lymphopoietic organs. The bone marrow and spleen contained erythropoietic, granulopoietic, and thrombopoietic elements. Lymphoid follicles, some with germinal centers, were observed in the spleen and in the lymph nodes, and the cortex of the thymus exhibited dense lymphoid cellularity.

So far, the ability to restore hemopoiesis has been proven for stem cells with trisomies 12 and 19. Further tests have demonstrated that these cells form almost normal numbers of colony-forming units in spleen tissue and in agar (*8*). However, stem cells with other trisomies may not possess the same survival capacity. In fact, there is evidence that stem cells with trisomy 13 or 16 cannot restore hemopoiesis permanently in lethally irradiated recipients (*9*).

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Larvae of Air-Breathing Fishes as Countercurrent Flow Devices in Hypoxic Environments

Abstract. *Larvae of the air-breathing teleost fish* Monopterus *are frequently exposed to periods of critical hypoxia, which they can survive because they have (i) dense capillary networks in the skin, (ii) a small blood-water barrier, (iii) an active pectoral fin mechanism that generates a posteriorly directed respiratory water current originating from the oxygen-rich surface layer, and (iv) a principal flow of blood that runs countercurrent to the water stream. Experimental data show that the larva as a whole is a functional analog of a fish gill lamella and that similar adaptive mechanisms are present in larvae of ancient fishes and some modern teleosts inhabiting permanently or periodically hypoxic waters.*

Adaptations in the gas exchange apparatus accompanying the transition from aquatic to terrestrial vertebrate life have been studied mainly in adult air-breathing fishes (*1*). Yet the most critical phase in the life history of fishes occupying periodically or permanently hypoxic environments is the larval stage before gills or air-breathing organs begin to function (*2*). I now present experimental evidence of a countercurrent flow mechanism by which larvae of some air-breathing fishes can cope with critically low oxygen concentrations in the water. The outer surface of the larva, along its entire length, takes up oxygen from the water. An adaptive advantage is gained by an arrangement that makes the stream of water over the larva and the stream of blood in the larva flow in opposite directions. Thus the larva as a whole is a functional analog of a fish gill lamella.

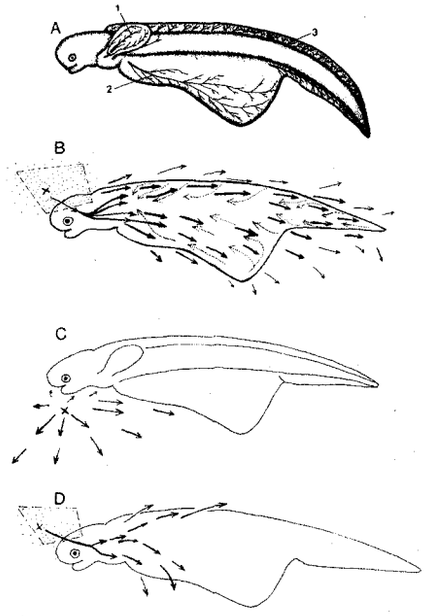

Fig. 1. (A) Larva of *Monopterus albus*, 4 days after hatching, with (1) large vascular pectoral fins, (2) well-developed respiratory capillary networks on the yolk sac, and (3) unpaired (median) fins. (B) Diagrammatic representation of water currents generated by pectoral fins (not included in the drawing) as indicated by heavy arrows. The stippled area is the region of influence of the pectoral fins; × indicates the placement of the dye (methylene blue); dotted arrows show the principal directions of blood flow; this pattern occurs when air equilibration of the water drops below 40 percent. (C) Fate of tracer dye when placed at X, outside the sphere of the pectoral fins (which are shown in this drawing). (D) Illustration of water currents generated by the pectoral fins as indicated by dye movements when air equilibration is above 40 percent; the major current takes a ventral direction after passing the hepatointestinal capillary network, while the dorsal current is only weakly developed so that the tracer dye is diffused (pectoral fins are not shown).

Members of the circumtropical Synbranchiformes are eel-shaped, air-breathing, amphibious teleost fish inhabiting swamps, rice fields, and permanent or temporary ponds, which become permanently or periodically hypoxic. When the larvae of the synbranchiform *Monopterus albus* of southeast Asia hatch (Fig. 1A), they have large muscular and vascularized pectoral fins that function as external gills (*3*). Dye tracer experiments (*4*) show that the active movements of the pectoral fins propel water from a well-circumscribed area anterodorsal to the head posteriorly along the length of the entire larva and its yolk sac (Fig. 1B). This ventilating mechanism, which draws the layer of water anterodorsal to the head posteroventrally, enables the buoyant larva to exploit the thin surface layer of water in which diffusion provides sufficient oxygen in an otherwise depleted water column. Dye tracer that is introduced anteroventral to the head does not get caught in the respiratory current because it is in an area outside the hydrodynamic sphere of the pectoral fins (Fig. 1C). The concentration of oxygen in the water has a strong effect on the frequency and nature of movements of the pectoral fins (*4*) and consequently on the velocity and extent of the respiratory water stream (Fig. 1, B and D). Ten to 13 days after hatching, the fish begins branchial and air respiration and the pectoral fins shrivel and drop off the body (*3*).

The larva of *Monopterus* has an extensive respiratory capillary network just below the epithelial surfaces of the un-

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paired (median) fins, the pectoral fins, and the yolk sac (Fig. 1A). In 1 mm^2 of skin surface, the average total length of vessels is 24.7 ± 1.9 mm (standard deviation). Direct observations in vivo of the illuminated larva show that the blood in these respiratory capillaries flows anteriorly, being channeled into the segmental vessels draining the median fin, the hepatovitelline respiratory vessels, and the subintestinal vein. Thus the blood below the thin epithelial surfaces of the larva flows in the opposite direction to the posteriorly directed respiratory water current produced by the action of the pectoral fins (Fig. 1B). The adaptive effectiveness of this countercurrent flow for gas exchange is often cited as a prime example of optimal design in various animal groups (*5*). The blood-water barrier as determined by light microscopy in *Monopterus* larvae varies from 8 to 15 μm, well within the range for gas exchange to occur.

The whole larva of *Monopterus* can function as a gill because it is equipped with (i) a dense capillary network over large areas of its body surface, (ii) a sufficiently small blood-water barrier, (iii) an active pectoral fin mechanism that generates a posteriorly directed respiratory water current originating from the oxygen-rich surface layer, and (iv) a principal flow of blood that runs countercurrent to the water stream.

The effectiveness of the countercurrent flow has been determined by measurements of the oxygen consumption of the larva in the countercurrent and parallel or concurrent (cocurrent) flow conditions at 10-minute intervals for 40 minutes. The larva is placed in a respiratory tube 5 mm in diameter through which water flows at a regulated rate of 130 ml per hour (*6*). The oxygen content of the water before and after passing the larva in the respiratory tube is then measured to determine the percentage of oxygen removed within a given unit of time. The larva is placed with its head facing the current to simulate the effects of countercurrent flow in the first series of experiments. In the second series of experiments the flow is reversed to the concurrent mode by placing the larva in the direction of the flow of water. In both series of experiments, the larvae remained motionless within the chamber, moving their pectoral fins slightly. Significant differences in the percentage of oxygen removed from the water during its passage over the larvae at the rate of 130 ml per hour have been found between the countercurrent and concurrent modes of flow (Fig. 2). During the countercurrent flow simulation 41.0 ± 2.8

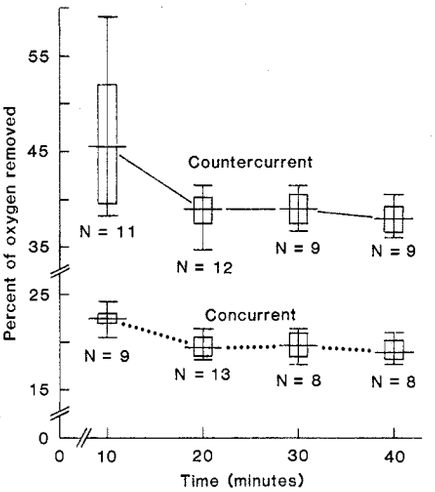

Fig. 2. Effectiveness of countercurrent flow in the removal of oxygen from air-saturated water by the larva of *Monopterus albus*. All measurements are made under identical temperature (28°C) and flow (130 ml per hour). Countercurrent simulation is accomplished by positioning the larva against the current in the respiratory tube. Concurrent simulation is made by positioning the larva with the current. During the entire procedure the larva remains motionless on top of the cotton base. The graph shows means (horizontal lines), ranges (vertical lines), and standard deviations (boxes). The initial high extraction coefficient during the first 10-minute interval is an artifact arising before the fish is acclimated to the experimental tube.

percent of the oxygen is removed within a 10-minute interval, whereas during the concurrent flow simulation, the value is reduced to 20.4 ± 1.1 percent. The difference can be atttributed solely to the effectiveness of the countercurrent flow in the removal of oxygen from the water by the larva.

The survival value of the countercurrent flow mechanism can be demonstrated in both field and laboratory studies. Under laboratory conditions, *Monopterus* larvae survive hypoxic, and even anoxic, conditions as long as they can exploit the surface layer of the water for the production of the countercurrent flow by moving their pectoral fins. However, when prevented from approaching the surface layer of the water, a larva invariably dies within 50 minutes in water having less than 20 percent air saturation because its countercurrent flow mechanism is rendered ineffective by the absence of oxygen-rich water immediately above and in front of its head (*4*). Thus under laboratory conditions the survival time of the larvae in hypoxic water is less than 50 minutes unless the countercurrent flow can be activated by access to the surface layer of the water.

In its natural habitat, *Monopterus* is subjected to frequent and wide fluctuations of oxygen, ranging from concentrations greater than that of air saturation to virtual anoxia. The larvae can survive the critically anoxic periods because they live in foam nests near the surface and can use the oxygen in the surface layer of the water by means of the countercurrent flow device. Similar adaptive strategies may be present in the larva of the Australian lungfish *Neoceratodus*, whose body surface is covered with cilia arranged in a pattern conducive to the generation of a flow of water countercurrent to the bloodstream (*7*).

Countercurrent flow mechanisms in larvae may have a broad evolutionary distribution, because other unrelated fish species inhabiting periodically hypoxic waters have larvae with elaborate respiratory capillary networks and mobile pectoral fins (*2*, *8*). Our knowledge of the respiratory and circulatory systems of the larval stage in which no functional gills are present is limited (*9*), so that the extent of the occurrence of countercurrent devices among teleosts is unknown. Countercurrent flow mechanisms optimize gas exchange, for which there is such a high selective premium that they have evolved independently in the gills of cartilaginous and bony fishes, crustaceans, and mollusks, all of which are aquatic breathers with a relatively high oxygen consumption (*5*). To this diverse evolutionary spectrum can now be added the larvae of several ancient fishes and some modern teleosts inhabiting periodically or permanently hypoxic waters.

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Growth Factors Modulate Gonadotropin Receptor Induction in Granulosa Cell Cultures

Abstract. *Epidermal growth factor and fibroblast growth factor inhibited follicle-stimulating hormone-dependent induction of luteinizing hormone receptor in cultured ovarian granulosa cells of the rat. In contrast, platelet-derived growth factor potentiated the induction of luteinizing hormone receptor by follicle-stimulating hormone. These results indicate that growth factors, well known for their effects on mitosis and DNA synthesis in cultured mammalian cells, are also able to modulate hormone-dependent differentiation in an endocrine target cell.*

Induction of receptor for luteinizing hormone (LH) is a critical aspect of granulosa cell differentiation and ovarian follicular development, because it is by this process that the follicle acquires responsiveness to LH and the capacity to ovulate and luteinize. Follicle-stimulating hormone (FSH) was established as an inducer of granulosa cell LH receptor in vivo (*1*) and the effect has now been documented in vitro under chemically defined culture conditions (*2*). In addition to their role as recipients of hormonal stimuli, granulosa cells of a number of species (rabbit, pig, human, and guinea pig, but not rat) are known targets for the mitogenic and growth-promoting actions of growth factors in vitro (*3*). The nature of the relation between growth factors and more highly differentiated granulosa cell functions has only begun to be appreciated (*4*). We report here that growth factors can modulate FSH-dependent granulosa cell LH receptor induction in vitro (*5*) and propose that such compounds may represent a physiologically relevant control system interacting with gonadotropic and other endocrine components to regulate ovarian cellular differentiation.

Granulosa cells from immature, diethylstilbestrol-primed rats were cultured for 3 days in the absence of FSH or growth factors to allow monolayers to establish. They were then cultured for another 3 days with or without purified human FSH, with or without growth factors, or with both FSH and growth factors. At the end of the 6 days, LH receptor binding was assessed by the specific binding of ^{125}I-labeled human chorionic gonadotropin (hCG) (*6*).

The effect of epidermal growth factor (EGF) on FSH-dependent LH receptor induction is shown in Fig. 1. In granulosa cells cultured in serum-free, chemically defined medium in the absence of FSH, LH receptor binding was comparable to that in cells before culture (data not shown). Treatment with FSH during the last 3 days of culture increased LH receptor binding nearly 15-fold ($P < .001$). Epidermal growth factor alone did not suppress LH receptor binding but it blocked the FSH-induced increase. The effect was dose-related with respect to both EGF and FSH. EGF completely inhibited LH receptor induction at 10 ng/ml but only partially inhibited it at 1 ng/ml. The inhibitory effect of EGF at 1 ng/ml was more pronounced at higher doses of FSH. Such a trend suggests that EGF may act, at least in part, to enhance any down-regulatory action of FSH on the newly induced LH receptor (*7*). The EGF did not compete with ^{125}I-labeled hCG in the binding assay (data not shown) indicating that the inhibitory action of EGF was exerted on receptor induction during the culture period.

Suppression of FSH-dependent LH receptor induction occurred in the absence of a pronounced growth-promoting or mitogenic effect of EGF, as assessed by the yield of cell protein after culture (*8*). This observation is in agreement with the reported resistance of rat granulosa cells to growth factor–stimulated mitogenesis (*3*).

The effect of EGF on progestin secretion was also investigated. The amount of 20α-dihydroprogesterone (20α-OH-P) in spent media from cells cultured with or without human FSH or growth factors during culture days 3 to 6 was determined by radioimmunoassay (*9*). We found that EGF (10 ng/ml) did not impair FSH-stimulated (250 ng/ml) progestin secretion: when expressed as micrograms of 20α-OH-P per milligram of cell protein per 3 days (mean ± standard error; $N = 3$) the basal level was 0.59 ± 0.01; with EGF the result was 0.49 ± 0.07; with FSH, 4.89 ± 0.19; and with FSH

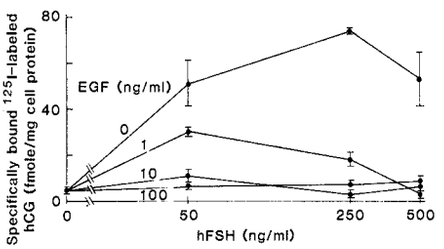

Fig. 1. The effect of EGF on FSH-dependent LH receptor induction. The results show the relation between dose and response for EGF and FSH. Ovarian granulosa cells were isolated from estrogen-treated immature female rats and incubated at 37°C in a humidified atmosphere of 5 percent CO_2 in air in multiwell plates (Falcon). The wells were first treated with human fibronectin (2 to 4 μg/cm^2, Collaborative Research, Waltham, Mass.) to facilitate cell attachment. Each well contained approximately 10^6 cells and 1.0 ml of culture medium consisting of McCoy's 5A supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml, Gibco) and insulin (25 mIU/ml, Eli Lilly, porcine sodium insulin, lot No. 050YB7). After 3 days the cultures received fresh 5A, antibiotics, insulin, and human follicle-stimulating hormone (*hFSH*) and EGF where indicated. After 6 days, specific binding of ^{125}I-labeled hCG was assessed. Total binding was determined in triplicate cultures with 75 ng of ^{125}I-labeled hCG in a final volume of 250 μl of culture medium containing 10 percent fetal calf serum. Duplicate cultures were incubated as described above following 30 minutes in the presence of a 250-fold excess of unlabeled hCG (APL, Ayerst Laboratories) to determine the nonspecific binding for each treatment group. After 2 hours at 37°C the binding reaction was terminated by washing with cold phosphate-buffered saline (PBS). Cells were scraped from the culture wells with a rubber policeman into 1 ml of cold PBS and centrifuged from the buffer. Radiologic counts were normalized on the basis of cell protein (protein assay kit No. 1, Bio-Rad Laboratories). The amount of labeled hCG bound was calculated from the specific activity of the labeled hCG preparation and a molecular weight of 47,500. Specific binding was taken to be the difference between total and nonspecific binding. Data points represent means ± standard error, $N = 3$.

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