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Culture, cryobanking and passaging of karyotypically validated native Australian amphibian cells

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ABSTRACT

This study describes the culture, cryobanking, thawing and passaging of karyotypically validated cells from two life history stages of the "least concern" Australian native amphibian species *Litoria infrafrenata*. Adult frog toe and tadpole macerates were generated from animals euthanized due to ill health following injury. Cultured cells proliferated and formed colonies after one to two weeks of culture. Cultures were cryopreserved in liquid nitrogen for a minimum of one month, thawed, passaged for expansion and karyotyped. Post-thaw karyotypes revealed the expected 2N = 24 diploid chromosome number in approximately 90% of all metaphase spreads. Further, metacentric, submetacentric and subtelocentric configurations were the same as previously described karyotype configurations obtained from living frogs of this same species. Using cryobanked and prospectively validated cell lines, conservation programs including assisted reproduction technologies and genomic, mitochondrial and proteomic mining initiatives may therefore be complemented with minimal or no disturbance to living and healthy animals.

1. Brief communication

Amphibians are experiencing a critical existence juncture, with approximately 32% of the currently recognised species under immediate threat [1]. The main driver of this crisis is human activity and the spread of chydridiomycosis [1]. Conservation programs involving habitat conservation and assisted reproduction techniques (ART) have demonstrated success in safeguarding many threatened species from imminent extinction. A greater number of complementary techniques, including cryobanking initiatives, are required [1].

Several amphibian cryobanks have been established and they hold tissue samples from critically endangered amphibians within their inventories [5]. However, examples of prospectively validated amphibian cultured cells that can be readily thawed and manipulated for application to ARTs such as somatic cell nuclear transfer (SCNT) are rarely described. It is intuitive to develop validated freezing protocols for viable cell recovery while living animals remain available.

Experimental tissues were sourced from animals immediately after euthanasia due to illhealth following injury. Relevant State government and institutional ethics, licenses and permissions were obtained, and all work was carried out in accordance with The Code of Ethics of the World Medical Association of The Declaration of Helsinki. All experimentation complied with the EU Directive 2010/63/EU for animal experiments. Tadpole macerates were produced as described below. A frog toe clipping was taken because this may represent a relevant source of scavenged tissue for future cultures of critically endangered animals studied in the wild [5].

Collected tissues were wiped with 70% v/v ethanol and washed in Amphibian Ringer's Solution (AR; Cold Spring Harbor Protocols [9]) at 4 °C. Tissues were stored at 4 °C for no longer than 4 h prior to further processing. Tissues were minced finely with scissors and incubated in 24 well plates (Falcon Multiwell™; GIBCO). It is understood that reports of successful amphibian cell culture are rare, that fully supplemented media may be superior to basic salt media and that several different techniques have been described for the successful culture of several different species (see Kouba et al., 2013 [5]). Culture was therefore performed in supplemented and diluted DMEM at room temperature according to Ferris et al., 2010 [3], but with 1000 units/mL penicillin (Sigma) and 1000 µg/mL streptomycin (Sigma) in place of gentamicin. Cell cultures were maintained for four days and then one half of the media was changed. The entire media was changed thereafter every one to two days.

Cells were trypsinized with 0.25% trypsin/0.02% EDTA for passaging or suspended in 100 µl of the same culture media but containing 10% DMSO in 1 ml cryotubes (Nunc®) for cryobanking. Cryobank cryotubes were placed overnight in a minus 80 °C freezer. The following day, cryovials were plunged into LN2 for long-term storage. Following retrieval from LN2, cryovials were place on ice and cells quickly thawed by rubbing the cryovial between finger and thumb until the last visible ice crystal had dissolved. Cryopreservation media was diluted in culture

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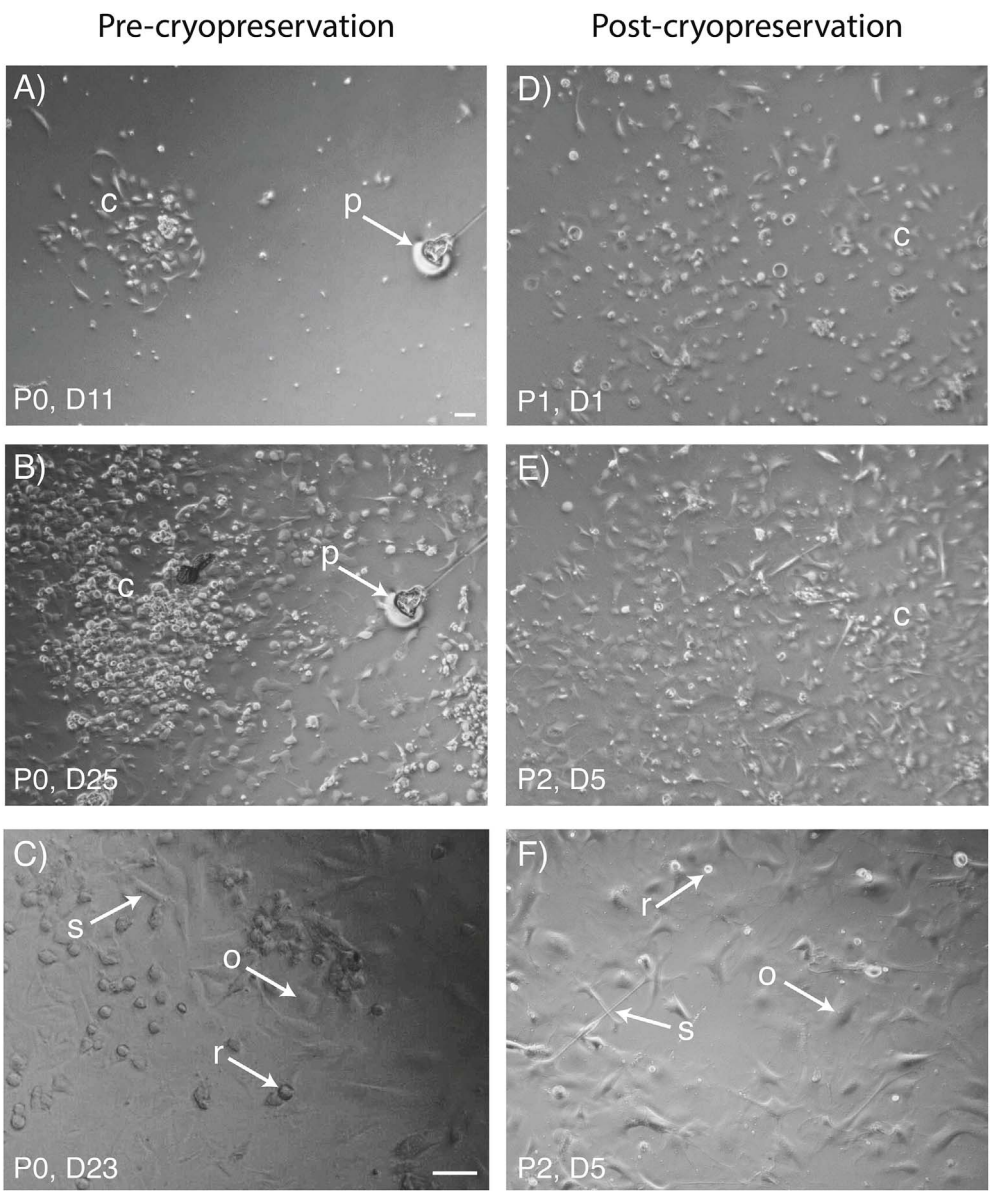

Fig. 1. Culture of a *Litoria infrafrenata* frog toe clipping. (A–C) Primary culture prior to cryopreservation (P0); (D–F) Passage 2 cells, post cryopreservation (P2); (A, B, D, E) low power; (C,F) high power. (A,B) P0 cells form an expanding cluster (c) adjacent to the reference plastic scratch (p). (C) P0 cells are either ovoid/polygonal (O) or elongated (s); rounded cells (r) are also observed. (D–E) Post-crypopreservation, P2 cells expanded to reach approximately 60% confluency by day 5 (D5). (F) Post-cryopreservation P2 cells are both ovoid/polygonal and spindle-shaped; rounded cells are also observed. LP and HP = 10× and 40× objectives, respectively, using a 10× eyepiece lens. Reference bar = 10 µM.

media so that the final DMSO concentration was not greater than 0.5%. Culture media was then changed with fresh media after 48 h. Both passaged cells and cryopreserved cells were reseeded in 24 well plates. By comparing culture plate surface area coverage before and after freezing, cell viability post freeze thaw cycle was estimated to be within the range of 30%–60% for both frog and tadpole cultures. Images of live cell cultures were captured using an Olympus IX70 – S8F2 inverted microscope using a ProgRes®C3 (Jenoptik, Germany) camera and ProRes® CapturePro Software Version 2.8.8.

Karyotyping was performed by treating cells for six to 8 h with 0.1 µg/ml KaryoMAX® colcemid (GIBCO) followed by staining with 40,60-diamino-2-phenylindole (DAPI; 500 ng/ml; Sigma) [8]. Slides were coverslipped with DAPI in Gelvatol mounting medium (Cold Spring Harbor Protocols [10]). The nomenclature for numbering chromosomes followed previous descriptions where the largest chromosome was labelled as chromosome 1, and the remaining were ordered according to descending chromosomal length; and in accordance with previous karyomaps prepared from primary and non-cultured cells of this species [4]. Chromosome arms were measured using Image J software with the Levan plugin. Metacentric, submetacentric and subtelocentric chromosomal designation are defined as a long arm to short arm ratios of 1–1.69, 1.7–2.99 and 3–6.99, respectively [6]. Imaging was performed at 1000 x under oil immersion using an Olympus BX60 microscope, colour CCD Leica DFC425C camera, and EL-6000 Leica