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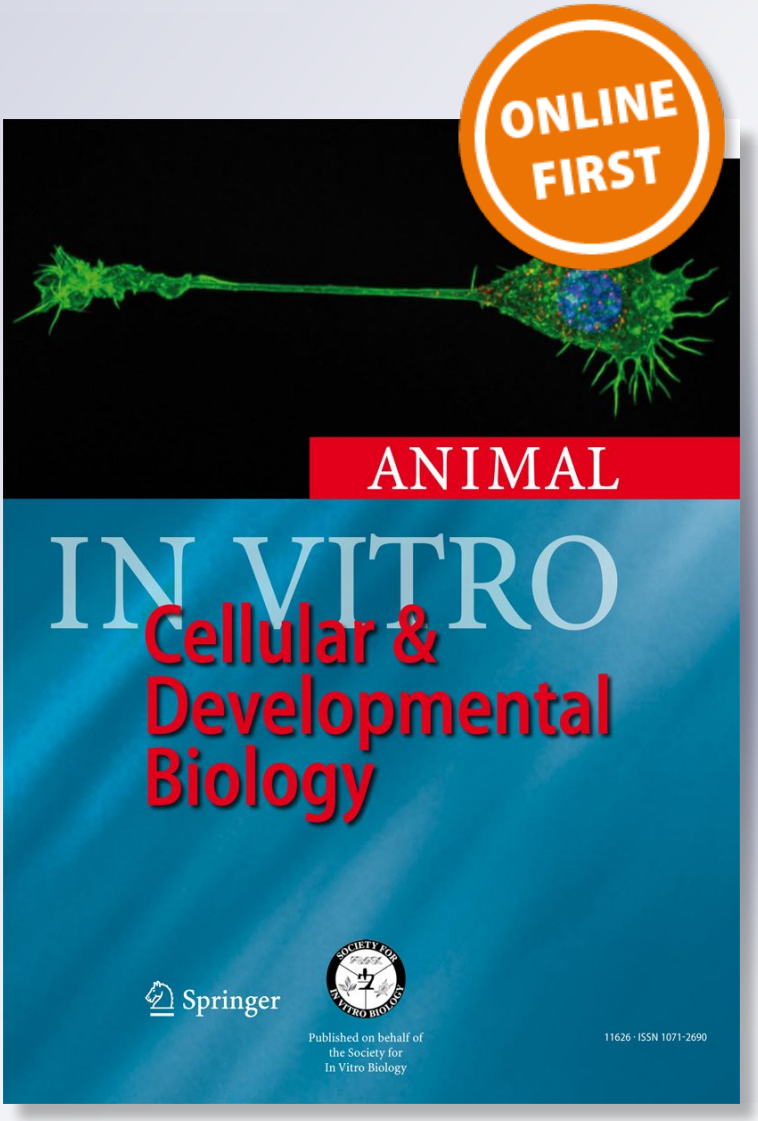

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REPORT

Establishing primary cultures of embryonic intestinal cells from the elasmobranch, *Leucoraja erinacea*

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Elasmobranch fish, including the sharks, skates, and rays have proved valuable animal models for biomedical and genomic studies (Luer 1989; Ballatori et al. 2003). However, few experimental resources are available for cellular studies in elasmobranch animal models. Over the past 20 years, several groups have cultured different tissues from elasmobranchs with mixed results (for a summary, see Table 1). Reported growth conditions for culturing elasmobranch tissues have varied in temperature (18–29 °C), in pH (7.1–7.6), and in the concentrations of salts, urea, and trimethylamine *N*-oxide (TMAO; Table 1). The use of a wide variety of culture conditions and basal nutrient media attests to the difficulties investigators have had in developing standard methods for maintaining elasmobranch tissues in vitro.

To date, only three cell lines have been established from elasmobranchs: a brain tissue cell line from *Carcharhinus falciformis*, a whole embryo mesenchymal line (SAE) from *Squalus acanthias* and a whole embryo line (LEE-1) from *Leucoraja erinacea* (Poyer and Hartmann 1992; Parton et al. 2007; Hwang et al. 2008). Here, we report the first successful attempt at creating an intestine primary cell culture system in the skate, *L. erinacea*.

Initiating Primary Cultures

Skate embryos (*L. erinacea*) were purchased from Woods Hole (MA) and cultured in circulating marine tanks composed of Instant Ocean™ at 16 °C. Embryos were sacrificed at stages 28–30 (Ballard et al. 1993). All procedures for animal use were in accordance with standards set by the NIH and approved by the Institutional Animal Care and Use Committee at Union College.

Spiral intestines were micro-dissected from killed embryos under sterile conditions and placed in LDF media. During dissections, care was taken to remove the liver, rectal gland, and distal hindgut. Spiral intestines were flushed five times on ice with LDF, using a 1 cc sterile syringe and 26-gauge needle to remove any debris in the lumen. Spiral intestines were placed in a fresh 1-mL drop of LDF and manually minced with sterile forceps and scalpel. The minced tissue with media was further disassociated by pipetting, spun, and washed three times with fresh LDF. The LDF skate media contains 50 % DME, 35 % L-15, 15 % F-12, 15 mM HEPES, 200 μg/mL penicillin, 200 μg/mL streptomycin, 25 μg/mL ampicillin, and 0.18 g/L sodium bicarbonate (Helmrich and Barnes 1999; Parton et al. 2007). pH was adjusted to 7.2 or 8.0 with sterile 1.0 N NaOH and filter-sterilized. A lower than usual bicarbonate concentration was used as elasmobranch cells are typically grown at low temperatures. In this study, cells were grown at 18 °C in an air atmosphere.

Initial plating with LDF (pH 7.2) and growth factors at 18°C were not successful. Cells failed to thrive and adhere to plastic ware. The application of a collagen I matrix to wells prior to plating cells significantly promoted cell adhesion and growth in initial cultures. For the collagen matrix, rat tail Collagen I (Gibco #A10483-01, Grand Island, NY) was gelled as instructed by the manufacturer and adjusted to a final pH of 8.0 with NaOH. To precondition the collagen and stabilize its pH, collagen was incubated for 24–48 h with LDF and growth factors prior to cell plating. Growth factors and supplements were added fresh to the LDF growth media prior to feeding cells: 2 % heat-inactivated FBS (Hyclone, Logan, UT), 10 μg/mL insulin (Sigma-Aldrich #I-5500, St. Louis, MO), 10 μg/mL transferrin (Sigma #T-1283), 10 nM selenous acid (Sigma #211176), 50 ng/mL EGF (R&D #236-EG), 10 ng/mL, FGF

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Table 1. Elasmobranch basal nutrient media

Basal nutrient media	Cells (species)	Temp.	Culture conditions									References
			Media	serum	salts	HEPES	$NaHCO_3$	Antibiotics	pH	Urea	TMAO	
	SRG–rectal gland (*S. acanthias*)	20°C	DME, 50 % F-12, 50 %	FBS, 5 %	$CaCl_2$, 3.9 mM $MgCl_2$, 2.5 mM NaCl, 94 mM	15 mM	21 mM	100 U/mL pen. 100 µg/mL strep.	7.4	300 mM	150 mM	Valentich, 1991
	Brain tissue (*C. falciformis*)	29°C	Opti-MEM I	FBS 10 %	NaCl, 188 mM	–[a]	–[a]	100 U/mL pen. 100 µg/mL strep. 0.25 µg/mL Fung. 50 µg/mL Genta.	7.2	333 mM	54 mM	Poyer and Hartmann 1992
	Heart, kidney, brain, testis—PC (*C. falciformis*)	26°C	Opti-MEM I[a] F-12[a] L-15[a]	FBS, 10 %	NaCl, 188 mM	–[a]	–[a]	50 µg/mL Genta.	7.1	333 mM	54 mM	Hartmann et al. 1992
MEM	Cartilage PC (*Raja porasa*)	24°C	MEM	FBS, 20 %		–[a]	–[a]	200 U/mL pen. 200 µg/mL strep.	7.6	–	–	Fan et al. 2003
	Immune cells (*S. tiburo*)	25°C	RPMI	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–	–	Walsh et al., 2006
LDF	SAE (*S. acanthias*)	18 °C	DME, 50 % L-15, 35 % F-12, 15 %	FBS, 2 %		15 mM	0.18 g/L	200 U/mL pen. 200 µg/mL strep. 25 µg/mL amp.	7.2	–	–	Parton et al. 2007Helmrich & Barnes, 1999
LDF	LEE-1	18 °C	DME, 50 % L-15, 35 % F-12, 15 % SW, 4 %	FBS, 2 %		15 mM	0.18 g/L	200 U/mL pen. 200 µg/mL strep. 25 µg/mL amp.	7.2	–	–	Hwang et al., 2008
LDF-SW	*e*Intestinal PC (*L. erinacea*)	18 °C	DME, 50 % L-15, 35 % F-12, 15 % SW, 16 %	FBS, 2 %		15 mM	0.18 g/L	200 U/mL pen. 200 µg/mL strep. 25 µg/mL amp.	8.0	200 mM	150 mM	This paper

PC primary culture, *SW* sea water

[a] Concentration not reported

(R&D Systems #233-FB, Minneapolis, MN), 2 mM L-glutamine (Gibco #35050061), 1:1,000 dilution Chemically defined lipids (Gibco #11905-031), 1:1,000 dilution MEM nonessential amino acids (Hyclone #SH30238.01), 1:1000 dilution MEM amino acids (Cellgro #25-030-Cl, Manassas, VA), and 55 μM beta-mercaptoethanol (Sigma #M-2650). In addition to growth factors and supplements, a final 10× concentration of Zonker antibiotic mix was added [100× Zonker contains 2.5 units of penicillin G (Sigma #P-7794), 2.5 g streptomycin sulfate (Sigma #S-9137), 2.5 g neomycin sulfate (Sigma #N-6386), 25,000 units bacitracin (Sigma #B-1025) and 5 mL 10× Hanks balanced salt solution made to 100 mL final volume]. The 10× Zonker antibiotic mix was kept on cells for the first 48 h to guard against any commissural bacteria, which may have colonized the intestine. After 48 h, the Zonker concentration was reduced to 5×, to prevent the development of antibiotic resistance.

Within 3–4 d of initial seeding on collagen, cells swelled into fluid-filled balloons. To determine the optimal osmolarity for growth of intestinal cells, several variables were tested: pH, and the addition of urea, TMAO, and seawater. Optimal growth was found with 84 % LDF at pH 8.0 and 16 % filter-sterilized seawater [(Frenchman's Bay, ME), containing 15 mM HEPES, 200 μg/mL penicillin, 200 μg/mL streptomycin, and 25 μg/mL ampicillin], combined with 150 mM TMAO and 200 mM urea and filter-sterilized (Table 1). These culture conditions are consistent with the only skate line established, but differ from reported conditions for shark (Table 1; Hartmann et al. 1992; Hwang et al. 2008). In particular, an increased pH is consistent with the pH 7.6 media used in *R. porasa* cartilage cultures, and evidence that elasmobranchs have blood pH ranges from 7.6–8.0 (Table 1) (Lai and Graham 1990; Fan et al. 2003; Brill et al. 2008). Culturing differences between skate and shark cells is likely due to the fact that the batoids and sharks are thought to be separate lineages, and thus may require different growth conditions (Winchell et al. 2004).

Cell Growth and Morphology

Several observations were key to successfully establishing primary cultures. The skate intestinal cells preferred dense plating. The manual dissociation of the intestines into small clumps was best, as opposed to enzymatic dissociation into single cell suspension (Fig. 1*A*). Approximately two to three spiral intestines were plated per 24-well plates. Clumps of cells that did not adhere to the collagen matrix after a few days were gently removed and seeded into new wells containing collagen. Primary cultures were fed weekly by removing half the volume of media and replacing with fresh LDF growth media. Replacing half the volume with fresh media was preferable to complete volume replacement with fresh media, likely because the cells were generating their own growth factors. When cells became confluent or appeared to stop dividing, cells were passaged (approximately every 4 wk). For passaging, cells were treated with trypsin for 5–10 min at room temperature. If trypsin failed to remove cells from the collagen matrix, the wells were further treated with 0.2 mg/mL of collagenase (Sigma #C2674) dissolved in LDF for 5–15 min at room temperature. Depending on the number of cells recovered, cells were either split into two wells, or cells from multiple wells were pooled into a larger or same-sized well. After passaging, the viability of cells was greatly reduced. This is consistent with previous studies which have reported up to a 70 % cell death after treating with trypsin (Hartmann et al. 1992).

Cell outgrowth from intestinal explants occurred within 48 h of seeding, establishing into the collagen matrix (Fig. 1*A*). Cells appeared fibroblast-like in appearance (Fig. 1*A*). Within a week, some explants formed tubes with beating cilia, and often "spun" any surrounding cell debris in the media (Fig. 1*B*). These tubes eventually settled down and adhered to the collagen matrix. Attempts were made to wean cells off the collagen matrix. Starting at passage 4, cells were plated onto wells without collagen. Cell division and viability was severely reduced when cells were cultured off the collagen matrix. In primary culture, cells survived for up to nine passages (8–9 mo).

Two populations of cells were observed once the explants were established. All explants exhibited cells with typical epithelial as well as typical fibroblast-like morphologies (compare Fig. 1*C* and *E*). No wells were observed initially with exclusively one cell type. Regions of flattened epithelial sheets were clearly visible in early cultures and had large nuclei (Fig. 1*C* and *D*). As passaging progressed, however, fibroblastic looking cells dominated the cultures and the epithelial cells disappeared (Fig. 1*E* and *F*). It is not clear if the fibroblastic looking cells overgrew the epithelial cells. In mammalian intestinal cultures, several strategies have been used to eliminate the overgrowth of epithelial cells by fibroblasts (Yeh and Chopra 1980; Chopra et al. 2010). Alternatively, it is possible that the fibroblastic looking cells simply appeared epithelial when they became dense enough to form tight junctions. Cell types cannot be based on morphology alone. Future characterization of cell types should include the assessment of immunohistochemical properties (including cytokeratins 18 and 19, E-cadherin, and vimentin expression) as well as functional properties (including alkaline phosphatase, aminopeptidase, and maltase activities) typical of intestinal epithelial cells (Quaroni et al. 1979; Yeh and Chopra 1980; Rusu et al. 2005).

The fact that fibroblastic looking cells dominated epithelial cells in culture suggests the two cells were competing for growth factors. In addition, we were unable to achieve consistent doubling rates, which ranged between 2 and

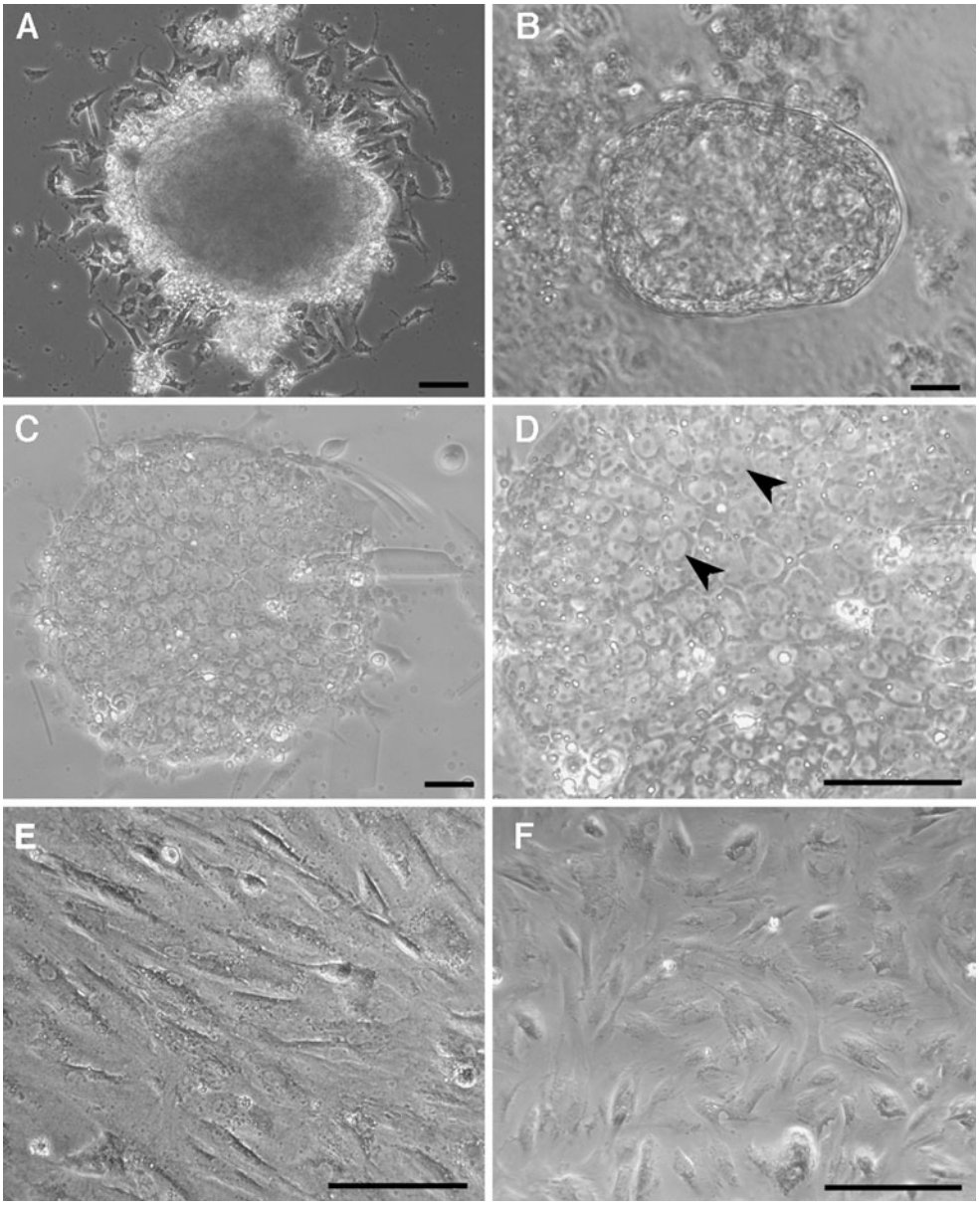

Figure 1. Explant cultures of spiral intestines from *L. erinacea* at different passages. (*A*) Cells growing out of an explant 48 h after seeding. (*B*) By day 6 of culturing, tubes formed with beating cilia. (*C*, *D*) Cells exhibiting typical epithelial-like morphologies contained enlarged nuclei. (*D*) A magnification of (*C*) highlights the enlarged nuclei (*arrowheads*). (*E*, *F*) Cells at passages P2 and P5, respectively, are fibroblastic looking. *Bar* 100 μm.

4 wk. To combat this, media removed from wells during feedings were kept as "conditioned media" and used to feed poorly performing wells. This helped extend the life of cultures, but was not sufficient to extend epithelial cell growth.

In order to maintain the growth and division of epithelial cells in culture, two main factors must be considered: extracellular matrix interactions and growth factors. Cell matrix interactions regulate gene expression, and collagen is essential for maintaining epithelial cells (Freshney 2000). However, collagen I from rat tail may not be the optimal matrix on which to grow elasmobranch cells, and attempts failed to successfully passage cells off collagen. The collagen matrix may be replaced with feeder cells derived from an elasmobranch. This would not only provide a more appropriate extracellular matrix for intestinal epithelial cells to grow, but would provide more appropriate growth factors to maintain the epithelial cell population in culture. Mitomycin C-treated LEE-1 cells from *L. erinacea* or SAE cells from *S. acanthias* are candidates on which to plate skate intestinal explants (Parton et al. 2007; Hwang et al. 2008). Additionally, the human recombinant growth factors used in our culture media are notoriously unstable and had to be replaced every fourth month (Hwang et al. 2008). The instability of growth factors along with their inappropriateness for marine elasmobranch cells would be further reconciled by supplementing growth media with growth factor-producing feeder cells.

The work presented is the first successful attempt at isolation and maintenance of elasmobranch intestinal cells.

Results from explant cultures in this study suggest that skate intestinal cells survive for up to 9 mo in culture. Further analysis of optimal culture conditions, especially the use of feeder cells to promote epithelial cell growth through a more robust extracellular matrix and source of growth factors is needed. The ability to establish and maintain intestinal cells is of potential use for understanding cellular mechanisms underlying environmental aquatic toxicity and stress physiology due to climate change, and would further complement whole animal studies (Ballatori and Villalobos 2002).

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