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New methods for the primary culture of gill epithelia from freshwater rainbow trout

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Abstract

Gill epithelia from freshwater rainbow trout can be grown in primary culture in Leibovitz's L-15 medium, by seeding freshly isolated gill cells on two successive days, from two different fish, directly onto permeable filter supports (DSI technique). This preparation allows the measurement of transepithelial resistance (TER) and exposure of the apical surface to freshwater, as *in vivo*. New culture methods were developed and evaluated, using TER as an indicator of epithelial integrity, in an effort to improve the utility of the preparation for proteomic and toxicological research. TER was not related to cell density or protein content in DSI epithelia. To eliminate bovine proteins, the 5% foetal bovine serum (FBS) normally required for epithelial development was replaced with trout plasma. While previously frozen trout plasma proved toxic, freshly collected heparinized plasma, provided by chronically cannulated adult trout, was not. The use of 5% fresh trout plasma supported a TER development curve identical to that with 5% FBS, a useful advance for proteomic research because foreign (bovine) proteins are eliminated. However, 10% plasma reduced TER development, and 100% plasma abolished it. The inhibitory effect on TER of high plasma levels was seen only early in epithelial development, and was exerted from the apical side, likely an effect on tight junction formation. Mature plasma-supplemented preparations mounted a TER rise in response to apical freshwater exposure comparable to that of FBS-supplemented epithelia. Yolk-sac fry extract was inhibitory to TER development, even in the presence of 5% FBS. Transfer of mature epithelia from 18 °C to 4 °C maintained stable TER and extended the useable lifespan by at least ten days, thereby facilitating storage of preparations for toxicity testing. A new method of growing epithelia, involving only a single seeding of cells from a single fish, directly onto filter inserts (SDSI technique), provided mature epithelia with much lower TER, a smaller TER response to apical freshwater, and lower cell density and protein content than DSI epithelia. These SDSI epithelia offer the advantage of multiple preparations grown directly from unique individuals for *in vitro* toxicity testing.

Abbreviations: DSI – double-seeded insert; FBS – foetal bovine serum; MRC – mitochondria-rich cell; PVC – pavement cell; SDSI – single directly seeded insert; SSI – single-seeded insert; TER (TEER) – transepithelial electrical resistance.

Introduction

Since the first report of a trout gill epithelium grown in primary culture on a permeable filter insert (Wood and Pärt 1997), the preparation has been used in more than a dozen studies (reviewed by Wood et al. 2002). Notably, it exhibits the highest transepithelial electrical resistance (TER, also known as TEER) reported in any

cultured epithelium and is the only cultured epithelium tolerant of prolonged apical freshwater exposure while being bathed basolaterally with a blood-like culture medium. Indeed, it generally exhibits an even higher TER during freshwater exposure. This cultured branchial epithelium has been employed as a model system for the freshwater gill in investigations of ionic transport (Wood et al. 1998), electrophysiology (Gilmour et al. 1998a, b), hormonal mechanisms (Kelly and Wood 2001b, 2002a), ammonia transport (Kelly and Wood 2001a), lipid metabolism (Hansen et al. 2002), protein synthesis (Smith et al. 2001), and toxicological responses (Sandbacka et al. 1999; Carlsson and Pärt 2001). During this period, there has been only one major methodological advance, the development of the double-seeded insert (DSI) preparation by Fletcher et al. (2000). This was an improvement over the original single-seeded insert (SSI) preparation of Wood and Pärt (1997) for several reasons.

First, the DSI preparation contains approximately 15% mitochondria–rich cells (MRCs) in addition to 85% pavement cells (PVCs), similar to the ratio in the intact gill. Recently we have also found the presence of a few mucous cells ($< 1\%$) in DSI preparations only. In contrast, the original SSI preparation is made up entirely of PVCs, the MRCs having died out during the initial 6–9-day phase of culture in flasks. Second, DSI epithelia offer a significant time saving. A mature SSI epithelium is obtained only after about two weeks, because the PVCs must first be cultured in flasks, then harvested from the flasks, and subsequently cultured on filter inserts for a further 6–7 days. In contrast, the DSI preparation does not require a flask culture stage. Freshly dispersed cells from the gills of one fish are simply seeded directly onto filters on Day 1, followed by a second set of cells from the gills of a second fish 24 h later on Day 2, with a mature DSI epithelium being attained in about one week. The higher TER and generally lower ionic and paracellular permeability of DSI epithelia relative to SSI epithelia may offer a third advantage (Fletcher et al. 2000).

In the present study, we attempted some further methodological improvements and evaluated their success. The first goal was driven by our long-term objective to perform proteomic analysis (Pandey and Mann 2000; Liebler 2002) on these preparations. Here there is a critical need to eliminate foreign proteins, so our goal was to see whether we could replace the foetal bovine serum (FBS, which had formerly been essential for success) with homologous trout plasma in the culture of DSI epithelia. We also took the opportunity to evaluate the effects of apical and basolateral plasma additions on TER at various times in epithelial development. Our second goal was to test whether development of the DSI preparation could be accelerated by an extract from trout yolk-sac fry, which has been reported to stimulate cell division in several fish cell lines (Collodi and Barnes 1990) and which is now recommended in the culture of various cells from zebrafish (Bradford et al. 2000). Our third and fourth goals were driven by our wish to exploit the preparation to a greater extent in aquatic toxicology. Here there is a need to extend the lifespan of the cultured preparation so that epithelia can be stockpiled for toxicity testing on demand, and also a need to directly capture the individual-to-individual variation seen in whole animal toxicity tests (Sprague 1969). Therefore, on the one hand we evaluated whether mature DSI epithelia could be preserved with stable TER by refrigerating at 4 °C, and on the other whether it was possible to culture gill epithelia from single animals by seeding only once, directly onto filters (SDSI – single directly seeded inserts). These new SDSI preparations were then compared with traditionally grown SSI and DSI epithelia. Finally, we evaluated whether differences in cell density or protein content could explain the very different TERs between the two directly seeded epithelial preparations (DSI *versus* SDSI).

Materials and methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*) were obtained from 'Farming Ittico Ticino' (Milan, Italy) and held in the aquarium facility at the Joint Research Centre (Ispra, Italy) in filtered running water which was withdrawn at a depth of 30 m from nearby Lake Maggiore. Water characteristics were [Na] = 116 μM, [Cl] = 112 μM, [K] = 4 μM, [Ca] = 375 μM, [Mg] = 133 μM, [DOC] = 1.8 mg C l^{-1}, pH = 7.2, conductivity = 0.18 mS/cm, temperature = 10.9–11.9 °C. Fish used for gill cell culture were juveniles, 6–12 months old (40–150 g), whereas fish cannulated as blood donors were adults, 3 years old (800–1500 g). During holding, fish were automatically fed with trout pellets (Hendrix SpA, Mozzecane, Italy) at a fixed ration of 1% body mass per day. Fish were starved for several days prior to use to prevent regurgitation during experimental procedures. Fertilized rainbow trout eggs were obtained from Alvdalslax AB (Alvdalen,

Sweden) and held in the aquarium facility at Ispra in 35 × 35-cm hatching trays. Yolk-sac fry approximately 2 weeks post-hatch were used for preparation of yolk-sac fry extract.

Isolation of gill cells

For all culture techniques, gill cells were isolated by the same methods described in detail by Kelly et al. (2000). Sterile technique was employed throughout, in a laminar flow hood. In brief, juvenile trout were sacrificed by decapitation, the gill filaments were removed by dissection, and then incubated for 3×10 min in standard phosphate buffered saline (without Ca or Mg) containing a cocktail of antibiotics and antimycotic agents (see Kelly et al. 2000). Gill cells were then separated by two cycles (2 × 20 min) of tryptic digestion. Cells were resuspended in Leibovitz's L-15 medium (with 2 mmol l^{-1} L-glutamine and phenol red; Gibco BRL, Life Technologies, Milan, Italy) plus 5% foetal bovine serum (FBS, mycoplasma screened; Gibco BRL, Paisley, U.K.). This L-15 + 5% FBS medium was supplemented with 100 i.u. ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 200 μg ml^{-1} gentamycin (all Gibco BRL, Life Technologies, Milan, Italy). Antibiotics were present for the first 4 days of subsequent culture, after which antibiotic-free media were used. Cultures were held in an incubator at 18 °C, except in tests where the effects of storage in a refrigerator at 4 °C were evaluated.

Double-Seeded Insert (DSI) preparations

DSI epithelia, which were used in the majority of experiments, were prepared by the methods of Fletcher et al. (2000), as described in detail by Kelly et al. (2000). On Day 1, freshly isolated cells were directly seeded, at a density of 3×10^6 cm^{-2}, onto permeable Falcon filter inserts (cyclopore polyethylene terephthalate filters; Becton Dickinson, Franklin Lakes, USA, pore density: 1.6×10^6 pores cm^{-2}, pore size: 0.45 μm, growth surface: 0.9 cm^2) in a Falcon 12-well plate. Apical volume was 0.8 ml, and basolateral volume was 1.0 ml. Twenty-four hours later, each insert was thoroughly rinsed with antibiotic-supplemented media until visual inspection ensured that the mucus layer was removed. At this time, new cells freshly isolated from a second fish were seeded at the same density onto the layer of cells already established in the inserts (visible under phase contrast). On the day following the second seeding, inserts were rinsed until the mucus layer was once again removed. At this point, any experimental changes in media composition (e.g., trout plasma, yolk-sac fry extract) were instituted (see Results for details). Aliquots of 1.5 ml (apical) and 2.0 ml (basolateral) of antibiotic-supplemented media were added to inserts and wells, and the preparations were returned to the incubator. Bathing medium was changed every subsequent 48 h, and antibiotics were removed at 96 h.

Single Directly Seeded Insert (SDSI) preparations

SDSI preparations were created simply using the first step (direct seeding of freshly isolated cells onto filters on Day 1 at a cell density of 3×10^6 cm^{-2}) of the DSI procedure. At 24 h, the mucus layer was rinsed away, and the apical and basolateral volumes set to 1.5 ml and 2.0 ml media (L-15 + 5% FBS), respectively. Media were changed again at 48 h, and every subsequent 48 h; antibiotics were removed from 96 h onwards. One or more SDSI preparations were routinely tried each time a batch of cells was isolated for DSI filters.

Single-Seeded Insert (SSI) preparations

SSI preparations were grown according to the two-stage methods developed by Wood and Pärt (1997), and described in detail by Kelly et al. (2000): 6–8 days of initial culture in Falcon 25-cm^2 flasks (initial seeding density = 5×10^5 cm^{-2}; media = L-15 + 5% FBS) until the cells were close to confluence, followed by re-trypsination and re-seeding (cell density = 5×10^5 cm^{-2}; media = L-15 + 5% FBS) onto filter inserts. Antibiotic-free media were used from Day 4 onwards of flask culture, and throughout the subsequent period of filter culture.

Freshwater exposure

In some experiments with mature epithelia (after Day 6 for SSI, Day 7 for DSI, and Day 8 for SDSI), the apical medium (1.5 ml) of the cultured epithelium was replaced by sterilized (0.2 μm Acrodisc filtration) fresh water (from Lake Maggiore, composition as above) to re-create the natural environmental conditions *in vivo*. To ensure a complete changeover, the apical compartment was rinsed 4 times with the sterilized lake water before the final addition. The rinses were repeated and the fresh water was renewed immediately prior to each measurement of transepithelial resistance.

Transepithelial Resistance (TER) measurements

TER was monitored daily or more frequently on all cultured preparations using STX-2 chopstick electrodes connected to an EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, Florida, USA) which had been custom-modified to measure TER as high as 100 kOhms cm^2. All measurements were made in triplicate, blank-corrected, and adjusted for the 0.9-cm^2 filter surface area. Appropriate blank corrections were determined using bare filter inserts freshly incubated with apical and basolateral solutions identical to those used in experimental preparations.

Measurements of cell numbers and protein content

These determinations were made on some DSI and SDSI preparations. Trypsin and 'stop' solutions were prepared as described by Kelly et al. (2000). Apical and basolateral media were aspirated, and the cultured epithelia were rinsed several times with PBS. Trypsin was added, 250 μl to the apical side and 1 ml to the basolateral side, while observing the cell layer under phase-contrast microscopy. As soon as the cells detached from the filters, the apical trypsin solution was aspirated into a 1.5-ml centrifuge tube containing 1.0 ml of ice-cold 'stop' solution. The apical surface of the filter was rinsed with an additional 250 μl of PBS, visually ensuring that any residual cells were collected; this PBS was also aspirated into the 'stop' solution. The cells were then gently spun down at $500 \times g$ for 10 min, and resuspended in an appropriate volume of PBS for cell counts using a hemocytometer (Sigma, St. Louis, USA). The exact volume of aliquots removed for cell counting was recorded gravimetrically. The remaining suspension was then frozen at -80 °C for later determination of protein concentration using the Bradford method, Sigma reagents, and bovine serum albumin (Sigma) as a standard. The protein content per epithelium was calculated taking into account the volume removed for cell counting.

Preparation of fresh trout plasma

Adult rainbow trout were fitted with indwelling dorsal aortic catheters by the method of Soivio et al. (1972) while under MS-222 anaesthesia. This allowed them to serve as repetitive blood donors without disturbance. The fish were allowed to recover from surgery for 48 h in individual 20-l aquaria fitted with aeration and flowing water (500 ml min^{-1}). Blood was withdrawn from the catheters, generally at 48-h intervals, to provide plasma for immediate use as a supplement in epithelial culture. Blood was slowly withdrawn (never more than 5 ml at a time per 1-kg fish), immediately heparinized at 50 i.u. ml^{-1} (sodium heparin, Sigma), and then centrifuged at $13000 \times g$ for 30 sec. Plasma was decanted, while the red blood cells were re-suspended in saline and returned to the fish. Individual fish were used for up to 6 bleeds. While hematocrit fell by up to 40%, plasma protein concentration, which is extremely well conserved in trout, never declined more than 15% (typically around 2.5 g 100 ml^{-1}, monitored by refractometry; Alexander and Ingram 1980). Plasma was sterilized by filtration through a 0.2-μm filter (Acrodisc, Gelman Sciences, Ann Arbor, USA), and kept on ice for no more than a few hours prior to use. While an effort was made to always use plasma from the same fish for the same set of cultured preparations, this was not always possible, and changes in donor in mid-experiment had no apparent ill effects.

Preparation of yolk-sac fry extract

Yolk-sac fry (2 weeks post-hatch) weighing a total of 2 g ($\sim$24 fry) were killed by concussion, blotted dry, and homogenized for 1 min in 8 ml of ice-cold PBS (without Ca or Mg), using a motorized dounce homogenizer (B. Braun, Saarbrücken, Germany) at 1350 rpm. The homogenate was centrifuged for 10 min at $500 \times g$. The supernatant was decanted and centrifuged again at $13000 \times g$ for 10 min. This second supernatant was sterilized by filtration through a 0.2-μm Acrodisc filter and immediately frozen at -80 °C in 0.5-ml aliquots. It was thawed only once, immediately before use.

Data analysis

Following the recommendations of Kelly et al. (2000), experimental comparisons were best made within a group of preparations prepared at the same time from the same batch of fish, thereby avoiding the problem of batch-to-batch variation in TER. While experiments were replicated between batches, representative results within a single batch have generally been displayed. Data have been reported as means + 1 SEM (where n = number of epithelia – i.e., filters). Comparisons between treatments were assessed using Student's two-tailed t-test, with a Bonferroni correction for multiple comparisons. Comparisons within treatments employed a paired Student's t-test for single

comparisons, or a Dunnett's test for repeated measures. Regression lines were fitted by the method of least squares, and the significance of Pearson's correlation coefficient (r) was determined. A significance level of $P < 0.05$ was employed throughout, unless otherwise noted. This study is based on more than 500 individual cultured epithelia from more than 60 fish.

Results

Substitution of trout plasma for FBS

In preliminary trials, we found that trout serum that had been frozen was toxic to trout gill epithelial cells, in agreement with earlier work (Pärt et al. 1993; Pärt and Bergström 1995), so all further experiments were conducted with freshly collected trout plasma. This was accomplished using large trout fitted with chronic indwelling arterial catheters as serial blood donors (see Methods). DSI epithelia grown with 5% fresh trout plasma added to L-15 media exhibited a pattern of TER increase over time that was identical to that seen in DSI preparations grown simultaneously with 5% FBS added to L-15 media (Figure 1). Thus, trout gill epithelia can be cultured successfully without the use of foreign proteins. In the example shown in Figure 1, both treatments reached a plateau TER of approximately 30 kOhms cm^2 by Day 8, whereas in preparations grown with 10% trout plasma, the rise in TER was greatly attenuated (to about 5 kOhms cm^2), and in preparations grown with 100% trout plasma, it was totally abolished. In both cases, phase contrast microscopy demonstrated a confluent carpet of live cells on the filters on Day 8, so it was the TER rise, rather than cell viability, that was inhibited. Very similar patterns were seen in three other experimental series varying greatly in absolute plateau TER – i.e., 5% plasma supported TER increases comparable to those supported by 5% FBS, while higher levels of plasma were inhibitory.

This apparent inhibitory effect of high concentrations of trout plasma was investigated further. When the culture medium was changed to 100% plasma on the basolateral, apical, or both surfaces of mature DSI epithelia at plateau resistance (TER > 20 kOhms cm^2, $n = 14$), there was never any substantive change in TER, and TER remained stable for at least 4 days (data not shown). However, when 100% trout plasma was added to epithelia that were just starting to show an initial increase in resistance during development (TER < 1 kOhms cm^2), TER was quickly lowered (Figure 2) and any further rise prevented as long as the 100% plasma remained present. Apical addition alone was sufficient to cause this effect. Thus, high concentrations of trout plasma appear to inhibit the development of high TER, but do not interfere with the maintenance of high TER once it has developed.

Preparations grown to plateau TER with L-15 plus 5% trout plasma were able to sustain 24 h of apical freshwater exposure just as well as preparations grown with L-15 plus 5% FBS. Both exhibited significant increases in TER, which had largely attenuated by 24 h (Figure 3). Preparations grown with 10% plasma actually exhibited a larger TER rise and sustained it more effectively during apical freshwater exposure, but started from a lower level (Figure 3). Control groups for all these treatments kept with unaltered (but renewed) apical media exhibited no significant change in TER over the 24-h experiment (data not shown). Preparations that had been cultured throughout in 100% plasma exhibited no increase whatsoever in their TER, which remained not significantly different from 0 kOhms cm^2 (Figure 3). However, when mature epithelia were grown with L-15 + 5% FBS or L-15 + 5% plasma and then switched to 100% plasma for 24–48 h, they were able to mount a typical TER increase during FW exposure, even when 100% plasma was retained on the basolateral surface (data not shown). Thus again, the inhibitory action of plasma on TER seems to be exerted only early in the development of the epithelium.

The influence of yolk-sac fry extract

When DSI epithelia were grown with a supplement of 5% yolk-sac fry extract (YFE) in addition to 5% FBS on both apical and basolateral surfaces, the normal pattern of TER increase over time was strongly inhibited (Figure 4). When epithelia were grown with L-15 + 5% YFE only, the rise in TER was essentially abolished. This inhibitory influence of YFE was effective even in mature epithelia, because substitution of 5% YFE for 5% FBS on Day 9 resulted in a progressive decrease in TER from the previous plateau (Figure 4). Interestingly, preparations that had been inhibited by 5% YFE only for as long as 8 days were still able to develop a modest increase in TER when 5% FBS was substituted on Day 9 (Figure 4), showing that the effect was at least partially reversible, and phase contrast microscopy demonstrated that a confluent carpet of live cells persisted.

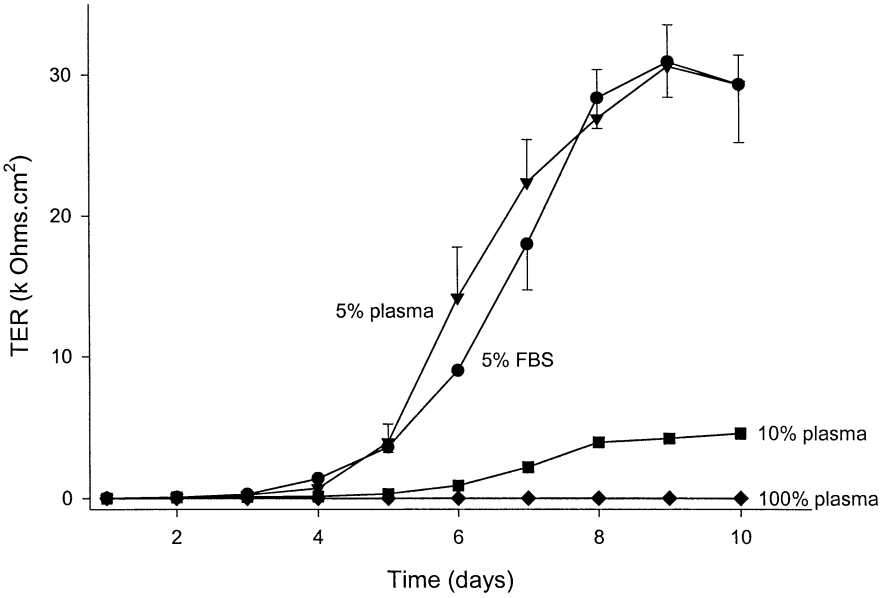

Figure 1. The increase in transepithelial resistance (TER) over time in a group of DSI epithelia grown with either L-15 + 5% FBS, L-15 + 5% trout plasma, L-15 + 10% trout plasma, or 100% trout plasma on both apical and basolateral surfaces. Means ± 1 SEM ($n = 6$ for all groups except 100% plasma, where $n = 5$). There were no significant differences at any time between the 5% FBS and 5% plasma treatments, and TERs in both were significantly greater ($P < 0.05$) than in the 10% plasma treatment from Day 5 onwards. TER in the 100% plasma treatment was never significantly different from zero.

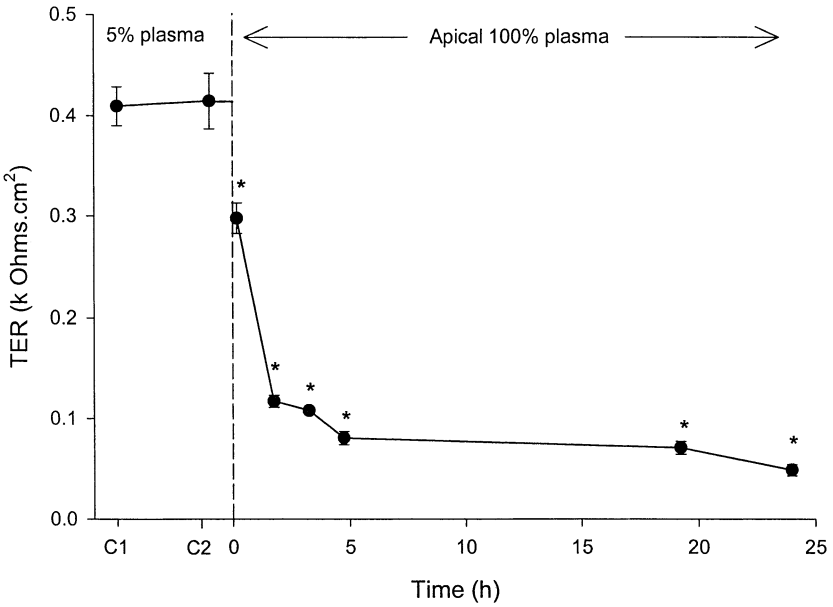

Figure 2. The influence on transepithelial resistance (TER) of changing the apical medium from L-15 + 5% trout plasma to 100% trout plasma in a group of DSI epithelia that were just starting to show an initial increase in TER during development. Means ± 1 SEM ($n = 5$). *indicates significant difference ($P < 0.05$) with respect to pre-changeover mean.

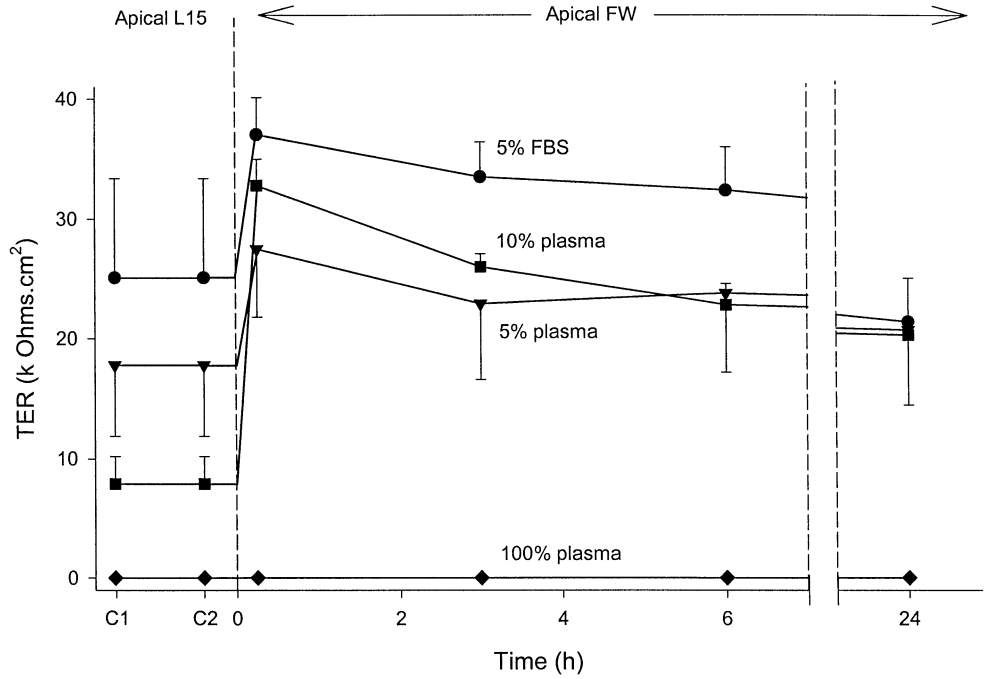

Figure 3. The influence, on Day 9, of 24-h exposure to apical freshwater (FW) on transepithelial resistance (TER) of a group of DSI epithelia grown with either L-15 + 5% FBS, L-15 + 5% trout plasma, L-15 + 10% trout plasma, or 100% trout plasma on both apical and basolateral surfaces. The respective media were retained on the basolateral surface throughout the 24-h exposure period. Means ± 1 SEM ($n = 6$ for all groups except 100% plasma, where $n = 5$). In all treatments except 100% plasma (no significant change), the increase in TER during FW exposure was significant ($P < 0.05$) through 6 h, and through 24 h in the 10% plasma treatment. There were no significant differences among the responses of the 5% FBS, 5% plasma, and 10% plasma treatments.

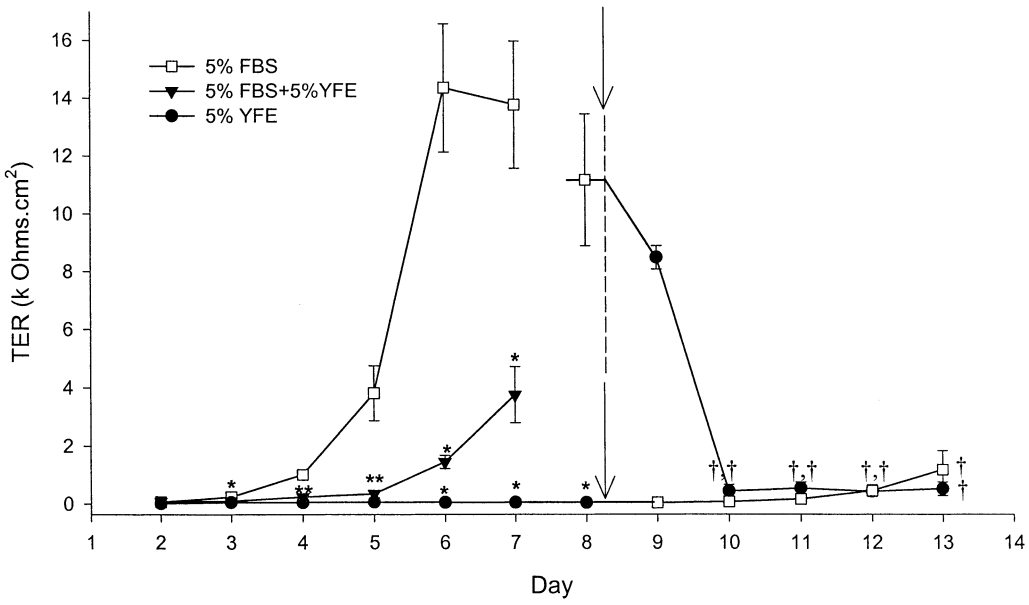

Figure 4. The influence of yolk-sac fry extract (YFE) on the development and maintenance of transepithelial resistance (TER) in a group of DSI epithelia. Epithelia were grown with either L-15 + 5% FBS ($n = 8$), or L-15 + 5% FBS + 5% YFE ($n = 8$), or L-15 + 5% YFE alone ($n = 6$) through Day 7–8. After Day 8, the medium was changed to L-15 + 5% FBS in the treatment previously exposed to L-15 + 5% YFE only, and to L-15 + 5% YFE only in a subset ($n = 3$) of the treatment previously exposed to L15 + 5% FBS. Means ± 1 SEM. *indicates significant difference ($P < 0.05$) with respect to the L-15 + 5% FBS treatment at the same time. †indicates significant difference ($P < 0.05$) with respect to the pre-changeover mean.

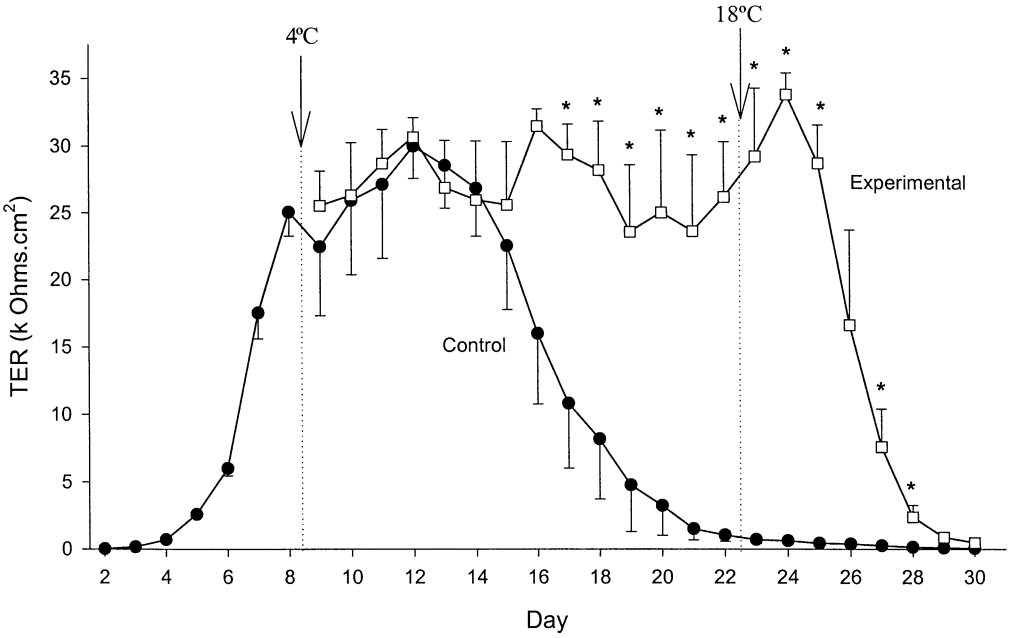

Figure 5. The pattern of normal TER change over the lifespan of a group of control DSI epithelia (grown in L-15 + 5% FBS on both apical and basolateral surfaces) at the normal incubation temperature of 18 °C ($n = 18$ until Day 8, $n = 5$–6 thereafter). The influence of transferring a subset ($n = 3$) from 18 °C to 4 °C at the plateau phase of TER (i.e., after Day 8) and then back to 18 °C two weeks later (after Day 22) on the pattern of TER change over time is also illustrated. Means ± 1 SEM. *indicates significant difference ($P < 0.05$) with respect to the control group. † indicates significant difference ($P < 0.05$) with respect to the mean at 4 °C on Day 22.

Preservation of cultured epithelia by storage at 4 °C

DSI epithelia, cultured at the standard incubation temperature of 18 °C, with L-15 + 5% FBS on both surfaces, exhibited a pattern of TER which was more or less normally distributed with respect to time (Figure 5). TER increased rapidly from Days 4 through 7 and reached a relatively stable plateau (25–30 kOhms cm^2) by Day 8, which continued through Day 14. The maximum value occurred on Day 12 (Figure 5). After Day 14, TER declined rapidly, although it remained significantly above zero through Day 30. Storage at 4 °C substantially prolonged the period of TER stability. When a subset of epithelia was transferred to a refrigerator at 4 °C after Day 8, TER stayed in the plateau region through Day 22. When these preparations were then transferred back to 18 °C, TER remained in the plateau region for another three days, thereafter declining but remaining significantly higher than the control group through Day 28.

Single Directly Seeded Insert (SDSI) epithelia – comparison with other types

It proved possible to culture gill epithelia by seeding freshly dispersed cells from a single fish directly onto a filter insert. Qualitatively, these SDSI preparations showed properties similar to those of the other two types of epithelia (DSI – double-seeded inserts, seeded directly twice in succession, with freshly dispersed gill cells from two separate fish; SSI – single-seeded inserts, seeded once, with cells derived from a single fish, which had first been grown in flasks for 6–9 days, then harvested and re-seeded onto filter inserts). Success with SDSI preparations was intermittent over the 5-month period during which these preparations were attempted, so their average TER profile against time has been compared with the overall average profiles of all DSI and all SSI preparations similarly cultured with L-15 + 5% FBS on both surfaces over the same time period (Figure 6). Of the three types of epithelia, SDSI epithelia exhibited the smallest TER increase over time, reaching a typical plateau of only 1.0–2.0 kOhms cm^2 by Day 7–8. TER was significantly lower than in DSI epithelia at all times from Day 4 onwards. SSI epithelia were intermediate, with significantly lower TER than in DSI preparations by Day 6. When SDSI epithelia were subjected to the standard 24-h challenge with apical freshwater, they exhibited a modest increase in TER, which was sustained only until 3 h (Figure 7). This was significantly lower

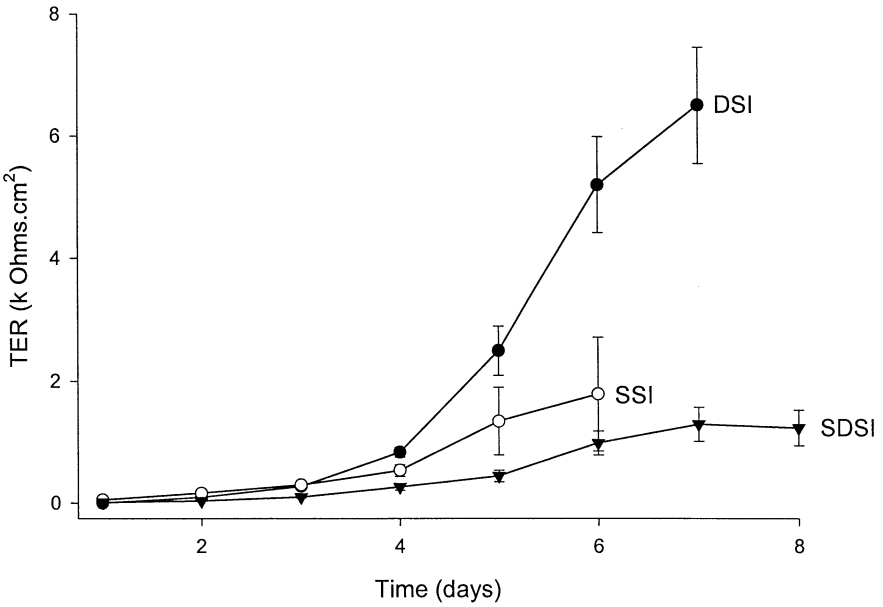

Figure 6. Changes in TER over time for three different types of cultured epithelia: DSI (double-seeded inserts, $n = 62$), SSI (single-seeded inserts, $n = 13$), and SDSI (single direct seeded inserts, $n = 21$). Means ± 1 SEM. This summary includes all preparations for which a complete data set was obtained until the last day shown, and for which both apical and basolateral media were L-15 + 5% FBS throughout. DSI and SDSI means were significantly different ($P < 0.05$) from each other from Day 4 onwards. SSI means were intermediate, and only significantly different from the DSI mean on Day 6.

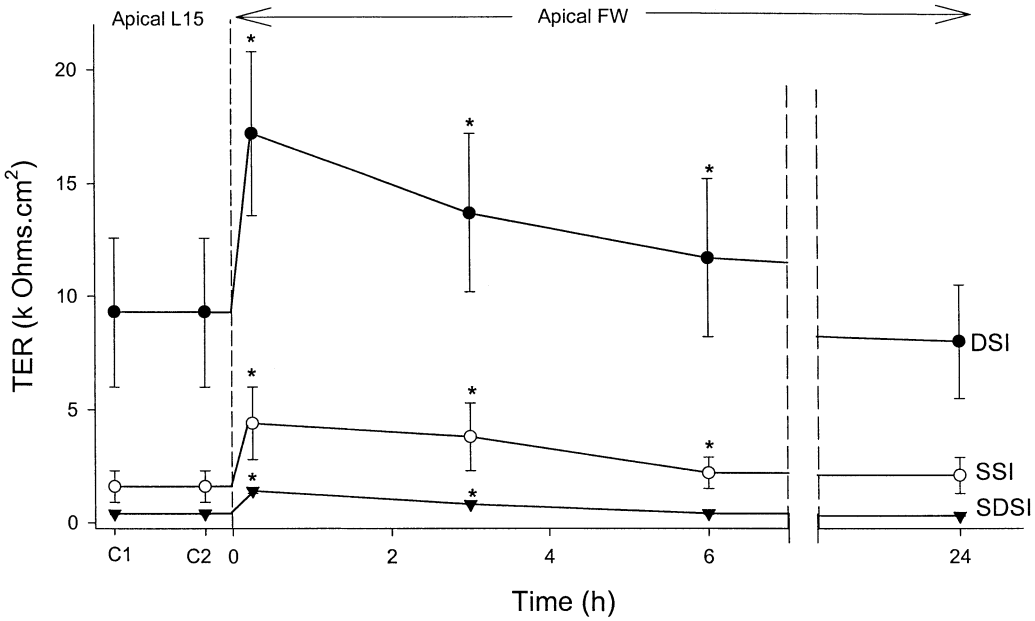

Figure 7. A comparison of the responses in transepithelial resistance (TER) to 24 h of freshwater (FW) exposure among mature SDSI ($n = 4$), SSI ($n = 4$), and DSI ($n = 12$) epithelia (Days 7–9). Means ± 1 SEM. The epithelia were grown from the same group of fish. DSI means were significantly different from both SSI and SDSI means throughout ($P < 0.05$), whereas the latter were not significantly different from one another at any time. The increase in TER upon FW exposure was significant through 6 h in DSI and SSI epithelia, but only through 3 h in SDSI epithelia.

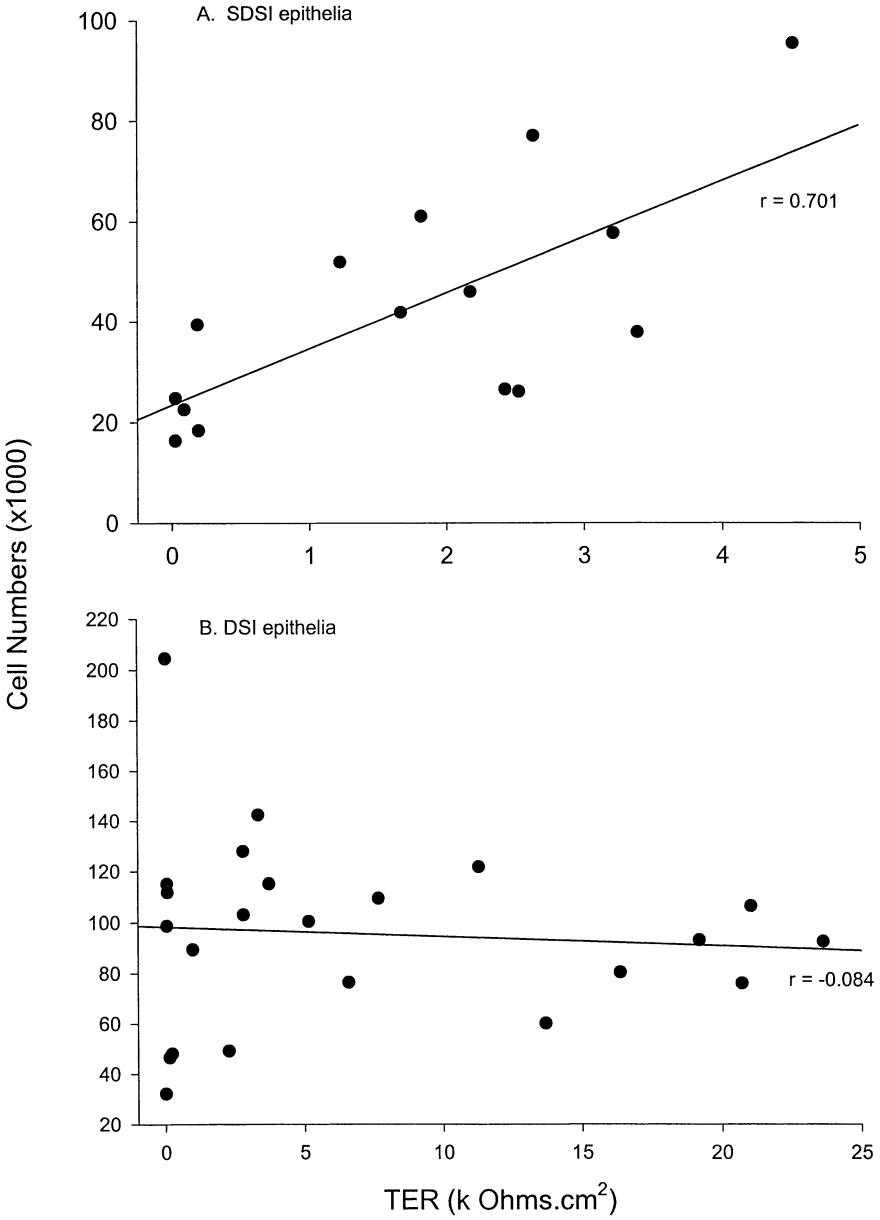

Figure 8. The relationships between transepithelial resistance (TER) and total cell numbers (growth area = 0.9 cm^2) in two types of cultured trout gill epithelia. (A) SDSI epithelia, $r = 0.701$, $P < 0.002$ ($n = 15$); and (B) DSI epithelia; $r = -0.084$, n.s. ($n = 24$).

throughout than in DSI epithelia; SSI epithelia were again intermediate.

The two types of directly seeded epithelia were further compared (Table 1). SDSI epithelia with a mean TER 75% lower than in DSI epithelia exhibited a protein content per insert that was also about 75% lower. Absolute cell numbers per insert were lower by about 55%. All these differences were significant (Table 1). In SDSI epithelia, there was a significant positive relationship ($r = 0.701$, $n = 15$, $P < 0.002$) between TER and cell numbers (Figure 8A). However, over a much wider range of TER (< 1–24 kOhms cm^2), there was no relationship ($r = -0.084$, $n = 24$, n.s.) between TER and cell numbers in DSI epithelia (Figure 8B); the same lack of relationship ($r = 0.195$, $n = 13$, n.s.) was seen if only those TER values < 5 kOhms cm^2 (i.e., the same as the SDSI range) were included in the analysis. Similarly, there was

a weak positive relationship ($r = 0.409$, $n = 15$, $P < 0.07$) between TER and protein content in SDSI epithelia, but no relationship in DSI epithelia ($r = -0.110$, $n = 10$, n.s; data not shown). Examination with mitochondrial fluorophores detected no MRC's present in SDSI preparations.

Discussion

Transepithelial resistance

In the present study, we focused on TER as our primary tool for assessment of the health of preparations. TER is now routinely used as an endpoint in toxicological investigations in both cultured mammalian epithelia (e.g., Faurskov and Bjerregaard 1999) and cultured gill epithelia (e.g., Sandbacka et al. 1999; Carlsson and Pärt 2001). The present study has demonstrated, at least in DSI epithelia (Figure 8B), that a high TER does not reflect a greater number of cells in the epithelium, or a greater protein content. Rather, it likely reflects a greater junctional tightness between the cells, in accord with the original work of Cereijido et al. (1981) showing a correlation between tight junction formation and greater TER in cultured mammalian epithelia. In earlier studies (reviewed by Wood et al. 2002) we have shown that TER provides an excellent measure of epithelial integrity in DSI and SSI preparations, reflecting both ionic and paracellular permeability. In preparations held under symmetrical conditions (culture medium on both surfaces), there are positive linear relationships between epithelial conductance (the inverse of TER) and permeability to polyethylene glycol-4000 (PEG-4000; a paracellular permeability marker), as well as between epithelial conductance and unidirectional ionic fluxes (Na^+ and Cl^-) in both SSI and DSI epithelia (Fletcher 1997; Wood et al. 1998; Gilmour et al. 1998b). Upon exposure to apical freshwater (asymmetrical conditions – e.g., Figure 7), TER increases because of a decrease in transcellular conductance, thought to reflect a closing of apical ionic channels. PEG-4000 permeability actually increases by a moderate amount at this time, while unidirectional leak rates of Na^+ and Cl^- show little overall change in the short term but increase in the longer term as TER and epithelial integrity both decline.

In future studies assessing the new methods and preparations developed here, it will be important to measure unidirectional ionic fluxes via the use of radiotracers. Of particular interest will be whether active transport of Na^+, Ca^{2+}, or Cl^- occurs. Knowledge to date on ionic transport in earlier cultured trout gill preparations has recently been summarized by Wood et al. (2002).

Trout plasma

Our primary goal in the plasma tests was to devise a method for growing gill epithelia that avoided the use of foreign proteins, specifically the mixture of unknown bovine proteins that are present in foetal bovine serum (FBS). We were successful in this regard, demonstrating that DSI epithelia exhibited comparable TER profiles when grown with fresh 5% trout plasma as when grown with 5% FBS. This finding runs counter to the generally accepted wisdom that fish sera are inferior to mammalian sera in supporting the culture of fish cells (Wolf and Quimby 1969; Maitre et al. 1986; Hightower and Renfro 1988), although there is one notable exception. Kocal et al. (1988) found that trout serum was superior to FBS in promoting the attachment and monolayer growth of trout hepatocytes in primary culture. Regardless, to our knowledge, this is the first time that unclotted, unfrozen plasma, freshly collected by catheter from chronically cannulated donor fish, has been employed. In practice, in a laboratory where chronic catheterization is a routine procedure, it is not difficult to keep a constant supply of fresh trout plasma available. Indeed, our impression is that plasma quality, in terms of its ability to support TER development, does not vary much from donor to donor, since changes in donor in mid-experiment had negligible effects on the development curves.

This is an important breakthrough, as the success of culture with homologous plasma opens the door to proteomic analysis on the cultured gill epithelium, which otherwise would be greatly confounded by the presence of bovine proteins. In the past few years, it has become clear that there are far more proteins than genes, and it is proteins, rather than messenger RNA, which are the proximate mediators of physiological response ('metabolomics'). As we enter the era of functional genomics, an important goal is to integrate its four components - genomics, transcriptomics, proteomics, and metabolomics (Butt et al. 2001). In fish gills, the approach was pioneered by Kultz and Somero (1996) using intact gobies exposed to different temperatures and salinities. However, cell culture systems may be better suited for this purpose, because they are less complex than the intact animal, and easily manipulated for differential protein display (Pandey

Table 1. A comparison of some properties of SDSI epithelia and DSI epithelia. Means ± SEM (*n*)

	SDSI	DSI
TER (kOhms cm^2)	1.74 ± 0.38 (15)	7.05 ± 1.67* (24)
Cell numbers $epithelia^{-1}$	42,150 ± 5912 (15)	95,662 ± 7742* (24)
Protein content $epithelia^{-1}$	12.07 ± 2.33 (14)	45.04 ± 2.83* (10)

* = $P < 0.05$

and Mann 2000). They can be used to target specific questions concerning fundamental processes of adaptation to environmental factors or to toxicological challenges. For example, we predict that DSI epithelia, because they contain only two cell types (PVCs and MRCs), will display fewer proteins than tissue taken from the intact gill with multiple cell types, and may provide a clearer picture of which proteins are directly up- or down-regulated by apical freshwater exposure, in contrast to those which change due to central hormonal controls.

An interesting sidelight of the plasma tests was the finding that a higher level (10%) of fresh trout plasma reduced the normal 'growth' of TER, while 100% prevented it entirely (Figure 1). This inhibitory effect was not seen if the plasma was applied after the preparations had matured to the plateau phase of TER, and did not alter the ability of mature epithelia to mount a TER increase (i.e., decrease in transcellular conductance) in response to apical freshwater exposure. Rather, the inhibitory effect was exerted on the initial development of TER, and apical addition alone was sufficient to cause this effect (Figure 2). Since the basolateral surface of the gill epithelium *in vivo* is likely in intimate contact with plasma, this asymmetry of action seems reasonable. In mammalian epithelial cell culture, there is considerable precedent for inhibitory effects of plasma or serum on TER, and these actions are generally attributed to the alterations in the integrity of tight junctions, though the specific plasma factors have not been identified. Obvious candidates are cytokines and interleukins, most of which have been found to weaken tight junctions (Walsh et al. 2000). In cultured canine kidney epithelial cells, the effects appear to be specific to the basolateral surface and involve a change in the organization of actin in the tight junctions (e.g., Conyers et al. 1990; Marmorstein et al. 1992). In cultured rat retinal pigment epithelial cells, the effects appear to be specific to the apical surface, occur when TER is relatively low, and involve a reduction in expression of zonula occludens-1 protein without alteration of actin (Chang et al. 1997a, b). The latter example appears more similar to the present observations, though the inhibition in the cultured retinal epithelium (Chang et al. 1997b) was not as fast (6 h) as in the cultured gill epithelium (< 1 h, cf. Figure 2). In the future, it will be of interest to evaluate the effects of plasma and specific cytokines on actin and zonula occludens-1 expression in tight junctions of the trout gill.

The properties of salmonid plasma, and/or the responses of salmonid gill cells to plasma, may be different from those of other teleosts. In preliminary trials of the present study, and in a number of previous studies with salmonid cells, frozen salmonid plasma had negative or toxic effects (Fryer et al. 1965; Collodi and Barnes 1990; Pärt et al. 1993; Pärt and Bergström 1995). However, in both Nile tilapia (Kelly and Wood 2002) and Atlantic sea bass (Avella and Ehrenfeld, 1997) gill epithelia have been cultured successfully using 10% homologous serum which had been previously frozen. Indeed, in the latter case, the 10% frozen sea bass serum promoted a higher degree of cellular differentiation and greater Cl- transporting capacity than did 10% FBS, though TER was only about 30% of that in FBS-cultured epithelia. The reason for these inter-species differences is unknown, but in future studies, it will be important to assess the effects of 5% and 10% fresh trout plasma on ionic transport in DSI epithelia.

Yolk-sac fry extract (YFE)

Growth and differentiation of cells in an epithelium are two different processes that tend to counteract each other (Dotto 1999). Growth is controlled by growth-promoting, or mitogenic, factors. These factors suppress differentiation. Differentiation is promoted by a variety of differentiation factors or differentiation-promoting stimuli such as interactions with the substrate (basal lamina) or other cells when growing cells reach confluence. Differentiation factors inhibit

Table 2. A comparison of the yield of SDSI, DSI, and SSI preparations per fish

	Number of fish[a]	Number of preparations	Success rate	Yield per fish
SDSI	1	50.0[b]	40%	20.0
DSI	2	50.0[b]	70%	17.5
SSI	1	48.0[c]	50%	24.0

[a]Assumes a typical yield of 135×10^6 cells per 80-g trout.
[b]Assumes a seeding density of 2.7×10^6 cells per 0.9-cm^2 filter on each seeding.
[c]Assumes an initial seeding density of 0.5×10^6 cells per cm^2 in 25-cm^2 flasks, and a yield after initial flask culture of 2×10^6 cells per flask, which are then seeded onto filters at a density of 0.45×10^6 cells per 0.9-cm^2 filter.

growth. An example is cortisol, which inhibited growth in gill cells (Pärt et al. 1993) but promoted differentiation of gill epithelia as judged from increases in TER (Kelly and Wood 2001b, 2002b; Wood et al. 2002). YFE was tested because it is reported to be mitogenic, stimulating the growth of several different fish cell lines in the absence of added serum (Collodi and Barnes 1990) and is routinely recommended as a supplement in the primary culture of cells from other teleosts (e.g., Bradford et al. 2000). In trout DSI preparations, it was clearly inhibitory to epithelial integrity, supporting no increase in TER at all in the absence of FBS, greatly reducing it in the presence of FBS, and reversing previous TER elevation when substituted for FBS (Figure 4). As with high levels of fresh plasma, these effects were not due to toxicity, and were at least partially reversible when YFE was replaced with FBS (Figure 4). As the major event occurring during the process of TER rise over time in DSI epithelia is likely not cell division (Figure 8B), but rather an increasing junctional tightness between the cells, these results indicate only that YFE is inhibitory to the latter process, in line with what is mentioned above. The mitotic activity of YFE likely retards the differentiated properties of the epithelium. However, since only one type of frozen YFE was tested, this does not eliminate the possibility that fresh YFE extract, or other YFE formulations, might be beneficial to epithelial integrity.

Storage at 4 °C

Storage of cultured epithelia at 4 °C was evaluated because this is a well known technique for the preservation and shipping of fish cell cultures (Wolf and Quimby 1969; Fryer et al. 1965; Lannan et al. 1984). The protective mechanism here is a dramatic slowdown of cell division and cellular metabolism, thereby greatly reducing the need for renewal of the culture medium (Wolf and Quimby 1962; Fryer et al. 1965; Plumb and Wolf 1971; Bols et al. 1992). When DSI epithelia at plateau TER were transferred to 4 °C, their lifespan was clearly extended by at least 10 days (Figure 5). To our knowledge, this is the first time that low temperature storage has been shown to preserve the integrity of a cultured epithelium. Since we do not believe that cell division is significant in the mature epithelium, it seems likely that cold temperature, by depressing metabolism, slows down or stops the normal cell aging process which eventually causes deterioration of transcellular and paracellular permeability, and thereby decrement of epithelial integrity. Two potential problems earlier identified in temperature work with fish cells are disassembly of the microtubular network (Tsugawa and Takahashi 1987) and altered membrane fluidity (Tsugawa and Lagerspetz 1990) at low temperature. It is not known whether these occurred in the present study, but if they did, they had no effect on TER.

The practical benefits for toxicological work, and potentially also for physiological studies, are obvious, because 4 °C storage will allow stockpiling of epithelia for testing on demand, and the accumulation of large numbers of preparations for tests involving multiple treatments. Because of these needs, the great majority of toxicological testing with fish cells in the past has been performed with continuous cell lines (Rachlin and Perlmutter 1968; Wolf and Quimby 1969; Bols et al. 1985; Marion and Denizeau 1985; Hightower and Renfro 1988), and primary gill cells, cultured as epithelia, have been employed only very recently, and only in small-scale tests (Sandbacka et al. 1999; Carlsson and Pärt 2001). Cell lines suffer the limitations of genetic uniformity, loss of differentiated character, and to date an inability to be cultured on permeable filter supports so as to permit TER measurements or apical freshwater exposure. The latter drawback prevents assessment of toxic effects on tissue level responses such as gill barrier properties. Furthermore, apical freshwater exposure is essential for preservation of contaminant speciation. In future experiments, it will be of interest to assess how much longer (beyond 10 days) the lifespan of DSI preparations can be extended at 4 °C, whether the same preservation works with SDSI preparations (see below), and whether toxicological sensitivity or physiological processes have been altered by this cold-temperature storage. For ex-

ample, changes in microtubular assembly (Tsugawa and Takahashi 1987), membrane fluidity (Tsugawa and Lagerspetz 1990), and heat shock protein induction (Hightower and Renfro 1988; Mitani et al. 1989; Bols et al. 1992) are all potential consequences of the return to 18 °C. Finally, it will be of interest to test whether low temperature preservation can extend the duration of tolerance to apical freshwater exposure.

Single Direct Seeded Insert (SDSI) epithelia

The SDSI technique was discovered by happenstance. When cells were left over from the first or second seeding of a batch of DSI preparations, they were seeded at the same density of 3×10^6 cm^{-2} onto spare filter inserts. Once it was realized that these preparations would actually develop TER, but at much lower rates and absolute levels than in DSI or SSI epithelia (Figure 6), their properties were examined. Overall success rate with SDSI preparations was about 40%, in contrast to about 70% with DSI preparations, and 50% with SSI preparations, resulting in a net yield of preparations per fish that was intermediate between SSI (highest) and DSI (lowest; Table 2). This potential for development of epithelia from single direct seeding of freshly dispersed trout gill cells onto filters had not been detected in earlier studies (Pärt et al. 1993; Wood and Pärt 1997; Fletcher et al. 1997). In practice, the technique appears similar to that of Avella and Ehrenfeld (1997) who grew cultured gill epithelia from sea bass by single direct seeding at comparable density, but in that species the final TER was much higher (12–13 kOhms cm^2 *versus* 1.0–2.0 kOhms cm^2)

The lower TER values of SDSI epithelia relative to DSI epithelia correlated with much lower cell numbers and protein content (Table 1), and within SDSI preparations, there was a significant relationship between TER and cell numbers (Figure 8A), in contrast to DSI epithelia where there was no such relationship (Figure 8B). We therefore cannot eliminate the possibility that the lower TER values of SDSI epithelia relative to DSI epithelia were due simply to lower protein content and/or lower cell numbers. However, we believe a more important difference may be the lack of MRCs in SDSI epithelia, for these are only incorporated on a second seed. In DSI preparations, MRC abundance is positively correlated with TER, and this factor is thought to explain the higher TER and lower paracellular permeability in DSI relative to SSI epithelia, with the MRCs contributing greater junctional stability (Fletcher et al. 2000).

The advantage of the SDSI approach for toxicology is the preservation of the individual, for the individual quantal response (reflecting phenotypic and genotypic variability) is the fundamental response unit in toxicological tests designed to protect populations (e.g., Sprague 1969). This variability is not available in cell lines that are clonal, is submerged by the mixing of individuals in DSI epithelia, but remains available in SSI epithelia, though the overall yield of preparations is lower and development time much longer in the latter. However, freshly dispersed cells from a single fish can yield approximately 20 SDSI preparations in 7–8 days (Table 2), allowing a large number of treatments (e.g., concentrations of toxicant) to be tested with replication. Thus, 10 individual fish that might die at one concentration in a standard *in vivo* LC50 test could otherwise provide enough SDSI epithelia to test all 10 concentrations, twice for each individual, in an *in vitro* test. The low absolute TER (Figure 6), and lower, shorter-lasting TER response (relative to SSI and SDSI epithelia) during apical freshwater exposure (Figure 7) may actually be an advantage in increasing sensitivity to toxicants. In our experience, low TER preparations have greater sensitivity to most disturbances than do high TER preparations. SDSI epithelia have this property naturally, so this is not a question of suboptimal performance, but rather an example of a more sensitive preparation. There is a potential here for eliminating *in vivo* testing, minimizing animal use, and maximizing test sensitivity, in accord with recent legislation.

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