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Research Article

Stabilization of gene expression and cell morphology after explant recycling during fin explant culture in goldfish

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ABSTRACT

The development of fin primary cell cultures for *in vitro* cellular and physiological studies is hampered by slow cell outgrowth, low proliferation rate, poor viability, and sparse cell characterization. Here, we investigated whether the recycling of fresh explants after a first conventional culture could improve physiological stability and sustainability of the culture. The recycled explants were able to give a supplementary cell culture showing faster outgrowth, cleaner cell layers and higher net cell production. The cells exhibited a highly stabilized profile for marker gene expression including a low *cytokeratin 49* (epithelial marker) and a high *collagen 1a1* (mesenchymal marker) expression. Added to the cell spindle-shaped morphology, motility behavior, and actin organization, this suggests that the cells bore stable mesenchymal characteristics. This contrast with the time-evolving expression pattern observed in the control fresh explants during the first 2 weeks of culture: a sharp decrease in *cytokeratin 49* expression was concomitant with a gradual increase in *col1a1*. We surmise that such loss of epithelial features for the benefit of mesenchymal ones was triggered by an epithelial to mesenchymal transition (EMT) process or by way of a progressive population replacement process. Overall, our findings provide a comprehensive characterization of this new primary culture model bearing mesenchymal features and whose stability over culture time makes those cells good candidates for cell reprogramming prior to nuclear transfer, in a context of fish genome preservation.

Introduction

Fish cell culture has been developed from a wide range of tissues [7–9,12,26,61]. Among them, fins were used in primary culture for studies relative to cellular biology [47,20,58], pathology [59,56,60,52], toxicology [2,3,58,61], cytogenetics, regenerative biology [33], and environmental biology [4]. Moreover, fin cell culture is of major interest in a context of preservation of fish genetic resources [31,6] as the cryopreserved cells have the potential to regenerate the original fish using the nuclear transfer technology [16,25,54].

Culture of explants taken from the fins leads to the production of the so called primary culture [48], and numerous cell lines,

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either finite or continuous, have been derived by subculturing the original culture over many tens of passages [23]. Primary cell cultures offer several advantages over cell lines such as the maintenance of the original cell–cell interactions and functional properties of the *in vivo* differentiated cells. For example, Hashimoto et al. [13] reported in goldfish that the sensitivity to the growth-promoting activity of carp serum detected in the fin cells from early passages was lost in the continuous RBCF-1 cell line. This line also lost its responsiveness to eurythermal growth [47]. However, the use of primary culture for comprehensive physiological or cellular studies still suffers from some limitation. First, the cell characterization in fin, or in the closely related tissue – the skin – , is often very rudimentary apart from few data on cell outgrowth and morphology [35,57,39,22,60,52] or from even scarcer data on the expression pattern of some marker genes [32,41]. Second, primary cell cultures often display low cell outgrowth rates [57]; low proliferation rates [41], massive cell losses over culture time [21,56] and difficulties to maintain the cells after three weeks [35] and after subculture [21]. These limitations are also a matter of concern when the fin explants are collected from rare or endangered fish from which very little material is available, and when genetic preservation and regeneration purposes rely on a stable and well characterized cell culture.

One mean to produce more cells from a given explant is to recycle the donor explant for another round of cell outgrowing, after the first primary culture. The concept of serial recycling of explants has been reported as a tool to provide an abundant and continuous supply of primary cells in fish gills [8,17]. The cell population outgrown from recycled gill explants were claimed to show better proliferation ability than the cells obtained from the initial fresh explants [17], although no experimental evidences were given. To our knowledge, no primary culture system using fin explant recycling was specifically described in the literature. It can be expected that such a system would be as promising as in gill. In this case, one major question is whether the produced cells would share the same origin and characteristics as the cells derived from fresh explants. Indeed, studies on cell physiology in primary culture require that the different cell populations included in the explant are well characterized to encompass the molecular phenomenon involved. It is also the case when these cells are used for fish restoration after nuclear transfer, because the donor nucleus reprogramming by the oocyte upon nuclear transfer is influenced by the initial features of the donor cell [5,49].

The objective of the present study was to develop a primary cell culture model for fin explants which would produce high numbers of cells with stable physiological stability and sustainability over time, in the perspective of using the cells for *in vitro* studies and for cryobanking prior to fish regeneration by nuclear transfer. Our strategy was to test whether the culture of fin explants recycled from fresh explants would improve the general status of the primary culture and the quality of the outgrown cells. The morphology, proliferation ability over time, and the expression pattern of marker genes were assessed on cells from recycled explants and compared to the cells cultured from the initial fresh fin explants. Because fin cells are mainly from epithelial and mesenchymal/fibroblastic origin [1], marker genes of these two cell types such as *cytokeratins* and type I *collagen* were chosen. In addition, the status of dedifferentiation of fin primary cells over culture time, previously suggested by Mauger et al. [32], was investigated using marker genes involved cell stemness and pluripotency such as *c-myc, sox2, nanog* and *pou2* [53].

With this approach, we showed that explant recycling is a powerful mean to obtain a whole new cell primary culture with most interesting characteristics such as a faster and more stable growing population with much less cell death than with fresh explants. Using molecular markers, we demonstrated that this culture with recycled explants was stable over the culture time, with a high expression of the mesenchymal marker (*collagen 1*). Finally, we described the kinetic of the changes occurring during a conventional culture with fresh explants that showed a radical evolution between day 7 and day 15 of the culture. This study provides a comprehensive description of the fin cells behavior in culture with two different explant systems, and gives new and unique information on the characteristics of the cells that can be used for future cellular biology purposes and conservation technologies.

Materials and methods

Biological material

Two years old goldfish (*Carassius auratus*) were obtained from outdoor ponds at the INRA U3E experimental facility (Rennes, France). Fish weighing 40 g on average were reared in 1 m^2 tanks with recycled water at a constant temperature of 14 °C under a photoperiod of 16 h of light and 8 h of dark. The fish were manipulated according to the guiding principles for the use and care of laboratory animals and in compliance with the French and European regulations on animal welfare, under the French registration authorization no. 78-25 (N. Chênais). Caudal fins were collected immediately after euthanasia by decapitation and processed within 10 min after collection.

In the experiment dealing with fin regenerative areas, a tiny piece of the caudal fin of anaesthetized adult goldfish was cut with sterile scissors, and the fish were put back in the rearing tank. After 8 days, the caudal fins were collected after fish euthanasia as described above. Only the regenerative area of the fin was taken for the *in situ* hybridization sections.

Two-month old goldfish fry were obtained after artificial fertilization and rearing in recycled tap water aquaria (20 °C) under 16 h light/8 h dark photoperiod. They were anaesthetized with a lethal dose of phenoxyethanol (90 μL/100 mL water), rinsed and immediately processed for the histological and molecular analyses.

Fresh and recycled explant cultures

Whole caudal fin pieces were cleansed from their mucus by thorough wiping. We observed that this wiping resulted in the loss of most mucus cells. The pieces were then washed 4 times with the sterile washing medium A made of Leibovitz L15 culture medium supplemented with Hepes 5 mM, $NaHCO_3$ 2 mM, gentamycin 100 μg/mL and amphotericin B 2.5 μg/mL, osmolarity 290 mOsm/kg, pH 7.3. The thinnest part at the extremity of the fin was cut into 7 mm^2 explants. The explants were mildly digested with collagenase (Sigma, C2674, 0.2 mg/mL, 30 min at room temperature) in 10 mL of sterile B culture medium (medium A supplemented with L-glutamine 2 mM and fetal calf serum 5%)