PROCEEDINGS

OF THE

NATIONAL ACADEMY OF SCIENCES

Volume 26 December 15, 1940 Number 12

THE DEVELOPMENT OF MELANOPHORES FROM EMBRYONIC MOUSE TISSUES GROWN IN THE COELOM OF CHICK EMBRYOS

By Mary E. Rawles

Department of Biology, Johns Hopkins University[1]

Communicated October 31, 1940

Transplantation experiments have demonstrated conclusively that migratory pigment-forming cells originate from the neural crest in amphibians[3] and in birds;[2,4,11] but so far no attempt has been made to determine whether or not the same is true in mammals. In their studies on the origin of pigment in birds Eastlick[4] and Ris[11] found the method of grafting embryonic tissue to the coelomic cavity of a developing chick embryo as used by Hamburger[5] particularly suitable. As far as the author is aware, tissues other than those of birds have not been grown in the coelom, but it is known that embryonic tissue of both the rat[7,9] and the mouse[10] will differentiate to a certain extent on the chorio-allantoic membrane of the chick. The possibility that the intracoelomic grafting method might be used with success for testing the origin of pigment cells in a mammal seemed too tempting to leave untried, therefore the following experiments were undertaken.

Experimental Procedure.—The mice from which the donor embryos were taken were of a strain known to produce only black offspring.[14] Embryos were used at ages ranging from $8^1/_2$ to 12 days, timed from the observance of the vaginal plug. They were removed from the uterus (under anesthesia) one at a time as needed. Those not used were fixed as controls for histological study. Each donor embryo was placed in warm sterile Locke's solution in a small Petri dish placed under a binocular dissecting microscope. After all membranes had been removed and the somites counted, small pieces (0.5 to 1 mm. in length) of skin ectoderm, plus some of the mesoderm lying directly beneath it, were isolated from various levels along the antero-posterior axis and implanted one at a time into the coelomic cavity of White Leghorn host embryos of 60 to 70 hours' incubation. White Leghorns were chosen as hosts for these experiments because of the fact

that they do not regularly exhibit pigment in their coelomic epithelium. Only 10 individuals out of 115 examined especially for this purpose on the 18th or 19th days of incubation showed even a trace of pigment. When present the pigment was more or less evenly distributed in the coelomic epithelium with greatest concentration in the region of the umbilicus. Examination of the pigmented region shows that nearly all of the melanophores had undergone degeneration, i.e., had retracted their processes, and persisted only as small dense balls of pigment (Fig. 5). For an account of similar degeneration in regenerated feathers and *in vitro* cultures see Hamilton.[6]

When the donors had well-developed limb buds (at 11 and 12 days), skin ectoderm plus adhering mesoderm from these regions as well as that from the head and trunk was tested. In the younger stages, however, the entire limb bud or longitudinal halves of limb buds were grafted, as were also pieces of somite and lateral plate with and without the neural tube. Aside from the intracoelomic implantations, a few grafts were made to the base of the developing wing bud. For this purpose skin ectoderm as free as possible of adhering mesenchyme was taken from the posterior head region of 10-day embryos (about 30 somites).

For making the isolations, steel beading needles with very fine points, sealed into glass tubing for handles, and a Bowman's iris knife were used. To facilitate handling, the pieces were stained by touching them lightly, before removal from the body of the embryo, with small glass rods having agar-coated tips stained with Nile blue sulphate.

The preparation of the host embryo was essentially that described previously for transplanting embryonic chick tissue to the body wall or to the limb bud.[13] A small rectangular piece of shell approximately 1 × 0.5 cm. was sawn and lifted out immediately over the body of the embryo whose position had been previously determined by candling. The shell membrane was picked away carefully under a binocular microscope, thus exposing the embryo lying on the yolk beneath. By touching the embryonic area with the stained tips of the above-mentioned glass rods, the membranes become visible immediately and can be very easily opened. Usually at the stages used the amnion had not grown over the posterior regions, so did not have to be dealt with. When the body wall was thus exposed a small slit was made with a glass needle in the ectoderm and somatic mesoderm just anterior to the leg bud region of either the right or left sides. The implant of donor tissue was transferred through the window to the host blastoderm in a capillary pipette of suitable size with a small amount of saline solution. It was pushed through the slit gently with a glass needle into the coelomic cavity. The original piece of shell was fitted into the window the edges sealed with melted paraffin and the egg returned to the incubator. During the operation and afterwards the egg was placed on a cotton pad in a

Syracuse watchglass to prevent rotation. The host embryo was removed and dissected usually on the 19th day of incubation. A thorough examination of the coelom was made for the graft and melanophores migrating from it. In all positive cases the grafts were fixed *in situ* in Bouin's fluid for histological study.

Observations.—When the hosts were dissected, it was found that the implanted mouse tissue had differentiated into a definite body or graft in 62 out of 101 cases. Except when entire limb buds or halves of limb buds had been taken originally, the grafts were small, rounded or oval-shaped bodies not exceeding 4 mm. in diameter, well vascularized and firmly attached to the wall of the coelom in the posterior region of the body. Often the free surface of the graft was connected to mesenteries, intestine, colacal wall or even to some other part of the coelomic epithelium by strands of connective tissue. Always the graft surface was covered with a thin, smooth, transparent layer of epithelium. The great majority of grafts contained well-developed hairs, very conspicuous when pigmented (Fig. 1), less so when unpigmented. Due to the fact that the implanted piece of skin ectoderm always tends to round up into a hollow ball or vesicle, the developing hairs are most often directed inwards towards the center of the graft.

The total age of the graft tissue when removed for study is equivalent to that of 3- to 7-day postnatal mice ($8^1/_2$ to 12 days + approximately 16 days in the coelom) and of strictly comparable differentiation. At these ages pigmented hairs can be seen in the normal mouse and with low magnification numerous melanophores scattered in the skin between the hairs are easily visible. Even at birth melanophores concentrated in the developing hair follicles and distributed in the skin between them can be found in the dorsal and lateral regions of the body. In 34 cases specific mouse melanophores densely crowded with coarse black melanin granules were found in the graft or migrating in the coelomic epithelium of the host in its immediate vicinity (Figs. 2, 3 and 4).

The larger size of the mouse melanophore as compared with that of the White Leghorn, and its extreme blackness make it very conspicuous and easy to detect in the coelomic epithelium. Usually there are two diametrically opposed processes ranging from 50 to 100 micra in length (Fig. 3). Occasionally several cells hang together and form long chains (Fig. 4).

Whether or not melanophores were produced by the grafted tissue was soon found to depend upon the age and the region of the embryo from which the implant was taken. Differences in these respects are indicated in the following paragraphs, grafts from older embryos being described first.

Twelve-Day Donors.—Skin ectoderm plus adhering mesoderm from all regions tested, namely, neck, shoulder, rump and limb buds, gave 15

successful grafts out of 20 transplants. All had well-developed pigmented hairs, many approximately 1 mm. long (Fig. 1). Histological study of one typical graft showed a vesicular arrangement of well-differentiated skin with numerous hairs growing out into a central cavity. Melanophores were seen in the subcutaneous tissue and dermal layer of the skin as well as in the hair follicles themselves. Although all of the grafts contained pigment, only 9 of the 15 showed migration of melanophores into the surrounding coelomic epithelium of the host and in none was it very extensive.[15]

Eleven-Day Donors (40–45 Somites).—Eleven grafts were recovered from 22 hosts examined. As in the preceding series, skin ectoderm and mesoderm from the head and trunk levels produced hairs with pigment, and in 5 of the 7 cases rather extensive migration of mouse melanophores into the host coelomic epithelium was observed. Unlike the preceding, however, skin ectoderm and mesoderm from the limb buds (4 cases, 2 examined histologically) gave well-differentiated hair follicles wholly without pigment: no melanophores were found either in the graft or in the host coelomic epithelium.

Ten to 10½-Day Donors (28–35 Somites).—In addition to transplanting

---

DESCRIPTION OF PLATE 1

Figure 1. Graft developed from small piece of skin ectoderm + adhering mesenchyme (0.8 × 0.5 mm.) from trunk at base of right fore limb bud of a 12-day mouse embryo, grown 16½ days in the embryonic coelom of a White Leghorn chick. Note mass of well-developed hairs fully pigmented. Photographed *in situ*. (× 10.)

Figure 2. Portion of coelom of White Leghorn host embryo (19¼ days) showing migration of mouse melanophores from graft (*G*). Note concentration of melanophores along walls of small blood vessels. Produced by implanting right half of neural tube and adjacent somite material at 25th somite level of 10-day (31 somites) mouse embryo to 70-hr. chick coelom. (× 10.)

Figure 3. Unstained whole mount of portion of coelomic epithelium of White Leghorn host embryo showing migrating mouse melanophores produced from implant of hind limb bud skin ectoderm (0.7 × 0.3 mm.) from 12-day mouse embryo, grown 16½ days in embryonic chick coelom. Graft (not shown) contained hairs fully pigmented. (× 85.)

Figure 4. Unstained whole mount of portion of coelomic epithelium of a White Leghorn host embryo showing migrating mouse melanophores produced from dorsal trunk skin ectoderm (0.7 × 0.4 mm.) of 45-somite (11-day) embryo, grown 17½ days in coelom. Note melanophores hanging together in long chains. (× 85.)

Figure 5. Unstained whole mount of portion of coelomic epithelium of a normal 19-day White Leghorn embryo showing degenerated melanophores with retracted processes. (× 85.)

Figure 6. Portion of wing of 17-day White Leghorn host embryo showing dense mass of mouse melanophores in the skin and subcutaneous tissue. Produced by implanting head skin ectoderm (1 × 0.4 mm.) from a 10-day (30-somite) mouse embryo to the wing bud region at 60 hrs. of incubation. (× 25.)

Figure 7. Same as figure 6 with greater magnification showing mouse melanophores at the periphery of mass. (× 85.)

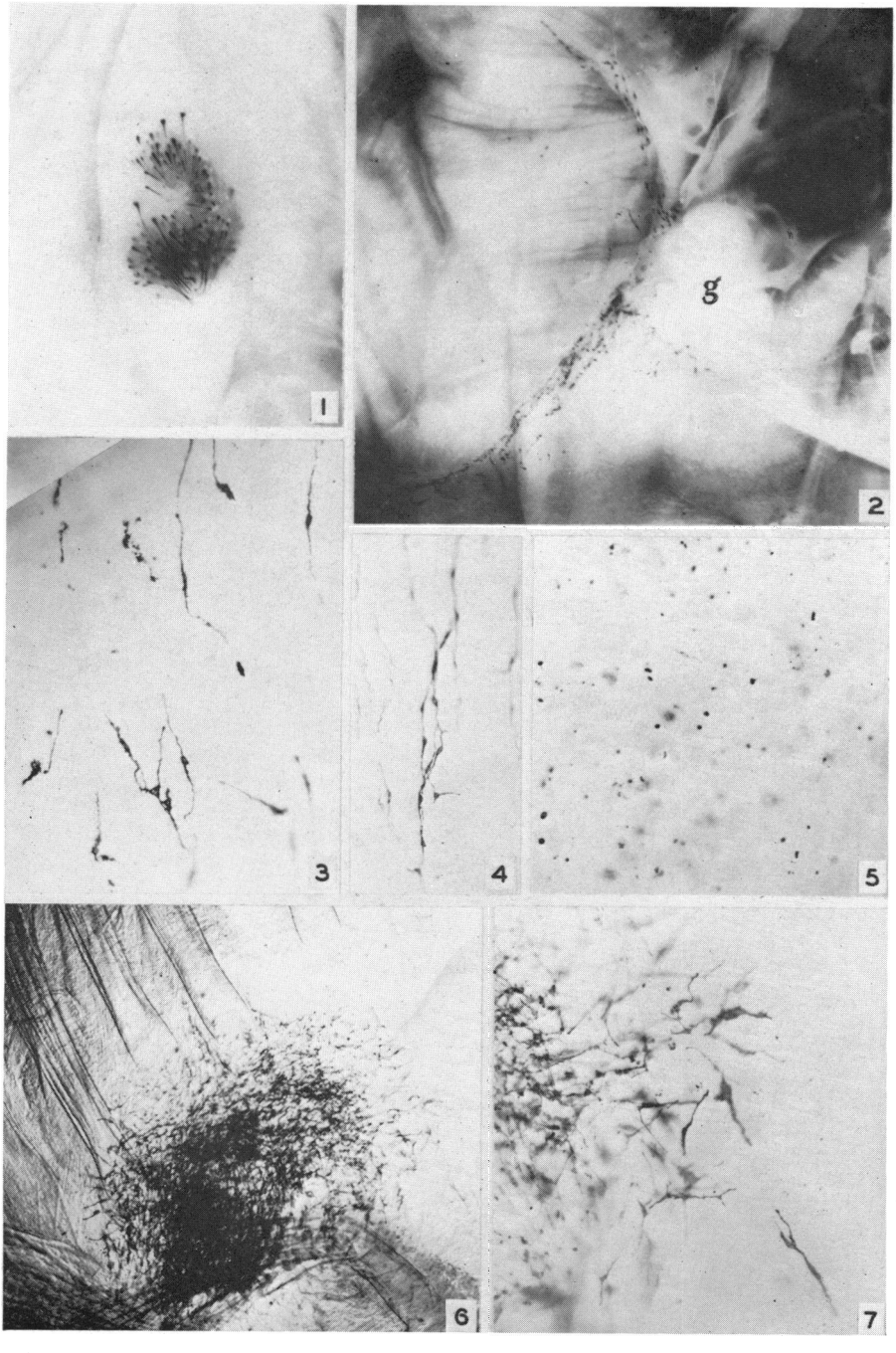

PLATE 1

skin ectoderm and subjacent mesoderm from various levels as done in the preceding series, grafts were made also of entire limb buds, longitudinal halves of limb buds, short segments of somite and somite plus the adjacent half of the neural tube. Thirty grafts were obtained from 40 hosts examined; 6 were studied histologically.

Head skin and trunk skin from the dorsal region, i.e., directly over the neural tube, at all levels produced melanophores both in the graft and in the coelomic epithelium about the graft. But no melanophores developed from either of the limb buds when grafted in their entirety or in part, although well-differentiated hair follicles were abundant. The entire limb bud (12 cases) showed a remarkably normal differentiation of skeletal parts, muscle, skin and hair.

The most beautiful and extensive migration of mouse melanophores into the coelomic epithelium came from grafts of somite including the neural tube taken from the leg bud level or just anterior to it. In the best of the six cases obtained, melanophores in large numbers had migrated for a distance of about 5 mm. in two directions along the walls of small blood vessels (Fig. 2). The grafts themselves showed no pigment either macroscopically or microscopically. In the two studied histologically small skin vesicles with well-differentiated skin were found but no hair follicles. The bulk of the graft consisted of a mass of central nervous tissue (spinal cord) showing strikingly normal differentiation.

Similar grafts obtained from the intact somite not including the neural tube showed no pigment either in the graft or in the coelomic epithelium (6 cases). Histological examination of two of these showed good development of skin and hair follicles. One in addition contained skeletal muscle and small cartilages.

In view of the fact that the developing wing bud of the chick has proved such a favorable site for melanophore migration in birds in general, it seemed desirable to make the same test with mouse melanophores. Thus small pieces of head skin ectoderm relatively free of adhering mesoderm were grafted to the wing bud region of White Leghorn host embryos of approximately 60 hours' incubation. In one case out of three, examination on the 17th day showed a small black spot 2 mm. in diameter, in the skin and subcutaneous tissue of the wing of the host at the implantation site (Fig. 6). When examined under a microscope the black area was found to be densely crowded with large branching mouse melanophores exactly like the normal and like those developed in the coelom (Fig. 7). None of the melanophores had migrated into the overlying feathers.

Eight and One-half to 9-Day Donors (15–20 Somites).—Embryos at this stage had not developed limb buds and were very fragile. Two types of transplants were made: those in which the somites and lateral plate were cut entirely free of the neural tube, and those in which the neural tube was

left attached. Levels from the 10th somite posteriorly were thus tested. Six grafts were recovered from eight hosts alive at the 19th day of incubation; two came from implants of pure somite and lateral plate and produced no pigment; the other four from implants of somite and lateral plate including neural tube. In none of the four containing neural tube was pigment found in the graft itself, but two showed typical mouse melanophores migrating in the coelomic epithelium around the graft site. In one, the melanophore migration was particularly pretty and extended for several millimeters along a strand of tissue attaching the graft to the cloacal wall. Even though the number of grafts analyzed in this series is small, the results are definitely in line with the others.

Discussion.—The results summarized above show that between $8^1/_2$ and 12 days of gestation the capacity of the grafted embryonic tissue to produce melanophores spreads rapidly both antero-posteriorly and medio-laterally. Grafts from the head region at all stages tested produced pigmented hairs consistently (no data on head skin of $8^1/_2$-day embryos), but whether or not grafts from the somites and limb buds produced hairs with pigment depended upon both age and body level. For example, at $8^1/_2$ days no melanophores developed from grafts of somite material when completely isolated from the neural tube; at 10 days skin ectoderm and mesoderm from somites of the anterior trunk levels including the fore limb region gave hairs fully pigmented while similar grafts from the hind limb region gave hairs entirely without pigment; at 11 days somite grafts from all levels including the posterior limb region produced hairs with pigment. Ten and 11-day limb buds, entire or in part, failed to develop pigment, although skin and hair follicles were abundant. By 12 days, however, pigmented hairs developed regularly from implants of skin + adhering mesenchyme from both limb buds.

This occurrence of well-differentiated hairs wholly without pigment from certain body regions at certain developmental stages suggests strongly that the melanophores migrate into the developing hair follicles from an outside source. That this source lies in or is closely associated with the neural tube is demonstrated by the fact that at those stages in which somite material failed to produce pigment cells, as in the hind limb region of a 10-day embryo, for example, the same regions would develop pigment if the implant was made to include the adjacent part of the neural tube.

The results of the present experiments parallel very closely those obtained from similar transplantation experiments in the chick. By grafting limb buds from chick embryos (24–30 somites) of pigmented breeds to the coelom of White Leghorn hosts of similar ages, Eastlick[4] was able to show that white feathers only were produced unless the implant included body wall material (skin ectoderm and mesoderm) up to the neural tube. Ris[11] obtained similar results by transplanting to the chorio-allantois as well as

to the coelom. Limb buds from embryos of pigmented breeds isolated at 72 hrs. developed white feathers at both sites while the same isolations later, i.e., at 90 hrs. or more, produced pigmented feathers in the graft.

Further evidence for the extra-epidermal origin of pigment cells in the chick, and their lateral migration from the neural tube region into the overlying ectoderm and into the limb buds, is obtained from the transplantation experiments of Willier and Rawles,[13] and Watterson.[12] Small pieces of skin ectoderm or pure mesoderm from embryos of one breed were implanted into the developing wing bud region of host embryos of a similar age (67–108 hrs.) but of a genetically different breed. Implants from the head or trunk regions at all ages tested produced an area of donor-colored feathers on the host at and about the site of implantation. But implants from the wing bud of embryos younger than 80 hours or from the leg bud earlier than 96 hours failed to produce pigment in the host feathers.

Using the same method of implantation to the limb bud, Dorris[2] obtained areas of pigmented skin and feathers in white hosts by implanting thin strips of the rising neural folds (neural crest region) anterior to the first somite from embryos of 3–10 somites of black breeds. Ris[11] correlated the development of pigment in grafts with the morphological appearance of the neural crest at the time of isolation. By transplanting portions of embryos (potentially pigmented) from various levels at successive stages in their development to the embryonic coelom of White Leghorns, he was able to show quite convincingly that the inclusion of the neural crest, migrating cells of the crest or presumptive neural crest, in the transplant was essential for the development of melanophores.

The similarity of the intra-coelomic grafts of mouse tissue with those obtained by Ris from chick and other bird implants is indeed striking. While the mouse melanophores do not migrate nearly as extensively in the White Leghorn coelomic epithelium as those from birds, they do nevertheless tend to wander out from the grafts and follow blood vessels, nerves and strands of connective tissue in much the same way. The failure of mouse melanophores to migrate to any extent in the wing bud, as observed in the one case obtained, is in contrast with the tremendous migration exhibited by bird melanophores in the same site.

The extensive work of Holmdahl[8] on the origin and development of the neural crest in birds and mammals shows how very much alike these two groups are in respect to this structure. In both it arises first in the region of the mid-brain at early somite stages, and develops posteriorly along the neural tube, migrating laterally as the age of the embryo increases. Histological examination of control mouse embryos at the stages and levels used for transplantation shows that the regions which produced pigment in the grafts coincide with the regions from which the neural crest had already migrated from the neural tube. At stages where in posterior levels

the crest had not migrated, or had not migrated sufficiently far to be included in the transplant ($8^1/_2$–10 days), no pigment resulted. Such facts strongly suggest that the same source and mode of migration of melanophores obtains in the mouse as in the bird.

In summary, the experimental as well as the morphological evidence shows that in the mouse, as in the chick, the melanophores arise from an outside source, the neural crest, presumably, and migrate into the skin and developing hair follicles. Further, it is demonstrated that the embryonic coelom of the chick is an excellent site for the growth and differentiation of implanted embryonic mammalian (mouse) tissue.

[1] The experiments upon which this report is based were carried out at the University of Rochester. The manuscript was prepared at Stanford University during a stay of four months in the laboratory of Professor C. H. Danforth. I am deeply indebted to him for the interest he showed in examining the material and for many helpful suggestions. My appreciative thanks are also due Professor B. H. Willier for a critical reading of the manuscript.

[2] Dorris, F., *Jour. Exp. Zoöl.*, **80**, 315–345 (1939).

[3] DuShane, G. P., *Ibid.*, **78**, 485–501 (1938).

[4] Eastlick, H. L., *Ibid.*, **82**, 131–158 (1939).

[5] Hamburger, V., *Ibid.*, **77**, 379–397 (1938).

[6] Hamilton, H. L., *Anat. Rec.* **78** (1940).

[7] Hiraiwa, Y. K., *Jour. Exp. Zoöl.*, **49**, 441–457 (1927).

[8] Holmdahl, D. E., *Zeit. mikro-anat. For.*, **14**, 99–298 (1928).

[9] Nicholas, J. S., and Rudnick, D., *Ibid.*, **66**, 193–256 (1933).

[10] Reed, S. C., and Alley, A., *Anat. Rec.*, **73**, 257–265 (1939).

[11] Ris, Hans, *Physiol. Zoöl.* (in press) (1941).

[12] Watterson, R. L., *Anat. Rec.*, **70**, Supp. 4, 100 (1938).

[13] Willier, B. H., and Rawles, M. E., *Physiol. Zoöl.*, **13**, 177–199 (1940).

[14] Heterozygous black females were crossed with homozygous black males (C57 black Bar Harbor strain) and the F_1 females from this mating back-crossed to the male parent for successive generations.

[15] A few trial cultures of skin ectoderm were grown *in vitro* in a medium of chick embryonic extract and plasma by Mr. Howard L. Hamilton. Both hair follicles and melanophores differentiated in the explants.