The Development of the Cardiac Outflow Tract in the Chick Embryo

by

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We accept this thesis as conforming to the required standard

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ABSTRACT

The development of cellular and extracellular elements of the cardiac outflow tract have been examined using indirect immunofluorescence and tissue culture techniques. In particular the role of fibronectin (FN) in the population of the outflow tract by cardiac neural crest cells has been studied. At stage 10 the truncus arteriosus consists of endothelium and myocardial cuff, separated by a layer of complex extracellular matrix (ECM). By stage 20 there are a few scattered mesenchyme cells in the ECM space. Using HNK-1, an antibody that recognizes neural tube derivatives, the migration of neural crest cells into the truncus was monitored. The neural crest cells begin migration caudally from the aortic arches at about Stage 25. The cells are seen in clusters, within the ECM of the tunica media. As development proceeds they migrate towards the heart down the length of the truncus arteriosus. Almost simultaneously septation of the truncus arteriosus into pulmonary and systemic pathways occurs. The HNK-1 immunoreactive cells become more dense and are organized into a laminar pattern by about stage 33. The cells that will become smooth muscle express the HNK-1 antigen only during their migration, whereas cells that become part of the nervous plexus of the heart continue to express it after differentiation. There is another population of cells in the tunica media that express the HNK-1 antigen early in development (Stage 10), which are not neural crest derived. This limits the utility of HNK-1 as a neural crest cell marker. An anti-FN antibody was used to follow the distribution of FN throughout this time. The FN shows up as randomly arrayed fibers before stage 15, that are not abundant at this time being mainly associated with cell surfaces. The fibers increase in abundance as the tract develops and appear to fill up the media as more cells appear. They are localized to the surfaces of the neural crest cells as well as the endothelium and adventitia, but the relative abundance within these layers changes dependent on what stage is examined. By stage 35 the fibers are more

abundant around the endothelium. The migrating neural crest cells express ß1 integrin at this time, a compontent of FN receptors. Truncus arteriosus explants were used to measure the migration of cardiac neural crest cell *in vitro*. FN and chick ECM substrates promoted migration and an anti-FN antibody inhibited migration. The conclusion is that FN and cardiac neural crest cells can function together *in vitro* and may function together *in vivo*. This study does not directly test the labeled pathway hypothesis of neural crest cell migration, already well established for other populations of neural crest cells however, it does support to the idea that cardiac neural crest cells may use similar cues and mechanisms to guide their migration.

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LIST OF ABBREVIATIONS

A-P septum	aortico-pulmonary septum
BSA	Bovine serum albumin
DAB	Diaminobenzidine
ECM	extracellular matrix
EDTA	Ethylenediamine tetra acetic acid
FITC	Fluoresceine isothiocyanate
FN	fibronectin
GAG	glycosamino glycan
GalTase	Galactosyl Transferase
GAR	Goat anti-rabbit IgG
HNK-1	Human natural killer cell antibody (antibody that recognizes neural tube derivatives)
HRP	Horseradish peroxidase
k	Relative molecular mass x 1000
LM	laminin
NRH	Non-relevant hybridoma
PBS	phosphate buffered saline
PMSF	Phenyl methyl sulfonyl fluoride
RAM	Rabbit anti-mouse IgG or IgM
RGDS	arginine, glycine, asparigine, serine
YIGSR	tyrrosine, isoleucine, glycine, serine, arginine

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INTRODUCTION

OVERVIEW OF HEART MORPHOGENESIS IN THE CHICK

Precardiac Cell Migration

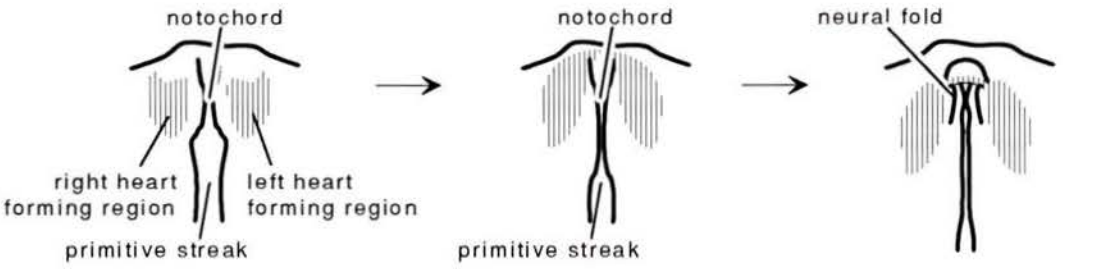
In vertebrates a complex circulatory system arises early in development and it is often described as the first functioning system of the embryo. A vital component of this system is the heart and it is a striking feature in the formation of the embryo. The heart develops from mesoderm in the neck region of the embryo and only later shifts into the chest. Development of the heart and circulatory system has been well studied by developmental biologists and there is a rich literature on the subject (Romanoff, 1960, De La Cruz et al., 1972; Goor and Lillehei, 1975; Rosenquist and Bergsma, 1978; Kirby and Waldo, 1990).

The first event in heart development is the migration of mesoderm cells, the precardiac splanchnic mesoderm. These precardiac cells are originally located in the two areas lateral and anterior to Henson's node between the ectoderm and endoderm (Linasek and Lash, 1986). Initially random movements of precardiac cells within the heart forming region begin, but soon the cells move anteriorly as a group (Linasek and Lash, 1986). The heart-forming cells, move anteriorly in an arc toward the midline of the embryo until they reach the gut (Linasek and Lash, 1986).

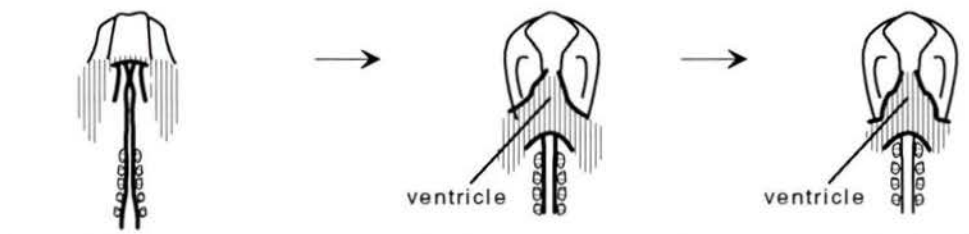
Formation of the Tubular Heart

The process of tubular heart formation begins at stage 8+ in chick development (Hamburger and Hamilton, 1951). The precardiac cells form 2 areas of thickened tissue on either side of the midline (Figure A). This is derived from mesoderm that has migrated from the precardiac region, and gives rise to myocardium which will form the muscular layer of the heart.

Figure A: Early heart formation showing the original location of the precardiac splanchnic mesoderm. Shading indicates the areas of heartforming mesoderm cells.



Stage 2 (6 to 7 hours) **Stage 3 (12 to 13 hours)** **Stage 7 (23 to 26 hours)**



Stage 8 (26 to 29 hours) **Stage 9 (29 to 33 hours)** **Stage 10 (33 to 38 hours)**

The endocardial (inner lining) cells appear between the myocardial layer and the endoderm. The endocardium also forms from sheets of mesoderm and lies as paired tubes. The endocardial tubes are formed on either side of the embryo by the splitting of the mesodermal sheets. Both are enclosed in the myocardium (or epimyocardium) (Figure B). The two tubes of the heart primordia are drawn together by the continued development of the foregut, and eventually fuse to form one tube. The endocardial components fuse with each other to form the blood carrying tube, and the myocardium fuses on its dorsal surface near the pharynx. The formation of a single tube called the primitive heart, occurs at about stage 8 or 9 (Manasek, 1968). The endocardium and myocardium are separated by a layer of extracellular matrix (ECM) called the cardiac jelly (Manasek, 1968) (Figure B). The heart primordium separates from the forgut, remaining attached by only a portion of the coelomic lining. At this point the anterior end of the heart is termed the truncus arteriosus, which will form the outflow tract. The posterior portion of the heart is made up of the bulbus cordis and the ventricle. The atrium is not yet differentiated.

Bending and Torsion of the Heart

Once the tubular heart is formed it elongates and as the anterior and posterior ends are fixed in position the tube begins to bend. The heart curves to the right, first forming a U-shaped curve and then an S-shaped curve. Also occurring at this time is torsion of the entire embryo, resulting in the rotation of the heart to the left and the entire embryo lying on its right side (Figure C).

Regional Differentiation

The regions of the heart by stage 11 consist of the sinus venosus, the atrium, the bulbus cordis, and the truncus arteriosus (Romanoff, 1960). This segmentation becomes apparent by the presence of superficial grooves before loop formation. The blood flow

Figure B: Stylized diagram of cross sections through the primitive heart of an early chick embryo, showing the three original layers of the tubular heart. The top view shows the paired heart primordia before fusion, and the bottom view shows the tubular heart after fusion.

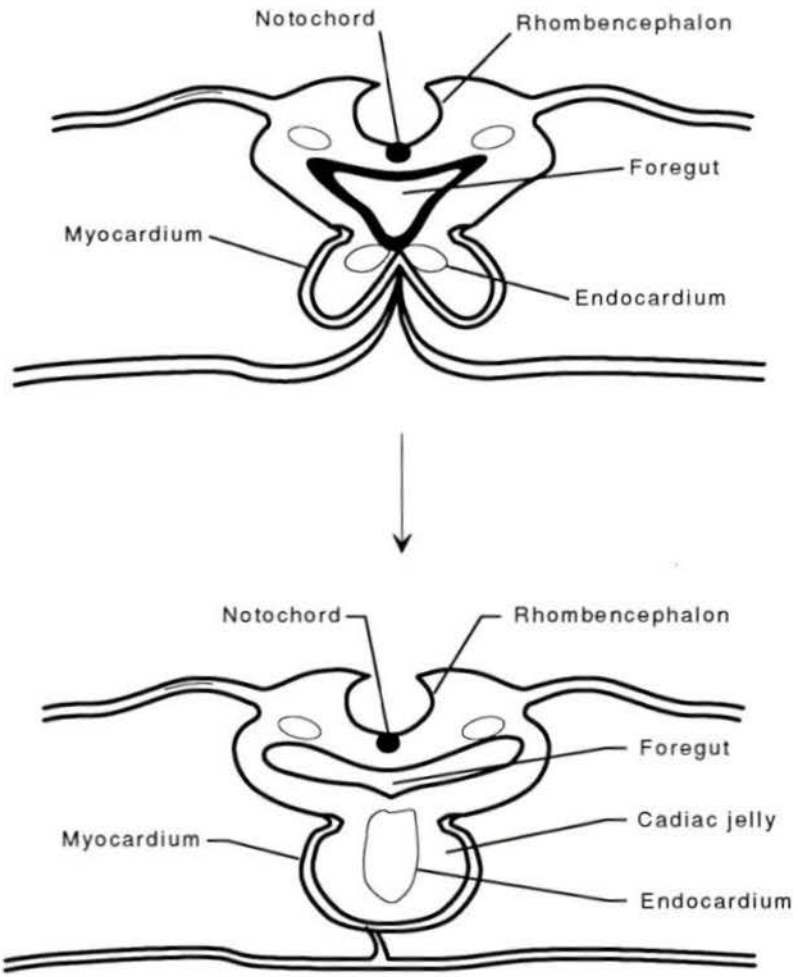
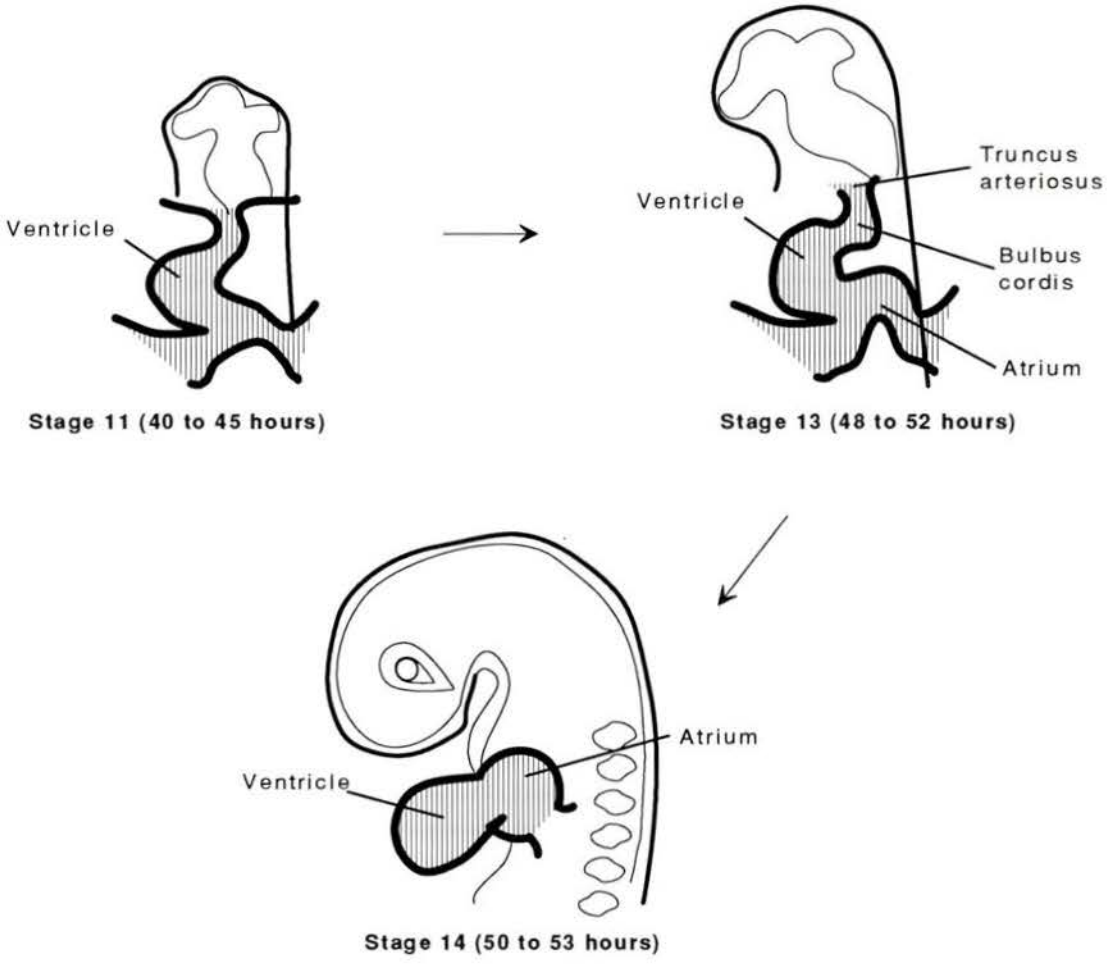

Notochord
Rhombencephalon
Foregut
Myocardium
Endocardium
Notochord
Rhombencephalon
Foregut
Cadiac jelly
Myocardium
Endocardium

Figure C: Diagram illustrating the partitioning and bending of the heart during later heart development, and the torsion of the entire embryo.


Ventricle
Stage 11 (40 to 45 hours)
Ventricle
Truncus arteriosus
Bulbus cordis
Atrium
Stage 13 (48 to 52 hours)
Atrium
Ventricle
Stage 14 (50 to 53 hours)

through the heart is from the yolk to the omphalomesenteric veins to the sinus venosus which is an enlargement at the entrance to the atrium. From there it flows into the atrium and the ventricle. As the blood leaves the heart it flows through the bulbus cordis and truncus arteriosus to the paired ventral aortae. These lead to the aortic arches and finally to the dorsal aorta which carries blood to the tissues (Manner, 1975).

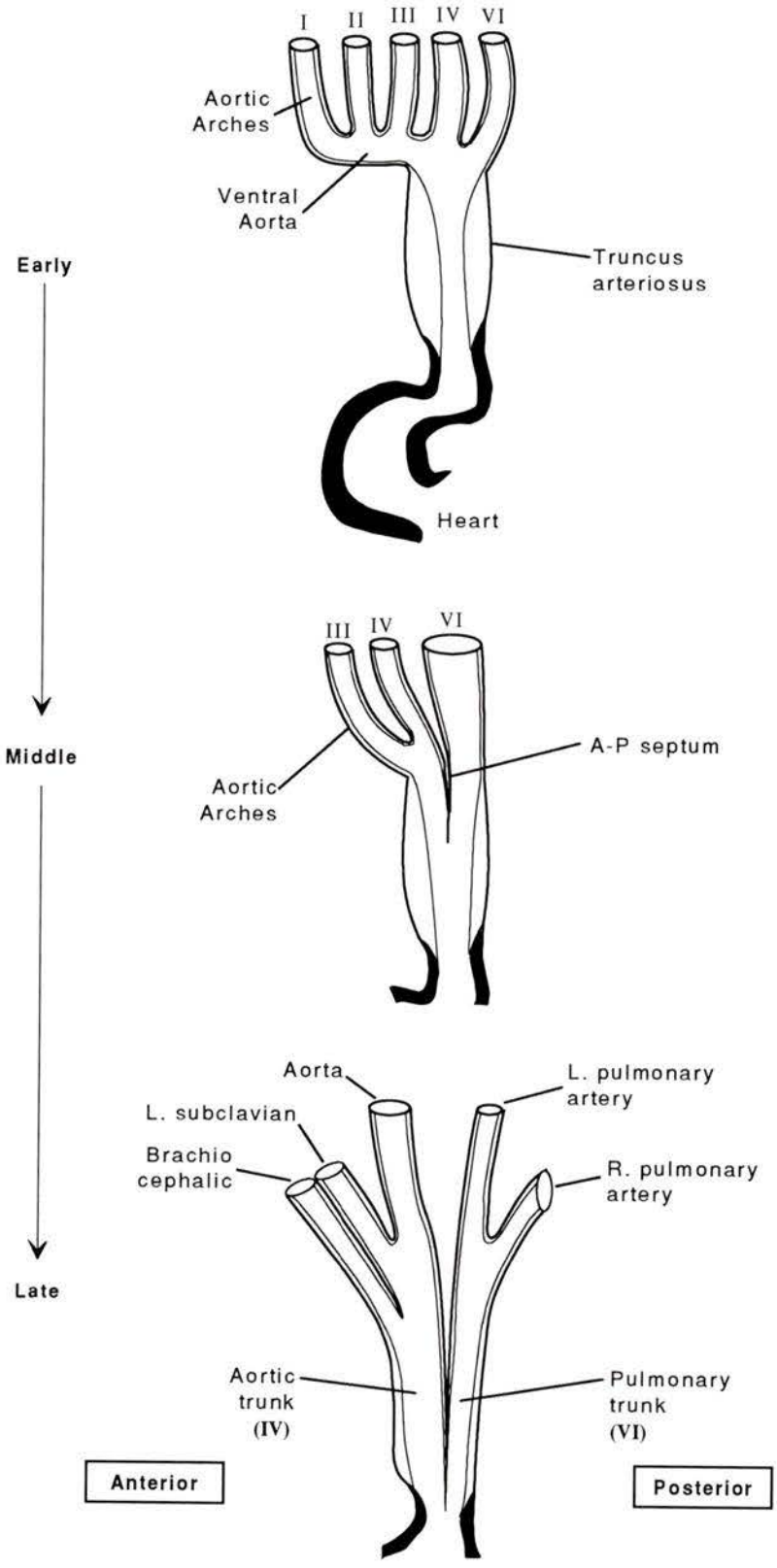
The Aorticopulmonary Septum

The blood flows naturally out of the truncus in two streams, which later become separated by the aorticopulmonary septum (AP septum) into two pathways (Romanoff, 1960). This is the structure that eventually divides the truncus into two tubes, the pulmonary and aortic trunks (Figure D). A dense aggregate of cells at the distal end of the truncus expands toward the heart forming a barrier between the right and left sides (Thompson and Fitzharris, 1979). It forms a spiral that turns from right to left, ventral to dorsal (Rosenquist, 1988). At the distal end the barrier is continuous with the ends of aortic arches IV and VI. . The pulmonary pathway eventually leads off the right ventricle and the aorta leads off the left ventricle. This septum in the truncus is continuous with the bulbar septum, the interatrial and interventricular septa which all form a barrier that divides the heart into two halves (Romanoff, 1960).

The Fate of the Aortic Arches

There are six aortic arches that form in succession, from anterior to posterior. Some remain and are remodeled, and some disappear during subsequent development (Manner, 1975). In general the aortic arches connect the ventral aortae to the dorsal aortae on each side of the pharynx. Arches I and II degenerate at about the time of AP septation. Arch III and part of the dorsal aorta become the internal carotid arteries. Arch IV remains intact only on the right side and becomes the adult aorta. Arch V is present only briefly, and is considered rudimentary in birds and mammals. Arch VI becomes the

Figure D: Diagram illustrating the process of outflow tract septation in the chick embryo.


I
II
III
IV
VI
Aortic Arches
Ventral Aorta
Truncus arteriosus
Heart
Early
Middle
Late
III
IV
VI
A-P septum
Aortic Arches
Aorta
L. pulmonary artery
L. subclavian
Brachio cephalic
R. pulmonary artery
Aortic trunk (IV)
Pulmonary trunk (VI)
Anterior
Posterior

pulmonary arteries, and part of the arch becomes the ductus arteriosus, which degenerates at birth. In the adult the aortic root gives off the aorta and the brachiocephalic and subclavian arteries, while the pulmonary root gives off the right and left pulmonary arteries (Figure D).

ORIGINS OF CELL POPULATIONS IN THE HEART AND OUTFLOW TRACT

Heart Wall Formation

Within the heart forming region the muscular myocardial cells as well as the endocardial cells are mesodermal in origin. The myocardial and epicardial layers are sometimes refered to as the epimyocardium since they are closely associated (Romanoff, 1960). The epimyocardium is separated from the endothelium by the cardiac jelly.

In the anterior continuation of the heart called the truncus, there is a similar arrangement of two cell layers. In this case there is the inner endothelium and an outer adventitial layer. These are separated by a layer of ECM as in the heart. There is also an extension of the muscular myocardial tissue which surrounds the proximal part of the truncus called the myocardial cuff. The cells of the middle layer, the tunica media, appear later in development.

Cardiac Cushion Cells

A major developmental event in cardiac morphogenesis is the formation and migration of a population of cells referred to as endocardial cushion cells (Crossin and Hoffman, 1991). At stage 17, when the cushions begin to develop, the endocardium and myocardium are still separated by the cardiac jelly. The cushion mesenchyme migrate as single cells into the cardiac jelly, and form protrusions into the A-V canal that will become the mitral and tricuspid valves (Bernanke and Markwald, 1982). These A-V pads also form the septa within the heart (Kinsella and Fitzharris, 1980).

According to the study by Bernanke and Markwald (1982) the endocardium is activated by stage 19 and the epithelial-mesenchyme transition can occur. At this time the endothelium undergoes reorientation, hypertrophy, and develops migratory appendages along the cardiac jelly boarder. The cushion tissue cells then migrate through the cardiac jelly toward the myocardium. Disruption of this normal sequence of events in cushion morphogenesis results in anomalies in the structure of the valves.

Endothelial derivatives in the Tunica Media

Cells which fill the tunica media of the outflow tract appear to be from two different sources. Cells thought to be endothelial in origin, may be the first to enter the tunica media (Thompson and Fitzharris, 1979). This unique mesenchyme cell population fills the proximal truncus in a radial direction at the same time as the expansion of the bulbus cordis (Thompson and Fitzharris, 1979). It is not clear whether these cells contribute to the vessel walls of the adult, as there is an influx of cells from the aortic arches which may displace them. This second population of cells that enters the tunica media is derived from the neural crest.

Cardiac Neural Crest Cells

Neural crest cells are a well studied example of a cell population with diverse fates that undergo extensive migrations during early development. The neural crest arises from the dorsal portion of the neural tube where cells release and begin migration along several defined routes (Kuratani and Kirby, 1991). The cells form diverse structures such as neurons, glial cells, endocrine cells and connective tissue elements. They have provided models for the mechanism by which cells undergo stereotypic patterns of migration and determination (Hall and Horstadius, 1988; Le Douarin, 1982; Weston, 1970). The mechanisms by which this phenotypic diversity is generated are

still unclear, but may involve a series of restrictions, resulting in the sequential appearance of various intermediate cell types (Ciment and Weston, 1985).

Cells from the portion of the neural crest between the midotic placode and the caudal limit of somite 3, termed cardiac neural crest, migrate into the cardiac outflow tract to form various parts of the heart and aortic arches (Kirby, 1990) (Figure E). The cardiac portion make significant contributions to the heart and outflow tract in the tunica media.

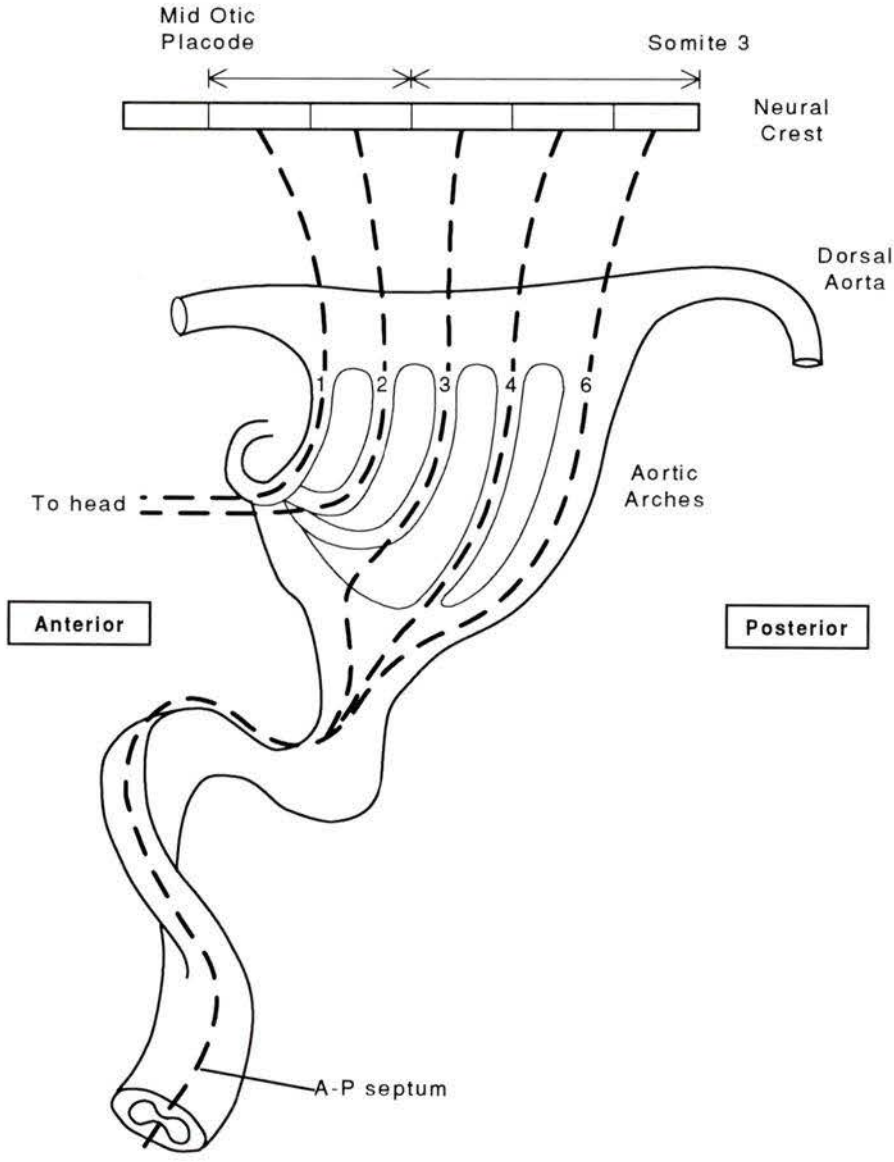
The cells of the cardiac neural crest have also been termed ectomesenchyme to emphasize the origin of the mesenchyme from ectoderm rather than its usual source of mesoderm. These cardiac neural crest cells leave the neural tube at approximately stage 10 or 11, and move into the pharyngeal region, before the pharyngeal arches are formed (Kuratani and Kirby, 1991). The cells appear to pause at this spot, giving rise to a second term, the circumpharyngeal crest. They then migrate into the outflow tract at about stage 25, with the major cellular contribution arising from the portion that pauses at aortic arch IV (Miyagawa-Tomita et al., 1991).

The cardiac neural crest contributes to components of the heart valves, septa in the heart and outflow tract, and walls of the major blood vessels leaving the heart. Ablation of this population of cells causes malformations of the heart and major vessels (Kirby, 1990). For example persistent truncus arteriosus results if the entire neural crest is removed. If a smaller portion is removed malformations such as double outlet right ventricle and transposition of the great arteries can occur (Kirby, 1990). If these portions are replaced by other neural crest cells, malformations will still occur.

COMPONENTS OF THE ECM

It is well known that the ECM, which is a meshwork of macromolecules, is synthesized early in development by various types of cells in the embryo. The ECM appears to act as a scaffold for cell migrations, and has been shown to influence such

Figure E: Illustration of the migration pathway as it is understood, for cardiac neural crest cells (modified from Kirby and Waldo, 1990).


Mid Otic
Placode
Somite 3
Neural
Crest
Dorsal
Aorta
1
2
3
4
6
To head
Aortic
Arches
Anterior
Posterior
A-P septum

activities as proliferation (Gospodarowicz and Ill, 1980), cell shape (Montesano et al., 1983), migration (Linasek and Lash, 1986) and adhesion (Dejana et al., 1983). ECM glycoproteins like fibronectin, collagens, and laminin, have been shown to be essential to cell movements and differentiation.

Fibronectin

Fibronectins (FN) are high molecular weight glycoproteins found in the ECM of all vertebrates examined. They appear to be well conserved molecules as there is a great degree of cross-reactivity between antibodies made against different species (Hynes, 1990) The insoluble form found associated with cells is called cell surface FN. FN is also found in a soluble form in body fluids, called plasma FN. All forms of FN are large glycoproteins with subunits of about 220,000 in apparent molecular weight, and are synthesized by a single gene (Hirano et al., 1983). Plasma FN is a disulfide bonded dimer. The two subunits are held together by a pair of interchain disulfides at the carboxy terminal end. Cellular FN is also a dimer but can occur in high molecular weight polymers or aggregates (Hynes, 1990). The molecule is folded into a series of globular domains, each specialized for particular functions. It contains fibrin, heparin, gelatin, and cell binding regions (Hynes, 1985). Thus it can interact with both cells and other components of the ECM. Previous studies on fibronectin suggest that it has a role in cell-cell contacts, cell adhesion, and cell migration (Couchman et al., 1982; Yamada et al., 1984; Lash et al., 1984). Its effect on cell migration is important in the developing embryo and examples of areas of study include gastrulation, neural crest cell migration, cardiac cell migration, and primordial cell migration (Hynes, 1990). The major cell binding domain of fibronectin is the RGDS (arg-gly-asp-ser) sequence (Piershbacher and Ruoslahti, 1984). Studies have shown that RGDS containing peptides resulted in partial disruption of the cell-ECM interaction (Boucaut et al., 1984).

Laminin

Laminin (LM) is a very large ECM glycoprotein formed from three different subunits, one of 400 k and two of 200 k (Chung, et al., 1979). It is an almost universal component of basal lamina synthesized by a variety of cell types (Timpl et al., 1979). LM and FN can codistribute in the same matrix fibrils, as detected by double label immunofluorescence (Hynes, 1990). LM, like most other ECM proteins promotes adhesion. LM mediated adhesion has been documented in hepatocytes, fibroblasts, and many other epithelial cell types (Hynes, 1990). A well characterized adhesion domain of laminin is the YIGSR (Tyr-Ile-Gly-Ser-Arg) domain (Graf et al, 1987), but LM is a multifunctional adhesion protein with several binding mechanisms. The YIGSR sequence is thought to bind a 67 k protein, but the role of this protein as a cell attachment receptor is uncertain. Evidence has accumulated to indicate that most cell attachment to laminin is mediated through integrins (Gehlsen et al., 1989).

LM probably serves many functions in the embryo. One possible function that has been investigated is its use as a substrate for neural crest migration. LM is found as well as FN along neural crest pathways *in vivo* (Krotoski et al., 1986: Rodgers et al., 1986). LM has been investigated extensively and has been found to have a positive effect on neural crest cell migration (Bronner-Fraser, 1986; 1988; Lallier and Bronner-Fraser, 1991). Conversely, an antibody against heparan sulfate and LM has been shown to cause abnormalities in neural crest cell migration (Bronner-Fraser and Lallier, 1988). Thus, LM may be an important molecule in some neural crest migrations.

Integrins

Receptor mechanisms have been described for LN and FN (von der Mark and Kuhl, 1985; Ruoslahte, 1988). Integrins are a widely expressed family of cell surface adhesion receptors that interact with many ECM molecules, including FN and LM. They are trans-membrane molecules that act as connections between the ECM and the cytoskeleton within the cell (Hynes, 1992). They appear to be the major receptors by which cells attach to the ECM. Some also mediate cell to cell adhesions (Hynes, 1992). There are α and ß subunits that make up each receptor. The α subunits vary in size between 120 and 180 K, and each is non-covalently associated with a ß subunit ranging from 90 to 110 K (Hynes, 1992). Individual integrins can bind more than one ligand, and FN and LM are commonly bound by integrins. Integrins on cells interact with FN and LM through the RGDS cell binding region (Gehlsen et al., 1989; 1992). The α5ß1 integrin is the most prominant integrin in the chick embryo, and its distribution closely parallels that of FN (Muschler and Horwitz, 1991). The expression of this receptor is greatly diminished in adult tissue, indicating that its main function is associated with developmental processes.

With the identification of integrins as a receptor for FN it has become possible to study their involvement in neural crest cell migration (Hynes, 1990). It was first shown by Bronner-Fraser (1985) that integrins are present on neural crest cells. *In vitro* addition of synthetic RGDS peptides will inhibit neural crest cell migration in culture (Boucaut, 1984). Also, antibodies to integrins have been shown to interfere with normal cranial neural crest migration *in vivo* (Bronner-Fraser, 1988).

THE ECM THROUGHOUT OUTFLOW TRACT DEVELOPMENT

Early Heart Development

During early heart development the precardiac mesoderm cells begin coordinated movement at the end of stage 5 (Linasek and Lash, 1986). It has been shown that communication between the mesoderm and the endoderm is necessary for normal precardiac cell migration (DeHaan, 1964). Cues for this migration probably occur through the ECM.

The start of directional movement is correlated with the appearance of cell associated FN (Linasek and Lash, 1986). The concentration of fibronectin also appears to be important, as it may enhance the formation of cell to stratum adhesion sites (Linasek and Lash, 1986). Thus, a difference in fibronectin concentration over the endoderm has been suggested to provide cues for precardiac cell movements. It appears that during early development fibronectin is probably an important molecule. Other extracellular molecules have also been studied. Heterogeneities in the ECM have been suggested by Lash et al. (1978), to regulate differentiation. It may be that common cell-associated molecules, by either qualitative or quantitative differences in their distribution, provide the necessary variability for diverse interactions to occur.

The Extracellular Matrix of the Pre-tubular Heart

As mentioned earlier, the heart goes through a phase where the two heart primordia are at the midline, but have not yet formed a single tube. The ECM in the heart forming region at this time is complex. Glycosaminoglycans (GAGs) and sulfated proteoglycans have been previously identified within this area (Manasek, 1970; Manasek et al., 1973). Drake et al. (1990) concentrated on collagens (Types I and IV), as well as FN and LM which have also been identified. Antigens to FN, collagens and LM are distributed unevenly. LM is associated with the basement membranes of the

associated endoderm and of the presumptive heart. Collagen IV is found in both the basement membrane of the endoderm, and the ECM between the endoderm and mesoderm. Collagen I and FN are also found in the ECM between the endoderm and mesoderm (Drake, 1990).

The functions of the ECM components of the pretubular heart have been examined. The ECM is integral to cell movements and plays important roles in the organization of primordial endothelial cells into tubes (Drake, 1990). The LM present in the endodermal basement membrane is thought to be involved later in the cardiac cushion cell migration in stage 20 (Davis, 1989). Thus there are many ECM components present at this time that perform a variety of functions.

The Cardiac Jelly

Manasek (1975) examined the cardiac jelly between stages 9 and 13. During this brief 12 hour period of development the presence of the tubular heart is established, the cells differentiate, and the heart becomes asymmetric with the formation of a D-loop. This D-loop eventually leads to chamber formation. The composition of the cardiac jelly appears to change as development proceeds, but is generally composed of acidic GAGs such as hyaluronic acid, glycoproteins such as FN, collagens Type I and VI, and sulfated proteoglycans (Manasek, 1975). The cardiac jelly also varies spatially, and appears to be divided into regions that relate to morphogenic events (Hurle et al., 1980).

Johnson et al. (1973) have carried out localization studies between stages 9 and 11 that have shown that the collagen components are incorporated during the period of cardiac jelly elaboration. At this time no fibroblasts are present. ECM molecules that are produced at this time come from three sources, the developing cardiac muscle, the endocardium, or the ventral foregut endoderm which it is attached to the heart at this point (Manasek, 1975).

Many possible functions of the cardiac jelly have been proposed. The cationic composition of the developing myocyte environment may be regulated by changing glycosamino glycan (GAG) synthesis within the cardiac jelly, due to the observations of varying Na^+ content (Klein, 1963). There are also structural contributions from the cardiac jelly. The fact that it has a composition of hyaluronic acid and collagen together gives it mechanical stability. Also it may regulate diffusion of particles, since the composition of the cardiac jelly determines the size of molecules that will diffuse through it (Manasek, 1975).

The matrix of the cardiac jelly changes during the time of migration of the mesenchyme from the endothelium. It has been noted by Bernanke and Markwald (1982), that the matrix contains a high amount of hyaluronate and a low amount of sulfated GAGs in proteoglycans. This situation is reversed in older cardiac jelly that has had cells migrate through it. Collagen is another ECM component that is affected by the cell migration. It was noted in an earlier study *in situ* that the early cushion tissue cells, through their filopoia, caused previously randomized collagen fibrils to become polarized bundles (Markwald, 1979).

Loeber and Runyan (1990) did a comparison of adhesion mechanisms involved in cardiac mesenchyme cell migration. They compared adhesion mechanisms of FN, LM, and galactosyltransferase (GalTase). As in other cell migration systems, there is evidence that FN and LM may play a role in the mesenchyme cell migration. They used a monoclonal antibody, JG22, that recognizes ß1 integrins to block the migration.

Davis et al. (1989) showed that YIGSR peptide could block migration of cardiac mesenchymal cells on LM. The small amount of migration that was not blocked was suggested to indicate that more than one mechanism is involved (Davis, et al., 1989). Support for this idea also comes from the great number of different molecules that have all been reported to effect the rate of migration of the cardiac mesenchyme cells.

A number of other studies on early cardiac mesenchyme focused on galactosyltransferase (GalTase) as a mediator of cell adhesion in cell migrations (Runyan et al., 1986, 1988). The addition of alpha-lactalbumin, which blocks GalTase, produced a dose dependent inhibition of cell migration (Loeber and Runyan, 1990). This was also supported by the use of a polyclonal against GalTase which produced a significant inhibition of cell migration. Therefore there must be more than one mechanism by which the mesechyme cells interact with the ECM. A great number of molecules have been reported to affect the rate of migration of the cardiac mesenchyme cells but it is still not known which ones are the most important.

The Neural Crest and its Contribution to the Outflow Tract

The ECM is also known to be important in neural crest cell migration and outflow tract septation later in cardiac development. FN has been implicated in the formation of both the AP septum and the arterial tunica media (Icardo, 1986). FN is present during the time of septation and is seen within the two prongs that extend into the truncal ridges, to eventually form the AP septum (Icardo, 1986). The intensity of immunofluorescent staining decreases once septation is complete (Icardo and Manasek, 1983).

Neural crest cells migrate into the area just prior to septation of the outflow tract. It is hypothesized that FN plays a role in this migration, as it does with other neural crest cell migrations. Studies of trunk neural crest migration suggest that neural crest cells utilize pre-formed pathways of FN which promote migration and may serve to guide the cells (Thiery et al., 1982). FN is found in all the pathways for neural crest migration in the chick (Newgreen and Thiery, 1980; Thiery et al., 1982). Crest cells migrate on FN coated substrates and appear to respond to gradients of FN using chemotaxis and chemokinesis (Greenburg et al., 1981; Newgreen et al., 1982). Neural crest cells express $\alpha 1 \beta 1$ integrin and antibodies to integrins, or RGD containing peptides interfere with cell

attachment to FN and crest cell migration *in vivo* (Bronner-Fraser, 1986); Krotoski, et al., 1986; Lallier and Bronner-Fraser, 1992). Antisense oligonucleotides to ß1 integrin and several α integrin subunites inhibit neural crest cell attachment to FN and LM substrates (Lallier and Bronner-Fraser, 1993). Other ECM components have been suggested to have a role in crest migration, particularly in the late phases of migration, but there is good support for the hypothesis that preformed pathways of FN have an essential role in trunk neural crest cell migration.

An important part of this hypothesis has been the observation that trunk neural crest cells do not synthesize cell surface FN. Trunk neural crest cells appear not to produce FN *in vitro* (Newgreen and Thiery, 1980) and in situ localizations with FN probes indicate that trunk neural crest cells do not contain significant FN mRNA (Ffrench-Constant and Hynes, 1988). Cells producing cell surface FN when injected into neural crest pathways do not migrate, whereas cells without cell surface FN readily translocate (Bronner-Fraser, 1982; 1984; 1985). It has been suggested that endogenously produced FN may mask guidance cues provided by exogenous FN (Ffrench-Constant and Hynes, 1988; Hynes, 1990). Thus, the idea that the migrating cells do not produce FN is central to this theory of neural crest migration, which is thought to also apply to other cell populations. There is new evidence that there may be variations on this basic idea in different cell populations (Burke et al., in preparation).

The cardiac neural crest migration is the final migration in cardiovascular development. Once the neural crest cells are in place no other cells come into the area. They begin to differentiate by about stage 28, and appear to secrete a unique ECM within the tunica media (Rosenquist et al., 1988).

Formation of the mature ECM

Once the neural crest cells have migrated to their final spot, they differentiate into smooth muscle cells and begin secretion of the mature ECM. Elastin is a major

component of all vessels and is the major component secreted at this time. Without the presence of the cardiac neural crest cells in the outflow tract this process will not occur sufficiently, and malformations will occur. Elastogenesis first begins at foci between the myocardial cuff and ectomesenchyme of neural crest origin (Selmin, 1991; Rosenquist et al., 1988). This begins at approximately stage 35 of development (Rosenquist, 1988). The process of elastogenesis proceeds downstream along the developing great arteries in an orderly sequence until there is a continuum of elastic tissue present in the tunica media of all the elastic vessels (Rosenquist et al., 1988). When the neural crest is removed, the rate of elastogenesis is slowed and the configuration of the elastic matrix is disordered. Thus it is clear that these cells play a significant role in directing this process (Rosenquist, 1989). Also, when the neural crest is removed, collagens I and III are coarsened and lack any distinct spatial order in the arteries (Rosenquist, 1989).

Since all the regions to which the cardiac ectomesenchyme contribute are destined to become elastic-walled vessels Rosenquist et al. (1988), have hypothesized that some factors related to the process of elastogenesis would be expressed only in the ECM associated with ectomesenchyme. This study by Rosenquist involved the examination of chick and quail embryos between stages 21 and 38 of development. The use of immunofluorescence and histochemistry allowed the location of two substances that are thought to be elastin precursors. The presence of these molecules distinguished the ectomesenchyme cells of the elastic arteries from cells in other areas.

It is clear from this discussion that the ECM within developing heart and outflow tract is a dynamic component, that changes throughout time and has many functions. Morphogenesis appears to require precise, sequential functioning of macromolecules in order to occur (Boucout, 1984). Thus the developmentally regulated appearance and disappearance of molecules is significant.

THE HNK-1 ANTIGEN

The HNK-1 antibody was originally made against a cell surface antigen on natural killer cells (Tucker et al.,1984). The carbohydrate moiety recognized by this antibody is expressed on cells of neural tube origin in the chick The antibody has proven useful in studies of migrating neural crest cells (Luider et al., 1992). This carbohydrate is shared by a family of adhesion molecules (Bronner-Fraser, 1987). The epitope is also found on mature neurons, Schwann cells, some leukocytes and a variety of other cells at specific times during development. This antibody has been used widely for tracing migration of neural crest cells along early pathways.

The antibody also recognizes adhesion molecules such as N-CAM, L1 and L2, therefore it is possible that molecules bearing the epitope may play a role in adhesion. Further evidence for this comes from explant studies where addition of HNK-1 caused neural crest cells *in vitro* to detach from a laminin substrate, and injection into embryos caused ectopic neural crest cells (Bronner-Fraser, 1987). This was not the case on a FN substrate and the results suggested that there was a limited window of susceptibility.

The HNK-1 antigen is also present early in the embryo associated with gastrulation and nerulation, and the expression is seen to change with development (Luider, 1992). For example, in association with mesoderm induction, it recognizes the inducing tissue before induction, but after induction it shows a mosaic pattern in the responding tissue (Canning, 1988). Thus, the antigen's temporal and spatial distribution is related closely to morphogenesis. This also appears to be the case in the developing cardiac outflow tract as indicated by the present study, where the majority of mesenchyme cells are positive for HNK-1 only during the migratory phase, while septation is occuring.

SUMMARY AND RESEARCH OBJECTIVES

There are many events occuring throughout the development of the circulatory system in the chick embryo, that appear to result from interaction between the ECM and various cell populations. For this study the morphogenic process that was examined was the formation of the outflow tract, a relatively late event in the development of the heart. There are profound changes that occur during truncal morphogenesis, that result in the rearrangement of mesenchymal cells into definite structures. The purposes of this study were to examine the distribution of HNK-1 immunoreactive mesenchyme in the outflow tract, along with the changes in distribution of FN in the ECM. Migration of neural crest cells on FN was analyzed to determine if FN has a role in cardiac neural crest migration that is similar to its role in trunk neural crest cell migration.

MATERIALS AND METHODS

CHICK EMBRYOS

Fertile White Leghorn chicken eggs were incubated at 102 ^{o}C in a humid environment, and turned daily. The embryos were staged according to Hamburger and Hamilton (1951). For dissection, a window was made in the egg shell, and the membranes were cut with fine scissors. Using an embryo spoon the embryo was lifted out of the egg and placed in Howard Chick Ringers (123 mM NaCl, 1 mM $CaCl_2$, ($2H_2O$), 5 mM KCl. The truncus arteriosus was dissected out by cutting at the aortic arches and at the ventricle. Stages ranging from 10 to 35 were used (33 hours to 8 days). The dissection was carried out using sterile technique in a tissue culture hood for the truncus explant experiments.

HISTOLOGICAL SECTIONS

The dissected conus arteriosus tissue was rinsed in fresh Ringers solution, and then in Cacodylate buffer (0.1M sodium cacodylate, 0.1M sucrose, 1 mM $CaCl_2$, pH 7.2). The tissue was then fixed for 1 hour in 2% Glutaraldehyde in Cacodylate buffer at room temperature. Once this was complete the tissue was rinsed thoroughly in cacodylate before postfixation in 1% osmium tetroxide in cacodylate buffer for 1 hour. The tissue was dehydrated in an ethanol series (30%,50%, 70%, 95%x2, 100%x2), and put through 3 changes of propylene oxide for 10 minutes each. Infiltration was carried out in 1:1 Epon 812 resin (21.3 parts Epon 812, 21.6 parts dodecenyl succinic anhydride, 7.1 parts nadic methyl anhydride, with 1.6 ml 2,4,6-trimethyl amino methyl phenol per 100 ml) and propylene oxide for 3 hours (open to air). Then it was left on a rotator over night in 3:1 resin to propylene oxide. Embedding was in pure resin in an embedding boat. The boat was placed in a 60^{o}C oven for 16-24 hours for curing. Once the resin was hard, the tissue was cut out in a small piece of resin, oriented and glued

with epoxy onto a stub. Serial sections were cut on a Sorvall MT5000 Ultra Microtome at 500 nm with a diamond knife. The sections were dried to the slide on a hotplate, then stained with Richardson's stain (Azure Blue and Methylene Blue) and examined under a light microscope.

Photographs were taken on T-Max 100 film using a Balzer 604 nm filter. The film was developed in D-76 and fixed in Hypam fixer according to standard darkroom procedures.

CRYOSTAT SECTIONS AND INDIRECT IMMUNOFLUORESCENCE

The dissected conus tissue was washed in fresh Ringer's buffer and transferred to OCT embedding medium. It was oriented so that cross sections could be obtained. This could then be stored at -80^{o}C until use. Serial sections were cut on an IEC cryostat model CTI at 7um at -25^{o}C. Alternate sections were examined with either anti FN (H3 or F5), HNK-1, anti ß1-integrin (Sigma Immunochemicals) or NRH (control) monoclonal antibodies. H3 and F5 are a monoclonal antibodies made in our lab against chicken FN. HNK-1 is a monoclonal antibody (ATCC), recognizing a cell surface antigen on neural crest derived cells as well as human natural killer cells. The anti-integrin monoclonal is against ß-1 chicken integrin (Sigma Immunochemicals), and recognizes an extracellular epitope of the avian integrin complex. The ß-1 subunit is made up of 803 amino acids and is a transmembrane glycoprotein. NRH is a non relevant antibody used as a negative control in these experiments. It is made against the plant protein rubisco (ribulose 1,5 bis phosphate carboxylase/oxygenase).

For antibody incubations the slides were flooded with the primary antibody solution and allowed to incubate overnight at 4^{o}C. The HNK-1 was used at a 1:200 dilution, and the H3 and NRH were used at 1:100 dilutions in phosphate buffered saline (PBS). The secondary antibody was either RAM-FITC IgG (with H3 and NRH) or RAM-FITC IgM (with HNK-1). Between treatments the slides were rinsed thoroughly

with PBS, and then mounted in 1:1 PBS:Glycerol with 6.25 mg/ml n-propyl gallate. The finished slides were examined with a Zeiss epifluorescence microscope at 495 nm.

In double stained preparations, the sections were incubated in one primary antibody (HNK-1 or anti-neurofilament 200) followed by the secondary specific to it (RAM-FITC of GAR-TRITC). Subsequently, the second primary antibody and its secondary were used. Photomicrographs were taken on T-Max 400 film and processed as above.

ANTIBODY PURIFICATIONS

The H3 and F5 antibodies were produced by IP injection of BALB/C mice with 10^3-10^4 hybridoma cells. The mice were left to produce ascites fluid for approximately 1 week. The ascites fluid was collected by perforating the abdominal wall with an 18 gauge needle, or by euthanizing the mouse, dissection of the body wall and aspiration of the ascites. The fluid was concentrated using an Amicon concentrator, and affinity purified with a protein A column (Pierce).

IN VITRO EXPLANTS

FN rich Extracellular matrix production

The primary chick fibroblastic cell cultures were prepared from day 16 to 18 chick embryonic aortas. The aortas were dissected and chopped into fine pieces before they were disaggregated with 0.025% trypsin (GIBCO) and 0.5% collagenase D (Boehringer Mannheim) at 37°C. The cells were resuspended in 1:1 mixture of Dulbecco's modified minimum essential medium and Ham's F-12 supplemented with 5% fetal bovine serum (Hyclone Laboratories) and allowed to settle on Petri dishes (Corning 25020-100). The cell culture supernatant was collected at day 6 in the presence of protease inhibitors (3 mM EDTA and 2 mM PMSF) and precipitated with 40% saturated $(NH_4)SO_2$ overnight at 4°C with gentle stirring.

After centrifugation at 15,000 g, for 30 minutes in a Beckman JA-21 centrifuge, precipitated proteins or were resuspended in 50 mM Tris HCl, 0.15 M NaCl, 2 Urea at pH 8.0, and dialyzed against this buffer overnight at 4°C. The protein solution, containing FN, was passed over a 10 ml immobilized gelatin agarose column (Pierce), washed with 50 mM Tris HCl, 0.5 M NaCl, at pH 7.5 and eluted with 50 mM Tris HCl, 8 M urea at pH 7.5 (Ruoslahti et al., 1982). The 2 ml fractions were collected and analyzed on 5% SDS-PAGE (according to Laemmli, 1970). The fractions containing FN were pooled and dialyzed against 50 mM Tris HCl, 2 M urea at pH 8.0 overnight at 4°C.

Coating Substrates

According to a dose response study using FN and chick ECM as stimulators of neural crest cell migration, doses of 0.5 µg/cm^2 of FN and 1.0 µg/cm^2 of ECM were coated as substrates on the bottom of 24 well plates (Corning) and left to air dry overnight at room temperature. The plates were rinsed twice with distilled water and allowed to air dry again before use.

Explant Cultures

Explants were taken from truncus tissue from Stage 28 embryos, from the region of the AP septum. These were dissected out using sterile technique. The tissue was rinsed with F5 containing PBS once. The concentrations of F5 were 0, 10, 20, 40, 60, 80 µg/ml. It was then bathed in culture medium (1:1 Dulbeccos modified Eagle medium with glucose and F-12 nutrient mix, pH 7.2) warmed to 37°C. The tissue was then minced into small pieces and approximately 3 pieces placed into each well of 24 well Corning culture plates. The experimental wells were coated with FN or ECM. Control wells were coated with 1 µg of BSA or PBS. The tissue was either incubated in culture medium alone (serum free), or culture medium containing anti-FN antibody (H3) or an

anti-LN antibody. The explants were cultured for 24 hours at 37°C in a CO_2 incubator, before measurements were taken under an inverted microscope.

Another group of experimental plates were set up with a Matrigel substrate (1:4 dilution with the culture medium). This is a commercially available gel-like substrate for cell cultures. It is a solubilized basement membrane preparation extracted from a mouse sarcoma cell line. Its major component is laminin, but it also contains collagen IV, heparan sulfate, proteoglycans, entactin, and nidogen (Collaborative Biomedical Products). It also contains TGF-ß, fibroblast growth factor, tissue plasminogen activator, and other growth factors that occur naturally in the mouse tumor. This substrate was stored at 4°C until just before use when it was allowed to warm to room temperature and solidify. One set of plates contained Matrigel alone, and one set of Matrigel with 5µg/well FN added.

WESTERN BLOTTING

SDS-PAGE was performed according to Laemmli (1970). The separating gels were 10% acrylamide(HNK-1 and anti-integrin) or 4% acrylamide(FN). The samples were placed in reducing sample buffer (with SDS) and boiled for 5 minutes prior to loading onto the gel. The gels were run at 150 mV. Proteins were electrophoretically transferred from the gels to nitrocellulose membranes using a constant voltage system. During this process the materials were kept cool with a circulating ice bath (approximately 4°C). The transfer buffer used contained 20% methanol, glycine and PBS. The nitrocellulose was blocked overnight in 5% milk, PBS and 1% Tween before incubation with the antibodies. The antibodies were diluted in this solution. The anti-FN primary antibody was diluted 1:100 and incubated at room temperature for 3 hours. The nitrocellulose was then rinsed three times in the PBS-milk solution (Blotto), and the secondary antibody (RAM-HRP) was added at 1:500 in Blotto. This was incubated at room temperature for 1 hour. The nitrocellulose was rinsed 3 times in Blotto, and 3

times in PBS before developing. The developer used consisted of 0.01 g DAB, 50 ml PBS, 3 crystals cobalt chloride, and 22µl of 30% hydrogen peroxide.

RESULTS

HISTOLOGY

The truncus arteriosus of the developing chick embryo begins as a tube with two distinct cell layers by stage 10. The inner endothelium is separated by a cell free space from the myocardial cuff. This cell free space is known to be filled with ECM and is the presumptive tunica media. Between stages 15 and 20 mesenchyme cells appear within the tunica media, scattered randomly within the ECM. The cells increase in density throughout this time (Figure 1, a-c). In later stages, there is a marked difference in the density of the mesenchyme when one compares regions proximal to the heart (Figure 1, d and e) with regions near the aortic arches or a comparison of early and late stages (Figure 1, h and i). There is a significant increase in the cell numbers at this time (Figure 2) which first appears as a gradient. In stage 24 embryos there are 0.2 to 0.4 cells/unit area in the proximal to middle regions, but the density is 0.7 near the AP septum. Stage 26 shows a similar pattern to that of Stage 24. By stage 30 there is no difference in the cell densities along the outflow tract, and the density is close to 0.7 cells/unit area.

Septation of the outflow tract is apparent first in the distal regions of the tract and it proceeds toward the heart in successive stages with a zippering mechanism. The AP septum is a barrier of tissue that separates the aortic and pulmonary tracts as they leave the heart. It continues the separation of the heart into left and right halves by the interventricular and interatrial septa. It arises from a different mechanism than these other septa though, being derived from cardiac neural crest cells (Kirby and Waldo, 1990). The AP septum has a distinct appearance when stained histologically (Figure 1, f). The cells are closely packed, and the staining is less intense than in the other mesenchyme cells.

Figure 1: Richardson's stained cross sections of the outflow tract during its development in the chick. (a) Stage 10 truncus, (b) Stage 15 truncus, (c) Stage 20 truncus. In (d) and (e) proximal and distal sections with respect to the heart from a stage 25 embryo showing the gradient of cells from the aortic arches to the heart. The AP septum is seen in (f) in a Stage 28 embryo, proximal to the heart. (g) shows the organization of the cells in the truncus of a Stage 28 chick, distal to the heart. Part of the AP septum is also visible between the vessels. Longitudinal sections of an early truncus (h) compared with a late truncus (i) show the differences in cell densities. Mag. for a,b,c,g is 80 x. Mag. for d,e,f,h,i is 156 x

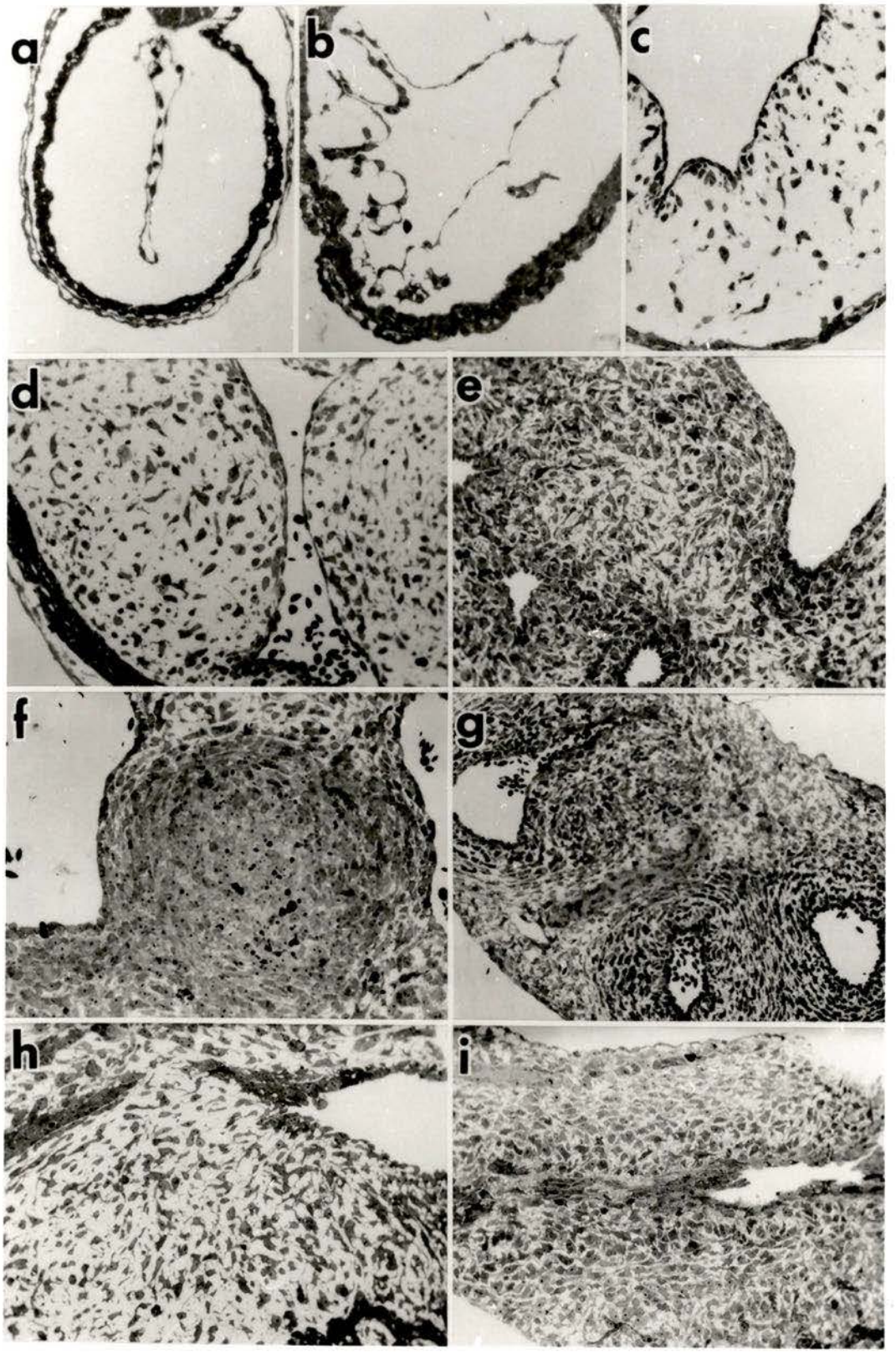
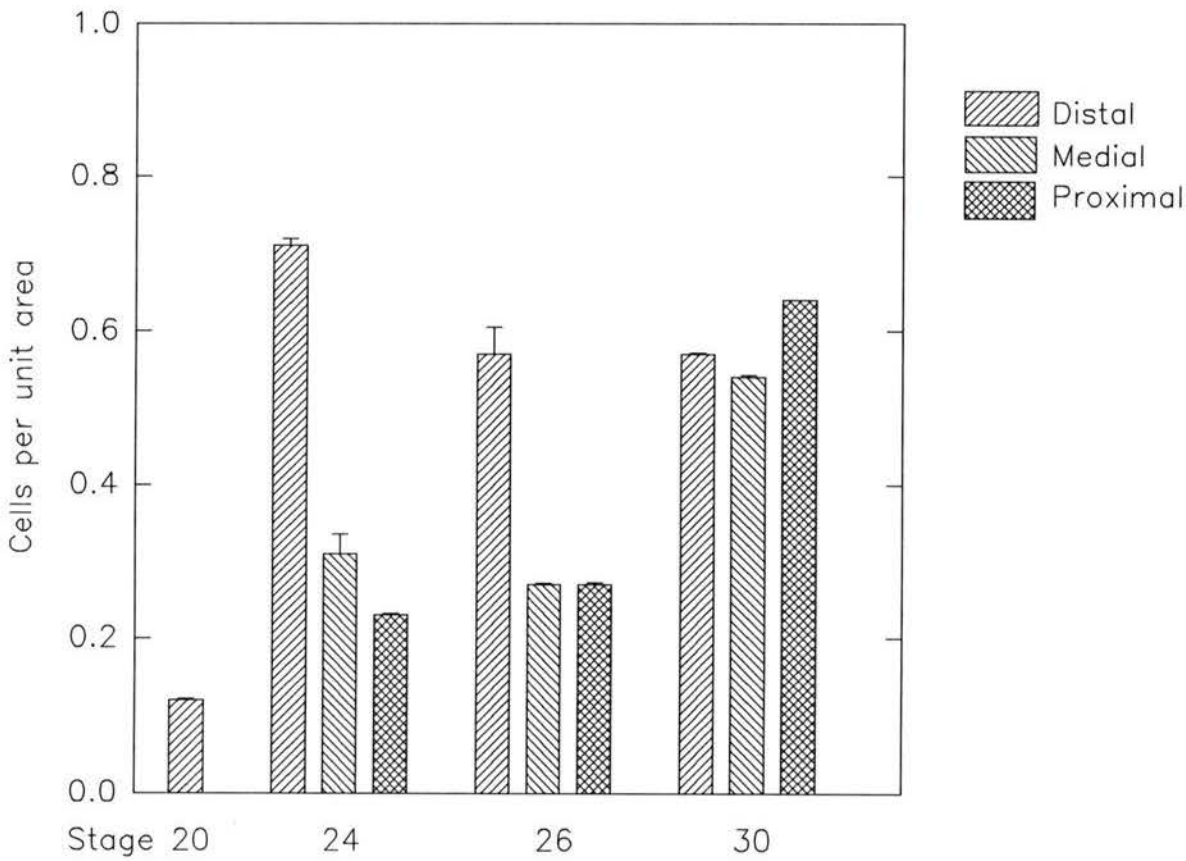

a
b
c
d
e
f
g
h
i

Figure 2: Histogram indicating the timing of migration of cells into the truncus. The cell densities (±S.E.) begin to increase at approximatelystage 24, and are evenly distributed along the length of the outflow tract by stage 30.


1.0
0.8
0.6
0.4
0.2
0.0
Cells per unit area
Stage 20
24
26
30
Distal
Medial
Proximal

At stage 28, at the distal end of the truncus, the cells begin to align themselves in concentric layers around the vessels (Figure 1, g). This appears to be a process that begins at the distal end and progresses towards the heart once the cells stop migrating.

WESTERN BLOTTING

In the western blot of outflow tract probed with F5 (Figure 3) a doublet of approximately 220 k consistently appears as well as several bands of lower apparent molecular weight. A range of early and late stages were sampled (Figure 3, lanes 3-7), and FN was present throughout the entire time. There appeared to be an increase in FN at approximately stage 25, that was consistent on repeated blots. The amount of protein was equalized for the lanes, so this may indicate a relative increase in the amount of FN at that stage. At stage 32 there was the consistent appearance of two lower molecular weight bands that reacted with the anti-FN antibody. These are thought to be break down products of the FN.

IMMUNOFLUORESCENCE

Frozen sections that have been prepared for indirect immunofluorescence with anti-FN antibody show thin fibrils of immunoreactive material in the tunica media of outflow tracts as early as stage 10. Figure 4 illustrates the approximate locations of cross sections of the truncus at stages 10, 15, 17, and 20 labeled with FN and HNK-1 antibodies. The fibrils are associated with the endothelial cells, the adventitia and the few scattered mesenchyme cells within the presumptive media. There are also spots of immunoreactive material within the media, associated with cells, which is not in the fibrillar form (Figure 5). As the mesenchyme increases in density the immunoreactive

Figure 3: Western blot of FN. Lane 1 contains molecular weight markers of 180, 116, and 84 kDa, lane 2 a FN marker, lane 3 Stage 18 truncus tissue, lane 4 stage 23 truncus tissue, lane 5 stage 26 truncus tissue, lane 6 stage 28 truncus tissue and lane 7, stage 33 truncus tissue. The lanes were loaded with equal amounts of protein, of 5 µg.

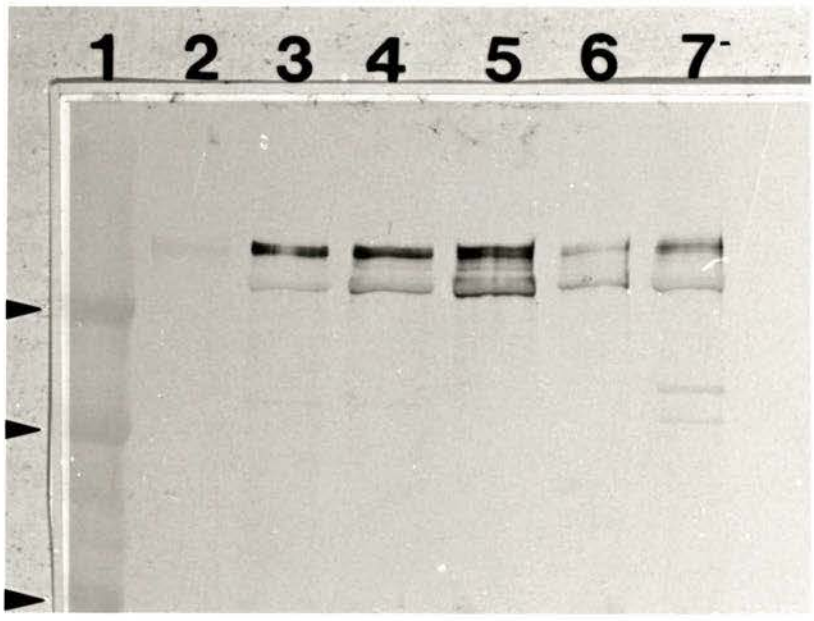

1
2
3
4
5
6
7

Figure 4: Diagram illustrating the approximate position of cross sections in stage 10 truncus tissue (a,b,c,d), in stage 15 tissue (b), and in stage 20 truncus tissue (b), in the following immunofluorescence plates of FN and HNK-1.

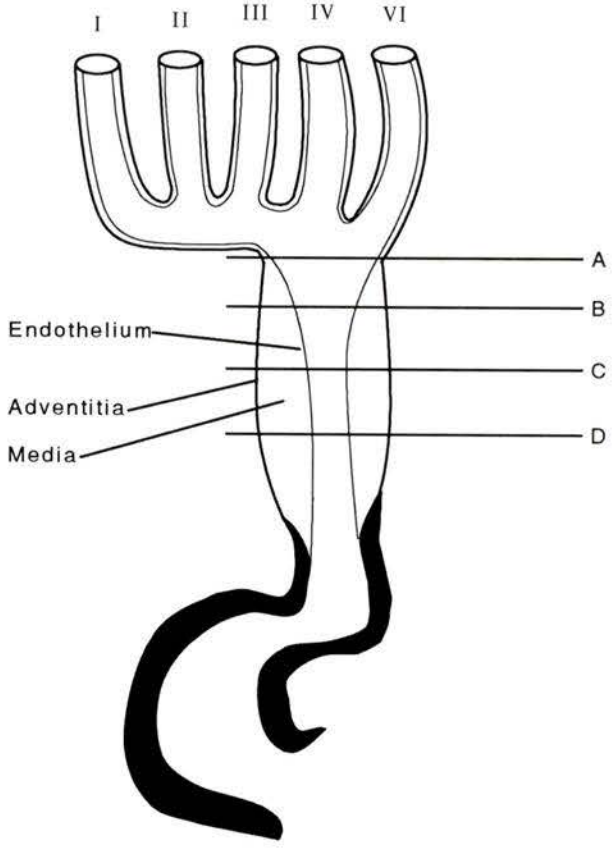
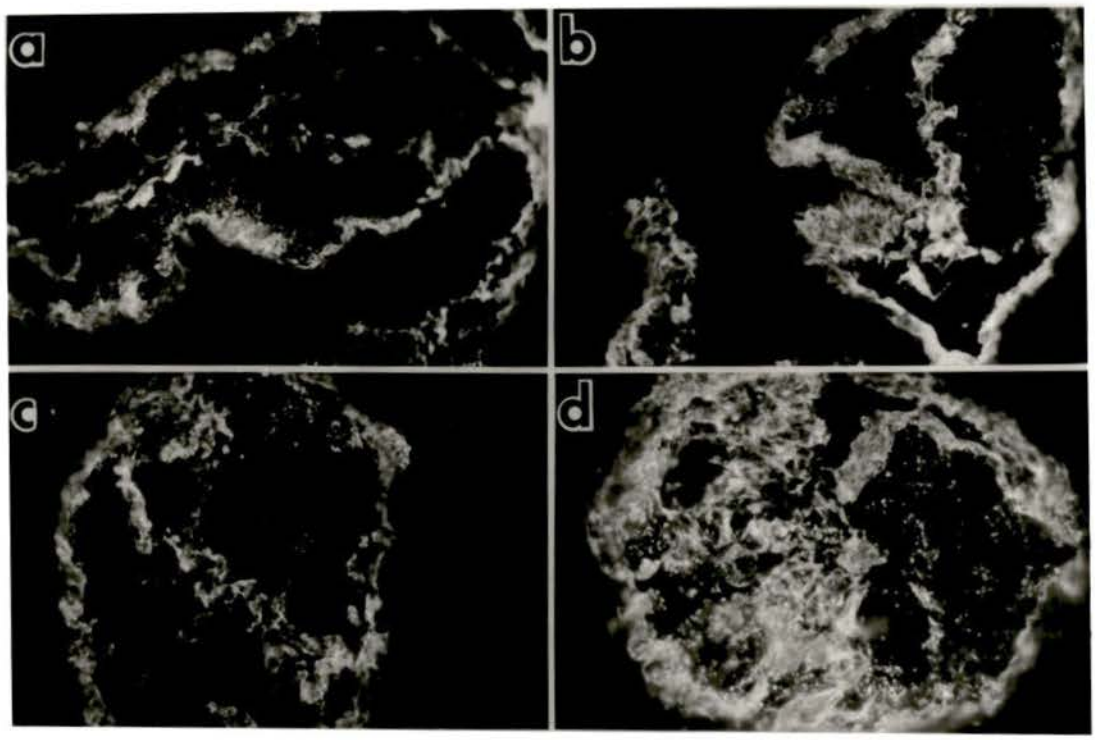

I
II
III
IV
VI
A
B
C
D
Endothelium
Adventitia
Media

Figure 5: Immunofluorescence preparations using anti-FN antibody, of stage 10 truncus tissue,(a) and (b), stage 15 (c), and stage 20 (d) truncus cross sections showing the pattern of FN distribution at these stages.

Mag. 80 x.


a
b
c
d

fibrils also increase in density. The fibers appear to fill in the media but are in random arrays (Figure 5, d).

Figure 6 is an illustration indicating the relative positions of cross sections in stage 25 truncus tissue. In stage 25 embryos the FN distribution differs dependent on where it is examined (Figure 7). From the heart to the A-P septum the FN is dense throughout the media as well as being associated with the adventitia. The lumen of only one vessel is visible (Figure 7, a and b). Distal to the A-P septum the FN is still present throughout the media, but it is more dense in association with the endothelium (Figure 7, c). This association remains after septation is complete.

Figure 8 is an illustration of the position of cross sections in stage 28 truncus tissue. By stage 28 the septation of the outflow tract is nearly complete, but there are still some differences in FN distribution proximal and distal to the heart. Near the heart, where the septation is not yet complete, there is abundant FN in the media as well as in association with the adventitia (Figure 9). The AP septum (Figure 9, b) is filled with FN as it is forming, and there is abundant FN between the newly forming vessels (Figure 9, c and d). In the region distal to the A-P septum the FN becomes organized into a regular array of lamellae (Figure 9, d), and this becomes the pattern along the length of the outflow tract by stage 33 (Figure 9, e). The FN is closely associated with the cells of the tunica media from stage 25 onward (Figure 9, f).

In frozen sections of stage 10 outflow tract prepared for indirect immunofluorescence with the HNK-1 antibody, there are cells associated with the endothelium and the adventitia that express the antigen. In the region nearest the heart there is a group of cells expressing the HNK-1 antigen just outside the outflow tract. This appears to be a portion of the forgut , which is attached to the outflow tract at this time of development. In sections near the aortic arches there are scattered cells within the endothelium and the adventitia that express the antigen (Figure 10, b-d). This arrangement remains until stage 15 (Figure 10, e). The cells that express the antigen at

Figure 6: Diagram illustrating the position of cross sections in stage 25 tissue stained with FN and HNK-1 in the following plates.

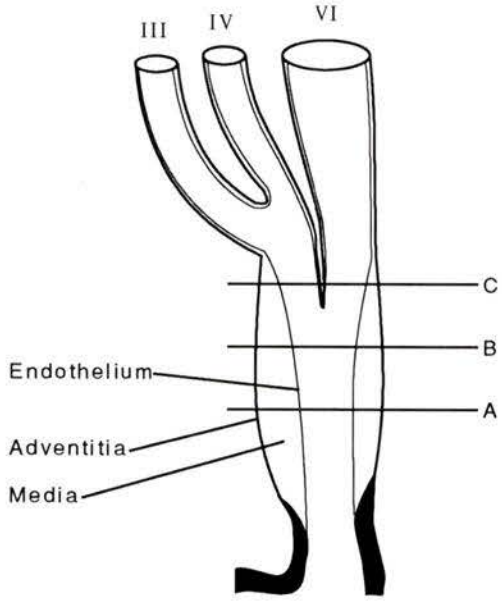

III
IV
VI
C
B
A
Endothelium
Adventitia
Media

Figure 7: Immunofluorescence preparation of stage 25 truncus tissue with the anti-FN antibody. Sections (a)-(c) are in a proximal to distal series. There are FN fibers filling the tunica media in all portions of the truncus at this stage. Note the association of bright staining fibers to the endothelium, once septation of the truncus has occured, beginning near the aortic arches in (c). Mag. 195 x.

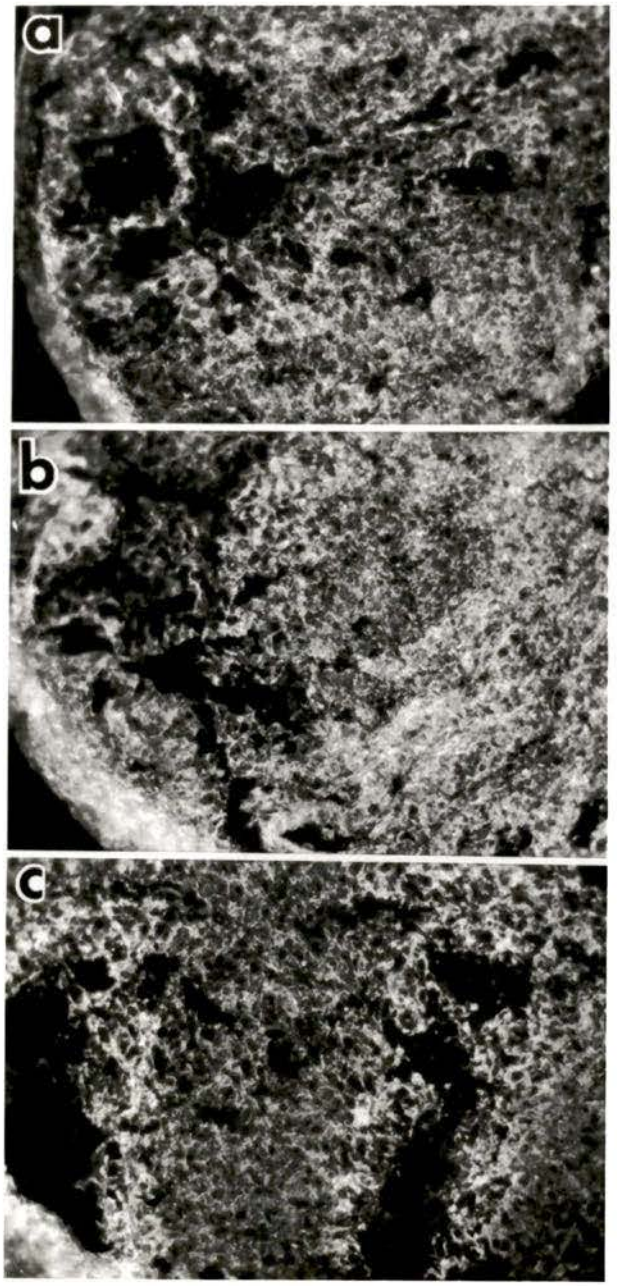

a
b
c

Figure 8: Diagram illustrating the position of cross sections in the stage 28 truncus stained with FN and HNK-1.

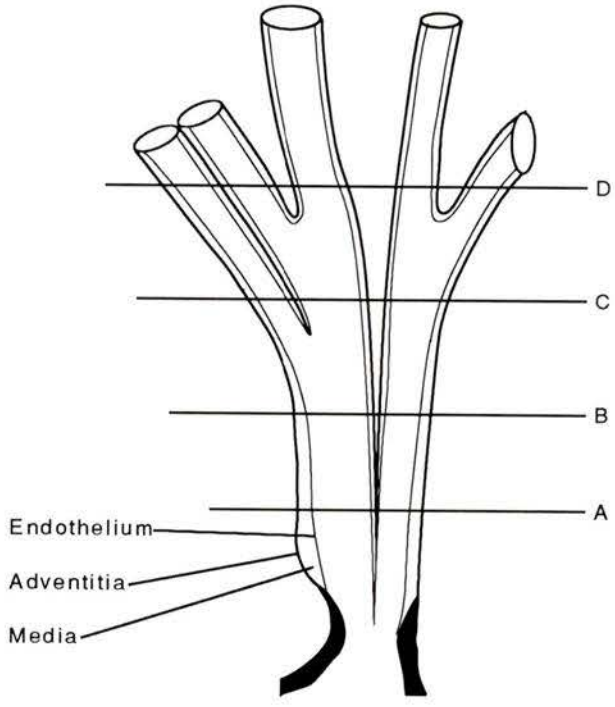

D
C
B
A
Endothelium
Adventitia
Media

Figure 9: (a)-(d) Immunofluorescence preparations of stage 28 truncus tissue showing the proximal to distal organization of FN. As septation progresses the FN becomes organized into lamellae around the vessels, (d), and becomes associated with the endothelium by stage 33 (e). (f) Higher magnification micrograph of FN associated with cells in the media of Stage 33 truncus. Mag. a,b,c,d,e 80 x. Mag. f 875 x.

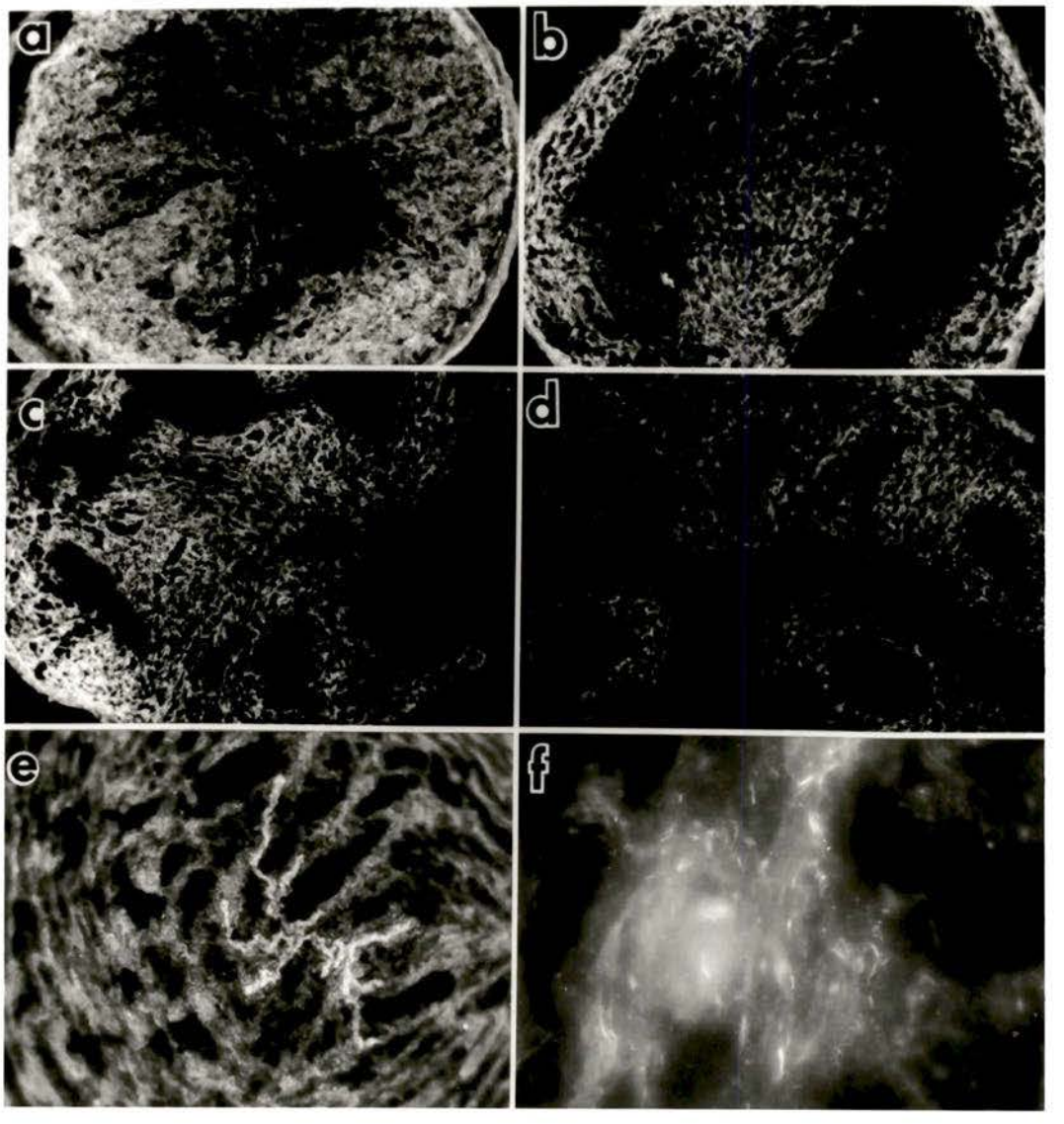
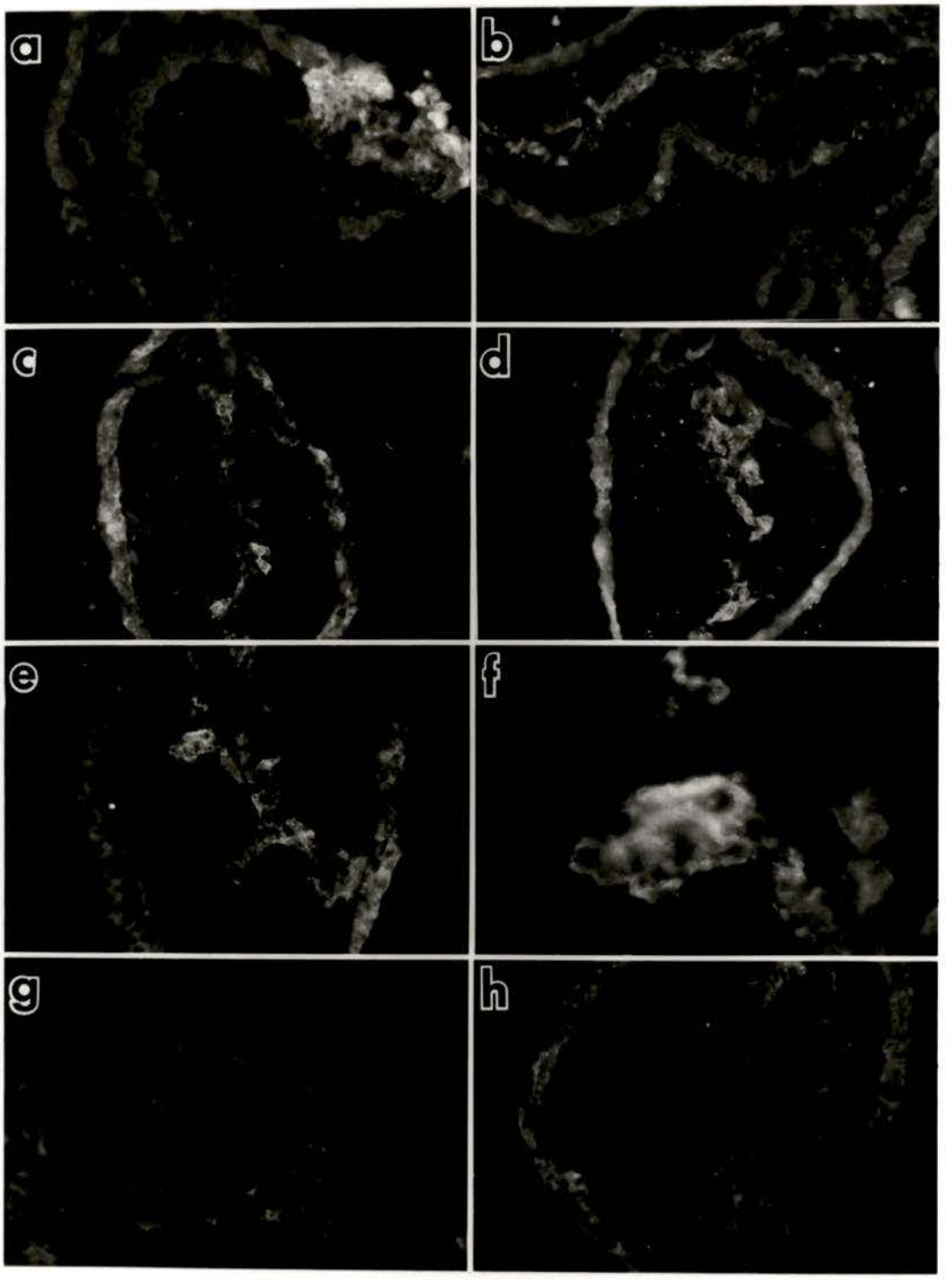

a
b
c
d
e
f

Figure 10: Immunofluorescence preparations using HNK-1 antibody, of stage 10 truncus (a)-(d), at points distal, middle and proximal to the heart. In (a) the mass of brightly staining cells is at the interface between the truncus and the foregut. (e) Stage 15 truncus showing a few scattered HNK-1 positive cells. A higher magnification is seen in micrograph (f), showing a mesenchyme cell associated with the endothelium, surrounded by a thick layer of HNK-1 labeled antigen. (g) Stage 17 truncus with few cells labeled by HNK-1 and (h) Stage 20 also with few labeled cells. Mag. a,b,c,d,e 80 x. Mag. f 875 x.


a
b
c
d
e
f
g
h

this time are typically brightly fluorescent with clear, sharp boundaries (Figure 10, f). Between stages 15 and 20 there are few immunoreactive cells scattered within the tunica media, but these stain poorly with the antibody (Figure 10, h). By stage 25 there is dense mesenchyme appearing in the distal regions of the outflow tract, that are immunoreactive with HNK-1 (Figure 11, c). The cells are a loosely adherent mass and the fluorescence is diffuse, showing no clear cell outlines. The media of the more proximal regions contain only a few immunoreactive cells at this time, similar to the earlier stages examined (Figure 11, a and b).

As the mass of dense mesenchyme approaches the heart (stage 28 to 30) the presumptive media becomes more uniformly immunoreactive (Figure 12). There appear to be two leading masses of cells (Figure 12, b), moving with septation. The HNK-1 immunoreactive mesenchyme fills in the spaces in the media as it moves into the outflow tract (Figure 12, c and d). By the time septation is complete, the entire media is filled with HNK-1 immunoreactive cells, and they become arranged in laminae similar to the FN (Figure 12, e and f).

Longitudinal sections of the outflow tract illustrate the influx of cells from the aortic arch region by stage 24 or 25. In stage 21 embryos the dense mesenchyme is not yet present in the region near the aortic arches (Figure 13). In stage 24 the mass of HNK-1 immunoreactive cells enter the outflow tract, and can be seen as a mass of closely associated cells that is most dense near the arches. This gradually spreads throughout the entire outflow tract as development proceeds.

Between stage 33 and stage 35 the HNK-1 immunoreactivity begins to weaken throughout most of the presumptive media. However, strongly immunoreactive patches of cells occur in the outer portion of the media immediately subjacent to the adventitia (Figure 14, a). These intensely fluorescent regions persist until after stage 35. In double labeling experiments with antibodies to the 200 k neurofilament antigen and HNK-1

Figure 11: Immunofluorescence preparation of stage 25 truncus tissue labeled with HNK-1. (a) Proximal to heart there are few faintly staining cells in the media, and the appearance of some staining in the mid region (b), just ahead of where septation is occurring. There is an increase in HNK-1 staining near the aortic arches where septation is occuring (c). Mag. 195 x.

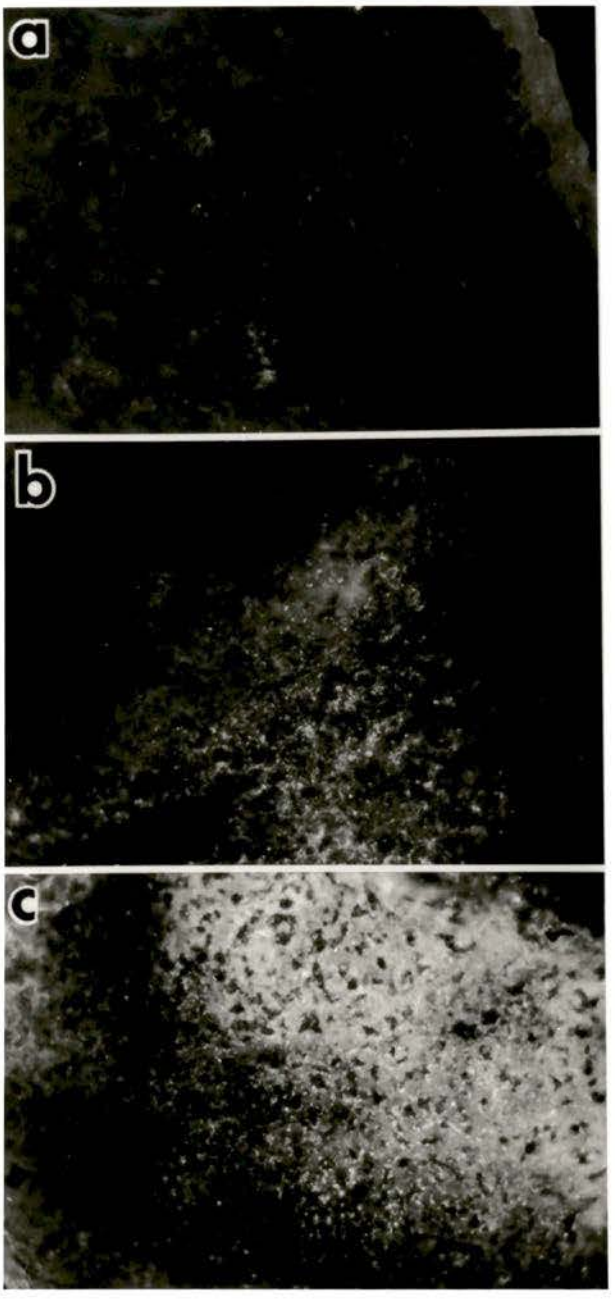

a
b
c

Figure 12: (a)-(d) Immunofluorescence preparations of stage 28 truncus tissue showing proximal to distal organization of HNK-1 antigen. Septation is almost complete and two areas of HNK-1 immunoreactive cells are seen near the heart (b). The cells fill in the media from the region of the aortic arches (c) and (d). By stage 33 the cells are becoming organized into lamellae and most are positive for HNK-1. (f) Higher magnification micrograph of cells in the media labeled with HNK-1. Mag. a,b,c,d,e 68 x. Mag. f 875 x.

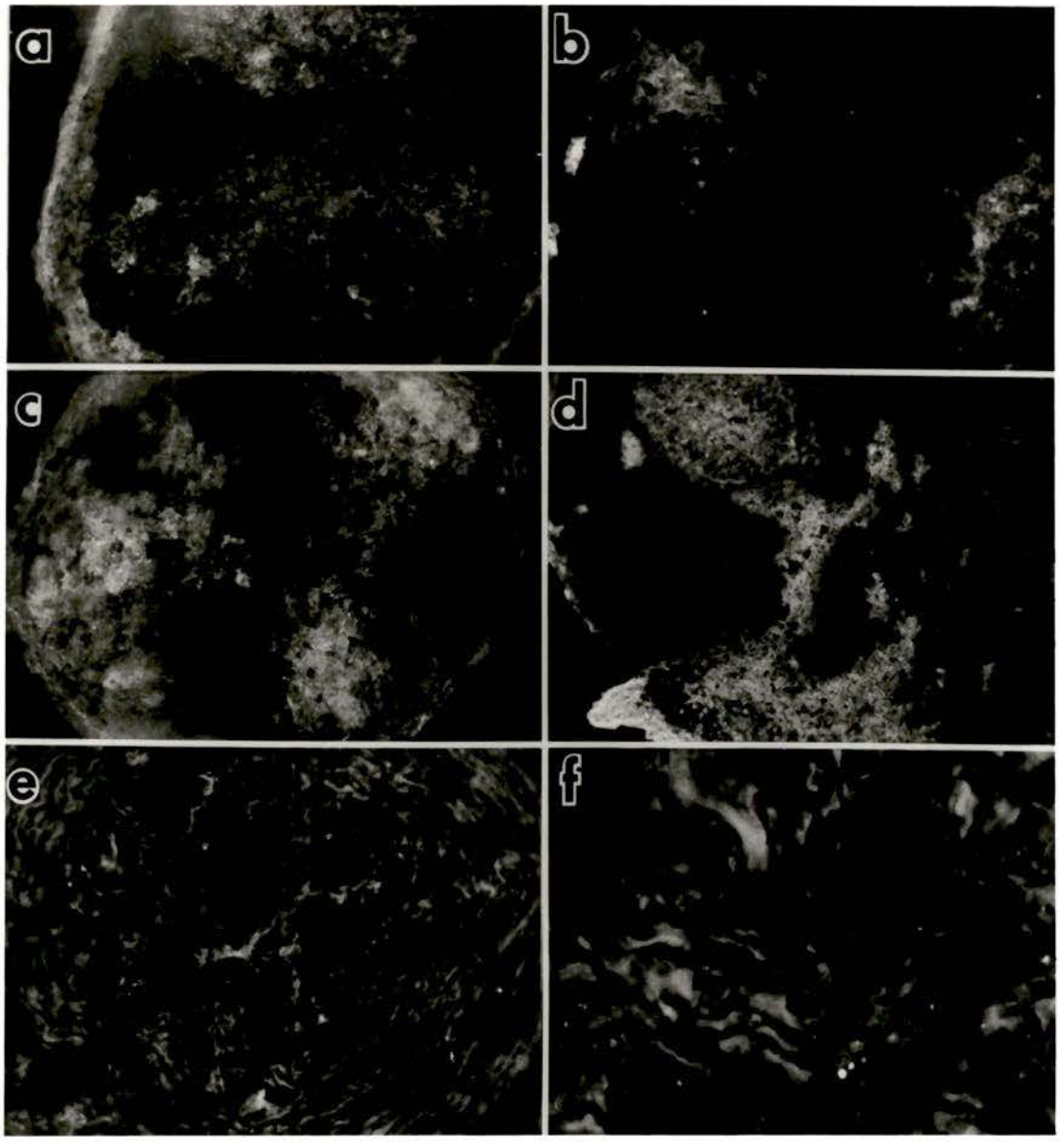

a
b
c
d
e
f

Figure 13: Immunofluorescence preparations of longitudinally sectioned outflow tracts. In the stage 21 outflow tract (a) dense mesenchyme is not yet present in the region nearest the aortic arches (to the left), whereas in the stage 24 outflow tract (b), the mass of HNK-1 positive cells has entered from the aortic arches. Mag. 60 x.

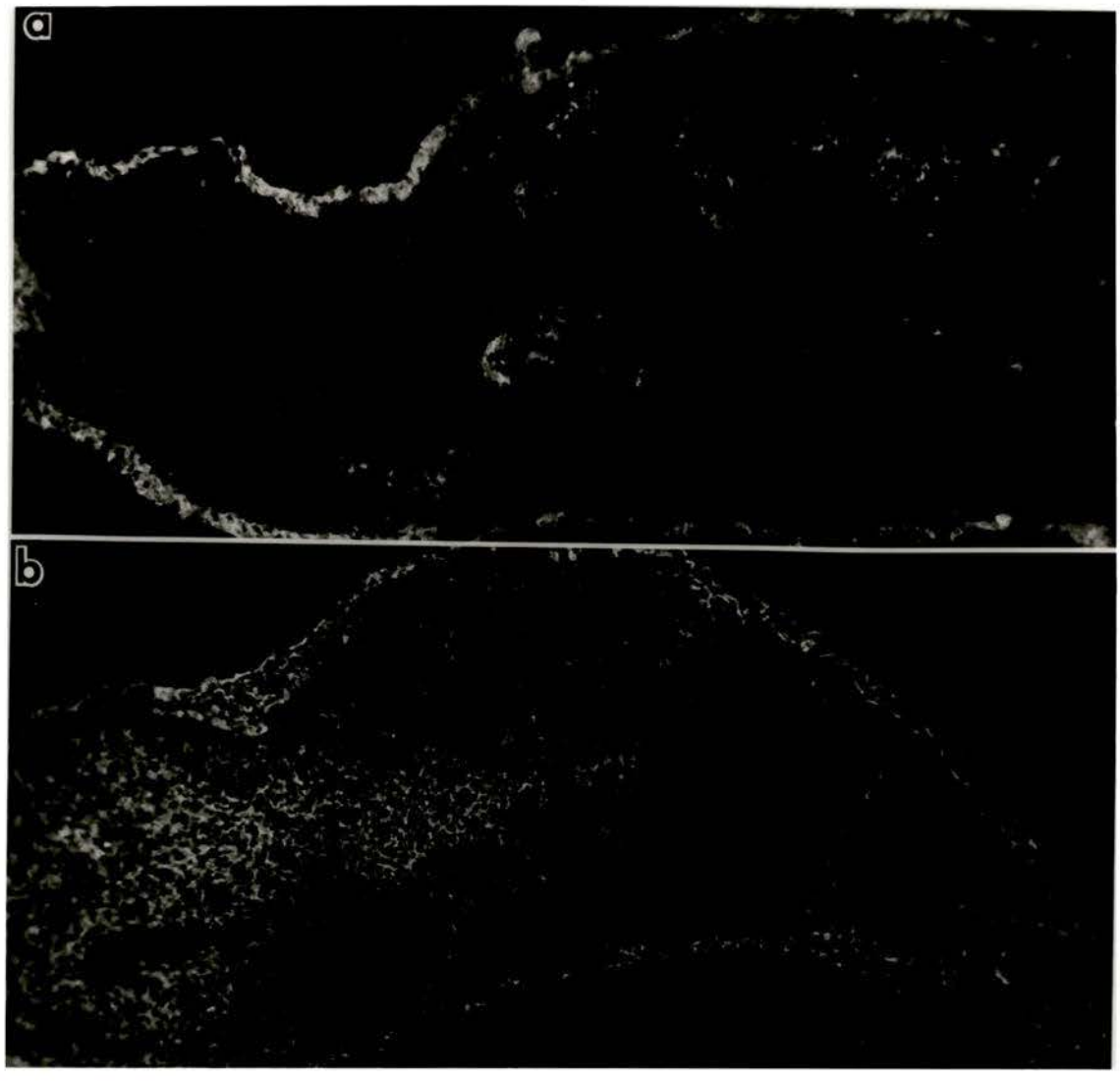
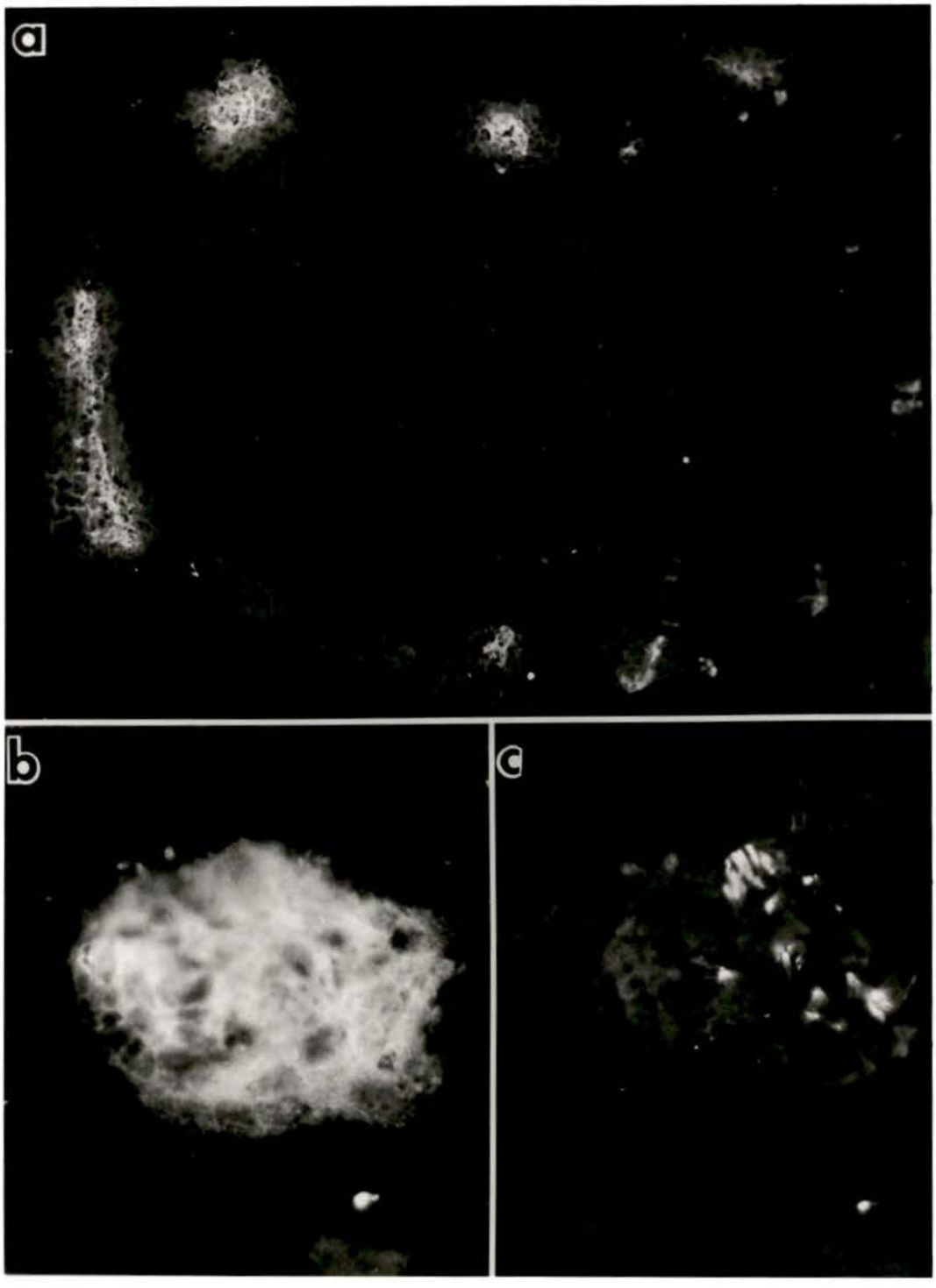

a
b

Figure 14: (a) Stage 35 truncus labeled with HNK. The labeling has become localized to a few distinct peripherally displaced areas. In double labeling experiments these HNK-1 positive areas (b) also react with anti-Neurofilament 200 antibody (c), indicating an association with nerves. Mag. a 80 x. Mag. b,c 1000 x.


a
b
c

antibodies, it can be seen that the patches of HNK-1 immunoreactivity surround axonal processes (Figure 14, b and c).

A stage 24 embryo was prepared for immunofluorescence with an anti ß1 integrin antibody (Figure 15). This shows clearly that the cells within the media express this receptor at the time of outflow tract septation. Cells seen in clumps within the media are outlined with fluorescent labelling.

CELL MIGRATION

In explant migration assays, the maximum distance migrated by cells on FN coated substrate was 5 times that on PBS, BSA or untreated substrate (Figure 16). The cells migrating from the explants are HNK-1 immunoreactive (Figure 17). The effect of the FN was titratable between 0 and 1 $\mu g/cm^2$ (Figure 18).. Migration from explants on FN coated substrates were significantly ($p<0.005$) inhibited when treated with anti-FN (F5). The inhibition was titratable between 0 and 60 µg/ml (Figure 19). In similar preparations in which anti-ß1 integrin was used as a blocking antibody, there also a significant decrease in the maximum distance migrated (Figure 16).

When soluble ECM components derived from chick aorta primary cultures were used in coating substrates, migration was similar to that on FN and titratable between 0 and 1 $\mu g/cm^2$ (Figure 20). Anti-FN was effective in blocking this migration. Anti-FN reduced the maximum distance migrated by cells to levels that did not differ from migration on untreated substrates. Anti laminin was less effective in blocking migration, and the reduction was not statistically signifigant. The combination of Anti-FN and Anti-LN had variable results.

On the Matrigel substrate neural crest cells did not migrate at all (Fgure 16). The cells did not attach to the substrate and flatten out the way they did on the two other

Figure 15: Immunofluorescence preparation of stage 24 truncus tissue, stained with anti-ß1 integrin antibody The mesenchyme cells within the tunica media express the integrin on their cell surfaces. Mag. 1500 x.

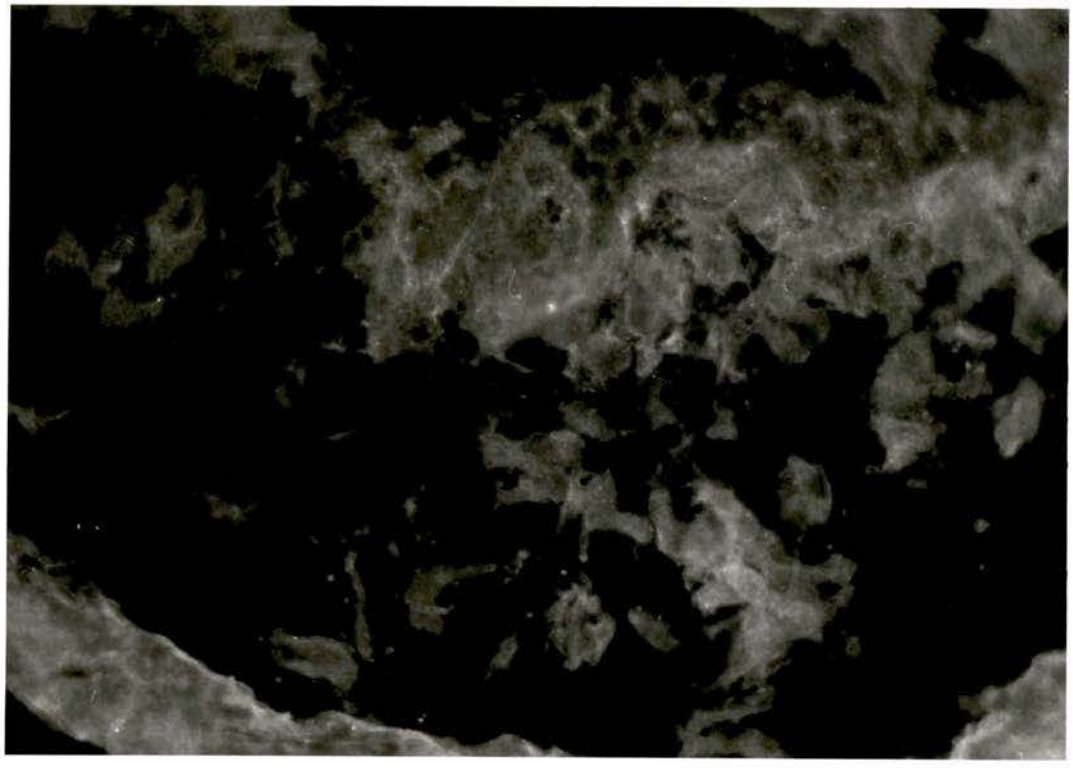


Figure 16: Histogram of the distance migrated (± S.E.) from tissue culture explants on PBS, FN, ECM, or Matrigel substrates. The cells migrated the farthest on FN and chick ECM substrates. Anti FN, Anti-ß1 integrin and anti LN antibodies were used to block the migration. Anti-FN had a strong effect on the migration in culture, and the anti-ß1 integin antibody also inhibited migration. The cells migrated on Matrigel with added FN, but not on Matrigel alone.

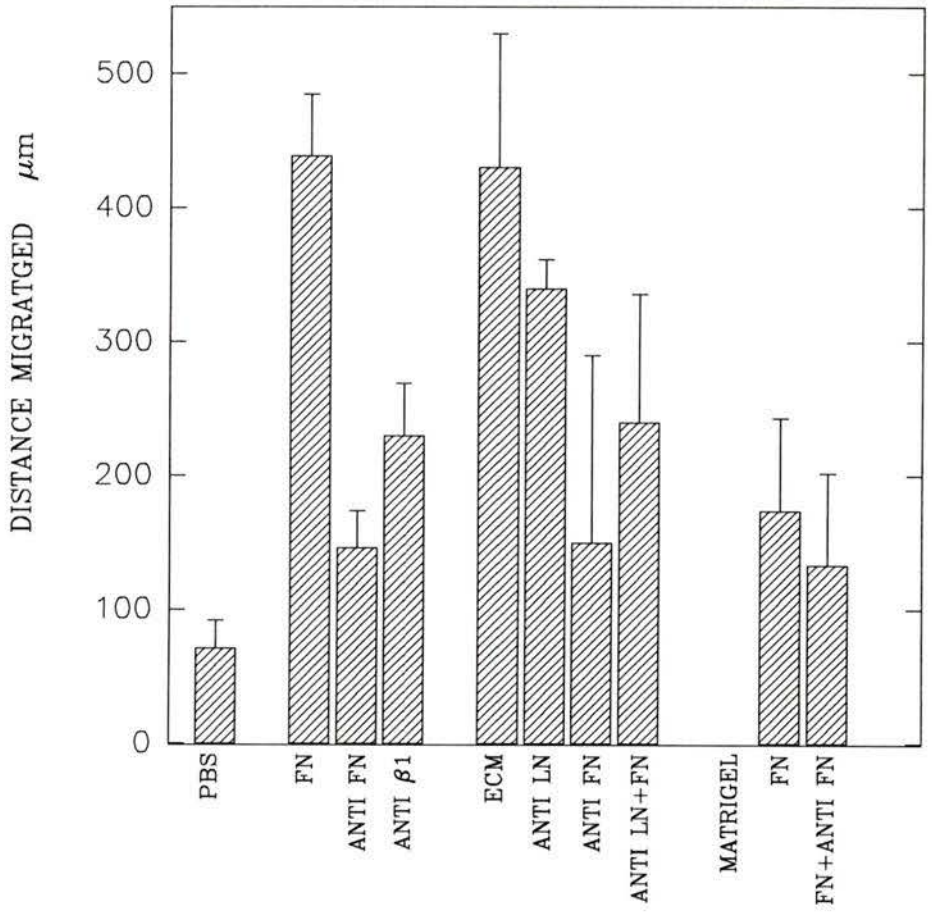

DISTANCE MIGRATGED μm
500
400
300
200
100
0
PBS
FN
ANTI FN
ANTI β1
ECM
ANTI LN
ANTI FN
ANTI LN+FN
MATRIGEL
FN
FN+ANTI FN

Figure 17: Tissue culture explant preparations seen in (a) under phase contrast microscopy, as migrating cells leave the tissue mass. The majority of the cells are HNK-1 immunoreactive, as seen by the immunofluorescence labeling in (b).

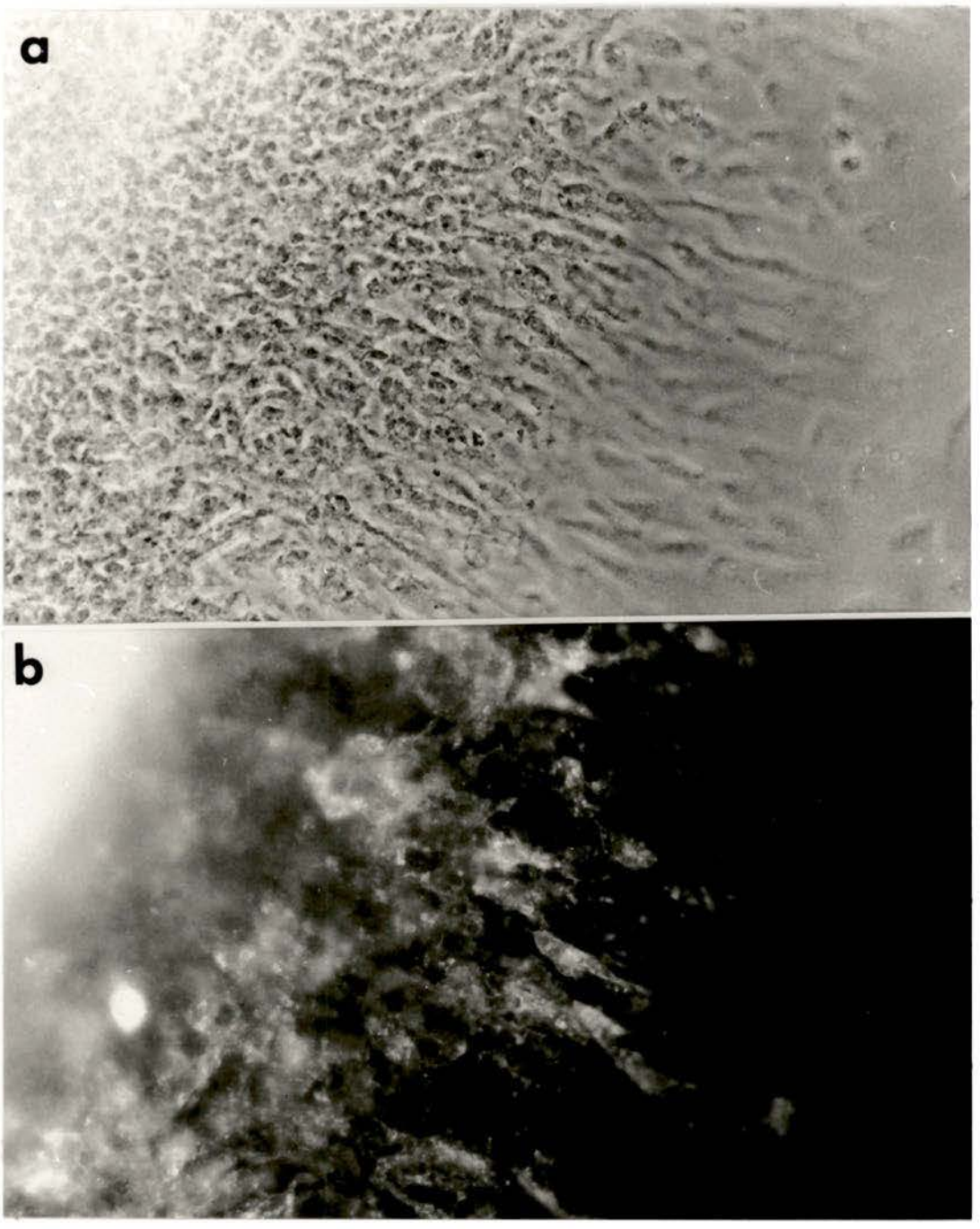

a
b

Figure 18: Results of cell migration assays in which the mean distance (± S.E.) cells migrated from explants is plotted against the concentration of FN used to coat the substrate.

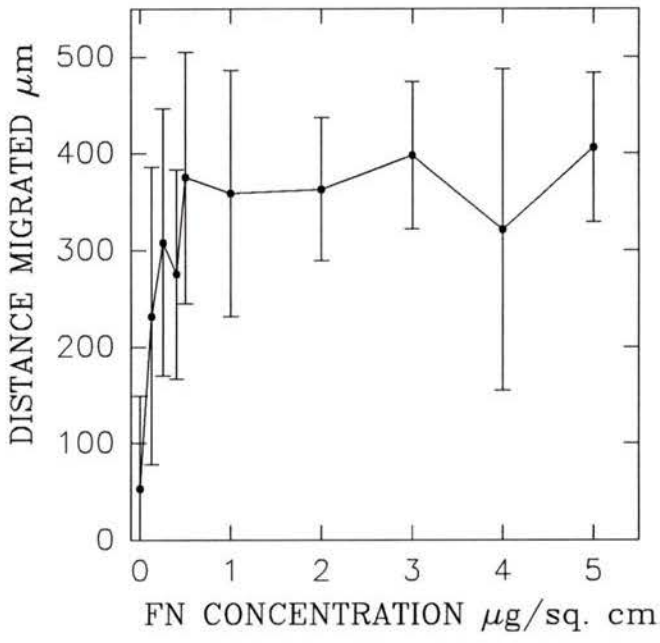

DISTANCE MIGRATED μm
500
400
300
200
100
0
0
1
2
3
4
5
FN CONCENTRATION μg/sq. cm

Figure 19: Results of experiments in which the distance cells migrated (± S.E.) on FN coated substrate is plotted against the concentration of anti-FN used to block migration.

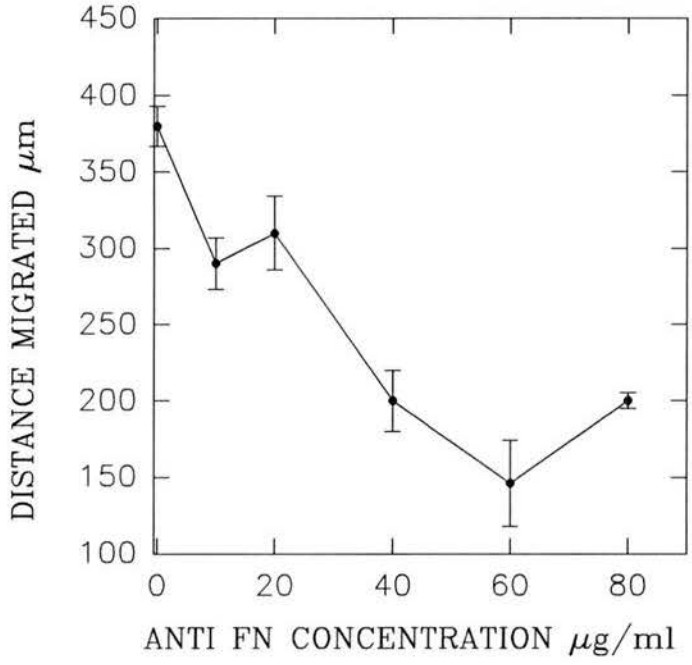
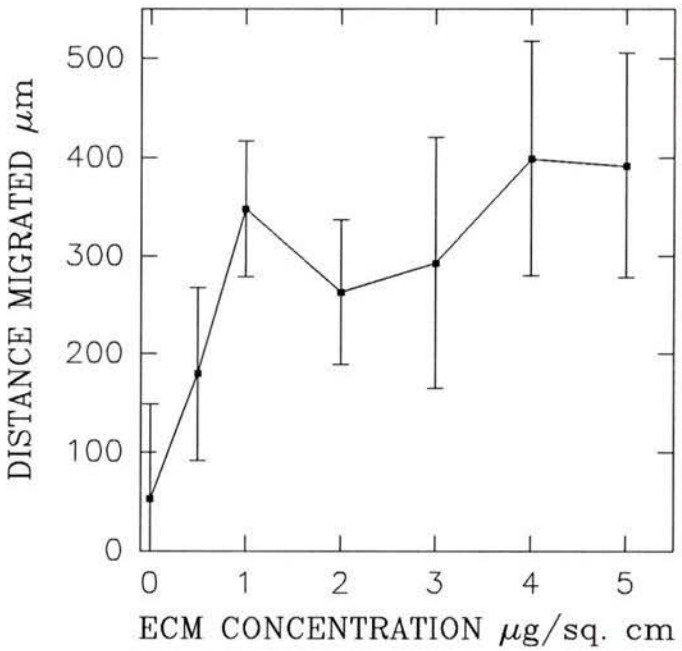

450
400
350
300
250
200
150
100
DISTANCE MIGRATED μm
0
20
40
60
80
ANTI FN CONCENTRATION μg/ml

Figure 20: Results of cell migration assay in which mean distance migrated (± S.E.) is plotted against the concentration of a crude ECM preparation used to coat the substrate.


DISTANCE MIGRATED μm
500
400
300
200
100
0
0
1
2
3
4
5
ECM CONCENTRATION μg/sq. cm

substrates. The cells did migrate when FN was added to the medium, and this migration was partially blocked by anti-FN antibodies.

DISCUSSION

The two cell poplations within the tunica media of the truncus arteriosus examined in this study, correspond to those seen in previous work. Thompson and Fitzharris (1979) used histological criteria to identify two populations of mesenchyme cells in this area of the outflow tract in the chick. They noted there was a population of cells that occupies the presumptive tunica media between stages 12 and 19 which was probably of endothelial origin. The second population of tunica media cells they noted, migrated caudally from the aortic arches later in development. Ablation and labeling studies have shown that cells derived from the neural crest, between the otic vesicle and the third somite, migrate into the major vessels of the heart and contribute to the cells forming the tunica media of the major arteries (LeLievre and Le Douarin, 1975, Kirby et al., 1983). Using the immunological markers HNK-1 and E/C 8+, Kuratani and Kirby (1992) have traced the migration of cardiac neural crest cells from their release between the otic placodes and somite 3 through their migration into the pharyngeal arches (stage 17). Using quail-chick chimeras, Miyagawa-Tomita et al., (1991) have documented the distribution of neural crest cells in aortic arches between stages 18 and 25. They report that clusters of quail neural crest cells were first found in the outflow tract by stage 23.

The present study has shown that a population of cells migrate caudally from the aortic arches into the outflow tract. These cells express HNK-1 antigens. The cells migrate into the tract beginning at about stage 24 to 25. They first form two clumps of cells in the truncus, and then fill in the entire tunica media. These cells are probably the same cells as were described by Thompson and Fitzharris (1979) and their entry into the outflow tract is consistent with timings of the early migration of cardiac neural crest cells described by Kirby (1987), Miyagawa-Tomita et. al., (1991) and Kuritani and Kirby (1992). Thompson and Fitzharris (1979) reported that the cells enter the outflow tract at stage 17 or 18, which differs from these observations and those of Miyagawa-Tomita

et. al. (1991) who also noted their arrival around stage 24.. The cells migrating from the arches uniformly express HNK-1 until about stage 33, when most of the cells become HNK-1 negative. The peripheral patches of HNK-1 immunoreactive cells seen in cross sections of the outflow tract after Stage 33 are associated with cells that express neurofilament antigens. It can be concluded that these are the neural crest cells which ablation and labeling studies have shown contribute to the tunica media of the major blood vessels and innervation of the heart.

Scattered cells express HNK-1 epitopes in the outflow tract, beginning at about stage 12. As the cardiac neural crest cells could not be in the outflow tract by this stage, these cells are probably a subpopulation of the early mesenchyme, which expresses the redundant carbohydrate epitope recognized by HNK-1. A similar situation exists in the developing gut (Luider et. al., 1992) where a population of enteric cells are immunoreactive with HNK-1 prior to colonization by neural crest cells. Luider et al. (1992) found that the non-crest derived cells express a combination of epitopes not expressed by neural crest cells. In particular two antigens (42 and 44 K) were found only on the HNK-1 positive cells not derived from neural crest. Comparisons of blots (Burke, et al., in preparation) from cardiac outflow tract of early embryos and late embryos shows the higher molecular weight bands are more abundant after stage 25, when the majority of HNK-1 cells are in the outflow tract (Burke et al., in preparation). There are also several immunoreactive bands with low molecular weight that do not increase or decrease in abundance in stage 25 and 28 embryos. This suggests that like the non-crest derived HNK-1 expressing cells of the developing gut, there are non-crest derived HNK-1 expressive cells in the outflow tract. These cells express low molecular weight antigens, not seen in migrating neural crest cells. These cells appear to be individual, isolated cells, which show a clear cell boundary and bright staining until stage 15. Thus, differences in immunofluorescent staining correspond to differences in the molecules that are expressed.

After stage 15 the staining becomes noticeably reduced. This may indicate a reduction in expression of the antigen by the mesenchyme throughout this period. Such changes in expression can signify important developmental events, as discussed earlier. A series of developmental stages on a Western blot may help to determine if the reduction seen in the immunofluorescent preparations is truely reflective of a reduction in expression.

The ECM between the myocardial cuff and the endothelium of the developing outflow tract contains FN as early as Stage 10. This is distributed as fibrils associated with cells, and as non-fibrillar material in the space of the presumptive tunica media. The FN fibrils are initially associated with the basal lamina of the adventitia and the endothelium, but by the time the cardiac neural crest cells have begun migration, FN fibrils are abundant and widely distributed throughout the layer. These fibrils remain in the tunica media throughout septation, and they are abundant. Once septation is complete the expression of FN by the cells in the tunica media changes. It appears to be decreased, according to the immunofluorescent images. Expression increases in association with the endothelium after septation. These findings confirm and extend the observations of Icardo and Manasek (1983) and Icardo (1985) who used immunofluorescence to localize FN in the outflow tract of embryos from about stage 20 to stage 35.

Using explants of stage 28 outflow tracts, it is clear that HNK-1 immunoreactive cells migrate to a greater extent on substrates coated with chick FN. The effect is titratable and can be blocked with antibodies to FN and the ß1 subunit of integrin. The ß1 integrin blocking was not as complete as the anti-FN blocking, indicating that there may be more than one type of integrin receptor present on these cells. The migration on soluble ECM from cultured aorta cells indicates that even when complex ECM substrates are used, FN appears to be a dominant component promoting migration. The anti-FN antibody had a greater effect than the anti-LM antibody on the complex

substrate. The anti-LM antibody used in this experiment was non purified ascites fluid, so there could be effects caused by the other proteins in the fluid. The lack of use of LM by the neural crest cells would be significant since in other areas of migration LN is thought to be a significant substrate for attachment (Bronner-Fraser, 1988).

Other constituents may be utilized by these cells as substrates for attachment and migration, but the role of these components is probably secondary to that of FN. Antibodies against some of the other ECM components were not used in this study, but it would be interesting to determine the extent of effect antibodies to such molecules as heparan sulfate and collagens would have. It would also be interesting to examine the effect of HNK-1 on these cultures, since it has been found to inhibit adhesion in other *in vitro* studies (Bronner-Fraser, 1987).

The explant experiment using Matrigel as a substrate illustrates the importance of FN. Matrigel is a commercially available gel containing mouse ECM components, but lacks FN. In this experiment, mouse Matrigel was not sufficient to induce migration of the neural crest cells. The addition of FN to the medium was enough to allow some migration of the cells, despite the fact that it was clearly not optimal conditions for the cells to migrate. Further evidence for the importance of FN comes from the observation that the migration of the cells was reduced after the addition of the anti-FN antibody.

Studies of trunk neural crest cell migration have led to the hypothesis that neural crest cells migrate along pre-formed pathways of FN. An important component of this model is that the neural crest cells do not synthesize FN, whereas cells associated with the pathways do. There are several lines of evidence that support this theory, especially regarding the trunk neural crest. Newgreen and Thiery (1980) showed using immunofluorescence, that only about 6% of explanted neural crest cells were positive for FN. The FN positive cells were only from cranial and caudal regions and were hypothesized to be a group of pioneer cells that migrated early using a different mechanism. Ffrench-Constant and Hynes (1988) showed with *in situ* hybridization that

trunk neural crest cells do not contain FN mRNA. The recent observations on cardiac neural crest cells in the outflow tract are contrary to these findings (Burke et al., in preparation). In situ localization using a FN probe indicates that all the cells of the tunica media contain FN mRNA and are probably synthesizing FN (Burke, et at., in preparation). According to this data the adventitia cells appear to express FN in early stages and by stage 30 do not bind the FN probe. This correlates well with the immunofluorescent images, which indicate that in late stages there is little FN associated with these cells. Neural crest cells releasing from the neural tube between otic placodes and somite three express FN mRNA and have cell surface FN.

In studies in which cells were injected into neural crest migratory pathways, Bronner-Fraser (1984) demonstrated that cells that synthesize FN did not translocate along the ventral route. Whereas, cells that did not have surface FN readily followed this pathway. Inert beads were used, coated with FN, and these were also unable to translocate while uncoated or BSA coated beads would translocate (Bronner-Fraser, 1984). As these studies indicate that cell surface FN is inhibitory to cells migrating along FN pathways, a simple model in which exogenous FN promotes and guides migrations will not explain the migratory behavior of the cardiac neural crest. In their study of the expression of FN Ffrench-Constant and Hynes (1988) showed that other forms of migrating mesenchyme such as endocardial cushion cells did produce FN and they suggest the neural crest model was not one generally applicable.

The present observations with respect to cardiac neural crest, are that FN appears to promote migration of these crest cells and serve as a substrate, but at the same time the cells probably actively produce FN (Burke et al., in preparation). It is clear that there is FN associated closely with the migrating cells, from immunofluorescent images. The FN associated with these cells may serve two functions. It may still promote migratory behavior, while it also maintains the neural crest cells as a cohesive mass. This property is seen in the immunofluorescent images of the HNK-1 labeled cells

migrating through the truncus at about stage 28. They appear in adherent masses of cells. It is not until they stop migration, and begin to differentiate into smooth muscle, that they are able to be visualized independently, and soon after loose their HNK-1 reactivity.

Due to the relationship of the HNK-1 antigen with cell adhesion molecules it has been hypothesized that all molecules bearing this epitope may be involved in cell adhesion (Kunemund, 1988). Since glycosylation and sulfation are important modifications to proteins during development (Luider, 1992), this is a possibility. It is significant that the cells of the outflow tract only express the antigen during morphogenesis. It is not understood how this carbohydrate epitope functions in relation to FN and integrin receptors, but it has been found that injection of integrin antibodies causes a similar effect in the embryo, and injection of both antibodies increased the percentage of anomalies (Bronner-Fraser, 1988). Thus it can at least be assumed that they are acting at different sites.

Jaskoll et al., (1991) used high-definition, time-lapse photomicrography to document the migrations of neural crest cells in vitro. They found neural crest cells move as a meshwork of synchronously pulsating cells that maintain contact with each other via several filopodia. Previous observations of neural crest cell migration *in vitro* indicated that cells moved as sheets (Bronner-Fraser, 1987; Noden, 1988; Erickson, 1988). It is possible that autonomously produced FN acts as a linker molecule, promoting adhesion between neural crest cells. Exogenous FN may still function as a substrate for the movement of the mass of neural crest cells. Rather than moving with the cells, it provides a fixed matrix through which the mass of neural crest cells move.

There are indications that the cardiac neural crest cells move in a mass and displace the mesenchyme cells that initially populate the outflow tract. If the cells migrate independently, one would expect intermixing of the neural crest cells with the endothelial derived mesenchyme. In quail-chick chimeras, in which the

rhombencephalic primordium was the graft, the walls of the vessels derived from the outflow tract are entirely made up of quail cells (Le Lievre and Le Douarin, 1975). In published micrographs of chimeras quail cells typically are in a mass not interspersed with host cells (Le Lievre and Le douarin, 1975; Miyagawa-Tomita et al., 1991). There are similar observations made in cranial neural crest cells where intermixing with mesenchyme appears not to occur (Noden, 1980; Hall and Horstadius, 1988). The origin of mesenchyme that first populates the outflow tract is not known for certain, but there are indications that it is derived from the endothelium (Thompson and Fitzharris, 1979; Bernanke and Markwald, 1982). In chimeras in which grafts were made to the splanchnic mesoderm that gives rise to the endothelium of the outflow tract, quail cells were restricted to the endothelium and only formed the underlying mesenchyme in endocardial cushions (Noden, 1991). Thus if the original population of mesenchyme cells is derived from the endothelium, they are ultimately displaced.

In preparations using HNK-1 in embryos older than stage 25 all of the cells of the tunica media are immunoreactive. This evidence does not mean that all of the cells are neural crest derived, as was previously believed, because of the presence of the early mesenchyme expressing HNK-1. The population of cells could still be of mixed origin, all expressing the HNK-1 antigens. Due to the fact that there are some endothelial derived cells that express the antigen before the neural crest cells arrive, it is difficult to distinguish between these cells. This problem can be partially overcome by using quail-chick chimeras, which has been done in previous studies, as mentioned above The apparent ability of the cardiac neural crest cells to displace the original population of mesenchyme, may be explained by the cells formation of a cohesive tissue because they are mutually adherent through cell surface FN.

It has recently been shown that cranial and trunk neural crest cells differ in their mechanism of attachment to ECM components (Lallier et al., 1993). Because cranial and trunk neural crest cells have different affinities for ECM components and different

divalent cation requirements, it is suggested that the two types of cell are distinct populations with different integrin receptors. The more research that is done on neural crest cells, the more it is recognized that they are not all equal and that there is great variation between the subpopulations. Cardiac neural crest cells are known to be determined relatively early, making them unique among the neural crest population which are generally thought to be plastic (Kirby, 1988). Cardiac neural crest cells are at the interface between the cranial and trunk populations and appear to differ in their capacity for FN synthesis, reinforcing the idea that neural crest cells are diverse, not only in their fates but also in the mechanisms they employ in migration.

The migration of neural crest cells is a well studied example of the movements of embryonic cells, a process typical of vertebrate development. A hypothesis that has developed from these studies is that exogenously produced FN provides a guidance mechanism for cell migration. There are examples of cell migrations in which, the migrating cells produce FN, unlike the migrating trunk neural crest cells (Ffrench-Constant and Hynes, 1988). The labeled pathway hypothesis has been formulated for one particular population of cells, and aspects of it apply to the cell migration observed in this study, but new data is being produced that indicate that this theory in its entirety cannot be transferred to all neural crest cell populations.

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