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1 # Fishing Cat Cell Biobanking for Conservation

2 Woranop Sukparangsi[1*], Ampika Thongphakdee[2*], Santhita Karoon[2], Nattakorn Suban Na
3 Ayuttaya[1], Intira Hengkhunthod[1], Rachapon Parkongkeaw[1], Rungnapa Bootsri[1], Wieware
4 Sikaeo[1]
5
6 **Affiliation**
7 [1]Department of Biology, Faculty of Science, Burapha University, Chon Buri, Thailand
8 [2]Wildlife Reproductive Innovation Center, Research Department, Bureau of Conservation and
9 Research, Zoological Park Organization under the Royal Patronage of H.M. the King, Bangkok,
10 Thailand
11
12 ***Correspondence**
13 Woranop Sukparangsi (woranop@go.buu.ac.th)
14 Ampika Thongphakdee (ampialaska@hotmail.com)
15
16 **Highlight**
17 Biobanking of viable cells is essential to provide long-term security of wildlife existence. Current
18 cell technology enables us to cultivate primary cells and adult germ cells from tissues of living and
19 postmortem fishing cats for cryopreservation. The primary cells exhibited limited proliferation and
20 cell senescence, which can be overcome by reprogramming the somatic cells toward pluripotent
21 state. Here we explored the challenge of tissue collection from fishing cat and several virus-free
22 approaches to induce cellular reprogramming in the fishing cat cells and provided insight into the
23 techniques and conditions to enhance cell expansion, which support the success of generation of
24 fishing cat cell biobank.
25
26 **Abstract**
27 Establishment of biobank to keep wildlife cells secure long-term conservation. Fishing cat
28 (*Prionailurus viverrinus*) is one of Vulnerable wild felids, currently under threaten by wetland
29 destruction and other human activities. Here we aimed to generate cell biobanking of fishing cats
30 by deriving various sources of primary cells from the living and postmortem animals and
31 enhancing their expandable potency by virus-free cellular reprogramming. We show that cells can

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be propagated from several tissues harvested from both living and dead fishing cats with different derivation efficiency. Testes from the postmortem animals contain several tissues that can be derived primary cells as well as putative alkaline phosphatase positive and SOX2 positive adult spermatogonial stem cells. Primary cells from ear pinna and abdomen sources can only be obtained from the living fishing cats. These primary cells exhibited sign of cell senescence after a few sub-cultures, limited its usability for downstream applications. This obstacle can be overcome by reprogramming via either nucleofection or liposome-based DNA/RNA delivery. The putative iPSC colonies as well as expandable induced cells from episomal-based reprogramming appeared to be a suitable choice for expansion of cells for cryopreservation. Thus, here we provide current conservation plan using cell technology for fishing cats and also recommendation of tissue collection and culture procedures for zoo researches to facilitate the preservation of cells from postmortem animals and living animals.

Key words: cryopreservation, biobank, fishing cat, wild felid, fibroblast, reprogramming

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47 **Introduction**

48 Distribution records of wild felid in Thailand via camera-trap surveys, radio-telemetry
49 studies, direct sightings, interviews as well as searches for signs from several efforts show the
50 presence of seven small to medium cat species including Asiatic golden cat *Catopuma temminckii*,
51 jungle cat *Felis chaus*, mainland clouded leopard *Neofelis nebulosa*, marbled cat *Pardofelis*
52 *marmorata*, leopard cat *Prionailurus bengalensis*, flat-headed cat *Prionailurus planiceps*, and
53 fishing cat *Prionailurus viverrinus* [1-4], and two large cat species including Indochinese tiger
54 *Panthera tigris corbetti* and leopard or panther *Panthera pardus* [2,5-6].

55 Fishing cat plays important roles in wetland ecosystem; however, in recent years, their
56 wetland habitats are destroyed by human threats such as prawn farming industry, illegally
57 established aquaculture ponds, human settlement, excessive hunting, deforestation, depletion of
58 fish stocks from over-fishing, and incidental poisoning [7-9]. The fishing cat is now included on
59 "Vulnerable" status (IUCN Red List of Threatened Species) [10] and CITES Appendix II and
60 protected by national legislation. Fishing cats in captivity at Zoological Park Organization of
61 Thailand are currently under conservation programs aimed to improve its breeding and produce
62 more offspring, which can be eventually re-introduced back to natural habitats. Rescuing of
63 endangered or vulnerable wildlife animals by breeding program at the zoo involves assisted
64 reproductive technology (ART), which become urgent trends to secure good genetics for long-
65 term conservation. The storage of valuable genetics of wild animals can be performed in several
66 ways including cryopreservation of gametes (spermatozoa and oocyte) and embryos for future *in*
67 *vitro* fertilization (IVF), artificial insemination (AI) and embryo transfer [11-13]. To date,
68 Santymire et al., 2011 and Thongphakdee et al., 2018 [14-15] provided better understanding of
69 reproductive biology in fishing cat and demonstrated a potential application in its captive breeding.
70 However, there are some restrictions for developing ART in fishing cat including restricted
71 number of fertile fishing cats, high variability of ovarian response to estrus and ovulation induction
72 for AI [15] and low fertilization success [16].

73 Another approach to secure good genetics of fishing cats as well as other wild animals is
74 to establish primary cells, in particular fibroblasts with some restricted degree of propagation and
75 stem cell lines such as embryonic stem cells (ESCs), mesenchymal stem cells (MSCs) and induced
76 pluripotent stem cells (iPSCs) with more capacity to self-renewal and differentiation. The
77 preservation of those cells in liquid nitrogen-containing cryotank generates invaluable wildlife

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78 biobank, or "Frozen Zoo" for future conservation approaches. *In vitro* culture of various cell types
79 also provides great benefit of reducing and replacement of using animals for experimentation in
80 3Rs (Reduction, Refinement and Replacement) model [17].

81 Tissues in primary culture can be harvested from either live animals or freshly dead/post-
82 mortem animals [18-20]. Fibroblast culture has already been achieved among several mammalian
83 species, mostly in mouse (*Mus musculus*) and human and in other mammals such as horse, dog,
84 drill, rhinoceros, and elephant [20-23]. In felid species, fibroblasts were derived in domestic cat
85 for iPSC generation and intraspecific feeder cells to support induction of iPSC and cat ESC
86 derivation and in wild cats including Bengal tiger, jaguar, serval and snow leopard for iPSC
87 generation [24-27]. Hence, in this study we aimed to establish cell biobanking for fishing cats to
88 preserve its genetics for long-term conservation. During a decade of fishing cat projects in our zoo
89 organization, here we reported the successful attempts to derive fibroblasts from different sources
90 including living and dead fishing cats in captivity and from natural resources. Here we also
91 examined the usability of our fibroblasts for cellular reprogramming and provided the insight into
92 current challenges to these approaches.

93

94 **Methods**

95 **Animal Ethics**

96 This project was permitted to perform under project "Development of fundamental science and
97 innovation for sustainable fishing cat conservation" with sub-project "Conservation of fishing cat
98 (*Prionailurus viverrinus*) by using innovation of stem cell technology" by wild animal ethics
99 committee of Zoological Park Organization of Thailand under the Royal Patronage of H.M. the
100 King (Protocol number: 630960000030 granted to PI of the project- A.T.). Permission to work
101 with wild fishing cats in the Natural Parks of Thailand was granted by Department of National
102 Park Wildlife and Plant Conservation-Thailand (Permission number: Tor Sor (in Thai)
103 0907.4/17939 (Issued date: 22/09/2021). Tissue collection from postmortem fishing cats and
104 during artificial insemination were performed by veterinarians at Animal Hospital Unit, Khao
105 Kheow Open Zoo (KKOZ), Zoological Park Organization, Chon Buri, Thailand. Cell culture was
106 performed at Tissue Culture Facility at Wildlife Reproductive Innovation Center (WRIC),
107 Research Department, Bureau of Conservation and Research, KKOZ and genetic materials and

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108 chemicals in this study were used under Certificate of Biosafety (22/2559) approved by Biosafety
109 Committee, Burapha University.
110

111 **Primary culture and cryopreservation**

112 Collected tissues (skin and testis) were washed with Dulbecco's Phosphate-Buffered Saline
113 (DPBS, ThermoFisher) containing Penicillin (10000 Unit/mL)-Streptomycin (10 mg/mL) (PS)
114 solution (Sartorius) and 500 µL 10X Amphotericin B (AmB) Solution (Sartorius) for 3 times.
115 Removal of subcutaneous adipose tissues and intact hairs (for skin tissues) was done to avoid
116 bacterial and fungal contamination and presence of lipid droplets. To activate the outgrowth of
117 fibroblasts from the tissues using wound healing process, several small cuts were made using
118 scalpel and the tissues were cut into approximately 2X2 mm. The excised tissues were then placed
119 (3-4 pieces) on gelatin (Attachment Factor, ThermoFisher)-coated dish (35 mm) or 12 well plates
120 already for 10 min for better explant attachment. Then warm complete fibroblast medium was
121 added to the dish/plate as lowest volume as possible to avoid tissue floating and ensure the
122 outgrowth. The primary culture was placed in the CO_2 incubator (5% CO_2 in humidified 95% air
123 at 37 ^{o}C). To avoid contamination, the cultures were monitored every day and medium changes
124 should be done every day without disturbing the explant attachment. The complete fibroblast
125 medium (50 mL) was composed of Dulbecco's Modified Eagle Medium (DMEM) high-glucose
126 medium (ThermoFisher and Sartorius), MEM Non-essential Amino Acid Solution (NEAA, Sigma,
127 Merck), Glutamax (ThermoFisher), Sodium Pyruvate (Sigma, Merck) and antibiotics PS-
128 antimycotics AmB (Sartorius), unless stated otherwise. For cryopreservation, the cells were
129 resuspended in freezing medium containing complete fibroblast medium with 10% dimethyl
130 sulfoxide (DMSO) or Recovery™ Cell Culture Freezing Medium (ThermoFisher) and placed in -
131 80 ^{o}C overnight using Mr.Frosty^{TM} Freezing Container and next day transferred to liquid nitrogen
132 for long-term storage. All cells in this study are currently stored in Biobank Liquid Nitrogen Tank
133 Facility under Zoological Park Organization (ZPO) of Thailand. Further detail of cell storage and
134 material transfer is available upon request to correspondence (A.T. or ZPO Research Bureau).
135

136 **MTS assay**

137 MTS colorimetric assay kit (Abcam) was used to quantify cell viability of transfected cells.
138 For nucleofection, the transfected cells were seeded into 96-well plate (10,000 cells/well) in the

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139 presence or absence of RevitaCell™ supplement (Gibco). RevitaCell™ supplement was added at
140 final concentration at 0.5X. At day 4 post nucleofection, fresh fibroblast medium was changed
141 prior performing MTS assay (200 uL/well). Then 20 uL MTS solution was added and incubate for
142 4 hours at 37 °C. Absorbance at 490 nm was measured by microplate reader (Thermo Scientific™
143 Multiskan™ GO Microplate Spectrophotometer) with Skanlt™ Software. Four independent
144 experiments with three technical replicates each were performed for measuring cell viability.
145

146 **Alkaline phosphatase live staining**

147 To identify pluripotent stem cells before cryopreservation for future uses, we used
148 Fluorescein-based Alkaline Phosphatase (AP) Live Stain (ThermoFisher) to detect activity of AP
149 in cell extracts from seminiferous tubules and epididymis. Briefly, we prepared diluted AP stain
150 (1:500) and Hoechst33324 mixed with medium and stained the cells for 20 minutes before imaging
151 with fluorescent microscope.
152

153 **Immunofluorescence**

154 To observe the presence of Spermatogonial Stem Cells (SSC), extracts from seminiferous
155 tubules from the fishing cat were fixed with 4% paraformaldehyde (PFA) for 15 mins at room
156 temperature and washed with DPBS for 3 times. In order to permeabilized the plasma membrane,
157 the fixed cells were treated with 0.1% Triton X-100 in PBS for 15 mins at room temperature and
158 washed with DPBS for 3 times with each time incubated for 5 mins. Then the cells were treated
159 with 1% BSA in DPBS for 1 hour at room temperature. The cells were treated with primary
160 antibodies SOX2 (AB5603, Merck), at dilution 1:300, for over-night at 4 °C and washed with
161 DPBS 3 times next day before secondary antibody staining. On the next day, the cells were stained
162 with Alexa Fluor 647 (ThermoFisher, 1:800) and Hoechst33342 (ThermoFisher, 1:200) in dark for
163 1 hr at room temperature and washed with DPBS 3 times. Fluorescent micrographs were taken by
164 inverted microscope Eclipse Ti-S Inverted Research Microscope (Nikon) and digital camera.
165

166 **Nucleofection**

167 Nucleofection™ programs in 4D Nucleofector™ system included CA-137 (specific for human
168 iPSCs and mammalian fibroblast with Primary Cell 2 (P2) and 3 (P3) solution kit), DS-150, EH-
169 100, EN-150, EO-114 (specific for mammalian fibroblast recommended by Lonza and compatible

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170 with P2 solution kit), FF-135 (specific for human fibroblast and dental palp cells [28-30]) and DT-
171 130 (specific for neonatal Normal Human Dermal Fibroblast, NHDF-neo cell lines compatible
172 with P2 solution and human fibroblast [31]). Small scale of 4D-NucleoFector™ kits (P2 Primary
173 Cell 4D-Nucleofector® X Kit S 32 RCT, V4XP-2032), composed of 16-well Nucleocuvette™
174 Strip and Primary Cell 2 (P2) solution, were used for nucleofection. To prepare fibroblast cell
175 transfection with small scale, the cells at 70% confluency were subcultured, counted by
176 hemocytometer, transferred 10^5 cells to new microcentrifuge tube, and then centrifuged at 90g for
177 5 minutes, supernatant was aspirated supernatant and cell pellet were gently resuspended in 20 µL
178 of P2 solution, 400 ng of pmaxGFP™ vector was added and gently mixed, transferred cell
179 suspension into Nucleocuvette™ Strip, nucleofected with programs of interest, kept the transfected
180 cell suspension at room temperature for 10 minutes, 80 µL of complete fibroblast medium (CF)
181 added to Nucleocuvette™, transferred transfected cells into non-coated 4-well plates containing
182 420 µL complete fibroblast medium per well, and incubating cells in a CO_2 incubator. GFP
183 expression was observed under fluorescence microscope.
184
185 **Senescence test**
186 Senescence β-Galactosidase Staining (Abcam) was used to detect sign of cell senescence
187 according to manufacturer's instruction. Briefly, the fibroblasts were washed once with DPBS,
188 fixed with fixative solution for 10 minutes and then washed with DPBS twice. The cells were then
189 stained with staining solution mix containing Solution A, Solution B and X-gal for overnight at 37
190 ^{o}C. On the next day, the stained cells were stored in 70% Glycerol before imaging with an inverted
191 microscope.
192
193 **Flow cytometric analysis**
194 To monitor transfection efficiency, GFP expression was detected by flow cytometry
195 (FlowSight® Imaging Flow Cytometer, Luminex). At day 4 post nucleofection, transfected cells
196 were washed once with DPBS, dissociated with 0.25% Trypsin-EDTA for 3 minutes, neutralizing
197 with fibroblast medium, spinning down cell pellet and resuspending in DPBS. Hoechst33342 (1
198 µg/mL) were then added to cell suspension to stain all cells. Flow cytometric data were analyzed
199 using FCS Express 7 software. GFP/Hoechst33342 cell populations were gated and transfection
200 efficiency was calculated from percentage of double positive cell populations with GFP and

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201 Hoechst33342 against total Hoechst33342 positive cells. Four independent experiments were
202 performed for measuring transfection efficiency.
203

204 **Reprogramming assay**

205 PiggyBAC transposon (PB) vector MKOS-mOrange (Gifted by Dr.Keisuke Kaji) contains
206 4 mouse reprogramming factors: C-Myc, Klf4, Oct4 and Sox2 under CAG-promoter and mOrange
207 reporter. The DNA vector was transfected by nucleofection. The transfected cells were cultured
208 with iPSC induction medium containing Advanced DMEM (ThermoFisher), 10% FBS, MEM
209 Non-essential Amino Acid Solution (NEAA, Sigma, Merck), Glutamax (ThermoFisher), Sodium
210 Pyruvate (Sigma, Merck) and antibiotics PS-antimycotics AmB (Sartorius) with 10 ng/mL human
211 Leukemia Inhibitory Factor (hLIF, Peprotech), unless stated otherwise. The cells were re-seeded
212 onto irradiated MEFs or other coating matrixes including Attachment Factor/gelatin
213 (ThermoFisher), Geltrex (ThermoFisher) and vitronectin (ThermoFisher). PB vector pC6F
214 (addgene: 140826 [32]) contains Tet-on system regulating the expression of polycistronic cassettes
215 of 6 reprogramming factors: human OCT4, SOX2, KLF4, CMYC, KLF2 and NANOG and
216 TdTomato reporter [32]. We transfected pC6F alongside with transposase vector (pCy43, Sanger
217 Institute) and PB rtTA vector (addgene 126034) using Lipofectamine 3000 (ThermoFisher) for
218 overnight. The transfection was performed for one time and on day 3, we selected cells carrying
219 pC6F construct with puromycin (2 ug/mL) and induced the expression of reprogramming factors
220 with Doxycycline in the iPSC induction medium. Episomal vector system is composed of pCXLE-
221 hSK (vector with SOX2 and KLF4; addgene: 27078), pCXLE-hUL (vector with L-MYC and
222 LIN28; addgene: 27080), pCXLE-hOCT3/4 (vector with OCT3/4; addgene: 27076), pCXWB-
223 EBNA1 (vector with EBNA1; addgene: 37624). These vectors were nucleofected into the cells.
224 The transfected cells were cultured in the iPSC medium, unless state otherwised. Commercial
225 media were also tested for reprogramming, including NutriStem hPSC XF Medium (Sartorius),
226 medium containing Knockout Serum Replacement (KOSR, ThermoFisher) and Essential 8
227 medium (ThermoFisher). For self-replicating RNA (srRNA) system, we generated RNA from T7-
228 VEE-OKSiM, T7-VEE-OKSiG, T7-VEE-GFP plasmids using *in vitro* transcription technique
229 with HiScribe™ T7 quick high yield RNA synthesis kit, according to manufacturer's protocol.
230 Synthesized RNA was modified by Vaccinia Capping System (NEB), mRNA cap 2'-O-
231 methyltransferase (NEB) and *E. coli* Poly(A) Polymerase (NEB). The fibroblasts were transfected

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232 with srRNA using Lipofectamine^{TM} MessengerMax^{TM} (ThermoFisher) according to adjusted Yo-
233 shioka & Dowdy method [33]. The transfected cells were cultured in iPSC medium with 200
234 ng/mL of recombinant viral B18R protein (R&D Systems). B18R was removal once the
235 transfected cells were ready to re-seed onto irradiated MEFs.

236

237 **Statistical analysis**

238 Data from three to four independent experiments (with at least three technical replicates)
239 are presented as the mean±SD (Standard Deviation). Statistical analyses were performed using
240 Student's t test to determine statistical significance between the groups.

241

242 **Results**

243 **Adult dermal fibroblast culture from abdominal dermis of living fishing cats**

244 Skin biopsy to collect dermis for producing fibroblast culture, an available source for
245 cellular reprogramming, from living fishing cats have been limited due to invasive procedures.
246 Within assisted reproductive technology (ART) program of the zoo, direct harvest of the dermis
247 in the abdominal skin of fishing cat during artificial insemination (AI) operation were conducted
248 (Figure 1Ai-iii), enabling us to derive dermal fibroblasts from a female fishing cat. As shown in
249 Figure 1B, outgrowth of the fibroblasts emerged around the edge of the tissue explants within five
250 days. Cell with epithelial character was not detected, indicating the absence of keratinocytes
251 (Figure 1B-E). The dermal fibroblasts in the primary culture expanded to reach its 90% confluency
252 at day 16 with homogenous fibroblastic morphology (Figure 1D). However, as the collection of
253 tissue from abdominal dermis was closed to subcutaneous layer containing rich adipose tissue,
254 lipid droplets were found during culture (Figure 1E). Secondary culture was done by removing
255 explant tissues and sub-cultured the fibroblasts into appropriate culture vessels (split ratio of 1:2;
256 Cell density at 2.0 X 10^4 cells/cm^2). Fibroblasts expanded to reach its 90% confluency within 4
257 days after the first passaging (Figure 1F). It is noteworthy that seeding the fibroblasts of fishing
258 cat at too low cell density generally led to less cell expansion. In early passages, the fibroblasts
259 exhibited spindle shape (Figure 1F) while at later time the cell area expanded in less
260 nucleus:cytoplasm ratio manner (Figure 1G). At the late passages, the fibroblast culture was
261 deficient due to longer trypsinization time needed for subculture and less cell expansion. By
262 removing supplements and measuring cell division rate with MTS assay, we also show the

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263 essential components of the fibroblast medium to support cell growth and division. As shown in
264 Figure 1H, removing either sodium pyruvate or non-essential amino acids or both did not reduce
265 cell proliferation while removing Glutamax caused a significant reduction of cell proliferation.
266 The effect became prominent from the complete removal of all supplements. Thus, additional
267 supplements did help to improve fibroblast cell expansion. In addition, increasing percent of fetal
268 bovine serum (FBS) from 10% to 20% did not significantly enhance cell proliferation (Figure 1H).
269 To improve the fibroblast culture, we next tested addition of basic Fibroblast Growth Factor (bFGF
270 or FGF2) and found that bFGF enhanced proliferation of the fibroblasts (Figure 1I-K).
271
272 **Adult dermal fibroblast culture from ear pinna of living fishing cats**
273 To preserve more genetic variation of fishing cats, cryopreservation of cell samples
274 collected from natural resources is required. Under permission of National Park of Thailand (in
275 Animal Ethics section), two fishing cats from nature were caught to collect samples including skin
276 biopsy. Due to nature of fishing cats in prey capture by swimming, we collected small skin tissues
277 from the ear pinna (Figure 2A,F), not other parts of abdominal and leg area, of anesthetized fishing
278 cats by veterinarian team. The skin tissue (Figure 2A, F) was kept cold (4°C) in complete fibroblast
279 medium during transportation to Tissue Culture Facility. The tissue explants (Figure 2B, G) were
280 cultured within 4-5 hours after the skin biopsy. Primary culture of freshly collected ear pinna tissue
281 showed that the fibroblast outgrowth can be observed within three days from most of explant
282 tissues (Figure 2C, D, H, I). However, epithelial-like keratinocytes were also expanded in
283 advanced DMEM (AD) medium (Figure 2D right, I right), but not in the fibroblast (FB) medium
284 (Figure 2C, H), which the proportion of keratinocytes/fibroblasts diminished after passaging in
285 DMEM-based medium (Figure 2E, J). The tissue explants from living fishing cat can be re-
286 explanted for at least three times and producing fibroblast culture sufficient to freeze for 30
287 cryovials (approximately 100,000 cells/vial), Table 1. It is noteworthy that cell cultures of tissues
288 collected from natural resources caused regular fungal contamination, even the presence of
289 Amphotericin B. We also compared duration of medium change and found that primary culture
290 with medium change everyday produced more fibroblasts while medium change every 2/3-day
291 period caused 100% contamination, shown as overall result in Figure 2.
292

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293 **Primary culture of tissues from postmortem fishing cats**

294 From a postmortem fishing cats at 3 months old (3FC) and 11 years old (11FC), we can
295 retrieve two parts of the body including ear pinna (both 3FC and 11FC) and testes (11FC). Explants
296 from the ear pinna were seeded after one day postmortem. We can derive fibroblasts from ear
297 pinna only from 3FC (Figure 3A-C) but cannot obtain any primary cells after prolonged culture
298 for a month from 11FC (Figure 3D). For tissue collection from testes, the fibroblasts can be
299 obtained from testicular wall and epididymis (Figure 3E, F). Comparing the testicular fibroblasts
300 between P.1 and P.4, we observed stronger signal of senescence in P.4 (Figure 3G, H). This
301 indicates that the usability of the adult fibroblasts from testis is limited to only early passages. We
302 also examined the extraction of cells from seminiferous tubules and epididymis to observe the
303 presence of spermatogonial stem cells (SSCs). Using immunofluorescence with an antibody to
304 detect SOX2 protein, we found SOX2-positive cells in the culture from seminiferous tubules,
305 indicating the plausible presence of SSCs (Figure 3I). We also found alkaline phosphatase positive
306 cells within the seminiferous tubule but not in the epididymis (Figure 3J). Thus, from postmortem
307 fishing cat, we can cryopreserve cells from various sources including testicular wall, epididymis
308 and putative SSCs from seminiferous tubules.

309

310 **Delivery of DNA vectors to hard-to-transfect adult fishing cat cells by nucleofection**

311 To enhance limited capacity of fibroblasts to proliferate, here we aimed to optimize DNA
312 delivery into the dermal fibroblasts of fishing cat to further apply for next step of reprogramming.
313 Nucleofection, performed by Nucleofector^{TM} (Lonza), has been used for non-viral transfection of
314 genetic materials with high efficiency into primary cells and hard-to-transfect cells. In this present
315 study, we conducted DNA delivery using 4D-Nucleofector^{TM} system. In general, each cell line
316 requires specific Nucleofector Solution (non-disclosed recipe) and particular program of electric
317 pulse to transfer DNA into cytoplasm and even nucleus of cells. Here we aimed to define optimal
318 Nucleofection^{TM} condition by testing seven programs specific for various types of mammalian cell
319 lines including human cells with recommended Primary Cell 2 (P2) solution specific for
320 mammalian dermal fibroblasts. Based on the expression of GFP, all programs could transfect
321 fishing cat fibroblast cells. Each program provided different cell viability and transfection
322 efficiency. At day 4 post nucleofection when the cells reach 90% confluency, differences in GFP
323 expression became obvious in that three programs including DS-150, EN-150 and FF-135 showed

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324 outstanding performance. However, after subculturing, the transfected cells with EN-150 and FF-
325 135 conditions still contained GFP expression (Figure 4). We used flow cytometry to confirm the
326 transfection efficiency and showed that program FF135 can transfect the best with 33.10-41.37%
327 GFP+ cells at day 4 post transfection (Figure 4B). The percentage of GFP+ cells reduced more
328 than half in Day 6 when the cells reach more than 90% confluency (Figure 4C).

329 After nucleofection, some abnormal character of fibroblasts could be detected. Different
330 degree of multinucleated cells appeared in the nucleofected culture within 24 hours. Among the
331 best two Nucleofector™ programs (EN-150 and FF-135) described earlier, FF-135 exhibited
332 higher degree of multinucleation (Figure 5A). However, this multinucleation could be eliminatied
333 by one-time subculture of FF-135 transfected condition (Figure 5B). In addition, to improve
334 survivor of cells after nucleofection, we treated transfected cells with RevitaCell™ Supplement
335 (RC), which contains ROCK inhibitor comparable to Y-27632 and Thiazovivin and found that
336 addition of 0.5X and 1.0X RC improved survived cells in similar manner, which two-fold higher
337 than non-treated transfected cells (Figure 5C).

338

339 **Challenge to obtain pluripotent stem cells for future conservation**

340 Reprogramming of fishing cat fibroblasts was aimed to enhance cell propagation ability
341 via the induction of pluripotent state. At first, we induced fishing cat fibroblasts with piggyBAC
342 transposon-transposase system by applying nucleofection strategy in previous section with mouse
343 reprogramming factors and mOrange as a reporter (Figure 6A). We can confirm the presence of
344 transfected cells with mOrange expression (Figure 6B) although the iPSC colonies cannot be
345 formed with this approach. In the second strategy of piggyBAC transposon-based reprogramming
346 on fishing cat testicular fibroblasts, we used Tet-on expression system of reprogramming factors
347 and cell selection using puromycin (Figure 6C). The expression of TdTomato allowed us to
348 monitor the expression of reprogramming factors. We found that with this approach iPSC-like
349 colonies with TdTomato expression emerged (Figure 6D). However, TdTomato-expressing
350 colonies was not expandable in iPSC medium with human LIF. We also applied Episomal-based
351 reprogramming using human reprogramming factors (Figure 6E). Episomal vectors-transfected
352 cells were treated with various conditions (Figure 6E). With this approach, fishing cat cells can be
353 expanded the most in FBS-based medium and with Geltrex (Figure 6F). The fibroblastic characters
354 were lost in Geltrex condition, becoming more epithelial characters (Figure 6F), but not in mouse-

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355 based feeder cells (Figure 6F). After prolonged culture until the third week of reprogramming,
356 iPSC-like colonies appeared in Geltrex (Figure 6F) but retained flatted epithelial like cells in
357 vitronectin (Figure 6F). However, the picked colonies from Geltrex cannot be propagated. Lastly,
358 we tested whether RNA based reprogramming can induce fishing cat cells based on Yoshioka et
359 al., 2013 and Yoshioka and Dowdy, 2017 [33-34]. We used self-replicating RNA expressing
360 OCT4, SOX2, KLF4, CMYC and GLIS1 proteins to induce the formation of iPSC colonies but
361 the same problems occurred from unexpandable clones (Figure 6G-H).
362 Thus, inducing and capturing pluripotency of fishing cats is still a main challenge.
363 However, with these reprogramming approaches and adjusted culture strategy, the fishing cat
364 fibroblasts can be reprogrammed toward expandable intermediate cells, in particular with episomal
365 vector based reprogramming (Figure 6), beyond the limitation of fibroblast passaging that we can
366 keep in cryopreservation for future reprogramming success.
367

368 **Discussion**

369 Fishing cat cell biobanking is currently in need of preserving its genetic back-up. Here we
370 reported the progress of our cryopreservation of fishing cats from several sources (summarized in
371 Table 1) and provide examples and challenges of using derived adult somatic cells for cellular
372 reprogramming. Embryonic or fetal fibroblasts are common sources for downstream applications
373 as the fibroblasts from embryos exhibited better regeneration process and wound/tendon healing
374 than adult fibroblasts [35-36]. However, embryo sources from wildlife are hard to be obtained.
375 Thus, adult cells are almost only option. In this study, the adult cells can be derived from tissues
376 of both living and postmortem fishing cats but various sources contributed differently, summarized
377 in Figure 7. From the living source, small pieces of ear pinna provided the highest number of
378 fibroblasts for cryopreservation. In contrast, the ear pinna from the postmortem animal contributed
379 to the fibroblast derivation the least. Testis collection from the postmortem fishing cat, on the other
380 hand, provided sufficient cryopreserved sources of cells including fibroblasts from testicular wall
381 and epididymis and mixed cell populations containing SOX2-positive SSCs from seminiferous
382 tubules. Thus, here we recommend collecting testes for cell culture from freshly dead wild animals
383 will provide a good source for biobanking. Although it was not unexpected that keratinocytes
384 appeared from the explant culture of ear pinna carrying intact epidermis, keratinocytes can be
385 eliminated after continuing culture in the medium of DMEM-high glucose with FBS as appeared

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386 in Vangipuram et al., 2013 for human fibroblast derivation and Siengdee et al., 2018 for Asian
387 elephant fibroblast derivation [20,37]. In addition to medium types, the procedure of cell culture
388 and supplementation also affected to the culture [20,37]. Either prolonged culture or more
389 passaging of fibroblasts of fishing cats leads to cell senescence and fibroblast is no longer usable
390 for other applications. The obstacles can be relieved by addition of bFGF, a common cytokine
391 known for its ability to support human fibroblast cell proliferation in dose-dependent manner
392 through ERK1/2 and JNK pathways [38], which enhanced cell proliferation in our culture. Hence,
393 frozen stock of early fibroblasts should be made as many as possible, which is also limited to the
394 amount of tissues that can be harvested.

395 In this study, we also show the presence of living SOX2+ spermatogonial cells from
396 postmortem fishing cat. It has been shown that some population of Sox2+ cells, representing a
397 type of adult stem cells in mice, can repopulate the testes with ablated spermatogenesis and restore
398 spermatogenesis [39]. Thus, the presence of SOX2+ cells in extracted seminiferous tubules from
399 the fishing cat open the possibility of adult stem cell derivation and maintenance or even reprogram
400 back to more potential stem cells. Other active adult stem cells in the freshly postmortem animals
401 might be present, which further investigation could help preserving more sources of postmortem
402 wild animals in captivity.

403 To deliver genetic material to fishing cat adult cells (e.g. for reprogramming) is another
404 challenge due to nature of hard-to-transfect cell type with low division rate compared to embryonic
405 sources. Nucleofection, an electroporation-based transfection method enabling direct DNA
406 delivery into nucleus, can solve the problem as we showed varied range of transfection efficiency
407 from different Nucleofector programs can indeed deliver DNA into the fishing cat cells. Similarly,
408 this technique has been shown to be one of the most suitable non-viral transfection methods to
409 deliver DNA into dermal fibroblasts in various species including human, rat and mouse [28,30-
410 31,40-42].

411 As the fibroblasts have limited capacity to expand, the preserved cells for better usability
412 is through the reprogramming of the fibroblasts to iPSCs, which have high cell potency to self-
413 renewal and differentiate to all types [43-44]. Felid reprogramming has already been done in some
414 wild cats and domestic cats with retrovirus/lentivirus-based approaches [24,27,45]. Since then
415 there is no success of non-integration approach of wild felid reprogramming. In this study, we
416 examined mouse/human reprogramming system to induce the fishing cat cells without using virus

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417 to deliver reprogramming factors. The iPSC colonies appeared from episomal vectors with human
418 OCT4, SOX2, LMYC, KLF4 and LIN28 [46] and piggyBAC transposon with human OCT4,
419 SOX2, KLF4, CMYC, KLF2 and NANOG [32]. However, putative iPSC clones cannot be
420 expanded under mouse iPSC (LIF) or human iPSC maintenance (bFGF) conditions; thus, keeping
421 pluripotent states or inducing the fully reprogrammed state of fishing cat is still a challenge. But
422 the reprogramming to partial state with these reprogramming factors also improved the expandable
423 capacity of the fishing cat cells, contributing more cryopreserved cells for biobanking.
424
425 **Conclusion**
426 Generation of biobanking to preserve genetics of fishing cats is limited due to availability
427 of obtaining samples from nature and captivity, fibroblast potency, delivery of genetic material to
428 hard-to-transfect cells and achieving fully reprogramming state. Nevertheless, we succeed in
429 preserving somatic cells from both living and postmortem fishing cats for future conservation
430 technology to prevent the extinction of fishing cats.
431
432 **References**
433 [1] Cutter P, Cutter PG. Recent sightings of fishing cats in Thailand. Cat News. 2010;51:26–7.
434 [2] Lynam AJ, Jenks KE, Tantipisanuh N, Chutipong W, Ngoprasert D, Gale GA, et al. Terrestrial
435 activity patterns of wild cats from camera-trapping. The Raffles bulletin of zoology. 2013;61:407-
436 15.
437 [3] Tantipisanuh N, Chutipong W, Ngoprasert D, Lynam AJ, Steinmetz R, Sukmasuang R, et al.
438 Recent distribution records, threats and conservation prio- rities of small cats in Thailand. Cat
439 News. 2014;8:36-44.
440 [4] Ash E, Kaszta Ż, Noochdumrong A, Redford T, Chanteap P, Hallam C, et al. Opportunity for
441 Thailand's forgotten tigers: assessment of the Indochinese tiger Panthera tigris corbetti and its prey
442 with camera-trap surveys. Oryx. Cambridge University Press. 2021;55(2):204–211.
443 [5] Ash E, Hallam C, Chanteap P, Kaszta Ż, Macdonald D, Rojanachinda W, et al. Estimating the
444 density of a globally important tiger (Panthera tigris) population: Using simulations to evaluate
445 survey design in Eastern Thailand. Biological Conservation. 2020;241.

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446 [6] Phumanee W, Steinmetz R, Phoonjampa R, Bejraburnin T, Bhumpakphan N, Savini T.
447 Coexistence of large carnivore species in relation to their major prey in Thailand. Global ecology
448 and conservation. 2021;32.
449 [7] Dugan P. Wetlands in danger: a world conservation atlas. New York: Oxford Univ. Press;
450 1993.
451 [8] Nowell K, Jackson P. Wild Cats. Status Survey and Conservation Action Plan. Switzerland:
452 IUCN; 1996.
453 [9] Parr JWK, Mahannop N, Charoensiri V. Khao Sam Roi Yot – one of the world's most
454 threatened parks. Oryx. 1993;27:245–249.
455 [10] Mukherjee S, Adhya T, Thatte P, Ramakrishnan U. Survey of the Fishing Cat Prionailurus
456 viverrinus Bennett, 1833 (Carnivora: Felidae) and some aspects impacting its conservation in
457 India. Journal of Threatened Taxa. 2012;4(14):3355-61.
458 [11] Pope CE, Gomez MC, Dresser BL. In vitro embryo production and embryo transfer in
459 domestic and non-domestic cats. Theriogenology. 2006;66(6-7):1518-24.
460 [12] Swanson A, Strawn E, Lau E, Bick D. Preimplantation genetic diagnosis: technology and
461 clinical applications. WMJ. 2007;106(3):145-51.
462 [13] Thiangtum K, Swanson WF, Howard J, Tunwattana W, Tongthainan D, Wichasilpa W,
463 Patumrattanathan P, Pinyopoommintr T. Assessment of basic seminal characteristics, sperm
464 cryopreservation and heterologous in vitro fertilisation in the fishing cat (Prionailurus viverrinus).
465 Reprod Fertil Dev. 2006;18(3):373-82.
466 [14] Santymire RM, Brown JL, Stewart RA, Santymire RC, Wildt DE, Howard J. Reproductive
467 gonadal steroidogenic activity in the fishing cat (Prionailurus viverrinus) assessed by fecal steroid
468 analyses. Anim Reprod Sci. 2011;128(1-4):60-72.
469 [15] Thongphakdee A, Tipkantha W, Punkong C, Chatdarong K. Monitoring and controlling
470 ovarian activity in wild felids. Theriogenology. 2018;109:14-21.
471 [16] Pope CE, Keller GL, Dresser BL. In vitro fertilization in domestic and non-domestic cats
472 including sequences of early nuclear events, development in vitro, cryopreservation and successful
473 intra- and interspecies embryo transfer. J Reprod Fertil Suppl. 1993;47:189-201.
474 [17] Doke SK, Dhawale SC. Alternatives to animal testing: A review. Saudi Pharm J.
475 2015;23(3):223-9.

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476 [18] Mestre-Citrinovitz AC, Sestelo AJ, Ceballos MB, Barañao JL, Saragüeta P. Isolation of
477 Primary Fibroblast Culture from Wildlife: the Panthera onca Case to Preserve a South American
478 Endangered Species. Curr Protoc Mol Biol. 2016;116:28.7.1-28.7.14.
479 [19] Praxedes ÉA, Borges AA, Santos MVO, Pereira AF. Use of somatic cell banks in the
480 conservation of wild felids. Zoo Biol. 2018.
481 [20] Siengdee P, Klinhom S, Thitaram C, Nganvongpanit K. Isolation and culture of primary adult
482 skin fibroblasts from the Asian elephant (Elephas maximus). PeerJ. 2018;6:e4302.
483 [21] Ben-Nun IF, Montague SC, Houck ML, Tran HT, Garitaonandia I, Leonardo TR, Wang YC,
484 Charter SJ, Laurent LC, Ryder OA, Loring JF. Induced pluripotent stem cells from highly
485 endangered species. Nat Methods. 2011;8(10):829-31.
486 [22] Gonçalves NJN, Bressan FF, Roballo KCS, Meirelles FV, Xavier PLP, Fukumasu H, et al.
487 Generation of LIF-independent induced pluripotent stem cells from canine fetal fibroblasts.
488 Theriogenology. 2017;92:75-82.
489 [23] Nagy K, Sung HK, Zhang P, Laflamme S, Vincent P, Agha-Mohammadi S, et al. Induced
490 pluripotent stem cell lines derived from equine fibroblasts. Stem Cell Rev Rep. 2011;7(3):693-
491 702.
492 [24] Dutton LC, Dudhia J, Guest DJ, Connolly DJ. Inducing Pluripotency in the Domestic Cat
493 (Felis catus). Stem Cells Dev. 2019;28(19):1299-1309.
494 [25] Serrano MA, Gomez MC, Lopez M, Dumas CL, Smith KE, Leibo SP, et al. Derivation of cat
495 embryonic stem-like cells from in vitro-produced blastocysts and their support by intraspecific vs.
496 interspecific feeder cells. Reprod FertilDev. 2006;18:210–211.
497 [26] Verma R, Holland MK, Temple-Smith P, Verma PJ. Inducing pluripotency in somatic cells
498 from the snow leopard (Panthera uncia), an endangered felid. Theriogenology. 2012;77(1):220-8,
499 228.e1-2.
500 [27] Verma R, Liu J, Holland MK, Temple-Smith P, Williamson M, Verma PJ. Nanog is an
501 essential factor for induction of pluripotency in somatic cells from endangered felids. Biores Open
502 Access. 2013;2(1):72-6.
503 [28] Chen Y, Liu S, Shen Q, Zha X, Zheng H, Yang L, et al. Differential gene expression profiles
504 of PPP2R5C-siRNA-treated malignant T cells. DNA Cell Biol. 2013;32(10):573-81.

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505 [29] Chen Y, Pitzer AL, Li X, Li PL, Wang L, Zhang Y. Instigation of endothelial Nlrp3
506 inflammasome by adipokine visfatin promotes inter-endothelial junction disruption: role of
507 HMGB1. J Cell Mol Med. 2015;19(12):2715-27.
508 [30] Lin M, Pedrosa E, Hrabovsky A, Chen J, Puliafito BR, Gilbert SR, et al. Integrative
509 transcriptome network analysis of iPSC-derived neurons from schizophrenia and schizoaffective
510 disorder patients with 22q11.2 deletion. BMC Syst Biol. 2016;10(1):105.
511 [31] Reichman S, Terray A, Slembrouck A, Nanteau C, Orieux G, Habeler W, et al. From confluent
512 human iPS cells to self-forming neural retina and retinal pigmented epithelium. Proc Natl Acad
513 Sci. 2014;111(23):8518–8523.
514 [32] Kisa F, Shiozawa S, Oda K, Yoshimatsu S, Nakamura M, Koya I, et al. Naive-like ESRRB+
515 iPSCs with the Capacity for Rapid Neural Differentiation. Stem Cell Reports. 2017;9(6):1825-
516 1838.
517 [33] Yoshioka N, Dowdy SF. Enhanced generation of iPSCs from older adult human cells by a
518 synthetic five-factor self-replicative RNA. PLoS One. 2017;12(7):e0182018.
519 [34] Yoshioka N, Gros E, Li HR, Kumar S, Deacon DC, Maron C, et al. Efficient generation of
520 human iPSCs by a synthetic self-replicative RNA. Cell Stem Cell. 2013;13(2):246-54.
521 [35] Kishi K, Okabe K, Shimizu R, Kubota Y. Fetal skin possesses the ability to regenerate
522 completely: complete regeneration of skin. Keio J Med. 2012;61(4):101-8.
523 [36] Tang QM, Chen JL, Shen WL, Yin Z, Liu HH, Fang Z, et al. Fetal and adult fibroblasts display
524 intrinsic differences in tendon tissue engineering and regeneration. Sci Rep. 2014;4:5515.
525 [37] Vangipuram M, Ting D, Kim S, Diaz R, Schüle B. Skin punch biopsy explant culture for
526 derivation of primary human fibroblasts. J Vis Exp. 2013;(77):e3779.
527 [38] Yun YR, Won JE, Jeon E, Lee S, Kang W, Jo H, et al. Fibroblast growth factors: biology,
528 function, and application for tissue regeneration. J Tissue Eng. 2010;2010:218142.
529 [39] Arnold K, Sarkar A, Yram MA, Polo JM, Bronson R, Sengupta S, et al. Sox2(+) adult stem
530 and progenitor cells are important for tissue regeneration and survival of mice. Cell Stem Cell.
531 2011;9(4):317-29.
532 [40] Bang JS, Choi NY, Lee M, Ko K, Lee HJ, Park YS, et al. Optimization of episomal
533 reprogramming for generation of human induced pluripotent stem cells from fibroblasts. Anim
534 Cells Syst (Seoul). 2018;22(2):132-139.

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535 [41] Pająk B, Kania E, Orzechowski A. Nucleofection of Rat Pheochromocytoma PC-12 Cells
536 with Human Mutated Beta-Amyloid Precursor Protein Gene (APP-sw) Leads to Reduced
537 Viability, Autophagy-Like Process, and Increased Expression and Secretion of Beta Amyloid.
538 Biomed Res. Int. 2015; 746092.
539 [42] Zhang Z, Slobodianski A, Arnold A, Nehlsen J, Hopfner U, Schilling AF, et al. High
540 Efficiency Low Cost Fibroblast Nucleofection for GMP Compatible Cell-based Gene Therapy. Int
541 J Med Sci. 2017;14(9):798-803.
542 [43] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and
543 adult fibroblast cultures by defined factors. Cell. 2006 Aug 25;126(4):663-76.
544 [44] Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of
545 pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;131(5):861-72.
546 [45] Zhou R, Comizzoli P, Keefer CL. Endogenous pluripotent factor expression after
547 reprogramming cat fetal fibroblasts using inducible transcription factors. Mol Reprod Dev.
548 2019;86(11):1671-1681.
549 [46] Okita K, Matsumura Y, Sato Y, Okada A, Morizane A, Okamoto S, Hong H, Nakagawa M,
550 Tanabe K, Tezuka K, Shibata T, Kunisada T, Takahashi M, Takahashi J, Saji H, Yamanaka S. A
551 more efficient method to generate integration-free human iPS cells. Nat Methods. 2011;8(5):409-
552 12.

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579 **Table 1** Primary culture of fishing cat tissues from different sources

Sources for tissue collection	Sex	Estimated age	Live or Postmortem animals	Days of the first appearance of fibroblast outgrowth	Number of re-explant	Number of vials for cryopreservation
Natural resources						
Ear pinna	Male	Young adult	Live	2-3 days	3-4 times	19
Ear pinna	Female	Young adult	Live	2-3 days	3-4 times	11
Captivity						
Abdominal tissues (AI)	Female	Adult	Live	15 days	0 time	7
Abdominal tissues (AI)	Male	Adult	Live	13 days	0 time	3
Ear pinna	Female	Adult	Postmortem	13 days	0 time	0
Ear pinna	Female	Baby	Postmortem	5 days	0 time	2
Testis	Male	Adult	Postmortem	3 days	2 times	5

580

581

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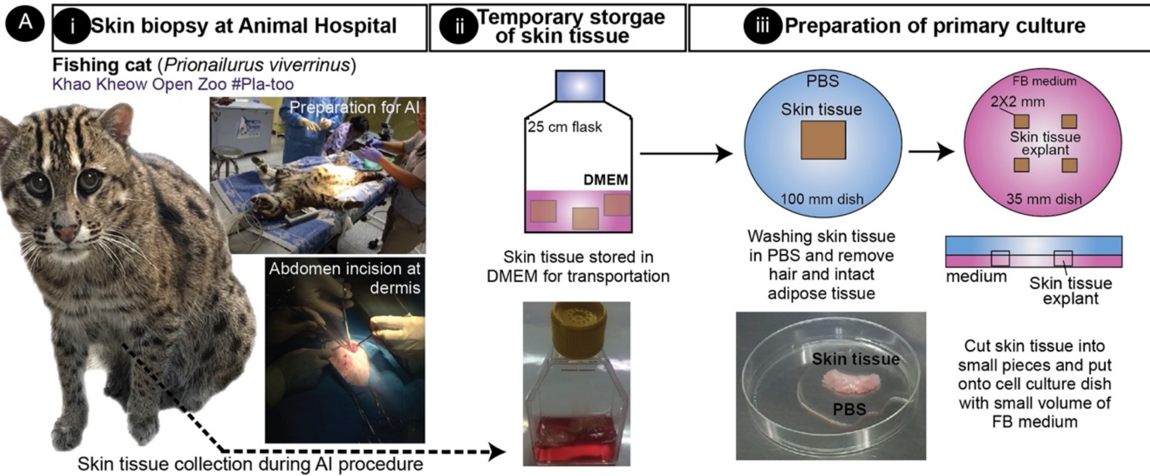
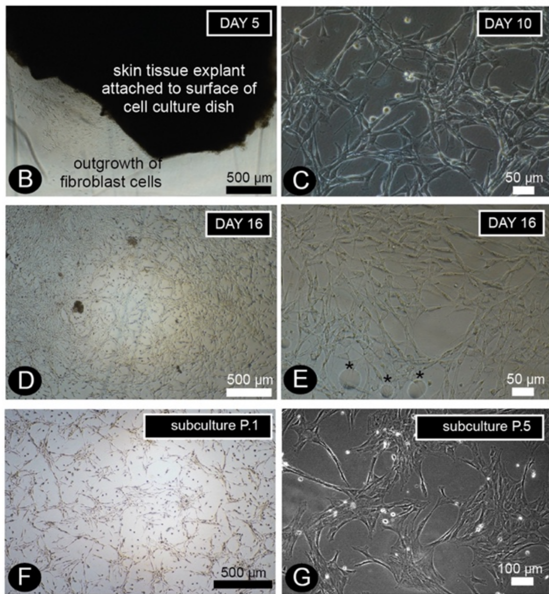
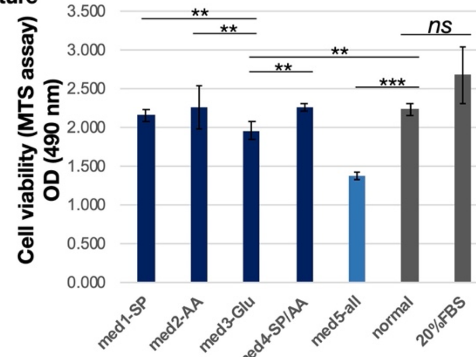
582 **Figures**



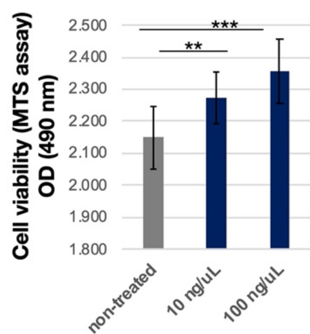


H **Type of medium for fishing cat fibroblast culture**

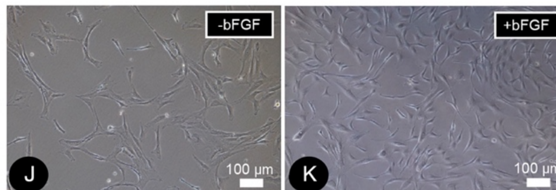


	med1-SP	med2-AA	med3-Glu	med4-SP/AA	med5-all	normal	20%FBS
DMEM (high-glucose)	+	+	+	+	+	+	+
Sodium pyruvate	-	+	+	-	-	+	-
Non-essential amino acid	+	-	+	-	-	+	-
Glutamax	+	+	-	+	-	+	+
Fetal bovine serum (%)	10	10	10	10	10	10	20
Pen/Strep	+	+	+	+	+	+	+
Amphotericin B	-			-	-	-	+

I **bFGF treatment**



Basic Fibroblast Growth Factor (bFGF) treatment



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584 **Figure 1. Primary culture of fishing cat cells and conditions supporting fishing cat cell**
585 **expansion**
586 A) Tissue collection and preparation for primary culture i) Skin biopsy was performed by
587 veterinarians during artificial insemination of fishing cat. ii) The skin tissues were then transported
588 to Tissue Culture Facility in DMEM or complete fibroblast (FB) medium with antibiotics. iii) The
589 skin tissues were washed with PBS and dissected into small pieces and the tissue explants were
590 cultured in FB medium. B) The adult fibroblasts were expanded from skin tissue explant as early
591 as day 5. C) Primary culture shows morphology of fishing cat fibroblast at day 10 D) Fibroblasts
592 expanded and reached 80% confluency within 16 days. E) Primary culture contained some oil
593 droplets (*) due to high subcutaneous fat connected to the dermis layer. F)-G) Culture of the adult
594 dermal fibroblasts after subculture from the primary culture. H) Adult dermal fibroblasts from
595 fishing cat at passage 2-4 were cultured in different medium conditions. Bar graph shows cell
596 viability of fibroblasts under different medium conditions at day 7 post treatment. The cell viability
597 was measured by MTS assay using spectrophotometer (at 490 nm). I) The fibroblasts were treated
598 with basic fibroblast growth factor (bFGF) at final concentration 10 ng/μL and 100 ng/μL. Bar
599 graph shows MTS assay-based cell viability of fibroblasts at day 4 post bFGF treatment. J)
600 Brightfield photograph shows morphology of fibroblasts without bFGF. K) Brightfield photograph
601 shows morphology of fibroblasts with bFGF at day 2 post treatment. Asterisks (** and ***)
602 indicate significant difference ($p<0.05$ and $p<0.01$ respectively, Student's t test) and "ns" indicates
603 not significant ($p<0.05$).
604

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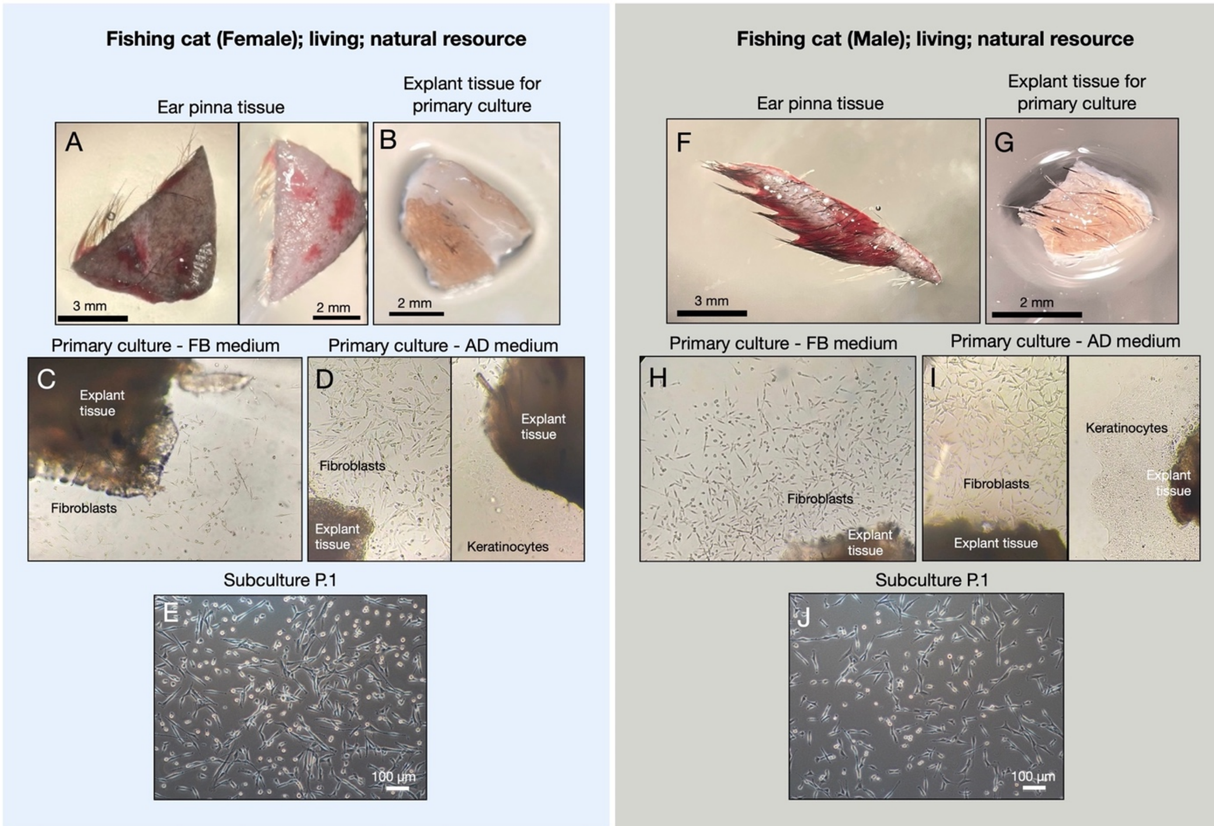

605
606 **Figure 2 Primary culture of fishing cat tissues collected from natural resource.** Ear pinna
607 tissues from female fishing cat (A) and male fishing cat (F) were collected and prepared for
608 primary culture. The collected tissues were washed and dissected into small pieces (explant) before
609 attaching on gelatin-coated culture dishes (B, G). Th explants were cultured in complete fibroblast
610 (FB) medium and Advanced DMEM (AD) medium (C-D, H-I). Fibroblast outgrowth was found
611 in both FB and AD medium (C-D, H-I) while Keratinocyte outgrowth was found only in AD
612 medium (D, I). E, J) Subculture at passage 1 (P.1) after primary culture reaching 90% confluency.
613

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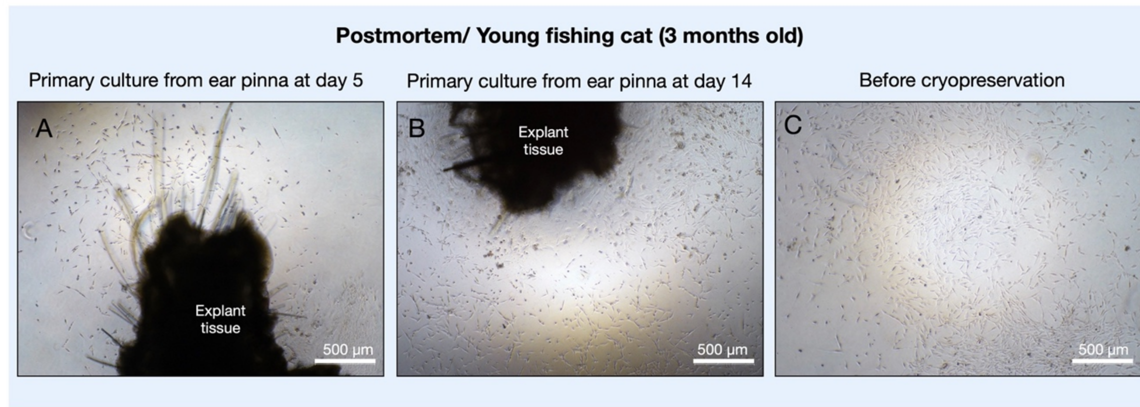

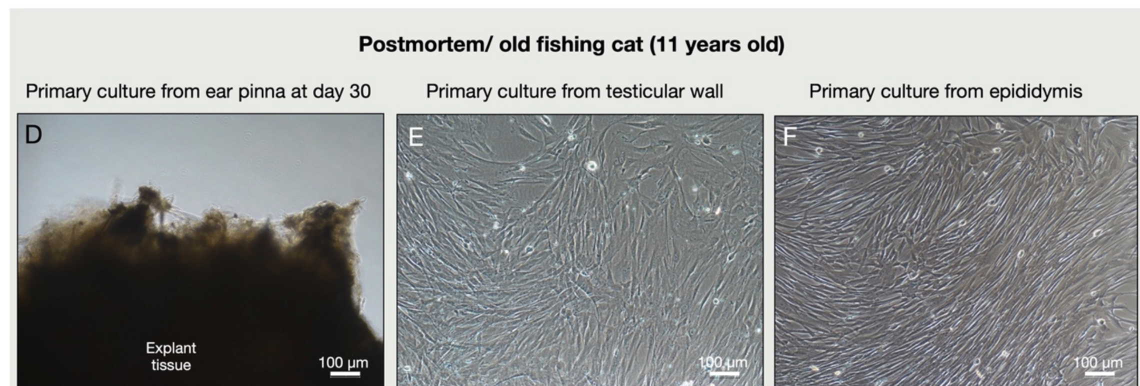

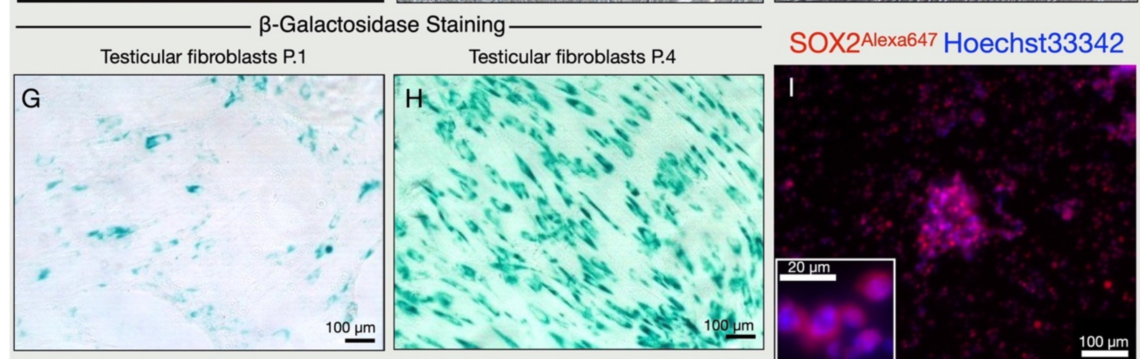

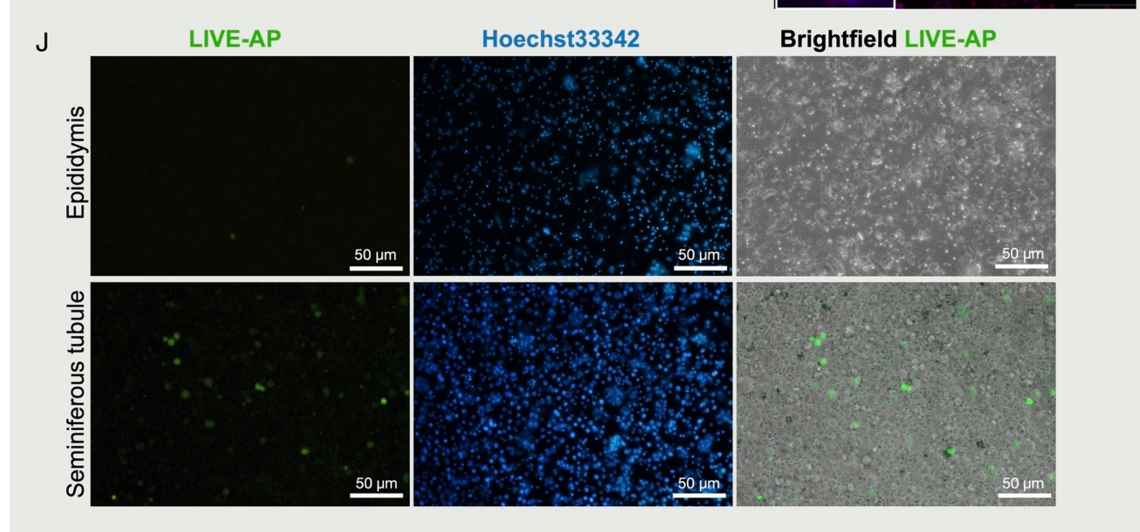

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615 **Figure 3 Primary culture of postmortem fishing cats** A-C) Primary culture of tissues collected
616 from young fishing cat at 3 months old. D-J) Primary culture of tissues collected old fishing cat at
617 11 years old. D) No cell expanded from ear pinna explant in primary culture at day 30. E)
618 Fibroblast outgrowth from testicular wall explants. F) Fibroblast outgrowth from epididymis
619 explants. G-H) Fibroblasts from the testes at passage 1 and passage 4 were tested for cell
620 senescence. I) Immunostaining of seminiferous tubule extract to detect SOX2 protein (Red,
621 Alexa647) with nuclear staining Hoechst33342. Inset shows zoom-up of SOX2 positive cells. J).
622 Live alkaline phosphatase staining was used to detect the presence of putative spermatogonial stem
623 cells (SSCs) from seminiferous tubules and epididymis.
624

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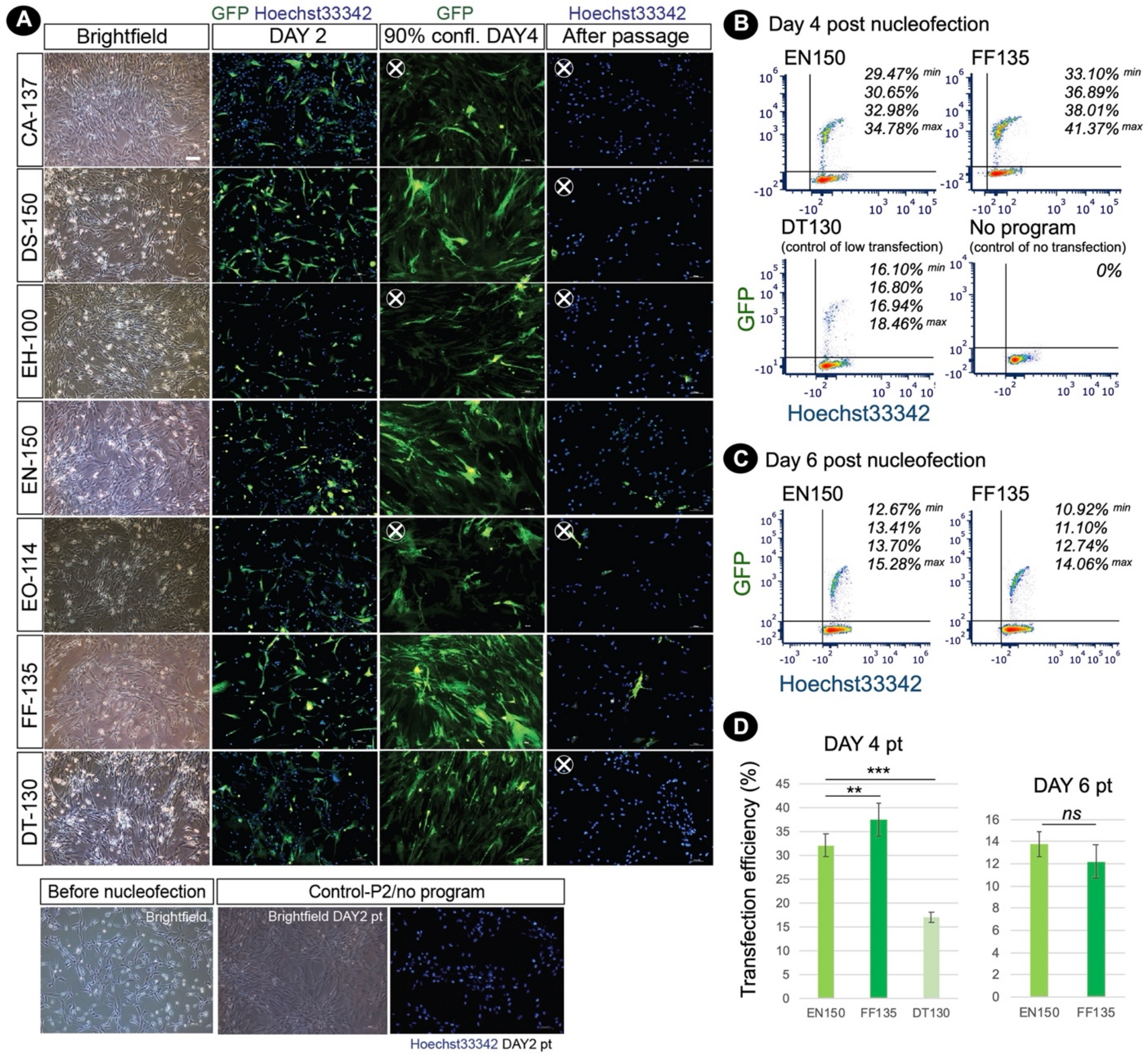

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627 **Figure 4 Nucleofection-based DNA delivery into adult dermal fibroblasts from fishing cat.**
628 A) Fluorescence photographs show the fibroblasts transfected with pmaxGFP expressing GFP by
629 using nucleofection method. Seven different nucleofection programs (CA-137, DS-150, EH-100,
630 EN-150, EO-114, FF-135 and DT-130) were examined. Photos were taken at day 2, day 4 (90%
631 confluency) and day 6 (day 2 after subculture) post nucleofection. Nuclei were counterstained with
632 Hoechst33342 (Blue). At day 4 post nucleofection, CA-137, EN-150 and EO-114 conditions
633 contained a smaller amount of GFP+ cells and were removed for further analysis (X). After
634 passaging, DS-150 and DT-130 condition shows a smaller number of GFP+ cells and were
635 removed for further analysis (X). B) Cells transfected with the best two nucleofection programs:
636 EN-150 and FF-135 were analyzed by flow cytometer to quantify transfection efficiency. The
637 representative contour plots show the percentage of double GFP+ and Hoechst33342+ cell subsets
638 (4 replicates) at day 4 post transfection. DT-130 program was used as a control of low transfection
639 and "No program" indicates a condition of no nucleofection in the presence of P2 solution and
640 pmaxGFP DNA. C) as in B), the contour plots show the percentage of double GFP+ and
641 Hoechst33342+ cell subsets (4 replicates) at day 6 post transfection or day 2 after subculture. D)
642 Graph shows transfection efficiency of nucleofection (mean ± standard deviation) at day 4 and day
643 6 post transfection calculated from percentage of double GFP+ and Hoechst33342+ cell subsets
644 (n=4, as shown in B). Asterisks (** and ***) indicate significant difference ($p<0.05$ and $p<0.01$
645 respectively, Student's t test) and "ns" indicates not significant ($p<0.05$). Abbreviation: pt, post
646 transfection; confl, confluency.

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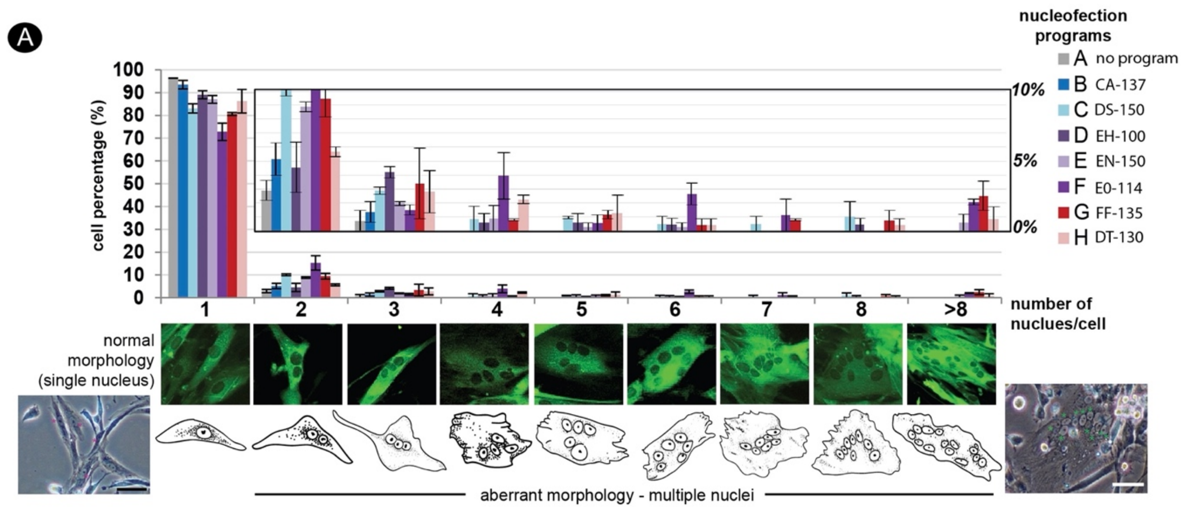

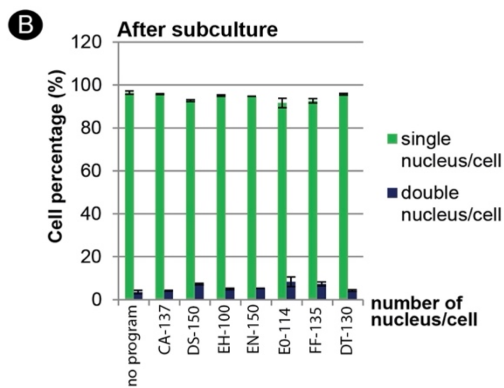

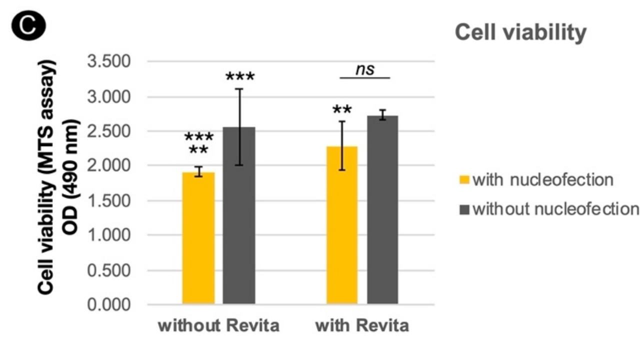

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649 **Figure 5 Effect of nucleofection on multinucleation and cell viability of adult dermal**
650 **fibroblast derived from fishing cat.** A) Multinucleation occurred in different levels from various
651 Nucleofector^{TM} programs. Top panel shows a graph depicting incidence of multinucleation from
652 different Nucleofector^{TM} programs. Y axis value represents the percent of transfected cells with
653 one or more nuclei per cell (mean ± standard deviation). X axis indicates the number of nucleus
654 per cell. Middle panel shows morphology of GFP expressing cells with different types of
655 multinucleation. Bottom panel shows brightfield images and cartoons of fibroblast cells with a
656 single nucleus and multiple nuclei. B) Graph depicts number of nucleus per cell as in A) from
657 subculture of the transfected cells at day 3 post passaging or day 7 post transfection. Subculture of
658 transfected cells removed multinucleation from all examined programs. C) Graph shows cell
659 viability of fibroblast cells after nucleofection with program FF-135 with or without RevitaCellTM
660 supplement. Cell viability was done by MTS assay and quantified with spectrophotometer at 490
661 nm as shown in Y axis. Mean indicates the average of absorbance values from four independent
662 experiments with three technical replicates each. Error bar indicates standard deviation. Asterisks
663 (** and ***) indicate significant difference ($p<0.05$ and $p<0.01$ respectively, Student's t test) and
664 "ns" indicates not significant ($p<0.05$).
665

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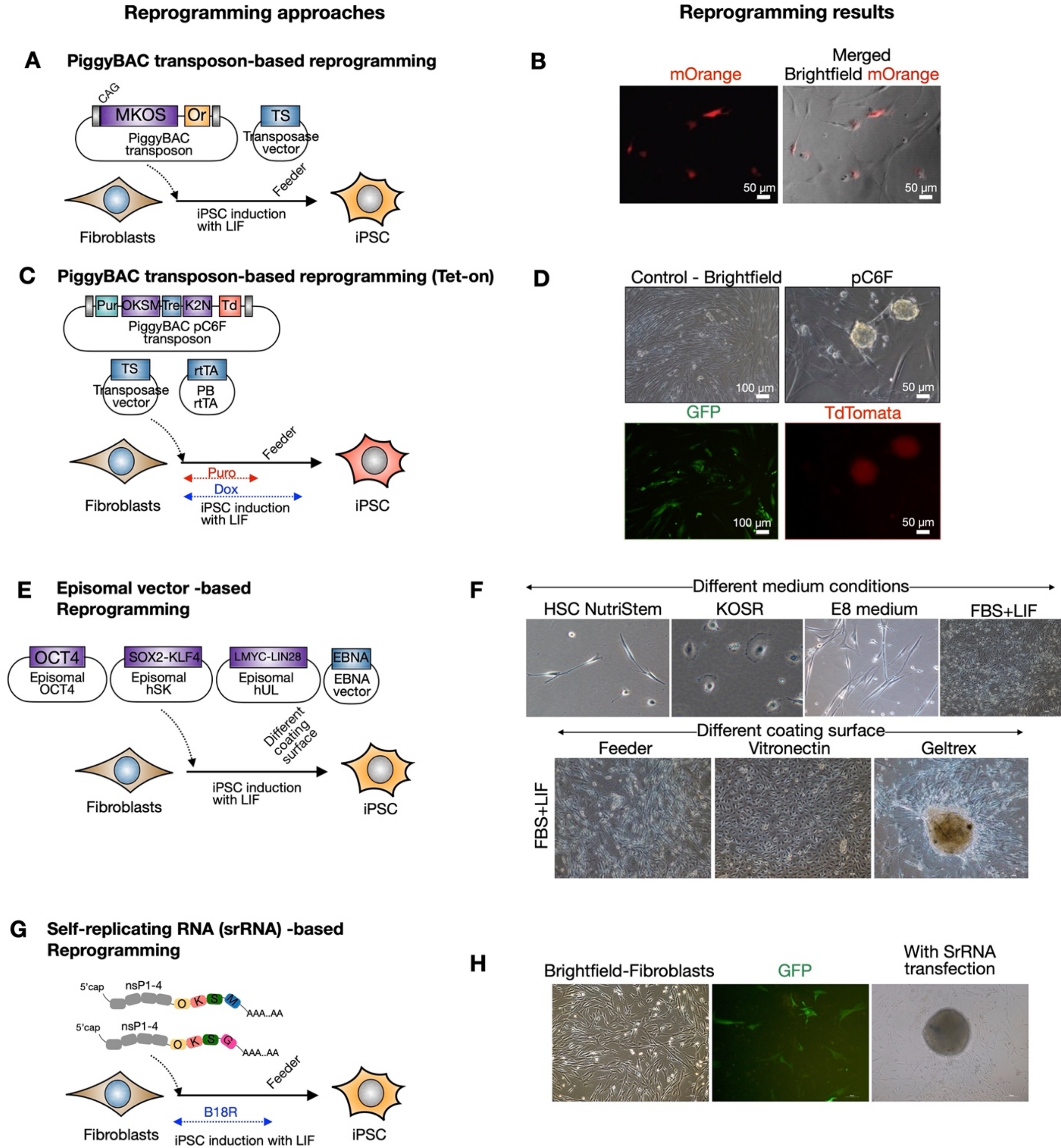

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668 **Figure 6 Cellular reprogramming of fishing cat cells via different virus-free approaches.** A-
669 B) Fishing cat fibroblasts were reprogrammed with PiggyBAC transposon carrying mouse C-myc,
670 Klf4, Oct4 and Sox2 (MKOS) and mOrange (Or) as a reporter, together with an expression vector
671 expressing transposase (TS). The fibroblasts expressed mOrange, indicating the successful
672 integration of piggyBAC transposon. C-D) The fibroblasts were reprogrammed with piggyBAC
673 transposon carrying inducible reprogramming cassette composed of human OCT4, KLF4, SOX2,
674 C-Myc (OKSM) and human KLF2 and NANOG followed by Ires-Tdtomato. The transfection
675 included transposase vector and piggyBAC expressing rtTA. The transfected cells were selected
676 with puromycin and the expression of reprogramming factors were induced via Tet-on using
677 Doxycycline. The iPSC-like colonies appeared with TdTomata expression. E-F) Reprogramming
678 of fibroblasts using episomal vectors. The transfected cells were tested in various media including
679 commercial HSC NutriStem and E8 medium, KOSR containing medium (KOSR) and media with
680 10% Fetal Bovine Serum (FBS) with LIF. The induced cells were also replated onto different
681 matrix including mouse irradiated feeder, vitronectin and Geltrex. G-H) RNA-based
682 reprogramming using self-replicating RNA (srRNA) expressing reprogramming factors. The
683 testicular fibroblasts were transfected with srRNA and cultured in the presence of B18R protein
684 before replating onto feeder cells. The iPSC-like colonies appeared in three weeks after
685 transfection.
686

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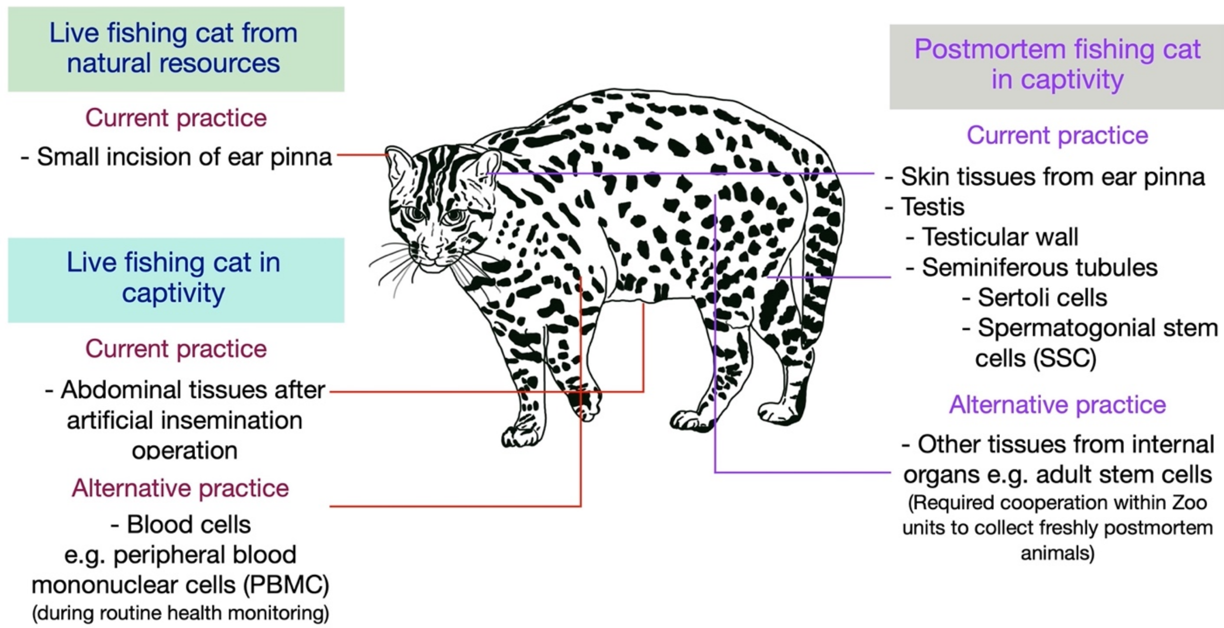

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688 **Figure 7 Tissue collection from fishing cat for conservation.** Schematic illustration showing
689 various sources of tissue collection for primary cell culture for cryopreservation in this study and
690 suggesting the alternative sources for primary cells from the fishing cats.