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Muscle-forming potential of the non-somitic cells of the early avian limb bud

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SUMMARY

It has recently been shown that the musculature of the chick wing arises by migration of cells from the somites, and that on morphological grounds this process begins at about stage 14.

We have carried out grafts of wing anlagen separate from the somites from quail donors to the extra-embryonic coelom of chicks, and find that anlagen from as early as stage 10 (11 pairs of somites) can give rise to muscle. We discuss the possible reasons for this finding, and conclude that in the absence of the cells normally giving rise to the musculature, mesodermal cells themselves can give rise to muscle.

INTRODUCTION

It has recently been demonstrated that the wing musculature of the chick is normally derived from the somites by invasion of cells arising from the dermo-myotome; and that these cells give rise to no other cell type. This has been shown by a variety of experiments exploiting the biological marker possessed by quail cells (Le Douarin, 1973). When quail somites are grafted into 2-day-old chick embryos in place of the somites of the wing region, the muscle cells are of quail origin, while all the other tissues, including the connective tissues of the muscles, are chick (Christ, Jacob & Jacob, 1974, 1977; Chevallier, Kieny, Mauger & Sengel, 1977; Chevallier, 1978). When quail limb somatopleural mesoderm is grafted in place of that of the chick, the muscle cells are chick, but all the other tissues are quail (Christ *et al.* 1977). When the somitic tissue opposite the wing is surgically removed, or inactivated by X-irradiation, muscle-less limbs can develop (Chevallier, Kieny & Mauger, 1978).

The time of invasion of the wing anlagen by the cells from the somites is clearly of major importance. This has been reported to occur at around stage 14 (stages according to Hamburger & Hamilton, 1951) on the basis of morphological examination of normal limbs, and of study of early limbs in which

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the somites have been replaced by those marked either by their quail origin, or by incorporation of radioactively labelled precursors (Chevallier, 1978). This conclusion has been supported by grafts of wing anlagen to the extra-embryonic coelom and chorio-allantoic membrane (CAM), where grafts from donors of stage 15 or later gave rise to structures containing muscle, while grafts from prior to this stage did not (Christ *et al.* 1977).

These findings are clearly of major importance for theories of control of cell differentiation in the limb, seeming to support lineage views as proposed by Holtzer and co-workers (Dienstmann, Biehl, Holtzer & Holtzer, 1974), rather than those such as Searls & Janners (1969), Zwilling (1966), and Caplan & Koutroupas (1973), who support the idea of multipotency of the cells of the early limb.

It also has implications for the role of positional information (Wolpert, 1969) in the formation of the muscles, in that it seems to suggest that this does not control cytodifferentiation itself, but rather the spatial organization of the muscle cells.

However, anomalous results arise during some of these experiments. For instance, when chick somites are grafted into quail rather than vice versa, the resulting limb muscles contain both chick and quail muscle cells (Chevallier *et al.* 1977). Again, when the somites are inactivated or removed, muscles still develop in some cases. The possibility exists that although wing muscle cells are usually of somitic origin, somatopleural cells can also give rise to muscle under certain conditions.

To investigate this, we have carried out a series of grafts of limb primordia solely to the extra-embryonic coelom, with particular attention to stages at and before the invasion of the primordia by the somite cells. The technique of grafts to extra-embryonic regions has of course been used before for the purpose of discovering the time at which the tissues of the limb become capable of self-differentiation (Murray, 1927; Hunt, 1932; Eastlick, 1943), but these results cannot now be relied upon, since as far as can be ascertained for experiments done before the development of accepted staging criteria, they were carried out after the critical period. This is certainly the case for one more recent study (Bradley, 1970).

MATERIALS AND METHODS

Our own breeding stock of Japanese quail (*Coturnix coturnix japonica*) were used as graft donors while commercially obtained Rhode Island Red × Light Sussex chick embryos served as hosts. Quail eggs were incubated from 40 to 72 h (i.e. stages 10–20) and the yolks tipped into sterile Ringer's Balanced Salt Solution (Paul, 1975), warmed to about 30 °C. The blastoderms were cut free and transferred to a dish of black wax, again under salt solution, where they were pinned out, and a piece incorporating the prospective wing anlagen on both sides and including the neural tube, was cut free (see Fig. 1). A careful record

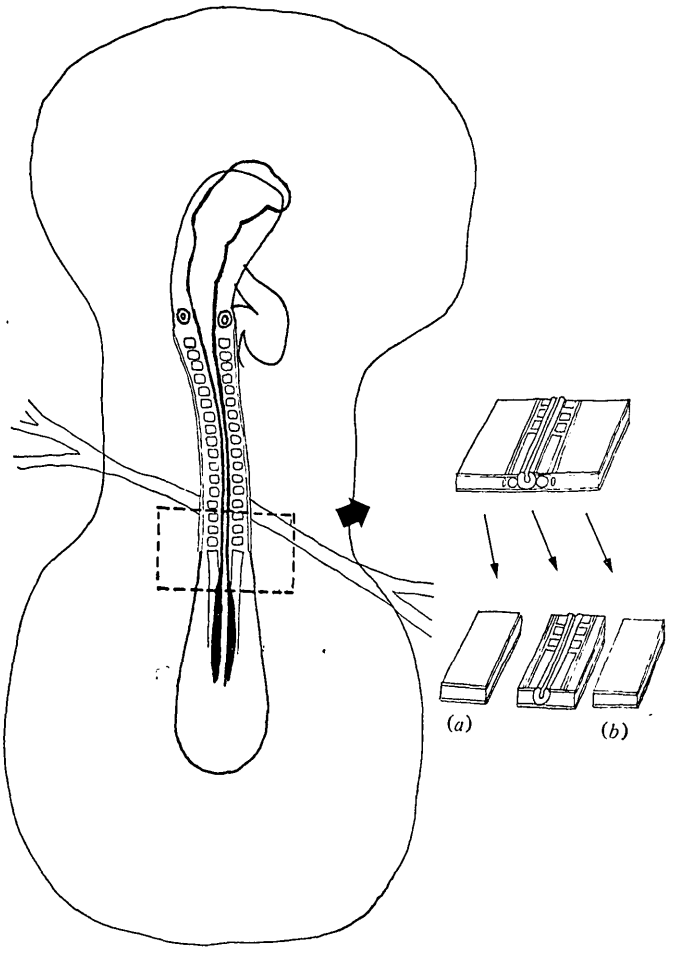

Fig. 1. Quail embryo of 17 pairs of somites. After a large piece incorporating both wing anlagen and the neural tube has been removed to a dish, the wing anlagen are carefully separated. (Anlagen marked (*a*) and (*b*).)

was kept of the number of pairs of somites each donor had possessed. The piece was then moved to a plastic culture dish, where the wing anlagen were carefully separated with electrolytically sharpened tungsten needles, taking care to exclude somitic material. In 15 cases, the remaining piece of neural tube and somitic mesoderm was fixed and embedded, so that subsequent sectioning could show that the somites remained intact.

Prior to stages 13–14, it is not possible to remove the anlagen without including some of the underlying splanchnopleural mesoderm, as the coelom which separates these two has not extended as far as the prospective wing region.

Chick hosts were incubated for 55–72 h, giving stages of about 13–17. A small hole was made in the air space, and a square window of 1 cm side removed

Table 1. *Results of coelom grafts by stage of donor*

Stage of donor	Somite number	No. of operations surviving	No. of grafts retrieved	No. with muscle
10	10–12	3	1	1
11	13–15	18	7	3
12	16–18	19	9	8
13	19–21	14	2	2
14–20	22	24	15	14

from above the embryo. The eggs were then resealed with Sellotape, and returned to the incubator till required.

Once a graft was prepared, a chick egg was opened, and the embryo moistened with medium 199. A mixture of one part Indian ink to three parts medium was injected into the yolk beneath the embryo with a fine angled hypodermic syringe needle, inserted outside the blastoderm. Normally, about 0·1 ml of this mixture sufficed to make the embryo visible. The vitelline membrane was now torn open with fine forceps, and the graft transferred to the embryo. A small slit was made in the lateral plate mesoderm of the host using a sharp tungsten needle, and extended downward through the somatopleural mesoderm until the coelom was reached. The graft was pushed into this pocket and the egg resealed and returned to the incubator.

Seven days later the host embryos were removed to saline, decapitated, and the body cavity carefully examined. Structures arising from the graft were removed and fixed in Smith's Formol-bichromate fixative, for staining by the Feulgen–Rossenbeck technique (Feulgen & Rossenbeck, 1924), and then double-embedded in wax. Seven μm serial sections were cut, and stained by Feulgen's method, except for one ribbon in each five or six, which was stained separately with haematoxylin and eosin to clarify morphological detail.

RESULTS

Out of 78 embryos surviving the operation, grafts were retrieved from 34. Of these retrieved grafts, 19 had been made from donors of stage 13 or earlier. Out of this 19, 14 were found on sectioning to contain muscle. Of the 15 grafts retrieved from later hosts, 14 contained muscle (see Table 1).

Figure 2 shows a graft obtained from a stage-12 donor sectioned to show the presence of muscle and cartilage. In all cases where muscle was present, quail muscle cells could be identified, showing that it originated from the graft (Fig. 3). Where muscle fibres were sectioned longitudinally, use of a system of crossed polarizing filters on a microscope with a revolving stage showed birefringence of the fibres and the presence of striations, confirming the mor-

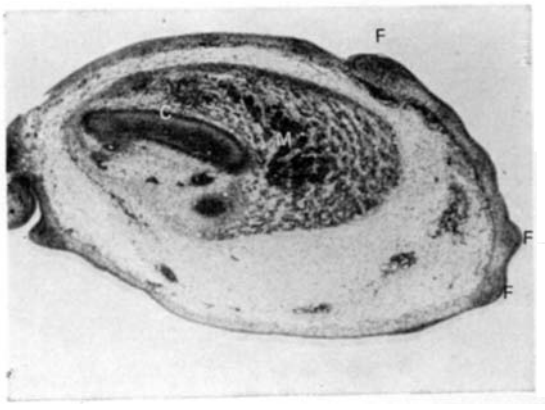

Fig. 2. 7 μm section through graft retrieved from the coelom, stained with haematoxylin and eosin. The donor was a stage-12 quail. M = muscle; C = cartilage; F = feather germs.

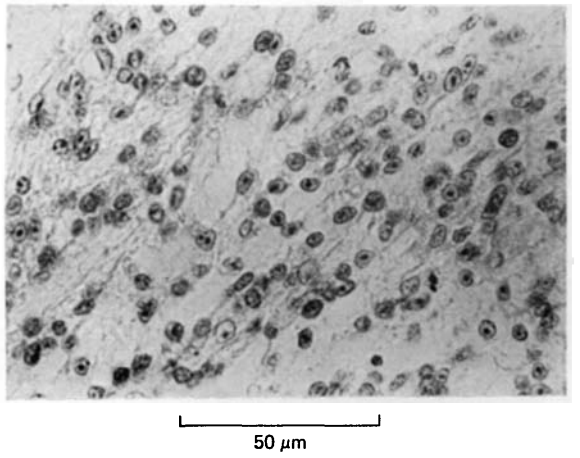

Fig. 3. Section, stained by Feulgen's method, of muscle from graft of Fig. 2 showing quail cell nucleoli.

phological identification of muscle (see Fig. 4). This phenomenon was observed in grafts taken from before and after stage 14.

Of the grafts where the donor neural tube and somites had been preserved, six gave rise to grafts, and four of these contained muscle. The donors in these cases could be observed to have intact somites all along their length (see Fig. 5). All of these cases were from before stage 14.

Only one of all the grafts made from before stage 14 showed normal development of the wing, the remainder forming roughly spherical structures some 2–3 mm in diameter, covered in feather germs. Since it has been demonstrated that development of normal wing morphology at these stages requires the presence

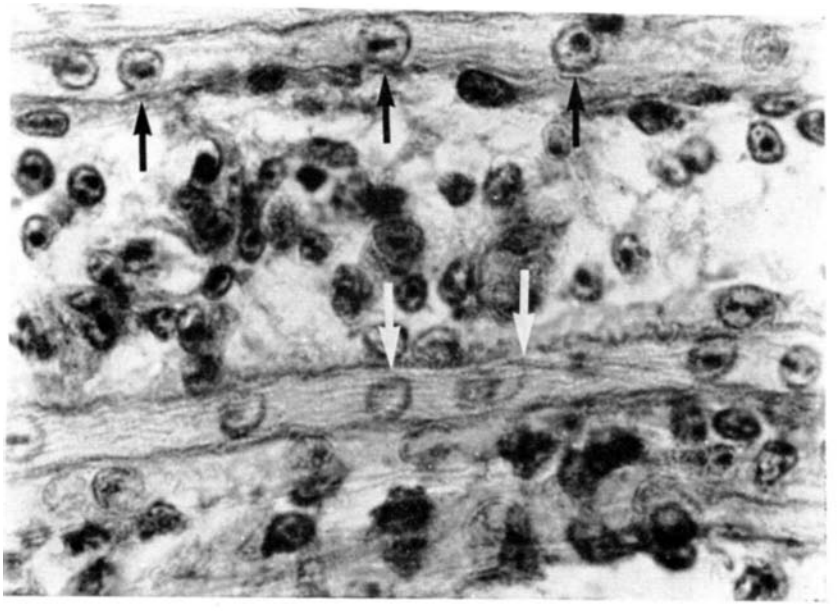

Fig. 4. Haematoxylin and eosin-stained section of graft retrieved from coelom, showing striations in the lower myotube (light arrows). The quail nucleoli are clearly visible in the upper myotube (black arrows). This section, arising from a stage-13 graft, was photographed by ordinary light.

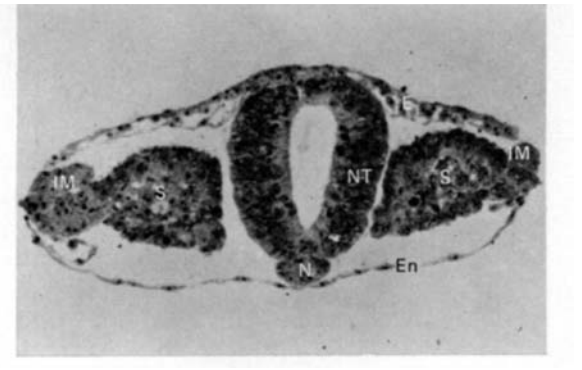

Fig. 5. Haematoxylin and eosin-stained section through donor piece remaining after the wing anlagen are removed. Note that the intermediate mesoderm which will give rise to the mesonephric tubule and which marks the boundary between the somites and the mesoderm is still intact. The left-hand side of this donor gave rise to the graft of Fig. 2. NT = neural tube; N = notochord; S = somites; IM = intermediate mesoderm; E = ectoderm; En = endoderm.

Table 2. *Results of coelom grafts by stage of host*

Stage of host	No. of grafts retrieved as % of no. of grafts surviving	No. containing muscle as % of no. of grafts retrieved
13	72	50
14	47	80
15	50	75
16	54	71
17	40	75
18–20	0	–

of somitic material (Pinot, 1970), it is clear that this single graft must be excluded from consideration, on the grounds that during its preparation some somitic material was included. This graft is not included in Table 1, or any of the above totals. Grafts made from later than stage 14 usually developed morphology at least reminiscent of the wing.

Secondary degeneration of muscle was occasionally observed, the muscle cells appearing vacuolated and showing fatty inclusions. This is due to the lack of innervation of the grafts in their new site (Eastlick, 1943). Only when healthy muscle cells could be seen at least somewhere in the graft, was it scored as having muscle.

The age of the host did not affect the ability of the graft to develop muscle (see Table 2). However, at stage 17 host age, retrieval of the graft becomes less common, and at stage 18 or later, grafts are never retrieved; perhaps because at these stages the coelom is much smaller, and the graft correspondingly more difficult to make accurately, or perhaps because the coelom walls are less capable of vascularizing the grafts at these stages.

DISCUSSION

We have demonstrated that when wing anlagen are removed before the reported time of invasion by somitic cells, they still contain cells having the ability to differentiate into muscle. The possibility that host somitic cells have invaded the grafts in their new site is excluded by our use of quail grafts. How then might this phenomenon arise? It is possible but unlikely in view of the results of Chevallier *et al.* (1978) that migration of cells occurs before stage 14. However, many of our results are obtained from stage 12 or earlier, which allows a considerable margin of error for mistakes in the staging. It is also possible that the muscle arises from the splanchnopleural tissue that our early grafts carry with them, and we are at present investigating this. The most interesting possibility, however, is that there is a real lability of the cells of the somatopleural mesoderm such that these cells can give rise to muscle which they never normally express, in the absence of the normal, somitically derived, muscle cells.

This would explain how muscle came to be present in some limbs where the somites had been removed or inactivated, and the anomalous results of chick to quail grafts, compared with quail to chick grafts, as described in the Introduction.

Our results differ from some previously reported (Christ *et al.* 1977), where the authors found that only grafts made from stage 15 or later gave rise to muscle, and some explanation must be made for this discrepancy. These authors base their conclusions on the pooled results of both coelom and CAM grafts, and it is possible that these two sites differ in their ability to evoke differentiation of cell types. For example, it is known that organ culture conditions which provoke good growth and differentiation of cartilage completely fail to provoke differentiation of muscle (Fell & Canti, 1935); and it may be that CAM grafts correspond more closely to these conditions. Again, it is not clear at what stage Christ *et al.* removed their grafts from the coelom, and it is possible that in some of them degeneration of the muscle due to the lack of innervation had already begun. Finally, in five of our early grafts we also failed to obtain muscle, for reasons which are not clear; so it is possible that some systematic difference in handling led Christ *et al.* to obtain a negative result.

Since, however, it seems that wing anlagen *can* give rise to muscle cells before invasion begins, the question arises of how this phenomenon is normally repressed. Perhaps there is a feedback effect of differentiating somitic muscle cells which suppresses further differentiation of somatopleural muscle cells.

These results also re-admit the possibility of a labile population of cells in the limb mesoderm, the likelihood of which had seemed reduced by the clarification of the somitic origin of the muscle cells in the normal musculature. The vexed question of lineage versus environmentally controlled differentiation remains open.

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