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Lens differentiation *in vitro* in the absence of optic vesicle in the epiblast of chick blastoderm under the influence of skin dermis

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SUMMARY

1. Dissociation and recombination experiments *in vitro* were found useful for analysing inductive tissue interactions involved in lens differentiation in the chick.

2. When the presumptive cephalic region (epiblast plus hypoblast) of the embryo at pre-definitive streak to one-somite stage is cultivated *in vitro* combined with the dermis isolated either from the dorsal skin of 6·5-day embryo or from the 13·5-day tarsometatarsal skin, a lens with fibres or lentoid is produced in the epiblast. In no case is there an optic vesicle present in the explant.

3. When the presumptive cephalic region (epiblast plus hypoblast) is cultivated without dermis, the lens is no longer formed.

4. If the epiblast alone, dissociated from the hypoblast of the presumptive cephalic region, is recombined with the dermis of the 6·5-day dorsal skin, lenses or lentoids fail to develop.

5. Cultivation of the epiblast alone cannot cause differentiation of the lens or lentoid.

6. The dermis can be replaced by other mesenchymes or embryonic organs: gizzard mesenchyme, mesonephros, sclerotome, liver and neural retina, though they are less effective than the dermis in producing lenses or lentoids in the epiblast.

7. It may therefore be concluded that the lens is induced *in vitro* by the actions of at least two factors: the epiblast first becomes competent under the specific influence of the hypoblast of the cephalic region. The lens will then differentiate from the competent epiblast by the non-specific action of various tissues such as the skin dermis, mesonephros, or sclerotome.

8. The primary stage of lens induction (action of the hypoblast on the epiblast) seems not yet completed by streak stage.

INTRODUCTION

Spemann (1901) was the first to test by direct experiments the possibility that lens formation might be influenced by the optic vesicle. Since surgical excision of the retinal rudiment in the early neurula of *Rana fusca* always resulted in the failure of lens formation, he concluded that lens formation is only possible under the influence of the optic vesicle. However, this conclusion soon proved to be unwarranted. The lens or lentoid developed in *Rana palustris* (King, 1905) and in *R. esculenta* and *Bombinator pachypus* (Spemann, 1912) even when the retinal

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rudiment had been removed. Similar results were also reported in the embryos of the newt, *Taricha torosa* (Jacobson, 1958).

The self-differentiating capacity of the presumptive lens ectoderm was further tested by cultivating the presumptive lens ectoderm alone, isolated from amphibian embryos of the early neurula stage by Perri (1934), by Woerdeman (1941), by de Vincentiis (1949), and by Jacobson (1958). They concluded that presumptive lens ectoderm could not form a lens by itself when isolated *in vitro*, though the ectoderm *in vivo* is capable of differentiating into lens in the absence of the optic vesicle. This may suggest that lens differentiation is dependent on the inductive action of some tissues other than the optic vesicle. Jacobson (1958) demonstrated that the lens formation occurred *in vitro* in the isolated presumptive lens ectoderm of the early neurula, only when this was cultured together with the subjacent entodermal archenteron wall. Okada & Mikami (1937) removed the primordial eye-cup from the embryo of *Triturus pyrrhogaster* before the lens was determined, and substituted other tissues for the optic cup. They demonstrated that the meso- or endoderm of the head region was very effective in inducing lens, whereas the ectoderm was effective only to a very small extent. Also, Liedke (1951) demonstrated that in *Ambystoma punctatum*, ventral gastrula epidermis could develop into a lens when it was transplanted into the presumptive lens area of the early neurula after removal of the presumptive lens ectoderm, but not when it was transplanted into the late neurula. Jacobson (1958) obtained similar results using *Taricha torosa* as material. He demonstrated that the presumptive lens ectoderm, when isolated from the early neurula and cultured with a retinal rudiment from the early neurula, failed to form lenses. The results of these experiments on amphibian embryos seem to indicate that lens formation may first be elicited by the action of the anterior endomesoderm during neurula stage long before the lens ectoderm comes to respond to the retinal stimulus.

In the chick, however, the evidence is rather fragmentary. Though the role of the optic vesicle in lens formation has been reported (Alexander, 1937; van Deth, 1940; McKeehan, 1951), the significance of the anterior endomesoderm for lens differentiation still remains uncertain.

During the course of the experiments in which the induction of feather germs from the recombined tissues of proamnionic epithelium and dorsal skin dermis was being investigated (Mizuno, 1970*a*, 1971), it was found rather unexpectedly that a fully differentiated lens formed from the isolated cephalic region of the streak stage embryo when this was combined with the dorsal skin dermis of older embryos. On the basis of this finding, further analysis of the nature of inductive tissue interactions involved in lens differentiation was attempted in the present study. Part of the results of this study has already been reported (Mizuno, 1970*b*).

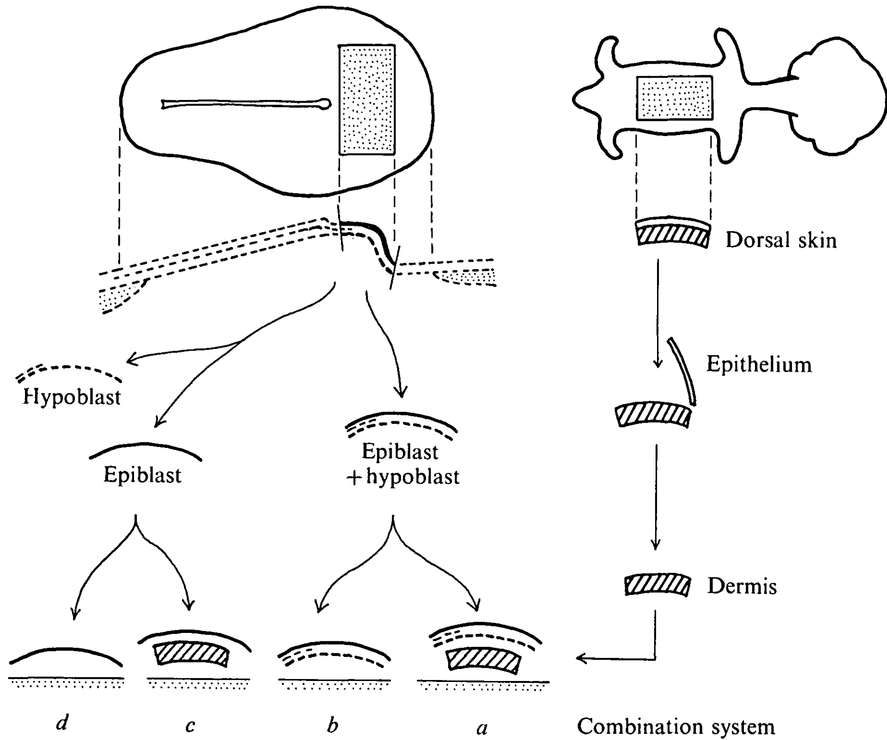


Fig. 1. Diagram showing the mode of combinations between the epiblast and hypoblast of the presumptive cephalic region from streak-stage embryo and the dermis of 6·5-day dorsal skin.

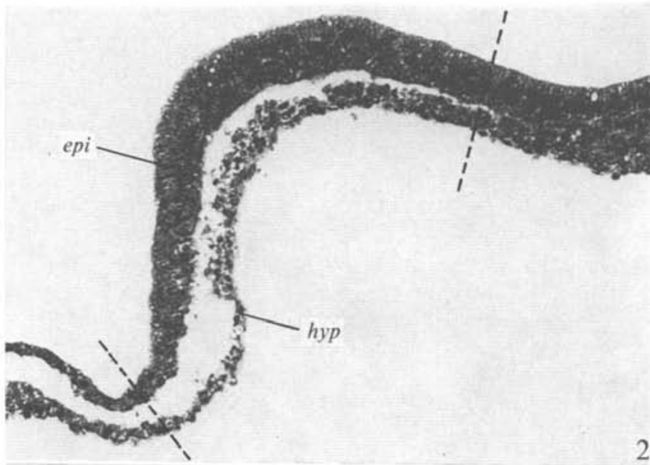
MATERIALS AND METHODS

Material

Embryos of the White Leghorn fowl (*Gallus domesticus*) were used as material throughout the experiment.

Isolation of tissue fragments

A piece of the blastoderm was dissected from the presumptive cephalic region of the embryo at pre-definitive streak to one-somite stage (Fig. 2). Epiblast of this area was separated mechanically from the underlying tissues (hypoblast with some mesoblast cells) in a Ca-Mg-free Tyrode's solution using watchmaker's forceps. The dermis of the 6·5-day dorsal skin and the 10- and 13·5-day tarsometatarsal skin was separated from the epidermis by brief treatment with a 0·5 % cold solution of trypsin, Difco 1:250. Mesenchyme from proventriculus, gizzard, and trachea was isolated with or without the aid of trypsin. Fragments of other organs such as heart, bulbus cordis, liver, somite, sclerotome, mesonephros, and Wolffian duct, were used entire without isolating a mesenchyme layer. Tissue fragments dissociated with the aid of trypsin were washed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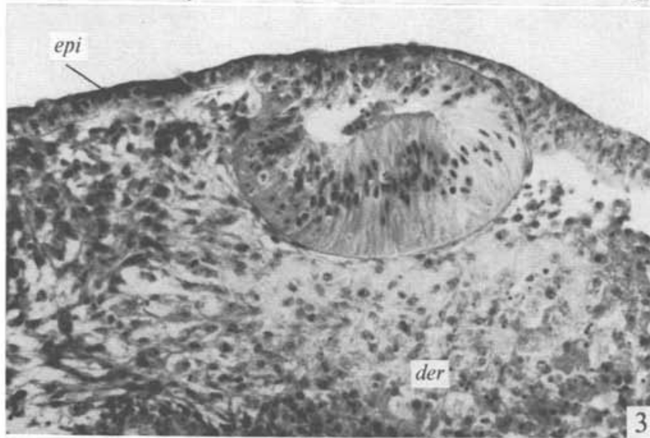
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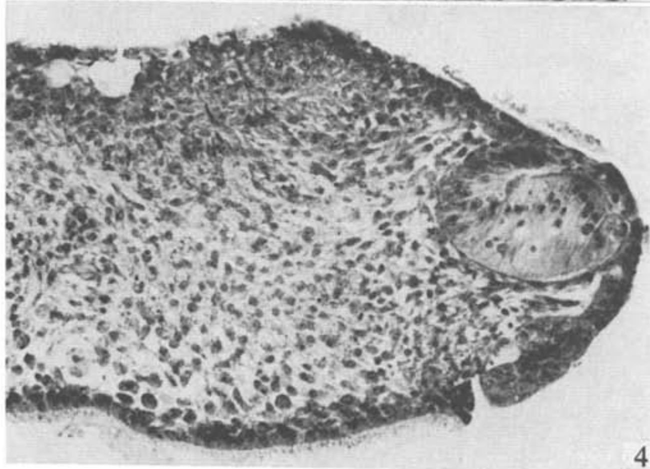
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repeatedly in trypsin containing embryo extract and horse serum in order to inactivate excess trypsin and finally in Tyrode's solution only.

Recombination of the separated tissues

A part of the cephalic region of the blastoderm (epiblast plus hypoblast) or of the epiblast was cultured combined with a fragment of mesenchyme or a piece of organ. The scheme of the general experimental method is shown in Fig. 1. Tissues were cultured, according to the procedure described by Wolff & Haffen (1952), in solid watch glasses at 37·5 °C for 6 days. The nutrient medium consisted of seven volumes of 1 % bactoagar (Difco) in Gey's solution, three volumes of filtered horse serum (Institut Pasteur), three volumes of 9-day eye-free chick embryo extract (15 %) and one volume of Tyrode's solution containing penicillin G (20000 i.u./cm³). The pH of the medium was 7·2.

Histological method

After cultivation, the explants were fixed in Bouin's fluid, embedded in paraffin, and sectioned at 5 μm. The sections were stained with Carazzi's glychémalum and eosin, following the usual histological techniques.

RESULTS

(1) *Combination of presumptive cephalic region of the blastoderm with 6·5-day dorsal dermis* (system *a*)

The presumptive cephalic region of the blastoderm consisting of the epiblast and the underlying hypoblast was removed from embryos of predefinitive streak stage to one-somite stage, and cultured *in vitro* directly on the explanted dorsal skin dermis of 6·5-day chick embryo (Fig. 1 *a*). Histological observations of the explants were made after 6 days' cultivation. The results are summarized in Table 1. It was rather striking to observe that this epiblast, which is embryologically very young and apparently neutral, gives rise to a ball of cells that transforms into an unmistakable lens, with lens capsule, epithelial cells, and with orientated, elongated lens fibres (Figs. 3–5). In some cases, the lenses

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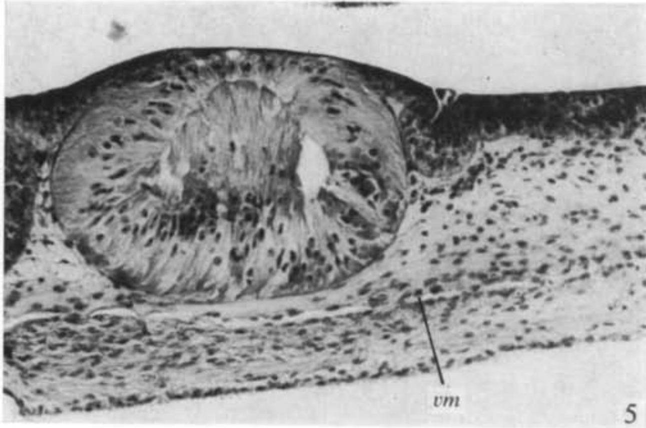
FIGURES 2–4

Fig. 2. Longitudinal section of the presumptive cephalic region of the head-process stage embryo. Dotted lines indicate the area used for culture. *epi*, Epiblast; *hyp*, hypoblast with some mesoblast cells. ×150.

Fig. 3. Six days after combination of presumptive cephalic region (epiblast plus hypoblast) from definitive streak-stage embryo with 6·5-day dorsal skin dermis (system *a*). A lens with fibres developed from the epiblast. *epi*, Epiblast; *der*, dermis. ×200.

Fig. 4. Six days after combination of presumptive cephalic region (epiblast plus hypoblast) from head-fold stage embryo with 6·5-day dorsal dermis (system *a*). There is no neural tissue. ×200.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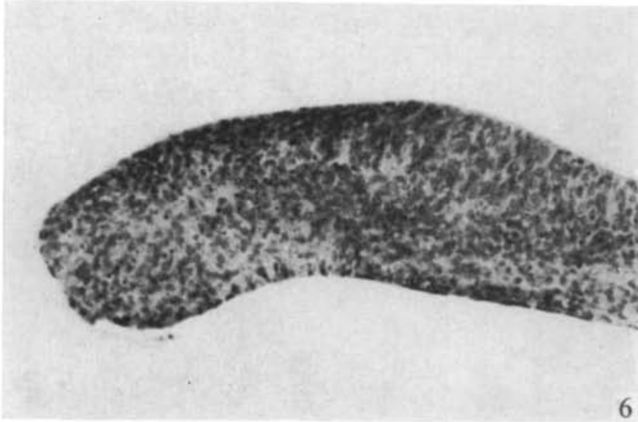
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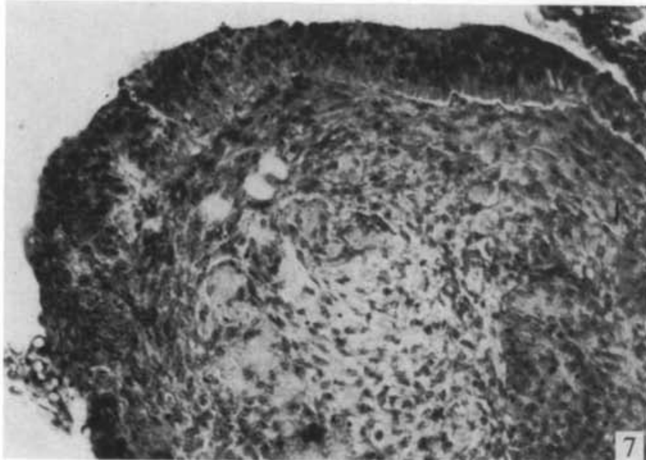
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Table 1. *Differentiation of epiblast of the presumptive cephalic region of pre-definitive streak to one-somite stage embryos, when it was combined with the underlying hypoblast and 6·5-day dorsal skin dermis, and cultured for 6 days* in vitro

Explants	No. of explants	Lens	Lentoid	Neural tissue	Optic vesicle
Epiblast + hypoblast + dermis	26	10	12	14	0
Epiblast + hypoblast	32	0	1	10	0
Epiblast + dermis	23	0	0	12	0
Epiblast	18	0	0	8	0

were found to be somewhat deformed, showing an unorganized mass of lens cells with or without the lens fibres. In no cases in this series of experiments, however, could the optic vesicle be found either around the lens or in other sites of the explants. The neural tissues were found to differentiate in some explants, but there seemed no causal relationship whatever between the development of the lens and the differentiated neural tissues. Apparently, the dorsal skin dermis from older embryos must have exerted some influence on the overlying epiblast to cause lens differentiation without any optic vesicle. To test this point, an attempt was first made to cultivate the blastoderm (epiblast plus hypoblast) without combining with dorsal dermis (system *b*). We then proceeded to system *c* in which the blastoderm deprived of hypoblast was combined with dorsal dermis, and then to system *d* in which the epiblast alone was cultivated. In the following, the details of each group of experiments will be described.

(2) *Cultivation of presumptive cephalic region of the blastoderm without dorsal dermis* (system *b*)

The blastoderm pieces (presumptive cephalic region) from streak stage to one-somite stage embryos consisting of the epiblast and the hypoblast with some mesenchymal cells were cultured alone directly on the medium for 6 days (Fig. 1*b*). Histological observations after 6 days' cultivation showed that the

FIGURES 5–7

Fig. 5. Six days after combination of presumptive cephalic region (epiblast plus hypoblast) from early definitive streak-stage embryo with 6·5-day dorsal dermis. A sheet of very thin vitelline membrane (*vm*) treated previously with trypsin was placed between the blastoderms and the dermis. Note a large lens with elongated fibres. × 200.

Fig. 6. Six days after cultivation of presumptive cephalic region (epiblast plus hypoblast) from definitive streak-stage embryo (system *b*). Neural differentiation, but no lens. × 200.

Fig. 7. Six days after combination of the epiblast of presumptive cephalic region from early definitive streak-stage embryo with 6·5-day dorsal dermis (system *c*). Neural differentiation, but no lens. × 200.

Table 2. *Differentiation of epiblast of the presumptive cephalic region of pre-definitive streak to one-somite stage embyos in the presence of the underlying hypoblast and of various mesenchymes or fragment of embryonic organs*

Combined mesenchymes or organs	No. of explants	Lens	Lentoid	Neural tissue	Optic vesicle
6·5-day dorsal dermis	26	10	12	14	0
10- and 13·5-day tarsometatarsal dermis	11	6	1	3	0
6-day proventricular mesenchyme	3	0	0	2	0
5-day gizzard mesenchyme	3	0	1	1	0
5-day lung mesenchyme	5	0	0	0	0
13·5-day tracheal mesenchyme	7	0	0	0	0
3- to 5-day mesonephros	19	2	1	7	0
3- to 6-day sclerotome	7	1	2	5	0
3- to 4-day liver	7	0	2	3	0
4- to 7-day neural retina	17	0	1	3	0
4- to 5-day heart	4	0	0	2	0
4-day bulbus cordis	4	0	0	2	0
3-day somite	6	0	0	2	0
5-day Wolffian duct	5	0	0	1	0

epiblast no longer differentiated into the lens without the combined dermis (though a very small lentoid appeared only in one case out of 32 explants) and that no optic vesicles were formed (Table 1). The neural tissues, such as diencephalic neural plate or telencephalon, were often differentiated from the epiblast (Fig. 6). These findings clearly indicate that the epiblast of the presumptive cephalic region can differentiate into neural tissues but hardly differentiates into lens when the dermis is omitted from the complete system (system *a*). Therefore, the dermis seems to be necessary for producing a differentiated lens, at least under the conditions of the present experiment, though it is not necessarily essential for neural differentiation.

(3) *Combination of epiblast of presumptive cephalic region and 6·5-day dorsal dermis* (system *c*)

Attempts were then made to cultivate the epiblast with the dermis. The epiblast of the presumptive cephalic region of the primitive streak stage (Hamburger & Hamilton's (1951) stage 4) was dissociated from the underlying hypoblast and then recombined with the 6·5-day dorsal skin dermis (Fig. 1*c*). The results are also shown in Table 1. It will be seen that in no case did the lens or lentoid appear in the explants despite the presence of the dorsal dermis. The neural tissues appeared in half of the cases, though (Fig. 7). From these results it is evident that the hypoblast of the presumptive cephalic area is a necessary factor in producing lens in the epiblast under the influence of the dermis, a rather remarkable fact considering that the hypoblast has hitherto been relatively neglected in tissue interactions.

(4) *Cultivation of epiblast of presumptive cephalic region* (system *d*)

Finally, both hypoblast and dermis were excluded from system *a*. The epiblast of the presumptive cephalic region from streak stage (Hamburger & Hamilton's stage 4) embryos was isolated from the underlying hypoblast and was cultured alone on the medium for 6 days (Fig. 1*d*). In this experiment, lens or lentoid was never observed to form, though differentiation of neural tissues, such as diencephalic neural plate and telencephalon, was often observed (Table 1). It can therefore be concluded that the epiblast of the streak stage is not able to differentiate into the lens by itself, but that it is already competent to develop neural tissues. This is in accord with the recent work of Eyal-Giladi & Wolk (1970), which demonstrated an inducing capacity of the primary hypoblast on the formation of neural tissues from the epiblast in the primitive streak stages of the chick embryo.

The results hitherto described, which are summarized in Table 1, seem to indicate that at least two factors, i.e. the primary hypoblast of the presumptive cephalic region and the dorsal skin dermis, are indispensable in inducing lens in the epiblast, at least under the conditions of the present investigation.

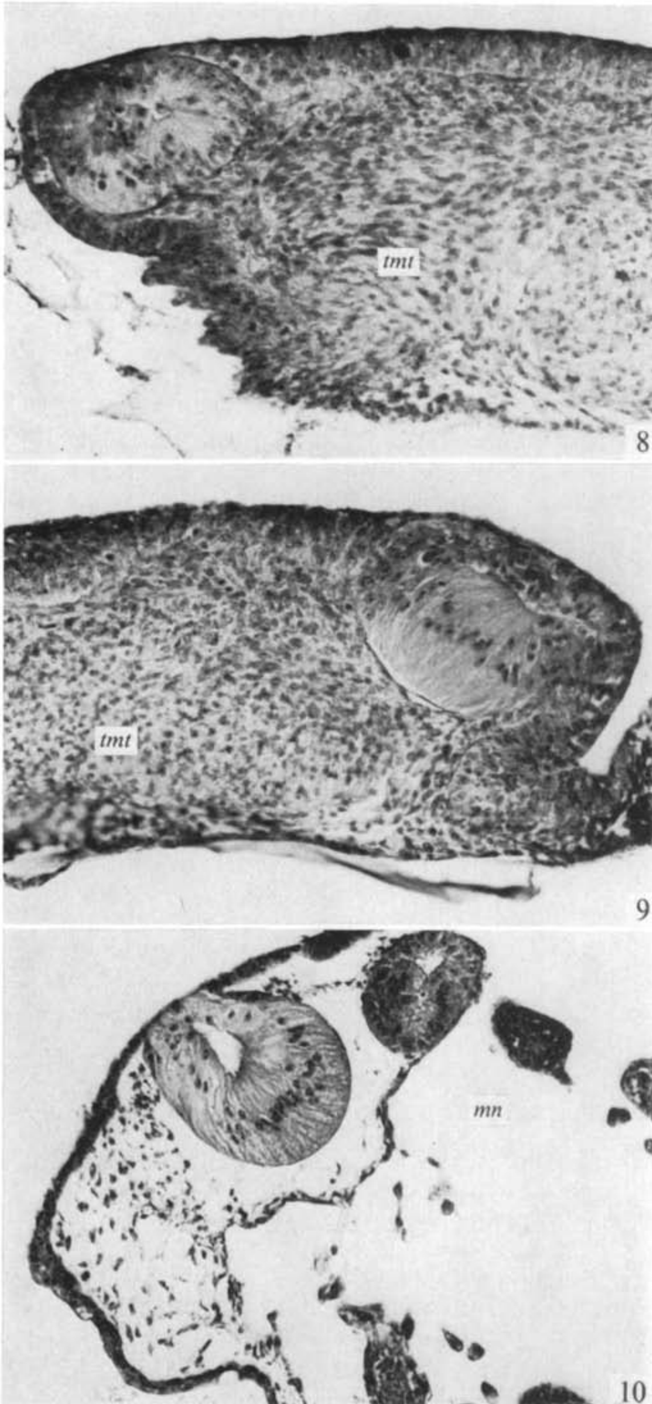
(5) *Combination of presumptive cephalic region of the blastoderm with diverse mesenchymes or fragments of organs*

The question next arises as to whether or not diverse mesenchymes other than the dorsal dermis could be equally effective in inducing lens differentiation. To answer this question, epiblast plus hypoblast of the presumptive cephalic region was cultivated on various mesenchymes or on fragments of organs. The results are summarized in Table 2.

When the blastoderm piece was cultured on dermis isolated from 10- or 13·5-day tarsometatarsal skin, differentiated lenses with fibres were induced only a little less frequently than in the case of the combined culture of the blastoderm with the 6·5-day dorsal skin dermis (Table 2 and Figs. 8, 9). This suggests that the tarsometatarsal dermis is as effective in producing lens as the dorsal dermis.

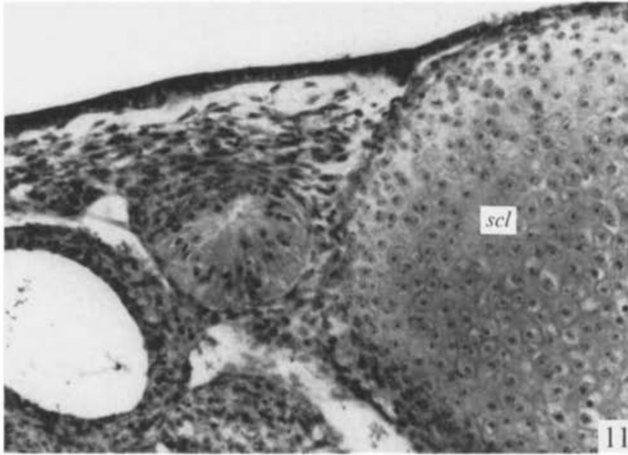
Mesenchymes derived from other organs or fragments of organs were also examined for their inducing capacity. Some were found to be effective and others were not (see Table 2). Three- to 5-day mesonephros (Fig. 10) and 3- to 6-day sclerotome (Fig. 11) could at least produce a low percentage of lenses with fibres. Three- to 4-day liver could elicit the development of some masses of lens cells (Fig. 12), and 4- to 7-day neural retina induced only one lentoid among 17 explants (Fig. 13). Five-day gizzard mesenchyme seems to have a slight capacity for producing lentoids. Other mesenchymes or organs examined, such as proventricular, lung and tracheal mesenchymes, or heart, bulbus cordis, somite, and Wolffian duct failed to produce lenses or lentoids.

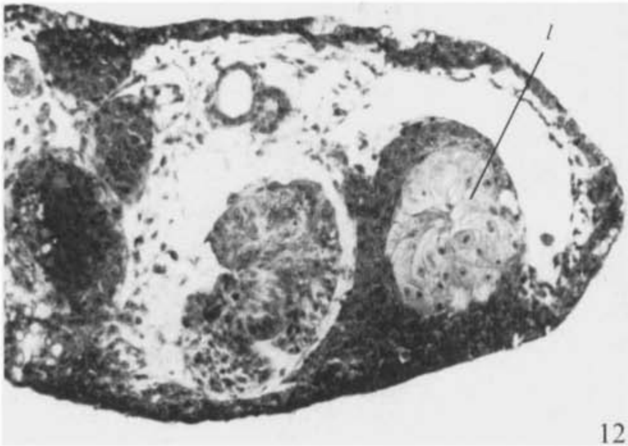
It appears therefore that the dermis can be replaced by some other embryonic organs, such as mesonephros, sclerotome, liver, gizzard and retina, though



Figs. 8, 9. Six days after combination of presumptive cephalic region (epiblast + hypoblast) from definitive streak-stage embryo with 13·5-day tarsometatarsal dermis (*tmt*). ×200.

Fig. 10. Six days after combination of presumptive cephalic region (epiblast + hypoblast) from definitive streak-stage embryo with 4-day mesonephros. Note a lens with fibres arising from the epiblast. Mesonephros (*mn*) is degenerative. ×200.





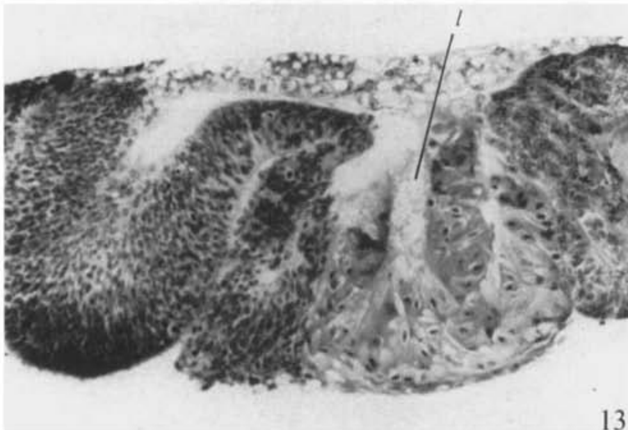


Fig. 11. Six days after combination of presumptive cephalic region (epiblast + hypoblast) from head-process stage embryo with 5-day sclerotome (*scl*). × 200.

Fig. 12. Six days after combination of presumptive cephalic region (epiblast + hypoblast) from head-process stage embryo with 4-day liver. Note a mass of lens cells (*l*) surrounded by liver cells. × 200.

Fig. 13. Six days after combination of presumptive cephalic region (epiblast + hypoblast) from intermediate streak-stage embryo with 4·5-day neural retina Some vacuolation has occurred in lentoid cell mass (*l*). × 200.

these provoke the formation of lenses or lentoids less frequently than does the dermis. This fact suggests that the action of the dorsal skin dermis in inducing the lens is by no means specific. It seems that the specificity of lens induction must rather reside in the primary hypoblast situated under the epiblast of the blastoderm, although the possibility cannot be ruled out that the complex, the hypoblast plus some mesoblast, is responsible for the specificity of induction.

DISCUSSION

The existence of a causal relationship between the optic vesicle and lens formation has been known for a long time. The literature on lens induction by the optic vesicle in the amphibians is voluminous. The same was also reported in the chick (Waddington & Cohen, 1936; Alexander, 1937; van Deth, 1940; McKeehan, 1951; Langman, 1956) and in the mouse (Muthukkaruppan, 1965). It is true that in normal development, lenses always appear in association with the optic cup. It is also known that the action of the optic cup is essential for the full realization of growth and differentiation of the amphibian lens (Woerdeman, 1953: for review, see Twitty, 1955), and that the morphological differentiation of the chick lens becomes incomplete after the optic vesicle has been partially extirpated, this incompleteness depending on the amount and quality of material removed (Génis-Gálves, Santos & Rios, 1967).

Nevertheless, the relation between lens development and optic cup development becomes rather complicated when we recall the fact that in some amphibians – *Rana palustris*, *R. esculenta*, *Bombinator pachypus*, *Xenopus laevis*, *Taricha torosa*, *Triturus vulgaris*, *T. pyrrhogaster*, *Hynobius nebulosus*, *R. japonica*, *R. limnocharis*, *R. rugosa*, and *Rhacophorus schlegelii* (King, 1905; Spemann, 1912; Balinksy, 1951; Jacobson, 1958; Becker, 1959; Tahara, 1962) – lenses or lentoids are formed even when the optic cup has been previously removed. This suggests that the eye cup is not the only tissue that is important for lens formation in normal developmental processes.

Twitty (1955) cites examples of the inductive action of the head mesoderm and the retinal rudiment, mainly in amphibian lens development. Okada & Mikami (1937) demonstrated that nose *Anlage*, ear-vesicle, brain, heart and liver substituted for the optic cup can induce lens in *Triturus pyrrhogaster*. Liedke (1951) reported that the influence of the head mesoderm is the factor which would prepare the epidermis for the subsequent induction coming from the optic vesicle in *Amblystoma punctatum*. According to Jacobson (1958), in some species, the optic cup fails to induce lens formation in the ectoderm which has not previously been affected by meso-endoderm of the head region. He demonstrated, using *Taricha torosa*, that the presumptive lens ectoderm isolated from the early neurula failed to form lens even on cultivation with a retinal rudiment.

In other groups of vertebrates, however, the role of the head mesoderm in inducing lens has not been well established. McKeehan's results (1954) dealing

with lens differentiation from the isolated lens primordia of 21- to 33-somite stage chick embryo without the optic vesicle, indicate that the lens rudiment has already been determined at these stages.

The present investigation, using chick embryos of early developmental stages, was aimed at finding whether or not the cephalic endomesoderm affects lens induction. The results of the experiments *in vitro* have shown that lens formation can be elicited by the action of at least two factors: (1) the hypoblast (plus some mesoblast cells) subjacent to the presumptive cephalic epiblast at streak stage to one-somite stage, and (2) the second factor coming from some tissues or organs other than developing optic vesicle, such as skin dermis, mesonephros, sclerotome, liver, gizzard or retina. This implies that the hypoblast first acts upon the undetermined epiblast, and this epiblast then becomes capable of responding to the second, non-specific stimulus coming from various mesenchymes. The action of this non-specific stimulus will be to reinforce the specific action of the hypoblast, and to accelerate the realization of full growth and differentiation of the induced lens.

Incidentally, the results of Coulombre & Coulombre (1971) are very interesting: when a piece of 5-day embryonic lens epithelium is implanted in place of the lens in 5-day-old chick embryo, the differentiated lens fibres originating from the epithelium elongate towards the vitreous body, regardless of the initial orientation given to the implanted epithelium. In the present study, the lens fibres always elongate towards the dermis recombined with the epiblast, suggesting that the dermis acts by controlling appropriate lens orientation in a similar way to that found in the eye region.

Though various embryonic organs are able to produce the second lens-inducing factor mentioned above, the dermis seems to be the most effective among them. Even retina obtained from 4- to 7-day embryo was not so effective in producing lens from the competent epiblast. Preliminary experiments have demonstrated that the second factor can act across a Millipore filter, and that alcohol treated dermis is still active in producing lens (T. Mizuno, unpublished data).

Doubt still exists, however, as to whether the inductive action of the optic vesicle in the chick can also be explained in the same way as in the lens induction *in vitro* observed in the present study. It is possible that the optic vesicle is a complete inducer for lens, possessing both the first and the second factors. This point must be clarified in a future study.

The significance of the present work is to provide evidence using dissociation and recombination techniques *in vitro* that at least two factors can be involved in lens differentiation in the chick; the first factor, derived from the hypoblast (plus some mesoblast cells) subjacent to the presumptive cephalic epiblast, might make the epiblast competent to react to the second factor. The second factor, experimentally demonstrated to come from various embryonic organs such as dermis and other mesenchymes, might stimulate non-specifically the

competent epiblast to cause lens formation, though the exact mechanisms of action of these factors are unknown. In addition, experimental data in the present investigation indicate that the primary step of lens induction (action of the hypoblast on the epiblast) seems not yet completed before the primitive streak stage, since lens could not develop if the epiblast alone isolated from the primitive streak stage was stimulated by the dermis.

This two-step mechanism of lens induction, if further verified, would also be of some use for the analysis of the mechanisms of the lens or lentoid formation from the iris in Wolffian regeneration, and from the neural retina (Moscona, 1957), and the tapetum (Dorris, 1938).

RÉSUMÉ

Différenciation de cristallin in vitro *en l'absence de vésicule optique dans l'épiblaste du blastoderme chez le Poulet sous l'influence du derme de la peau*

1. Des expériences de dissociation et réassociation *in vitro* ont permis l'analyse des interactions tissulaires intervenant dans la différenciation du cristallin chez le Poulet.

2. Lorsque la région céphalique présomptive (épiblaste plus hypoblaste) d'un embryon dont le stade de développement est intermédiaire entre le stade ligne primitive prédéfinitive et le stade 1 somite est associée *in vitro* au derme de la peau dorsale d'un embryon de 6,5 jours ou bien au derme du tarsométatarse de 13,5 jours, on observe le développement dans l'épiblaste de cristallins avec fibres ou de lentoïdes. Dans tous les cas, la vésicule optique est absente de l'explant.

3. Quand la région céphalique présomptive (épiblaste plus hypoblaste) est cultivée sans derme, aucun cristallin ne se forme.

4. Si la région céphalique présomptive est dissociée en épiblaste et hypoblaste, l'épiblaste seul étant réassocié au derme de la peau dorsale d'embryons de 6,5 jours, cristallins ou lentoïdes ne se développent jamais.

5. L'épiblaste cultivé seul ne donne lieu à la formation de cristallin ou de lentoïde.

6. Le derme peut être remplacé par d'autres mésenchymes ou organes embryonnaires: le mésenchyme de gésier, le mésonéphros, le sclérotome, le foie et la rétine. Ils provoquent dans l'épiblaste, en moins grand nombre cependant, la formation de cristallins ou de lentoïdes.

7. On pourrait donc conclure que l'induction d'un cristallin *in vitro* nécessite au moins deux facteurs: premièrement l'épiblaste devient ≪ compétent ≫ sur l'action spécifique de l'hypoblaste de la région céphalique. Puis il peut se différencier en cristallin sous l'action stimulatrice non-spécifique de différents tissus tel que le derme de la peau, le mésonéphros ou le sclérotome.

8. La première phase de l'induction du cristallin (action de l'hypoblaste sur l'épiblaste) n'a pas encore eu lieu au stade de la ligne primitive.

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