THE BEHAVIOR AND MORPHOLOGY OF URODELE PIGMENT CELLS *IN VITRO*

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Our laboratory is interested in the role of extracellular matrix (ECM) molecules in the morphogenesis of the neural crest. We use a number of approaches to investigate this problem: 1) the pathways of neural crest migration are determined using cell specific markers, 2) the composition of the surrounding ECM is determined using histological techniques, 3) neural crest cells are confronted with isolated ECM components *in vitro*, and 4) the ECM or neural crest cell surface is altered *in vivo*, and associated changes in the distribution of the neural crest cells is determined. The results of such experiments are helping us to answer questions concerning the molecular basis of extracellular control of embryonic cell motility and differentiation.

As one model system for these studies, we have chosen the control of pigment cell pattern formation in amphibian embryos. This system has a number of inherent advantages over avian and mammalian systems: 1) the embryos are readily accessible and amenable to a variety of surgical manipulations, 2) there are several types of easily identifiable pigment cells that facilitate study of tissue-specific cell segregation and differentiation, 3) pigment cells (especially melanophores) can be observed in transparent embryos, permitting direct videoanalysis of cell migration, and 4) there are a variety of mutants and species differences that can be used to study pigment pattern formation. One stumbling block in these studies has been the development of reliable tissue culture methods that permit analysis of urodele pigment cell migration and differentiation in the presence of isolated ECM molecules. We would like to report here our success in culturing both melanophores and xanthophores isolated from the California newt, *Taricha torosa*, on both tissue culture plastic and in three-dimensional collagenous artificial ECM. The behavior and morphology of these cells *in vitro* has permitted us to speculate about the nature of the controls of xanthophore and melanophore patterning in *T. torosa* embryos.

There is a substantial literature from the laboratories of V.C. Twitty and his colleagues on the development of pigment cell patterns in the three species of *Taricha* found in California (eg. Twitty, 1936, 1945; Twitty and Bodenstein, 1939, 1944; Twitty and Niu, 1948). One of these species, *T. torosa*, has been studied extensively, in part due to the striking nature of the melanophore pattern and in part because of its availability in the San Francisco Bay area. Melanophores first appear in *T. torosa* scattered over the surface of the somites at stage 35 (the axolotl stages of development of Schreckenberg and Jacobson [1975] are used with *Taricha*, since the embryos are almost indistinguishable). Over a period of several days, these cells segregate into two stripes. One stripe forms at the base of the dorsal fin along the apex of the somites; a second stripe, which is less pronounced than the dorsal stripe, forms along the somite-lateral plate mesoderm border. In our laboratory, we

have determined the distribution of xanthophores during the development of melanophore stripes using the NH_4OH-induced pteridine fluorescence technique (see Epperlein and Claviez, 1982; Tucker, 1986). Xanthophores first appear scattered among the melanophores over the surface of the somites, and are later found as individual cells between the two melanophore stripes and in the dorsal fin. We were interested in determining if xanthophores were found in the dorsal fin because of the influence of ECM on pigment cell differentiation or due to the ability of xanthophores and not melanophores to enter the glycosaminoglycan (GAG) -rich environment of the dorsal fin.

In the axolotl, the premigratory neural crest sits as a cord along the neural tube. This cord can be dissected free of surrounding tissue and is a potent source of pigment cells (Keller and Spieth, 1984). The neural crest in T. torosa is not as accessible at this stage, making it difficult to remove the premigratory crest without neural tube contamination. As a source of T. torosa neural crest cells, we chose to dissect fragments of neural folds from stage 15-18 neurulae. Embryos were washed briefly in 20% ethanol and rinsed in sterile 20% Steinberg's solution. Pieces of neural folds approximately 250 um long (as long as the fold is wide) were removed with sterile tungsten needles from the region of the hindbrain-neural tube border. Embryos were immersed in 100% Steinberg's solution during the dissection to promote healing of the wound. These embryos were then returned to 20% Steinberg's solution or tap water and raised in order to compare the timing of the appearance of pigment cells in vitro and in vivo. There are a number of advantages in using fragments of neural folds instead of explanting the dorsal half of the neural tube of older (stage 24-26) embryos. After a little practice, it is possible to remove fragments with very little epidermal or neural plate contamination, and contralateral fragments can be compared after being cultured under different conditions. Neural fold fragments also seemed to adhere to a variety of substrata more readily than neural tube fragments, and generally provided more consistent results.

Neural fold fragments were cultured on collagen gels to which we had added various ECM components. Collagen gels were constructed using modifications of procedures described in detail elsewhere (Elsdale and Bard, 1972; Tucker and Erickson, 1984; Turley et al., 1985). In brief, 8 parts collagen (Vitrogen 100, from Collagen Corporation, Palo Alto, CA; lot #84K139), 1 part 2X L-15 Leibovitz's medium (GIBCO, Grand Island, NY; lot #60P7430; from powder), and 1 part 0.142N NaOH were combined with 1/2X L-15 medium containing 10% fetal calf serum (GIBCO, lot #28P9410) to provide a final collagen concentration of 750 ug/ml. The final concentration of the fetal calf serum was approximately 6%. For some experiments, 2.5 mg/ml chondroitin sulfate (CS; from shark cartilage, Sigma, St. Louis, MO, lot #102F-0418) or 1 mg/ml hyaluronate (HA; from human umbilical cord, Sigma, lot #34F-0649) were added to the 1/2X L-15. The purity of the GAGs was determined previously (Erickson and Turley, 1983). Antibiotics and antimycotics (gentamicin sulfate, from Sigma, and Fungizone, from GIBCO) were also added to the collagen solution, which was kept on ice until neural folds were ready for explantation. The collagen solution was gelled for 10 min at 37°C, and neural fold fragments were placed on top of the gel. All cultures were maintained at 16°C in a humid environment.

TABLE 1

T. TOROSA NEURAL CREST DIFFERENTIATION IN VITRO

CULTURE CONDITIONS[1]	PIGMENT CELLS APPEAR (DAYS)	DAYS IN VITRO	MELANOPHORES/ EXPLANT, OR % PIGMENT CELLS
COLLAGEN GEL			
1/2X L-15	6	10	26.6 (5)[2]
1/2X L-15 1 mg/ml HA	8	10	1.1 (7)
T.C. PLASTIC			
1/2X L-15	8/9	12	50% (5)[3]
1/2X L-15 2 mg/ml CS	9	12	43% (3)
1/2X L-15 1 mg/ml HA	12	12	8% (3)

[1]See text for precise culture conditions.
[2]Number of melanophores/explant (number of explants analyzed).
[3]Percent pigment cells (number of explants analyzed).

Cells migrated from the neural fold explants approximately 48 hr after explantation. Melanophores became visible in the cultures of collagen alone after 6 days (Table 1), which corresponded with the time that melanophores appeared in embryos as well. Xanthophores appeared in these cultures 2 to 3 days later. After 13 days, xanthophores had migrated significantly farther than melanophores from the edge of the explant (Figure 1). Xanthophores tended to be bipolar or rounded in shape, whereas melanophores were highly arborized. When CS was present in the collagen gel, the distance that both xanthophores and melanophores migrated from the explant was significantly reduced, with the end result being that almost no melanophores invaded the gel. Cells were found both on the surface of the collagen, as well as inside the gel. When HA was present in the gel, there was a dramatic reduction in the number of pigment cells, and the time of appearance of the pigment cells was delayed by 48 hr (Table 1). Cells did not invade the collagen, but instead spread as a sheet on the surface of the gel.

To determine if the delay in chromatophore differentiation was the result of HA interfering with neural crest-substratum interactions, or if HA was acting independently at the cell surface, neural fold fragments were explanted onto tissue culture plastic dishes (Corning Glass Works, Corning, NY) in 1/2X L-15 medium with 10% fetal calf serum (Keller and Spieth, 1984). After the explant had attached and cells had started to

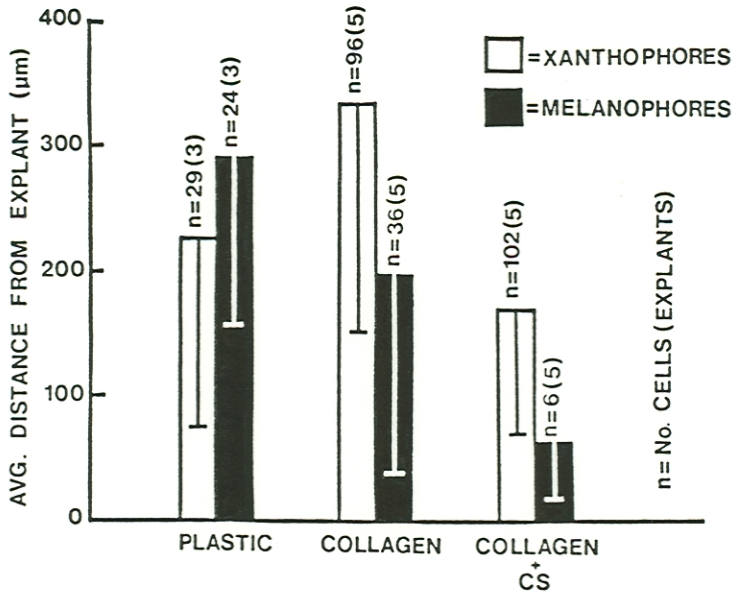

Figure 1. The distance that xanthophores and melanophores migrate from explants after 13 days in culture. On plastic, melanophores migrate significantly farther ($p < 0.1$) than xanthophores. In contrast, in collagen gels and in collagen gels that contain chondroitin sulfate (CS) xanthophores migrate significantly farther ($p < 0.01$) than melanophores.

migrate onto the plastic (approximately 48 hr), the medium was exchanged with serum-enriched medium containing 1 mg/ml HA, 2 mg/ml CS, or enriched 1/2X L-15 medium alone. The medium with or without the GAGs was replenished every 48 hr.

As was seen in the collagen gels, HA inhibited the time of appearance and number of pigment cells when it was present in the solution alone (Table 1). Since HA does not bind in appreciable amounts to tissue culture plastic (Erickson and Turley, 1983), this suggests that HA is acting atthe surface of the neural crest cells,perhaps by inhibiting access of a serum factor to the cell surface. It is interesting to note that fetal calf serum was necessary for chromatophore differentiation; when no serum was present, there was a significant increase in the number of neurites in the cultures, but there were no pigment cells.

Our results lead us to speculate as to how the ECM may control the distribution of xanthophores and melanophores in T. torosa. The dorsal fin contains considerably more GAG (both HA and sulfated GAG) than the space between the somites and ectoderm where the pigment cells appear in vivo (Tucker and Erickson, 1986). Perhaps neural crest cells that enter the tail fin are inhibited from differentiating by HA. Xanthophores would be able to enter the tail fin later (after differentiating elsewhere) but not melanophores--we have shown that this is the case for these two cell types in GAG-rich collagen gels. Thus GAGs in the ECM are controlling pigment cell pattern formation by providing regions of differential adhesion to pigment cells with different migratory capacities, as well as by inhibiting neural crest cell differentiation.

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