J. Embryol. exp. Morph. **81**, 169–183 (1984)
Printed in Great Britain © *The Company of Biologists Limited 1984*

Development of the iris in the chicken embryo

II. Differentiation of the irideal muscles *in vitro*

By PATRICIA A. FERRARI AND WILLIAM E. KOCH

Department of Anatomy, School of Medicine, University of North Carolina, Chapel Hill, NC 27514, U.S.A.

SUMMARY

The developmental capabilities of the iris rudiment in the chicken embryo, as well as the role of tissue interactions in the differentiation of the iris, were investigated *in vitro*.

Sectors of the intact iris from 7½- through 9-day embryos (stages 32 through 35) lost their morphological organization *in vitro*, but were capable of normal histodifferentiation. The pigmentation of the epithelium increased, and muscle differentiation occurred. Developing muscle was identified using immunocytochemistry with antiserum against chicken muscle myosin; this procedure permitted positive identification of myoblasts, myotubes, and muscle fibres in cultures in which histological features alone were equivocal. The proportion of irideal explants which developed muscle increased with the age of the embryo, and correlated with the incidence of epithelial buds and epithelial cells in the stroma.

Irideal mesenchyme from stage-32 through stage-35 embryos was already populated with stromal epithelial cells when isolated, but growth and muscle differentiation in these cultures compared poorly with that in the intact iris *in vitro*. Isolated irideal epithelium (stages 32 through 37) demonstrated even more limited muscle differentiation *in vitro*, suggesting reciprocal interaction between irideal epithelium and mesenchyme during development. Irideal epithelium was also cultured in direct association with non-irideal mesenchyme from various embryonic organ rudiments, but muscle differentiation was not enhanced.

INTRODUCTION

Though the iris rudiment first becomes morphologically distinct with its outgrowth from the rim of the optic cup, its histodifferentiation is marked by the development of the sphincter and dilator pupillae muscles and by the pigmentation of the irideal epithelium. Descriptive studies of the differentiation of the sphincter and dilator muscles from the irideal epithelium (Brini, Porte & Stoeckel, 1964; Imaizumi & Kuwabara, 1971; Lai, 1972*a*,*b*; Tamura & Smelser, 1973; Ferrari & Koch, 1984), and of melanogenesis in the epithelial layers (El-Hifnawi & Hinrichsen, 1975) can offer only limited insight into the factors which operate during irideal differentiation. Nevertheless, in an ultrastructural study, El-Hifnawi (1977) observed mesenchymal cells contacting the posterior irideal epithelium during melanogenesis and concluded that differentiation of the epithelium relied upon interaction with mesenchyme. While those observations alone do not establish that epithelial melanogenesis depends upon mesenchymal influence, experimental studies of many organ systems have shown that

epithelial–mesenchymal interactions are important for tissue differentiation (see e.g. Koch, 1967; Lawson, 1974; Tyler, 1983). In addition, it has long been recognized that differentiation of some epithelia is supported by heterologous mesenchyme as well as homologous mesenchyme (Auerbach & Grobstein, 1958; Grobstein, 1968).

Previous experimental studies of iris development have addressed primarily the determination of the iris-ciliary body rudiment, which seems to require presence of the lens (McKeehan, 1961; Stroeva, 1963, 1967; Genis-Galvez, 1966). In Stroeva's experiments, pigmentation in the inner layer of the optic cup was taken as a definitive criterion of iris differentiation, though El-Hifnawi & Hinrichsen (1975), have reported features of melanogenesis in the inner layer at least 2 days prior to the outgrowth of the iris. These observations suggest that one aspect of irideal histodifferentiation, the pigmentation of the posterior epithelium, is initiated in the iris-ciliary body rudiment before the definitive iris rudiment is formed. This is supported by the fact that pigmentation in the inner epithelial layer is not a feature unique to the iris; this layer later becomes pigmented in a portion of the first ciliary process as well. For this reason, we chose to emphasize the development of the sphincter and dilator muscles as representative of irideal histodifferentiation.

In this study, the developmental capabilities of the iris rudiment and isolated epithelium and mesenchyme were examined *in vitro*. Identification of developing muscle was enhanced by using immunocytochemistry with myosin antiserum (Ferrari & Koch, 1984). Our findings from study of the intact iris and isolated irideal tissues suggest that epithelial–mesenchymal interactions function in the differentiation of the iris.

MATERIALS AND METHODS

Fertilized chicken eggs from a commercial hatchery were incubated at 37·7 °C in a humidified forced-draft incubator.

1. *Dissection procedures*

Chicken tissues

Eggs were cleaned with 80 % ethanol; the embryos were transferred to Tyrode's solution and staged according to their development (Hamburger & Hamilton, 1951). Only tissues from embryos with the same degree of eye development were used in a single experiment.

Irises were obtained from chicken embryos of $7\frac{1}{2}$ through 9 days incubation (stages 32 through 35); corneas were obtained from 8- and 9-day embryos (stages 33 and 35). The cornea was stripped from the eye using fine forceps, and transferred to a mixture of Tyrode's solution and horse serum (T/HS, 1: 1 by volume). Corneal mesenchyme was isolated by dissection and stored for later use in a chamber of 5 % CO_2 in air at room temperature. The remainder of the anterior

portion of the eye was removed intact and placed in T/HS. The anterior segment of the eye was hemisected by a cut passing through the choroid fissure, and before the halves were separated, a second cut was made bisecting each half. The resulting quadrants were designated *a* through *d* starting with the quadrant adjacent and temporal to the choroid fissure (*a*) and proceeding around the eye to the quadrant adjacent and rostral to the choroid fissure (*d*; Fig. 1). At this point, each quadrant included a portion of the iris, ciliary body, lens, and vitreous body (Fig. 2). The sectors of the iris were then carefully cut from the adjacent tissues and either reserved intact or placed into a cold (4 °C) solution of 3 % trypsin-pancreatin (3: 1 by weight; Difco Laboratories, Detroit, MI) in calcium- and magnesium-free Tyrode's solution (TP). The irideal epithelium and mesenchyme were separated in this solution with gentle manipulation and rinsed and stored in T/HS.

Mouse tissues

All mouse tissues were obtained from Brown Belt stock mice. Pregnancies were timed from the day a copulation plug was detected; this was designated as day 0 of gestation. On the appropriate day, a pregnant female was sacrificed by cervical dislocation. The uterus was isolated and placed into sterile Tyrode's solution. The embryos were then removed from the uterus and transferred to T/HS. Dissections were carried out in T/HS; mesenchyme and epithelium of the embryonic rudiments were separated in TP. Mesenchyme was isolated from 11-day metanephric rudiments, from 13-day submandibular salivary gland rudiments, and from mandibular incisors of 16-day rudiments. Tissues were stored in T/HS at room temperature in an atmosphere of 5 % CO_2 in air.

2. *Culture procedures*

The culture procedure was similar to that devised by Grobstein (1956). The culture assembly consisted of a disc of Millipore filter (THWP, 0·30 or 0·45 μm porosity, 25 ± 5 μm thick; Millipore Corp., Bedford, MA) cemented to one side of a plexiglass ring with two glass rods cemented to the opposite side of the ring. The sterilized assemblies were placed over the wells of a depression slide within a Petri dish. The tissue was placed upon a thin layer of congealed nutrient agar (1 % agar in an equal volume of the culture medium) which coated the filter surface. In some experiments, another thin layer of agar was added after the tissue was positioned. The culture medium (Moscona, 1962) consisted of Eagle's basal medium (Gibco, Grand Island, NY) supplemented with 1 % L-glutamine (200 mM, Gibco), 10 % foetal calf serum (Gibco), 3 % 11-day chicken embryo extract (Cameron, 1950) and 100 units/ml penicillin–streptomycin (Gibco). Each well of the depression slide was filled with medium so that the fluid level contacted the filter. The tissues were incubated at 37·5 °C in a humidified atmosphere of 5 % CO_2 in air. The culture medium was changed or supplemented with fresh medium every 48–72 h for the duration of the culture period, usually 7 or 8 days.

Culture of the intact iris

Sectors of the intact iris from 7½- through 9-day embryos (stages 32 through 35) were cultured *in vitro*. At stage 32, only quandrants *a* and *b* were explanted, whereas all quadrants from subsequent stages were used. The iris was positioned with either its epithelial (posterior) or its mesenchymal (anterior) surface contacting the agar substratum.

Culture of isolated irideal tissues

Irideal mesenchyme was isolated from 7½- through 9-day embryos (stages 32 through 35) and cultured *in vitro*. Isolated irideal epithelium from 7½- through 11-day embryos (stages 32 through 37) was also cultured.

Culture of irideal epithelium and non-irideal mesenchyme

Irideal epithelium from 7½- to 10-day chicken embryos (stages 32, 33, 35, and 36) was cultured in direct association with non-irideal mesenchyme from various sources. Mesenchyme for the combined cultures was isolated from corneas of 8- and 9-day chicken embryos (stages 33 and 35), and from the following organ rudiments in mouse embryos: metanephric kidney of the 11-day embryo; submandibular salivary gland of the 13-day embryo and mandibular incisor of the 16-day embryo.

Cultures of the isolated irideal epithelium and cultures of the isolated non-irideal mesenchyme from the mouse embryos served as controls for the recombination experiments.

3. *Histological procedures*

All cultures were fixed in Bouin's fluid (Lillie, 1954), dehydrated in graded ethanols, cleared in toluene, and embedded in Paraplast. Serial sections 5 μm thick were cut and stained with haematoxylin and eosin or stained immunocytochemically. The primary antiserum used for the immunocytochemistry was rabbit antiserum to chicken skeletal muscle myosin (rA-cMyosin; Antibodies Inc., Davis, CA). The immunocytochemical staining procedure and the specificity controls for the method and primary antiserum are described in an earlier report (Ferrari & Koch, 1984). As in that study, we refer to positive immunocytochemical staining as muscle protein immunoreactivity (MPI).

RESULTS

Though outgrowth of the iris begins by stage 30 (Ferrari & Koch, 1984), sectors of the iris were not large enough to adequately dissect from adjacent tissues prior to stage 32. At stages 32 and 33, only quadrants *a* and *b* could be isolated and maintained in culture. Beyond stage 33, all quadrants could be

isolated, but most cultures were set up with tissue from quadrants *a* or *b* to allow for more valid comparison with earlier stages. We did not culture iris or irideal mesenchyme from developmental stages greater than 9 days, or irideal epithelium from stages beyond 11 days, in order to ensure that there was no differentiation of muscle prior to explantation. In an earlier study on the development of the iris *in vivo* (Ferrari & Koch, 1984), muscle protein immunoreactivity in the developing sphincter muscle was evident by 11 days of incubation and in the developing dilator muscle by 13 days.

Differentiation of the intact iris in vitro

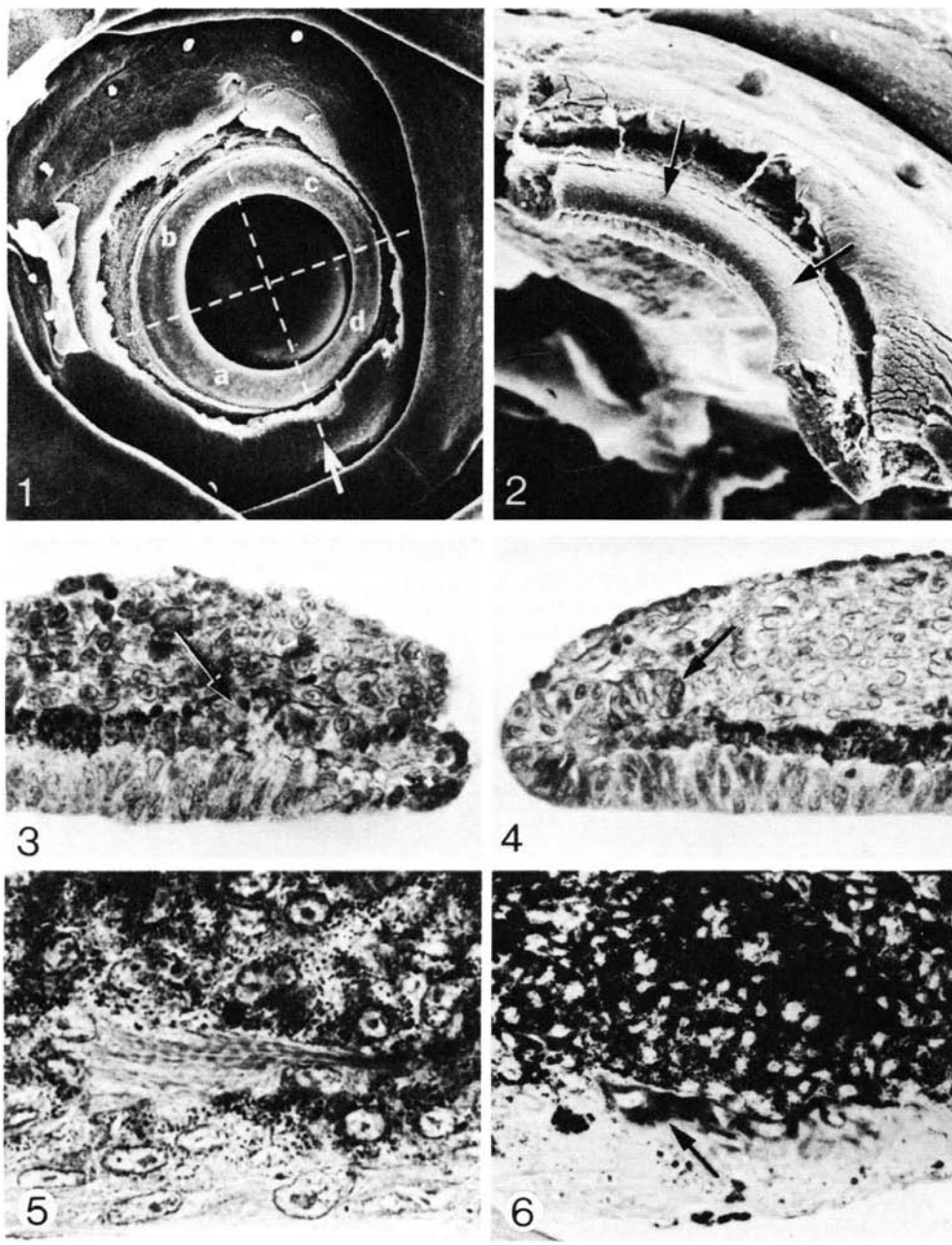
Sectors of the dissected iris at stages 32 through 35 were fixed and sectioned to serve as controls (Figs 3, 4). Beginning at stage 32 *in vivo*, foci of the anterior epithelium near the pupillary margin grow into the stroma forming epithelial buds (Ferrari & Koch, 1984). Just prior to bud formation, cells of the anterior epithelium at the site appear quite large and lie in disarray (Fig. 3). During subsequent stages, more epithelial buds form around the iris, and as they enlarge, they extend farther into the irideal stroma (Fig. 4). Melanogenesis continues in the anterior epithelium during this period (stages 32 through 35), but the posterior and marginal epithelium are still unpigmented at stage 35 (Fig. 4).

Table 1. *Differentiation of muscle in irideal tissues* in vitro

	Stage					
Tissue	32 (7½ day)	33 (7½–8 day)	34 (8 day)	35 (9 day)	36 (10 day)	37 (11 day)
A. *Intact iris*						
Total number of cultures	9	9	21	36		
Cultures with myotubes/MPI	3	7	17	35		
% Positive cultures	33%	78%	81%	97%		
B. *Isolated irideal mesenchyme*						
Total number of cultures	6*	7	14	34		
No. of cultures with myotubes/MPI	1†	3†	8	22		
% of cultures with myotubes/MPI	17%	43%	57%	65%		
C. *Isolated irideal epithelium*						
Total number of cultures	6	15	15	16	10	13
No. of cultures with myotubes/MPI	0	3	3	1	0	0
% of cultures with myotubes/MPI	0%	20%	20%	6%	0%	0%

* Six cultures were explanted, but three disintegrated and did not survive the culture period.
† Single cells demonstrating MPI.

The morphology of the iris did not persist *in vitro*. The epithelium in cultures from all stages usually lost its layered organization, though the pigmentation increased substantially (Figs 5 and 6). Cultures from each stage developed muscle with increasing frequency in explants from older embryos (Table 1, A). Striated fibres were identified in cultures stained routinely (Fig. 5) or using immunocytochemistry (Fig. 6). Myotubes were most evident following



immunocytochemical staining, but occasionally myotubes were seen which were not positive for MPI. These myotubes were usually small and seen in cultures from earlier stages. In general, MPI was present in the periphery of small myotubes and throughout the cytoplasm in larger ones. Very large myotubes with abundant cytoplasm were common in explants from stage 35, and these could be identified easily following routine staining (Fig. 7). Large myotubes were less abundant in stage-34 explants and rare from stage-32 or stage-33 explants.

Differentiation of irideal mesenchyme in vitro

Sectors of isolated irideal mesenchyme were fixed and sectioned to serve as controls (Figs 8, 9). At early stages, it was difficult to identify stromal epithelial cells (Fig. 8), but at later stages, epithelial cells were present throughout the stroma (Fig. 9). The development of irideal mesenchyme *in vitro* also varied with the age of the explant. Mesenchyme from stage-32 and -33 (7½- to 8-day) embryos failed to develop; the explants progressively diminished in size and often completely disintegrated. The explants from stages 32 and 33 did not develop myotubes or muscle, although four cultures contained single cells demonstrating MPI (Table 1, B, and footnote b). Explants of isolated mesenchyme from stage-34 and -35 embryos usually survived the culture period intact, and in most cases there was differentiation of muscle. Nevertheless, significantly fewer cultures of isolated mesenchyme developed muscle compared to explants of the intact iris (Table 1, A and B). Myotubes were identified by morphological characteristics in conventionally stained sections (Fig. 10) or by immunocytochemistry (Fig. 11).

Note: Tissue preparation for the scanning electron micrographs (Figs 1 & 2) is outlined in Ferrari & Koch, 1984.

Fig. 1. Scanning electron micrograph showing the right eye of a 10-day (stage-36) chick embryo following removal of the cornea. The approximate position of the choroid fissure is indicated (arrow); dotted lines delineate the quadrants formed after further dissection. These are labelled *a* through *d*, starting with the quadrant (*a*) adjacent and temporal to the choroid fissure and proceeding around the eye to the quadrant (*d*) adjacent and rostral to the choroid fissure. ×22.

Fig. 2. Scanning electron micrograph of a quadrant of the anterior segment of the eye of a 9-day (stage-35) embryo. The cornea and lens have been removed; the iris is indicated by the arrows. ×62.

Fig. 3. Histological section of the iris from a 7½-day (stage-33) embryo. The anterior epithelium near the pupillary margin has lost its epithelial organization (arrow). ×460.

Fig. 4. Histological section of the iris from a 9-day (stage-35) embryo clearly showing an epithelial bud (arrow). ×460.

Fig. 5. Histological section of an iris from a 7½-day (stage-33) embryo after one week *in vitro*. A striated muscle fibre is evident in the centre of the field. ×1140.

Fig. 6. Immunoperoxidase-stained section of a culture of the iris from a 7½-day (stage-33) embryo. The stained fibre (arrow) is multinucleated and lies in close proximity to the pigmented epithelium. Antiserum: rA-cMyosin; dilution: 1:5000. ×460.

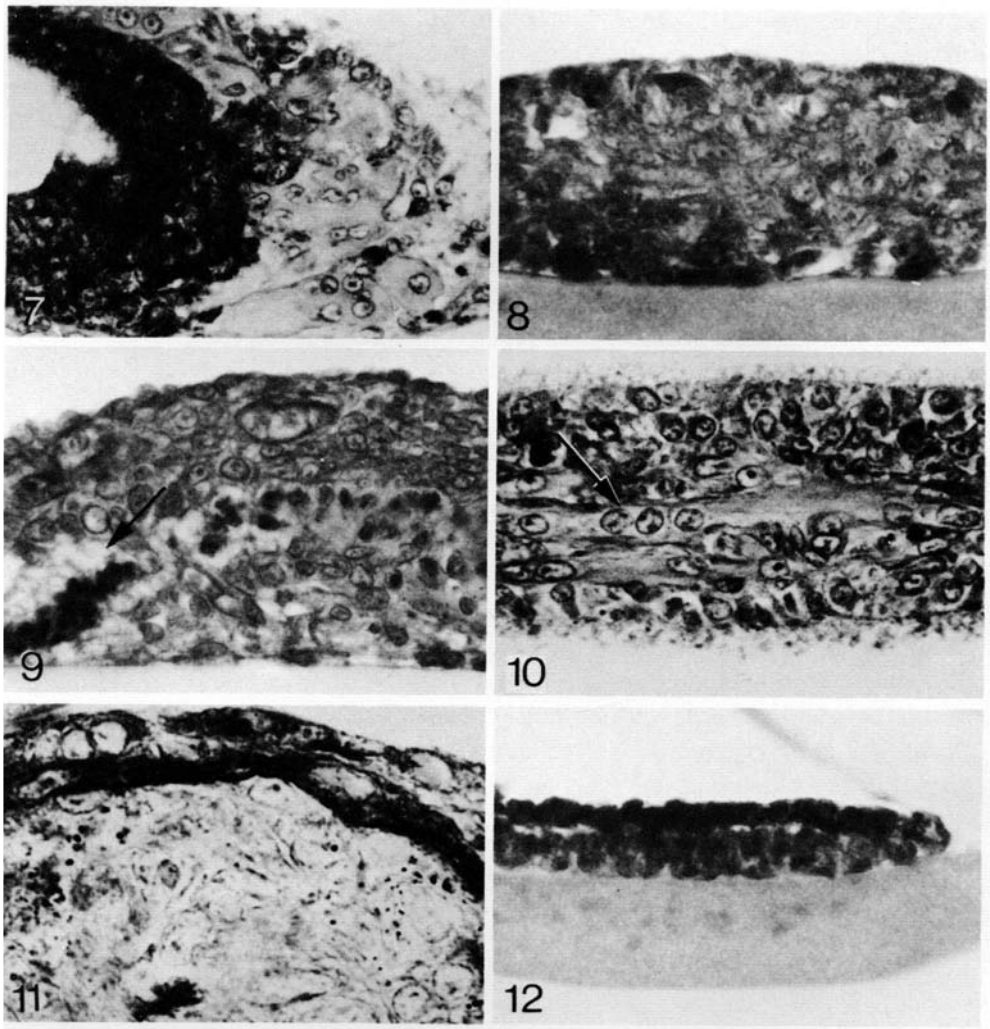


Fig. 7. Photomicrograph showing a culture of the iris from a 9-day (stage-35) embryo. Many large myotubes are seen in cross section. ×460.

Fig. 8. Histological section of isolated irideal mesenchyme from a 7½-day (stage-32) chick embryo prior to culture. ×600.

Fig. 9. Isolated irideal mesenchyme from a 9-day (stage-35) chick embryo before culture. An epithelial bud is indicated by the arrow; the centrally located stromal epithelial cells have darkly stained nuclei. ×600.

Fig. 10. Histological section of a culture of isolated mesenchyme from an 8-day (stage-34) embryo. Myotubes are shown at the arrow. ×540.

Fig. 11. Immunoperoxidase-stained preparation of cultured irideal mesenchyme from a 9-day (stage-35) embryo. A stained fibre is obvious in the upper portion of the field. The round granules at the extreme left and right sides of the field are pigment granules. Antiserum: rA-cMyosin; dilution: 1:10000. ×1140.

Fig. 12. Histological section of isolated irideal epithelium from a 7½-day (stage-32) embryo prior to culture. The tissue rests upon a Millipore filter. ×466.

Most cultures also contained small areas of sparsely pigmented cells (Fig. 11), but this was not unexpected since some of the epithelial cells populating the irideal stroma at the time of explantation included pigment granules (Fig. 9).

Differentiation of irideal epithelium in vitro

The complete separation of irideal epithelium from the stroma was assessed during dissection, and also documented histologically in control sectors of isolated irideal epithelium (Fig. 12). Cultures of isolated irideal epithelium from

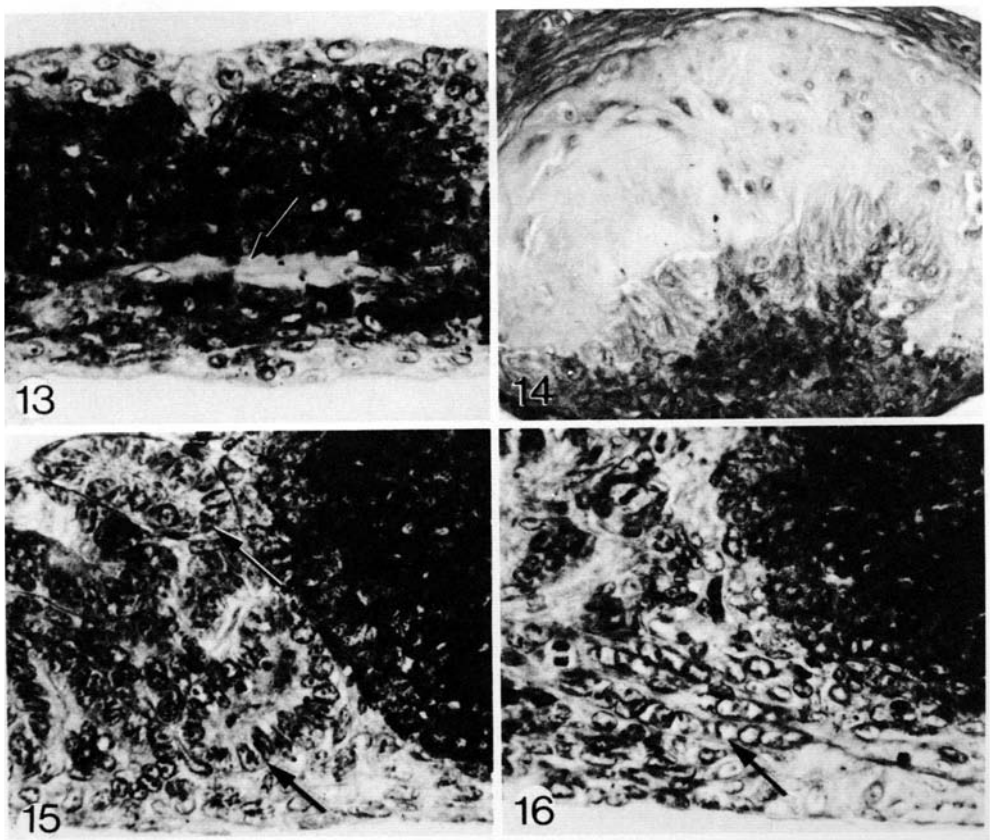


Fig. 13. Histological section of a culture of irideal epithelium from a 7½-day embryo. A myotube is shown at the arrow. ×620.

Fig. 14. Section of a culture of irideal epithelium with corneal mesenchyme; both tissues are from a 9-day (stage-35) chick embryo. Note the abundant extracellular material produced by the corneal mesenchyme. ×370.

Fig. 15. Histological section through a culture of irideal epithelium from a 9-day (stage-35) chick embryo with renal mesenchyme from an 11-day mouse. Nephrogenic tubules (arrows) were formed in the mesenchyme. ×430.

Fig. 16. Photomicrograph of a culture of irideal epithelium from a 9-day (stage-35) chick embryo with mesenchyme from the salivary gland of a 16-day mouse. Myotubes (arrow) are visible in the mesenchyme. ×430.

7½- through 11-day embryos (stages 32 to 37) usually remained healthy and showed significant increases in size and pigmentation. The epithelium from certain stages was capable of limited muscle differentiation (Table 1, C). Myotubes were demonstrated in explants from stages 33 and 34 and in a single culture from stage-35 tissue. Occasionally large myotubes with multiple nuclei were evident (Fig. 13), but most myotubes were small, and could be identified only by MPI. Myotubes or muscle were never seen in explants from stages 32, 36, or 37.

Differentiation of irideal epithelium and non-irideal mesenchyme in vitro

Isolated irideal epithelium from 7½- to 10-day embryos (stages 32, 33, 35 and 36) was cultured in direct association with non-irideal mesenchyme from chick cornea and murine incisor, kidney and salivary gland. The non-irideal mesenchyme was also cultured in isolation to serve as controls for the combined explants. All cultures were assessed for the presence of muscle.

In the recombinant cultures, both the irideal epithelium and the non-irideal mesenchyme from all sources remained healthy, and increased in size during the culture period. As in the cultures of irideal epithelium alone, the irideal epithelium in the recombinant cultures became darkly pigmented and usually did not maintain an epithelial organization apparent in sections (Figs 14–16).

The combined explants of irideal epithelium with mesenchyme from chick cornea or murine incisor or kidney never developed myotubes or muscle, and in general, the effect of the recombination was more apparent on the development of the mesenchyme. In the 16 explants of irideal epithelium combined with corneal mesenchyme, the mesenchyme produced abundant extracellular material (Fig. 14). The amount of extracellular material in control cultures of isolated corneal mesenchyme appeared unchanged during the culture period. Incisor mesenchyme was explanted with irideal epithelium in 12 cultures, and the mesenchyme remained healthy and increased in size but without differentiation. In contrast, isolated incisor mesenchyme in control cultures was poorly maintained *in vitro* and diminished in size. The control cultures of isolated renal mesenchyme also failed to develop *in vitro*, but when renal mesenchyme was combined with irideal epithelium, kidney tubules formed in the mesenchyme in all 12 cultures (Fig. 15).

Irideal epithelium was also cultured with mesenchyme from the murine salivary gland rudiment. In 15 of 26 explants, the mesenchyme contained myotubes which could be identified histologically (Fig. 16) or by immunocytochemistry. However, myotubes also developed in four of seven control cultures of isolated salivary mesenchyme. In many cases, living cultures of salivary gland mesenchyme alone or combined with irideal epithelium could be observed to contract. Contractile activity was never observed in any irideal cultures which did not contain salivary mesenchyme.

DISCUSSION

The results of these studies indicate that the intact iris rudiment will grow and differentiate *in vitro*, but without retaining the same level of organization that is present *in vivo*. Nevertheless, the pigmentation of the epithelium increases and, with increasing frequency in older explants, muscle differentiation occurs. These experiments do not establish when determination of the iris occurs, i.e. when the tissues first exhibit an inherent ability to differentiate. Though the pigmentation of the inner layer of the optic cup is a criterion which has been used in some studies to indicate the determination of the iris (Stroeva, 1963, 1967) it has never been shown that the formation of the iris rudiment, and its subsequent differentiation, necessarily follows that step. Melanogenesis occurs in the inner epithelial layer of the iris-ciliary body rudiment prior to outgrowth of the iris (El-Hifnawi & Hinrichsen, 1975) and, in the adult chicken, the inner epithelium of both the iris and a portion of the first ciliary process are pigmented (Ferrari, personal observation). Thus pigmentation of this layer is not a unique feature of irideal determination or histodifferentiation, unlike the differentiation of the sphincter and dilator muscles from the irideal epithelium.

In this study, 7½-day embryos were the youngest from which the intact iris rudiment was isolated, and one third of these explants formed muscle *in vitro*. The proportion of cultures which formed muscle progressively increased with explants from older embryos. This pattern corresponds to the increase in size and distribution of epithelial buds over the same period, and it seems likely that the ability of the irideal explant to form muscle *in vitro* is related to the incidence of buds and stromal epithelial cells.

Explants of isolated irideal mesenchyme also showed features of differentiating muscle at each age explanted, though a lower proportion of positive cultures was seen when compared with explants of the intact iris. Furthermore, there were fewer myotubes and immunoreactive cells in the cultures of isolated mesenchyme than in the cultures of the intact iris. It is possible that during culture of the intact rudiment the epithelium continually contributes cells which differentiate into muscle, and the more extensive differentiation of muscle in the intact rudiment *in vitro* may be due to the fact that there were more muscle cell precursors. Other differences between the cultures of the isolated mesenchyme and the intact rudiment indicate that the mesenchyme requires the presence of the epithelium for its maintenance as a tissue. At early stages, irideal mesenchyme frequently was not maintained *in vitro*; the tissue simply disintegrated during the culture period. Since quadrants of the iris vary greatly in size between 7½- and 9-day embryos, the mass of the younger explants was increased by pooling mesenchyme, but without improvement in survival or differentiation. It is likely that more cultures from older embryos remained intact not because of the larger size of the original explant, but because of the presence of more stromal epithelial cells. The epithelium of the iris and the stromal epithelial cells seem

to support the maintenance of the mesenchyme as a tissue, while at the same time, the mesenchyme enhances the differentiation of muscle.

The latter point was considered by examining the ability of the irideal epithelium to give rise to muscle in the absence of the irideal mesenchyme. Since it is known from the study of various embryonic organs that epithelia are dependent upon an association with mesenchyme for continued development (Grobstein, 1968), it was unexpected that isolated epithelial cultures would form muscle *in vitro*. However, very few of the cultures developed muscle, and then only from embryos between stages 33 and 35. It remains unclear why the epithelium at only certain stages expressed the capability for muscle differentiation in the absence of mesenchyme. One possibility is that the epithelium itself changes after stage 35, and develops an absolute requirement for mesenchyme. It is also possible that the dissection and separation of the tissues changes with the growth of the iris and the enlargement of the epithelial buds, allowing more complete separation of the ridge of the sphincter muscle precursor cells from the epithelial layer at later stages. Dissection alone would not account for the complete failure to develop muscle beyond stage 35, since the dilator muscle differentiates from the entire anterior epithelium. In either case, it is clear that the irideal mesenchyme has an important effect upon the differentiation of muscle in the iris. Though the epithelial cultures remained healthy in the absence of mesenchyme, the proportion which developed muscle was very low compared to cultures of the intact iris or of mesenchyme alone.

The most direct test for interactions of the epithelium and mesenchyme in the development of an organ would be to recombine both tissues *in vitro*. However, since the irideal mesenchyme already possesses a population of epithelial cells, the differentiation of these stromal epithelial cells would mask any response of the epithelium to the mesenchyme. Consequently, muscle differentiation was assessed by culturing irideal epithelium with non-irideal mesenchyme from chick and mouse embryos. Numerous studies have established that morphogenesis and histogenesis can be supported *in vitro* by recombining epithelium and mesenchyme from different organs within the same species (e.g., chick-chick recombinations, see Coulombre & Coulombre, 1971; Tyler, 1983) or from different species (e.g., chick-mouse recombinations, see Houissant & Le Douarin, 1968; Coulombre & Coulombre, 1971; Kollar & Fisher, 1980; chick-rabbit recombinations, see Propper, 1969). In testing the tissue requirements for differentiation in irideal epithelium, non-irideal mesenchyme was chosen from both chick and mouse embryos.

Chick corneal, and murine incisor and metanephric mesenchyme were chosen for the recombination experiments because each supports the differentiation of an epithelium that does not form muscle *in vivo*. Corneal and incisor mesenchyme are derivatives of neural crest and thus similar to irideal mesenchyme which is also derived from neural crest (Johnston *et al.*, 1979). Corneal mesenchyme was also chosen for its ability to form abundant collagenous extracellular

material, since it has been suggested that components of extracellular matrices influence muscle differentiation *in vitro* (Konigsberg, 1970; Ketley, Orkin & Martin, 1976; Yamada, Olden & Hahn, 1980). In each of these cases, the non-irideal mesenchyme failed to induce muscle differentiation in the irideal epithelium. Nevertheless, other interactions between the recombined tissues were observed. Irideal epithelium enhanced the growth of incisor mesenchyme and supported tubule formation in kidney mesenchyme. Grobstein & Parker (1958) have reported that metanephrogenic mesenchyme will form tubules when grafted into the anterior chamber of an adult eye, but neither iris nor cornea from the adult eye has been shown to support tubule formation *in vitro* (Grobstein, 1955). However, kidney mesenchyme does form tubules *in vitro* in response to embryonic dorsal spinal cord (Auerbach & Grobstein, 1958) and like the spinal cord, the irideal epithelium is derived from a neuroepithelium. Murine salivary gland mesenchyme was selected for recombination because of its association with and possible influence upon an epithelium which forms myoepithelial cells *in vivo*. It has not previously been reported that isolated salivary mesenchyme forms muscle *in vitro*, but in this study, myotubes were observed in most explants of salivary mesenchyme whether cultured alone or with irideal epithelium. Other studies of isolated salivary mesenchyme from 13-day mouse embryos (Grobstein, 1953) or from 14-day mouse embryos (Lawson, 1974) have not reported the formation of myotubes. In the present study, muscle differentiation may have been enhanced by the agar substratum which prevented spreading of the explant. Lawson (1974) used a similar substratum, but he cultured salivary mesenchyme from the 14-day mouse embryo. It may be that the capability for myotube formation in isolated salivary gland mesenchyme does not extend beyond the 13-day embryo, or simply that in Lawson's study too few cultures were examined to detect a limited capacity for muscle differentiation. In any case, murine salivary gland mesenchyme was apparently unaffected by culture with irideal epithelium, whereas the development of chick corneal mesenchyme and murine incisor mesenchyme was enhanced. In the case of murine metanephric mesenchyme, the irideal epithelium clearly permitted the differentiation of kidney tubules. Despite these interactions, the non-irideal mesenchyme that we tested did not support differentiation of irideal muscle.

In conclusion, these experiments establish that even in the absence of normal morphogenesis, the intact iris is capable of histodifferentiation *in vitro*. Neither isolated irideal mesenchyme nor irideal epithelium demonstrated the capability for muscle differentiation exhibited by the intact iris, and this suggests that epithelial-mesenchymal interactions function in irideal differentiation *in vitro*.

The authors are grateful to Ms Daynise Skeen for technical assistance. This investigation was supported in part by USPH Research Grant DE 04580 from the National Institute of Dental Research.

REFERENCES

AUERBACH, R. & GROBSTEIN, C. (1958). Inductive interaction of embryonic tissues after dissociation and reaggregation. *Expl Cell Res.* **15**, 384–397.

BRINI, A., PORTE, A. & STOECKEL, M. E. (1964). Développement ultrastructural des muscles iriens chez l'embryon de poulet. *Bull. Mem. Soc. Fr. Ophthalmol.* **77**, 488–497.

CAMERON, G. (1950). *Tissue Culture Technique*. 2nd Edition, p. 55. New York: Academic Press.

COULOMBRE, J. L. & COULOMBRE, A. J. (1971). Metaplastic induction of scales and feathers in the corneal anterior epithelium of the chick embryo. *Devl Biol.* **25**, 464–478.

EL-HIFNAWI, E. (1977). Interaction between mesenchymal cells and the posterior iris epithelium in chick embryos. *Anat. Embryol.* **151**, 109–118.

EL-HIFNAWI, E. & HINRICHSEN, K. (1975). Melanogenesis in the pigment epithelium of chicken embryos. I. Topogenesis of pigment in the iris anlage. *Anat. Embryol.* **147**, 177–187.

FERRARI, P. A. & KOCH, W. E. (1984). Development of the iris in the chicken embryo. I. A study of growth and histodifferentiation utilizing immunocytochemistry for muscle differentiation. *J. Embryol. exp. Morph.* **81**, 153–167.

GENIS-GALVEZ, J. M. (1966). Role of the lens in the morphogenesis of the iris and cornea. *Nature* **210**, 209–210.

GROBSTEIN, C. (1953). Epithelio-mesenchymal specificity in the morphogenesis of mouse submandibular rudiments *in vitro*. *J. exp. Zool.* **124**, 383–413.

GROBSTEIN, C. (1955). Tissue interaction in the morphogenesis of mouse embryonic rudiments *in vitro*. In: *Aspects of Synthesis and Order in Growth*, (ed. D. Rudnick), pp. 233–256. Princeton: Princeton University Press.

GROBSTEIN, C. (1956). Trans-filter induction of tubules in mouse metanephrogenic mesenchyme. *Expl Cell Res.* **10**, 424–440.

GROBSTEIN, C. (1968). Developmental significance of interface materials in epitheliomesenchymal interaction. In: *Epithelial-Mesenchymal Interactions*, (ed. R. Fleischmajer & R. E. Billingham,), pp. 173–176. Baltimore: Williams and Wilkins.

GROBSTEIN, C. & PARKER, G. (1958). Epithelial tubule formation by mouse metanephrogenic mesenchyme transplanted *in vivo*. *J. natn. Cancer Inst.* **20**, 107–119.

HAMBURGER, V. & HAMILTON, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49–92.

HOUISSANT, E. & LE DOUARIN, N. (1968). La differenciation de l'endoderme hepatique de poulet associe au mesenchyme metanephritique de l'embryon de souris. *C.r. hebd. Séanc Acad. Sci., Paris* **267**, 201–202.

IMAIZUMI, M. & KUWABARA, T. (1971). Development of the rat iris. *Invest. Ophthalmol.* **10**, 733–744.

JOHNSTON, M. C., NODEN, D. M., HAZELTON, R. D., COULOMBRE, J. L. & COULOMBRE, A. J. (1979). Origins of avian ocular and periocular tissues. *Expl Eye Res.* **29**, 27–43.

KETLEY, J. N., ORKIN, R. W. & MARTIN, G. (1976). Characterization of chick embryo skeletal muscle collagen. *Expl Cell Res.* **99**, 261–268.

KOCH, W. E. (1967). *In vitro* differentiation of tooth rudiments of embryonic mice. I. Trans-filter interaction of embryonic incisor tissues. *J. exp. Zool.* **165**, 155–170.

KOLLAR, E. J. & FISHER, C. (1980). Tooth induction in chick epithelium: Expression of quiescent genes for enamel synthesis. *Science* **207**, 993–995.

KONIGSBERG, I. R. (1970). The relationship of collagen to the clonal development of embryonic skeletal muscle. In: *Chemistry and Molecular Biology of the Intercellular Matrix*, Vol. 3, (ed. E. A. Balazs), pp. 1779–1810. New York: Academic Press.

LAI, Y.-L. (1972*a*). The development of the sphincter muscle in the iris of the albino rat. *Expl Eye Res.* **14**, 196–202.

LAI, Y.-L. (1972*b*). The development of the dilator muscle in the iris of the albino rat. *Expl Eye Res.* **14**, 203–207.

LAWSON, K. A. (1974). Mesenchyme specificity in rodent salivary gland development: The response of salivary epithelium to lung mesenchyme *in vitro*. *J. Embryol. exp. Morph.* **32**, 469–493.

LILLIE, R. D. (1954). *Histopathologic Technic and Practical Histochemistry*, p. 46. New York: Blakiston.

MCKEEHAN, M. S. (1961). The capacity for lens regeneration in the chick embryo. *Anat. Rec.* **141**, 227–230.

MOSCONA, A. A. (1962). Analysis of cell recombination in experimental synthesis of tissues *in vitro*. *J. cell. comp. Physiol.* **60**, 65–80.

PROPPER, A. (1969). Competence de l'epiderme embryonnaire d'Oiseau vis-a-vis de l'inducteur mammaire mesenchymateux. *C.r. hebd. Séanc Acad. Sci., Paris*. **268**, 1423–1426.

STROEVER, O. G. (1963). The role of the lens epithelium in the induction of iris and ciliary body tissue. *Dokl. Acad. Nauk. SSSR* **151**, 977–980.

STROEVA, O. G. (1967). The correlation of the processes of proliferation and determination in the morphogenesis of iris and ciliary body in rats. *J. Embryol. exp. Morph.* **18**, 269–287.

TAMURA, T. & SMELSER, G. K. (1973). Development of the sphincter and dilator muscles of the iris. *Arch. Ophthalmol.* **89**, 332–339.

TYLER, M. S. (1983). Development of the frontal bone and cranial meninges in the embryonic chick: An experimental study of tissue interactions. *Anat. Rec.* **206**, 61–70.

YAMADA, K. M., OLDEN, K. & HAHN, L.-H. E. (1980). Cell surface protein and cell interactions. In: *The Cell Surface: Mediator of Developmental Processes*, (ed. S. Subtelny & N. K. Wessells), pp. 43–78. New York: Academic Press.