Fig. 6A,B).

To examine closely whether α_5-null cells are able to assemble FN matrix, we cultured cells from day 8.5 embryos from heterozygous intercrosses and stained them with antiserum against FN. In the embryonic cell cultures, two morphologically distinct cell types were obtained. One type looked fibroblastic, adhered to plastic tissue culture dishes and spread well; the other cell type was more rounded and formed clusters. These clustered cells did not adhere well to the culture dish unless a monolayer of the fibroblastic cells covered the dish. There was no obvious difference in growth properties between cells from normal and mutant embryos. Both cell types assembled extensive FN matrices when confluent, and the matrices formed by the mutant cells were indistinguishable from those produced by wild-type cells (Fig. 6C,D).

Focal contact formation and migration of α_5-null cells

The fibroblastic cells derived from α_5-null mutant embryos were also stained with antibodies against vinculin and talin to examine the formation of focal contacts by these cells when plated on FN.The mutant cells formed focal contacts

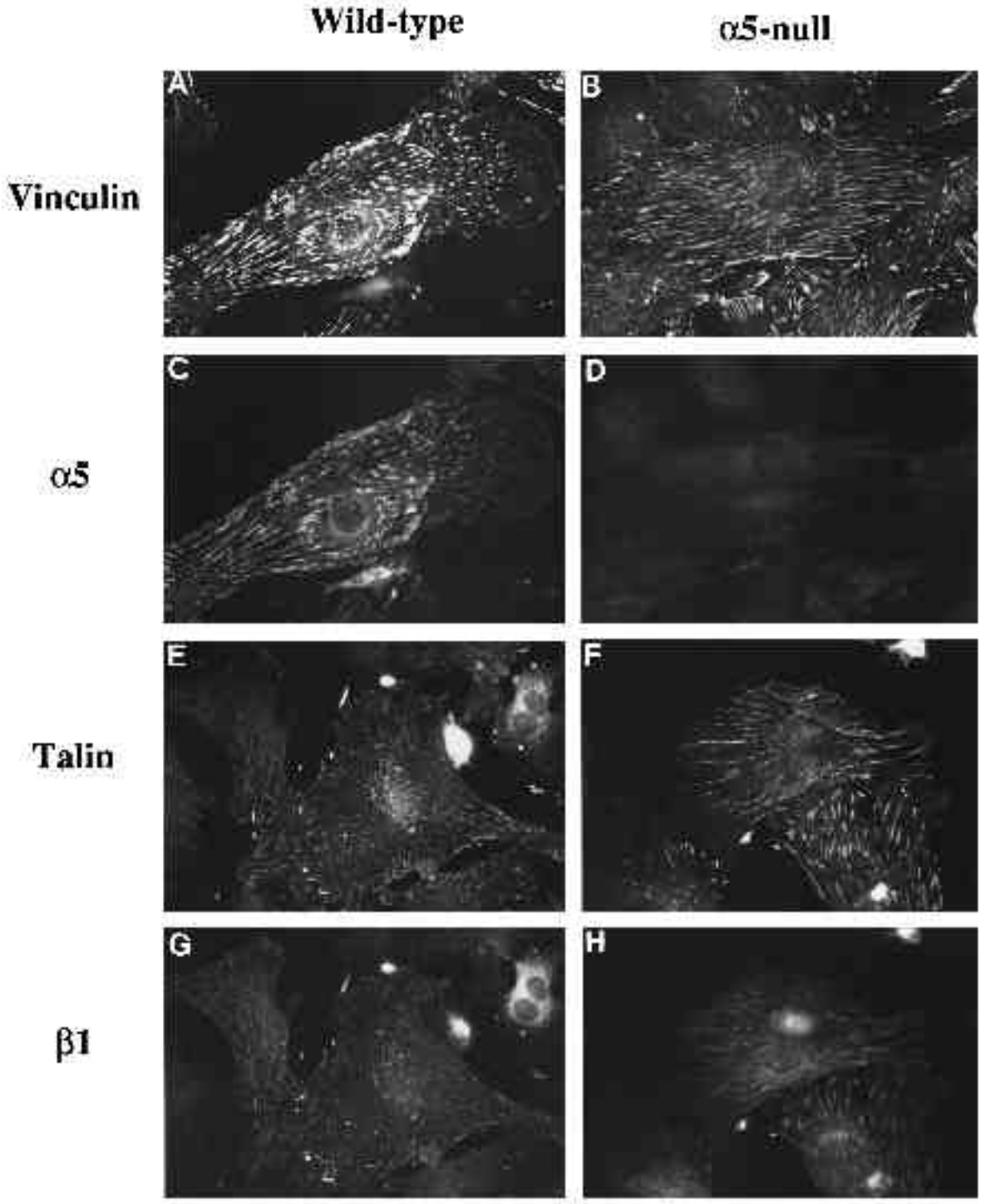

Fig. 7. Immunofluorescence showing formation of focal contacts by cells derived from wild-type and α_5-null embryos. Cells derived from wild-type (A,C,E,G) and α_5-null (B,D,F,H) embryos were cultured on coverslips coated with FN and stained with antibodies against vinculin (A,B), α_5 integrin (C,D), talin (E,F), and β_1 integrin (G,H). Double label staining was carried out for vinculin and α_5 integrin, and for talin and β_1 integrin. Notice that the mutant cells formed focal contacts similar to those of the wild-type cells, and α_5 was not expressed in the focal contacts of the mutant cells.

indistinguishable from those of wild-type cells, and both vinculin (Fig. 7A,B) and talin (Fig. 7E,F) were present in these focal contacts. We also tested for the presence of α_5 and β_1 integrins in the focal contacts by double-label immunofluorescence staining using antibodies against α_5 and vinculin, as well as β_1 and talin. While β_1 integrin was present in the focal contacts of both wild-type and mutant cells (Fig. 7G,H), α_5 was present only in the focal contacts of wild-type cells, but absent from mutant cells (Fig. 7C,D).

Next, we examined the ability of the fibroblastic cells to migrate on FN, using a modified Boyden chamber assay. In this assay, a membrane of appropriate pore size was placed

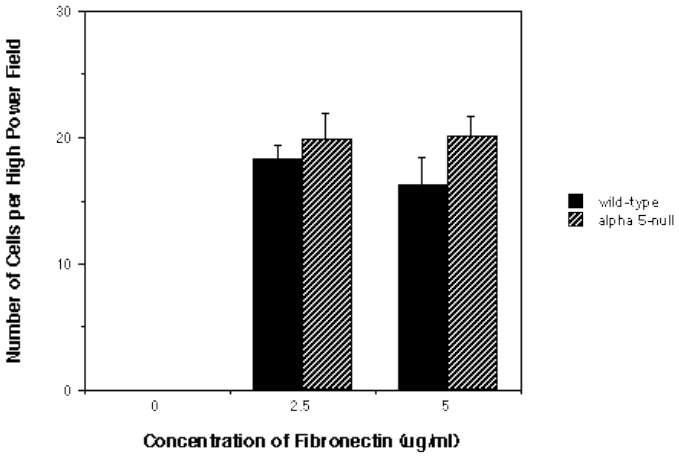

Fig. 8. Migration of cells derived from wild-type and α_5-null embryos. Cell migration assays were carried out as described in Materials and Methods. Two independent experiments were performed for each of the wild-type or α_5-null cells. In each experiment, cells were tested in duplicate wells with 0, 2.5 or 5 μg/ml of FN. The number of migrated cells per high power field (HPF, ×200) was counted (15 nonoverlapping HPF were counted per well). The mean values for the two independent experiments for the wild-type and the mutants cells were then calculated. Black bars, wild-type cells; shaded bars, α_5-null cells; the thin lines above the bars represent the standard deviations.

between the adhesive ligand and the cells to be tested. Cells that are able to migrate on FN pass through the membrane when FN is added to the opposite side of the membrane. The results are summarized in Fig. 8, which shows that, for both wild-type and α_5-null cells, few cells migrated through the membrane in the absence of FN, while significant numbers of cells did so when FN was added. Statistical analysis showed that there were no significant differences between wild-type and mutant cells in their abilities to migrate in a FN-dependent fashion.

Thus, three properties of the α_5-null cells, previously suggested to involve or require $\alpha_5\beta_1$ integrin, were normal, showing clearly that this integrin is not essential for assembly of a FN-rich matrix, assembly of focal contacts on FN substrates or migration in response to FN.

DISCUSSION

Generation of mice with null mutations in the α_5 integrin gene has allowed us to investigate the functions of this integrin subunit in vivo. The only integrin in which the α_5 subunit is known to occur is $\alpha_5\beta_1$, a receptor specific for the RGD cell adhesion site of FN. The embryonic lethality of homozygous α_5-null embryos establishes that this integrin plays one or more crucial roles in embryogenesis. This is so despite the fact that there are at least six other integrins that can mediate adhesion to FN, including several others which, like $\alpha_5\beta_1$, bind to the RGD site (Hynes, 1992; Vogel et al., 1990; Busk et al., 1992; Elices et al., 1991). Therefore, despite this multiplicity of integrin receptors for FN, $\alpha_5\beta_1$ is not functionally redundant in the intact organism. Either it performs some function(s) in which another integrin cannot function or it is expressed by cells that fail to express functionally equivalent integrins, or both.

Examination of the phenotype of α_5-null homozygous mutant embryos reveals some morphogenetic processes that can proceed without $\alpha_5\beta_1$ and others that require it. The distinctions between the two groups could not be predicted from prior results on functions of $\alpha_5\beta_1$ in cultured cells or from the distribution of $\alpha_5\beta_1$ in embryos, in so far as it has been determined. Functions that do not require $\alpha_5\beta_1$ produced by the embryos include implantation, despite strong expression of α_5 by invading human cytotrophoblasts (Damsky et al., 1992), and the initial phases of gastrulation and neurulation, despite earlier results implicating FN, β_1 integrins and RGD recognition in gastrulation of amphibians and birds (Boucaut et al., 1984; Darribère et al., 1988, 1990; Harrisson et al., 1993). In those studies, injection of antibodies or peptides blocking the interactions of integrins with FN blocked mesodermal ingression and migration. An early embryonic function of $\alpha_5\beta_1$ was also suggested by studies on *Xenopus*, which showed that $\alpha_5\beta_1$ is highly expressed throughout early embryonic development (Whittaker and DeSimone, 1993). However, it is not yet clear whether the expression of $\alpha_5\beta_1$ is the same in mouse embryos. Our results show extensive mesodermal ingression and movement in α_5-null mouse embryos, suggesting that $\alpha_5\beta_1$ is not required in these processes. Another possibility is that other integrins have compensated for the α_5 functions in early mouse embryogenesis.

Considerable further morphogenesis occurs in α_5-null embryos, including formation of notochord, somites and heart, considerable development of the brain, optic and otic anlagen and formation of branchial arches (Figs 3 and 4). This degree of morphogenesis is markedly in excess of that shown by embryos homozygous for a null mutation in FN, the only known ligand for $\alpha_5\beta_1$ (George et al., 1993). In the FN-null embryos, notochord and somites do not form and heart formation is variable. Thus, the receptor mutation is milder than the ligand mutation, a result perhaps best explained by the involvement of another FN receptor, as will be discussed later.

The α_5-null embryonic lethal phenotype, while milder than that of FN-null mutants, is not completely unrelated. While the anterior part of the α_5-null embryos develops relatively normally up to day 8.5 of gestation and up to about the level of the tenth somite, the posterior regions of these embryos are highly defective. There is an absence of posterior somites and a general deficit in paraxial mesoderm and the neural tube becomes kinked. Although the anterior part of the embryo undergoes turning, the posterior part does not. The defects in mesoderm, somites, neural tube and turning are all more extreme (and extend more rostrally) in the FN-null embryos (George et al., 1993). Both FN-null and α_5-null mutants also show defects in vascular development, although again the defects due to absence of $\alpha_5\beta_1$ are less severe than those due to the absence of FN. In both mutants blood islands form apparently normally and hematopoiesis occurs at day 8.0-8.5. Subsequently the mesodermal and endodermal layers of the yolk sac separate and the primitive blood cells come to lie in sacs between the two germ layers and also leak into the exocoelomic space (Fig. 5). However, the separation of mesoderm and endoderm is less severe in

α5-null mutants than in the FN-null mutants, where it is almost complete (George et al., 1993) in contrast with the 'bubbly' appearence seen in α5-null yolk sacs (Fig. 5). FN-null embryos contain very few, if any, red blood cells even when they do form heart and blood vessels. In contrast, the α5-null embryos all form hearts and a vascular system, which contains primitive blood cells (Fig. 4), but the blood vessels are distended and leaky. It seems clear that FN is essential for proper vasculogenesis and that, while α5β1 is also necessary for proper formation and maintenance of blood vessels, some other FN receptor can suffice to support significant vasculogenesis (Drake et al., 1992).

It is of some interest that erythropoiesis proceeds in both FN-null and α5-null mutant embryos. Earlier work has shown the presence of α5β1 on early cells of the erythroid lineage, which adhere well to FN (Patel and Lodish, 1986; Vuillet-Gaugler et al., 1990), and it has been reported that adhesion to FN is required for erythroid differentiation of murine erythroleukemia cells (Patel and Lodish, 1987). Clearly adhesion to FN is not required and α5β1 is not essential for erythropoiesis in the embryonic cohort of blood cells.

Given the partial overlap of the phenotypes caused by null mutations in FN and α5, we examined some of the FN-related properties of cells that have been suggested to depend on α5β1. Although several studies of cultured cell lines have implicated α5β1 in the assembly of FN-rich extracellular matrices (see Introduction for references), we found that FN-rich matrices assemble apparently normally both in vivo in α5-null embryos and in vitro around cells cultured from such embryos (Fig. 6). These results show that the α5-null phenotype is not simply a consequence of failure to assemble a FN-rich matrix. The data show clearly that α5β1 is not necessary for FN matrix assembly. It remains possible that α5β1 plays a significant role in matrix assembly in some cells but not others and/or that other FN receptors substitute in the situations we have examined. The ability of cells cultured from α5-null embryos to assemble β1 integrin-containing focal contacts on FN (Fig. 7) shows that these cells do express other β1 integrins, probably including other FN receptors.

The undiminished ability of α5-null cells to migrate in a FN-dependent fashion (Fig. 8) also supports this supposition. Other possible FN receptor integrins include α3β1 (Wayner and Carter, 1987; Elices et al., 1991), α4β1 (Wayner et al., 1989; Guan and Hynes, 1990; Mould et al., 1990; Mould and Humphries, 1991) and αvβ1 (Vogel et al., 1990). The status of α3β1 and αvβ1 as FN receptors has been questioned (Hynes et al., 1989; Bodary and McLean, 1990; Dedhar and Gray, 1990) and they have not been shown to mediate migration on FN. α4β1, which recognizes an alternatively spliced segment of FN (Guan and Hynes, 1990; Mould et al., 1990), has been implicated in neural crest migration since these cells do migrate in response to fragments of FN recognized by α4β1 (Dufour et al., 1988). Further work will be necessary to determine which of these integrins or other FN receptors function in the α5-null cells and to investigate the migration of specific cell types such as neural crest and primordial germ cells which are thought to use FN as a substrate for migration (Heasman et al., 1981; Rovasio et al., 1983; Dufour et al., 1988; see Hynes, 1990 for review).

Although α5 appears not to be necessary for assembly of FN-rich matrices or focal contacts or for migration in vitro, at least in the cells tested, the embryonic defects show that it is needed for several aspects of embryogenesis. This could be because of its involvement in these functions in specific cell types or because of involvement in other functions. As mentioned in the Introduction, some in vitro results suggest that α5β1 may be involved in the control of gene expression (Werb et al., 1989), differentiation (Adams and Watt, 1990) and cell proliferation (Giancotti and Ruoslahti, 1990) and could play important roles in proliferation and/or differentiation of embryonic cell types. The primitive streak undergoes extensive proliferation (MacAuley et al., 1993) and the mesodermal deficits we observe could arise from reduced proliferation, movement or differentiation of cells generating the posterior trunk. It will be of particular interest to investigate cell proliferation in the mesoderm of α5-null embryos and the expression of specific markers of differentation of different subclasses and derivatives of mesoderm.

It will also be important to investigate further the properties of heterozygous α5β1-null mice. Although these mice appear normal and healthy up to one year of age, one might expect them to be more susceptible to tumorigenesis. α5β1 is lost from several transformed cells (Plantefaber and Hynes, 1989), selection for α5-deficient cells leads to increased tumorigenicity (Schreiner et al., 1991) and transfection of α5 cDNA into tumorigenic cells leads to reduced tumorigenicity (Giancotti and Ruoslahti, 1990). Therefore, it appears that loss of α5β1 can lead to tumorigenesis and that mice possessing only one functional α5 allele might be susceptible to loss of α5β1 and thus to development of tumors.

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