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A STUDY ON THE CAPACITY OF NORMAL AND *TALPID*^{3} MUTANT MYOGENIC CELLS TO MIGRATE IN FOWL EMBRYONIC WING BUDS

by

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Thesis submitted for the degree of

Doctor of Philosophy,

University of Glasgow,

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DECLARATION

I hereby declare that this thesis is my own composition and that except where otherwise stated, the experimental work was performed by me alone.

None of the material in this thesis has been submitted for any other degree.

Kenneth K. H. Lee

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This thesis is dedicated to my parents.

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SUMMARY

1. The ability of myogenic cells to migrate within chick wing bud was investigated using quail cell markers. Fragments of quail wing buds containing myogenic cells of somitic origin were excised from stage 20-26 H.H. embryos and orthotropically implanted into chick wing buds *in ovo*. The chimaeric limbs were fixed for analysis at daily intervals. It was not until after the second day of implantation that quail cells started migrating out of the graft sites. Quail chondrocytes were found at the graft sites but they were non invasive. After the third day of implantation, quail cells were detected in most of the muscle regions in the zeugopod, but it was not until after the fourth day that quail cells were discernible in the autopod. Myogenic cell migration was exclusively in a proximodistal direction, with very little or no concurrent movement in the opposite direction.

2. There have been suggestions that quail cells are more invasive than chick cells (Chevallier *et al*, 1977; Bellairs *et al*, 1981). Therefore, to establish whether or not the observed myogenic cell movements in quail into chick transplantations were an artefact of cellular interaction between these two cell types, a series of homospecific transplantations were performed. Chick wing fragments (staged 20-25 H.H.) were labelled with

tritiated thymidine and inserted into unlabelled chick wing buds *in ovo*. In addition, quail wing fragments were also transplanted in the same manner into chick (heterospecific), so that the effectiveness of tritium, as a marker, could be assessed. After 4 days of post incubation, moderate levels of myogenic cell movement were detected in 4 out of the 5 homospecific transplantations performed. Myogenic cell movement is therefore not an artefact of interaction between chick and quail cells since movements also ensue in chick into chick transplants. Nonetheless, heterospecific transplantations revealed that the full extent of myogenic cell migration cannot be detected using autoradiography because tritiated thymidine is subject to dilution.

3. *Talpid*^{3} mutant cells are apparently more adhesive to each other than normal cells (Ede & Flint, 1975a) so *talpid*^{3} material has been used in this study to investigate the relationship between cellular adhesion and myogenic cell movement. Normal and *talpid*^{3} wing fragments were transplanted into quail wing buds and allowed to develop for 5 days. Extensive myogenic cell migrations were detected in 10 out of the 14 normal chick into quail transplantations. In contrast, only 2 suspected cases of myogenic cell movements were detected in 11 of the *talpid*^{3} into quail transplantations. *Talpid*^{3} myogenic cells

therefore have a reduced capacity for movement under these experimental conditions. Histological study of 9 day *talpid*[3] limbs showed that all of the myocytes in *talpid*[3] muscles and chondrocytes in *talpid*[3] cartilages were randomly orientated and developmentally retarded, while their normal counterparts were aligned in neat columns and well differentiated.

4. The ability of normal and *talpid*[3] limb mesenchymal cells to translocate inside hydrated collagen lattice cultures was examined. Computer analysis of various cell parameters (leading edge, trailing edge and cell centre), taken from time-lapse films of cells in locomotion, did not reveal any significant difference between the mode or rate of normal and *talpid*[3] cell movements. Normal and *talpid*[3] leading edges traversed through collagen substrata with an average speed of 0.8 µm/min and 0.73 µm/min respectively. In both genotypes, the trailing edges were moving significantly faster than the leading edges; the tail does not overtake the head because in both genotypes the cells use the 'inchworm' mode for locomotion and trailing edge is immobilized for a period.

5. The forces which the cells exert on collagen substrata as they migrate out of the limb explants have remarkable powers of generating collagen patterns. The

collagen patterns in turn influence the shape, orientation and directionality of migrant cells, e.g. when two normal or _talpid_[3] limb explants were placed at some distance from each other inside a collagen lattice, aligned collagen tracks were always formed between them; cells migrating on these collagen tracks were apparently contact guided. I propose that this tractional structuring is the mechanism by which collagen fibres become organised between the somites and the limb somatopleure and that the aligned collagen fibres in turn direct the migration of somitic cells into the limb.

6. The effect on limb mesenchymal cell movements in collagen cultures of serum, fibronectin, hyaluronic acid and chondroitin sulphate separately and in various combinations was investigated. (1) The presence of serum in culture medium was found to be essential for maintaining cell locomotion in collagen lattices: in its absence cells did not migrate. (2) Addition of fibronectin (100 µg/ml) without serum did not support cell movement. (3) Hyaluronic acid (0.5 mg/ml and 2 mg/ml) in the presence of serum did not enhance or inhibit cell locomotion. (4) Chondroitin sulphate (1 mg/ml and 2 mg/ml) in the presence of serum inhibited cell movement in a graded manner: the higher the concentration of chondroitin sulphate the longer the time required for cells to generate traction lines

between double explants in collagen lattices. In sum, some factor present in the serum - not fibronectin - is essential for cell locomotion. Hyaluronic acid probably plays a permissive role, while chondroitin sulphate, whose presence usually signals the onset of differentiation, might have a role in stopping limb mesenchymal cell movements.

CHAPTER I

MYOGENESIS IN THE DEVELOPING LIMB BUD

1:1 PREFACE

What can be more curious than that the hand of a man, formed for grasping, that of a mole for digging, the leg of a horse, the paddle of the porpoise and the wing of the bat should all be constructed on the same pattern and should include similar bones, and in the same relative positions?

Charles Darwin

On the Origin of Species

1:1:1 General Introduction

The complexity and diversity of limb structures in various vertebrates is indeed immense, yet they all share the same pentadactyl pattern. Looking at it from the point of view of an embryologist, the developmental mechanism involved in specifying these structures must be relatively simple to remain unaffected by evolution. This apparent 'simplicity' is probably the reason why the embryonic limb bud is the subject of intense investigation.

Apart from patterning, the limb also exemplifies many of the fundamental problems of developmental biology, such as differentiation, cell migration and induction. Among the vertebrates, the avian limb bud is

the system which has been most widely studied recently, because of its convenience in being available at all times and easily accessible for a wide range of experimental operations. The chronology of chick embryogenesis has been established (Hamburger & Hamilton, 1951) and is adopted universally to standardize experiments involving avian materials.

The wing bud first appears at stage 16 Hamburger & Hamilton (H.H.) as a thickening of the somatopleure. Histologically, the early limb bud is composed of an apparently homogeneous population of mesenchymal cells enclosed in an ectodermal jacket. The ectoderm is made up of two layers - a cuboidal basal layer and a squamous peridermal layer. As the limb bud increases in length along its proximo-distal axis, the distal tip thickens to form a well defined ridge (Saunders, 1948a). The ridge is apparently essential for maintaining the outgrowth of the limb, because its removal will lead to truncation of the distal elements (Summerbell, 1974; Summerbell & Lewis, 1975).

It is from the mesenchyme that cartilage, muscles and connective tissues develop. One central problem is to explain how these tissues are laid down in the correct position and at the correct time. According to Wolpert's positional information hypothesis, the limb is initially homogeneous; how each cell differentiates is dependent on its position in the limb. Each cell will receive a particular positional value - perhaps based

upon systems of chemical gradients, so that whether a cell decides to become a chondrocyte or myocyte is dependent on information regarding its position and its interpretation of that information (Wolpert, 1971).

Although the avian limb bud has been investigated intensively, it still produces surprises. For instance, contrary to what was originally thought, it is now known that muscle cells do not originate from the limb's somatopleure but from somites (Chevallier et al, 1977; Christ et al, 1977). So contrary to Wolpert's hypothesis, determination, with respect to myogenic cells is not specified by positional information within the limb bud because the fate of these cells has already been determined in the somites. Muscle patterning is therefore dependent on how these somitic cells spatially arrange themselves inside the limb. Recent findings have highlighted the possible involvement of active cell movement in this process (Ede et al, 1984; Gumpel-Pinot et al, 1984). The main objectives in this thesis are to confirm that myogenic cells migrate within avian limb buds, and if so to explore how genetic and environmental factors affect this activity.

1:1:2 The somitic contribution to limb morphogenesis

The possibility of somitic material contributing to limb development has been investigated in a wide variety of animals. In reptiles, long somitic processes have been seen penetrating and releasing

somitic cells in the mesenchyme of limb rudiments (Reynaud & Adrain, 1975; Reynaud, 1977; Goel & Mathur, 1977). Accordingly, these workers proposed that the reptilian limb was made up of 2 cell lines, one originating from the somatopleural mesenchyme, and the other from the somites.

In mammals, contribution of cells from the ventro-lateral edge of the somites has been described ultrastructurally in the mouse (Houben, 1976; Milaire, 1976). During the 28 somite stage discrete regions in the caudal area of the ventro-lateral somite edge undergo cytological modifications. A few stages later, these same regions elongate, forming cellular trails which invade the mesenchyme in the proximal half of the limb.

In fish, in the trout, 4 somites have been reported to contribute to the development of the pelvic fin, with somitic cells forming muscles and endoskeleton, while somatopleural cells form the dermal ray (Geraudie & Françios, 1973).

Similar studies have also been carried out on amphibians (Amono, 1960; Bourgeois & Houben, 1975) and the mole (Milaire, 1962), but the chick system has been most intensively studied. As early as 1895 chick somitic cells were implicated in the development of the wing musculature (Fischel, 1895) and subsequent research has supported this suggestion (Murray, 1928; Grim, 1970; Gumpel-Pinot, 1974). Nevertheless, there have been

conflicting reports that limb myogenesis is independent of any involvement of somitic material (Saunders, 1948b; Seno, 1961; Pinot, 1970), but most of the experiments from which this conclusion was drawn were based on carbon marking and coelomic grafting, and doubts have arisen over the reliability of these methods (Theiler, 1957; Kenny-Mobbs, 1985).

The discovery of the quail marker has made life much easier for experimental embryologists concerned with the fate of developing tissues (Le Douarin & Barq, 1969; Le Douarin, 1972). As a direct consequence the fate of somites has been mapped using chick/quail chimaeras. All the skeletal musculature in the limb was found to be of somitic origin, whereas dermis, cartilage, smooth muscles, tendons, epi- and peri-muscular connective tissues were all of somatopleural origin (Christ _et al_, 1974, 1977, 1979; Chevallier _et al_, 1977; Kieny _et al_, 1981). According to Chevallier (1979) all of the intrinsic and extrinsic muscles of the wing and scapular girdles came from somites 12-20 while muscles of the leg and pelvic girdle came from somites 26-32.

Myogenic precursors normally start migrating into the wing's somatopleural mesenchyme at stage 14 H.H. (Jacob _et al_, 1978) and into the leg anlagen at stage 16H.H. The somites and the limb somatopleural mesenchyme are separated by a 20-30 µm. gap. The gap is filled with transversely aligned collagenous fibres

and at stage 14 H.H. lots of individually elongated somitic cells can be observed migrating on these fibres. The migration is apparently independent of the myotome, contradicting Kaestner's (1892) idea that the myotome contributes part of itself in limb myogenesis.

1:1:3 Myogenesis

More is known about the development of the structure and spatial arrangement of skeletal elements than of developing muscles, due to the sheer number of muscles involved. The chick wing alone is estimated to contain about 50 distinct muscles and 30 tendons. Luckily, the tedious work of reconstructing the spatial arrangement of these structures has already been carried out (Romer, 1927; Wortham, 1948; Sullivan, 1962; Seichert *et al*, 1982). There is general agreement amongst these workers that the limb musculature is derived from 2 muscle masses located in the dorsal and ventral portion of the early limb bud. Between stages 27-32 H.H. these muscle masses start splitting up in a chronological manner (Shellswell & Wolpert, 1977). In the zeugopod the muscles always start cleaving from the middle of the rudiment and from there on extend proximo-distally in a binary fashion.

Very little is known about the early location of myogenic precursors prior to stage 25 H.H. because the mesenchymal cells are histologically homogeneous. Thorogood (1972, 1973) tried targeting fluorescently

labelled antiserum against actinomyosin but was unable to detect any sign of activity until stage 28 H.H. When the fluorescent labels were distributed in the periphery beneath the dorsal and ventral ectoderm. Medoff & Zwilling (1972) claimed to have detected myosin as early as stage 24 H.H. before the muscles have differentiated histologically. Attempts have also been made to determine the early location of muscle blastema through the study of cell packing (Thorogood & Hinchliffe, 1975).

During a recent NATO conference on somite development (Glasgow, 1986), Solursh described a specific marker which can label early myogenic precursor cells - a monoclonal antibody raised specifically against desmin. To my great surprise, he reported that between stages 21-24 H.H. desmin positive cells are located within the central core and are excluded from the avascular zone that extends around the limb ectoderm. This is contrary to the general belief that early myogenic precursors are distributed in the periphery as dorsal and ventral analgen and not as a single mass in the central core of the limb. Solursh made sure that desmin antibodies specifically labelled myogenic cells by substituting chick somites with quail somites to demonstrate that the the antibodies specifically tagged onto quail somitic cells which have migrated into the limb (Solursh et al, 1985; Solursh, 1986). Strangely enough, the distribution of desmin

labels appear to correlate well with cholinesterase activity at corresponding stages of development (Vanittanakom & Drews, 1985).

1:1:4 Cell lineage verses multipotency

Many early investigators believed that the limb mesenchymal cells are initially homogeneous and that it is not until stage 25 H.H. that these cells enter different pathways of development (Zwilling, 1968; Schacter, 1970; Finch & Zwilling, 1971). These cells were also believed to be multipotent and capable of expressing a variety of tissue types with respect to their spatial location in the limb bud (Wolpert, 1971; Caplan & Koutropus, 1973). Measurements of carbohydrate metabolism led Medoff (1967) to conclude that all of the cells in the limb bud initially display chondrogenic properties and that only at later stages of development are some cells shunted to form muscles. Searls & Janner (1969) investigated multipotency by isolating labelled blocks of presumptive chondrogenic tissue (central core of the limb) from stage 24 H.H. limb buds and transplanting them into soft tissue forming regions. These authors found that up to 70% of the grafts can apparently regulate according to their new position in the host, suggesting that pre-stage 24 H.H. limb mesenchymal cells are equivalent. Since it has already been demonstrated that early myogenic cells are distributed in the central core between stages 20-25

H.H. (Solursh *et al*, 1985) it is difficult to see the validity of this observation.

Dienstman *et al*, (1974) regarded the limb bud not as being homogeneous but heterogeneous, comprising cells from different lineages. Indeed, heterospecific somite transplantation studies have confirmed this by revealing how all the limb's skeletal musculature are derived from somites while cartilage, connective tissues and tendons are all obtained from somatopleural mesenchyme (Christ *et al*. 1974; 1977; Chevallier *et al*, 1977; Beresford, 1983). There is more recent evidence backing the lineage concept (Swalla & Solursh, 1986). Sasse *et al* (1984) employing CSAT mono-clonal antibodies, which preferentially attach to myogenic cells, demonstrated that stage 20-22 H.H. limbs contained 2 subpopulations of cells, one myogenic and the other chondrogenic. Moreover, the proximal half of stage 22-25 H.H. limb buds is now known to contain 5 subpopulations of cells with different surface properties while in the progressive zone there are 4 (Cottrill *et al*, 1986).

There have been suggestions that under certain experimental conditions somatopleural cells have the potential to form muscles (Chevallier *et al*, 1978). This potential can be tested by isolating virgin somatopleure from limb forming regions which have not been invaded by somitic cells (i.e. before stage 14 H.H.) and culturing them, in isolation, on chorioallantoic membranes or as coelomic grafts. The

test has produced conflicting results, with Murray (1928); Christ et al (1979) and Christ & Jacob (1980) all maintaining that somatopleural cells are incapable of forming muscle while McLachlan & Hornbruch (1979) and Mauger et al (1980) found somatopleure to be developmentally liable and capable of expressing myogenic potentials. It has now been confirmed that somatopleurally derived limb mesenchyme does not have the potential to form muscles (Kenny-Mobbs, 1985). Furthermore, muscle formation is stage related and dependent on the presence of somites. This author also pointed out that misleading results can arise if pre-stage 14 H.H. somatopleure is not isolated carefully to prevent contamination from somitic material.

Experiments have also been carried out to establish whether or not myocytes can transdifferentiate into chondrocytes. Kieny (1980), through a series of heterospecific somite transplantations, constructed bispecific limbs in which all the muscles were made up of quail cells while the rest were chick cells. She dissociated these limbs, made them into mixed aggregates, and then stuffed these aggregates back into ectodermal jackets for transplanting into chick hosts. In effect, cells in the aggregates found themselves in a new spatial arrangement, i.e. a presumptive myogenic cell might have found itself in a prospective cartilage forming region. From sections of these fully developed transplants, quail cells were found in the cartilage,

which suggest that under certain experimental conditions myogenic cells have the ability to transdifferentiate into chondrocytes. This is further substantiated by findings that muscle explants grown in contact with demineralised bone matrix can occasionally produce cartilage (Nathanson *et al*, 1978; Nathanson & Hay, 1980; Nathanson, 1986).

The distal tip of an early chick limb bud is apparently homogeneous and free of myogenic cells (Newman & Mayne, 1974; Newman, 1977; Newman, 1980; Brand *et al*, 1985). The length of this somitic cell-free zone is estimated to be 300 µm long (Newman, 1981) and the somatopleural cells within it are believed to be pluripotent, capable of differentiating either as chondroblasts or fibroblasts (Newman, 1977). Newman (1977) hypothesized that the choice between the two different expressions is determined by the presence or absence of cell contacts. If cells establish contact with each other then chondroblasts will be formed whereas cells that are kept apart will become fibroblasts.

1:1:5 Format of the thesis

This investigation has been undertaken to determine whether or not skeletal myogenic cells have the capacity to migrate within the avian limb bud, to explore the nature of this movement, and to study the effects of altered genetic properties and

microenvironmental conditions upon it.

Chapter I is a general introduction and review of literature relating to relevant aspects of limb myogenesis. The origin and distribution of the skeletal musculature is discussed along with currently and previously held concepts.

Chapter II The ability of skeletal myogenic cells to migrate within the avian limb bud is investigated by transplanting blocks of quail mesenchyme containing myogenic cells into chick wing buds *in ovo*, using the quail cells as markers.

Chapter III It has been suggested that quail cells are more invasive than chick cells (Bellairs *et al*, 1981), and this would affect our interpretation of the results. In order to determine whether or not myogenic cell movement is an artefact of heterospecific interactions in our system, homospecific transplants were also created, using chick wing mesenchyme labelled with tritiated thymidine as implants into unlabelled chick wing buds.

Chapter IV is a general introduction and review of literature relating to the *talpid*^{3} mutant. All aspects of the mutant's abnormalities are described.

Chapter V The relationship between cellular adhesion and myogenic cell movement is investigated. Talpid[3] limb mesenchymal cells are known to be more adhesive than normal in certain situations (Ede & Flint, 1975a); hence, cells from mutant limb buds have been used to determine whether increased cellular adhesion has any effect on myogenic cell movement. Talpid[3] wing fragments were transplanted into quail wing buds in ovo and the results compared with normal chick wing fragments transplanted in the same manner.

Chapter VI The dynamics of normal and talpid[3] cell locomotion was investigated in vitro. Limb explants from both embryos were cultured in collagen lattices and cells migrating out from these explants recorded on time-lapse films. Activity of normal and talpid[3] cells was analysed and compared. The ultrastructure of normal and mutant cell surfaces were compared and the effects of cell traction on the alignment of collagen fibres investigated.

Chapter VII The effects of a number of extracellular matrix components - fibronectin, hyaluronic acid and chondroitin sulphate (all found in abundance in the limb's connective tissues) - on cell locomotion were examined, to establish what role they may play in myogenesis.

Chapter VIII Includes general conclusions to be drawn from the investigations and a discussion of their contribution to problems of limb morphogenesis.

CHAPTER II

A STUDY ON SKELETAL MYOGENIC CELL MOVEMENT IN THE DEVELOPING AVIAN LIMB BUD

2:1 INTRODUCTION

There are still considerable doubts as to whether skeletal myogenic cells actually migrate within the avian limb bud. The migration of myogenic precursor cells from the ventro-lateral edge of somites into the somatopleural limb mesenchyme has been well documented (Chevallier *et al*, 1976, 1977, 1978; Chevallier, 1979; Christ *et al*, 1974, 1977, 1983), but what happens to these cells once inside the limb is still unclear. Do they carry on migrating within the limb bud and actively participate in pattern formation, in a way analogous to cell sorting experiments (Moscona & Moscona 1952; Burdick, 1970; Steinberg, 1970), or do they lose their capacity for movement and get to their destined locations through growth and passive reshuffling?

Searls (1967) made an attempt to establish whether the limb bud harboured a mobile population of cells by transplanting blocks of tritiated thymidine-labelled chick limb mesenchyme into different locations in other limb buds. The procedure was unable to reveal any signs of cell displacement, leading many workers to assume that cell movement did not have a role in limb morphogenesis. Tritiated thymidine, however, is

subjected to dilution, especially in rapidly proliferating tissues, so it is possible that migration was missed as a direct consequence (Lee & Ede, 1985). Carins (1965) used mouse limb mesenchyme instead of tritium and, like Searls, was unable to find signs of cell movement.

These markers have now been superseded by the quail marker (Le Douarin, 1973) which, being biological, is inherently more stable and not subject to dilution. When Searls' experiment was repeated with the quail marker, migration was indeed detected, and specifically for myogenic cells; skeletal myogenic cells were found to be capable of migrating extensively and exclusively in a proximo-distal direction within the host limb buds (Wachtler et al, 1981, 1982; Gumpel-Pinot et al, 1984). Nevertheless, these results are inconsistent with findings obtained by Tickle et al (1978) and Fisher & Solursh (1979). These workers were unable to detect any signs of cell movement, and the first group felt confident in using the system as a control when invasive tissue types were compared.

Examination of the methodologies of these two different sets of workers suggests that it may be the time interval between transplantation and fixation, which is important in determining whether or not cell movement is discernible. Those that did not find cell movement fixed their limbs between 1-2 days after implantation, whereas the groups that did, fixed theirs

between 2-8 days [Wachtler *et al*, 1981 (2-3 days); Wachtler *et al*, 1982 (5-8 days); Gumpel-Pinot *et al*, 1984 (3-4 days)].

In an attempt to resolve this question, I have followed the progress of implanted quail cells through successive days of implantation, using the grafting procedures described by Gumpel-Pinot *et al* (1984). A core of quail limb mesenchyme was removed from the wing bud of a 3.5 day embryo and transplanted into the wing bud of a 4 day chick embryo. The chimaeric limbs were allowed to develop for up to 5 days, fixing specimens for analysis at daily intervals.

2:2 MATERIALS AND METHODS

2:2:1 Materials

Normal chick embryos were obtained from matings between *talpid*^{3} hens and cockerels kept in this laboratory. Fertile quail eggs (*Coturnix coturnix japonica*) were also obtained from flocks maintained in this laboratory. The stageing of both chick and quail embryos was in accordance with Hamburger and Hamilton (H.H.) (1951). Eggs were incubated at 37°c to reach the desired stages of development, between 20-26 H.H.

2:2:2 Transplantation

The objective of the procedure was to introduce into the interior of a chick wing bud a fragment of quail wing tissue containing myogenic cells of somitic

origin either in the process of migration or capable of migrating (Refer to figure 1a).

Preparation of host embryos

With forceps, a 1 cm hole was made at the blunt end of the chick egg, allowing direct access to the embryo. The shell membrane around the embryo was then removed and also a small section of the amnion around the wing bud. Using an emporte pièce, which consists of fine forceps with an incorporated circular knife Hampé (1959), a 300-400 µm hole was punched into the host's wing bud. The hole was then made ready to receive an implant with the removal of excess tissue around the edge of the hole.

Preparation of donor implants and implantation

Quail eggs were opened at the blunt end and embryos transferred into a perti dish filled with sterile tyrode solution. The amnion was then removed from the embryo and, using the emporte pièce, a core from the central posterior region of the wing bud was extracted. With a loop made from tungsten wire, the graft was slowly teased up to the surface of the tyrode. The surface tension created within the loop was of sufficient strength to capture the graft.

The loop containing the graft was then introduced into the host egg. By inserting a blunt tungsten needle, the graft was pushed out of the loop and implanted into the hole made in the host's wing bud;

the graft was not orientated in any way.

2:2:3 Histology

Operated limbs were fixed in Carnoy's fixative, formula b (Humason, 1972). Controlling the fixation process was found to be vital for the staining reaction to succeed. Best results were obtained from limbs fixed for 1 hour, longer durations tended to leach DNA out of the cells, resulting in understaining. Fixed limbs were dehydrated through a graded series of alcohols, cleared with toluene and embedded in paraffin wax. The limbs were sectioned at 5 µm, longitudinally, so that if any cell movement should occur the maximal distance of displacement would be detected. The resultant serial sections were then mounted onto subbed slides, to prevent sections from falling off during hydrolysis in HCL. The mounted sections were either stained with haematoxylin and eosin (H & E) or with the Feulgen reaction.

The protocols are described below:-

Feulgen reaction

(1)	Dewax in Histosol	5 min.
(2)	95% Alcohol	3 min.
	90% Alcohol	3 min.
	75% Alcohol	3 min.
	50% Alcohol	3 min.
	30% Alcohol	3 min.
	Water	3 min.

(3)	5N HCl at room temp.	50 min.
(4)	Wash in Water	10 min.
(5)	Schiff's solution 4°c	90 min.
(6)	Wash in 3 baths of sodium metabisulphate	3 min.
(7)	Wash in water	20 min.
(8)	30% Alcohol	3 min.
	50% Alcohol	3 min.
	75% Alcohol	3 min.
	90% Alcohol	3 min.
	95% Alcohol	3 min.
	100% Alcohol	3 min.
(9)	Clear in Histosol	10 min.

Haematoxylin and Eosin

(1)	Dewax in Histosol	5 min.
(2)	95% Alcohol	3 min.
	90% Alcohol	3 min.
	75% Alcohol	3 min.
	50% Alcohol	3 min.
	30% Alcohol	3 min.
	Water	3 min.
(3)	5N HCL Room Temp	50 min.
(4)	Wash in Water	10 min.
(5)	Harris Haematoxylin	10 min.
(6)	Differentiate in a weak acid bath	3 min.
(7)	30% Alcohol	3 min.

	50% Alcohol	3 min.
	75% Alcohol	3 min.
	90% Alcohol	3 min.
	95% Alcohol	3 min.
	Eosin	30 sec.
	100% Alcohol	3 min.
(8)	Clear in Histosol	10 min.

<u>Subbing solution</u>

1 g. of gelatin in 1 ltr of hot distilled water, cool and add 0.1 g. of chromium potassium sulphate.

<u>Carnoy fixative</u>

Absolute alcohol	60ml
Chloroform (BDH.)	30ml
Glacial acetic acid (BDH.)	10ml

2:2:4 <u>Analysis</u>

Quail cells were easily differentiated from chick cells with both Feulgen and H & E stains because during interphase; the chick nuclei stain homogeneously (fig 1b), while the nucleoplasm of quail nuclei remains clear but containing one or two heavily stained clumps of nuclear material (fig. 1c).

The graft sites in operated limbs were defined by the presence of quail chondrocytes, which were found not to move and to remain exclusively in one particular location. The distance between the boundary of the implant and the furthermost cluster of migrant quail cells was measured and taken to represent the total distance for cell displacement. Quail cells were only

identified as being myogenic cells if they were located within muscle regions.

2:3 RESULTS

2:3:1 1 day after implantation

The majority of the implants were located at the level of the humerus (fig. 2). The centres of these implants were very compact and were made up exclusively of quail cells. Towards the periphery, the cells were less compact and quail cells were found intermingling with the chick cells (fig. 2b). In 3 cases, the quail cells had differentiated into chondrocytes and took the form of nodules (fig. 2b). These nodules were integrated into the chick cartilage, but a clear boundary separating the two cell types was always present. During this period of development, the humerus, radius and ulna had become established. The apical ectodermal ridge was still present but was displaced to one side of the limb apex.

At this stage of development, quail cells were usually confined within the graft site. Only one of the specimens exhibited cell displacement (fig. 3), and in this, quail cells were found 294 μm from the graft site. These displaced cells were found to be located within one of the muscle regions directly in front of the graft site and quail cells were also found in the intermediate mesenchyme leading towards the colonized muscle; the displacement was in a distalward direction

without any concurrent backward or lateral movement. These results and results for subsequent days are summarized in table 1.

2:3:2 2 days after implantation

After 2 days of implantation, the chick's humerus, radius and ulna had increased in length, with the metacarpals beginning to develop. Myogenic regions were also more numerous, probably due to growth and the muscle splitting processes. The majority of the quail chondrocytes were either integrated into the chick's humerus or into the posterior half of the ulna. The quail chondrocytes did not intermix with the chick chondrocytes (fig. 4b) and were never found distal or proximal to the graft site.

The boundaries of the implants were no longer distinct, so the overall dimensions of the quail chondrocyte regions were taken to represent the confines of the graft site. This was felt to be justified on the basis that quail chondrocytes do not move and remain exclusively in one location. In this series, 5 out of the 6 transplants showed a moderate level of cell movement (fig. 4), with quail cells found along the whole length of the zeugopod, located within myogenic regions and the intermediate mesenchyme (fig. 4c, d). Backward movement was also detected in 2 cases, but the distances were short when compared with distances covered during distalward movement.

2:3:3 3 days after implantation

The chimaeric limbs have further increased in size, with the humerus, radius and ulna becoming more elongated. The carpals and metacarpals were now more prominent, with muscles also developing at these sites. All of the quail chondrocytes in these 6 transplants were found to have bonded onto the chick's humerus and were not found proximal or distal to this location. In 5 out of the 6 replicates, quail cells which had moved out of the graft sites (fig. 5) were detected in most of the myogenic regions along the whole length of the zeugopod (fig. 5c, d); although muscles were found in the metacarpals, no quail cells were present in these regions (fig. 5b). Displaced quail cells were now progressively restricted to myogenic regions, with fewer cells found distributed in the intermediate mesenchyme. This suggests that the quail cells found in the intermediate mesenchyme during the 2nd and the 3rd days of implantation were probably myogenic cells.

5 out of the 6 specimens exhibited myogenic cell movement, which was predominantly distalward towards the apex of the limb, with very little backward or lateral movement (fig. 5e). The distances covered by these myogenic cells are summarized in table 1. It must be noted that these measurements may be an over estimation of the actual distances actively migrated by these cells, since quail cells were already found in the

myogenic regions of the zeugopod during the 2nd day of post-incubation; the process of growth, should not be disregarded as having an influence on the overall displacement of these myogenic cells.

2:3:4 4 days after implantation

Quail cells were now found within the myogenic regions of the metacarpals (fig. 6b), and occurring in 5 out of the 8 transplants which exhibited cell displacement (fig. 6). This suggests that the quail cells found within the myogenic regions of the zeugopod, after the 3rd day of implantation were still capable of undergoing further migration. The fact that muscles were already present at the metacarpals of 3 day post-operative limbs and were colonized by quail cells during the 4th day, suggests that the quail cells got there through active cell movement rather than passively through growth.

2:3:5 5 days after implantation

The muscle pattern was now essentially that of an adult. 3 out of the 5 transplants performed displayed extensive cell movement (fig. 7) with quail cells found in the muscle regions of the autopod and zeugopod (fig. 7b, c). Tendons found in the distal regions of the limbs were composed exclusively of host cells, even if the muscles to which they attached were chimaeric (fig. 7b). 2 of the transplants failed to show any sign of

cell movement; this may be attributed to the implants being located behind developing cartilage, which impedes forward cell movement.

Table 1

1 DAY AFTER IMPLANTATION

Code	Stages of Donors	Stages of Hosts	Distalward movement	Backward movement
1QC1	23	22	294 µm	no movement
2QC1	22	22	no movement	no movement
3QC1	22	22	no movement	no movement
4QC1	21	22	no movement	no movement
5QC1	21	22	no movement	no movement
6QC1	21	24	no movement	no movement

2 DAYS AFTER IMPLANTATION

Code	Stages of Donors	Stages of Hosts	Distalward movement	Backward movement
1QC2	25	24	500 µm	no movement
2QC2	25	23	471 µm	150 µm
3QC2	25	23	588 µm	no movement
4QC2	22	24	588 µm	294 µm
5QC2	23	24	no movement	no movement
6QC2	24	22	559 µm	no movement

3 DAYS AFTER IMPLANTATION

Code	Stages of		Distalward	Backward
	Donors	Hosts	movement	movement
1QC3	25	23	1,176 µm	no movement
2QC3	25	24	1,235 µm	no movement
3QC3	25	24	1,294 µm	no movement
4QC3	22	23	1,051 µm	no movement
5QC3	20	22	589 µm	no movement
6QC3	21	23	no movement	no movement

4 DAYS AFTER IMPLANTATION

Code	Stages of		Distalward	Backward
	Donors	Hosts	movement	movement
1QC4	20	25	706 µm	200-300 µm
2QC4	21	22	1,470 µm	no movement
3QC4	21	22	1,645 µm	no movement
4QC4	26	22	no movement	no movement
5QC4	26	21	500 µm	no movement
6QC4	26	22	no movement	no movement
7QC4	26	22	1,606 µm	no movement
8QC4	23	22	2,000 µm	529 µm
9QC4	22	22	1,059 µm	no movement
10QC4	23	22	1,624 µm	no movement

5 DAYS AFTER IMPLANTATION

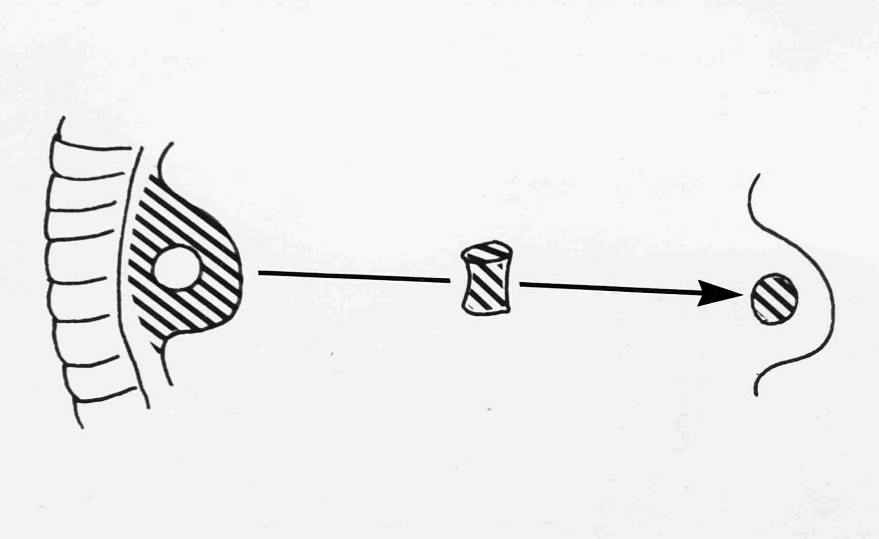
Code	Stages of Donors	Stages of Hosts	Distalward movement	Backward movement
1QC5	23	22	no movement	no movement
2QC5	23	23	no movement	no movement
3QC5	22	22	2,647 μm	no movement
4QC5	22	22	1,923 μm	no movement
5QC5	22	22	2,353 μm	no movement

Figure 1

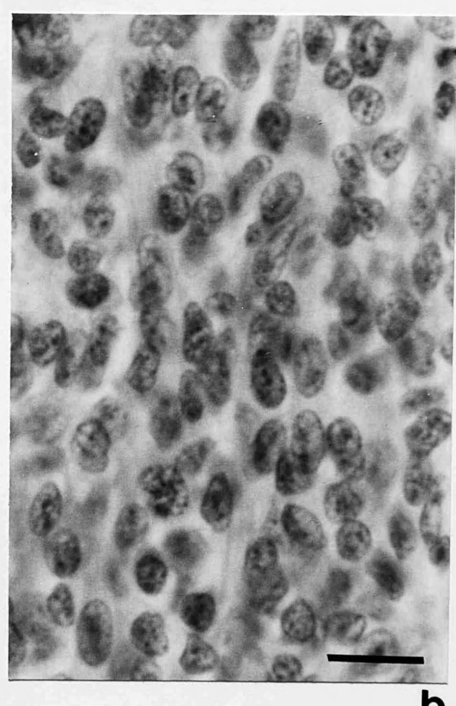
a Method of grafting a fragment from quail wing bud (hatched) into a chick wing bud *in ovo*.

b Chick cells. Bar = 10 μm

c Quail cells



a



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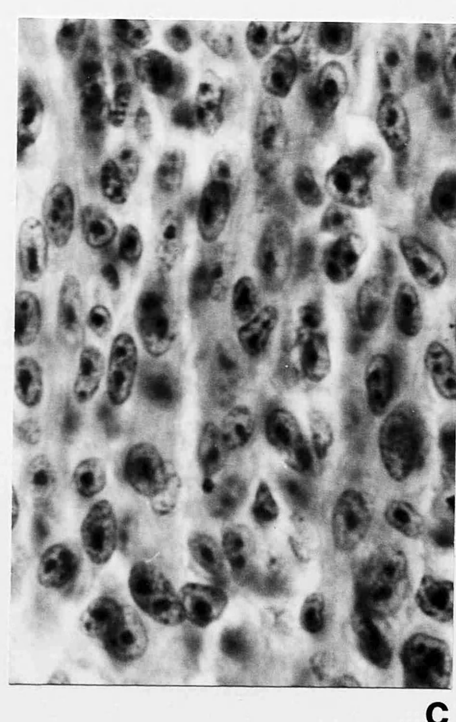


c

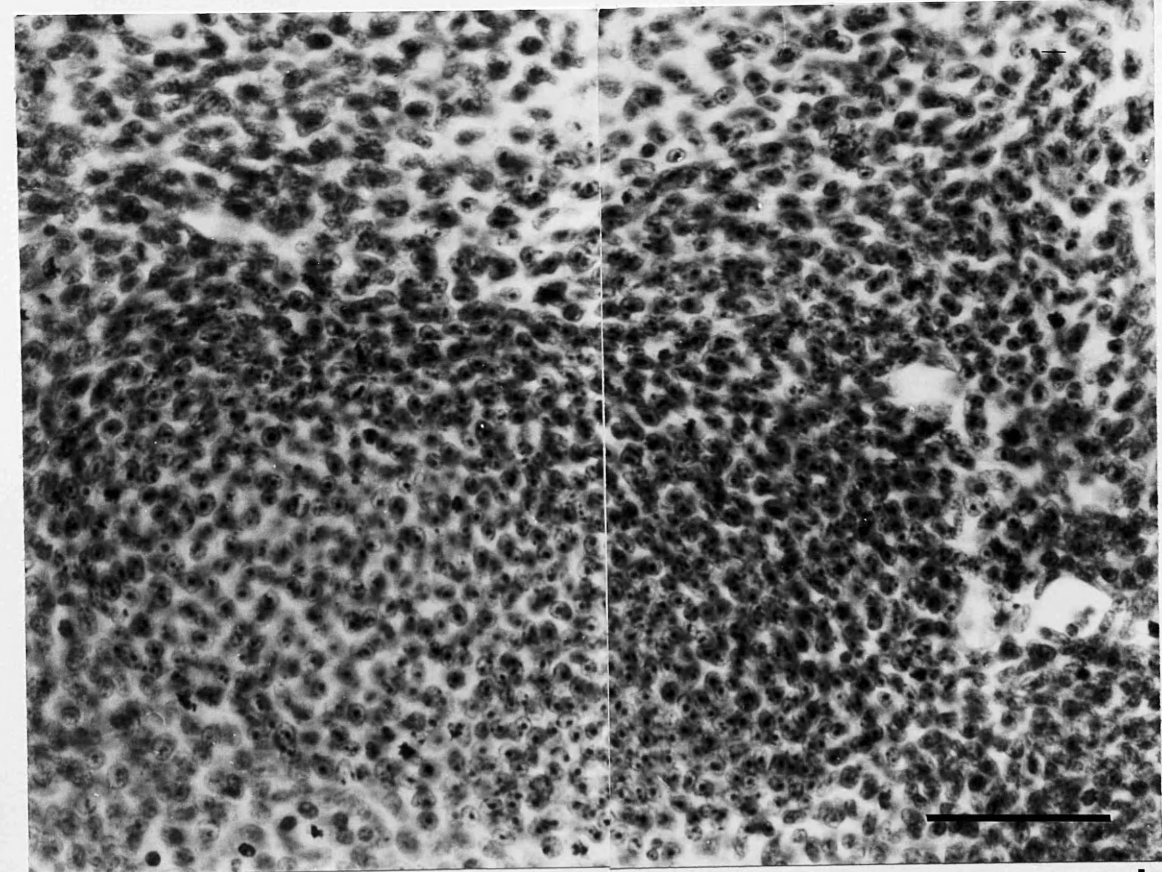
Figure 2

1 day after implantation

a General view of a quail into chick transplant Framed area denotes the graft site.
Bar = 250 µm

b Enlargement of the framed region in (a).
Bar = 50 µm

a



b

Figure 3

a The only example of myogenic cell movement observed after 1 day post-incubation.

Quail cells (arrows) can be detected some distance from the graft site(G).

Bar = 50 μm

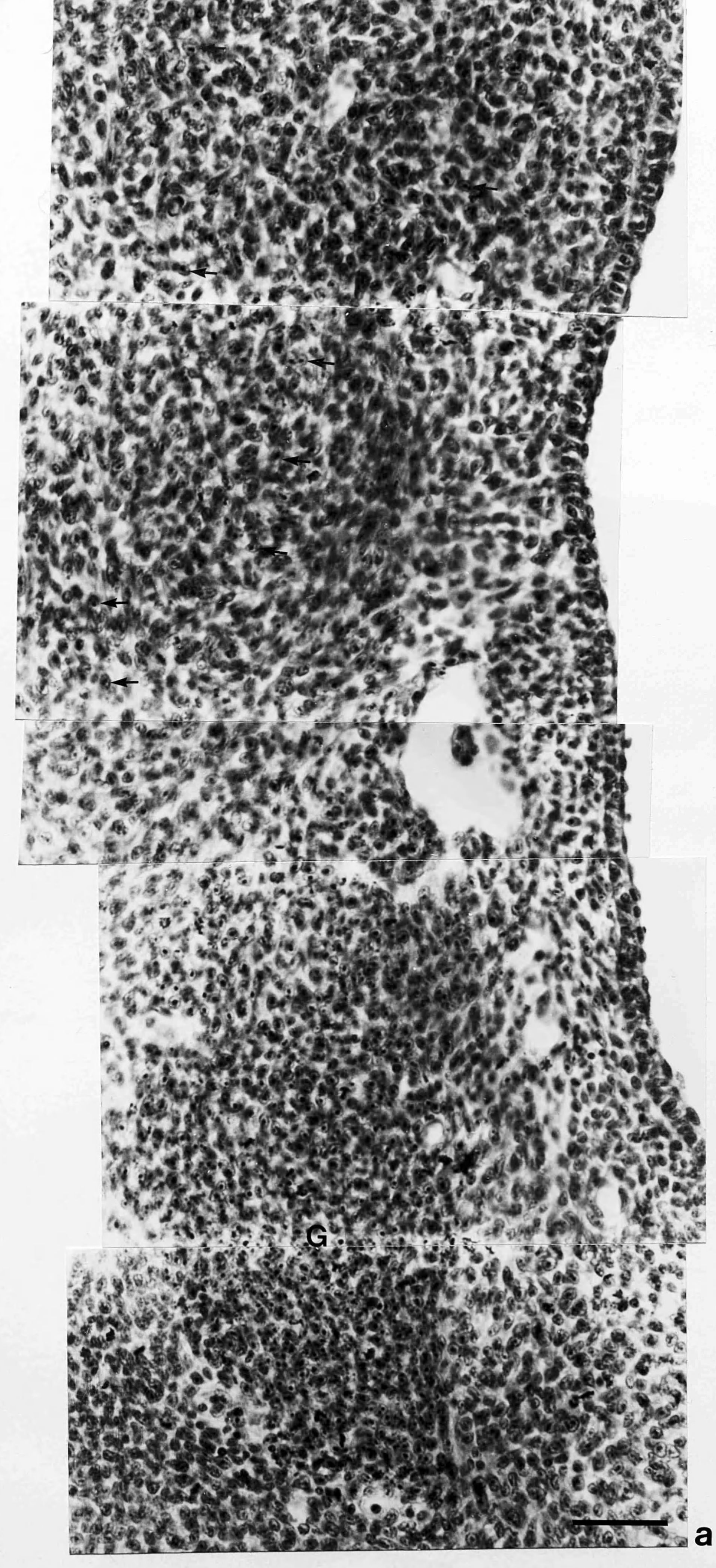

G
a

Figure 4

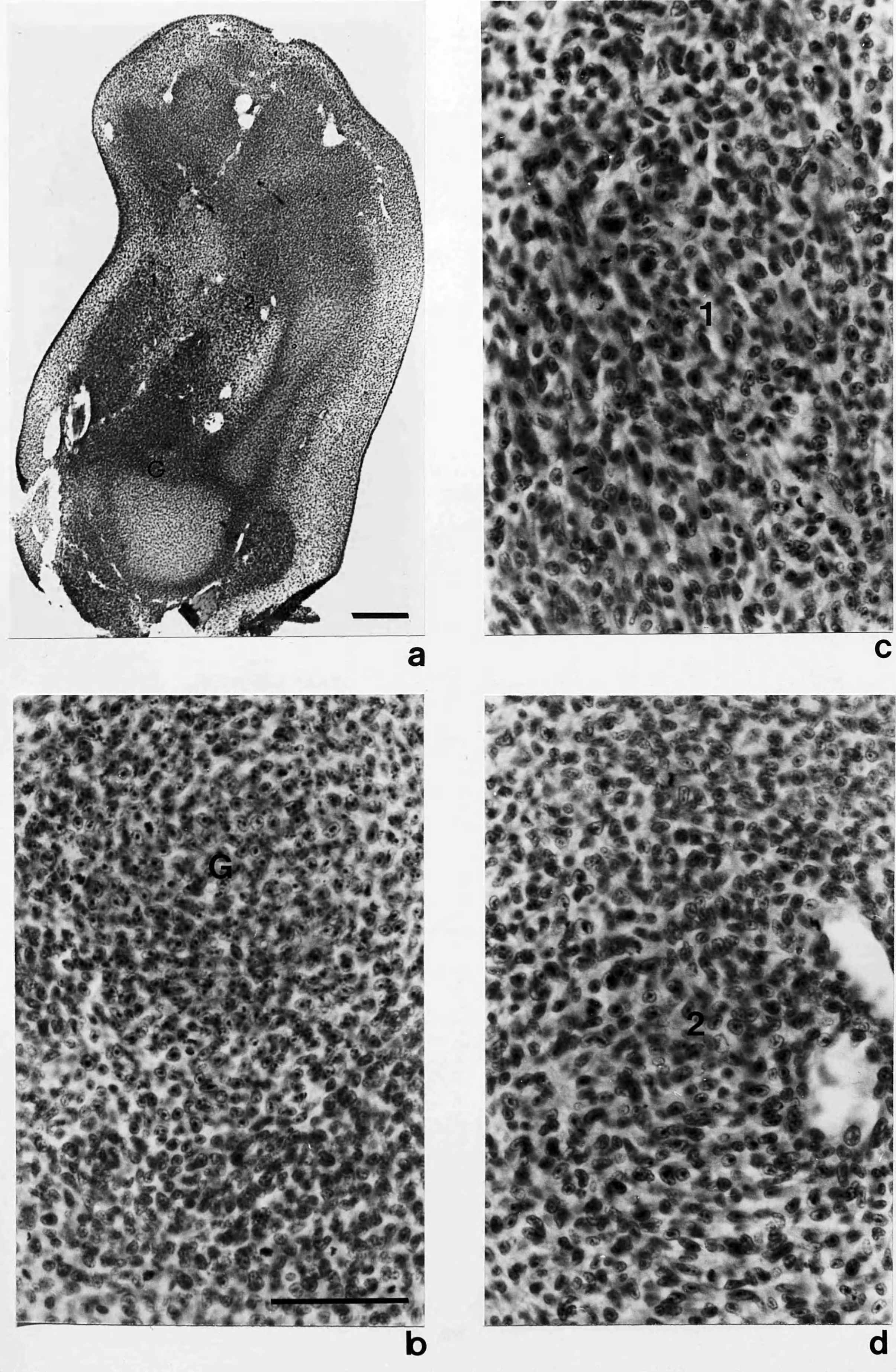
2 days after the implantation

a General view of the transplant. The graft site(G) is located at the level of the humerus. 1 and 2 are muscle regions where quail cells are found.
Bar = 200 µm.

b Quail chondrocytes found at the graft site(G). Note that they remain segregated from chick chondrocytes (hosts).
Bar = 50 µm.

c Muscle labelled 1 in (a) mixed chick and quail cells.

d Muscle labelled 2 in (a) mixed chick and quail cells.



1
2
G
a
1
c
G
b
2
d

Figure 5

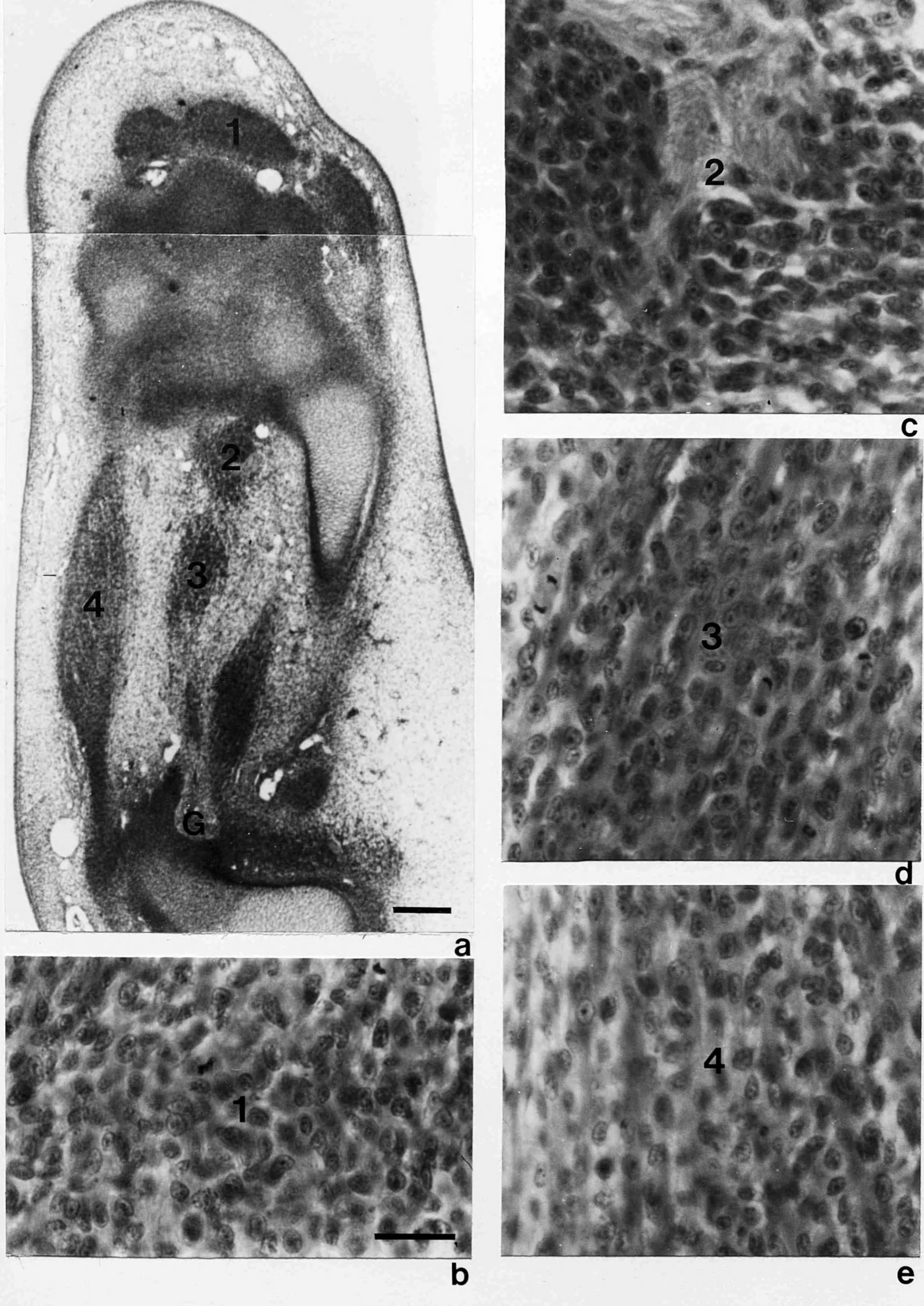
3 days after implantation

a General view. Various muscle regions are labelled 1-4. G is the graft site.
Bar = 200 μm

b Quail cells are not found in the muscles of metacarpal regions, labelled 1 in (a).
Bar = 20 μm

c-d Quail cells are found in muscles labelled 2 and 3 in (a).

e Quail cells are not found in muscle labelled 4 in (a). ie no lateral movement.



1
2
3
4
G
a
1
b
2
c
3
d
4
e

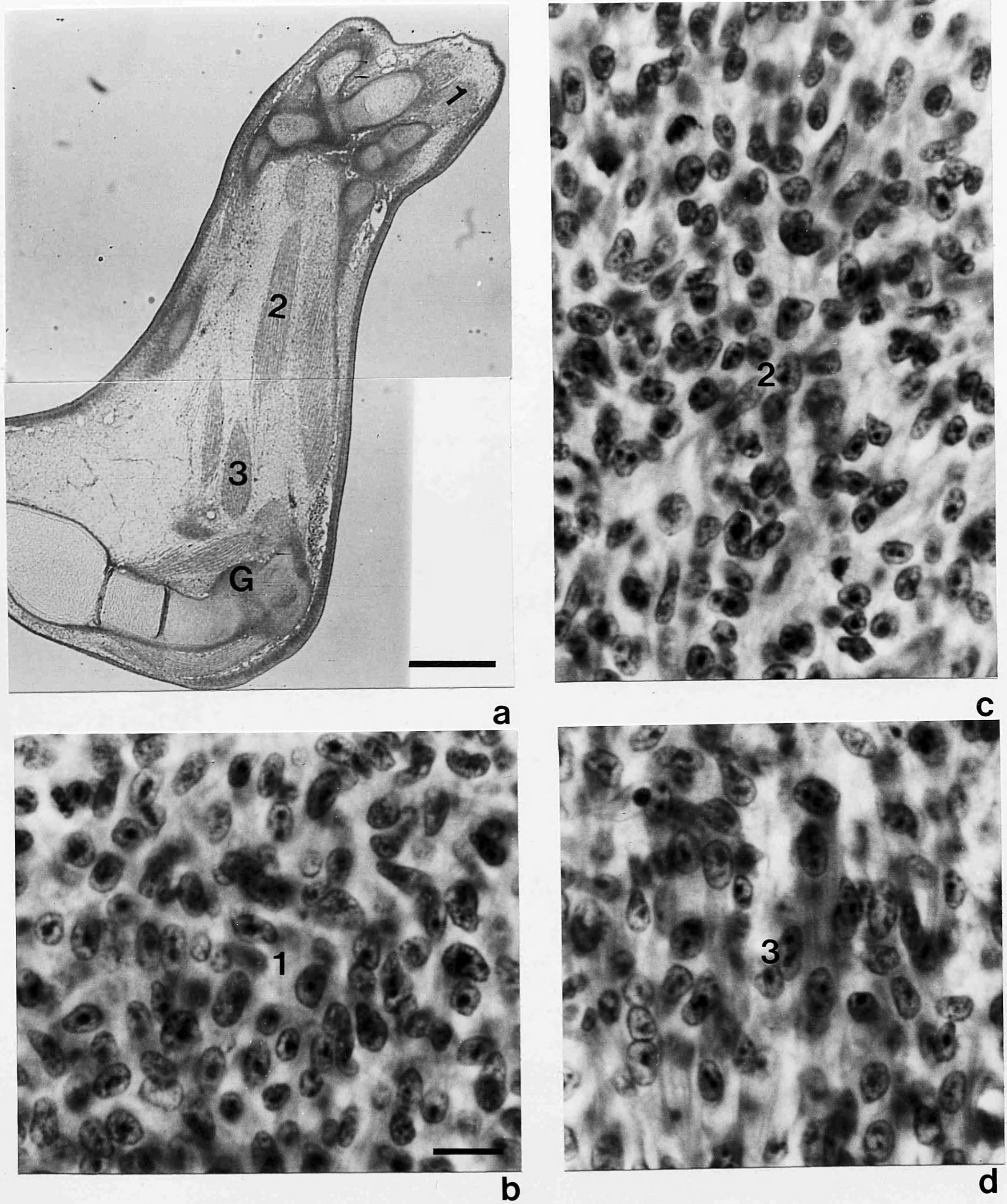
Figure 6

4 days after implantation

a General view. 1-3 labelled muscle regions containing quail cells. G is the graft site.
Bar = 300 µm

b Quail cells are now found in the muscles of metacarpal regions.
Bar = 10 µm.

c-d Muscles labelled 2 and 3 in (a), mixed chick and quail cells.



1
2
3
G
a
c
2
1
b
3
d

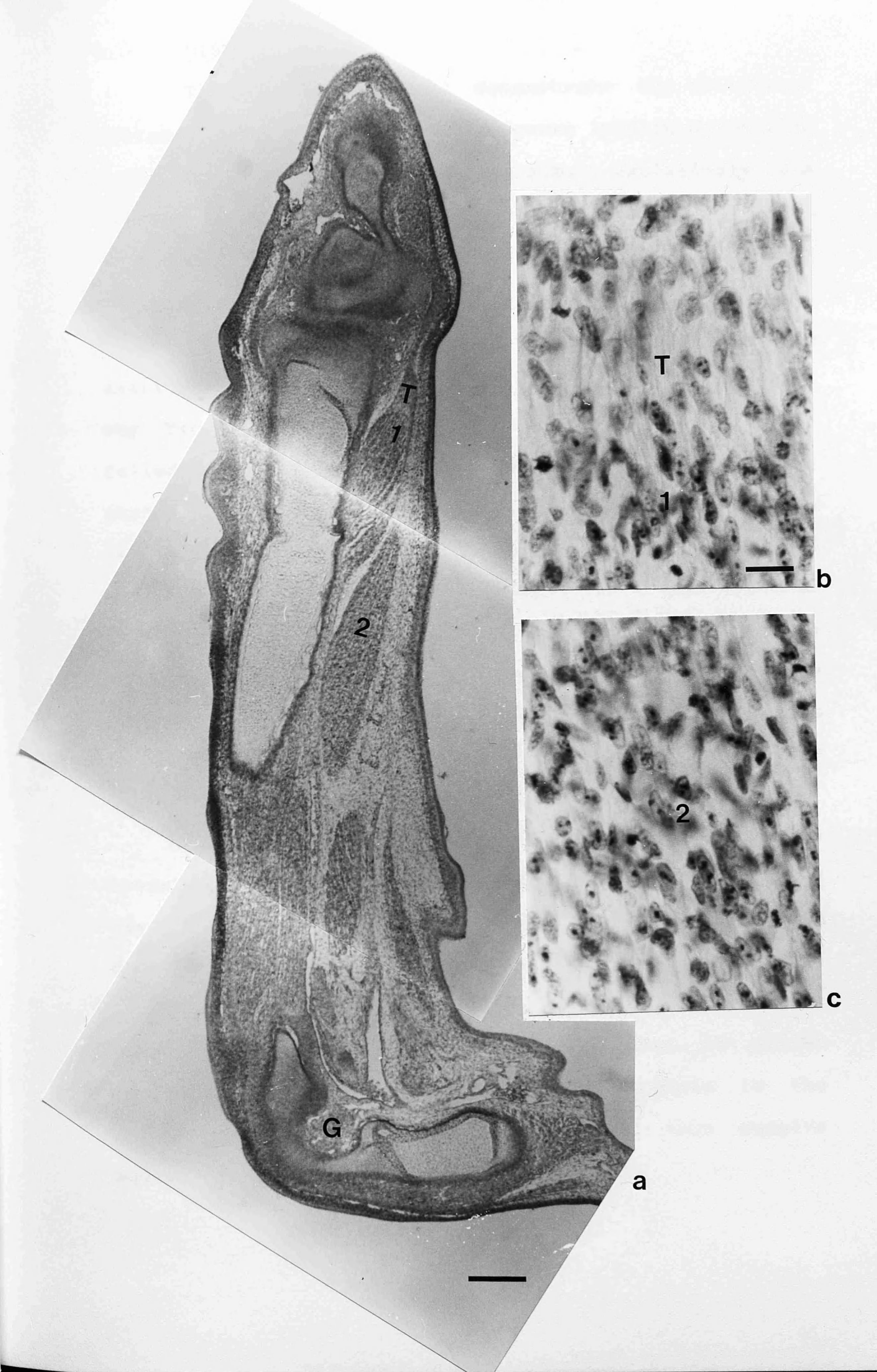
Figure 7

5 days after implantation

a General view. 1 and 2 labelled muscle regions containing quail cells. G is the graft site. T is a piece of tendon.
Bar = 200 µm

b Chimaeric muscle labelled 1 in (a) is attatched to a tendon (T). The tendon is composed entirely of chick cells (host).
Bar = 10 µm

c Muscle labelled 2 in (a) mixed chick and quail myogenic cells.


T
1
2
G
a
T
1
b
2
c

2:4 DISCUSSION

The results clearly demonstrate the ability of skeletal myogenic cells to migrate within avian limb buds. Moreover, migration is almost exclusively in a proximo-distal direction, with very little concurrent backwards movement. This is consistent with the results obtained by Gumpel-Pinot *et al* (1984). In general, myogenic cells do not start migrating out of grafts until the second day of implantation, which may explain why Tickle *et al* (1978) and Fisher & Solursh (1979) failed to pick up cell movement. Judging from the photographs of Tickle *et al* (1978) the number of quail cells they introduced were relatively small, so another possible reason why cell movement was missed might be the fact that there were not enough cells to make subsequent detection possible. In my experiments the emporte pièce was employed, which has the distinct advantage that both the size of the graft and hole made to receive it is constant throughout all replicates.

A possible contribution of growth towards the overall displacement of myogenic cells should not be discounted, since myoblasts must obviously be able to divide to generate the numbers required for these cells to fill the myogenic regions. Finding an effective control to take growth into account is almost impossible; however there are several clues in the results which suggest active rather than passive movement.

(1) If the observed displacement was solely attributed to growth then one would expect cells to be displaced all around the graft instead of specifically in a proximo-distal direction.

(2) The fact that quail cells found in the intermediate mesenchyme during the early stages of implantation later become confined within muscles is consistent with the idea that myogenic cells are actively colonizing these regions.

(3) During the 3rd day after operation chick muscles are established in the metacarpal regions but are devoid of quail cells. Twenty four hours later quail cells are discernible in the muscles, suggesting that these cells got there through active movement rather than being passively pushed there through growth, since muscles in these regions have already been established.

The migratory capacity of myogenic cells at different stages of development has already been investigated (Mauger & Kieny, 1980). Quail myogenic cells from stages 24-27 H.H. when heterospecifically transplanted into the somitic regions of 2 day chick embryos were capable of invading newly formed limb buds and participate in myogenesis. More intriguing is the

ability of myogenic cells to migrate exclusively in a proximo-distal direction. Several mechanisms which might account for this are considered next.

2:4:1 Influence of the apical ectodermal ridge

The apical ectodermal ridge (AER) has been described as an inductor of limb outgrowth (Saunders, 1948a) and the nature of its inductive influence has subsequently been investigated (Hampé, 1959; Dhouailly & Kieny, 1972; Summerbell _et al_, 1973). The grafting of an additional AER to the lateral surface of the limb has been shown to result in a secondary outgrowth (Zwilling, 1956). If a strip of AER is placed over the surface of a thin multi-layer of limb mesenchymal cells _in vitro_, cells will be attracted to migrate and accumulate beneath it (Globus & Vethamany - Globus, 1976).

The importance of the AER with respect to myogenic cell movement has been demonstrated by Gumpel-Pinot _et al_ (1984). When these workers transplanted a fragment of quail wing mesenchyme into a chick wing bud, extensive migration was observed but when the AER was excised migration was inhibited. The insertion of an impermeable barrier behind the AER also has the same effect (H Jacob, personal communication). It is not unlikely that myogenic cell movement is directed by some form of chemotactant synthesized by the AER. Indeed, Wachtler (1984) has also drawn the same conclusion regarding the migration of epidermal

melanoblasts in avian embryonic wing buds. Again, these cells are able to migrate exclusively in a proximo-distal direction. However with an impermeable barrier inserted beneath the AER, directionality is lost and cells are capable of moving in both directions. Perhaps relevant to this, the AER can apparently synthesize a diffusible factor which can delay cell death in the distal limb mesenchyme (Cairns, 1975; Bell, 1980).

2:4:2 Nudging

One way a cell or a group of cells could maintain their directionality over a period of time is via nudging. This phenomenon was first demonstrated in Fundulus deep cells (Tickle & Trinkaus, 1976; Trinkaus, 1978). When a Fundulus deep cell is nudged with a probe, a large bleb is usually formed on the cell's opposite side. The bleb will generally extend to form a lobopodium or flatten to form a lamellipodium (Tickle & Trinkaus, 1977). If these cells are in close contact with each other, the nudging signal can be transmitted to the next cell and others down the line. Accordingly these authors proposed that such contact-induced blebbing could confer a sense of directionality to a moving population.

Fibroblasts in culture also appear to respond to touch (Chen, 1981). For example, if a probe nudges the fully extended trailing edge of a fibroblast, the

structure will rapidly retract and within seconds is followed by a greatly increased surge of activity at its leading edge. A repetition of this process will not only increase movement 10-30 fold but it will also encourage fibroblasts to persist in moving in a certain direction. Since limb mesenchymal cells and fibroblasts translocate in a similar manner _in vitro_, perhaps it may be that such a mechanism might have a role in coordinating myogenic cell movement in the limb.

Ede (1980) has suggested that the outward growth of the limb bud involves some element of mesenchymal cell movement in a distal direction, whether active or passively imposed by ectodermal constriction of a growing population of cells. This general distalward drift of mesenchymal cells might impose directionality on the migrating myogenic cells.

2:4:3 Electric fields and galvanotaxis

It has long been known that organisms generate electric fields in regions of intensive cellular activity, such as in amphibian limb regeneration (for review, see Borgen, 1984). Most migratory and developmentally active cells, such as fibroblasts and neural crest cells are known to respond to applied electrical fields in cultures (Cooper & Keller, 1984; Stump & Robinson, 1983). For example, when steady electric fields are set up across conventional or collagen cultures containing quail somitic fibroblasts,

the cells respond by realigning their body axis perpendicular to the field. Furthermore, the fibroblasts will start migrating towards the cathode by producing extensive lamellipodia (Nuccitelli & Erickson, 1983). This phenomenon has also been observed in cultures of embryonic _Xenopus_ muscles (Hinkle _et al_, 1981).

Borgen (1984) proposed a model for amphibian limb development based solely on ionic flux. He hypothesized that local electrophysiological changes in the embryonic epidermis will set up an electric field . This will then direct mesenchymal cells to migrate and accumulate beneath the epidermis until a bud-like structure is formed. If electrical gradients were to be found in avian limb buds, it might serve as a guide for directing myogenic cell movement.

2:4:4 Contact guidance and extracellular matrices

The most consistent feature of contact guidance lies in the behaviour pattern of the cells. Cells undergoing contact guidance show an equal tendency to translocate in two opposite directions provided that other behavioural influences are absent. If a fragment of limb mesenchyme is explanted onto an aligned network of fibronectin, myogenic cells emigrating from the explant will be predominantly orientated with their long axes parallel to the fibre axis. Cells will assume an elongated shape and will only move along the axis of

these fibres (Turner _et al_, 1983).

Cells from limb explants can also be contact guided by collagen fibres (Chapter 6). More importantly, the pre-existence of aligned collagen fibres is not necessarily a prerequisite for contact guidance because, through cell traction, the cells have the ability to bring random network of collagen matrix into alignment (Stopak & Harris, 1982). Although this mechanism can not explain unidirectionality, it is possible through interaction with another system (eg. chemotaxis) to restrict the choice of direction open to a migrating cell.

2:4:5 What determines muscle patterning?

In order to understand the genesis of muscle pattern it is important to know how far the process is determined by the specific character of the myoblast and how far by the intrinsic structure of the limb.

It is now known that there are species specific differences between chick and quail wing muscles (Jacob _et al_, 1983). The extensor medius brevis muscle (EMB) in the chick wing is well developed, while in the quail the muscle primordium degenerates after 12 days of incubation. Another species specific difference is the flexor digitorum superficialis muscle (FDS), which is long and thin in the chick; in the quail this muscle is very much larger. Jacob _et al_ (1983) performed a series of quail brachial somite substitutions in chick hosts:

the chimaeric wings which formed, produced normally developed chick-like FDS and EMB muscles. The inability of quail myoblasts to exert their influence on chimaeric muscle patterns in chick hosts must suggest that it is the intrinsic make up of the limb rather than the specific character of the myoblasts which is important in determining muscle pattern.

Apart from muscles, the embryonic limb is made up of a loose meshwork of connective tissues, cartilage, nerves, tendons and an ectodermal covering. In the light that myogenic cells appear to be 'redundant' with regard to patterning, it is vital to establish how the different components in the limb interact with muscles. This will be discussed in the following sections.

2:4:6 Connective tissues and muscles

The role of connective tissue in the patterning of chick limb musculature has been investigated by modifying the temporal relationship between these tissues (Chevallier & Kieny, 1982). Quail myogenic cells from the somitic mesenchyme or limb premuscular mass were transplanted into the dorsal regions of a muscle-cell deprived chick wing bud (Lewis *et al*, 1981) between stages 22-27 H.H. Results showed that the connective tissue at first regulates the proximo-distal migration of myogenic cells and then moulds the muscles in the zeugopod and autopod, provided that myogenic cells have accrued before stage 27, which corresponds to

the first muscle splitting process in the zeugopod. Furthermore, myogenic cells have to accomplish normal somito-somatopleural migration before they can respond to the organizing influence of the connective tissue (Chevallier & Kieny, 1982).

If flank somatopleure from a quail embryo is substituted for leg somatopleure in a chick embryo (Jacob & Christ, 1980), the pattern of the striated muscles which form in the somatopleure correspond to the source of the graft (flank) and not to the position within the host (leg). This implies that it is the connective tissue rather than myogenic tissue which determines muscle patterning. Nevertheless, myogenic cells are not inert. Studies on the _crooked neck dwarf_ mutant have shown that myogenic cells must interact with connective tissue if muscle patterns are to be maintained (Mauger _et al_, 1983).

2:4:7 Cartilage and muscles

It is possible to produce a limb devoid of muscles simply by destroying the somites adjacent to the limb bud, prior to somito-somatopleural migration. When the limb is examined, the cartilage pattern is found to be unaffected (Chevallier _et al_, 1978; Lewis _et al_, 1981). It therefore appears as if cartilage development is independent of muscle development.

The inverse relationship between these 2 tissues has also been examined, using teratogens (Tanaka _et al_

1967; Landauer & Salam, 1973). 6-aminonicotinamide is a drug that specifically affects cartilage development but not muscle (Seegmillar, 1977). McLachlan (1980) used this teratogen to demonstrate a direct relationship between the length of the cartilage and the length of the muscle. A reduction in the length of the ulna was found to be followed by a corresponding shortening of the muscles, but the width of affected muscle and muscle patterns (in terms of numbers of muscles) remained unaffected. Reduction in the length of a mouse embryo's autopod with 6-mercaptopurine has also been demonstrated not to affect muscle patterning (Bogusch, 1980).

In sum, the lengths of muscles along the proximo-distal axis are dependent on the length of the cartilage, but muscle patterning, although in the strict sense this should include muscle length, is independent of cartilage development.

2:4:8 Nerves and muscles

The genesis of nerve patterns in avian limbs shows some parallelism with that of the muscles, in that pattern to a large degree is specified by the environment rather than being an inherent cellular characteristic. For example, if a limb bud is grafted onto a foreign site, alien nerves which invade it follow exactly the same routes taken by nerves during normal innervation (Hamburger, 1939). The topography of the limb must therefore play a major role in specifying the

pattern of the nerves. Since muscle is one of the major constituents, knowing what part muscles play in specifying the nerve patterns and vice versa is therefore important.

In the absence of muscles, the main mixed trunk of nerves and all cutaneous branches appear to develop normally, but the terminal branches that normally innervate muscles are absent (Lewis, 1980; Lewis _et al_, 1981). In addition, innervation is not altered in the presence of chimaeric muscles even though the motor endplates are modified (Jacob _et al_, 1983). These findings support the conventional view of the independence of nerve and muscle development (Hunt, 1932; Hamburger & Waugh, 1940). It must be pointed out that this independence is not absolute because the maintenance of established muscles requires the presence of nerves, in whose absence it would undergo atrophy (Lewis, 1980); likewise the development of terminal nerves requires the presence of muscle endplates (Lewis _et al_, 1981).

It has been suggested that the splitting of leg muscle masses is caused by their invasion by nerves, which separate and push the masses apart (Wortham, 1948). This idea has subsequently been disproved because in the complete absence of nerves, muscles divided normally (McLachlan _et al_, 1976; Shellswell, 1977).

2:4:9 Tendons and muscles

The distal tip of the limb bud is devoid of myogenic cells (Newman, 1977, 1981). If these tips are grafted into the flank of embryos (Shellswell & Wolpert, 1977) or cultured on chorioallanotic membranes (Brand et al, 1985), tendons usually develop normally despite the absence of muscles in these tissues. Nevertheless, the appearance of tendons is transitory because these structures generally degenerate after 9 days of incubation (Christ et al, 1979; Kieny & Chevallier, 1979; Brand et al, 1985). In sum, the initial development of muscles and tendons is independent of each other but if tendons are to be maintained, the presence of muscles is essential.

2:4:10 Ectodermal covering and muscles

In transfilter culture experiments, it has been shown that chondrogenic expression in limb mesenchyme from embryos prior to stage 17, requires the presence of ectoderm. Apparently, this is dependent on direct contact between the ectoderm and the mesenchyme (Gumpel-Pinot, 1980, 1981). On the contrary, myogenic expression of stage 15-17 mesenchyme is independent of the ectoderm (Gumpel-Pinot, 1980). Myogenic differentiation in older limb mesenchyme appears to require the presence of ectoderm. Searls & Smith (1982) found that intact limb buds from stage 19-21 embryos in culture can develop with the formation of both cartilage

and muscles. However if the limb ectoderm is removed, the limb mesenchyme will only differentiate as cartilage and connective tissue.

2:4:11 Blood vessels and muscles

The early limb bud was at one time believed to be composed of a single homogeneous population of uncommitted mesenchyme. The physical location of the blood vessels was thought to produce just the necessary variation in environment required to determine the commitment and subsequent distribution of chondrocytes and myocytes (for review, see Caplan, 1981). Regions rich in capillaries were postulated to be the site of myogenesis, while poorly nourished regions were associated with chondrogenesis (Caplan & Koutropus, 1973). Although the theory is inadequate with respect to myogenic determination, since the limb bud is now know to be heterogeneous, there may however, be some substance to linking the spatial location of blood vessels with the distribution of myogenic cells. For instance, at stage 21 desmin-positive (ie. myogenic) cells are absent from the avascular zone that extends around the ectodermal hull, but present in the central core region where the venous network has become established (Solursh *et al*, 1985). Accordingly these workers hypothesized that early patterning of myogenic cells in the limb was influenced by chemotatic and mitogenic stimuli from the already patterned vasculature.

CHAPTER III

AN AUTORADIOGRAPIC STUDY ON MYOGENIC CELL MOVEMENT IN HETEROSPECIFIC AND HOMOSPECIFIC TRANSPLANTATIONS

3:1 INTRODUCTION

If quail mesenchymal cells are transplanted into chick wing buds, extensive myogenic cell movements are observed (Wachtler *et al*, 1981; Gumpel-Pinot *et al*, 1984). However, when the same experiment is repeated with tritiated thymidine-labelled chick wing mesenchyme instead of quail wing mesenchyme, no migration is discernible (Searls, 1967; Stark & Searls, 1973). In the first example the transplant is heterospecific while the latter is homospecific; could it be that cell movement is not a normal part of limb morphogenesis, but that it has arisen as a direct consequence of the interactions between chick and quail cells?

It has always been taken for granted that chick and quail cells are of sufficient similarity to warrant use of quail as a marker in heterospecific transplantations (Le Douarin & Barq, 1969; Le Douarin, 1972). Recent results obtained by Bellairs *et al* (1981) contradicted this view. They have shown that quail endoblasts penetrated early hypoblasts twice as efficiently as those in reciprocal experiments. Chevallier *et al* (1977) have also shown disparity between the behaviour of chick and quail cells. When

brachial quail somites were orthotopically implanted into chick embryos, the resulting adjacent limb musculature consisted entirely of quail cells, whereas in the converse procedure the muscles were composed of a mixture of chick and quail cells. These findings seem to suggest that quail cells are more invasive than chick cells, and if this were true our results, especially those reported in chapter 2, would have to be interpreted with this in mind.

It is a possibility that cell movements do occur within homospecific transplants but are not discernible when reliance is placed on tritiated thymidine as a marker, because misinterpretation due to tritium dilution is a notorious problem. In an attempt to resolve this problem 2 different series of experiments were set up;

(1) In order to determine whether tritiated thymidine is subject to dilution in the conditions of these experiments, quail wing mesenchymes were labelled with this marker and then implanted into chick wing buds. Because quail cells are themselves markers, it was therefore possible to tell with certainty whether or not migration had taken place. If it had, then the same sections could be monitored for the presence of tritiated thymidine beyond the graft site. It was then possible to assess the extent of tritium dilution.

(2) In order to determine whether myogenic migrations were evident in homospecific transplants,chick wing mesenchymes were labelled with tritiated thymidine and then inserted into unlabelled chick wing buds. This is a repeat of Searls' experiment, but taken a step further; instead of fixing the experimental limbs after 2 days of post-incubation, in this case limbs were fixed after 4 days, since this is the time interval required to express the full extent of myogenic migration (see chapter 2).

3:2 MATERIALS AND METHODS

3:2:1 Autoradiography

The embryos used in this series of experiments were staged between 20-25 H.H. Twenty-four hours prior to transplantation, a small window was made at the blunt end of the donor egg shell. 20 µl of Methyl H^3 Thymidine (activity 24 ci/mmol, Amersham) was then injected onto the chorio-allantoic membrane, with the embryo given a second dose 12 hours later. Using the procedure described in chapter 2, cores of radioactively labelled quail (hetrospecific transplants), or chick (homospecific) wing mesenchyme were transplanted into unlabelled chick wing buds. The legs of donor embryos were removed and processed in order to determine whether or not the limb mesenchyme was properly labelled. Sections taken from the legs showed all of the

mesenchyme to be well labelled and that the tissues were healthy, indicating that these dosages were satisfactory.

The operated embryos were incubated for a further 4 days, after which the wings were removed and fixed in Carnoys fixative for 40 minutes. The limbs were then embedded in wax and sectioned at 5 μm. Sections were mounted onto slides cleaned with chromic acid and subbed in a mixture of 5% gelatin and 0.05% chrom. alum. solution. Slides were then coated with Kodak AR10 stripping film (Gahan, 1972). Heterospecific sections containing labelled quail cells were prestained using the Feulgen reaction before photographic emulsion was applied. This was to ensure that the acid used during the Feulgen reaction did not leach out the silver grains on the emulsion.

All solutions were filtered before use, in order to reduce the level of background radiation, induced by the presence of dirt. Exposed sections (4 weeks) were allowed to reach room temperature prior to development, to prevent condensation from affecting this process. Autoradiographs were then developed in Kodak D19 developer for 5 min at 20°c, washed with running water for 30 sec and then cleared in Kodak fix F-24 for 1 min.

3:3 RESULTS

3:3:1 Quail wing mesenchyme labelled with tritiated thymidine transplanted into chick wing bud

Quail cells are natural markers; so the effect of labelling these cells with tritiated thymidine, would be to create a double marker. Applying this double marker system, it was possible to compare the effectiveness of the natural marker against the autoradiography technique, in showing up cell movement. Two transplantations were performed in this series, with both of the implants located at the level of the humerus.

Sections were examined under light and dark field illumination, light field for determining the presence of Feulgen positive quail cells and dark field for detecting the silver grains of tritiated thymidine in labelled quail cells. Under light field illumination, quail chondrocytes were found at the graft sites of both of the transplants (fig. 1j). Cells with Feulgen positive spots were also detected within myogenic regions along the whole length of the zeugopod and autopod (fig. 1g-i). The maximum distances travelled by these quail myogenic cells are summarized in table 1.

The same sections were also examined under dark field illumination, and showed the quail chondrocytes to be well labelled with tritiated thymidine (fig. 1f). Each of the quail chondrocytes had an average silver

grain count of 14 per nuclei. Quail myogenic cells on the other hand, had a lower labelling count, with an average grain count of 7 for cells close to the graft site. The intensity of labelling in quail myogenic cells progessively decreased towards the upper half of the zeugopod until the level of labelling was indistinguishable from background levels (fig. 1e-c), making it impossible to pick up quail cells in the myogenic regions of the autopod (fig. 1c). Although the full extent of myogenic cell movement was not discernible using autoradiography, it was still possible to detect moderate level of movement (table 1).

3:3:2 Labelled chick wing mesenchyme transplanted into unlabelled chick wing bud

Five transplantations were performed, with 3 of the implants located at the posterior tip of the ulna and 2 at the humerus. Labelled chondrogenic cells were found at the graft sites of 4 of the implants and typical of quail/chick transplants; a sharp border was present, separating donor chick chondrocytes from host chick chondrocytes.

Labelled chick cells were also detected in muscle regions distalward from the graft site, intermixing with unlabelled myogenic cells. Although myogenic cells were not as well labelled as chondrogenic cells, it was still possible to detect labelled cells amongst most of the myogenic regions in the lower half of the zeugopod (fig.

2a). A moderate level of labelled cell displacement was detected in 4 of the limbs, as summarized in table 1.

The spreading pattern of labelled chick myogenic cells (homospecific transplantation, fig. 2a and 3a) were very similar to those found in quail/chick chimaeras (heterospecific, fig. 2b and 3b), so that, extrapolating from these results, it was possible to conclude that myogenic cell migration does indeed occur in homospecific transplants. However, probably as a result of tritiated thymidine dilution, the full extent of this activity cannot be demonstrated with the autoradiography technique.

Table 1

Tritiated thymidine labelled quail wing mesenchyme transplanted into chick wing buds.

Code	Stages of donors	Stages of hosts	Estimation of maximum distal cell movement: Feulgen reaction	Estimation of maximum distal cell movement: Autoradiography
HTQ1	21	23	1,176 µm	1,000 µm
HTQ2	22	24	2,235 µm	590 µm

Tritiated thymidine labelled chick wing mesenchyme transplanted into chick wing buds.

Code	Stages of donors	Stages of hosts	Feulgen reaction	Autoradiography
HTC1	20	23	-	1,057 µm
HTC2	20	21	-	565 µm
HTC3	22	25	-	no movement
HTC4	22	25	-	706 µm
HTC5	22	25	-	565 µm

Figure 1

A single longitudinal section from a tritiated thymidine-labelled quail into chick transplant after 4 days post-incubation. The section was examined under dark and light field illumination.

a) A camera lucida drawing of the section showing the extent to which quail cells can be detected under light field illumination. Different myogenic regions are labelled 1-3 and correspond with fig. **g-i** respectively. G is the graft site.

b) A camera lucida drawing of the same section observed under dark field illumination. Again different myogenic regions are labelled 1-3 and correspond with fig. g-i respectively. Note that autoradiography does not show up quail cells in the distal myogenic regions (1).

c-e) Myogenic regions observed under dark field. Note that it becomes progressively more difficult to detect labelled cells (arrows) in the distal regions. Bar = 25μm.

g-i) Myogenic regions under light field. Quail cells can be detected in all the myogenic regions.

f&j) The graft site.

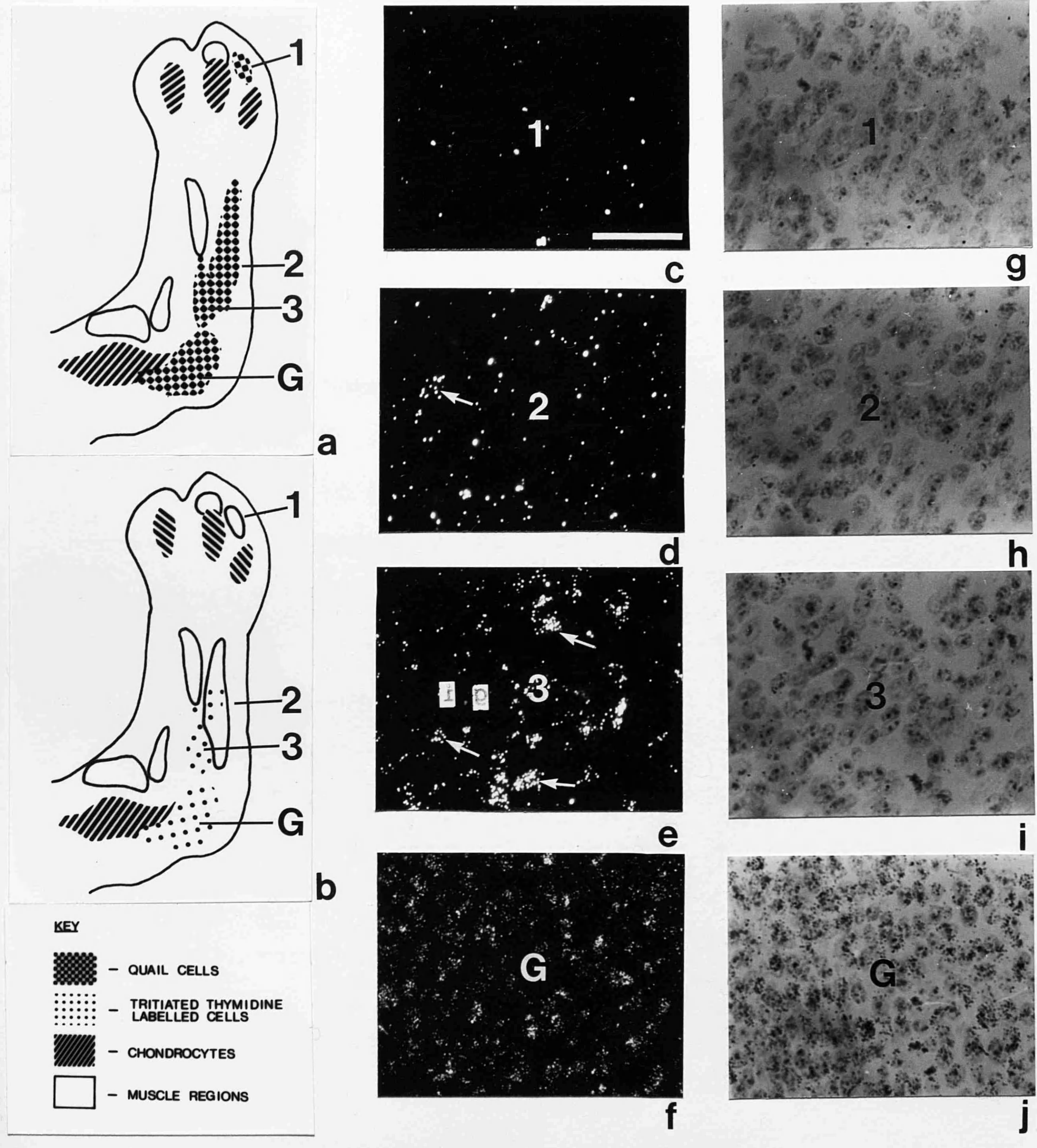

1
2
3
G
a
1
2
3
G
b
KEY
- QUAIL CELLS
- TRITIATED THYMIDINE LABELLED CELLS
- CHONDROCYTES
- MUSCLE REGIONS
1
c
2
d
3
e
G
f
1
g
2
h
3
i
G
j

Figure 2

a) A tritium-labelled homospecific transplant after 4 days post-incubation. Myogenic cells (arrows) can be seen some distance from the graft site (G). Bar = 100 μm.

b) A tritium-labelled heterospecific transplant after 4 days post-incubation. The graft site (G) is well labelled and contains predominantly quail chondrocytes. In contrast quail myogenic cells (arrows) are not very well labelled but can still be detected some distance from the graft site.

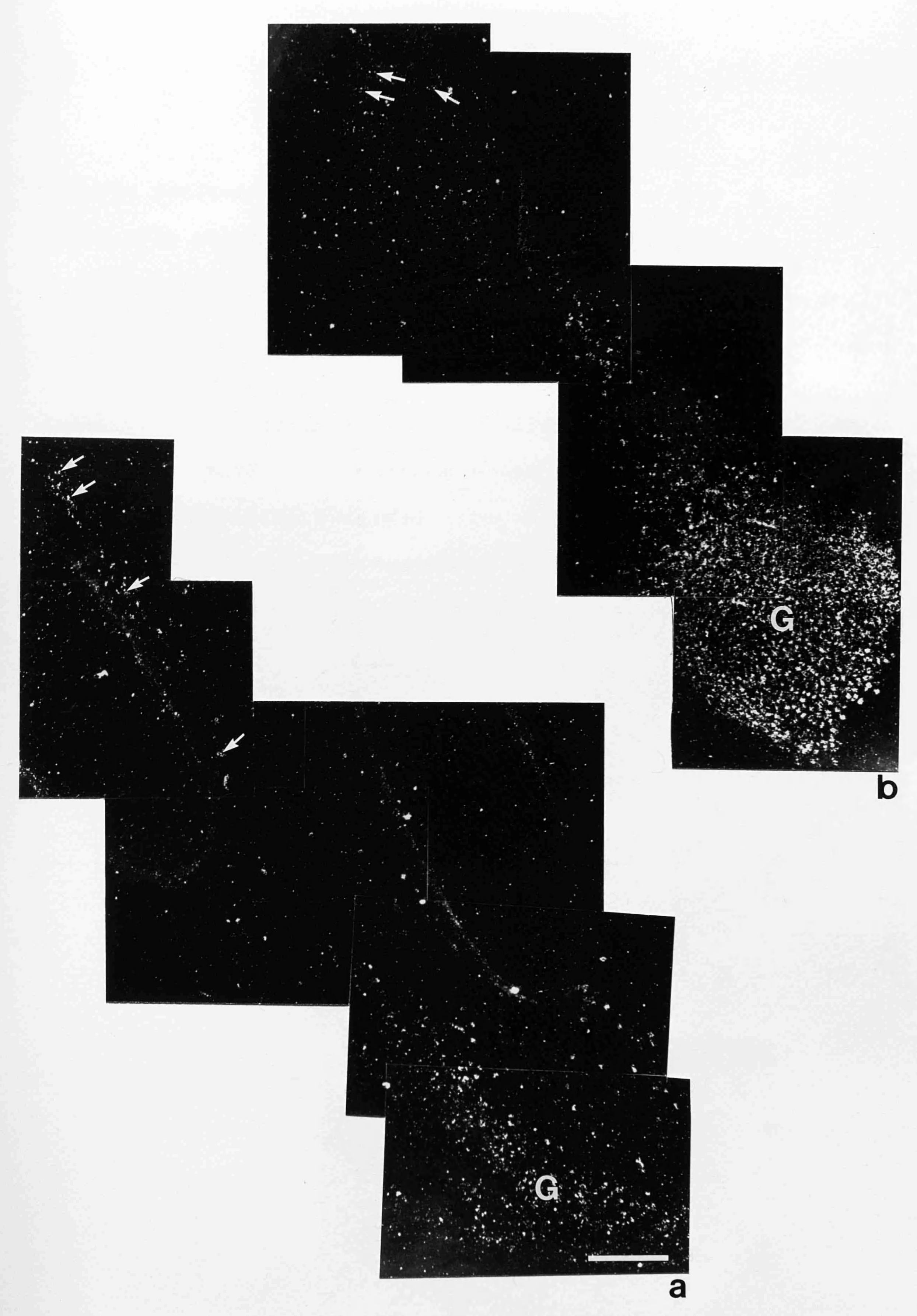
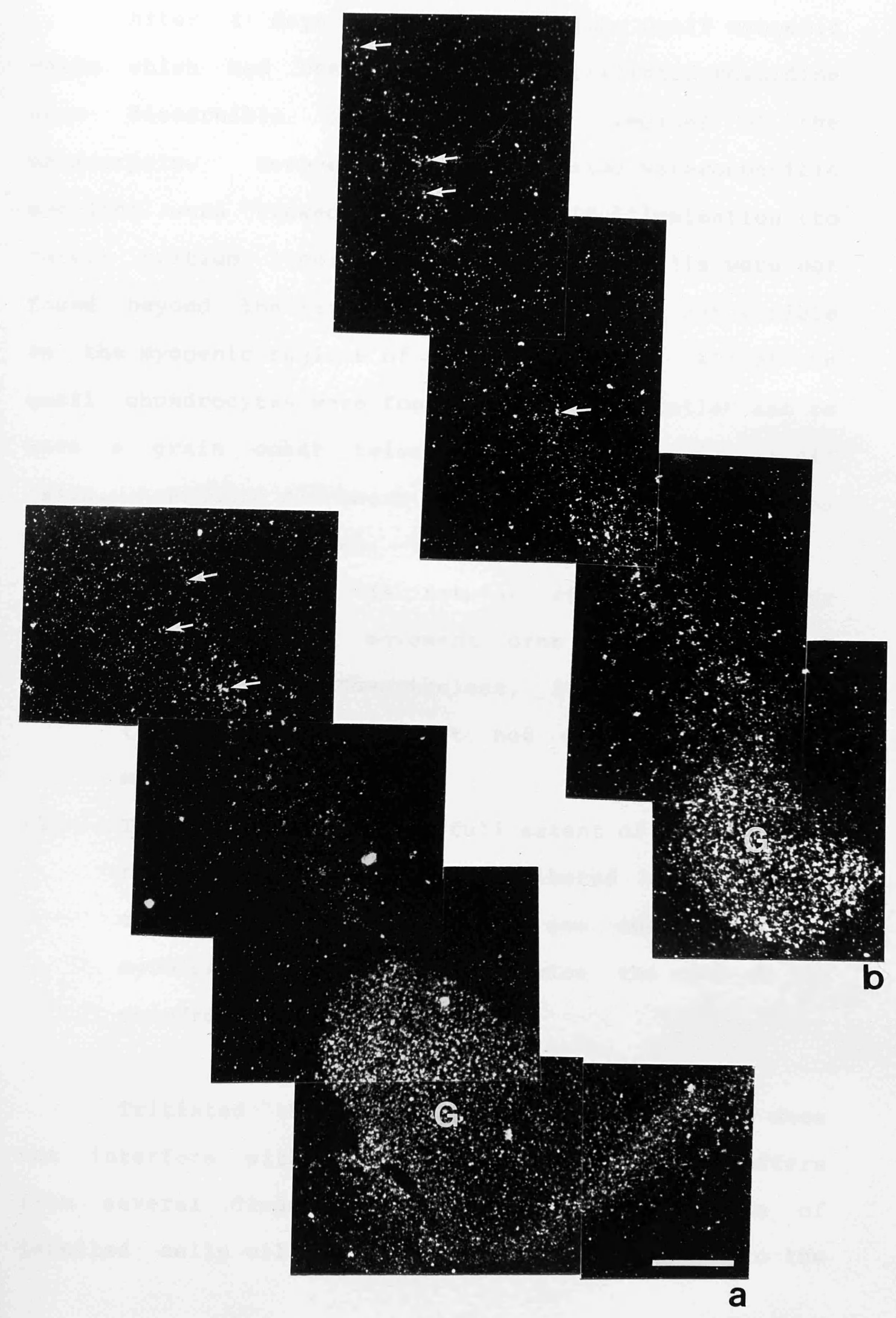

G
b
G
a

Figure 3

a) A tritium-labelled homospecific transplant after 4 days post-incubation. G is the graft site and arrows denote the location of myogenic cells. Bar = 100 μm.

b) A tritium-labelled heterospecific transplant after 4 days post-incubation. Note that the spreading pattern of labelled myogenic cells in homospecific and heterospecific transplants are very similar in appearance.


G
G
b
a

3:4 DISCUSSION

After 4 days of post-incubation, quail myogenic cells which had been labelled with tritiated thymidine were discernible in the muscle regions of the metacarpals. However, when these same heterospecific sections were viewed under dark field illumination (to detect tritium labelled cells) migrant cells were not found beyond the level of the zeugopod (ie. not visible in the myogenic regions of the metacarpals). All of the quail chondrocytes were found to be well labelled and to have a grain count twice as high as that of myogenic cells. Putting all these facts together, the following conclusions may be drawn.

(1) Autoradiography is not an efficient method for studying cell movement over long periods of incubation. Nevertheless, it is still possible to show moderate, but not the full extent of, myogenic cell movement.

(2) The reason why the full extent of migration is not detected may be attributed to thymidine dilution, especially when one considers that myoblasts proliferate at twice the rate of the chondrocytes.

Tritiated thymidine has a long half life and does not interfere with cell behaviour; however, it suffers from several disadvantages, for example: cytosis of labelled cells will release tritiated thymidine into the

surrounding tissue, consequently tritium can be picked up by these tissues; phagocytes can engulf labelled cells and carry it away from the graft site, so that these could be mistaken for migrant cells, and, finally, the problem of tritiated thymidine dilution (for review, see Trinkaus & Gross, 1961).

In homospecific transplants (i.e. chick into chick), moderate levels of myogenic migration were detected in 4 out of 5 replicates, and the spreading pattern of labelled myogenic cells correlated well with those found in heterospecific transplants. Combining this with what is known about the behaviour of myogenic cells in quail into chick graft operations, it appears highly likely that myogenic cell movement occurs in the normal limb system, and is not an artefact of cellular interactions between chick and quail cells.

There are further indications from the literature to support the suggestion that myogenic cell movement is a naturally occuring morphogenetic event rather than an attribute of heterospecific transplantation. For example studies carried out by Newman (1977), Rutz et al (1979, 1982), Rutz & Hauschka (1983) and Seed & Hauschka (1984), in which cells from different proximo-distal levels have been cultured in vitro to produce identifiable cell types have shown there to be a proximal-distal gradient of the number of myogenic cells within both leg and wing buds. This is consistent with the idea of myogenic cells gradually invading the distal

regions of the limb.

Moreover, it appears that the myoblast population in early limb buds is not homogeneous but consists of two subpopulations of muscle colony-forming (MCF) cells, which differ in their clonal morphologies (Bonner & Hauschka, 1974; White _et_ _al_, 1975). The two subpopulations are: (1) early MCF cells which give rise to colonies with few myotubes and (2) late MCF cells which produce colonies with many long myotubes, each containing hundreds of nuclei. These subpopulations have subsequently been shown to come from different progenitors (Womble & Bonner, 1980). In addition, late MCF precursors do not start migrating into the wing somatopleural mesenchyme until stage 16 H.H., while early MCF cells do so at stage 15H.H. (Seed & Hauschka, 1985).

A similar study has also been carried out on the arm and leg buds of human embryos. In the leg, early MCF cells first appear in the proximal regions of day 41 embryos, and progessively these cells are found in more distal regions of day 45 embryos. This suggests that myogenic cell migration is also a feature of human limb morphogenesis.

3:4:1 Cell sorting and micromass cultures

Moscona & Moscona (1952) have investigated the ability of disrupted tissues from avian limb buds to reorganise themselves into their normal spatial

arrangements. They mixed equal concentrations of presumptive limb chondrogenic and myogenic cell suspensions together and then reaggregated them. Sections of these aggregates after 3-4 days of development showed an inner core of cartilage surrounded by an outer layer of myoblasts; exactly what one finds in an intact limb. The re-establishment of these spatial patterns is brought about by cell sorting, a process which involves active movement (Armstrong & Parenti, 1972). The tendency to always find chondrocytes in the middle of aggregates (Elton & Tickle, 1971; Armstrong & Niederman, 1972; Ede & Flint, 1972) and myocytes on the outside might be due to the inherent invasive nature of myogenic cells. From my results, I have already shown chondrogenic cells to be relatively immobile, and it may be that the inside-outside relationship between chondrocytes and myocytes arises as a direct consequence of differential mobility. Although this is speculative, further hints in support of it have come from studies of micromass cultures.

Under high density culturing conditions, limb mesenchymal cells can differentiate into cartilage, connective tissues and muscles (Caplan, 1977). Even though these micromass cultures are composed of many cell layers, myogenic cells are usually found in the top layers (Ahren et al, 1977, 1979). How these cells come to be located there was investigated by Solursh (1984),

with the aid of time-lapse cinematography. He found numerous bipolar cells, which had previously been identified as myoblasts, penetrating and then migrating onto the surface of the cultures. These bipolar shaped cells were found to be very active and capable of undergoing extensive movement. The ability of dissociated limb mesenchymal cells to 'self assemble' might therefore be a consequence of myoblasts actively migrating away from aggregates undergoing chondrogenesis. It may be that this mechanism occurs also *in vivo*, because in early limb buds myogenic precursors are distributed in the central core (Solursh *et al*, 1985) and it is only much later that they become located in the periphery.

3:4:2 Cell Adhesion

Bellairs *et al* (1981) explained the differences they found in the behaviour of chick and quail cells in terms of adhesiveness. They pointed out that the reason why chick endoblasts find it more difficult to penetrate quail hypoblast than in reciprocal experiments, is that quail cells are more cohesive than chick cells.

It is appropriate that cell sorting experiments have been used as a means for determining cell adhesiveness. According to Steinberg's theory, if the adhesiveness between 2 cell types are the same then cells within the aggregate will remain thoroughly mixed. If however, one type is more adhesive than

another then cells would segregate out homotypically. If quail cells are more adhesive than chick cells, as suggested by Bellairs et al (1981), then one would have expected them to sort out. Shamslahidjani (1980), investigated this possibility by mixing and reaggregating equal concentrations of chick and quail cells together, packing them into an ectodermal jacket and then grafting the whole construction into a chick host. She was unable to find any evidence of cell sorting, and both cell types were found to be thoroughly mixed. On the other hand, during a recent conference on somite development; Sanders (1986) presented evidence suggesting that quail and chick somite cells do sort out from one another. I was not convinced by his slides, because it required a great deal of imagination to see the 'clumps' of quail cells which he said was the product of cell segregation.

In sum, any results obtained from quail into chick transplantation must be vindicated by the converse procedure. In chapter 5, it is described how chick wing mesenchymes have been implanted into quail wing buds; in terms of the invasiveness of myogenic cells, results obtained from these experiments are compatible with quail into chick graft operations, so that the results presented in chapter 2 may be considered to be valid.

CHAPTER IV

THE TALPID^3 MUTANT

4:1 INTRODUCTION

Investigations using genetic mutations have contributed a great deal towards the elucidation of developmental processes. Appropriate utilization of the mutations affecting development in fowl as experimental tools have contributed by revealing mechanism responsible for some of the normal morphogenetic events that occur during the life history of an individual. For example, application of the *wingless* mutant has revealed how important it is that the ectoderm and the mesenchyme should interact, for the limb to develop normally (Zwilling, 1974). Certain aspects of cell behaviour and morphology in one particular mutant, the *talpid*^{3} mutant, are especially appropriate for use in further investigation into properties essential for myogenic cell movement. This chapter is solely devoted to describing the *talpid*^{3} mutant and will provide some background for the next chapter where the mutant is experimentally utilized.

Talpid^3 is an autosomal recessive lethal mutant found in fowl. A majority of the affected embryos usually die within 6 days of incubation and very rarely will they survive beyond 12 days. In the homozygotic embryo the gene produces a complex pattern of

pleiotropic abnormalities throughout the whole body (compare fig. 1 with fig. 2). Some of these anomalies are shared with 2 other mutants which have been presumed to be alleles: Cole's talpid (Inman, 1946) and talpid^2 mutant (Abbott *et al*, 1960).

4:1:1 Talpid^3 head

In the head the eyes are drawn together towards the midline. Likewise the maxillary processes are also drawn together and are additionally fused, instead of forming a normal parallel pair of lateral condensations. This may explain why the upper beak of talpid^3 mutants is always absent. The pituitary is missing and embedded in the head mesenchyme are a number of ectopic lenses (Ede & Kelly, 1964a). As far as the head is concerned, both talpid^3 and Cole's mutant share the same deformities, whereas talpid^2 mutant is perfectly normal.

4:1:2 Talpid^3 flank

The vertebral column is shortened and is accompanied by fusion of adjacent vertebrae (Ede & Kelly, 1964b). Cartilage-replacement bone fails completely to appear in the ribs, coracoid and ilium (Hinchliffe & Ede, 1968). Substantial subcutaneous oedema and the failure of the body wall to close ventrally around the viscera are also characteristic of the mutant.

4:1:3 Talpid^3 limb buds

The most dramatic effect of the gene is illustrated in the mutant's limb bud. The talpid^3 limb bud is mushroom shaped, with a shortened proximo-distal axis and an elongated antero-posterior axis (fig. 2). At the tip, the apical ectodermal ridge is highly extended but histochemical analysis of the structure revealed normal levels of metabolic activity (Hinchliffe & Ede, 1967). Strangely, cell death is absent from both the anterior and posterior borders (Hinchliffe & Ede, 1967).

Fusion of the skeletal elements is widespread throughout the talpid^3 embryo and it is not exempted from the mutant's limbs. In the wing the humerus is extremely short, and the radius and ulna are fused together as a single plate. The metacarpals and carpals, too, are also stuck together, to form a second plate. Ede (1982) attributed these defects to the inability of talpid^3 prechondrogenic cells to condense and segregate in the normal manner. Both the fore and hind limbs have a greater than the normal number of digits (usually numbering between 6-7), and because of this, the talpid^3 embryo has been classified as a polydactylous mutant.

The effect of the gene is not limited to the mesenchyme but is also prevalent in the ectoderm. For instance, when normal whole wing mesenchyme is cultured in a talpid^3 ectodermal jacket all the proximo-distal

sequence of skeletal elements are produced normally, but the carpals are bent backwards (Shamslanidjani, 1980): this suggests that the mutant's ectoderm is restricting the outward extension of distal elements probably through the inflexibility of its ectodermal jacket (For review, see Ede, 1980.).

Practically no work has been done on muscle development in the *talpid*^{3} limb. Only Thorogood (1972) briefly touched on the subject. In the leg bud, the author found that myogenesis was retarded, and that cells within the muscle anlagen were small, "married" together, disorientated and with very little intercellular space. A lack of cellular orientation seems to be a common feature of *talpid*^{3} tissues and is readily discernible in the mutant's cartilage (Hinchliffe, 1965), feather papilla (Ede *et al*, 1971), myotome (Ede & Kelly, 1964b) and sclerotome (Ede & EL-Gadi, 1986). The mutant's limb mesenchymal cells also appear to be more adhesive to plastic substrate and to each other than normal (Ede & Agerbak, 1968; Ede & Flint, 1975a) and have a reduced capacity for movement *in vitro* (Ede & Flint, 1975b). Ede (1976) believes that much of the mutant's abnormal development can be explained in terms of increases in cellular adhesion and reduction in cellular mobility.

Figure 1

The normal embryo

a Stage 24 H.H.

b Stage 26 H.H.

c Stage 28 H.H.

Bar = 5mm

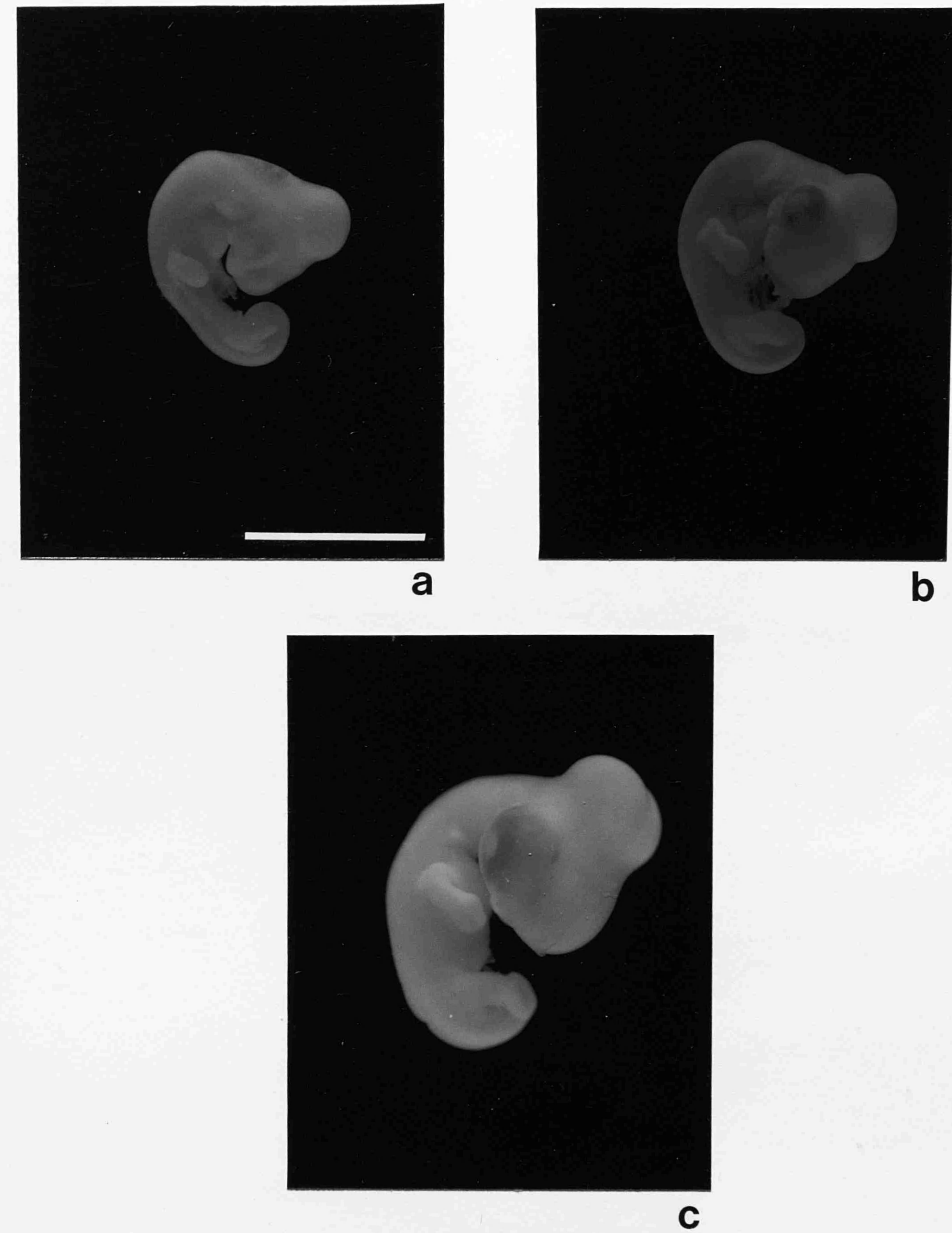


Figure 2

The *talpid*^{3} embryo

a Stage 24 H.H.

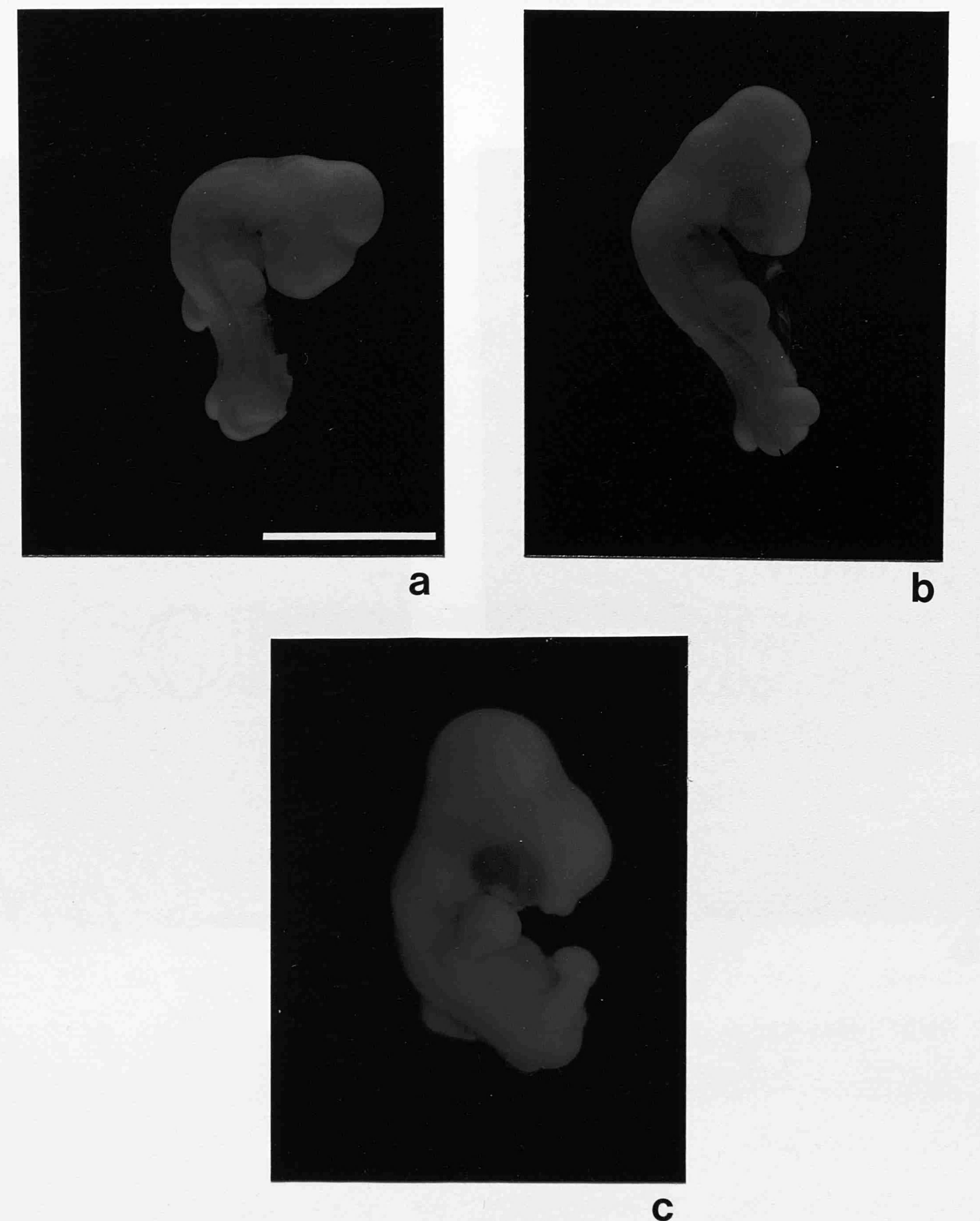
b Stage 26 H.H.

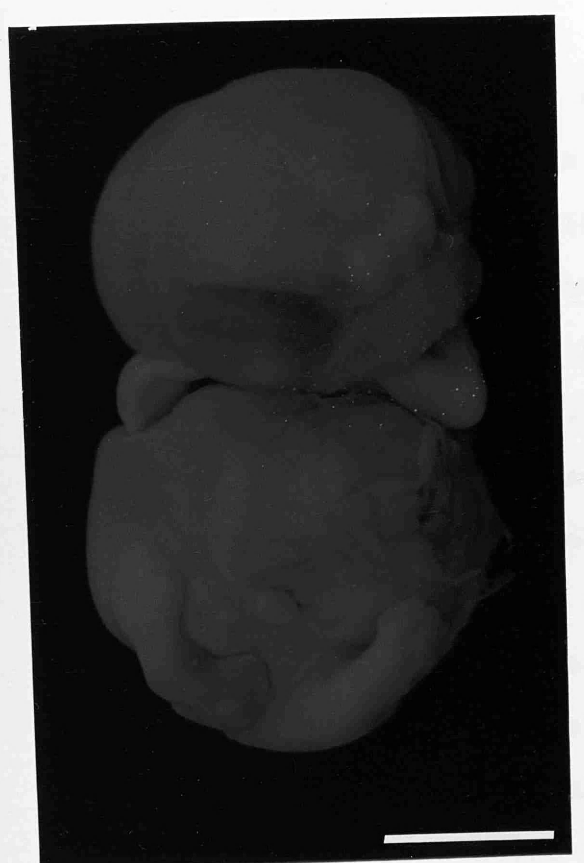
c Stage 28 H.H.

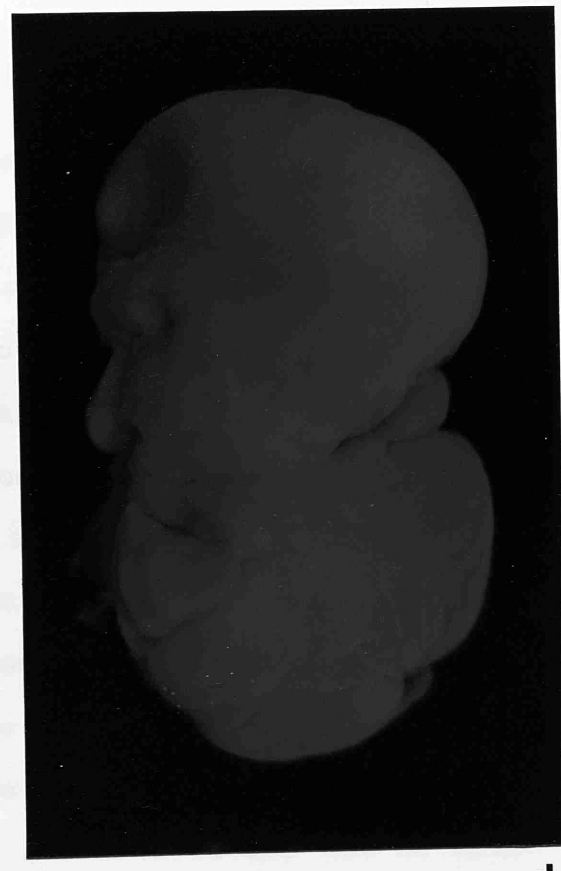
Bar = 5mm

d 12-days

Bar = 1cm


a
b
c





d

CHAPTER V

The capacity of talpid^3 myogenic cells to migrate in heterospecific transplantations

5:1 INTRODUCTION

Cell adhesion is a basic cellular property of fundamental importance to multicellular organisms. Not only does it have a role in keeping tissues together but it also ensures that various types of motile cells begin moving at the right time, end up in the right places and stay there upon arrival. According to Trinkaus (1984) "Cell adhesion not only accompanies cell locomotion, it is necessary for it. A cell must adhere to a substratum strongly enough to gain the traction necessary for its movement, but not too strongly, else it would become stuck, or too weakly, else it would slip. And of course, it must also be able to de-adhere from the same substratum, else it would be immobilised and not be able to move farther. In sum, in order to move along, a cell must be able to adhere to the proper degree and de-adhere constantly in a coordinated fashion." Since cell movement is an integral part of limb myogenesis, it is therefore important to establish whether or not "a proper degree of adhesion" is essential for this purpose.

The *talpid*^{3} mutant can help in this quest to elucidate the relationship between cellular adhesion and locomotion because certain aspects of the histology of the *talpid*^{3} limb suggest that cell to cell adhesion may be abnormal. For example, whereas chondrocytes within normal cartilage are aligned in neat columns, in the mutant these cells are randomly orientated as if they were incapable of moving to achieve normal alignment (Hinchliffe, 1965). *Talpid*^{3} limb mesenchymal cells are known to be more adhesive to one another than cells from normal limb buds (Ede & Agerbak, 1968; Ede & Flint, 1975a, b) and this is believed to be the primary cause of the mutant's abnormal limb morphology. Hence, application of *talpid*^{3} material provides a rare opportunity to investigate the effects of altered cellular adhesion on skeletal myogenic cell migration by allowing us to determine whether increased cell adhesion enhances or impairs cell movement in this system.

Two different sets of transplantations were performed in this investigation. Fragments of normal wing mesenchyme were excised from 4-day chick embryos and transplanted olthotropically into quail wing buds *in ovo* using the method previously described. These served as controls against which the identical procedures using *talpid*^{3} wing fragments were compared. Histological studies were made on unoperated normal and *talpid*^{3} 9-day limbs for comparison, embedded in wax, sectioned

at 6 µm and stained with H & E.

5:2 MATERIALS AND METHODS

5:2:1 Materials

Fertile *talpid*^{3} eggs were obtained from matings of known carriers (*ta*^{3}/+) kept in this laboratory (University of Glasgow) and at the West of Scotland College of Agriculture. *Talpid*^{3} embryos (*ta*^{3}/*ta*^{3}) were easily identified by their eyes drawn forward towards the midline, their fused maxillary processes and their fan shaped limb buds (fig. 1a). Heterozygotes (*ta*^{3}/+) are indistinguishable from homozygous normals and treated as being normal chick embryos. Quail (*Coturnix coturnix japonica*) eggs were produced from a stock maintained in the laboratory. Eggs were incubated at 38°c and the stageing of normal development was determined in accordance with Hamburger and Hamilton (1951); the scheme for stageing *talpid*^{3} embryos was devised by Hinchliffe (1965).

5:2:2 Transplantation

Experiments were performed on hosts ranging from stages 20 - 25 H.H. and donors 20 - 26 H.H. A 1 cm hole was made at the blunt end of the quail egg shell, making the embryo directly accessible for manipulation. The shell membrane on top of the embryo was then removed and also a small section of the chorion around the quail wing bud. Using an emporte pièce, a core of mesenchyme

was removed from the wing bud. The resultant hole was then made ready to receive either a normal or _talpid_^{3} implant. (Refer to figure 1b).

Normal and _talpid_^{3} chick grafts were also prepared with the emporte pièce so that the graft would fit perfectly into the host's wing bud. After implantation a small piece of shell membrane was placed directly over the wound to reduce the leakage of amniotic fluid and enhance the survival of the quail embryo (Fyfe & MacMillan, 1983). The operated eggs were then sealed with sellotape and incubated for a further 5 days.

5:2:3 Histology

The procedures have been described in Chapter 2. Briefly, all operated limbs were fixed in Carnoy fixative for 1 hour and sectioned at 5 µm. Sections were mounted onto subbed slides, hydrolysed in 5N HCL at room temperature for 45 minutes and stained in Schiff's solution for 1 hour. Sections were not counterstained, because counterstaining tended to mask the results.

5:2:4 Analysis

Quail cells were easily distinguished from chick cells after staining with the Feulgen reaction. The chick nuclei were stained homogeneously with a few lightly stained Feulgen positive spots. The quail nuclei in contrast, usually have a clear nucleoplasm

with one or two large heavily stained clumps of Feulgen positive material; sometimes three or four clumps were seen. Quail cells, being more positively identifiable, were easily discernible in a chick background; however, in the reciprocal situation, it was very difficult to distinguish chick cells in a quail background. In addition in control sections, some of the myogenic cells could have been taken for chick cells.

In order to estimate the size of error introduced by misidentification of this sort, a double blind test was set up, in which two persons who were unfamiliar with the appearance of chick or quail myogenic cells were asked to count the number of 'chick' cells they saw within a given quail control section. The area of the field of observation was 0.09 mm^2, with the myogenic field chosen at random. The observers were asked to count the number of suspected chick cells and then the total number of cells within the field. The results, summarized in bar charts 1 and 2, showed that up to 11% of the quail myogenic cells could be mistaken as chick.

To check that the observers were not biased towards an average count, sections containing chick grafts were added intermittently, when the number of chick cells counted would be expected to increase providing no such bias occurred. Both observers did show this increase, so bias towards an average count was ruled out.

From the results, it was possible to

quantitatively determine whether a given quail myogenic region contained chick cells or not, by applying the rule that a suspected chick containing region must have a count greater that 11% to be accepted as a positive result.

5:3 RESULTS

5:3:1 Normal chick wing mesenchyme transplanted into quail wing bud

14 chick into quail transplantations were performed. The surgical intervention did not affect the gross appearance of the resulting limbs.

5:3:2 Cartilage derivatives

Chick chondrocytes were found at the graft sites of all 14 operated limbs, 13 of which were located at the level of the humerus and the remaining 1 located at the posterior tip of the ulna. These chick chondrocytes were integrated into the quail host cartilage overall, but there was always a clear demarcation between them (fig. 2c).

3 of the limbs exhibited chondrogenic pattern regulation, in which the profile of the quail humerus (fig. 2a), into which chick grafts had been incorporated, had a normal appearance. The donors of these embryos were of stage 21 - 22 H.H. and the hosts 22 H.H. In the remaining specimens, chondrogenic development was ectopic and, as described in chapters 2

and 3, donor chondrocytes were never found outside the graft sites.

5:3:3 Muscle derivatives

Chimaeric myogenic regions, consisting of donor chick myocytes and host quail myocytes were found at the graft sites of all 14 operated quail wing buds. Chick myocytes were usually the predominant inhabitants of these regions and frequently these regions were made up purely of chick cells. In contrast to the relative immobility of the chondrocytes, chick myogenic cells were found to be very invasive and could be found at some distance from the graft site. 10 out of the 14 operated limbs exhibited extensive myogenic cell movement, as summarized in table 1.

Translocation was always in a distalward direction towards the apex and only in 2 cases was some backwards movement detected, and then only of the order of 200-300 μm. Chick myogenic cells could migrate from as far back as the level of the humerus, to become eventually located within the muscle regions of the metacarpals, a distance of some 2,100 μm (fig. 2).

There was no correlation between the age of the donor and the extent to which myogenic cells could translocate because myogenic cells from stage 26 H.H. embryos migrated equally as well as those from stage 21 H.H. (table 1). In one instance a piece of tendon was attached to a chimaeric myogenic region (fig. 2b),

region 4; no chick cells were found in this structure, supporting the view that muscles and tendons are derived from separate cell lineages (Shellswell & Wolpert 1977). In 4 cases there was no indication of cell movement, but in these cases the grafts were located behind developing cartilage which may have prevented the myogenic cells from advancing distally.

5:3:4 *Talpid^3 wing mesenchyme transplanted into quail wing bud*

11 *talpid^3* into quail transplantations were performed. 2 of these chimaeric limbs had slight gross abnormalities, which was probably the result of graft development rather than the effect of surgical intervention.

5:3:5 *Cartilage derivatives*

Talpid^3 chondrocytes were found at the graft site of all 11 transplants. In 10 cases, the *talpid^3* chondrocytes had fused onto the quail cartilage and, as in normal implants typically, there was a clear demarcation separating the two cell types, where little intermixing occurred. All *talpid^3* chondrogenic development was ectopic (fig. 3b), with no cases of pattern regulation. The chondrocytes did not migrate and remained exclusively at the graft site.

5:3:6 Muscle derivatives

At the graft site, muscle regions comprising *talpid*^{3} and quail myogenic cells were frequently found. The abnormalities associated with *talpid*^{3} cells did not affect the ability of the *talpid*^{3} myoblasts to fuse with quail myoblasts during the formation of myotubes (fig. 3c). Muscle pattern regulation, which frequently took place in normal transplants, never occurred; on the contrary, all *talpid*^{3} muscle patterns were abnormal.

Only 2 out of the 11 operated limbs showed moderate levels of myogenic cell movement (table 2), in neither case as extensive as those found in normal transplants. In these cases, the identification of myogenic regions as chimaeric was not very clear, so that a cell count had to be performed to verify that *talpid*^{3} cells were present. Figure 3 shows one of the limbs suspected of exhibiting a moderate level of myogenic cell movement. A cell count was performed on all the distal myogenic regions in this chimaeric limb; myogenic region 1 (fig. 3d) had a count of 13.8% and region 2 (fig. 3e) a count of 12.8%. These percentages were significant to warrant a positive identification, since the double blind test suggests that maximum error of misidentification is around 11%. More distal muscle regions did not show any signs of containing *talpid*^{3} myocytes (fig. 3f).

The remaining 9 specimens exhibited no myogenic

cell movement. The *talpid*^{3} myocytes appeared simply to grow and differentiate at the graft site, doubling the size of the implant. However, it must be stressed that 'no movement' should not be taken literally since *talpid*^{3} cells at these regions were often mixed with quail cells, indicating that limited local movements should not be discounted.

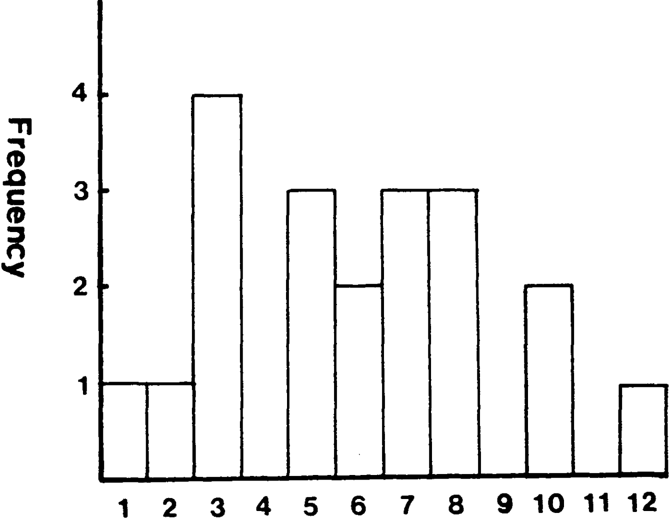
5:3:7 Histology of normal and *talpid*^{3} 9-day limbs

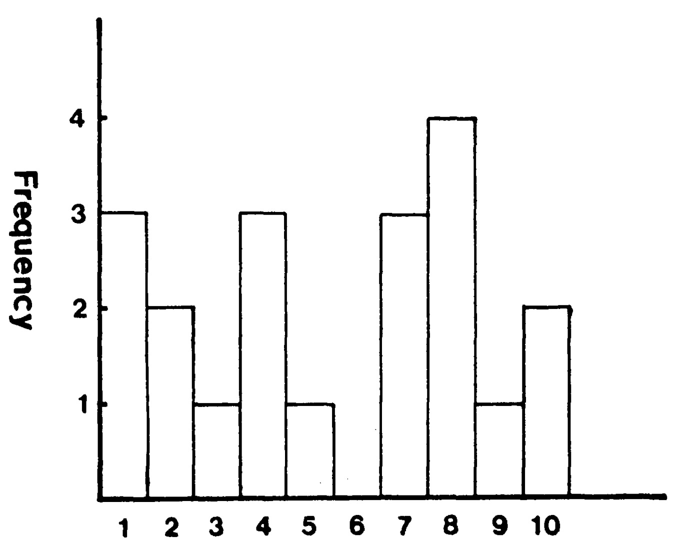
Compared with normal 9-day limbs, *talpid*^{3} limbs are severely shortened proximo-distally, but antero-posteriorly, *talpid*^{3} limbs are such broader (compare fig. 4 with fig. 5). Muscles are found in the autopod and zeugopod of *talpid*^{3} limbs, but whereas muscles in normal limbs exist as separate entities, at this stage of development (fig. 4), a majority of *talpid*^{3} muscles are present as short broad bands (fig. 5). The broad-banded appearance of *talpid*^{3} muscles is primarily due to their inability to split in the correct chronological order: while 3 muscle anlagen are present in the stylopod of normal stage 25-26 H.H. limb buds, only 2 are correspondingly found in the mutant (fig. 6).

All myogenic cells within *talpid*^{3} muscle anlagen appear randomly orientated (fig. 5c, d) and even more so in the distal regions of the mutant's limb (fig. 5b). Normal myogenic cells, on the other hand, are aligned parallel to the proximo-distal axis (fig. 4b, c). Differentiation of *talpid*^{3} myogenic cells in the

metacarpal regions are developmentally retarded, with the distinct absence of myotubules (fig. 5b). On the contrary, myotubules are well formed in the metacarpal regions of normal limbs (fig. 4b). The development of _talpid_[3] chondrocytes also appear to be retarded; furthermore, within the cartilage, these cells are randomly orientated (fig. 5e) instead of aligning normally in neat columns (fig. 4d).

Histograms showing the frequency and percentage of myogenic cells one could mistake as chick cells in a quail control section.


Frequency
4
3
2
1
1 2 3 4 5 6 7 8 9 10 11 12
Percentage of suspected chick cells (%).


Frequency
4
3
2
1
1 2 3 4 5 6 7 8 9 10
Percentage of suspected chick cells (%).

CHICK INTO QUAIL

Code	Stages of donors	hosts	Distalward movement	Backward movement	Graft sites
1C	22	22	824 μm	no movement	humerus
2C	26	25	1,235 μm	no movement	radius
3C	24	22	944 μm	no movement	humerus
4C	20	22	2,188 μm	no movement	humerus
5C	24	20	1,882 μm	200-300 μm	humerus
6C	24	22	no movement	no movement	humerus
7C	22	21	1,177 μm	no movement	humerus
8C	24	22	3,117 μm	no movement	humerus
9C	21	22	2,100 μm	no movement	humerus
10C	22	22	1,647 μm	no movement	humerus
11C	22	22	no movement	no movement	humerus
12C	22	22	2,178 μm	no movement	humerus
13C	22	22	no movement	no movement	humerus
14C	22	22	no movement	200-300 μm	humerus

(Table 1)

TALPID^3 INTO QUAIL

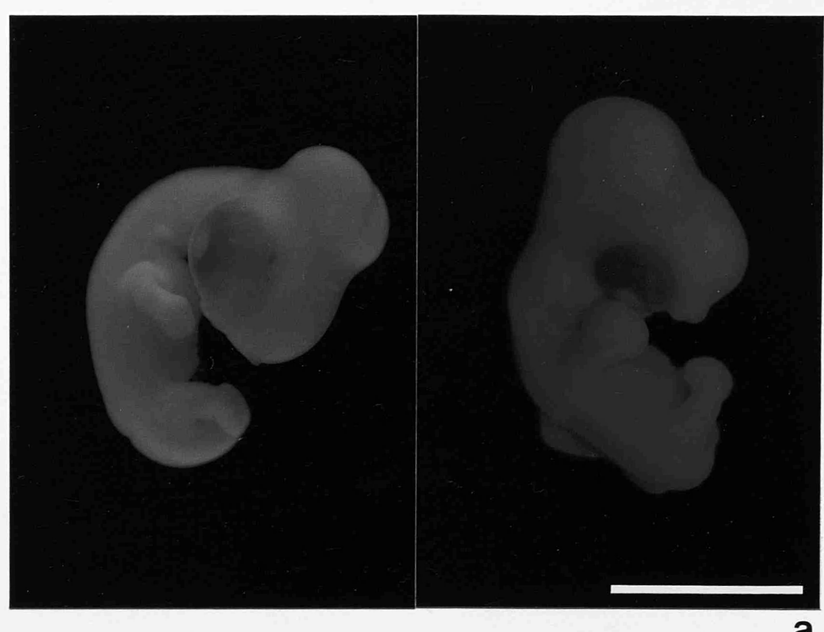
Code	Stages (H.H) donors	hosts	Distalward movement	Backaward movement	Graft sites
1T	26	22	no movement	no movement	humerus
2T	20	22	no movement	no movement	ulna
3T	20	22	706 µm	no movement	humerus
4T	20	22	no movement	no movement	humerus
5T	22	20	no movement	no movement	humerus
6T	26	25	no movement	no movement	radius
7T	24	22	no movement	no movement	humerus
8T	20	21	no movement	no movement	ulna
9T	20	21	no movement	no movement	humerus
10T	20	21	no movement	no movement	humerus
11T	22	22	617 µm	no movement	humerus

(Table 2)

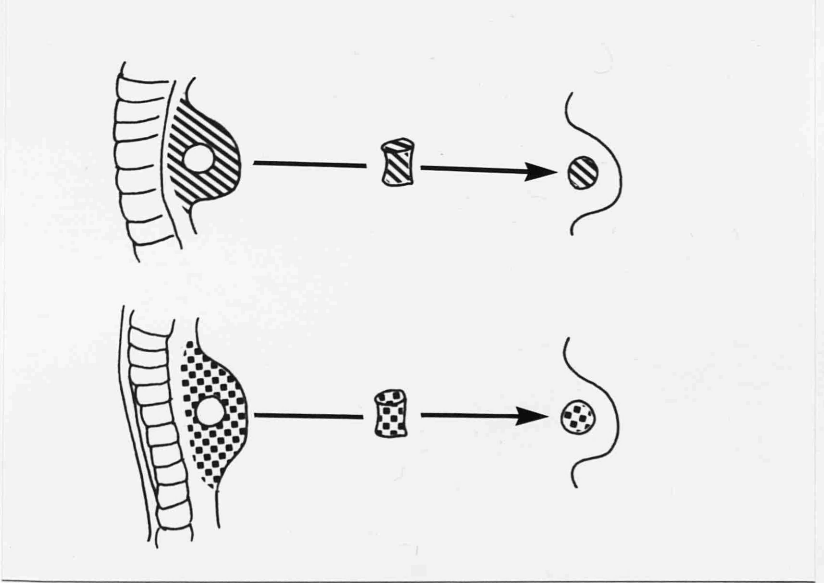
Figure 1

a Stage 28 H.H. normal (left) and *talpid*^{3} (right) chick embryos. Bar = 5 mm.

b Method of grafting fragments from normal (hatched) and *talpid*^{3} (squares) chick limb buds into quail limb buds *in ovo*.



a



b

Figure 2

Normal chick into quail transplant after 5 days post-incubation.

a General view of the transplant. G is the graft site. 1-7 are labelled muscle regions where chick cells are found. T is a tendon. Bar = 200 µm.

b Tendon (T) contains only quail cells even though the muscle to which it attaches is chimaeric. Bar = 10 µm.

c Chick chondrocytes found at the graft site. Note that they remain segregated from quail chondrocytes (host).

d Muscle labelled 6, mixed chick and quail myogenic cells.

e Muscle labelled 7, mixed chick and quail myogenic cells.

f Muscle labelled 4, similarly contains chick cells mixed with quail cells.

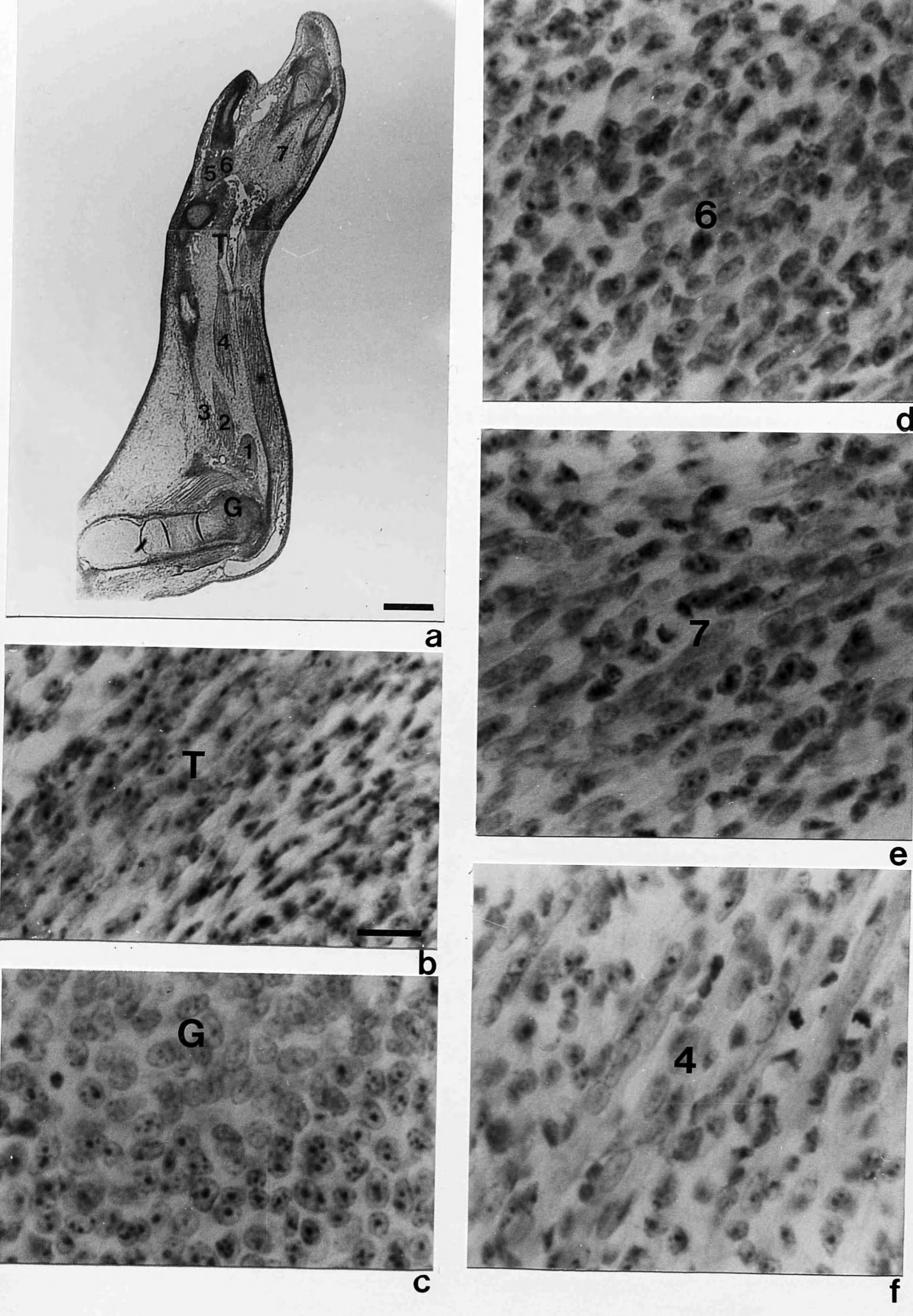

5
6
7
T
4
3
2
1
G
a
T
b
G
c
6
d
7
e
4
f

Figure 3

Talpid^3 into quail transplant after 5 days post incubation.

a General view of the transplant. G is the graft site. Distal myogenic regions are labelled 1-5. Bar = 200 µm.

b Talpid^3 graft site containing nodules of talpid^3 cartilage (tc). Bar = 100 µm.

c At the graft site talpid^3 (arrows) and quail myogenic cells can fuse together to form myotubes. Bar = 10 µm.

d Muscle labelled 1, suspected of containing talpid^3 cells (arrows). Bar = 10 µm.

e Muscle labelled 2, suspected of containing talpid^3 cells (arrows).

f Muscle labelled 5, contains only quail host cells.

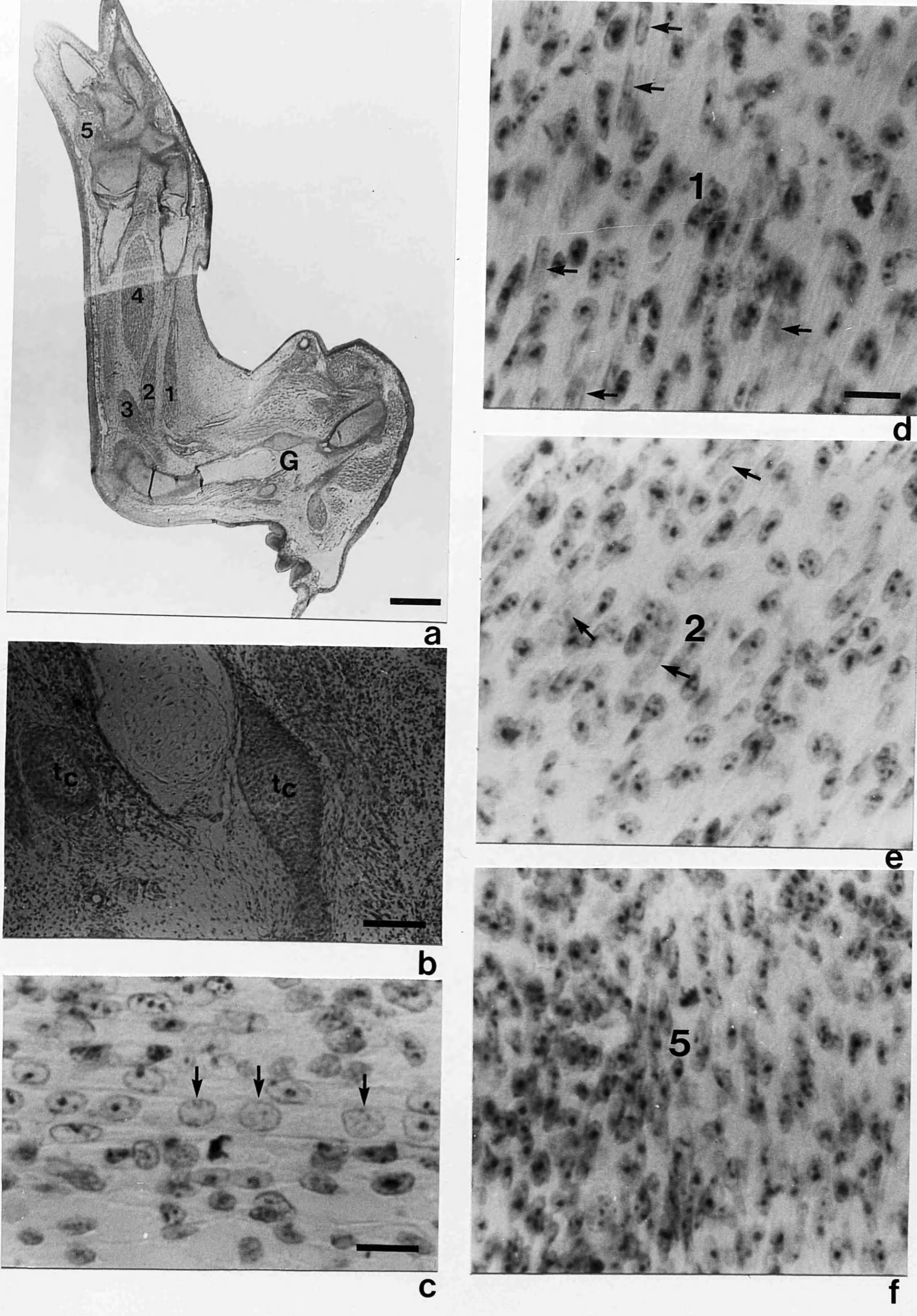

5
4
2
1
3
G
a
tc
tc
b
c
1
d
2
e
5
f

Figure 4

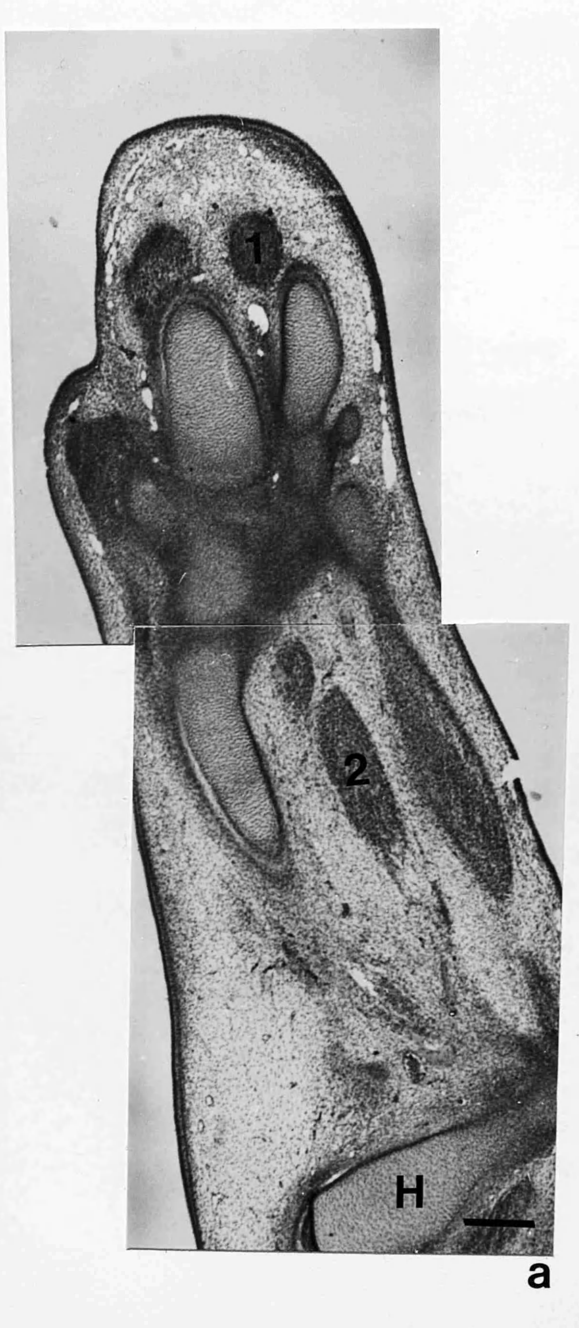
Longitudinal section of a normal 9-day unoperated chick wing.

a General view of the wing. 1-2 are muscle regions. H is the humerus. Bar = 100 µm.

b Muscle region labelled 1. Note myogenic cells are aligned along the proximo-distal axis. Bar = 50 µm.

c Muscle region labelled 2. Bar = 50 µm.

d Chondrocytes are orientated at right angles to the humerus' main axis.


1
2
H
a

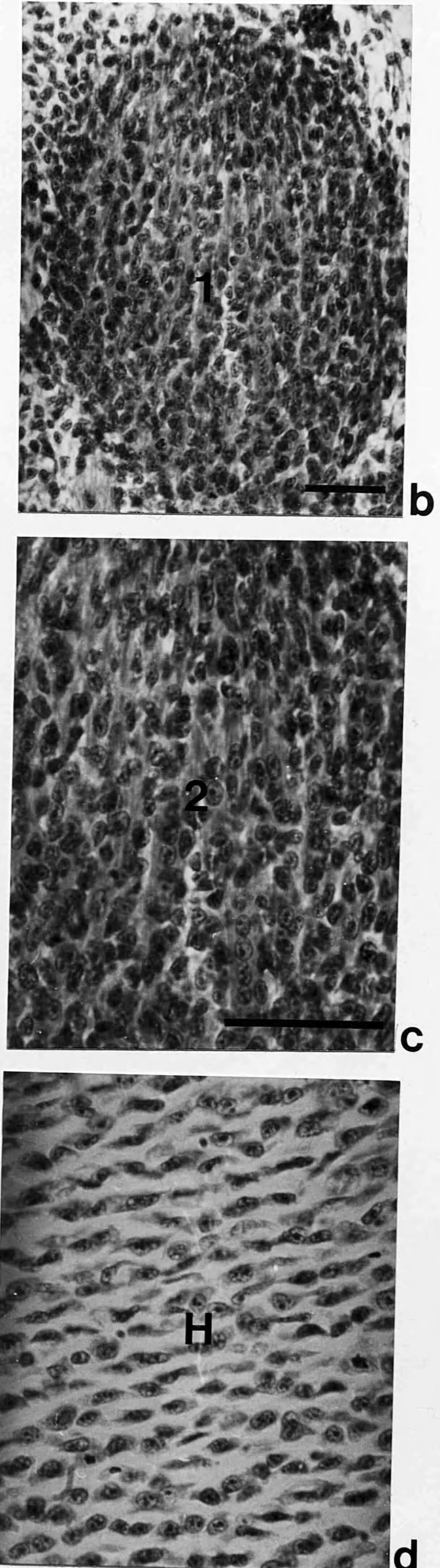

1
b
2
c
H
d

Figure 5

Longitudinal section of a *talpid*^{3} 9-day unoperated wing.

a   General view. 1-3 are different muscle regions in the limb. H is the humerus. Bar = 100 µm.

b-d   *Talpid*^{3} muscles labelled 1-3 respectively. Note myogenic cells are disorientated. Bar = 50 µm.

e   Chondrocytes in *talpid*^{3} humerus are round and randomly orientated.

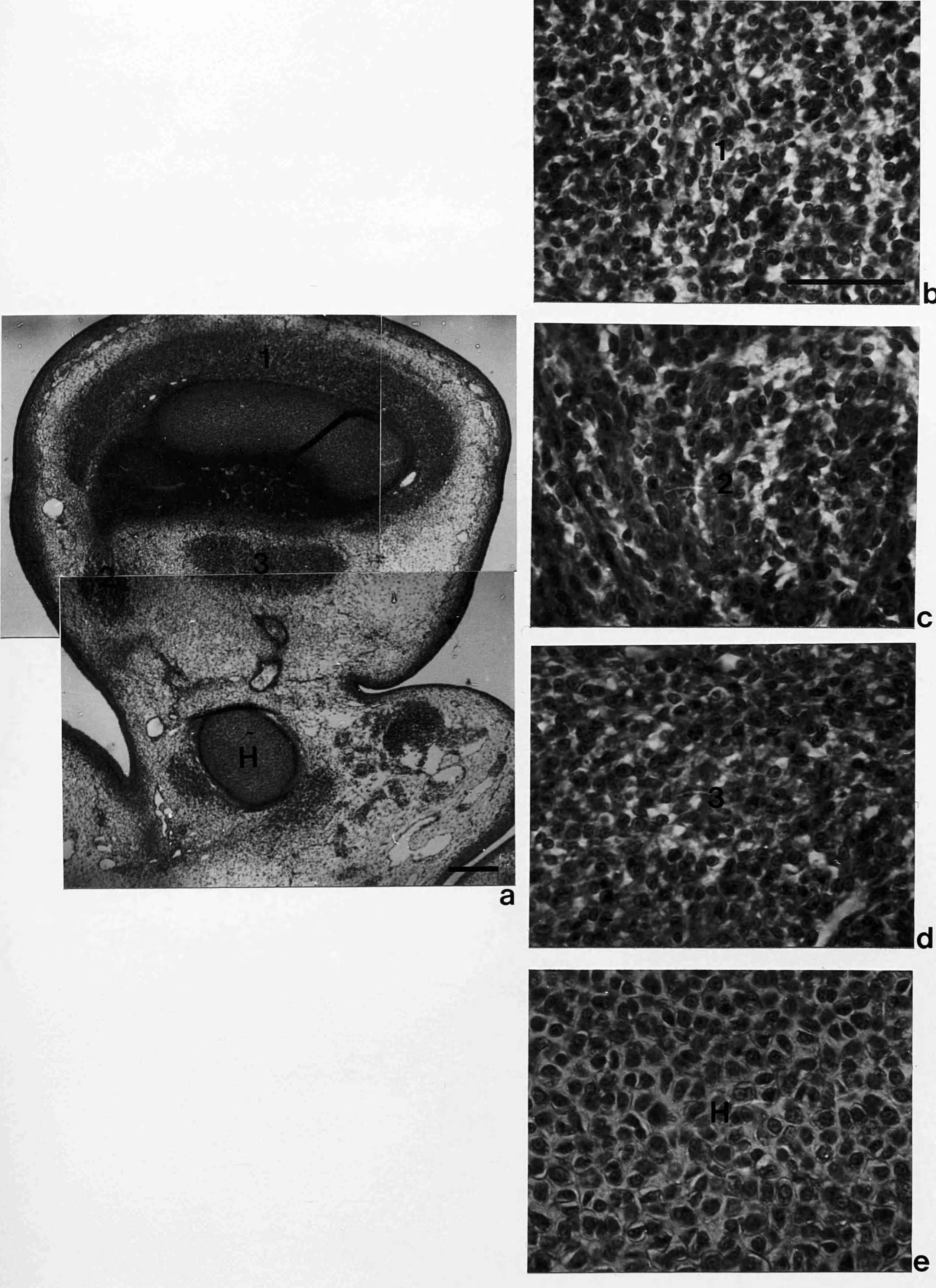

1
3
H
a
1
b
2
c
3
d
H
e

Figure 6

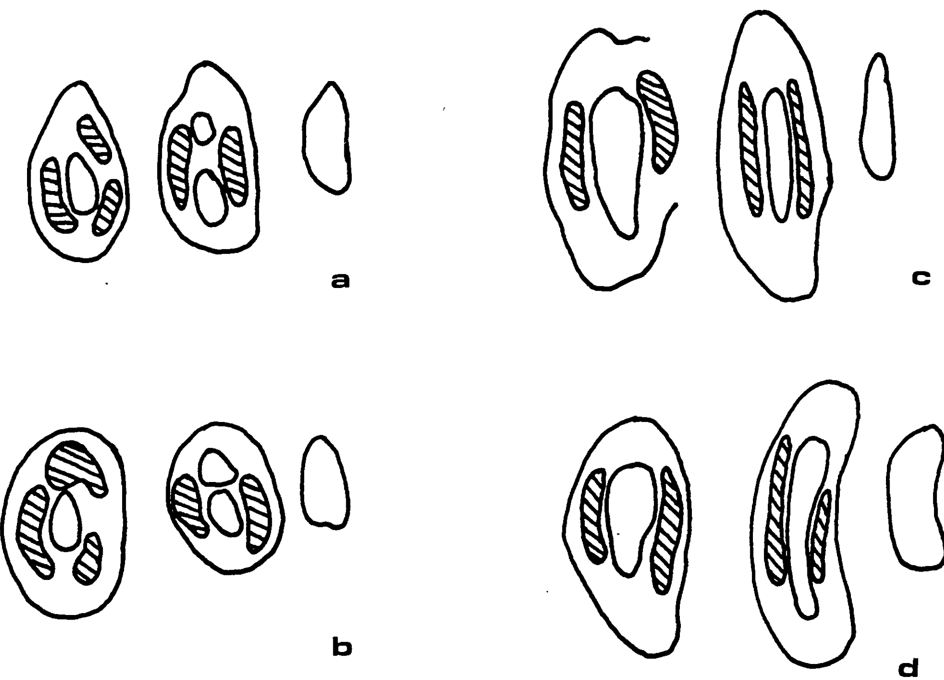
Camera lucida drawings from transverse serial sections of normal and talpid^3 limb buds; displaying muscle splitting between stages 25-26 H.H. Hatched areas represent myogenic regions.

a Normal stage 25 H.H. wing bud.

b Normal stage 26 H.H. wing bud.

c Talpid^3 stage 25 H.H. wing bud.

d Talpid^3 stage 26 H.H. wing bud.



a
b
c
d

5:4 DISCUSSION

The results have clearly demonstrated a difference between the capacity of normal and talpid^3 myogenic cells to migrate within avian limb buds. Normal chick myogenic cells are usually evident in the zeugopod and autopod of quail hosts after 5 days of implantation, while talpid^3 myogenic cells are predominantly restricted to the graft site, with only 2 suspected cases of limited movement. Nevertheless, limited local movement in talpid^3 transplants should not be discounted, since talpid^3 graft sites are usually twice the normal size, within which chimaeric myogenic regions are always present. The existence of these chimaeric regions must imply at least some degree of movement; moreover, the fact that muscles are present within unoperated talpid^3 limb buds dispells any notion of a total lack of mobility, because somite-somatopleural migration is a prerequisite of limb myogenesis. Furthermore, the appearance of muscles at all levels in talpid^3 9-day limbs suggest that talpid^3 myogenic cells are capable of migrating inside talpid^3 limb buds. The distance traversed by these cells, however, is comparatively short because adult talpid^3 limbs are considerably shorter than normal adult limbs.

It must be stressed that limb myogenesis involves 2 phases of movement, the first being the migration of myogenic precursors from the somites to the limb's

somatopleural mesenchyme (Christ et al, 1977) and in the second, the translocation of myogenic cells within the limb bud (Gumpel-Pinot et al, 1984). The environments through which these myogenic cells have to migrate, during these different phases are radically different. In the first phase, myogenic cells are translocating on an aligned meshwork of extracellular fibres, moving as individuals, unopposed by other cells (Jacob et al, 1978, 1979). On the contrary, during the second phase, myogenic cells are moving within a pool of mesenchymal cells with which they will have to interact.

The dilemma of explaining the presence of muscles within talpid[3] limbs, in spite of our results showing these cells to have a reduced capacity for movement, is made easier if one regards myogenic cell migration in terms of its environment. It is conceivable that the ability of talpid[3] myogenic cells to locomote within an extracellular environment, unopposed by other cells, is perhaps equivalent to its normal counterpart (for further discussion see Chapter VI), hence explaining the source of myogenic cells in the talpid[3] limb bud; however, once these cells get inside the limb, they will have to interact with the somatopleural mesenchymal cells, perhaps it is through this interaction that cell movement is restricted in talpid[3] limbs. This conjecture is made on the basis that one of the most common features of the talpid[3] embryo, as revealed in histological sections, is the tendency of mesenchymal

cells to stick together. This is especially evident during myotome formation and sclerotomal cell dispersion (Ede & El-Gadi, 1986). Ede & Flint (1975a) have shown *in vitro* that *talpid*^{3} mesenchymal cells are much more adhesive to one another than normal cells; furthermore, *talpid*^{3} cells have also many more filopodial processes on their surfaces. It is possible that larger numbers of filopodia in the mutant present more potential sites for adhesion between themselves and - in the case of *talpid*^{3} into quail transplantations - quail cells. *Talpid*^{3} myogenic cell movement in quail limb bud is probably hampered by increased cell-to-cell interaction thereby allowing competing quail cells to fill up all vacant myogenic sites.

Ede & Law (1969) pointed out that a computer model of limb development based solely on cell division, even in the form of a gradient, cannot create a simulated limb with sufficient elongation without the introduction of a cell movement component: if the moving component is left out, a simulation resembling that of a *talpid*^{3} limb will be synthesized. All the results presented in this thesis, so far, are in agreement with these computer models - that movement is involved in normal limb morphogenesis and a lack of it with abnormal development. Ede & Law (1969) left out one essential point, i.e. specifying what cell type is involved in movement; they assumed that all mesenchymal cells were capable of this activity. My results have

shown otherwise: locomotion is an exclusive property of myogenic cells. It is possible that *talpid*^{3} myogenic cell's reduced capacity for movement is one of the primary factors responsible for the shortening and fan-shape of the *talpid*^{3} limb bud.

5:4:1 Are *talpid*^{3} cells more adhesive?

There is some controversy over whether *talpid*^{3} cells are in fact more adhesive than normal. According to Moscona & Moscona (1952) and Steinberg (1970), when cells from different tissue types are dissociated, mixed in suspension and then reaggregated together, the most adhesive cell type will be found in the interior of the aggregate while the least adhesive at the periphery. If the adhesiveness between two different cell types is equivalent then cells within the aggregate will not sort out and remain thoroughly mixed. Working in accordance with this principle, Niederman & Armstrong (1972) mixed a suspension of *talpid*^{2} limb mesenchymal cells with normal limb mesenchymal cells and found these cells to remain evenly distributed in aggregates. This led them to conclude that normal and *talpid*^{2} limb mesenchymal cells are equally adhesive and that it is highly likely the same will also apply to *talpid*^{3} cells. However, the amount of evidence suggesting *talpid*^{3} cells to be more adhesive is extensive and very compelling. For instance, Shamslahidjani (1980) repeated Niederman & Armstrong's experiment with *talpid*^{3} material and was

able to show segregation: within the aggregate, talpid[3] limb mesenchymal cells were arranged as clumps in the centre surrounded by normal limb mesenchymal cells, which is consistent with Steinberg's hypothesis that the most adhesive cell type will be found in the centre of aggregates. Earlier evidence came from studies on the sizes and shapes of aggregates from normal and talpid[3] cell suspensions (Ede & Agerbak, 1968; Ede & Flint, 1975a) - normal aggregates were found to be large and irregular in shape, while talpid[3] aggregates were small and round (Ede & Agerbak, 1968) and this was interpreted in terms of tighter adhesion of talpid[3] cells to each other. Electron micrographs of talpid[3] aggregates revealed a tendency for cells in these aggregates to wrap closely around each other, to form tight spheres. Normal cells on the other hand adhere to one another very loosely (Ede & Flint, 1975a), forming loose lumpy aggregates which quickly joined with other aggregates to form the larger aggregates observed.

In order to establish whether or not abnormal cell movement is the common factor responsible for the wide spectrum of pleiotropic anomalies seen in the talpid[3] mutant, and also to make doubly sure that a lack of cell movement is the reason why talpid[3] cells are not found in the distal myogenic regions of quail buds in talpid[3] transplants, one must find out whether this deficiency affects other talpid[3] organs. Talpid[3] chondrogenesis and somitogenesis have been

studied as described below.

5:4:2 Somitogenesis

Relative changes in the adhesiveness of cells have often been suggested as playing a role in embryonic differentiation (Ede & Agerbak, 1968; Bellairs & Portch, 1977; Bellairs *et al*, 1978). Significant changes in cellular adhesion have been found between segmented and unsegmented chick somitic mesenchyme (Bellairs *et al*, 1978). Segmented mesenchymal cells derived from stage 12 embryos were discovered to be significantly more adhesive than cells derived from unsegmented mesenchyme. Accordingly, these workers pointed out that "increases in cell-to-cell adhesiveness of the mesenchyme play a role in the progress of segmentation".

Two major processes prevail during somitogenesis: the dispersal of sclerotomal cells and the formation of the myotome. Sclerotomal cells can apparently migrate ventrally and medially away from the somites (Flint, 1977), in a process that involves active cell locomotion (Chernoff & Lash, 1981). Ede & El-Gadi (1986) reported that *talpid*^{3} sclerotomal cells are reluctant to move and they tend to stick together, so that these cells appear as clumps rather than being evenly dispersed. This 'reluctance' to move is probably a manifestation of *talpid*^{3} cells' inability to move away from each other, because of the increased cell-to-cell adhesion. Similar findings have also been reported in mouse *amputated* mutants (Ede & Flint, 1978), and as with *talpid*^{3},

amputated sclerotomal cells also tend to clump together.

During the formation of normal myotome, cells from the dorso-ventral tip migrate along the ventral surface of the dermatome to establish the myotome layer (Ede & El-Gadi, 1986). In the talpid[3] mutant, myotome formation is drastically transformed; the medial portion of the myotome is bulged out and clumped together, giving a 'multilayer' appearance (personal observation), myotomal cells are disorientated and mingle with the sclerotome, and the myocoele is not obliterated (Ede & El-Gadi, 1986). Moreover, the muscles which develop from the myotome are randomly aligned (Ede & Kelly, 1964b). Again this might be another example of abnormal cell adhesion and movement affecting development.

5:4:3 Chondrogenesis

Chondrogenic condensation is one of the first features that one notices when a limb bud starts to differentiate (Fell, 1925). The cell density apparently increases in these condensations (Thorogood & Hinchliffe, 1975) but it is not accompanied by cell division (Ede et al, 1975). Consequently, Ede (1982) saw the condensation process as being analgous to aggregating slime moulds, where prechondrogenic cells become attracted to move towards a central focus. Ede & Wilby (1981) established, through studies on the orientation of Golgi apparatus in prechondrogenic cells, that 2 different phases of active movement are involved

in the formation of normal chondrogenic condensations; with cells moving towards the condensation centre during the first phase and some form of "cell sorting" where non-chondrogenic cells withdraw from the centre in the second.

The most striking defect in the _talpid_[3] limb is the presence of fused skeletal elements. _Talpid_[3] chondrocytes have the ability to condense in a 'normal' fashion, but somehow, the condensations themselves fail to segregate, so that in the case of the limb, the ulna and radius are fused together as a single plate. Golgi orientation studies on _talpid_[3] condensations have revealed that the movements of _talpid_[3] cells towards and away from the chondrogenic centre are defective (Ede & Wilby, 1981; Ede, 1982); conceivably, this might be the reason why _talpid_[3] cartilages are fused together and chondrocytes within these structures failed to align. This association between cell orientation and movement is also evident in the muscles of developing _talpid_[3] limbs. It seems most likely that the inability of _talpid_[3] myocytes to align in the limb is a manifestation of defective myogenic cell movement.

CHAPTER VI

LOCOMOTION OF NORMAL AND TALPID^3 LIMB MESENCHYMAL CELLS CULTURED IN COLLAGEN LATTICES

6:1 INTRODUCTION

Myogenesis can be regarded as a developmental act (for discussion see Ede & El-Gadi, 1986), comprising of a sequence of subacts in which each transitional phase of development is revealed. Hitherto, studies on the movement of myogenic cells into and through the limb bud have relied on analysis of specimens fixed at intervals after implantation of marked cells. This technique does not reveal continuous changes in e.g. morphology and direction and rate of movement in particular groups of myoblasts. The dynamic nature of myogenic cell locomotion can only be investigated by observation on living cells, maintained in conditions where the movement can be recorded.

Monitoring myogenic movement directly in the avian limb bud is out of the question since the tissues are not sufficiently translucent, although it is possible to do so in the teleost fish fin (Wood & Thorogood, 1984). One way of overcoming these problems is to analyse cell locomotion *in vitro* following explantation. A piece of tissue from the region of interest is excised, and transferred into a plastic culture dish with culture medium. In a matter of hours,

cells will start migrating out of the explant and may easily be observed and recorded using time-lapse photography. This method has already been used to examine the migratory characteristics and factors influencing the spreading and migration of avian limb mesenchymal cells (Ede & Flint, 1975b; Bell & Ede, 1978) and mouse forelimb mesenchymal cells (Kwasigroch & Kochhar, 1975). These studies have yielded information about the mode of cell locomotion _in vitro_ and it is possible by a process of extrapolation, to gain some insight into how cells move _in vivo_.

A major difference, however, exists between cells migrating _in vivo_ and _in vitro_: the substratum. The homogeneous planar substratum provided by tissue culture dishes provides highly artificial conditions which do not exist within the embryo. _In vivo_, cells are moving in a three-dimensional space, constantly in contact with like and unlike cell types, within a loose connective tissue space which is filled with amorphous extracellular material. The drawbacks of using conventional cultures are nicely summed in a passage taken out of Elsdale & Bard's (1972) paper -

> "Snatched from a life of obscurity and installed in contemporary glass and plastic palaces, cells are in danger of becoming Pygmalion's proteges. Housed in more traditional residences constructed of water and collagen instead of plastic or glass, do cells lead primitive, less cultured

lives?"

Since a plastic substratum is neither spacially nor biochemically physiological, Elsdale & Bard tried to compensate for this by culturing cells on or within collagen lattices. The lattice provides a three-dimensional substratum for cells to migrate on; moreover, collagen is one of the main proteins found in vertebrate embryos, so to a far greater extent the system is more like the embryonic situation.

Culturing on collagen lattices has now been applied in many different systems; neutrophil leucocytes (Lackie, 1982); neurites (Ebendal, 1982); fibroblasts (Schor, 1980); chondrocytes (Zanetti & Solursh, 1986); neural crest cells (Tucker & Erickson, 1984); cardiac myocytes (Bernank & Markwald, 1982; Borg et al, 1983, 1984) and sertoli cells (Tung & Fritz, 1986). This present investigation is in many respects a repetition of Ede & Flint's (1975b) work, but instead of culturing normal and talpid[3] limb explants in plastic culture dishes, the explants are embedded within collagen lattices. The differences in the shape of cells cultured on these two types of substrata are well known: e.g. fibroblasts cultured on a plastic substratum are very flat and move by ruffling lamellipodia, on the other hand fibroblasts on collagen lattices are bipolar shaped and move by extending pseudopodia and filopodia (Bard & Hay, 1975; Tomasek et al, 1982a; Bard & Elsdale, 1986). Ede & Flint's work requires revision in this

respect, since cell shape is an obvious determinant of cell locomotion which in turn is determined by the type of substratum. The objectives of the experiments in this chapter are as follows:-

(1) To determine the mode of normal and _talpid_[3] limb mesenchyme locomotion in a three-dimensional substratum.
(2) To verify whether _talpid_[3] cells have a reduced capacity for movement in these conditions.
(3) To determine whether _talpid_[3] cells can be contact guided, since one of the main defects in the mutant embryo is the failure of its cells to align in a normal fashion.
(4) To ascertain whether there are any surface features peculiar to _talpid_[3] mesenchymal cells.

The protocol of the experiment is relatively simple: a core of wing mesenchyme is extracted from a normal or a _talpid_[3] embryo, this is then embedded in a meshwork of rat-tail collagen and placed inside a culture dish. Next, culture medium is added to maintain the explant and also to prevent the lattice from drying up. In addition, sometimes 2 explants are placed within the same lattice. This will usually create the classical "2 centre effect", first described by Weiss (1934): if 2 explants are positioned at some distance from one another, orientated fibres are generated by

cell traction which connect the closest points of contact between the 2 tissues. This will provide an opportunity to determine whether or not normal and talpid[3] mesenchymal cells can become contact guided when they encounter these aligned fibres.

6:2 MATERIALS AND METHODS

6:2:1 Preparation of explants

Normal and talpid[3] embryos used in this series of experiments, were staged between 21-23 H.H. With an emporte pièce, cores of wing fragments were obtained from the central proximal portion of the embryonic wing bud. The ectoderm at both ends of the cores was excised with fine tungsten needles. The prepared explants were then left in tyrode solution for approximately 10 min until things were ready for them to be inserted into collagen lattices.

6:2:2 Preparation of collagen solution

A freshly obtained rat tail was sterilised in 70% alcohol for 10 min. and then skinned. Strands of tendons were stripped off the tail and dropped into a sterile beaker. 50 ml of 0.5M acetic acid along with a magnetic stirrer were than placed into the beaker. The mixture was left to extract in the fridge at 4°c for 2 days.

After 2 days the viscous extract was poured into centrifuge tubes and centrifuged at 2,300 r.p.m. for 1

hr., to remove unwanted pieces of blood vessels and muscles. The supernatant was then carefully pipetted into sterilised (u.v. light) visking tubings and dialysed against two batches (1 ltr. each) of 0.1 strength medium 199 (1 x). Dialysis was carried out in the cold under constant agitation from a magnetic stirrer for a period of 48 hr. During the second phase of dialysis, the pH of the bathing medium was readjusted to pH 4 with M/20 HCl in order to prevent the collagen from precipitating. Finally the collagen extract was recentrifuged through a 0.3 μm filter at 2,300 r.p.m. to remove unwanted debris and bacteria. The resultant stock solution could be stored in the fridge for periods of up to 6 months.

0.5M acetic acid = 3.5 ml glacial acetic acid
96.5 ml distilled water

6:2:3 Construction of collagen lattice

The objective was to get the dissolved collagen to reaggregate into native bundles, inside a fluid filled medium (Medium 199 + 10% foetal calf serum). This was achieved by raising the pH of the acidified collagen stock solution to physiological levels. It was at this point of the proceedings that explants were introduced, so that the tissues could become enmeshed inside a three-dimensional collagen lattice.

Solutions required:

(1) Collagen extract.

(2) Medium 199 (10 X).

(3) 10% foetal calf serum.

(4) N/7 NaOH.

(5) Culture medium (Ham + 10% foetal calf serum) (penicillin + streptomycin).

Construction of the collagen lattice was performed inside a sterile beaker which had been inserted into a trough filled with ice. The ice slowed down the rapid reaggregation process of the collagen, allowing time for explants to be inserted before the collagen could gel. 1 ml of acidified collagen solution was added to the sterile beaker along with 0.1 ml of Medium 199 and 0.1 ml of 10% foetal calf serum. NaOH was then added in a drop-wise fashion into the yellow mixture until a stable pink colour change appeared. The pink colour indicated that the pH had reached physiological levels (7.6). A blob of the pink solution was then pipetted on to a 35mm. plastic culture dish (Falcon) and it was at this point that the explant was inserted.

When required, precipitation was speeded up by placing the mixture containing the explant into an incubator at 37°c for 10 min. When aggregation was completed, the hydrated collagen lattice had a soft agar gel appearance and could be inverted without causing any mechanical disturbance. 2ml of culture medium was added to prevent the gel from drying up.

6:2:4 Determination of collagen concentration

The concentration of the collagen extract was determined as the total protein concentration. Procedures devised by Lowry _et al_ (1951) were applied. A test kit containing all the necessary reagents is available from Sigma chemicals and was used in this case.

Procedure

Biuret reagent was reacted with various known concentrations of bovine albumin. Next Folin and Ciocalteu's phenol was added, resulting in a range of blue colour changes. The graded blue mixtures were then read in a spectrophotometer transmitting at 725 µm. This allowed the construction of a standard calibration curve against which the collagen extract, which had also undergone the same reaction, was compared. Collagen concentration was estimated to be 946 µg total protein/ ml.

6:2:5 Scanning electron microscopy

The 60X magnification used in the time lapse photography was not sufficient to show the finer details of migrating mesenchymal cells, so scanning electron microscopy was adopted for analysis of individual cell morphology.

Fixation for S.E.M

The gels were fixed in 2.5% glutaraldehyde (made up in 0.1M cacodylate buffer, pH 7.4) for 4hr at room

temperature. They were then soaked in 3 changes of 0.1M cacodylate buffer for 1 hr to wash away any remaining fixative. Next, 1% osmium tetroxide was added to post-fix the gels at room temperature for 40 min. After this treatment, distilled water was substituted for the fixative and, using very fine tungsten needles, some of the gel directly above the explants was slowly teased away to expose the underlying mesenchyme. Fixation hardens the fragile gel sufficiently to allow the manipulation to be carried out without causing the lattice to collapse.

Processing

Exposed gels were dehydrated through a graded series of alcohol (30%, 50%, 70%, 90%, absolute), 5 min at each stage but 15 min for absolute alcohol. The gels were then quickly transferred into an aluminium boat filled with absolute alcohol and loaded into a critical point dryer. The collagen lattice is very fragile because the whole structure is supported by fluids, therefore every dehydration process must be stringently controlled in order to maintain the structure of the collagen lattice. Inside the critical point dryer, the absolute alcohol was slowly replaced by liquid carbon dioxide; this process took about 45 min. The critical point drying chamber was heated up to above 38°c in order to increase the internal pressure of the chamber to 1400 p.s.i. Under these critical conditions, the liquid carbon dioxide was converted into a gas which was

slowly let out of the chamber to complete the dehydration process.

The dehydrated gels were mounted onto 13 mm aluminium stubs with double sided sellotape. These specimens were then coated with gold/palladium in a Polaron E1500 sputter coater to a thickness of 500 Å. Since the heat generated during the coating process could cause the gels to collapse, the process was carried out under refrigeration. Finally, the prepared specimens were examined using a Phillips scanning electron microscope and photographed on Ilford FP4 film, with a Rollei 120 mm camera.

6:2:6 Preparation of whole limbs for S.E.M

In order to determine whether what occurs *in vitro*, has any bearing on events *in vivo*, wings from normal and *talpid*[3] embryos (stage 22-24 H.H.) were also processed for S.E.M. for a comparable S.E.M. study. The limbs were fixed in 2.5% glutaradehyde for 4 hr., after which they were divided into equal halves along the meridian of the proximo-distal axis. Further processing procedures were the same as those described for collagen cultures.

6:2:7 Filming

At various time intervals, the cultures were removed from the incubator to a hot room regulated at 38°c. Inside the hot room, individual culture dishes

were slotted into customised perspex chambers. Each of the chambers has a gas inlet and a gas outlet through which a mixture of 95% air and 5% Co_2 gas was passed, in order to simulate the gaseous environment of the incubator and to maintain the pH of the cultures. The whole set up was mounted on the stage of a Wild M40 inverted microscope linked to a Bolex cine camera. The explants were filmed between 24-72 hr of incubation with a magnification of 60X, using colour Kodachrome 40 16mm film. The time interval between each frame of film was 60 sec. A heat filter and a blue filter were inserted into the filming apparatus to prevent the cultures from being heat killed and also to enhance the image under photography.

6:2:8 Analysis of cell translocation

Developed 16 mm colour films were analysed frame by frame using a 224-A-MK3 data analyser. Projected images from the projector were deflected off a mirror mounted at 45° beneath a glass top table. Tracing paper was placed directly over the deflected image on the glass and certain parameters of cells in motion were recorded.

Criteria of analysis:-

Selection of cells for analysis: To get an accurate estimation of the speed at which a cell can travel, cells were only selected for analysis if they were moving.

Cells which had made contact with other cells during translocation were disregarded in case phenomena such as cell contact inhibition should distort the analysis. Cells which had undergone mitosis were also not examined.

<u>Cell Parameters</u>: To allow for changes in cell shape during locomotion, 3 different reference points were selected: the leading edge, the trailing edge, and the centre of the cell. One of the major advantage of using collagen lattice, as opposed to cells grown on plastic surfaces, was the ability of the cell to retain its polarlity during translocation, so that one did not have to worry about leading processes suddenly appearing from the sides of the cell body, as is often the case on plastic.

<u>The time interval between each reference point</u>: Reference points were selected on the basis that there would be sufficient distance between them to be measured, but this distance would not be so great that

significant changes would be missed. The optimum time interval between each reference point was found to be 10 min, so that since our films were taken at speeds of 60 sec/frame, points on every 10th frame were recorded.

Individual steps taken by the leading edge, trailing edge and cell centre were recorded onto tracing paper over a period of 110 min for each cell recorded. In each track there were 12 points and 11 steps, since the selected time interval between each point was 10 min. Tracing paper with recorded cell tracks was placed onto a graphic digitizing tablet (Summagraphic Ltd, Bit Pad One) connected to a BBC computer. A scale bar from a single frame of a stage micrometer served to calibrate the digitizer in microns. A programme called LEE written by Dr. D. Neil and with slight modification by myself was employed to measure the distances between each reference point. The software automatically assigned an X,Y coordinate value to each of the reference points.

The values were then loaded into a second programme called RMS-KL3, originally written by Dr. J. Lackie and modified by Dr. M.Huxham and myself to accept and handle the dimensions of my data. The programme was able to calculate the distance for cell displacement,

speed, persistence and diffusion coefficient. Speed (s), persistence (p) and diffusion coefficient (R) are represented by the formula R=2.S.S.P. The programme operates by calculating the root mean square displacement between sequential positions and alternative positions; the reciprocal of root mean square displacement is then plotted against the reciprocal of time. The gradient of this line gives an estimate of speed, while the intercept on the X-axis gives an estimate of persistence (for reviews see; Lackie & burns, 1983; Lackie & Wilkinson, 1984; Wilkinson *et al*, 1984).

6:2:9 Statistical analysis

The data is non parametric because the sample size is small, so the Mann-Whitney U-test (MW U-test) was applied instead of the conventional t-test. Various values from the parameters of normal and *talpid*^{3} cell movement were compared, using the U-test, to determine whether there were any significant differences. The MW U-test operates as follows: values from 2 sets of data are lined up in an ordered fashion, starting from the lowest values and ending with the highest values. C values are then assigned to the number of odd values in overlapping regions. For example, if we have 2 samples A and B (fig. 1), in front of 11 there is one B, so a C value of 1 is given. In front of 12 there are two Bs, so the C value is 2. The sum of the C values will

therefore be 3. The Mann-Whitney statistic U value is the greater of two quantities, C and (N1.N2 - C), in this case 3 and (6 x 7) - 3 = 39 respectively. From the statistical tables, 39 is significantly different, with P > 0.01.

Sample A = 1,4,6,8,10,12; NI = 6

Sample B = 9,10,11,14,16,18,19; N2 = 7

Sample	A	A	A	A	B	A	B	A	B	B	B	B	B
	1	4	6	8	9	10	11	12	12	14	16	18	19
C	0	0	0	0	0	0	1	0	2	0	0	0	0
values										total C = 3			

(fig. 1)

6:3 RESULTS

6:3:1 Morphology of normal mesenchyme in collagen lattice

After 48 hours of incubation, the explants had expanded and many migrating cells were seen at their borders. The activities of these cells were studied, using time lapse photography and scanning electron microscopy. These techniques were employed to complement each other, so that morphology could be related to function.

Cells found near the periphery of the explants

were of 3 basic shapes: (1) an elongated spindle (fig. 2), (2) pear-shaped (fig. 3) and (3) spherical. Each of these shapes represents a transitional phase of cellular activity. The elongated spindle-shaped cell arises as a direct consequence of the leading edge crawling forward while the trailing edge remains lagging behind; accordingly the cell becomes highly stretched and elongated. It is quite easy to distinguish the leading edge from the trailing edge, even in scanning electron micrographs, because the leading edge usually has more than one pseudopodium while the trailing edge has a single 'pseudopodium'.

The pear-shaped cell is so called because of its rounded trailing edge. This shape is a derivative of the elongated spindle cell, a product of the trailing edge retracting into the cell centre, thereby leaving either a remnant of a tail or a rounded back end. The pear-shaped cell can also be a permanent rather than a transitional structure, especially when it is involved in movement which does not require drastic changes in morphology. When cells undergo mitosis or a period of inactivity, their shape is usually spherical (fig. 2a).

It is impossible to tell whether a cell at the periphery of an explant is myogenic or fibroblastic, because in collagen cultures both cell types are bipolar in appearance. This is the reason why I have referred to them as mesenchymal cells rather than assigning them the name of a specific cell type. On the

contrary, it is quite easy to distinguish between the two if cells are grown on a planar glass substratum (fig. 4). Under these conditions, the fibroblasts are typically fan shaped, with ruffling lamellae at their leading edge. The myogenic cells, on the other hand, are very elongated and spindle shaped, with very fine but sometimes flat projections emanating from both ends of its polar axis. Turner _et al_ (1983) used these differences as the criteria for distinguishing myoblasts from fibroblasts. One other important difference between explants grown under these 2 different conditions, is that cells start migrating out of explants grown on glass at least 12 hr. earlier than those grown in collagen lattices.

Numerous bipolar cells are usually found on the surface of explants grown in collagen lattices. These cells can climb over each other and give the general impression of being highly active (fig. 5a). Furthermore, these cells appear very much like the bipolar cells Solursh (1984) described on the surfaces of micromass cultures, which he demonstrated were in fact myoblasts. As well as bipolar cells there are spherical cells, some of which, according to Zanetti & Solursh (1986), could be chondrocytes.

6:3:2 Morphology of talpid^3 mesenchyme in collagen lattices

Time lapse observations of the behaviour of

talpid^3 cells did not reveal any peculiarities distinguishing them from normal cells. This is reflected in observations on the morphology of normal and *talpid*^{3} mesenchyme, with both showing the same 3 basic cell shapes (fig. 6 & 7). Although both genotypes shared the same basic morphologies, there were, nevertheless, several exceptional cases, where *talpid*^{3} mesenchymal cells produce more pseuodopodia from their leading edges than would normally be expected. This is dramatically illustrated in figure 8. Examination of the *talpid*^{3} explant surface did not reveal any differences, and, as with normal explants, a great number of bipolar cells were found interspersed with spherical cells (fig. 5b).

6:3:3 Normal and *talpid*^{3} distal mesenchyme *in vivo*

Contrary to the cylindrical appearance of mesenchymal cells in collagen lattices, *in vivo*, both the normal and *talpid*^{3} distal mesenchymal cells (stage 22-24 H.H.) appear to be very flat and *en masse*, like "heaps of corn flakes" (fig. 9), as by Ede *et al* (1974). Scanning electron micrographs of *talpid*^{3} distal mesenchymal cells give the impression that these cells are sending out more thread-like filipodial processes from their cell surfaces than normal mesenchymal cells in the corresponding regions (fig. 9b). The cell surface of normal mesenchyme appears to be smoother and less interrupted by outgrowth of these

processes (fig. 9a). Filopodia emanating from one cell can be seen making contact with the surfaces of neighbouring cells. A detailed study carried out by Ede et al (1974) suggested that the filopodial processes sprouting from normal distal mesenchyme are very much longer than those from talpid^3 distal mesenchyme, and that normal processes can make contact with cells several diameters away.

The morphology of cells from the distal regions of normal and talpid^3 wing buds in vivo bears no resemblance to that of cells found in the collagen lattice. Apart from the obvious difference in cell shape, the very fine thread like processes are not discernible in collagen cultures. There are 2 possible reasons why this might be the case: (1) Distal mesenchymal cells are predominantly chondrogenic precursors (Newman, 1977) and constitute a population of cells not normally associated with locomotion, hence these cells would not be expected to be found outside the explants from which a majority of the scanning electron micrographs of cells in collagen lattices were taken. (2) It is also possible that in collagen cultures, the fibres hide the filodopia from view.

6:3:4 Normal and talpid^3 proximal mesenchyme in vivo

The general appearance of normal and talpid^3 proximal mesenchymal cells in vivo is clearly dissimilar (fig. 10). The talpid^3 cell surfaces are constantly

interrupted by outgrowth of filopodia, giving these cells a very ragged appearance (fig. 10c & d). On the contrary, the cell surfaces of normal proximal mesenchyme have a smoother outline with fewer interruptions from filopodial outgrowths (fig. 10a & b).

Both normal and talpid[3] proximal mesenchymal cells comprise of 3 different phenotypes: (1) the very flat stellate cells, reminiscent of cells found in the distal regions of the wing bud, (2) the spherical, and (3) bipolar shaped cells (fig. 11) which are reminiscent of cells grown in collagen cultures. However in the case of the bipolar cells, there is one major difference between the cells found in the collagen cultures and those in the proximal portion of the limb, i.e. the presence of the thread like filopodia; generally, bipolar cells in the limb do not produce very many of these processes.

It is difficult to establish with certainty to what extent the fibrous processes are cytoplasmic and what extracellular, but close inspection of the magnified cell surface suggests that most of the processes are cellular rather than extracellular. The only region where the amount of collagen found is similar to that found in the in vitro culture system is at the basement membrane (fig. 12), which is composed of a fine mat of collagen fibrils, covered with globular droplets of extracellular material (fig. 12b & c).

6:3:5 Cell traction and matrix realignment

Cores of mesenchymal cells extracted from the central portions of normal and *talpid*^{3} wing buds were inserted into collagen lattices either as a single explant or double explants (2 explants side by side, separated by a gap of 1 - 3 mm). After 20 hours of incubation, cells started emerging from the borders of these explants. At first, cell migration was restricted to the bottom layer of the explant core and it was only after a further 24 hours that cells started emerging from planes higher up. A combination of this activity and cell growth have the effect of altering the arrangement of collagen fibres around the margins of the explants. The once randomly distributed native fibres are reorganised into parallely aligned bundles which radiate out from the margins where the cellular activities are most intense (fig, 13). These aligned collagen bundles can stretch out several millimetres in front of advancing cells in both normal and *talpid*^{3} explants, and as a consequence, any cells encountering these regions will effectively be migrating on an aligned substratum.

Cells which advanced on to these aligned collagen tracts, readjusted their own alignment so that their cell bodies became polarized along the axis of the fibres (fig. 14). Time-lapse observations showed that cells locomoting on these tracts were restricted to moving forward or backward along the axis of these

fibres, like a train unable to move off its tracks. When a cell reversed direction, the leading edge usually became the trailing edge and vice versa (fig. 15 & 16). These observations suggest the possibility of limb mesenchymal cell migration being subjected to the influence of contact guidance _in vivo_ and if this is the case for normal cells, it will also apply to _talpid_[3] cells (fig. 14).

In situations where a collagen lattice contains two explants, migratory activities are at their most intense between these structures. This is reflected in the fact that aligned collagen bundles are rapidly generated between the two explants; it appears as if the explants are using each other as fixed sites for exerting traction (see fig. 13b & d). Cells migrate actively along these tracts from one explant to another (fig. 14a) so that usually, after 4 days of incubation, the two tissues have coalesced into a single tissue mass. Classically this has been called the "2 centers effect" (Weiss 1934).

6:3:6 _Mode of normal locomotion_

Two modes of cell movement were observed, one in which it was accompanied by deformation in cell shape and a second which involved only a very slight modification in shape. In the first form, the pseudopodia and filopodia of the leading edge crawled forward, while the trailing end remained lagging behind,

adhering to the collagen substratum. The advancement of the leading edge was not restricted by the relative immobility of the trailing edge (Graph 1 between 30 - 50 min and 70 - 90 min). This had the overall effect of stretching out the cell into a very elongated spindle shape and, when the extension reaches its maximum, the tensions created were evidently sufficient to break the adhesion between the trailing edge and the substratum. With the bonds broken, the trailing edge advanced rapidly forward into the cell body until most of the tapered tail disappeared and a pear-shaped cell was formed (fig. 17). A second cycle of this form of movement could be repeated, by the leading edge advancing forward again and the trailing edge extending slightly backward. It took about 2 bursts of this type of activity to cover one maximum cell length.

In the second form of motion, involving only a slight change in cell shape (fig. 15), the advancement of the leading edge was followed by the advancement of the trailing edge, generating a wiggling motion, very much like a Chinese sand pan. In this case, the trailing edge was usually rounded and did not give the impression of being as adhesive as in the first form. This may explain why the actions of the leading and trailing edges are mutually dependent.

Because cells could indulge in both forms of movement during a filming sequence, it was not possible to separate out the 2 forms of motion for analysis and

then still have sufficient footage to make comparison with other cell tracks. For this reason both forms of motions were compounded together as a single track and taken to represent general cell movement.

6:3:7 Rate of normal locomotion

Because cell shape changes during locomotion, selecting a single parameter such as the centre of the cell, as is often the convention, does not reflect the actual dynamics of cell movement. For this reason, the leading edge and the trailing edge, as well as the cell centre, were measured in this experiment in order to give a better representation of the dynamics of cell locomotion.

The normal leading edge usually consisted of one or two pseudopodia and these crawled forward constantly, with an average speed of 0.81 µm/min (table 1). The rapid retraction of the trailing edge did not usually prevent the advancement of the leading edge (Graph 1, 10-30 min and 50-70 min; Graph 2, 0-20 min and 50-70 min). The trailing edge moved with an average speed of 1.5 µm/min (table 1). Both sets of data were statistically analysed using the Mann-Whitney non parametric U test. The speed of advancement of the normal trailing edge was found to be significantly faster ($P > 0.001$, table 3) than the leading edge. This was surprising, because logically this should result in the tail catching up with the head. In fact this did

not happen because the trailing edge had a dormant period of inactivity, which allowed the leading edge to advance further forward to re-establish the original cell shape, prior to another round of tail retraction (Refer to graph 1 and 2, where the areas below the trailing edge line and above the leading edge line correspond with changes in cell shape).

Comparisons of the speed of advance of the cell centre with that of the leading or trailing edge showed no significant difference between cell centre and leading edge but a significant difference ($P<0.01$, table 3) between cell centre and trailing edge. This suggests that the displacement of the cell centre i.e. the overall cell mass, is more related to the advancement of the leading edge than to the trailing edge.

The persistence (directionally) of all 3 parameters is extremely high because most of the cells are moving on aligned collagen fibres; however these data should be interpreted with caution, since the duration of filming was relatively short, so that the cells will not have been given sufficient time to show up any deviations of direction over longer periods.

6:3:8 Mode of *talpid*^{3} locomotion

Talpid^3 cells, like normal cells, showed 2 modes of movement, one where the leading edge moves independently of the trailing edge and a second form where there is dependency, see figures 18 and 19.

6:3:9 Rate of talpid[3] locomotion

The talpid[3] leading edge travelled with an average speed of 0.73 μm/min., the trailing edge at 1.77 μm/min. (table 2). Comparison of these 2 sets of parameters using the Mann-Whitney U test (M.W. U test), showed them to be significantly different ($P<0.001$, table 3) with the trailing edge moving at a much faster rate than the leading edge. This is very much in the same mode as normal locomotion, as can be seen when the distances travelled by the leading and trailing edges are plotted against time (graph 3 & 4). The M.W. U test also shows the movement of the overall cell mass to be mutually dependent on the locomotion of the leading edge (table 3). The persistence for leading edge, trailing edge and cell centre is very high, which is expected, since most of the cells from which the measurements were taken were migrating on aligned collagen fibres. However, the same proviso regarding the short distance followed as for normal cells is applicable.

6:3:10 Comparison of normal and talpid[3] parameters

The speed of movement of the leading edge, trailing edge and cell centre of normal and talpid[3] mesenchymal cells was compared, using the M.W. U test. No significant differences were to be found between the 6 different sets of data (table 3), so it appears that in the circumstances of these observations talpid[3] cells move equally fast. If the films were unlabelled,

it would be impossible to distinguish convincingly between the two genotypes.

TABLE 1

NORMAL LEADING EDGE

Track	M.Steps (μm/10min)	Steps	Displacement (μm)	Speed (μm/min)	Persistence	R
1	8.11	11	78.60	1.03	5825.93	3.41
2	6.49	11	64.29	0.83	1978.43	0.76
3	5.57	11	58.14	0.70	1604.11	0.43
4	4.50	11	49.09	0.66	3555.42	0.86
5	7.01	11	77.88	0.78	7530.35	2.57
6	4.51	11	51.24	0.53	11369.53	1.81
7	5.65	11	61.35	0.74	3404.51	1.05
8	4.56	11	54.00	0.53	-6734.19	-1.03
9	6.81	11	72.80	0.95	2741.04	1.36
10	8.09	11	72.84	1.20	469.97	0.38
11	7.87	11	91.08	1.02	1398.73	0.80
12	6.68	11	78.26	0.79	-36094.61	-12.63

(Average speed = 0.81μm/min)

NORMAL TRAILING EDGE

Track	M.Steps (μm/10min)	Steps	Displacement (μm)	Speed (μm/min)	Persistence	R
1	11.85	11	101.41	1.86	537.08	1.04
2	8.56	11	83.24	1.22	881.73	0.72
3	9.88	11	117.02	1.91	637.78	1.30
4	8.86	11	102.88	1.16	1615.05	1.22
5	9.76	11	80.08	2.15	214.83	0.55
6	5.20	11	47.27	0.88	374.82	0.16
7	7.77	11	84.34	1.81	523.33	0.95
8	5.61	11	50.09	1.09	259.98	0.15
9	9.00	11	78.45	1.41	896.40	0.99
10	6.15	11	70.60	0.82	781.57	0.30
11	11.39	11	118.66	2.59	161.80	0.60
12	7.77	11	91.92	1.10	765.07	0.52

(Average speed = 1.5 μm/min)

NORMAL CELL CENTRE

Track	M.Steps (μm/10min)	Steps	Displacement (μm)	Speed (μm/min)	Persistence	R
1	8.22	11	86.02	1.15	2086.20	1.53
2	7.02	11	72.50	0.87	28688.33	12.09
3	6.39	11	75.24	0.95	1732.65	0.88
4	5.82	11	68.59	0.73	3226.17	0.95
5	7.92	11	85.21	1.26	662.96	0.59
6	3.88	11	43.57	0.53	1037.98	0.16
7	6.41	11	74.04	0.91	2117.98	0.98
8	4.01	11	46.57	0.53	3521.69	0.55
9	6.98	11	69.40	1.07	1480.73	0.94
10	5.70	11	66.13	0.80	890.00	0.32
11	9.84	11	114.86	2.50	205.89	0.71
12	6.36	11	75.43	0.71	6635.72	1.84

(Average speed = 1μm/min)

TABLE 2

TALPID^3 LEADING EDGE

Track	M.Steps (µm/10min)	Steps	Displacement (µm)	Speed (µm/min)	Persistence	R
1	3.99	11	39.41	0.69	533.29	0.14
2	3.24	11	30.41	0.53	1741.39	0.27
3	2.87	11	31.78	0.55	573.07	0.10
4	5.20	11	58.31	0.75	1406.13	0.44
5	4.44	11	51.01	0.54	6472.41	1.04
6	8.29	11	96.01	1.01	13276.43	7.47
7	6.93	11	56.29	0.88	1529.75	0.66
8	6.56	11	76.16	0.77	11800.57	3.84
9	5.50	11	40.20	1.08	212.02	0.14
10	6.27	11	72.78	0.80	1711.63	0.61
11	2.16	11	25.02	0.39	1158.19	0.10

(Average speed = 0.73µm/min)

TALPID^3 TRAILING EDGE

Track	M.Steps (µm/10min)	Steps	Displacement (µm)	Speed (µm/min)	Persistence	R
1	7.08	11	53.64	0.92	6904.19	3.23
2	7.16	11	45.40	1.14	548.74	0.40
3	6.45	11	62.36	1.53	451.38	0.59
4	10.94	11	129.28	2.97	165.23	0.81
5	4.86	11	57.94	0.74	2495.63	0.75
6	9.38	11	86.31	1.21	-7627.88	-6.16
7	11.66	11	88.01	1.81	619.87	1.12
8	7.38	11	73.55	1.37	867.89	0.90
9	12.19	11	83.24	2.35	161.93	0.50
10	5.59	11	51.09	4.12	37.93	0.36
11	7.05	11	83.15	1.35	409.32	0.41

(Average speed = 1.77µm/min)

TALPID^3 CELL CENTRE

Track	M.Steps (µm/10min)	Steps	Displacement (µm)	Speed (µm/min)	Persistence	R
1	5.17	11	51.62	0.87	1178.86	0.50
2	3.17	11	44.72	0.50	3943.05	0.54
3	5.04	11	59.00	0.83	2216.65	0.86
4	7.41	11	83.22	1.21	410.51	0.33
5	5.83	11	67.12	0.77	-10176.27	-3.35
6	7.79	11	90.14	0.97	-2451.21	-1.28
7	7.18	11	63.32	0.96	1787.01	0.92
8	6.94	11	80.62	1.06	2755.82	1.72
9	4.13	11	49.04	0.65	1151.48	0.27
10	4.91	11	57.43	0.96	242.11	0.12
11	5.40	11	63.79	0.68	2135.70	0.55

(Average speed = 0.86µm/min)

TABLE 3

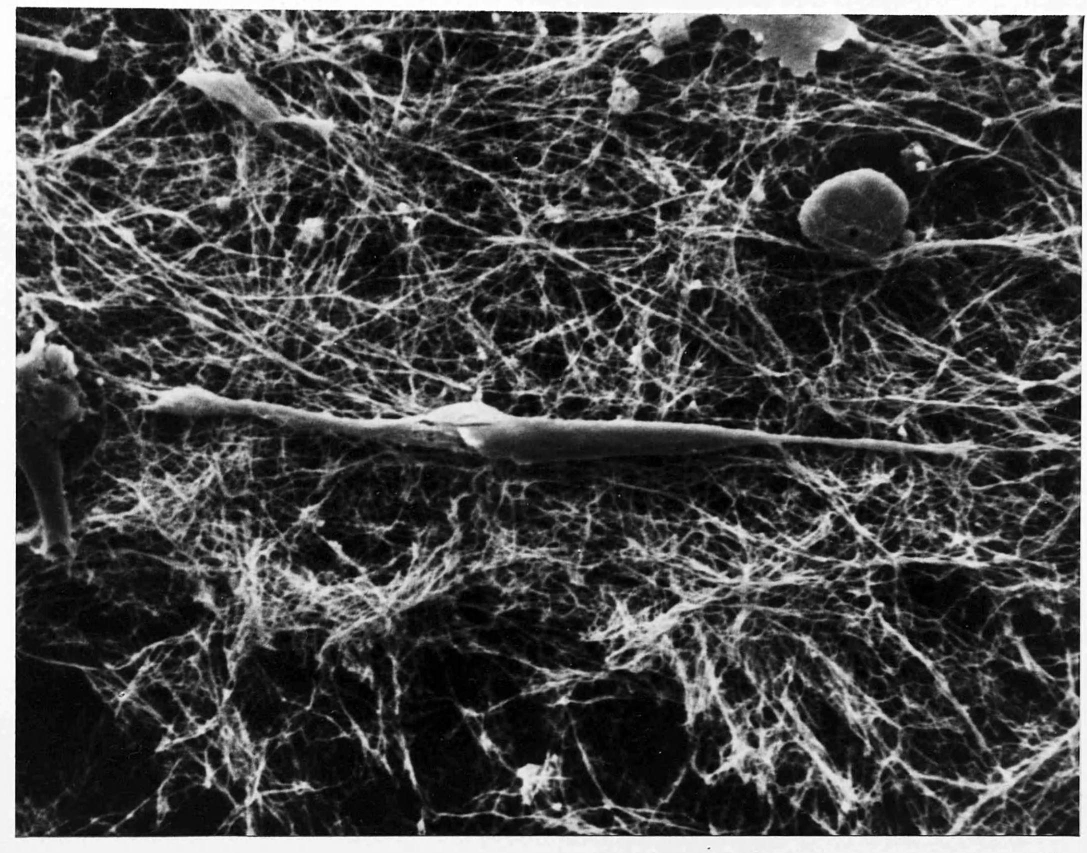
Mann-Whitney U test

SAMPLE1	SAMPLE 2	U	N	Result
Normal leading edge (speed)	Normal trailing edge (speed)	U1=132 U2=12	N1=12 N2=12	Significant difference $P<0.001$
Talpid^3 leading edge (speed)	*Talpid*^{3} trailing edge (speed)	U1=113 U2=8	N1=11 N2=11	Significant difference $P<0.001$
Normal leading edge (speed)	Normal cell centre (speed)	U1=87.5 U2=86.5	N1=12 N2=12	No Significant difference
Normal trailing edge (speed)	Normal cell centre (speed)	U1=28 U2=116	N1=12 N2=12	Significant difference $P<0.01$
Talpid^3 leading edge (speed)	*Talpid*^{3} cell centre (speed)	U1=79.5 U2=41.5	N1=11 N2=11	No Significant difference
Talpid^3 trailing edge (speed)	*Talpid*^{3} cell centre (speed)	U1=14.5 U2=106.5	N1=11 N2=11	Significant difference $P<0.01$

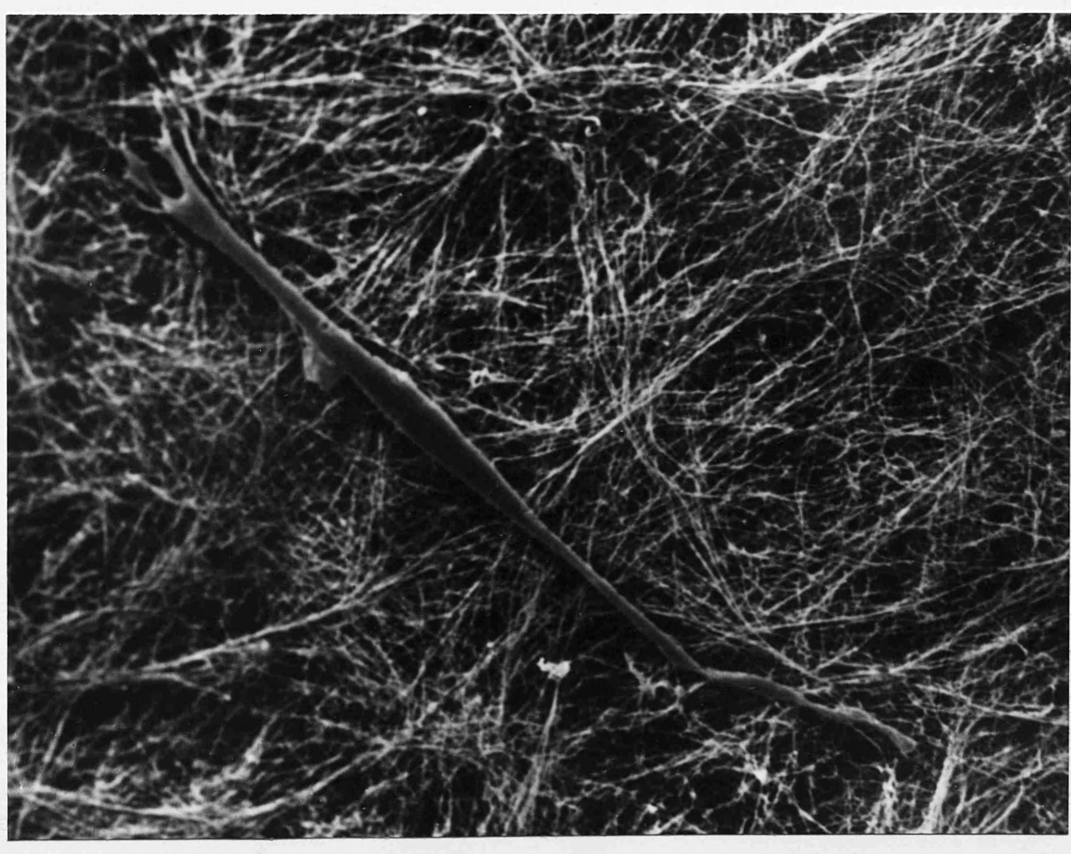
Normal leading edge (speed)	*Talpid*^{3} leading edge (speed) No Significant difference	U1=53 U2=79	N1=12 N2=11
Normal leading edge's persistence	*Talpid*^{3} leading edge's persistence No significant difference	U1=63 U2=69	N1=12 N2=11
Normal trailing edge (speed)	*Talpid*^{3} trailing edge (speed) No significant difference	U1=72.5 U2=59.5	N1=12 N2=11
Normal trailing edge's persistence	*Talpid*^{3} trailing edge's persistence No significant difference	U1=55 U2=77	N1=12 N2=11
Normal cell centre (speed)	*Talpid*^{3} cell centre (speed) No significant difference	U1=60.5 U2=71.5	N1=12 N2=11
Normal cell centre's persistence	*Talpid*^{3} cell centre's persistence No significant difference	U1=50 U2=82	N1=12 N2=11

Figure 2

a - b SEM of normal elongated, spindle-shaped limb mesenchymal cells (800X). A spherical cell is found at the top right corner of (a).



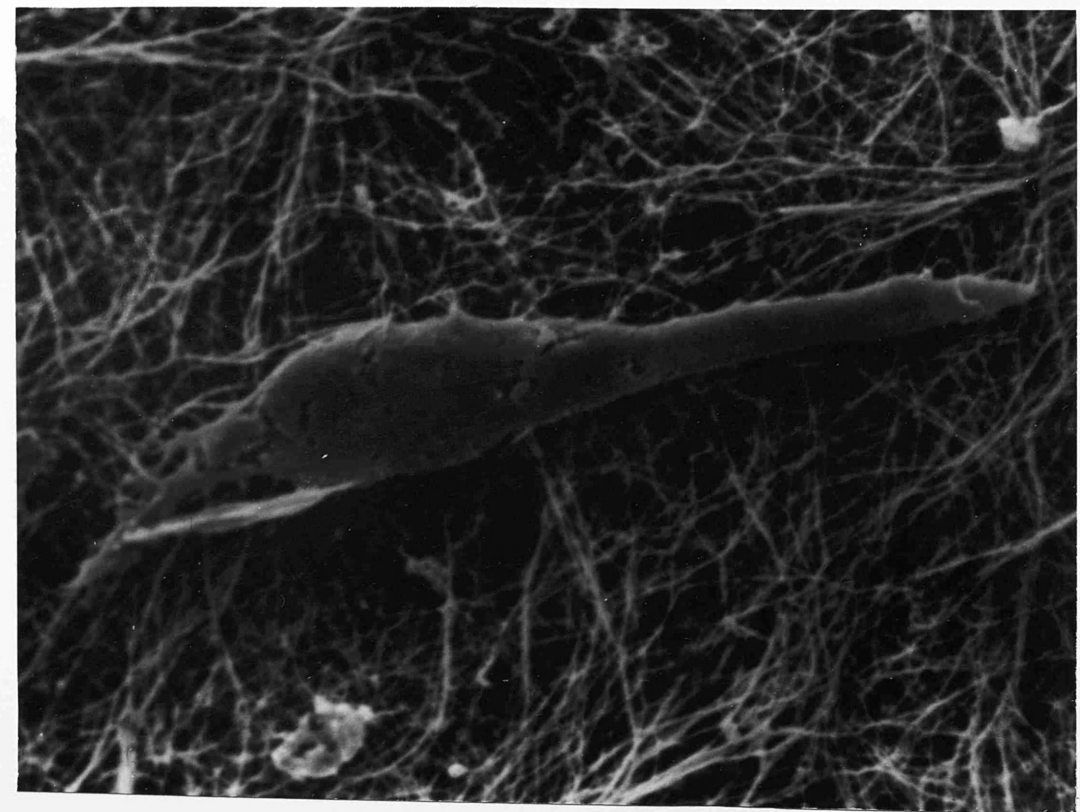
a

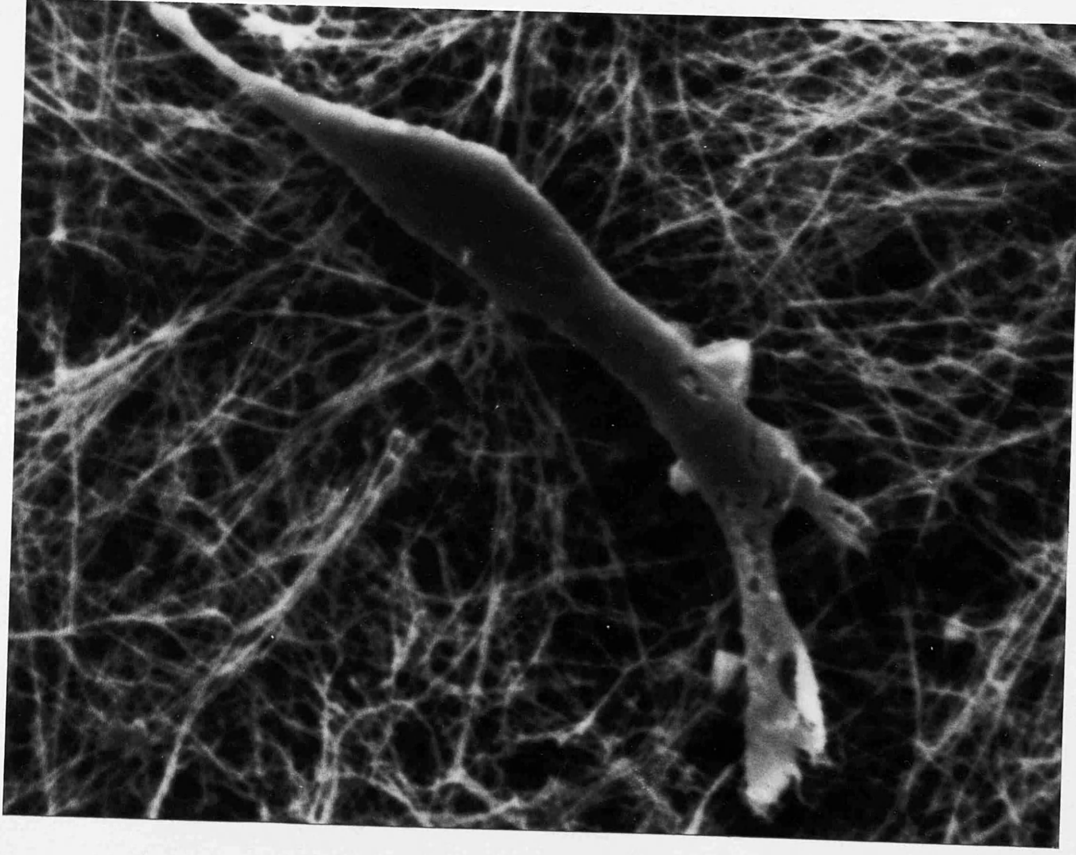


b

Figure 3

a - b SEM of normal pear-shaped limb mesenchymal cells (1600X).





b

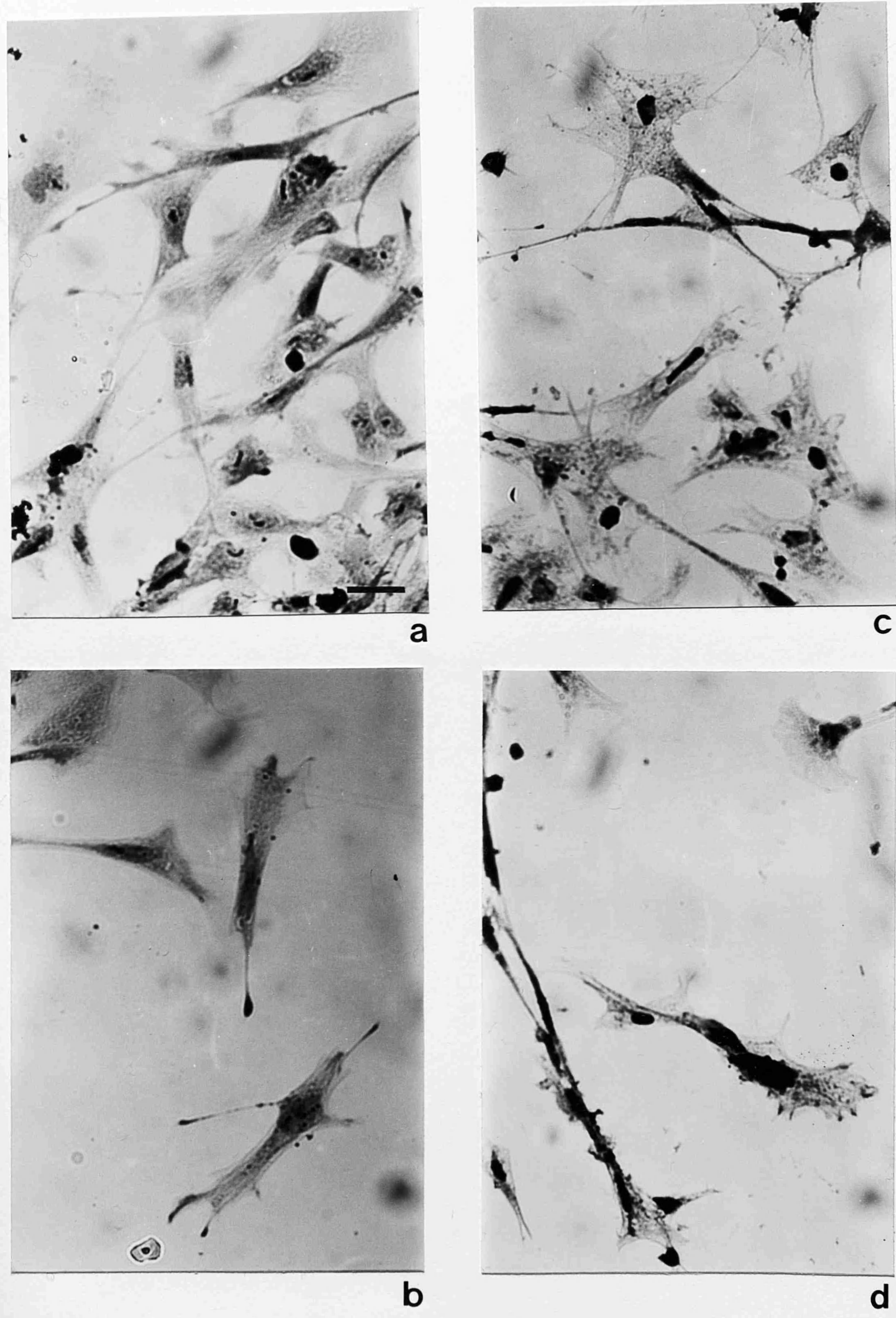
Figure 4

Cells spreading out from normal and *talpid*^{3} limb explants after 24 hr on glass substrata.

a - b Normal limb mesenchymal cells.

Bar = 10 μm

c - d *Talpid*^{3} limb mesenchymal cells.



a
c
b
d

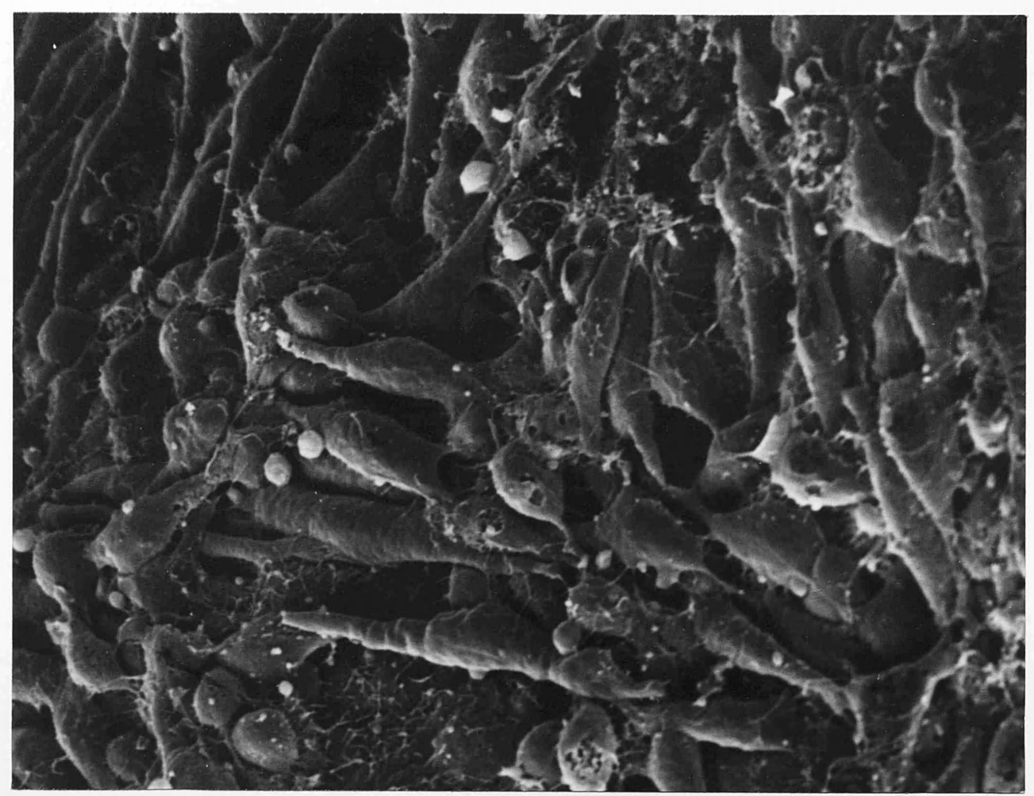
Figure 5

SEM of the surfaces of normal and *talpid*[3] explants after 48 hr of incubation inside collagen lattices.

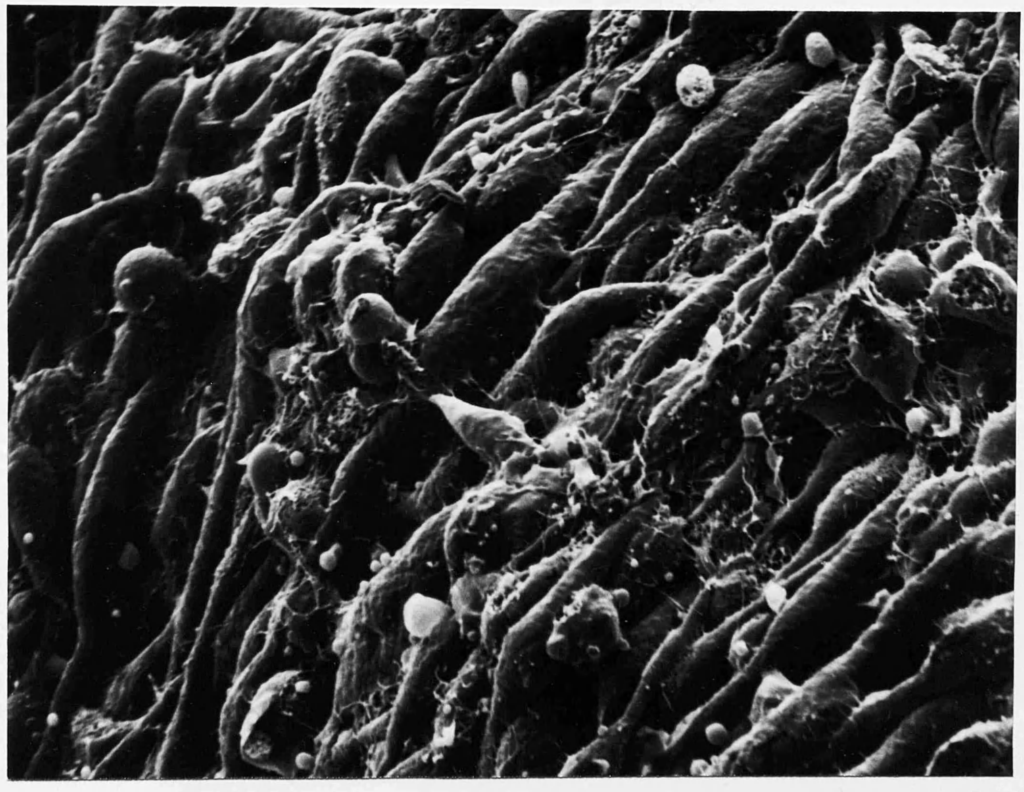
a Normal explant surface (800X).

b *Talpid*[3] explant surface (800X).

Note the presence of bipolar and spherical cells.



a



b

Figure 6

a - b SEM of *talpid*^{3} elongated, spindle-shaped limb mesenchymal cells (800X).

a

b

Figure 7

a - b SEM of *talpid*^{3} pear-shaped limb mesenchymal cells (1600X).

a

b

Figure 8

a SEM of a *talpid*^{3} limb mesenchymal cell producing more pseudopodia from its leading edge (3200X) than would a normal cell

b SEM of a *talpid*^{3} limb mesenchymal cell producing more pseudopodia from its leading edge (800X) than normal.

a

b

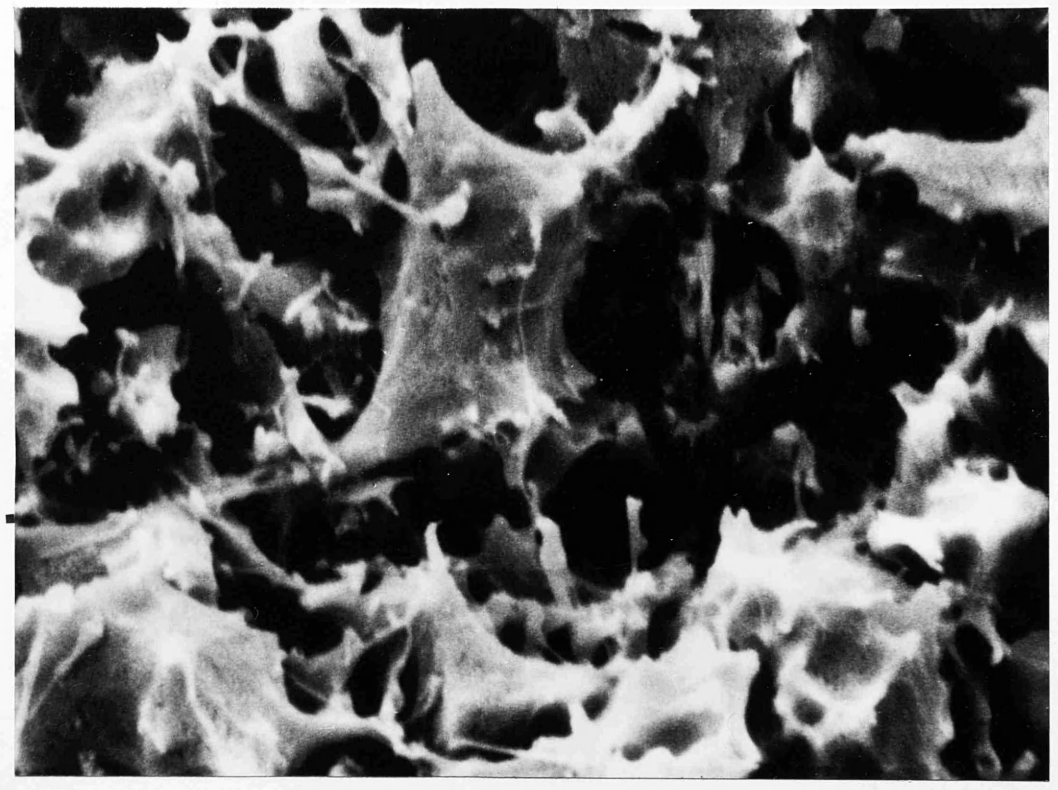
Figure 9

SEM of the distal regions of normal and *talpid*^{3} stage 23 H.H. wing buds.

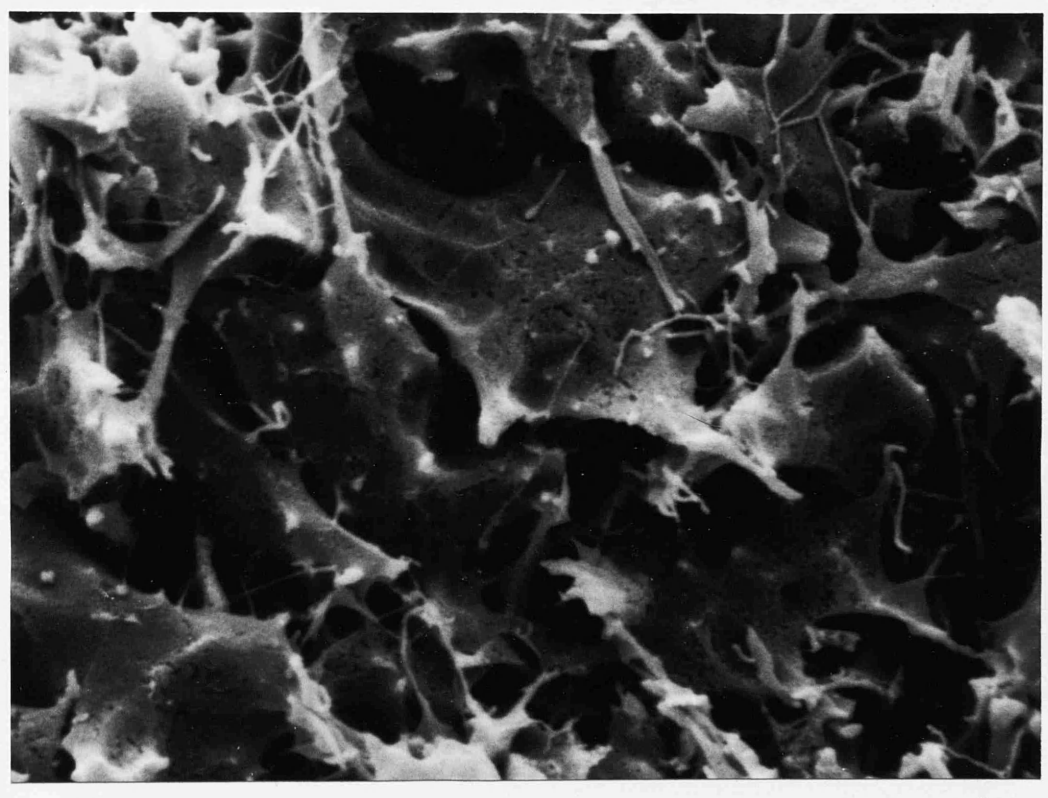
a Normal distal mesenchyme (3200X).

b *Talpid*^{3} distal mesenchyme (3200X).

Note the presence of filopodia.



a



b

Figure 10

SEM of the proximal regions of normal and *talpid*^{3} stage 23 H.H. wing buds.

a Normal limb mesenchyme(1600X).

b Higher power of (a) showing the presence of bipolar and spherical cells (3200X).

c *Talpid*^{3} limb mesenchyme (1600X).

d Higher power of (c) showing the presence of bipolar and spherical cells (3200X). Note the bunches of short spikey cytoplasmic processes present in *talpid*^{3} cells.

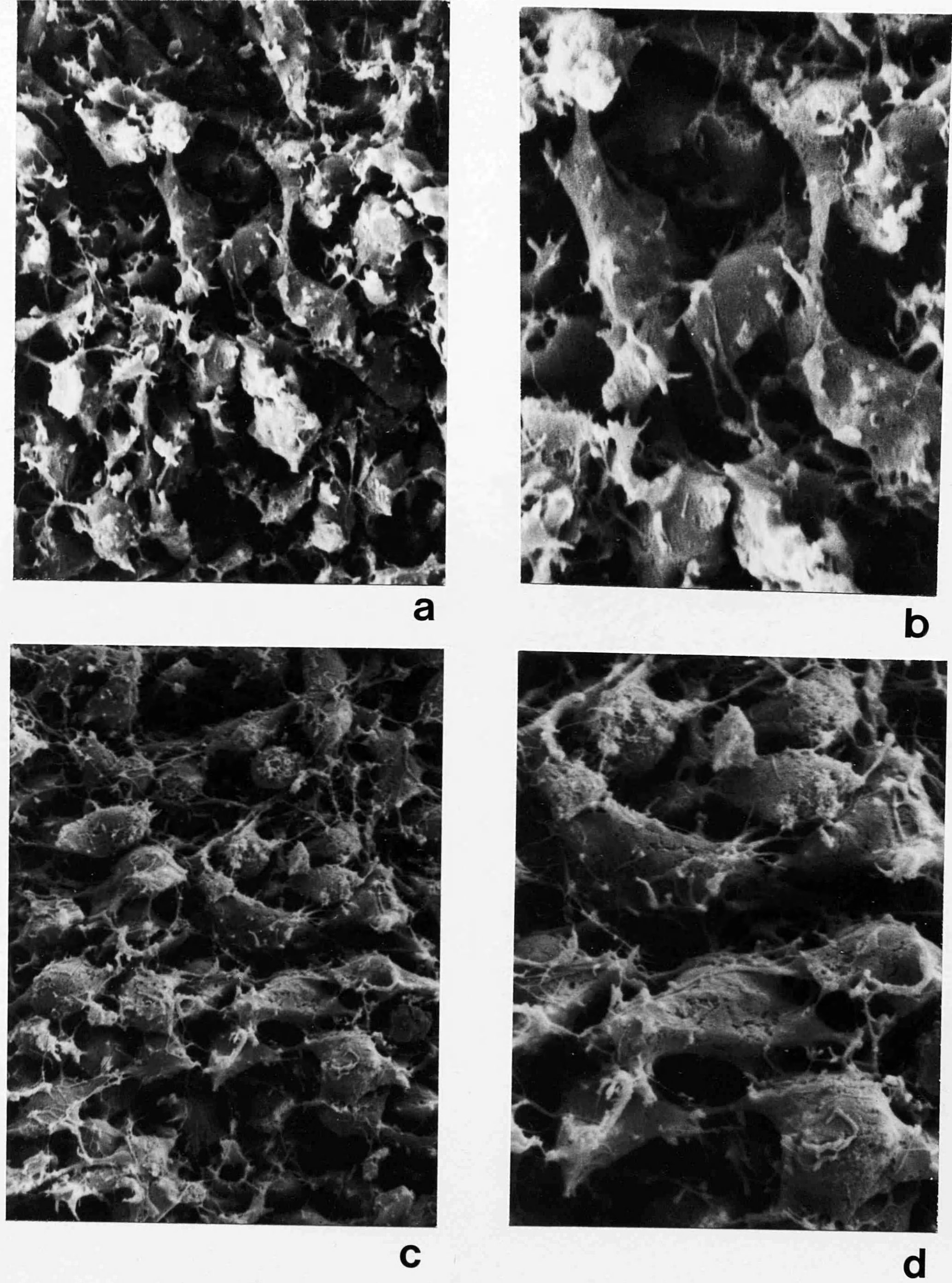
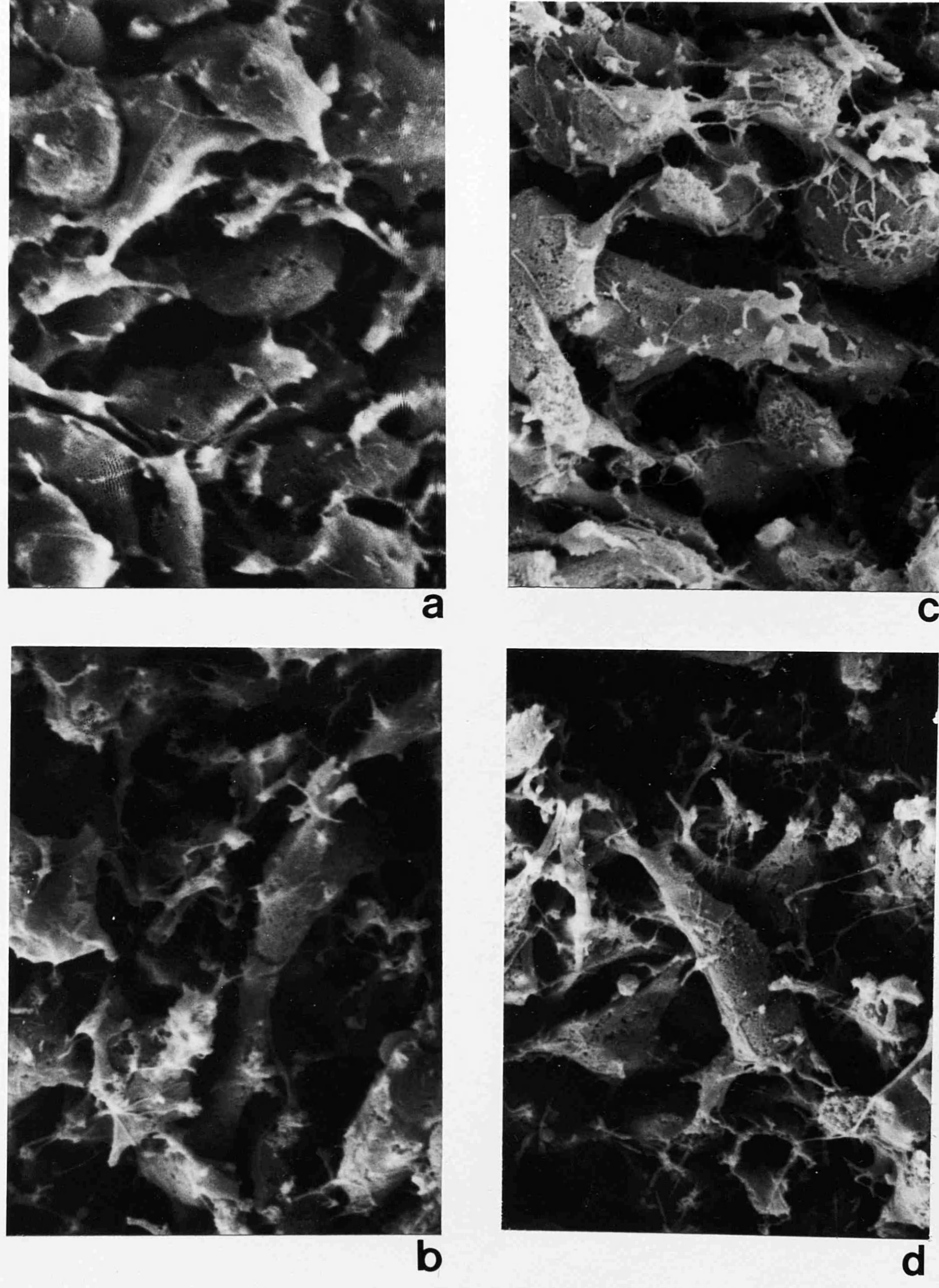

a
b
c
d

Figure 11

SEM of the proximal regions of normal and *talpid*^{3} stage 24 H.H. wing buds; showing the presence of bipolar and spherical cells.

a - b Normal limb mesenchyme (3200X).

c - d *Talpid*^{3} limb mesenchyme (3200X).



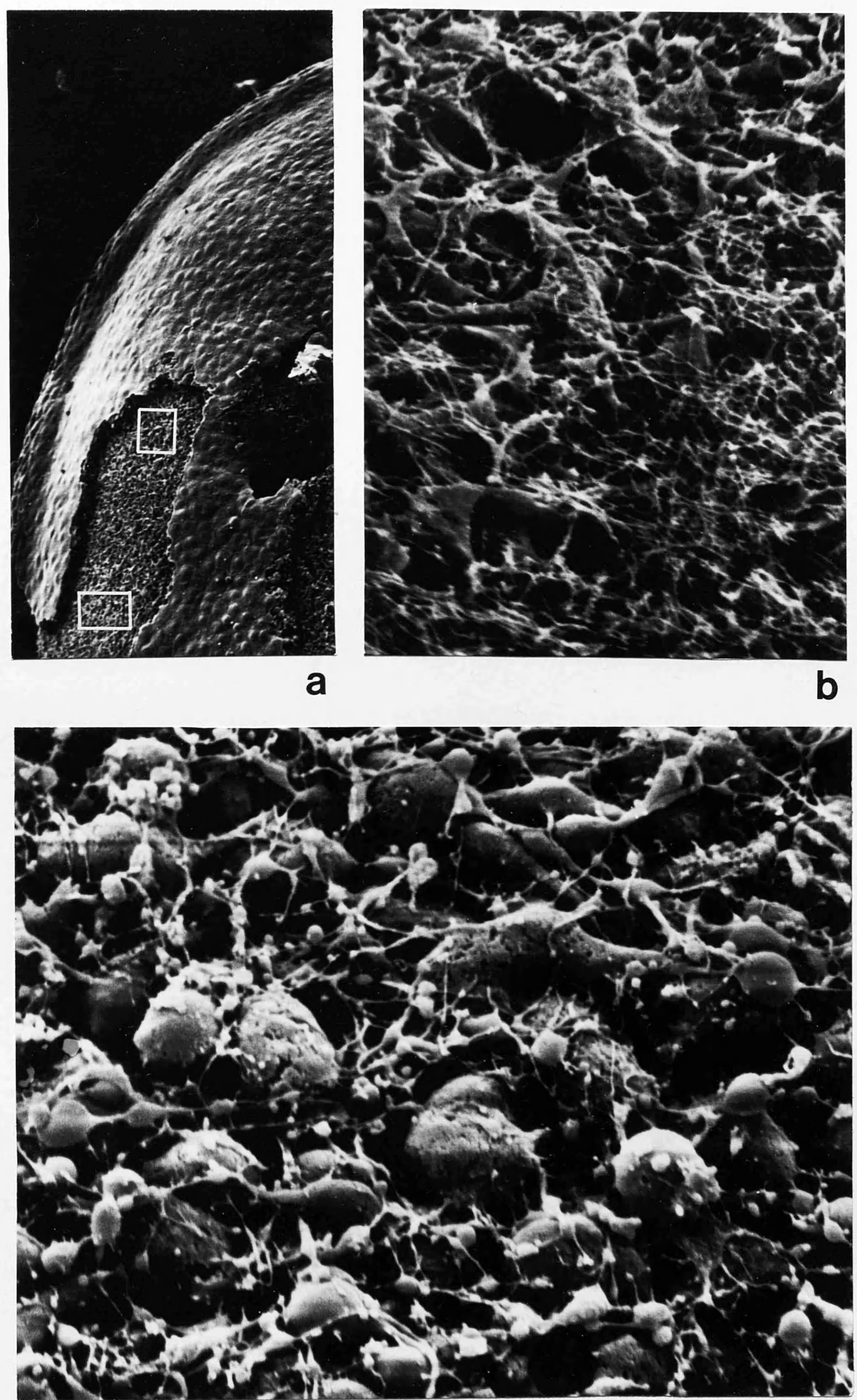
a
c
b
d

Figure 12

SEM of a normal stage 22 H.H. wing bud.

a General view of the wing bud (50X). Part of the ectoderm has been removed to expose the basement membrane (framed regions).

b - c Higher magnification of the framed regions. Demonstrating the presence of extracellular fibres and globular droplets of extracellular materials (1600X).



a
b
c

Figure 13

Single/double limb explants cultured inside collagen lattices for 48 hr.

a Single normal limb explant. Aligned tracts of collagen fibres (arrows) have formed, which extend outwards from the explant towards the margin of the collagen lattice.
Bar = 400 µm

b Double normal limb explants. A dense tract of aligned collagen (arrows) has formed between the explants.

c Single *talpid*^{3} limb explant behaving just like its normal counterpart.

d Double *talpid*^{3} limb explants. Aligned collagen tracts (arrows) have formed between the explants.

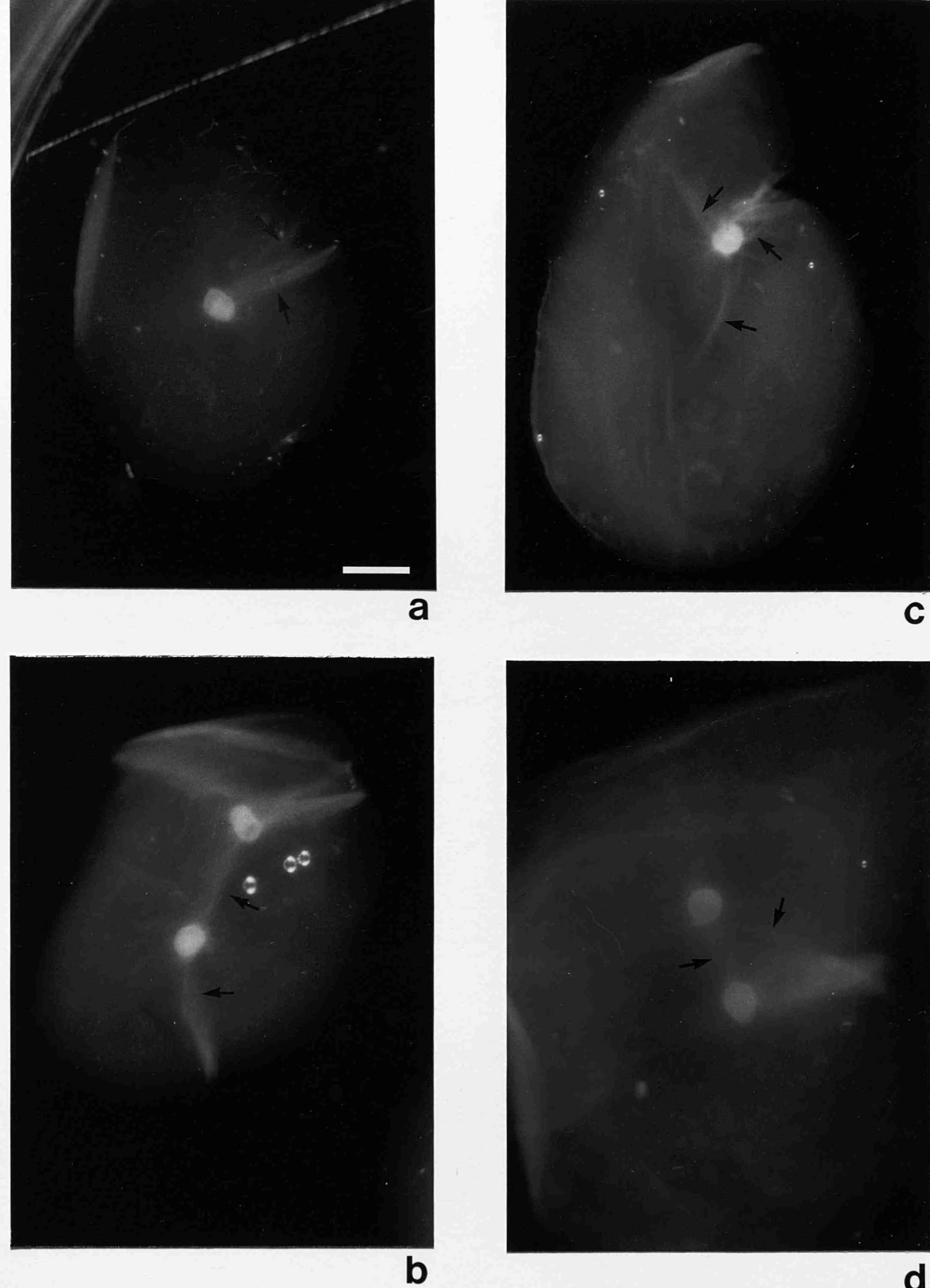

a
c
b
d

Figure 14

SEM of the region between 2 talpid[3] limb explants grown in a collagen lattice for 72 hr.

a General view (50X).

b Higher power of bottom frame in (a). Cells are streaming out of the explant. They are aligned parallel to the axis connecting both explants (400X).

c Higher power of top frame in (a). Cells streaming out of the explant are bipolar and aligned parallel to each other (400X).

a

b

c

Figure 15

Tracing from a time-lapse film of a normal limb mesenchymal cell moving through aligned collagen fibres. Time interval between each outline is listed on the 2 vertical lines which are reference points.

The cell is originally moving from left to right. Between 110 -260 min, the cell begins to move in the opposite direction; cell polarity remains unchanged i.e. the trailing edge becomes the leading edge and vice-versa.

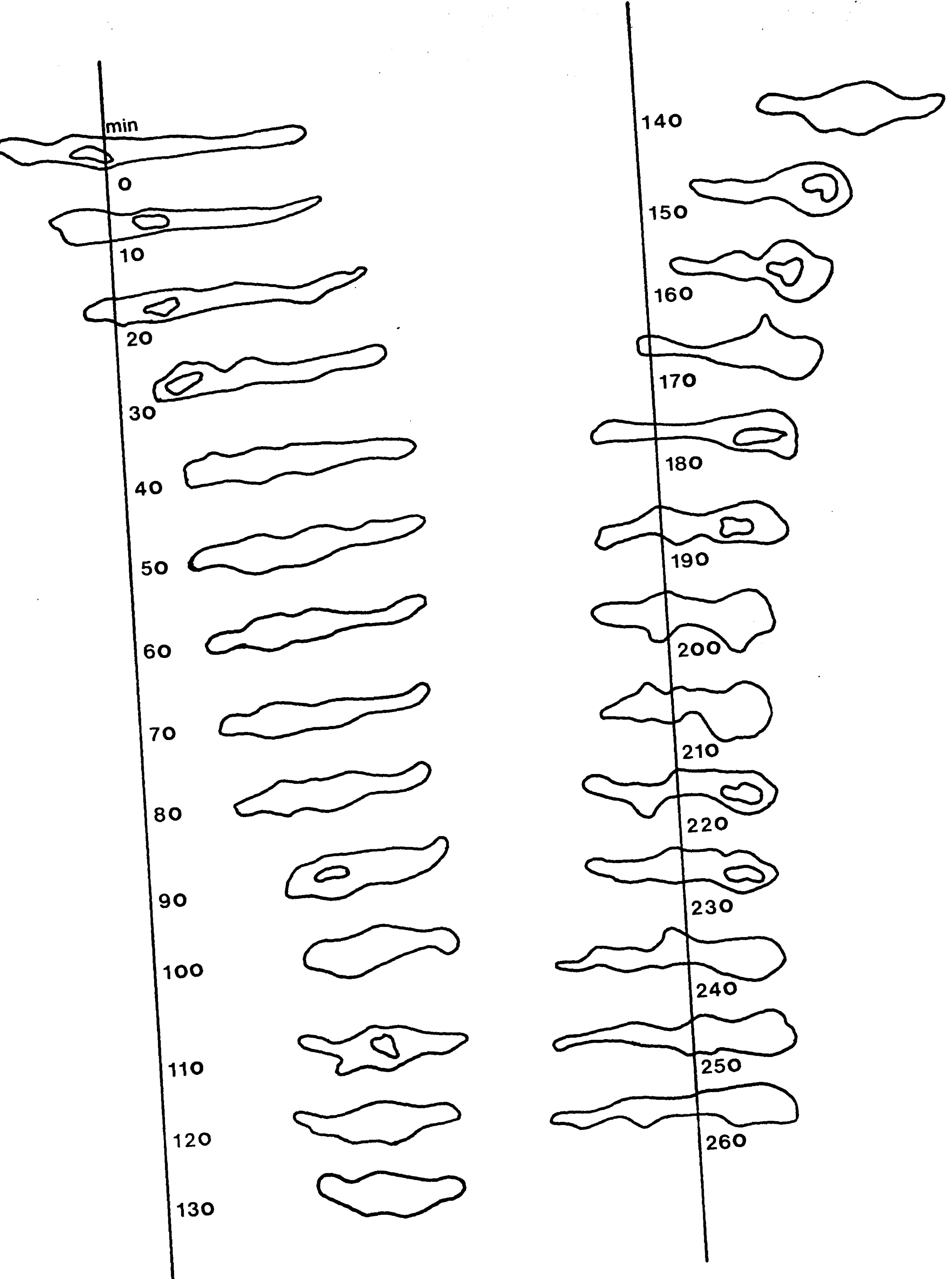

min
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230
240
250
260

Figure 16

Tracing from a time-lapse film of a *talpid*[3] limb mesenchymal cell moving through aligned collagen fibres. The time interval between each cell outline is listed on 2 vertical lines which are reference points.

Between 55-120 min, the cell begins to move in the opposite direction (left to right). Again the trailing edge becomes the leading edge and vice versa, while cell polarity is retained.

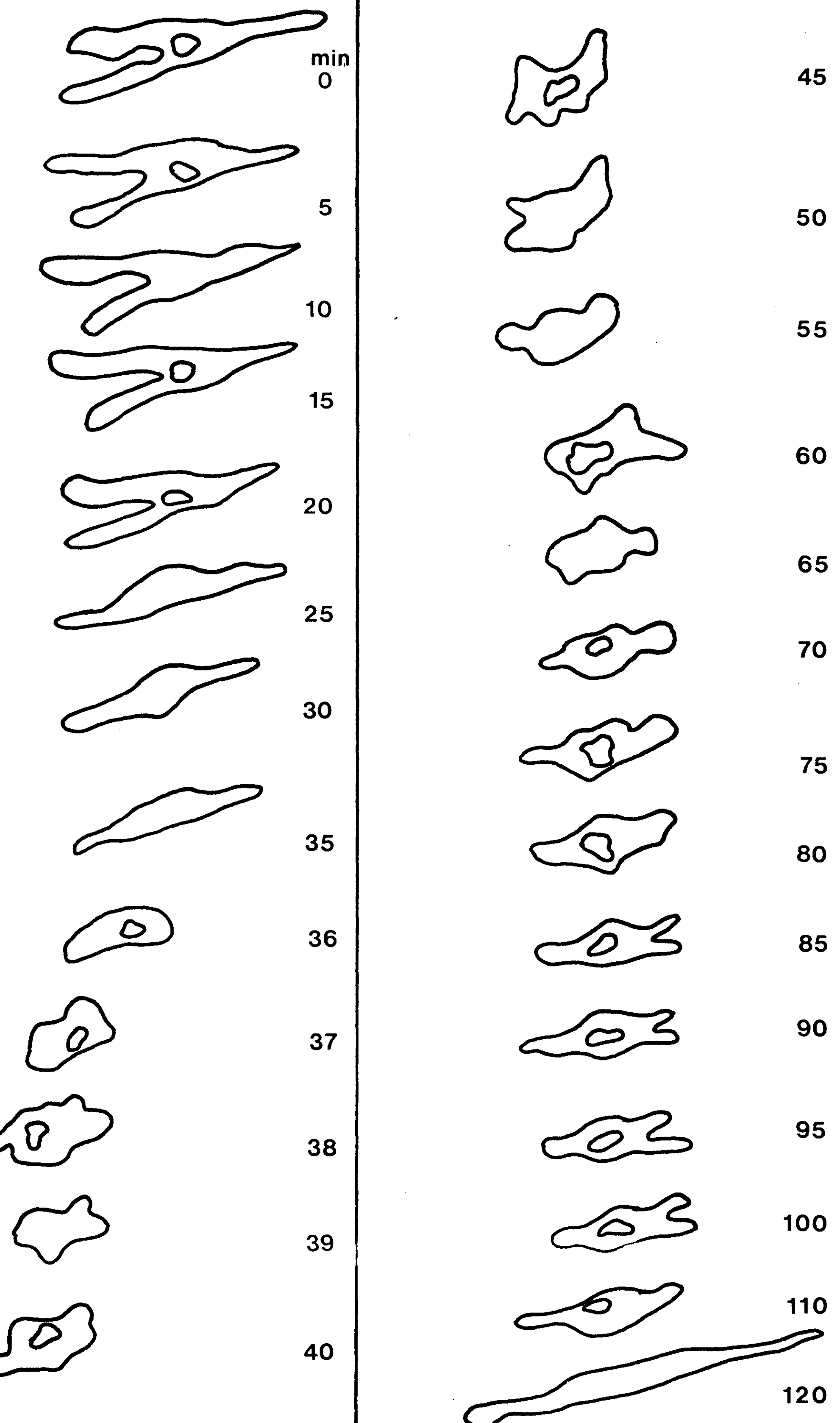

min
0
5
10
15
20
25
30
35
36
37
38
39
40
45
50
55
60
65
70
75
80
85
90
95
100
110
120

Figure 17

Outlines of 2 normal mesenchymal cells from time-lapse films. Note how rapidly the trailing edge retracts into the cell body.

min
0
1
2
3
4
5
6
7
8
9
10
11
0
1
2
3
4
5
6
7
8
9

Figure 18

Time-lapse tracing of 2 *talpid*^{3} limb mesenchymal cells migrating inside collagen lattices.

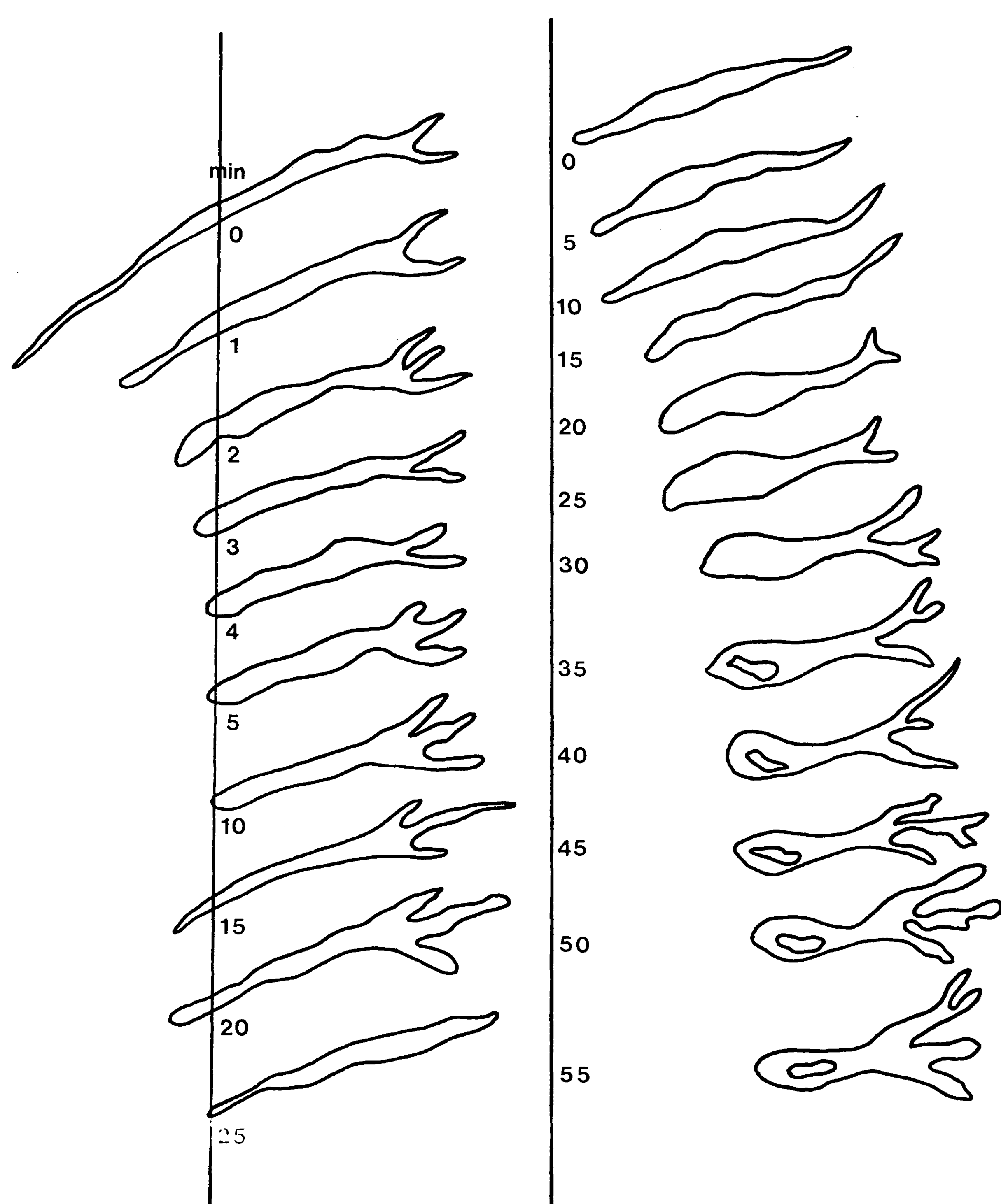
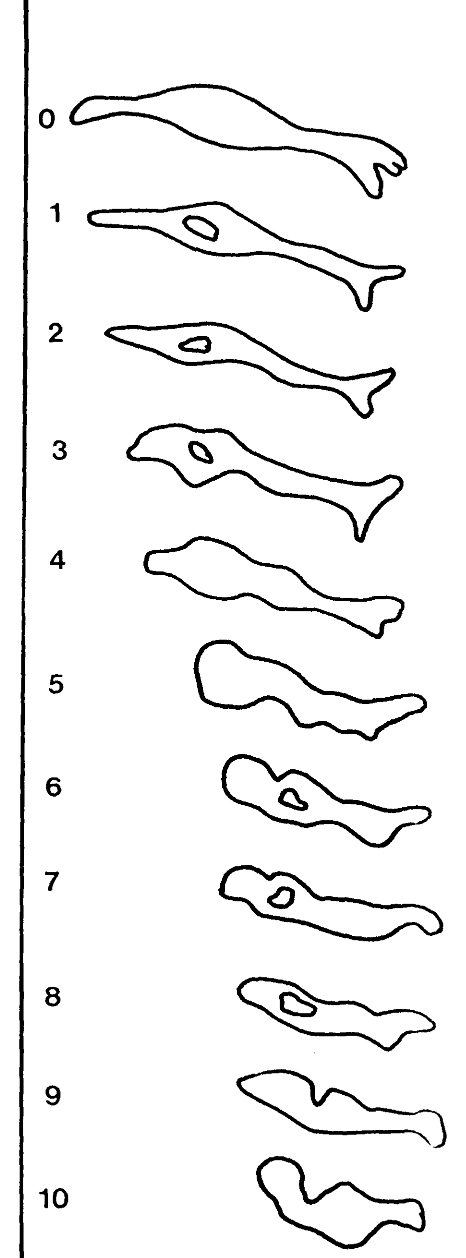

min
0
1
2
3
4
5
10
15
20
25
0
5
10
15
20
25
30
35
40
45
50
55

Figure 19

Drawing traced from a time-lapse film of 3 *talpid*^{3} limb mesenchymal cells migrating on aligned collagen fibres.



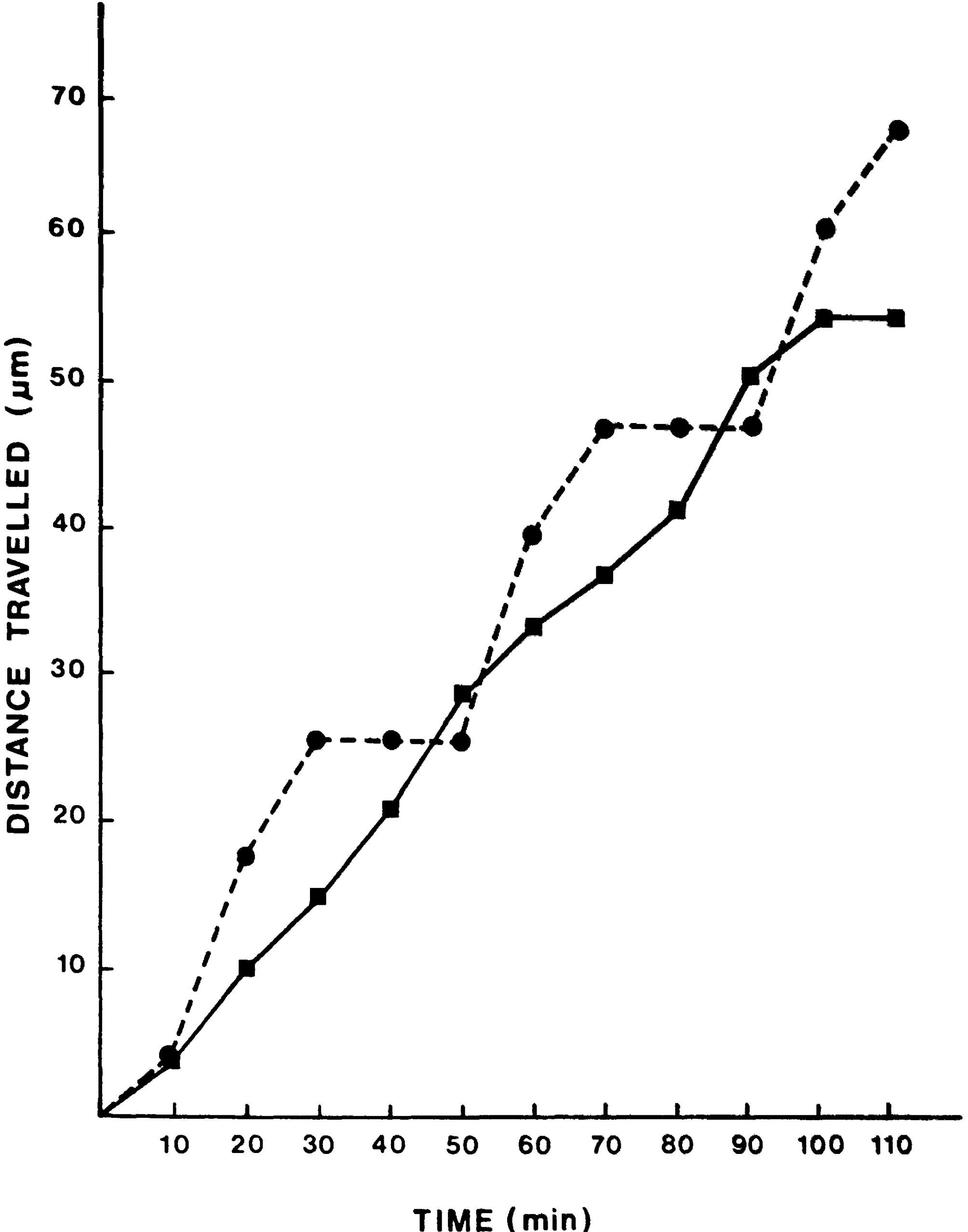
min 0 1 2 3 4 5 6 7 8

0 1 2 3 4 5 6 7 8

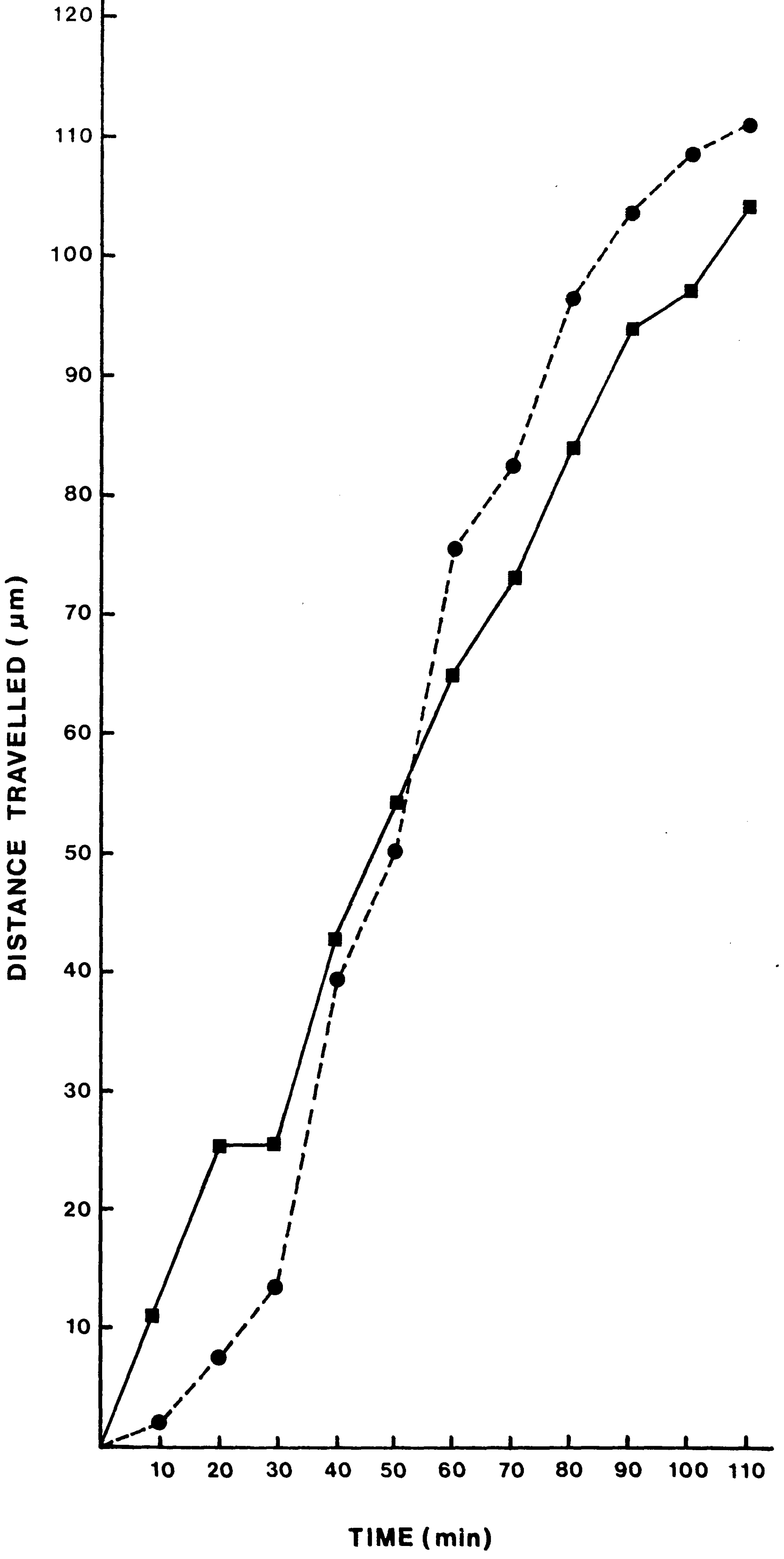
Graph 1 and 2

Abscissa: time (min) cells were observed in locomotion; ordinate: distance travelled (μm) by the leading edge (■) and the trailing edge (●). The translocation activity of normal limb mesenchymal cells in collagen lattice cultures was recorded with time-lapse cinemicrography and the relationship between the rates of advancement of the leading and trailing edges during locomotion was compared.

Note that the advancement of the leading edge is not restricted by the relative immobility of the trailing edge (graph 1, between 30-50 min and 70-90 min). The rapid retraction of the trailing edge does not prevent the advancement of the leading edge (graph 1, 10-30 min and 50-70 min; graph 2, 0-20 min and 50-70 min).



graph 1

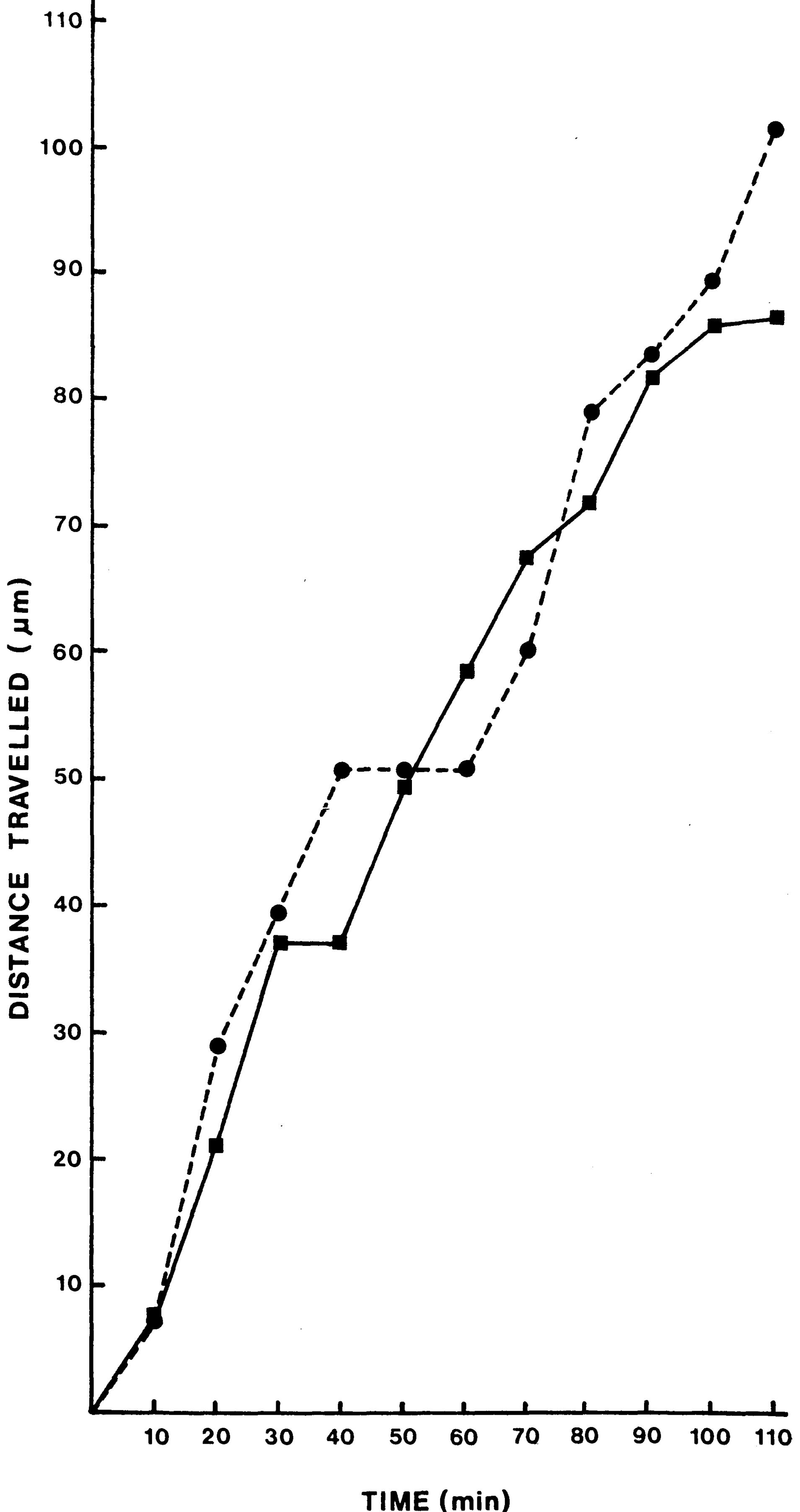


graph 2

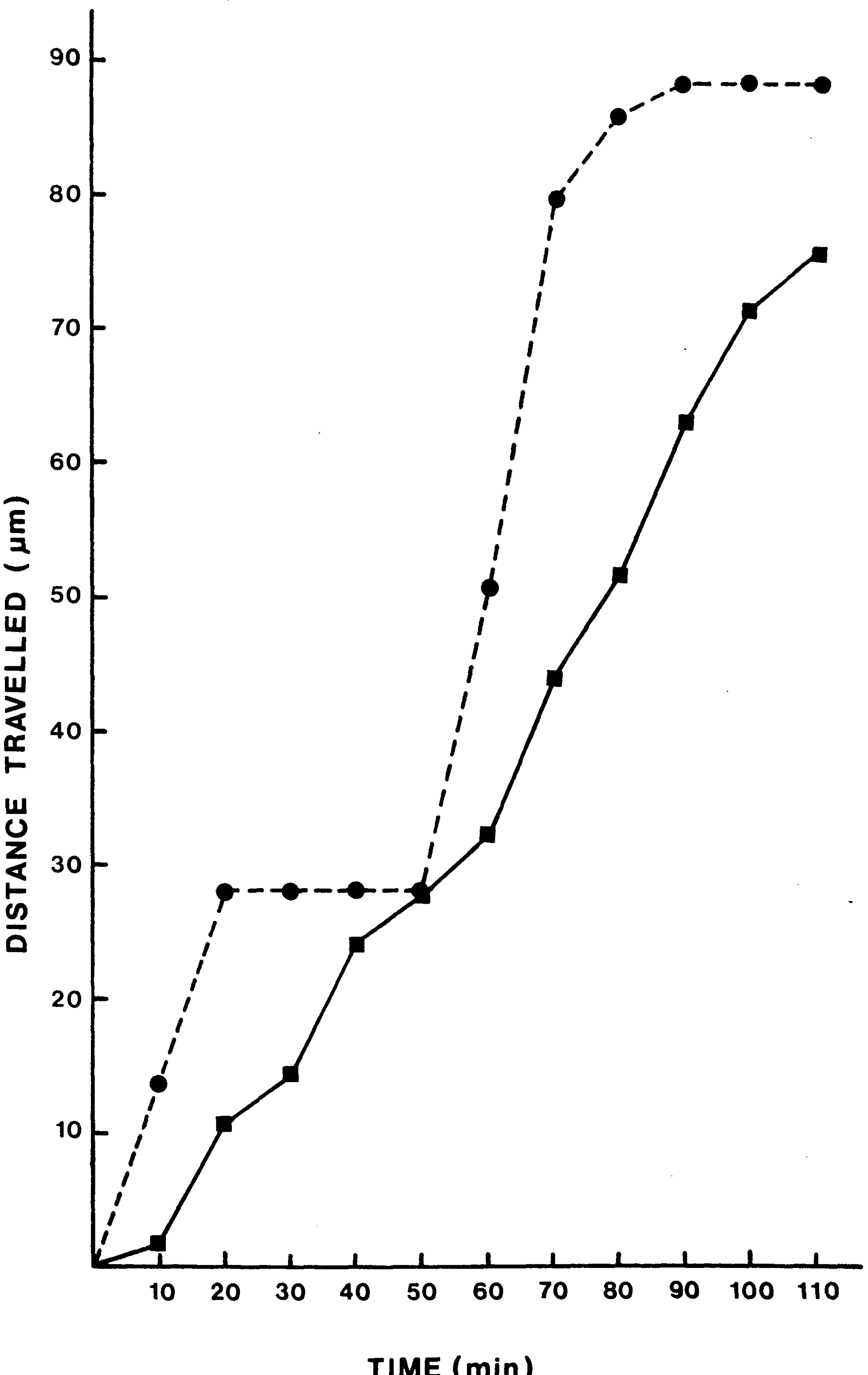
Graph 3 and 4

Abscissa: time (min) cells were observed in locomotion; ordinate: distance travelled (µm) by the leading edge (■) and the trailing edge (●). Comparison of the rates of advancement of the *talpid*[3] leading and trailing edges during locomotion.

Note that the advancement of the leading edge is not restricted by the relative immobility of the trailing edge (graph 3, 40-60 min and graph 4, 20-50 min). The rapid retraction of the trailing edge does not prevent the advancement of the leading edge (graph 3, 0-30 min and 60-110 min; graph 4, 0-20 min and 50-70 min).



graph 3



graph 4

6:4 DISCUSSION

Detailed measurements of the speed of normal and talpid^3 limb mesenchymal cell locomotion in collagen lattices have not revealed any significant difference between them. This appears to contradict the findings in Chapter 5 where talpid^3 myogenic cells were demonstrated to have a reduced capacity for movement in the limb. However, these inconsistencies are not altogether unexpected since Ede & Flint (1975b) also found no difference in the actual speed of movement between these two genotypes; they discovered that talpid^3 cells spent longer periods at rest than normal cells, and that consequently talpid^3 cells travelled less distance over long periods. I was unable to confirm this, because the duration of my filming was relatively short, but the apparent reduction or absence of talpid^3 myogenic cell movement in quail wing buds can be explained in terms of these cells spending longer periods at rest: the mutant's inactivity would allow time for host myoblasts to proliferate and migrate to fill up any vacant muscle sites before talpid^3 cells had a chance to do so, most of the talpid^3 cells remaining near the graft site.

Ede & Flint (1975b) estimated normal limb mesenchymal cells travel on plastic substrates with an average speed of 0.28 µm/min and talpid^3 mesenchymal cells 0.20 µm/min. My estimates are roughly 3 times as high, with the normal leading edge translocating with an

average speed of 0.81 µm/min and *talpid*[3] leading edge 0.73 µm/min. Therefore it seems that cells are capable of moving much faster in collagen lattices than on flat planar substrata.

6:4:1 Cell traction and collagen alignment

Cell locomotion must involve the exertion of a rearward force upon the substratum in its immediate surroundings. Harris (1982) coined the word 'traction' to refer to this rearward force, which the cells must apply during locomotion and spreading. *In vitro*, cell tractions are usually exerted upon solid surfaces such as glass or plastic or on deformable surfaces such as collagen and silicone rubber. When fibroblasts are cultured on flexible silicone rubber, the traction the cells exert as they move compresses the underlying surface of the substratum into a series of easily visible wrinkles (Harris *et al*, 1980; Harris *et al*, 1981). Hitherto, the same effect has also been observed as cells migrate out of normal and *talpid*[3] limb explants, but instead of wrinkles, a series of aligned collagen tracts are generated around the borders of these explants and may run some distance towards the margins of collagen lattices. This phenomenon has also been noted in many other systems: palate mesenchymal cells (Venkatasubramanian *et al*, 1980); somitic mesenchyme (Sanders & Prasad, 1983) and cardiac cushion tissue cells (Bernanke & Markwald, 1982).

Weiss (1934) was the first to report the effect of culturing 2 explants on a deformable substratum: "If two cultures somewhat distant from each other are put in one of those extremely thin 'plasma membranes in the frame', an extensive growth of cells appears along the connecting line of the two cultures, as if each fragment were attracting the cells of the other.......It can be shown that merely the presence of two active growth centers necessarily results in the formation in the medium of a structural pattern of such arrangement as to offer the cells a straight connection between the two centers as a preferential pathway." Indeed, this is what also happens if two normal or talpid[3] explants are cultured inside a collagen lattice: collagen fibres are always preferentially realigned parallel to the axis connecting the explants, moreover, cells also prefer to migrate along these aligned fibres.

Weiss (1934) proposed that the formation of these collagen patterns are the direct result of actively growing cells absorbing water from surrounding extracellular matrices to shrink and thus create different patterns according to where shrinking is induced. My results have shown otherwise: collagen realignment is attributed solely to tractional forces, created, as cells start to migrate out of the explants. In this sense, the phrase "2 centers effect" which Weiss used to describe this phenomenon should really be called "2 tractional fields".

The substitution of one of the two explants with a fixed point such as a polystyrene cylinder can also create the phenomenon (Stopak & Harris, 1982). The effect of a fixed point is similar to that of a neighbouring explant in that cells migrating from an explant produce axial alignment between themselves and the fixed point because the fixed point resists the distorting forces the explant exerts upon the matrix.

During embryogenesis, collagen and other extracellular fibres such as fibronectin are somehow arranged in a variety of anatomical patterns. In the limb, dense bundles of aligned collagen fibres form tendons connecting muscle to bones, and in between the somites and the limb somatopleural mesenchyme, the space is filled with transversely aligned collageneous fibres. If one regards a somite as being an explant and the somatopleure as being a fixed point, it is then possible to see cell traction as being the mechanism responsible for generating the aligned collagen fibres in the spaces separating the somites from the limb somatopleure. In fact traction forces not only have the ability to generate collagen patterns but the collagen in turn dictate the sequential action of the cells. (Refer to contact guidance section 6:4:2.)

In a very elegant experiment, Stopak & Harris (1982) extracted the tibiotarsus and the tarsometatarsus from a 10 day chick embryo and embedded them in a collagen lattice. Along each side of these long bones

were arranged 2 parallel rows of muscle explants. After 6 days of postincubation the muscle explants had coalesced and were stretched out, resembling the bellies of true muscles; the cartilages at the same time acted as fixed points for exerting traction and as sites for muscle attachment. Accordingly, these authors proposed that muscle prepatterning in the chick limb is physical rather than chemical and ~~and~~ that it is directly determnined by the position and alignment of myogenic cells. This suggestion is highly probable, especially since it is now known that myogenic cells migrate within avian limb buds (Ede et al, 1984; Gumpel-Pinot et al, 1984).

6:4:2 Contact guidance

As cells migrate out of limb explants and onto axially aligned collagen fibres, which they themselves have generated, one is struck not only by the definite orientation of cells along these organised fibres but also by the fact that cells moved much farther in that particular direction than in any other. The ability of aligned collagen fibres to determine the orientation and shape of cells, and in addition the direction of their movement, is consistent with the phenomenon of contact guidance (Weiss, 1961; Weiss & Taylor, 1956; Dunn & Ebendal, 1978).

Contact guidance has been shown to operate during the initial migration of myogenic cells from the

somites to the limb somatopleure (Jacob _et al_, 1978; 1979). Between the spaces of somites and limb somatopleure, collagen fibres are arranged preferentially in a medio-lateral direction. Individual myogenic precursor cells migrating on these fibres are spindle-shaped and are aligned parallel to these fibres; furthermore, filopodia rather than ruffling membranes are found on the leading edges of these cells. These observations may be readily related to the results obtained in this chapter.

There are other good examples in the literature of contact guidance operating _in vivo_ e.g. (1) Stuart & Moscona (1967) reported that during the formation of chick feather papillae, a lattice of aligned collagen fibres was found in the skin, which they believed preceded the appearance of dermal condensation; they also presented evidence that the lattice functions in directing the migration of dermal cells. Ede _et al_ (1971) repeated this analysis using both normal and _talpid_^{3} mutant cells and concluded that a lattice did exist, but that it was initiated by the appearance of early condensations, whose development was then reinforced by cell movement along the lattice. Collagen staining in the _talpid_^{3} skin was found to be less intense than in normal and the fibres not so clearly aligned; dermal cells lying over these fibres themselves lacked clear orientation. (2) Contact guidance is also a feature of avian eye development (Bard & Higginson,

1977). The corneal stroma of the avian eye comprises a complex arrangement of collagen bundles. During the 6th day of development, fibroblasts invade the primary corneal stroma. In the posterior portion of the stroma, fibroblasts are found to be aligned by an orthogonal array of collagen fibres. (3) Directed mesenchymal cell movement has been observed in the fins of teleost fish (Wood, 1982; Wood & Thorogood, 1984). In the distal sector of the fin, there is an array of collagen fibres, aligned along the proximo-distal axis and arranged parallel to one another at regular intervals. When mesenchymal cells migrating distally encounter these fibres, they realign accordingly; moreover, once aligned on the fibres, the speed of their movement is increased.

6:4:3 Cell surface and adhesion

Ede & Flint (1975b) reported that *talpid*[3] limb mesenchymal cells cultured on plastic produce many more microspikes from their cell surfaces than normal cells and have suggested that these are involved in cellular adhesion. They proposed that it was an excessive production of these adhesive points which led to *talpid*[3] cells spending a much longer time at rest. Indeed, microspikes have been shown to participate in cell-substratum adhesion (Tuffery, 1972; Collin, 1978) through focal contacts (Izzard & Lochner, 1980). Microspikes, however, are not characteristic of cells grown within collagen lattices; instead of microspikes,

pseudopodia and filopodia are produced. These cylindrical structures apparently have the ability to adhere directly to collagen fibres (Goldberg, 1979; Sanders & Prasad, 1983), though fibronectin can also mediate in this process (Johansson & Höök, 1980)

In terms of morphology, undifferentiated mesenchymal cells in the wing bud approximate more to cells grown in collagen than on plastic, because *in vivo* cytoplasmic extensions appear as elongated filopodia rather than as microspikes. One of the most striking features of *talpid*^{3} cells *in vivo* is their ragged appearance as shown in my SEM. Compared with normal cells, the mutant cells produce a prolific array of filopodia all around their cell surfaces. If, as has been suggested, filopodia are involved in cell adhesion, then *talpid*^{3} cell-to-cell adhesion would be very much enhanced, and it may be this interaction which limits locomotion of *talpid*^{3} myogenic cells in the limb *in vivo*.

Talpid^3 limb mesenchymal cells migrating in collagen lattices behave remarkably like normal cells: not only can these mutant cells be contact guided but the speed and mode of their movement is relatively normal. If one likens this situation to *talpid*^{3} somito-somatopleure migration, it will explain the presence of myogenic cells inside the *talpid*^{3} limbs even though results from *talpid*^{3} into quail transplantations suggest *talpid*^{3} myogenic cells have a

reduced capacity for translocation within the limb (Refer to Chapter V).

CHAPTER VII

CELL MOVEMENT AND THE EXTRACELLULAR MATRIX

7:1 INTRODUCTION

Increasingly the extracellular matrix is regarded not merely as some form of inert support but as having an important role in modulating and integrating the behaviour of cells (for review see Hay, 1981). Moreover, the very fact that connective tissues (where extracellular matrices are particularly abundant) determine muscle pattern in avian limbs (Chevallier & Kieny, 1982) makes it even more important to know how specific components in the matrix influence cellular activities. Apart from collagen, whose influence has already been discussed in detail, there are 2 other classes of molecules - proteoglycans and glycoproteins. In this study I will be concentrating on the specific effect of 3 molecules, fibronectin (a glycoprotein), hyaluronic acid and chondroitin sulphate (proteoglycans).

7:1:1 Fibronectin

Fibronectin is a large glycoprotein dimer consisting of 2 chains, of approximately 220,000 daltons each, linked by disulphide bonds (Hynes & Yamada, 1981). It is involved in a bewildering range of cellular interactions which include cell adhesion

(Hynes, 1981), cytoskeletal organisation (Hynes & Destree, 1978; Singer, 1979), migration (Tucker & Erickson, 1984), differentiation (Swalla & Solursh, 1984), contact guidance (Turner et al, 1983; Thiery et al, 1984) and chemotaxis (Greenberg et al, 1981). Apart from its ability to interact with cells, it is also capable of interacting with hyaluronic acid, cartilage proteoglycans, collagen, plastic, gelatin, heparin and many other substances (Hynes & Yamada, 1981). Fibronectin, especially, has a high affinity for collagen and is believed to mediate in cell-collagen adhesion (Hahn & Yamada, 1979). Fibronectin has been detected by immunofluorescence in basement membranes and a variety of adult and embryonic connective tissues.

In vivo, it has been put forward as a prime candidate for directing the movement of cells during morphogenesis (Thiery et al, 1984). With this idea in mind , the distribution of fibronectin has been investigated in chick (Dessau et al, 1980; Melnick & Mauger, 1984) and in mouse (Silver et al, 1981) limb buds: Fibronectin was found to be unevenly distributed in the limb (Tomasek et al, 1982b) and distinctly absent from myogenic regions. This is consistant with the idea that migratory cells synthesise very little fibronectin (Newgreen & Thiery, 1980). One of the exercises in this chapter will be to determine whether fibronectin has any effect on cell migration from limb implants.

7:1:2 Hyaluronic acid

Hyaluronic acid is a high molecular weight polyanion which is highly negative charged. In solution this glycosaminoglycan can expand to one thousand times its volume in its dehydrated form (Laurent, 1970). Hyaluronic acid occurs in low concentrations in many tissues and has been isolated from notochord, spinal cord and somites (Kvist & Finnegan, 1970).

A general pattern has emerged from studying the relationship between the appearance of hyaluronic acid and morphogenetic events, i.e. that hyaluronic acid is ubiquitous during the period of development in which cell migration and proliferation predominate. The subsequent removal of hyaluronic acid by hyaluronidase usually signals the onset of differentiation. In the development of the chick cornea, hyaluronic acid is the principle glycosaminoglycan synthesized at a time when the primary stroma starts to swell and mesenchyme is beginning to invade it. As the cornea begins to clear (differentiation), the level of hyaluronic acid decline as the hyaluronidase level rises (Toole & Trelstad, 1971). Similar associations have also been reported in amphibian limb regeneration (Toole & Gross, 1971) and fracture repair of long bone (Maurer & Hudack, 1952). Accordingly, Toole & Trelstad hypothesised that hyaluronic acid has the ability to modulate the behaviour of invading mesenchymal cells by providing a

substratum over which these cells could migrate.

In the limb bud, hyaluronic acid is not evenly distributed. More hyaluronic acid is found in the periphery than in the central core (Singley & Solursh, 1981). In addition, its concentration varies in a graded manner along the proximo-distal axis, with the highest concentration just beneath the AER.

7:1:3 Chondroitin sulphate

Chondroitin sulphate is a high molecular weight linear polysaccharide. There are 3 commonly occuring forms of chondroitin sulphate: 0-sulphate, 4-sulphate and 6-sulphate (Couchman *et al*, 1984). These molecules are usually attached to a protein core; up to 100 chondroitin sulphate chains can attach to a single protein core of molecular weight $2\text{-}3 \times 10^5$ (Hardingham *et al*, 1984). Chondroitin sulphates are usually associated with cartilage (Vasan & Lash, 1979) but have now also been found in loose connective tissues surrounding muscles, nerve fibres and blood vessels (Couchman *et al*, 1984).

What makes chondroitin sulphate interesting is its inverse relationship with hyaluronic acid synthesis. *In vivo* chondroitin synthesis does not generally begin until the level of hyaluronic acid drops (Toole, 1972). In fact the addition of hyaluronic acid to the medium of chondrocyte cultures results in a reduction of chondroitin sulphate synthesis (Solursh *et*

al, 1974; Solursh et al, 1980; Vasan et al, 1984). The appearance of chondroitin sulphate has been linked with differentiation, a phase not normally associated with cell movement. Does this imply that chondroitin sulphate has an inhibitory influence on cell mobility?

In this chapter I report on how all of these 3 molecules influence the migration of mesenchymal cells from limb explants embedded in collagen lattices.

7:2 MATERIALS AND METHODS

7:2:1 Construction of collagen lattice

Rat tail collagen was extracted in the manner described in Chapter 6. To create a collagen lattice 1 ml of acidified collagen extract was added to a sterile beaker embedded in ice. Into this was introduced 0.1ml of medium 199(10X), 0.1 ml of foetal calf serum (omitted in no serum experiments) and the necessary amount of NaOH required to turn the whole mixture pink. A drop of the pink solution was pipetted onto the surface of a 13mm glass disc, inside a 35mm falcon plastic dish. Explants taken from the central portion of normal chick limb buds (St. 21-24 H.H) were then inserted into the gel before the solution solidified. Once the collagen has gelled 2ml of culture medium was added. It was decided that the best way to introduce the extracellular matrix material was through the medium.

7:2:2 Addition to culture medium

Fibronectin (bovine plasma, Bioprocessing), hyaluronic acid (grade I human umbilical cord, Sigma) and chondroitin sulphate (grade III shark cartilage, Sigma) were all reconstituted in HAM culture medium. Various combinations were added to the medium and these are listed in accordance with their appearance in the results.

(1) Collagen lattice + HAM + serum (control).

(2) Collagen lattice + HAM.

(3) Collagen lattice + HAM + fibronectin (100 µg/ml).

(4) Collagen lattice + HAM + serum + hyaluronic acid (0.5 mg/ml and 2 mg/ml).

(5) Collagen lattice + HAM + serum + chondroitin sulphate (1 mg/ml and 2 mg/ml).

7:2:3 Analysis

A majority of the cultures were recorded on time-lapse films. The times required for explants to generate aligned collagen tracts were also recorded. From experience, the speed at which these traction lines appear usually represents how actively cells are migrating out of explants. This was one of the parameters used to assess how various culture media affected cell movement.

7:3/4 RESULTS AND CONCLUSIONS

7:3/4:1 Migration in the presence and absence of serum

As described in the previous chapter, normal mesenchymal cells in the presence of serum usually start migrating out of explants after 24 hr and extensively after 48 hr of incubation (fig. 1a). Around the periphery of both single and double explants, extensive reorganisation of collagen fibres takes place (fig. 2a, b). Aligned collagen tracts radiated out of explants and the classical '2 centres effect' was also created (fig. 2b).

In the absence of serum the cells failed to migrate (fig. 1b); consequently no aligned collagen tracts were generated (fig. 2c,d). This enforces the idea that cell traction is required to generate these tracts. A scattering of cells were frequently found around the borders of these explants but their appearance was distinctly abnormal. These cells were nodule-like and were not flattened out into the typical bipolar shape. Finally, time-lapse observations revealed these cells to be incapable of active locomotion.

7:3/4:2 Migration in the absence of serum and presence of fibronectin

Its generally believed that the low level of fibronectin present in serum (Hayman & Rouslanti, 1979) is the active ingredient necessary for cells to attach

and migrate in culture (Hydes & Yamada, 1982). Sanders & Prasad (1983) discovered that mesenchymal cells failed to migrate out of explants in collagen lattices if the tissues were cultured in the presence of anti-fibronectin antiserum or fibronectin-depleted serum. If fibronectin is indeed the active ingredient, then in the absence of serum a supplement of fibronectin should be able to promote cell locomotion. I have set up 10 such culture; none showed any signs of cell movement. Cells found scattered around the periphery of explants had a morphology similar to that described in the absence of serum (fig. 3a), which suggests that factors present in the serum as well as fibronectin are necessary for cell locomotion. Surprisingly, cells were able to adhere and migrate prolifically on fibronectin coated filters in the absence of serum; migration was so intense that cells invaded the pores in the filters and out onto the underside of the filters (fig. 3b). Uncoated filters did not promote cell adhesion and migration.

Why fibronectin-coated filter and collagen should have different effects is puzzling. According to Hahn & Yamada (1979), the adhesion of cells to type I collagen requires both a site on fibronectin that binds to the cell surface and a second site that binds to collagen. The possibility that in the absence of serum the cells become unhealthy and therefore unable to respond to fibronectin in collagen lattice is eliminated, since

cells are able to respond on fibronectin coated filters. The fact that fibronectin is introduced into the culture medium and not the collagen lattice itself might have something to do with it; perhaps fibronectin is unable to diffuse into the collagen lattice. This seems unlikely because the addition of chondroitin sulphate in the same manner is able to exert an effect on cell mobility (see 7:3/4:3). The most likely explanation is that in the absence of a serum factor, collagen and fibronectin might interact in some way to mask adhesion sites necessary for cell attachment and subsequent movement.

[NOTE: The ability of limb mesenchymal cells to migrate on fibronectin-coated filters was observed in the course of studies designed to investigate the possible role of chemotaxis in this system, using Venkatasubramanian & Solursh (1984) methodology of sandwiching the filter between 2 chambers. The chemotactant is usually placed in the bottom chamber and the cells to be tested in the top. Much time was spent in attempting to set up the control with a suspension of limb mesenchymal cells without serum in the top well and culture medium without serum in the bottom well. Under such conditions in my experiments, mesenchymal cells attached to the filter and migrated through its pores with great speed. In contrast,

Venkatasubramanian had not observed any penetration of the filter. In order to prevent cells from moving through the filter with such speed, the pore size was reduced from 8 µm to 3 µm, cell concentration was reduced from 10^6 cells/ml to 10^5 cells/ml and incubation time cut from 6 hr to 4 hr but cells still came through the filter. Consequently, for a lack of a control, these experiments were abandoned, but these observations highlight the ability of fibronectin to promote cell attachment and migration].

In the chick limb, fibronectin is not uniformly distributed: very low concentrations are found in the myogenic regions while in their immediate surrounding they are relatively high (Tomasek _et al_, 1982 b; Kieny & Mauger, 1984). This is not unexpected, since other migratory cells have been shown to synthesise very little fibronectin; for example, neural crest (Newgreen & Thiery, 1980) and primordial germ cells (Heasman _et al_, 1981). Orientated fibronectin is capable of contact guiding limb myogenic cells (Turner _et al_, 1983) and it has been suggested that the migration of cells from the premuscular masses and subsequent splitting are directed by orientated fibronectin synthesized by the connective tissues (Chiquet _et al_, 1981). Although no orientated fibronectin tracts have been found in the limb bud, it

is not pure conjecture to suspect that cell traction is capable of propagating these tracks.

7:3/4:3 Migration in the presence of serum and hyaluronic acid

Two different concentrations of hyaluronic acid (0.5 mg/ml and 2 mg/ml) were tested in this part of the experiment. Neither concentrations had any influence on the spreading of explants or cell migration. In both cases, cells were equally efficient in producing aligned collagen bundles (used as an indicator of cellular activity) as those without hyaluronic acid (fig. 4). Once cells have migrated onto these aligned tracts, they become contact guided (fig. 4). This contrasts with Fisher & Solursh's (1979) findings that 2 mg/ml hyaluronic acid delayed cells from migrating out of limb explants. Sanders & Prasad (1983) found, on the contrary, 2 mg/ml hyaluronic acid enhances outgrowth of segmented somitic mesoderms. Concentrations as high as 20 mg/ml have been reported not to effect neural crest cell migration (Newgreen et al, 1982).

For a long time hyaluronic acid has been associated with mesenchymal cell dispersion (Toole, 1972, 1973). Hyaluronic acid is a negatively charged polymer that attracts positive anions (Oster et al, 1985); the ionic pressures created are sufficient to swell intercellular spaces. Although the results presented here did not show hyaluronic acid to have any

influence over the rate of cell movement, this does not mean that the molecule cannot play a permissive role by keeping intercellular spaces open and maintaining locomotion.

Singley & Solursh (1981) studied the distribution of hyaluronic acid in early chick limb buds; between stages 19-24 they found a progressive increase in the density of hyaluronic acid in the peripheral regions and a low density in the central core. During stage 26, hyaluronic acid concentration is reduced in the myogenic areas but not in the connective tissue-forming regions. This correlates well with the spatial distribution of myogenic precursors. In the wing, between stage 21-24 H.H., myogenic precursors are located in the central core (Solursh _et al_, 1985); by stage 25 H.H. these cells have moved out to the periphery, where they form the dorsal and ventral muscle anlagen. It may be that the initial high levels of hyaluronic acid in the peripheral regions promote myogenic cells to 'migrate' from the central core to the periphery by making more intercellular spaces available. Likewise, if, as has been suggested, after stage 26 H.H. hyaluronic acid is still present in the connective tissues mixed with collagen-like fibres (Singley & Solursh, 1981), this might further help to facilitate myogenic cell movement in the proximo-distal direction.

7:3/4:4 Migration in the presence of serum and chondroitin sulphate

Chondroitin sulphate has an inhibitory effect on cell locomotion. Cells in 1 mg/ml of chondroitin sulphate require 72 hr to generate traction lines (fig. 5a) comparable to those in 24 hr control cultures (fig. 2b). If the concentration of chondroitin sulphate is increased to 2 mg/ml, there will be further inhibition: compare figure 5a with 5b; the traction lines in 2 mg/ml cultures are much weaker than those in 1 mg/ml at the same stage of development. Although chondroitin sulphate delays the onset of cell movement, it does not appear to affect cell morphology. Cells on the surface of explants are typically bipolar in shape (fig. 6a). Chondroitin sulphate has also been shown to inhibit neural crest movement in collagen lattices (Tucker & Erickson, 1984).

Chondroitin sulphate and hyaluronic acid have been reported to have different effects on myogenesis of chick leg muscles in culture (Kujawa & Tepperman, 1983): chondroitin sulphate-coated dishes were shown to promote cell adhesion and myogenesis, while hyaluronic acid-coated plates allowed proliferation but inhibited differentiation. Along with my results, this may imply that hyaluronic acid has a role in maintaining cell proliferation and migration while chondroitin sulphate is associated with reduction in movement and differentiation?

7:3/4:5 Contact inhibition

Cells migrating in collagen do not appear to be contact inhibited (fig. 7). Time-lapse films showed that cells contacted one another freely without contact paralysis or change of direction. Cells have the ability to underlap and overlap other cells; however it must be stressed that the closeness of these overlapping cells relative to one another cannot be estimated, since the cells were observed in a single plane.

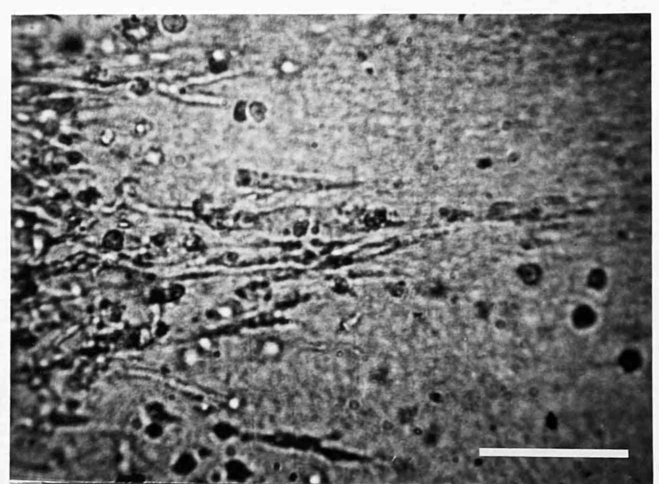
Abercrombie (1970) defined contact inhibition as the failure of a cell to continue moving in a direction that would carry it over the surface of another. Clearly, this does not appear to be the case with cells migrating in collagen lattices. Bard and Hay (1975) succeeded in observing one case of contact inhibition of cell movement following the collision of two fibroblasts in the cornea. In planar cell culture, contact inhibition is manifested as a cessation of protrusive activity and a local retraction of pseudopodia from the sites of collision (Abercrombie & Dunn, 1975). Cells in a collagen lattice, on the other hand, carry on migrating in the same direction following collisions simply by moving over or under the other cell. This difference in cell behaviour between conventional and collagen cultures may be explained in terms of availability of substrata. In a collagen lattice, direction of cell movement is flexible since substratum is available in three-dimensions; cells locomoting in

conventional cultures, however , are restricted , since substratum is available only on a single flat plane. Very little evidence is presently available regarding the contact-mediated control of mobility *in vivo*.

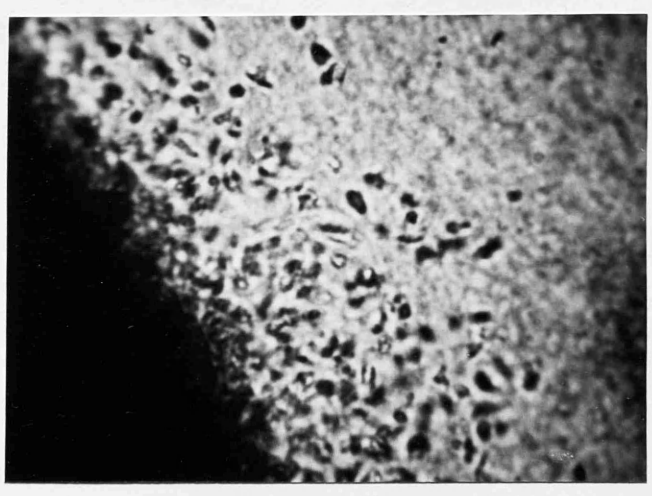
Figure 1

a A frame from a time-lapse film of normal mesenchymal cells migrating from a limb explant suspended in a collagen lattice with serum. Note that some cells are in focus while others are not. This results from cells emigrating from the limb explant at various depths within the three-dimensional substratum of collagen, after 48 hr of incubation. Bar = 50μm.

b A frame from a time-lapse film of a normal limb explant suspended in a collagen lattice without serum. Although some cells are found around the periphery of the explant, these cells are non-motile.



a



b

Figure 2

Normal single/double limb explants embedded in collagen lattices and cultured in the absence or presence of serum.

a Single explant culture, with serum after 48 hr. Note the presence of traction lines (arrows) which can stretch from the explant to the margins of the collagen lattice. Bar = 400 µm.

b Double explant cultures, with serum after 48 hr. Traction lines (arrows) are formed between explants and also around the borders of the explants.

c Single explant culture, without serum after 48 hr. No traction lines are present.

d Double explant cultures, without serum after 48 hr. No traction lines are present.

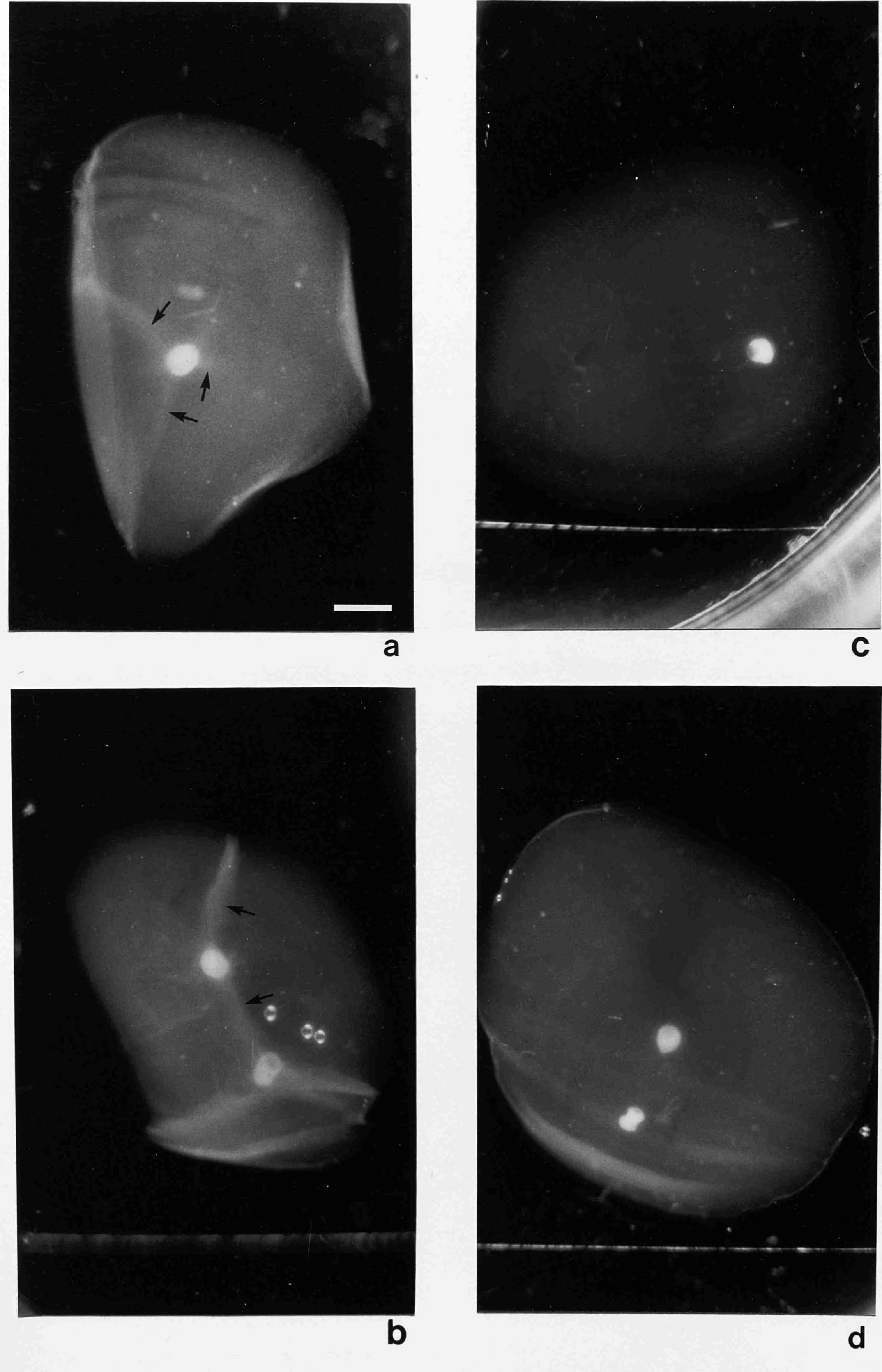
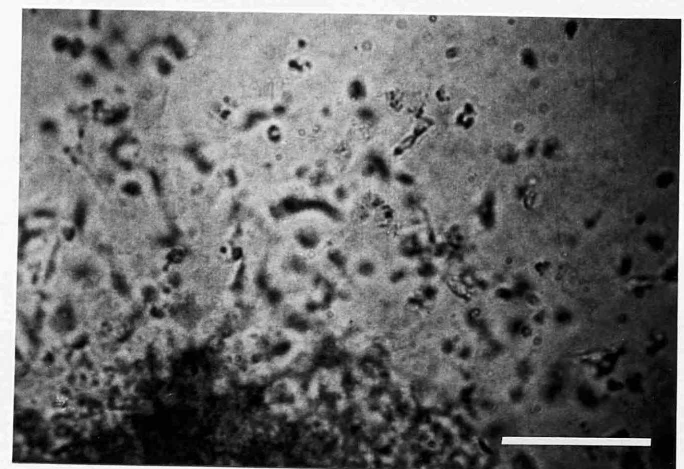

a
c
b
d

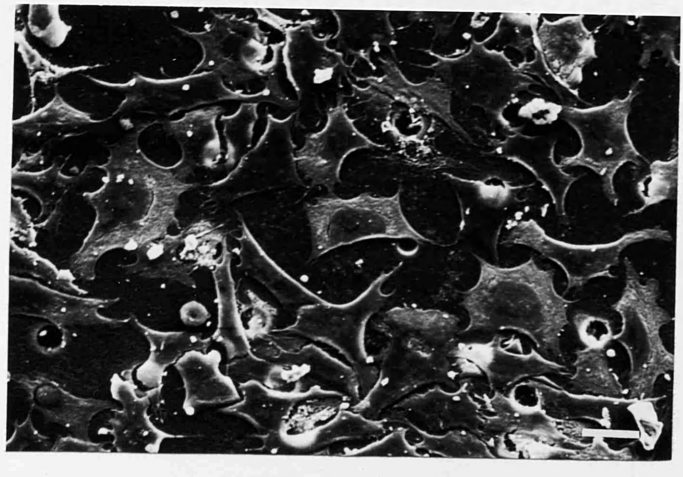
Figure 3

a A frame from a time-lapse film of a normal limb explant suspended in a collagen lattice without serum, but in the presence of 100 µg/ml fibronectin. Although cells are scattered around the explant these cells are non-motile. After 48 hr of incubation. Bar = 50 µm.

b SEM of the underside of a fibronectin coated nucleopore filter. Limb mesenchymal cells migrate through the pores on the filter, in the absence of serum. After 6 hr of incubation. Bar = 16 µm.



a

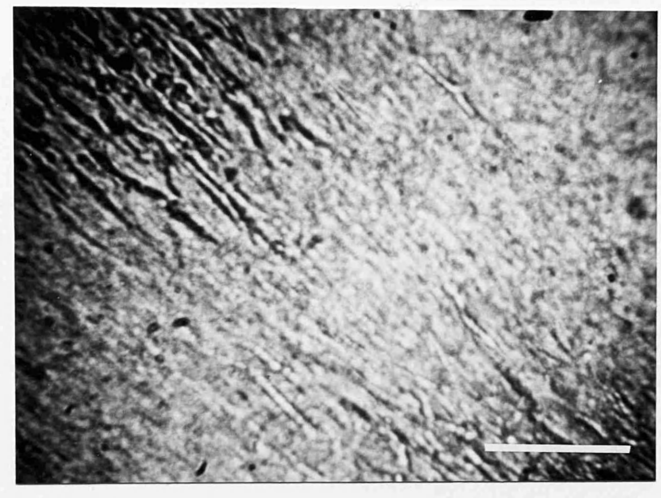


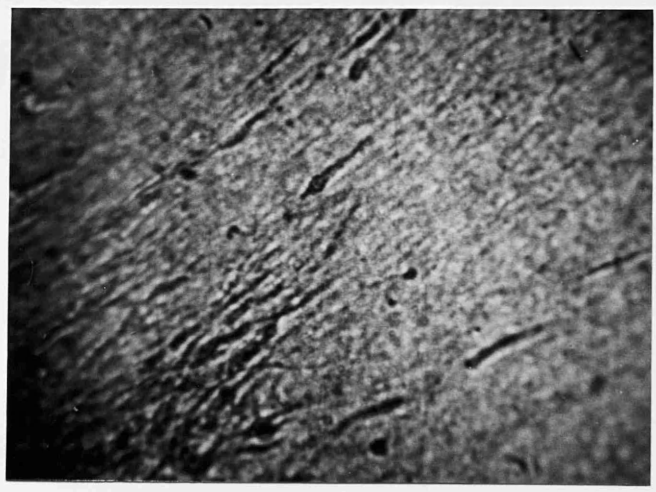
b

Figure 4

a A frame from a time-lapse film of normal limb mesenchymal cells migrating in-between 2 limb explants suspended in a collagen lattice, with serum and 0.5 mg/ml of hyaluronic acid. Cell locomotion is unaffected in the presence of hyaluronic acid. Region between 2 limb explants showing both the alignment of cells and collagen fibres. Bar = 50 μm.

b A frame from a time-lapse film, showing aligned collagen fibres with cells orientated along them. Cell movement is unaffected despite the presence of 2 mg/ml hyaluronic acid.





b

Figure 5

Double limb explants embedded in collagen lattices and cultured in the presence of serum and chondroitin sulphate (C.S.).

a Double explant cultures, with serum and 1 mg/ml of C.S. after 72 hr of incubation. [In the absence of C.S. (fig. 2b) it would normally take 48 hr to generate traction lines with intensity comparable to those shown in this figure. Therefore 1mg/ml of C.S. delays cell movement from limb explants for 24 hr]. Bar = 400 µm.

b Double explant cultures, with serum and 2mg/ml of C.S. after 72 hr of incubation. Formation of traction lines are further delayed with increasing concentration of C.S. Note the weakness in intensity of collagen tracts (arrows).

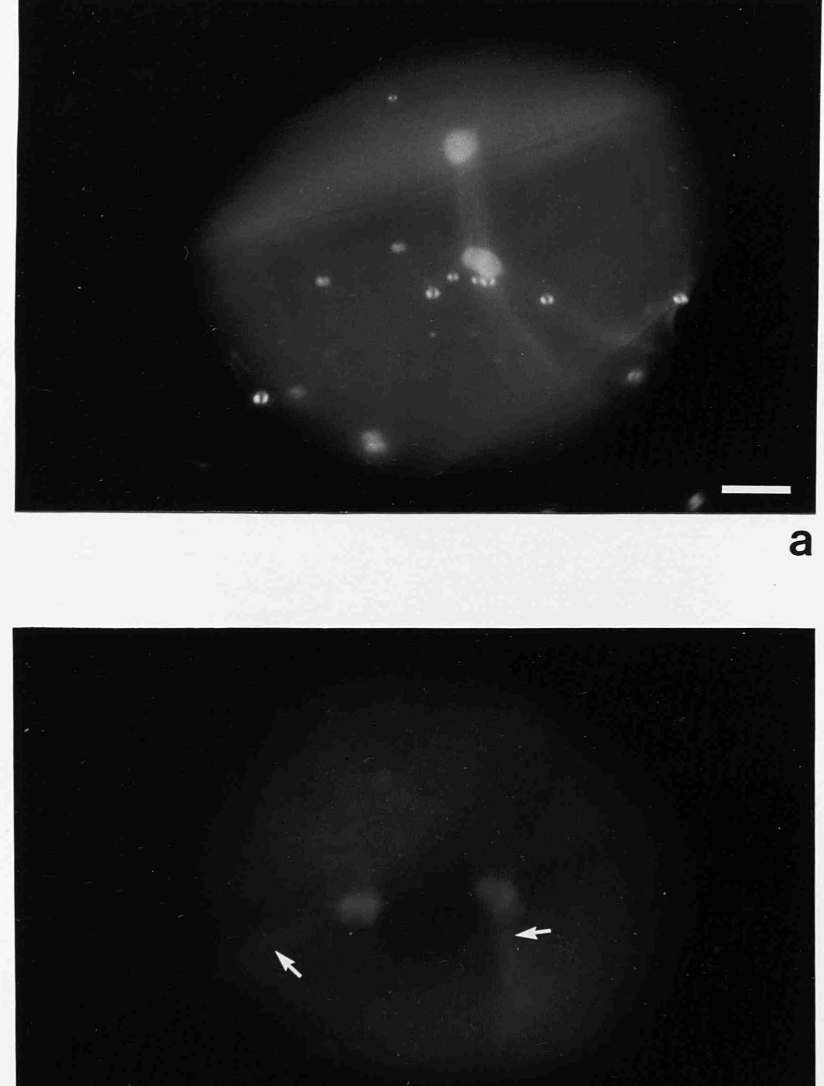
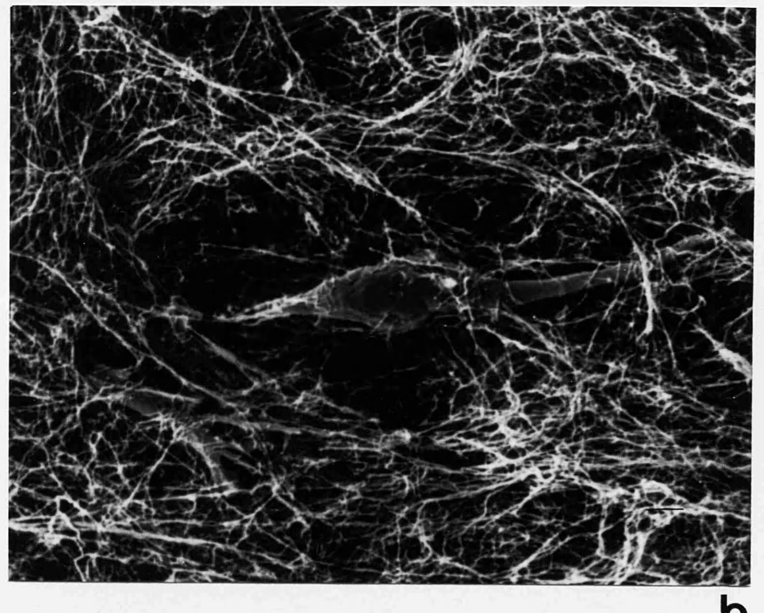

a
b

Figure 6

a SEM of the surface of a normal limb explant in the presence of serum and 2 mg/ml of chondroitin sulphate. Cell morphology is unaffected in the presence of chondroitin sulphate (800X). After 72 hr of incubation.

b SEM of a normal limb mesenchymal cell in the presence of serum and 2 mg/ml of chondroitin sulphate(800X).

a

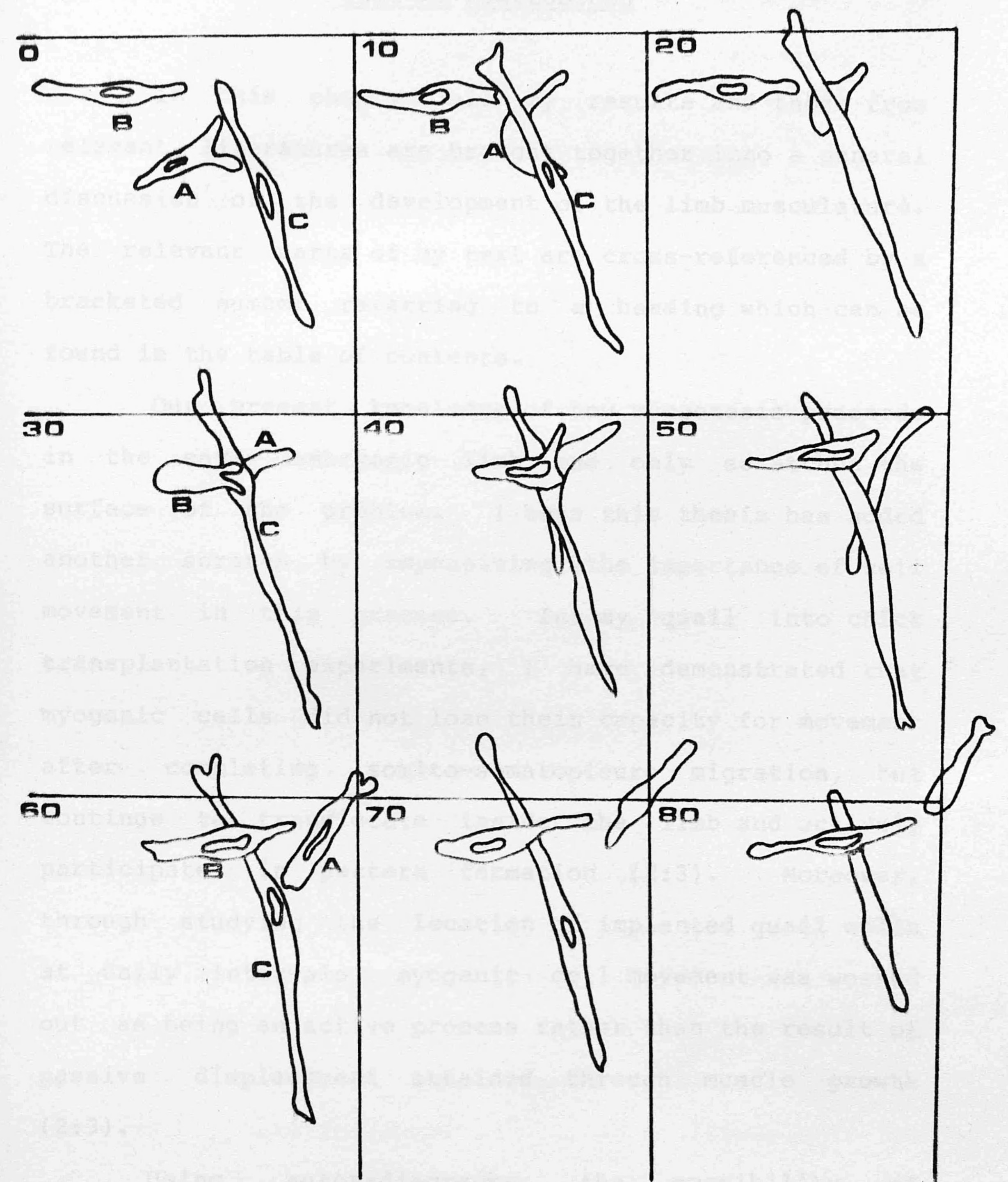


b

Figure 7

Tracing from time-lapse films of cells translocating inside a collagen lattice. The time sequence between each frame is 10 min. The lines are reference points.

0 min	Cell A is about to make contact with cell C.
10 min	Cell A underlaps cell C.
20 min	Cell B is about to make contact with cell C.
30 min	Cell B overlaps cell C.
50 min	Cell A starts to move away from cell C, pulling the main axis of cell C slightly to the right as it does so.
60 min	Cell A moves away from cell C. There appears to be a pause in the activity of cell B.


0
B
A
C
10
B
A
C
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30
A
B
C
40
50
60
B
A
C
70
80

CHAPTER VIII

GENERAL CONCLUSIONS

In this chapter all my results and those from relevant literatures are brought together into a general discussion of the development of the limb musculature. The relevant parts of my text are cross-referenced by a bracketed number referring to a heading which can be found in the table of contents.

Our present knowledge of how myogenesis proceeds in the early embryonic limb bud only scratches the surface of the problem. I hope this thesis has added another scratch by emphasizing the importance of cell movement in this process. In my quail into chick transplantation experiments, I have demonstrated that myogenic cells did not lose their capacity for movement after completing somito-somatopleure migration, but continue to translocate inside the limb and actively participate in pattern formation (2:3). Moreover, through studying the location of implanted quail cells at daily intervals, myogenic cell movement was worked out as being an active process rather than the result of passive displacement attained through muscle growth (2:3).

Using autoradiography, the possibility of myogenic cell movements being an artefact of heterospecific interaction between chick and quail cells

was eliminated when it was discovered that movements also occurred in homospecific chick into chick transplantations (3:3). It must however be said that autoradiography is not a good system for studying cell movement which requires long periods of incubation, because tritiated thymidine is subject to dilution (3:3). It is now abundantly clear that the limb bud is not a closed system but is invaded by a variety of cell types: pigment cells (Wachtler, 1984), axons (Stirling & Summerbell, 1977) and osteoclasts (Jotereau & Le Douarin, 1978).

The avian limb bud - and presumably limb buds in other systems - is made up of a loose mesh work of mesenchymal cells stacked up like heaps of corn flakes (6:3). The large amount of space between these cells make the limb especially fitted to supporting cell movement. Limb mesenchymal cells have the remarkable ability to squeeze through extremely narrow spaces - e.g. I have shown they can penetrate 3 µm nucleopore filters easily - through their ability to deform: myogenic cells will therefore have no problem in infiltrating limb tissues. Tickle (1982) suggested that cells migrate through limb tissues by pushing cells out of the way and if necessary breaking cell-to-cell contacts. Interestingly, in most of my transplantation studies there were always a few transplants which did not exhibit cell movement. In these cases the majority of the grafts were found behind chondrogenic elements:

this might imply that myogenic cells were prevented from moving because they found it difficult to push chondrogenic tissue aside, and this suggests that myogenic cells migrating through the limb do so along paths of least resistance.

Morphogenetic movement is apparently not only important in myogenesis but it is also important for establishing the overall shape of the limb bud. Ede & Law (1969) devised a model for normal limb growth by introducing various aspects of cell behaviour that they thought were important into a computer simulation. They pointed out that simulations based primarily on cell proliferation even in the form of a disto-proximal gradient, with increased cell division distally, cannot generate a model with sufficient elongation. In order to produce elongation a distalward cell movement component has to be included in the simulation. These authors, however, did not provide any experimental evidence that limb mesenchymal cells moved in a distalward direction inside the limb or specify what cell type was involved. My transplantation experiments supply this evidence, showing myogenic cells to migrate exclusively in a proximo-distal direction inside the limb bud and that only myogenic cells, and not somatopleure derivatives such as chondrocytes and connective tissue cells, are capable of locomotion (2:3, 3:3 and 5:3).

If cell movement is omitted from Ede and Law's

computer simulation a broad, short, fan-shaped model resembling a *talpid*^{3} limb is formed. Again, my results have provided more evidence in support of the model by demonstrating that when fragments of *talpid*^{3} wing mesenchyme were implanted into quail wing buds *in ovo*, *talpid*^{3} myogenic cells either failed to migrate or showed a reduced capacity for movement (5:3). In view of the connection between the model and my experimental results, it would be interesting to determine whether reduced myogenic mobility is responsible for the mutant's disturbed limb morphology.

Talpid^3 myogenic cells' inability to migrate inside quail limb buds is not the result of simple incapacity for movement. *In vitro*, *talpid*^{3} limb mesenchymal cells moved through collagen lattices as fast as their normal counterparts (6:3). This can only imply 2 things: (1) *talpid*^{3} and quail cells have interacted with each other in some way to prevent mutant cells from moving, (2) an integral part of cell locomotion *in vitro* might have escaped my methods of detection.

SEM observations of *talpid*^{3} limb mesenchymal cells revealed that *in vivo* these cells produce many more filopodia than normal cells, and since filopodia have been implicated with cellular adhesion (Ede & Flint, 1975a, 1975b), the larger numbers of these processes might offer mutant cells more potential sites for cell-to-cell adhesion. Perhaps *talpid*^{3} cells

stick more firmly to quail cells than would normal cells, and it is this which is the basis of the mutant's diminished mobility inside quail limb buds.

Ede & Flint (1975b) have shown that *talpid*^{3} limb mesenchymal cells *in vitro* spent a longer time at rest than normal cells, consequently over a period of time normal cells travelled significantly further than mutant cells. Unfortunately, because of technical problems this was not confirmed in collagen lattices and only periods of active movement were filmed, giving an imperfect record of the cell's capacity for movement over longer periods. I suggest that it is likely that a combination of increased cell-to-cell adhesion coupled with the mutant's longer rest periods are the factors responsible for *talpid*^{3} myogenic cells' relative immobility in quail wing buds.

Rather similar observation on cell movement has been made in the *amputated* mouse mutant in which morphogenesis is disturbed by abnormalities of cell-to-cell contact behaviour (Flint & Ede, 1978a, 1978b, 1982). Flint & Ede (1982) reported that *amputated* cells migrating from somite explants moved more slowly than normal cells because they spent more time at rest. Moreover, the *amputated* cells, like the *talpid*^{3} cells, appear to produce many more filopodia than the normal cell.

The fact that myogenic cells migrate inside the avian limb bud will have special implications on any

models formulated to explain patterning in the limb. Traditional models such as the zone of polarizing activity (Tickle _et al_, 1975; Summerbell & Tickle, 1977) and the polar coordinate model (Javois & Iten, 1981, 1982, 1986), based on the concept of positional information, are especially affected. According to these models pattern specification relies on the acquisition of a "positional address" according to a cell's physical location in the limb and interpretation of this "positional value" will lead to appropriate cytodifferentiation. It is difficult to see how such a system can operate with respect to muscle pattern since myogenic cells are actively migrating inside the limb bud and will therefore have "no fixed address". Nonetheless, the system might act on the chondrocytes and the connective tissue cells because these cells are relatively immobile and come from the same cell lineage (2:3 and 5:3).

In chapter 6, I discussed the tractional forces generated by moving limb mesenchymal cells, pointing out that the force is so strong that a collagen lattice , in which collagen fibrils are randomly orientated, becomes converted into highly aligned linear tracts between contending groups of mesenchymal cells. Such collagen tracts between explants of normal or _talpid_^{3} limb tissues _in vitro_ can be several centimetres long. It is important to understand that it is the tractional forces exerted by the limb mesenchymal cells, rather than

simple contraction which accompanies cell locomotion, which distorts the collagen substrata. Collagen fibrils can be pulled inwards past the cells for essentially unlimited distances - far greater than the length of a single mesenchymal cell (6:3 and 7:3).

Aligned collagen fibrils have a positive influence on the orientation and shape, and more importantly the directionality, of the migrant cells which generated these patterns in the first place. In sum, tractional forces can create a cascade of events in which not only the structure of the cells' surroundings is altered, but also the subsequent behaviour of migrant cells is determined. I believe this cascade of events arises during the somito-somatopleural migration of myogenic cells. The scenario probably proceeds like this: as myogenic cells start to migrate out of somites, they pull the collagen fibrils between the somites and the limb somatopleure into alignment, using the somatopleure as a fixed point for exerting traction. These aligned fibrils, which are known to exist _in vivo_ (Christ _et al_, 1986), in turn direct the myogenic cells to move into the limb somatopleure, probably through contact guidance.

Whether tractional forces have a role in limb myogenesis is still speculative. However, several models have recently been proposed to explain patterning in the limb based primarily on the physical properties of the extracellular matrix and on the ability of

tractional forces to generate large patterns (Oster *et al*, 1983, 1985). The connection between an absence of myogenic cell movement and of orientation in the *talpid*^{3} limb (5:3) suggests that there might be a role for tractional forces in normal muscle cell alignment.

Up to now, all available evidence suggests that it is the connective tissue which determines muscle patterning (Noden, 1983a, 1983b, 1984), probably mediated through extracellular matrices which the connective tissue cells synthesize. Hyaluronic acid and chondroitin sulphate, 2 molecules commonly found in the matrices, were tested for their competence to alter the behaviour of migrant cells in collagen lattices. Hyaluronic acid at concentrations of 0.5 mg/ml and 2 mg/ml neither enhanced nor inhibited limb mesenchymal cell movement (7:3). Chondroitin sulphate on the other hand inhibited cell locomotion in a graded manner (7:3).

Hyaluronic acid's influence is probably permissive, supporting cell movement by creating cell-free spaces (Solursh, 1976). It is now known that hyaluronic acid is distributed in a graded fashion along the proximo-distal axis of chick wing buds, with the highest accumulation beneath the AER. This is readily related to the fact that myogenic cells migrate exclusively in a proximo-distal direction (2:3). Chondroitin sulphate's ability to inhibit cell locomotion *in vitro* makes it an ideal candidate for signalling myogenic cells when to stop moving in the

limb, and since the molecule is mainly synthesized by chondrocytes, it might also serve to partition myogenic tissue from chondrogenic tissue by not allowing myogenic cells to penetrate the latter.

More than anything else in the culture medium, serum is vital for the support of cell locomotion in collagen lattices (7:3) and it has been suggested that fibronectin is the vital ingredient it provides in other *in vitro* cultures (Turner, 1983). In collagen lattices the most important ingredient in the serum with respect to cell movement is evidently not fibronectin, because in the absence of serum a supplement of fibronectin cannot restore cell movement in this system (7:3). This is puzzling, since fibronectin in the absence of serum can restore cell movement on nucleopore filters (7:3). My results consequently throw little light on the role of fibronectin, but it may still be important. It has been claimed that extracellular formation alone is sufficient to drive translocation by a biophysical process that may play a role in cellular migration during embryogenesis (Newman, 1985). Nonuniform collagen-fibronectin matrices can apparently entice chick limb mesenchymal cells to translocate from regions lacking in fibronectin to regions rich in fibronectin. The cells can travel several millimetres in a matter of minutes without expressing a morphology indicative of active locomotion, moreover nonliving particles also share this capacity. Newman (1985) called this

phenomenon "matrix-driven translocation". The fibronectin can apparently generate a driving force that can passively attract cells to move towards it.

Matrix-driven translocation has already been shown in avian embryos. For instance, if latex beads or cells with no intrinsic motile behaviour are injected into avian somites, they will become selectively guided along neural crest pathways (Bronner-Fraser, 1982, 1984, 1985). The introduction of fibronectin coated beads or cells capable of synthesizing fibronectin have the opposite effect and will not be induced to translocate (Bronner-Fraser, 1985). Coincidentally fibronectin is not uniformly distributed in avian limb buds (Tomasek _et al_, 1982b; Kosher _et al_, 1982). Myogenic regions specifically lack fibronectin while the surrounding tissues are rich in fibronectin. This would be just the right sort of environment to permit matrix-driven translocation. Whether or not this phenomenon has a role in myogenic cell movement remains to be investigated. My results suggest that if this mechanism does have a role, it will probably be acting through a balance of forces, with cell-to-cell interaction on one side and cell-to-matrix on the other.

This discussion and problems presented in this thesis suggest a number of areas for further investigation:

(1) Just how myogenic cells maintain their

proximo-distal directionality while in motion, in the limb, is still unclear. The AER has been implicated (Gumpel-Pinot _et al_, 1984; Christ _et al_, 1986) but the extact nature of its influence - whether chemotactic or through physical contact - remains to be elucidated. One possible way of finding out would be to transplant fragments of quail wing mesenchyme into chick wing buds and then partition the AER from the rest of the limb with barriers of different permeability. If myogenic cell movement failed to occur in the presence of an impermeable barrier, but occurred in the presence of a barrier that allowed diffusion but not cell-to-cell contact, then the AER's influence would have to be chemotactic.

(2) The location of myogenic cells between stages 18-25 H.H. in normal and _talpid_^{3} wing buds has not yet been established. It would now be possible to do so using desmin monoclonal antibodies. A comparison of the distribution of myogenic cells in normal and _talpid_^{3} embryos at these early stages might provide valuable clues as to how dorsal and ventral premuscular masses are formed.

(3) Why _talpid_^{3} myogenic cells are unable to migrate inside quail wing buds is still unclear.

Apparently it is not because *talpid*^{3} cells lacked the basic capacity for movement, but, as I have suggested, some form of increased cell-to-cell contact between *talpid*^{3} and quail cells. To decide whether this is indeed the case, I suggest labelling fragments of normal and *talpid*^{3} wing mesenchyme with tritiated thymidine and implanting them into quail wing buds *in ovo* ; then cutting thin sections of these chimaeric limbs at daily intervals to assess whether there are any quantitative differences in cell-to-cell contacts between *talpid*^{3}/quail and normal/quail cells, using autoradiography to identify donor cells.

(4) Cell-to-matrix interaction plays an important role in modulating the behaviour of migrant cells. A survey on how individual extracellular matrix components are codistributed with myogenic cells in normal and *talpid*^{3} limbs, using fluorescent antibodies, would therefore provide invaluable information on the function of the extracellular matrix with respect to myogenic cell movement.

(5) Finally the development of other organ systems in the *talpid*^{3} embryo obviously calls for further exploration. In particular, the development of

tongue muscles and branchiometric muscles; which all have somitic origins (Deuchar, 1958; Noden, 1983a). Information on how these myogenic cells interact with tissues in their surroundings would undoubtedly help to elucidate how myogenesis proceeds in general and within the limb bud.

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