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Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs

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SUMMARY

Neuropilin is a type 1 membrane protein, which is highly conserved among *Xenopus* frog, chicken and mouse. The extracellular part of the neuropilin protein is composed of three unique domains, each of which is thought to be involved in molecular and/or cellular interactions. In mice, neuropilin is expressed in the cardiovascular system, nervous system and limbs at particular developmental stages. To clarify the roles of neuropilin in morphogenesis in vivo, we generated mouse embryonic stem (ES) cell clones that constitutively expressed exogenous neuropilin, then produced chimeras using these ES cell clones. The chimeras overexpressed neuropilin and were embryonic lethal. The chimeric embryos exhibited several morphological abnormalities; excess capillaries and blood vessels, dilation of blood vessels, malformed hearts, ectopic sprouting and defasciculation of nerve fibers, and extra digits. All of these abnormalities occurred in the organs in which neuropilin is expressed in normal development. The variety of abnormalities occurring in these chimeric embryos suggested diverse functions of neuropilin in embryonic morphogesesis, which may be ascribed to multiple interaction domains identified in the molecule. Correct spatiotemporal expression of neuropilin seems to be essential for normal development of the cardiovascular system, nervous system and limbs.

Key words: cell surface molecule, embryonic stem (ES) cell, chimeric mouse, overexpression, abnormal morphogenesis, cardiovascular system, nervous system, limb.

INTRODUCTION

The identification and characterization of cell surface molecules that mediate cell interactions is important in the understanding of mechanisms of organization of multicellular tissues and organs. Neuropilin (previously known as A5) is a type I membrane protein which was first identified in the *Xenopus* tadpole nervous system (Takagi et al., 1987), then in chicken (Takagi et al., 1995) and mouse (Kawakami et al., 1996). Cloning of neuropilin cDNAs from *Xenopus* (Takagi et al., 1991), chicken (Takagi et al., 1995) and mouse (Kawakami et al., 1996) has revealed that the primary structure of this molecule is highly conserved among these vertebrate species. Neuropilin is distinctly different to other known cell surface molecules that mediate cell-cell interactions or adhesion. The extracellular part of the molecule is composed of three unique domains referred to as a1/a2, b1/b2 and c, each of which is expected to be involved in molecular and/or cellular interactions. The a1/a2 domains are homologous to the noncatalytic regions of the complement components C1r and C1s (Leytus et al., 1986; Mackinnon et al., 1987), the interaction domains in bone morphogenetic protein-1 (BMP-1; Wozney et al., 1988) and in the *Drosophila* dorsal-ventral patterning protein Tolloid (Shimell et al., 1991), and the carbohydrate-binding proteins involved in the binding of spermatozoa to the zona-pellucida (Sanz et al., 1991, 1992a,b). The b1/b2 domains of neuropilin are homologous to the C1 and C2 domains of the coagulation factors V and VIII (Toole et al., 1984; Jenny et al., 1987), the discoidin 1-like domain in the receptor tyrosine kinase DDR (Johnson et al., 1993). The central portion of the c domain of neuropilin has a conserved amino acid sequence designated as the MAM domain contained in the metalloendopeptidases meprins and the receptor protein tyrosine phosphatase μ (Beckmann and Bork, 1993). The complex domain structure of neuropilin suggests that this cell surface molecule serves in a variety of cell-cell interactions by binding to a variety of molecules. Recently, we showed that neuropilin can mediate cell adhesion by a heterophilic molecular interaction in an in vitro model system (Takagi et al., 1995).

Our previous studies in *Xenopus* have indicated several roles of neuropilin in the development of the nervous systems. Neuropilin/A5 has been demonstrated to possess neurite outgrowth-promoting activity by in vitro model experiments. Outgrowth of the neuropilin-expressing retinal fibers and trigeminal nerve fibers but not of the neuropilin-negative vestibulocochlear nerve fibers was promoted when they were co-cultured with neuropilin-expressing L cells prepared by transfection with the *Xenopus* neuropilin cDNA (Hirata et al., 1993). We also reported that neuropilin and the other neuronal cell surface molecule referred to as plexin (previously B2: see

Takagi et al., 1987; Ohta et al., 1992, 1995) were differentially expressed in the *Xenopus* olfactory fiber subclasses, suggesting that neuropilin as well as plexin may play roles in selective fasciculation of olfactory fibers (Satoda et al., 1995). Furthermore, we showed that neuropilin-expressing retinal fibers transplanted at ectopic positions in *Xenopus* embryos preferentially grew into neuropilin-expressing brain regions (Fujisawa et al., 1989), suggesting the involvement of neuropilin in target recognition. Despite these findings, however, the roles of neuropilin, particularly in vivo, have remained unclear.

To obtain better insight into the functions of neuropilin, manipulation of its gene expression in vivo seems to be an effective approach. Our recent studies in mice in which gene manipulation is available have indicated that the expression of neuropilin is restricted to particular neuronal circuits, and at particular stages of development (Kawakami et al., 1996). Moreover, as will be reported in this paper, neuropilin is expressed in a temporally restricted manner in the cardiovascular system, limb buds and mesenchyme of mouse embryos. Thus, ectopic or excess expression of neuropilin in developing mouse embryos is expected to cause morphological abnormalities in the tissues or organs in which neuropilin is expressed in normal development and thereby enable us to deduce the roles of the molecule in morphogenesis in vivo.

In this study, we generated embryonic stem (ES) cell clones that constitutively expressed neuropilin, by transfection with the mouse neuropilin cDNA transcribed from β-actin promoter, then produced chimeric mice using these ES cell clones. We report here that chimeric embryos overexpressing neuropilin showed excess capillaries and blood vessels, abnormal hearts, ectopic sprouting and defasciculation of nerve fibers or extra digits, and were embryonic lethal. These results suggest that neuropilin is functional in vivo and plays important roles in the development of not only the nervous system but also the cardiovascular system and limbs.

MATERIALS AND METHODS

Construction of overexpression vectors

To express neuropilin constitutively in ES cells and in chimeric embryos produced using these ES cells, we prepared two constructs, Miw171KT and Miw171EP (see Fig. 1). Miw171KT: A cDNA fragment corresponding to the protein-coding region of the mouse neuropilin cDNA (348th-3119th bases of the clone M171; Kawakami et al., 1996) was made by PCR, its nucleotide sequence was confirmed and then this cDNA was inserted into the eukaryotic expression vector Miw (Suemori et al., 1990). Miw171EP: An insert ranging from bases 175 to 3637 of the clone M171 (including 5′ and 3′ non-translated regions of the neuropilin cDNA) was digested with restriction enzymes *Eco*RI and *Pst*I, and inserted into the Miw vector. The Miw vector contains RSV enhancer and chick β-actin promoter, and is expected to promote insert gene expression ubiquitously.

Isolation of neuropilin-expressing ES cell lines

ES cell line E14 (Hooper et al., 1987; Thompson et al., 1989) derived from a 129/Ola male mouse blastocyst was used. E14 cells and its subclones were cultured on mitomycin C-treated NHL7 cells (Sawai et al., 1993), following the procedures described by Robertson (1987). ES cells (1×10^7 cells) were trypsinized, washed three times with ice-cold Ca^{2+}- and Mg^{2+}-free Hanks' saline, then suspended in 0.5 ml of the saline supplemented with 1 mM of $CaCl_2$ and $MgCl_2$. The cell suspension was mixed with 20 μg of the Miw171KT or Miw171EP (linearized by digestion with *Nde*I), and 5 μg of pSTneoB (Katoh et al., 1987; linearized by digestion with *Cla*I) in an electroporation cuvette, placed on ice for 10 minutes, electroporated at 200 V and 960 μF using a gene pulser (Bio-Rad) on ice for 10 minutes, then plated on culture dishes. After 2 days, neomycin (G418; at 400 μg/ml) was added to the cultures. The surviving colonies were cloned on the 7th day. To examine the expression level of neuropilin, the ES cell clones were immunostained with an anti-mouse neuropilin antibody (Kawakami et al., 1996).

Blastocyst injection

For blastocyst injection, C57B/6×C3H F1 (BCF1) were used. ICR mice were used as pseudopregnant females for injected blastocysts. Timed mating was set up under a 12 hour light/dark cycle (lights on 7 a.m.-7 p.m.). Noon on the day on which a copulation plug was found was designated as 0.5 day postcopulation (0.5 dpc). Blastocyst injection and transfer of embryos to pseudopregnant recipient females were carried out following the procedures described by Robertson (1987).

Immunohistochemistry

Immunohistochemical detection of neuropilin protein using an anti-mouse neuropilin antibody was carried out as described elsewhere (Kawakami et al., 1996). Briefly, the DNA region encoding amino acids 483-856 of mouse neuropilin was cloned into pET-3c (Studier, 1990) to obtain recombinant protein. After immunizing a rabbit with the recombinant protein, antiserum was collected and the Ig fraction specific for neuropilin was purified by affinity chromatography using the recombinant protein as a ligand (Oliver et al., 1988). Pregnant mice were deeply anesthetized with nembutal (Dinabot), then embryos were removed and fixed with 4% paraformaldehyde in 10 mM PBS (pH 7.0) at 4°C overnight. Cryostat sections (10 μm thick) were collected on poly-L-lysine-coated glass slides, and processed for immunostaining. Endogenous peroxidase activity was quenched with 0.2% hydrogen peroxidase in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) at room temperature for 30 minutes. After blocking with 5% skimmed milk in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 30 minutes, the sections were incubated overnight with the anti-mouse neuropilin antibody [1:200 dilution of the stock solution (0.35 mg/ml) with 1% skimmed milk in TBST]. Then, the specimens were incubated with biotinylated anti-rabbit IgG (1:200, Amersham) and with streptavidin-HRP complex (ABC elite kit, Vectastain). HRP activity was detected with diaminobenzidine.

Immunostaining for neurofilaments was performed using a mixture of polyclonal antibodies against the chicken 150×10^3 M_r and 200×10^3 M_r neurofilaments (Bio Med Tek).

The sections were counterstained with 0.02% toluidine blue.

In situ hybridization

Detection of neuropilin mRNA by in situ hybridization was carried out as described elsewhere (Kawakami et al., 1996). Briefly, ^{35}S-labeled antisense RNA probe was transcribed from the subcloned *Eco*RI-*Bam*HI fragment of the neuropilin cDNA clone M51 (nucleotides 1791-2913, see Kawakami et al., 1996). The RNA probe was subjected to alkaline hydrolysis to reduce its mean length to about 100 bases and used as a probe at a final concentration of 10^8 disints/minute/ml (10^7 dpm per slide). After hybridization, the sections were dehydrated, coated with NTB-2 emulsion (Kodak) containing 1% glycerol, exposed for 7 to 10 days at 4°C and developed. The sections were counterstained with toluidine blue.

Staining of skeletal structures

Embryos at 14.5-15.5 dpc were fixed with 3.5% paraformaldehyde at 4°C overnight, then stained with 0.5% alcian green in 3% acetic acid for 3 days. Differential destaining was achieved by dipping the specimens into a solution containing 1% HCl and 70% methanol. The

specimens were dehydrated with 100% methanol, then cleared and stored in a 1:2 mixture of benzyil alcohol and benzyil benzoate.

DiI labeling

The spinal cord and dorsal root ganglia (DRGs) were dissected out as a single piece from the fixed embryos, then small crystals of the lipophilic dye 1,1-dioctadecyl-3,3,3′,3′-tetramethlindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, USA) were put on the DRGs. The specimens were kept in the fixative for 1-2 days, then examined under an epifluorecence microscope (Olympus BHT-RFK) equipped with a rhodamine filter.

RESULTS

Production of chimeric embryos with exogenous neuropilin

We transfected E14 cells with either neuropilin cDNA construct, Miw171KT or Miw171EP (Fig. 1) and isolated three ES cell clones that stably expressed neuropilin; KT21 and KT55 for Miw171KT, and EP40 for Miw171EP. Immunoblot analysis indicated that the neuropilin proteins expressed in clones KT21, KT55 and EP40 had the same molecular weight as that of mouse embryos (data not shown).

These three neuropilin-expressing ES cell clones were injected into blastocysts of C57BL/6×C3H F_1 (BCF1) to generate chimeric mice. In this study, the chimeric mice produced using KT21 and KT55 clones were referred to as KT21 and KT55 chimeras, respectively, or collectively as KT chimeras. The chimeric mice produced using the EP40 clones are referred to as EP chimeras. As the BCF1 mice contained many more melanin granules in the retinal pigmented epithelium than 129/Ola mice from which the ES cell line E14 is derived, chimeric mice were easily discriminated from non-chimeric siblings by external observation of the eye pigmentation.

Immunostaining and in situ hybridization analyses indicated that the descendants of the injected ES cells within the chimeras expressed exogenous neuropilin during embryogenesis. The exogenous expression of neuropilin was more prominent in the KT than in the EP chimeras, although the levels of exogenous neuropilin were different among chimeras depending on the ratio of the ES cells contributing to embryo formation. In normal embryos at the age of 12.5 dpc, neuronal cells in the dorsolateral part of the diencephalon did not express neuropilin (Fig. 2A). Only capillaries were stained with anti-neuropilin antibody. In the EP chimeric embryos at the same age, however, several neuropilin-positive neuronal cells were observed (Fig. 2B), indicating ectopic expression of neuropilin. In controls, the floor plate was a normal site for neuropilin expression (Fig. 2C). In the EP chimeras, a part of the floor plate was often heavily stained with anti-neuropilin antibody (Fig. 2D) suggesting excess expression of neuropilin. In normal embryos at 12.5 dpc, neuropilin was expressed in limited parts of the nervous system, cardiovascular system and mesenchymal cells (Fig. 2E; for detailed expression patterns of neuropilin, see the later parts of this paper). While, in KT21 chimeric embryos, almost all parts of the body except the epidermis and endoderm derivatives were stained strongly with anti-neuropilin antibody (compare Fig. 2F and E). In situ hybridization analysis also confirmed the ectopic and excess expression of neuropilin; in the spinal cord of the normal embryos at 12.5 dpc, in situ hybridization signals for neuropilin mRNA were prominent in the motor neuron pool but weak in the dorsal horn (Fig. 2G), while in the KT21 chimeric embryos at same stage, in situ hybridization signals were expanded over all parts of the spinal cord (Fig. 2H).

Mortality of chimeric embryos

As listed in Table 1, we recovered a total of 26 KT chimeric embryos at ages of 12.5 dpc, 14.5 dpc and 15.5 dpc (from 20 pregnant dams), but no living chimeric embryos at more advanced stages or mice were obtained. In the uteri of the pregnant dams at 12.5-15.5 dpc, many traces of implantation and dead embryos were found. In the EP chimeras, we recovered 5 living chimeric embryos (3 at 12.5 dpc, and 2 at 17.5 dpc; from 3 pregnant dams), but no living chimeric embryos of more advanced stages of development or mice. These findings indicate that ectopic and/or over expression of neuropilin is embryonic lethal.

Anomalies in the cardiovascular system

The cardiovascular system is a normal site of neuropilin expression. Immunohistochemistry using anti-neuropilin antibody and in situ hybridization analyses indicated that endothelial cells of capillaries and blood vessels expressed neuropilin (Figs 2A,E, 3A,B). The immunostaining and in situ hybridization signals in the walls of blood vessels were wider than the thickness of the endothelium, indicating that mesenchymal cells surrounding blood vessels also express neuropilin (Fig. 3B). The expression of neuropilin in endothelial cells and mesenchymal cells was detected as early at 8.5 dpc and persisted throughout the fetal and early postnatal period. Neuropilin was also expressed in the endocardial cells of the embryonic heart (Fig. 2E). In the adult cardiovascular system, the expression of neuropilin was extremely weak or almost nonexistent, except in the atria (data not shown).

The KT chimeric embryos collected at 12.5 dpc exhibited

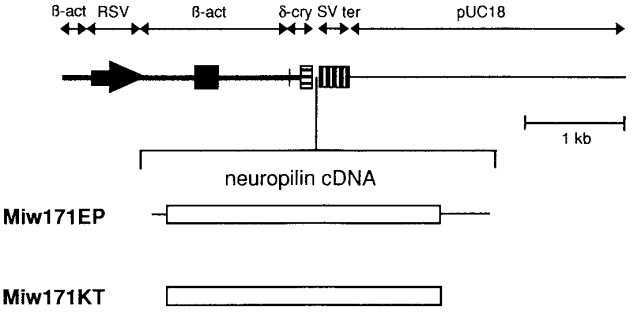

Fig. 1. A schematic representation of DNA constructs for constitutive expression of neuropilin. Mouse neuropilin cDNAs Miw171EP (including 5′ and 3′ non-translated regions) and Miw171KT (neuropilin-coding region only) were inserted between δ-crystallin (δ-cry) and SV termination signal (SV ter; a box shown with vertical strips) of the Miw expression vector (Suemori et al., 1990). Both β-actin (β-act) and δ-crystallin sequences are shown by solid lines. The exons of β-actin and δ-crystallin are shown by a filled box and a box with horizontal stripes, respectively. The RSV LTR sequence (RSV) is shown by a solid arrow and pUC18 plasmid vector sequence by a thin line.

various types of anomalies in the vascular system; excess capillaries or vessels, dilation of vessels and hemorrhaging. The abnormalities in the vascular system were often detected by external observation of embryos. The chimeric embryos usually appeared more red in color than their normal counterparts when they were recovered from the uterus. Some chimeric embryos showed hemorrhaging, mainly at the head or neck (Fig. 3D). Histological examination showed that these chimeric embryos possessed more capillaries or vessels than their normal counterparts and, sometimes, also dilated vessels, even though these abnormalities were not quantified in the present study. In embryos with severe anomalies, excess and dilated vessels deformed the brain vesicles (Fig. 3E,F). In this study, 10 of 15 chimeric embryos at 12.5 dpc (4 of 6 KT21 chimeras, and 6 of 9 KT55 chimeras; see Table 1) were judged to have excess capillaries and/or blood vessels, by either external viewing or histological examination. 8 of these embryos (4 each of KT21 and KT55 chimeras) exhibited hemorrhaging, which seemed to be a secondary effect of the malformations in the vascular system.

The disordered vascular system was often accompanied by malformation of the heart. In 5 KT chimeric embryos collected at 12.5 dpc (4 KT21 chimeras, and 1 KT55 chimera; see Table 1), the atrium was more expanded than that of normal embryos, and the wall of the atrium was thin. In 3 of these embryos (2 KT21 chimeras, and 1 KT55 chimera), the musculature of the ventricles was extremely thin (compare Figs 2E and 3G). In one chimeric embryo, the ventricular septum disappeared and was replaced with trabecula-like tissues (Fig. 3G). The atrial septum was formed in all KT chimeric embryos examined.

The EP chimeric embryos collected at 12.5 dpc also showed excess capillaries and vessels and the thinning of the ventricular wall (see Table 1). The abnormalities in the cardiovascular system in the EP chimeric embryos, however, were less severe than those in the KT chimeras, probably reflecting lower expression of exogenous neuropilin in the EP chimeras. Neither the KT nor EP chimeric embryos collected after 14.5 dpc showed clear defects in the cardiovascular system, suggesting that chimeric embryos suffered severe abnormalities in the cardiovascular system died at earlier developmental stages.

Anomalies in the nervous system

In normal development in mice, neuropilin is expressed in limited parts of the central nervous system (CNS) (Kawakami et al., 1996) and in the peripheral nervous system (PNS). Here, we describe neuropilin expression in the parts in which anomalies occurred in chimeric embryos. In the 12.5 dpc normal embryos, neuropilin was expressed in cells of the motor neuron pool, dorsal horn and DRG (Fig. 2G). The spinal nerves (Figs 2E, 4A,D), dorsal funiclus (Fig. 2E), vagal and trigem-

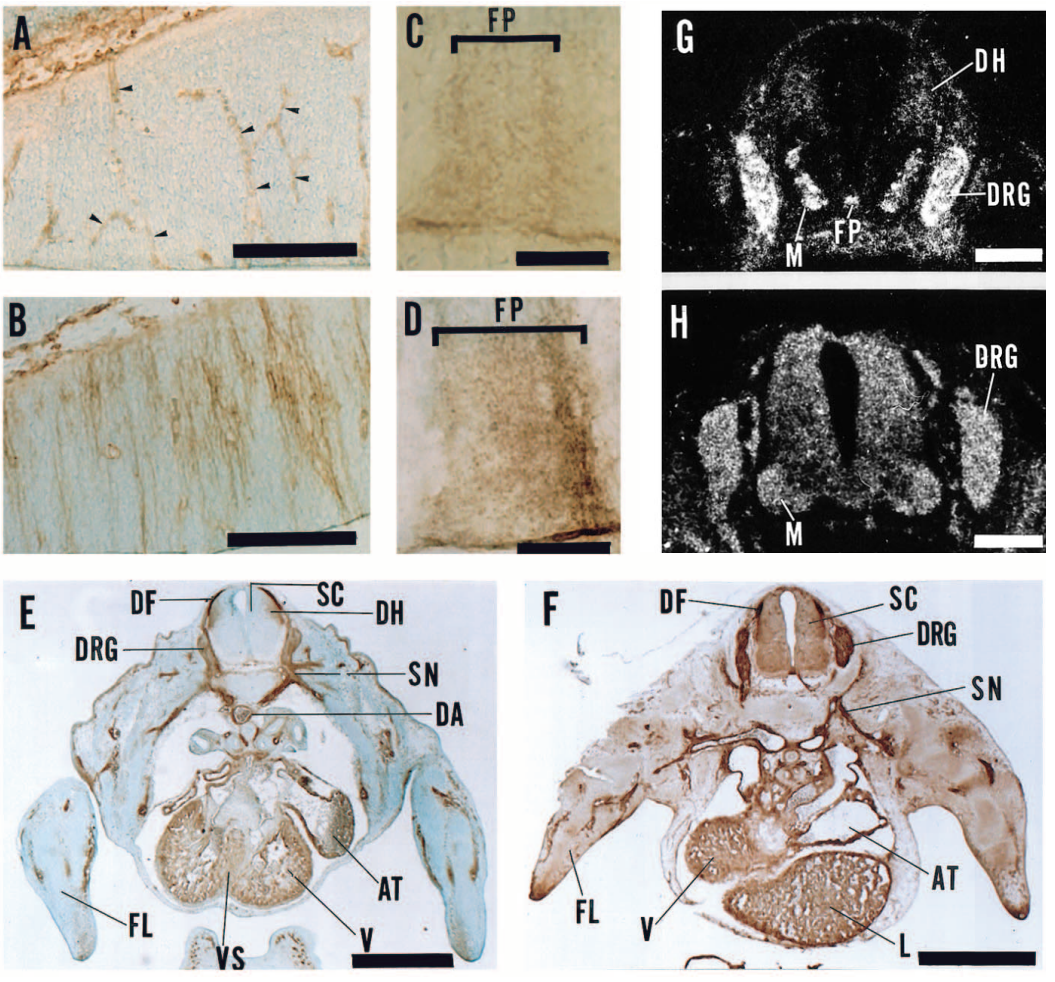

Fig. 2. Expression of neuropilin in normal and chimeric mouse embryos at 12.5 dpc. Neuropilin protein was detected using anti-neuropilin antibody (A-F). Neuropilin mRNAs were detected by in situ hybridization (G,H; dark-field images). (A,B) Expression of neuropilin in the dorsolateral part of the diencephalon of normal (A) and EP chimeric (B) embryos. Arrowheads (in A) show capillaries expressing neuropilin. (C,D) Expression of neuropilin in the floor plates (FP) of normal (C) and EP chimeric (D) embryos. The right side of the floor plate in the EP chimeric embryo (D) was strongly stained with anti-neuropilin antibody. (E, F) Expression of neuropilin at the level of the forelimbs in normal (E) and KT 21 chimeric (F) embryos. (G,H) Neuropilin mRNAs in normal (G) and KT21 chimeric (H) embryos. FL, forelimb; SC, spinal cord; DF, dorsal funiculus; DH, dorsal horn; M, motor neuron pool; FP, floor plate; DRG, dorsal root ganglion; SN, spinal nerve; DA, dorsal aorta; AT, atrium; V, ventricle; VS, ventricular septum. Scale bars: 100 μm (A,B), 50 μm (C,D), 1 mm (E,F), 200 μm (G,H).

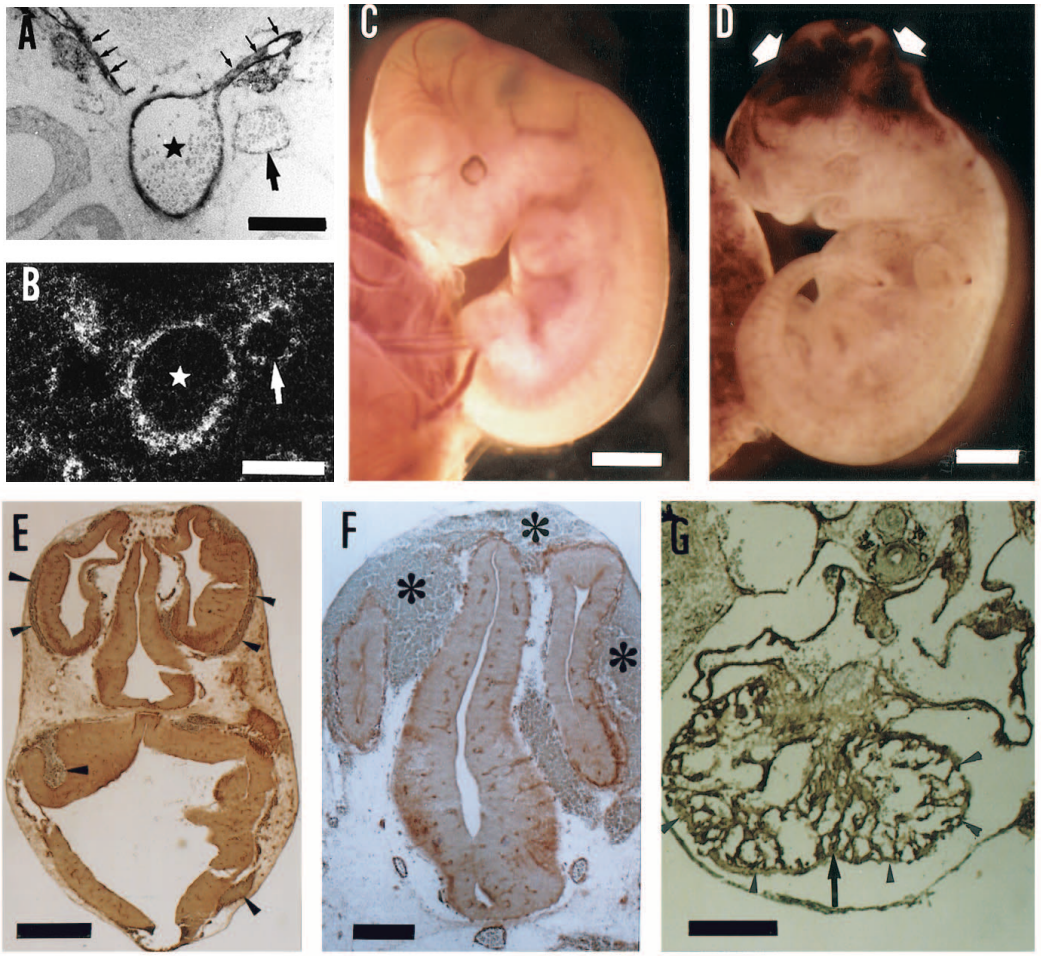

Fig. 3. Neuropilin expression and abnormalities in the cardiovascular system of embryos at 12.5 dpc. (A,B) Expression of neuropilin in blood vessels of normal embryos, detected by immunostaining with anti-neuropilin antibody (A) and by in situ hybridization (B; dark-field image). asterisks, dorsal aorta; small arrows, arteria; large arrows, veins. (C,D) External appearances of normal (C) and KT21 (D) embryos. Arrows in D indicate hemorrhaging. (E,F) Sections of a KT21 chimeric embryo stained with anti-neuropilin antibody. Arrowheads in E indicate dilated blood vessels. Asterisks in F indicate hemorrhage. Dilated vessels deformed the brain vesicles. (G) The heart of a KT21 chimeric embryo stained with anti-neuropilin antibody. The atrium (AT) was expanded, the ventricular septum was replaced with trabecula-like tissues (an arrow), and the musculature of the ventricles (arrowheads) was thin. Scale bars: 200 μm (A,B), 1 cm (C,D), 500 μm (E), 250 μm (F), 500 μm (G).

inal nerves (data not shown) were stained positively with anti-neuropilin antibody, indicating localization of neuropilin protein on the cell surface of these nerve fibers. Cells surrounding the spinal nerves (Fig. 4B), the vagal nerve and esophagus (Fig. 4C) also expressed neuropilin. As the Schwann cells do not express neuropilin in chicken (Takagi et al., 1995), the neuropilin-expressing cells surrounding the peripheral nerves seemed to be mesenchymal cells.

In the KT chimeric embryos, two types of morphological abnormalities were found in the peripheral nervous system. Firstly, the spinal nerve fibers often sprouted at incorrect positions. In 3 chimeric embryos (2 KT21 chimeras at 12.5 dpc, and 1 KT55 chimera at 12.5 dpc; see Table 1), the spinal nerve fibers sprouted at a point dorsal to the bifurcation of the communicant root to the sympathetic ganglia (Fig. 4E). In normal embryos, no sprouting of the spinal nerve fibers occurs at this position (Fig. 4D). Secondly, in the KT chimeric embryos, fasciculation of the peripheral nerve fibers was often more sparse than that in normal embryos. The spinal nerves were sometimes defasciculated (compare Fig. 4D and E). The disorganization of fiber bundling was more clearly seen in the vagal nerve. In normal embryos, the right and left vagal nerves were separated from each other, and formed distinct fiber bundles outside of the esophagus (Fig. 4F), while, in KT chimeric embryos at 12.5 dpc, the left and right vagal nerves were completely defasciculated and not distinguishable from each other (Fig. 4G). Defasciculation of the vagal nerves was observed in 3 chimeric embryos (2 KT21 chimeras, and 1 KT55 chimera; see Table 1). Fiber bundling of the other cranial nerves such as the trigeminal and lingual nerves also appeared more sparse than that in normal embryos (data not shown).

To examine fiber bundling in the CNS, the central DRG fibers of 3 KT chimeric embryos at 12.5 dpc (2 KT21 chimeras, and 1 KT55 chimera) were retrogradely labeled with the lipophilic dye DiI. In all of these embryos, pathways for dye-positive DRG fibers within the dorsal funiculus were considerably disorganized (Fig. 4I) as compared to ones in normal embryos (Fig. 4H).

The KT chimeric embryos collected at 15.5 dpc and the EP chimeric embryos at any developmental stage showed no clear abnormalities in the nervous system, probably due to low levels of exogenous neuropilin expression.

Anomalies in limbs

Mesenchymal cells of limb buds also expressed neuropilin in normal development. At 10.5 dpc, neuropilin was expressed uniformly in the limb bud mesenchyme (Fig. 5A). After the occurrence of precartilaginous mesenchymal condensation (after 12.5 dpc), the cells within the condensations stopped expression of neuropilin (Fig. 5B). As development proceeds, expression of neuropilin was gradually limited to the connective tissues surrounding limb cartilage or bones.

Most of the KT21 and EP chimeric embryos showed extra digits (Fig. 5C) which were always observed at the anterior side of the hindlimb. Nine of 10 KT21 chimeric embryos (5 of 6 at 14.5 dpc, 3 of 3 at 15.5 dpc, and one dead embryo recovered at 17.5 dpc; see Table 1) possessed extra digits. Among these, 5 embryos had extra digits in both sides of the hindlimb. All the EP chimeric embryos at 17.5 dpc (2 living and 1 dead; see Table 1) also possessed extra digits in the anterior side of the hindlimb. The chimeric embryos generated using KT55 cells (2 embryos at 15.5 dpc; see Table 1) had no extra digits.

The alcian green staining of the chimeric embryos with extra digits showed that the hindlimbs had redundant pieces of cartilage at the anterior side; in some hindlimbs, the proximal and distal phalangeal cartilage of the first digit were duplicated (Fig. 5D) and, in the others, each of the medial cuneiform cartilage, the first metatarsus cartilage, and the first phalangeal cartilage was duplicated (Fig. 5E). Some hindlimbs exhibited duplicated medial cuneiform and first metatarsal cartilage, and triplicated proximal and distal phalangeal cartilage of the first digit (Fig. 5F). The numbers of cartilage elements in the extra digits differed between the right and left hindlimbs of the same embryo. In this study, no morphological abnormalities were observed in the forelimbs in chimeric embryos, except in one KT21 chimeric embryo at 17.5 dpc in which the proximal phalangeal cartilage of the second and third fingers were fused (data not shown). No abnormalities were observed in the skeletal system other than the limbs.

DISCUSSION

In this study, we generated chimeric mouse embryos using ES cells constitutively expressing the membrane protein neuropilin and showed that most of the chimeric embryos with excess and/or ectopic expression of neuropilin exhibited anomalies in the cardiovascular system, nervous system or limbs, and died in utero. The abnormalities occurred in the present chimeric embryos are not due to the intrinsic properties of the ES cells, since the parental ES line E14 does not produce any analogous abnormalities (Sawai et al., 1993). Moreover, in this study, we produced chimeric mouse embryos using three different ES cell clones; the KT21 and KT55 clones for the neuropilin DNA construct Miw171KT and the EP40 clone for the construct Miw171EP (see Fig. 1), and detected similar anomalies in these chimeras; the KT21 and KT55 chimeras showed disorganization in the cardiovascular and nervous systems, and the KT 21 and EP chimeras exhibited extra digits. These results indicate that the anomalies observed in the chimeras were caused by the exogenous neuropilin expressed by the ES-derived cells.

Table 1. Phenotypes of chimeric embryos

ES cell clone	Stages of embryos	Number of chimeras examined	Abnormalities (embryos with abnormalities / embryos analyzed)
KT21	12.5	6	Excess capillaries (4/6) Hemorrhaging (4/6) Heart malformation (4/4)†·‡ Ectopic sprouting of spinal nerves (2/2)† Defasciculation of vagal nerves (2/2)† Irregular dorsal funiculus (2/2)‡
	14.5-17.5	9+1†	Extra digits (9/10)
KT55	12.5	9	Excess capillaries (6/9) Hemorrhaging (4/9) Heart malformation (1/1)§ Ectopic sprouting of spinal nerves (1/1)§ Defasciculation of vagal nerves (1/1)§ Irregular dorsal funiculus (1/1)§
	15.5	2	None
EP40	12.5	3	Excess capillaries (2/3) Heart malformation (2/3)
	17.5	2+1*	Extra digits(3/3)

*Dead embryos; †·‡·§The same respective embryos.

An important finding obtained in the present study is that all of the abnormalities occurred in the organs in which neuropilin is expressed in normal development. As the expression of neuropilin in these organs is limited at particular stages of normal development, the excess and/or persistence of neuropilin expression after the stage when in normal development it would have ceased appeared to results in abnormal morphogenesis. In the normal development of limbs, neuropilin is expressed widely in the limb mesenchymal cells, then disappears in the cells of the precartilaginous mesenchymal condensations. Thus, it is likely that not only the excess expression of neuropilin in the limb mesenchymal cells but also the prolonged expression of the molecule in the cells of the precartilaginous mesenchymal condensations disturb the normal limb morphogenesis.

Another important observation obtained in this study is that the exogenous neuropilin expression caused embryonic death. The disordered development of the cardiovascular system observed in the chimeric embryos seemed to be the main cause of the lethality of embryos; chimeric embryos with more exogenous neuropilin may suffer more severe abnormalities in the cardiovascular system, resulting into embryonic death at earlier developmental stages. It seems worthy of note that only embryos with low levels of exogenous neuropilin expression and with the resultant minor anomalies in the cardiovascular system and probably in the other organs survived until the later stages of development and were analyzed in the present study. The finding that chimeric embryos collected after 14.5 dpc did not show clear defects in the cardiovascular or nervous systems may support the possibility. The extra digits occurred in most of the KT21 and EP chimeric embryos with excess neuropilin, but not in the KT55 chimeric embryos in which strong expression of exogenous neuropilin was expected. These results, however, do not indicate that the KT55 chimeras developed normal limbs. Rather, only 3 living KT55 chimeric embryos were obtained after 13.5 dpc suggesting that the KT55 chimeras could potentially produce extra digits but died at stages before limb development due to severe abnormalities in the cardiovascular system.

Our findings raise the question of how the overexpression of neuropilin causes developmental aberration. The abnormalities that occurred in the chimeric embryos affected the nervous system, cardiovascular system and limbs, which are formed as the results of quite different developmental processes. Molecular cloning studies of neuropilin in *Xenopus* (Takagi et al., 1991), chicken (Takagi et al., 1995) and mouse (Kawakami et al., 1996) have indicated that the extracellular segment of the neuropilin protein is composed of three domains, each of which is expected to be involved in different

molecular and/or cellular interactions, suggesting multiple roles of the molecule. Thus, it seems unlikely that a single function of neuropilin is responsible for all the range of the abnormalities observed in the chimeric embryos. We will discuss below the effect of overexpressed neuropilin in morphogenesis in each organ.

Nervous system

Our previous studies in *Xenopus* have shown that neuropilin promotes neurite outgrowth in vitro (Hirata et al., 1993). We also showed that the subclasses of the *Xenopus* olfactory fibers differentially expressed either neuropilin or the other cell surface molecule named plexin and their pathways were sorted depending on the expression levels of these two molecules (Satoda et al., 1995). From these findings, we predicted that neuropilin is involved in neuronal cell interaction, and plays roles in guidance and fasciculation of nerve fibers. The observation of ectopic sprouting and defasciculation of nerve fibers of the PNS and CNS in the chimeric embryos with excess neuropilin support the above hypothesis.

Our recent study in an in vitro model system indicated that neuropilin interacts with heterotypic ligands (Takagi et al., 1995). However, very little is known about neuropilin ligands. Also, we cannot exclude the possibility that neuropilin interacts homophilically. Thus, it is open to question how neuropilin regulates interaction of nerve fibers and why excess neuropilin disorganizes growth and fasciculation of nerve fibers. At least, the present observation that neuropilin is normally expressed in both the peripheral nerve fibers and mesenchymal cells surrounding them enables us to speculate that neuropilin mediates interaction not only between nerve fibers but also between nerve fibers and their surrounding cells. It is possible that, in the chimeras, excess expression of neuropilin in both the peripheral nerve fibers and the mesenchymal cells disrupts the appropriate balance of the fiber-fiber and fiber-mesenchymal cell interactions, resulting into ectopic sprouting or defasciculation of the nerve fibers. Several studies have indicated that non-neuronal tissues around the spinal nerves play roles in guidance and patterning of the nerves (Landmesser, 1984; Tosney, 1988; Lance-Jones and Dias, 1991). A similar disordered cellular interaction may occur in the CNS of the chimeric embryos; excess neuropilin in the dorsal spinal cord may alter the growth and fasciculation of the DRG fibers, resulting into disorganization of the fiber pathway in the dorsal funiculus.

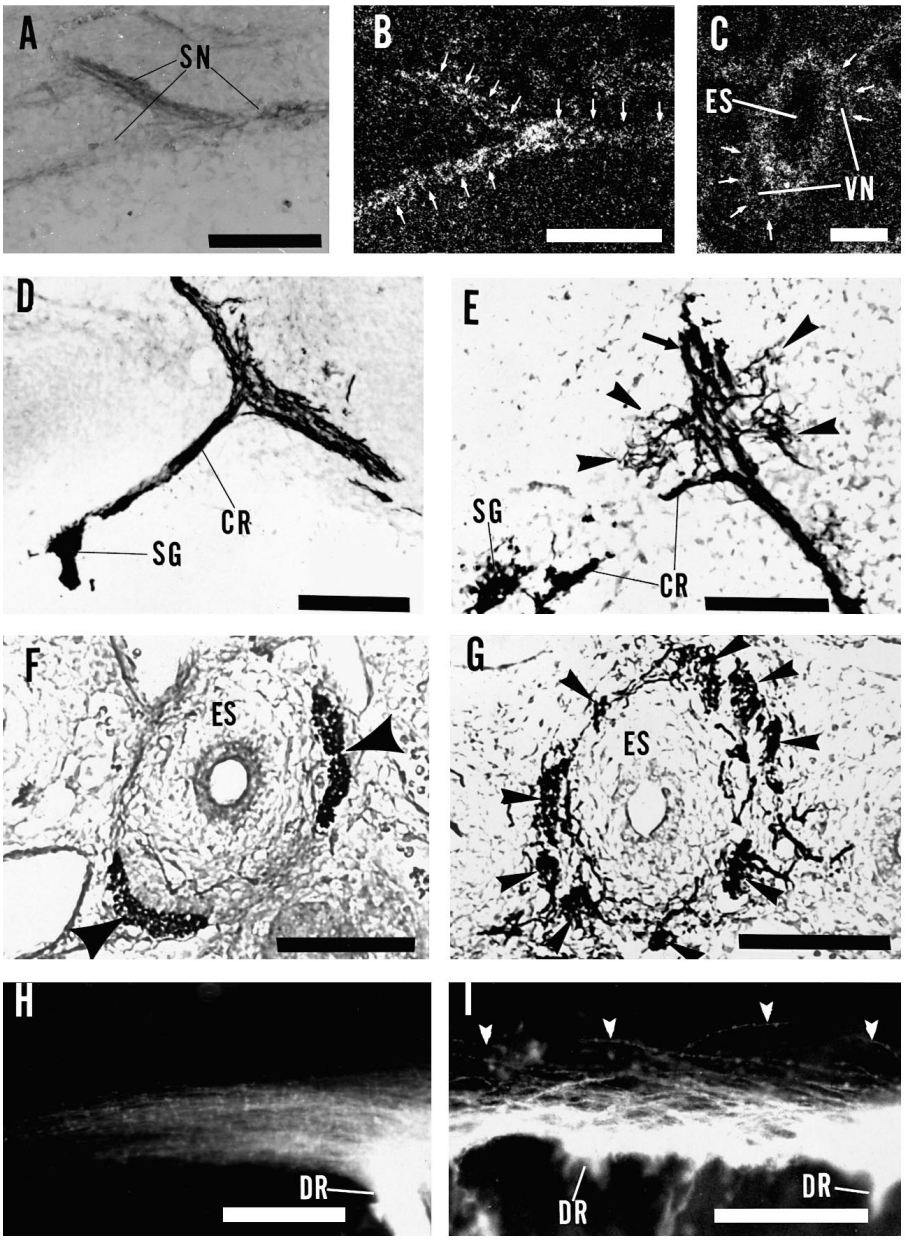

Fig. 4. Neuropilin expression and abnormalities in the nervous system of embryos at 12.5 dpc. (A) Expression of neuropilin in the spinal nerve (SN) of a normal embryo, detected by immunostaining with anti-neuropilin antibody. (B) Expression of neuropilin mRNAs in cells (arrows) surrounding the spinal nerve of a normal embryo, detected by in situ hybridization (dark-field image). A and B are adjacent serial sections. (C) Expression of neuropilin mRNAs in cells surrounding the esophagus (ES) and vagal nerves (VN; arrows) of a normal embryo, detected by in situ hybridization (dark-field image). (D,E) The spinal nerves of normal (D) and KT21 chimeric (E) embryos stained with anti-neurofilament antibody. In the chimeric embryo, the spinal nerve was partially defasciculated (an arrow in E), and sprouted ectopically (arrowheads in E). CR, communicant root; SG, sympathetic ganglion. (F,G) Vagal nerves (arrowheads) of normal (F) and KT21 chimeric (G) embryos around the esophagus (ES) stained with anti-neurofilament antibody. In the chimeric embryo, both the right and left vagal nerves were defasciculated. (H,I) DiI-filled fibers of DRGs within the dorsal funiculus of normal (H) and KT21 chimeric (I) embryos. In the chimeric embryo, fiber bundling was irregular (arrowheads in I). DR, dorsal root. Scale bars: 100 μm (A-I).

Cardiovascular system

The chimeric embryos with excess neuropilin exhibited excess capillaries and vessels, dilation of vessels, and abnormal hearts, suggesting the involvement of neuropilin in morphogenesis of the cardiovascular system. The capillaries and vessels are formed as a result of different morphogenetic processes such as the proliferation and differentiation of endothelial cells, the migration of endothelial cells and the formation of capillary tubes. More complicated processes are required for heart formation. Though the present studies did not show which steps of the morphogenesis of cardiovascular system are controlled with neuropilin, some explanations are possible for the observed abnormalities.

It has been reported that proper interaction of endothelial cells with surrounding mesenchymal cells, smooth muscle cells or extracellular matrix is necessary for the proliferation, differentiation and migration of endothelial cells (Yang and Moses, 1990; Sato et al., 1990). Excess expression of neuropilin in the endothelial cells and/or their surrounding cells might disrupt correct cellular interaction, resulting into the excess capillaries and vessels, or the dilation of vessels. The other possibility is that neuropilin modulates the activities of cytokines. Several studies have shown that the production and the migration of endothelial cells, and also capillary tube formation are regulated by the growth factor TGFβ (Robertis et al., 1986; Yang and Moses, 1990; Sato et al., 1990). Also, TGFβ plays a role in the epithelial-mesenchymal cell transformation to form valves and septa of the embryonic heart (Potts and Runyan, 1989). The a1/a2 domains in the extracellular segment of neuropilin are homologous to BMP-1 (Wozney et al., 1988) and Tolloid (Shimell et al., 1991) which are expected to bind to precursors of TGFβ via these domains and proteolytically cleave the precursors into active forms (Finelli et al., 1994). Thus, it seems possible that neuropilin modifies the activity of TGFβ or TGFβ-like cytokines. The excess angiogenesis and the disordered hearts observed in the chimeric mice might be due to the up- or down-regulation of the activities of TGFβ-like cytokines as a result of overexpression of neuropilin.

Limbs

Most of the chimeric embryos overexpressing neuropilin showed extra digits, suggesting that limb morphogenesis is very sensitive to the exogenous neuropilin expression. Several studies have shown the importance of cell-cell and cell-substratum interactions in limb morphogenesis (Leonard et al., 1991; Jiang et al., 1993; Ide and Wada, 1994). Thus, excess and/or ectopic expression of neuropilin might modify interactions of limb mesenchymal cells to form abnormal limbs. Growth factors belonging to the TGFβ superfamily such as

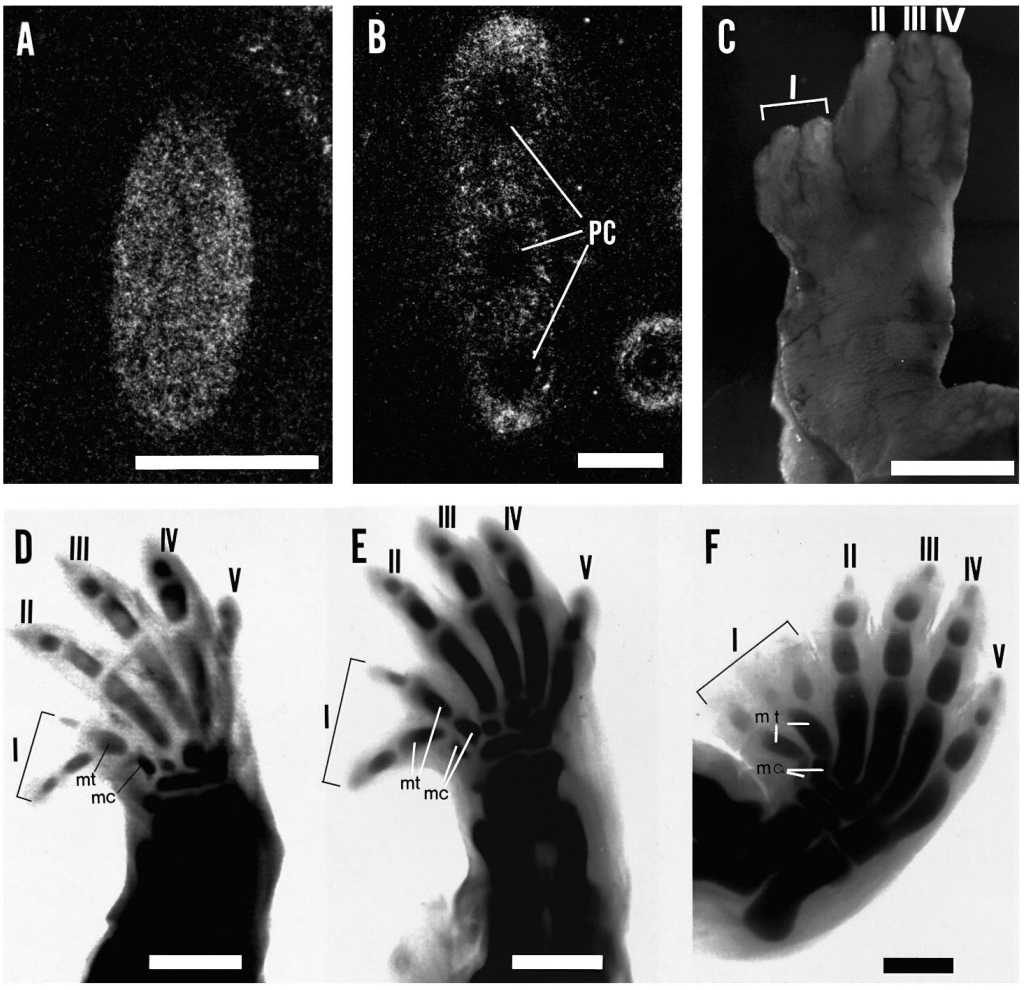

Fig. 5. Neuropilin expression and abnormalities in hindlimbs. (A,B) Expression of neuropilin in hindlimb buds of normal embryos at 10.5 dpc (A) and 12.5 dpc (B) detected by in situ hybridization (dark-field image). Sections were cut along the rostrocaudal axis of the limb buds. The upper and left in each figure are the anterior and dorsal sides of the limb buds, respectively. Neuropilin mRNA was not detected in the areas of precartilaginous mesenchymal condensation (PC). (C) External appearance of the right hindlimb of the EP chimeric embryo at 17.5 dpc. The first digit (I) was triplicated. II, III and IV indicate the second, third and fourth digits, respectively. The fifth digit is behind the fourth toe and cannot be seen in the figure. (D-F) Cartilage patterns of hindlimbs of KT 21 chimeric embryos at 15.5 dpc (D,E) and at 17.5 dpc (E,F), stained by alcian green. I, II, III, IV and V indicate the first, second, third, fourth and fifth digits, respectively. mc, medial cuneiform cartilage; mt, first metatarsal cartilage. Scale bars: 250 μm (A,B), 1 cm (C), 500 μm (D-F).

TGFβ1, TGFβ2, BMP-2, BMP-4 and Activin A are assumed to regulate proliferation of limb mesenchymal cells, precartilaginous condensation of mesenchymal cells and cartilage or bone morphogenesis (Hayamizu et al., 1991; Jones at al., 1991; Leonard et al., 1991; Jiang et al., 1993; Niswander and Martin, 1993). Thus, the excess or ectopic expression of neuropilin in the limb mesenchymal cells might modify cytokines functions and disturb normal patterns of proliferation or condensation of the cells, resulting in the formation of redundant pieces of cartilage in their digits. It is still unclear why the anomalies were limited to the anterior side of the hindlimbs.

In conclusion, the present results indicated that neuropilin is an active cell surface protein which regulates morphogenesis in vivo. Diverse morphological abnormalities occurred in chimeric mice with excess or ectopic neuropilin expression indicating that neuropilin is a multifunctional molecule capable of regulating various aspects of morphogenesis. Although determination of the precise roles of neuropilin in each step of morphogenesis at the molecular level require further analysis, the correct spatiotemporal patterns of neuropilin expression were shown here to be essential for the growth and fasciculation of nerve fibers, formation of the cardiovascular system and pattern formation of limbs.

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