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DETERMINING INFLUENCE OF CULTURE MEDIA AND DOSE-DEPENDENT SUPPLEMENTATION WITH BASIC FIBROBLAST GROWTH FACTOR ON THE *EX VIVO* PROLIFERATIVE ACTIVITY OF DOMESTIC CAT DERMAL FIBROBLASTS IN TERMS OF THEIR SUITABILITY FOR CELL BANKING AND SOMATIC CELL CLONING OF FELIDS*

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Abstract

Dermal fibroblasts are commonly used as donors of genetic material for somatic cell nuclear transfer in mammals. Basic fibroblast growth factor (bFGF) is a cytokine that regulates proliferation and differentiation of different cell types. The study was aimed at optimizing the cell culture protocol for cat dermal fibroblasts by assessing the influence of culture media and different doses of bFGF on proliferation of fibroblasts and their viability in terms of cell banking and somatic cloning of felids. In Experiment I, skin biopsies of domestic cats were cultured in DMEM (D) and/or DMEM/F12 (F), both supplemented with 5 ng bFGF/ml (D-5, F-5, respectively). After the primary culture reached ~80% of confluency, the cells were passaged (3–4 times) and cultured in media with (D-5, F-5) or without (D-0, F-0) bFGF. To determine the optimal doses of bFGF, in Experiment II, secondary fibroblasts were cultured in DMEM with 0 (D-0), 2.5 (D-2.5), 5 (D-5) or 10 (D-10) ng bFGF/ml. The results showed that in D-5 the cells proliferated faster than in D-0, F-5 and F-0. Due to their poor proliferation, passages IV were not performed for cells cultured in F-0. In experiment II, a dose-dependent effect of bFGF on proliferation of cat dermal fibroblasts was found. In D-5 and D-10, the cells exhibited higher ($P<0.05$) proliferation compared with D-0. In D-2.5 the cells showed a tendency to proliferate slower than in D-5 and D-10 and at the same faster than in D-0. In conclusion. DMEM supplemented with bFGF provides better proliferation of domestic cat dermal fibroblasts culture than DMEM/F12. Supplementation of culture medium with bFGF has a beneficial effect on cat dermal fibroblast proliferation and could be recommended for addition to culture media.

Key words: cat, skin, fibroblasts, cell culture, basic fibroblast growth factor

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Establishment and banking of somatic cell lines contributes to the conservation of animal genetic resources, especially those threatened with extinction. Cryobanked cells can be used for somatic cell nuclear transfer (SCNT) many years after a genetically-valuable animal is dead. Banked cells constitute an excellent source of material for conducting diverse fundamental as well as applied research studies. It is worth noting that germ cell lines and even gametes were generated from induced pluripotent stem cells (iPSCs) that had been produced by epigenetic reprogramming (i.e., molecular dedifferentiation) of fetal or adult fibroblast cells (Zhu et al., 2012; Ishii, 2014). Results of these experiments raise hope and create opportunities for utilizing somatic cells in embryo production, especially when applied to animals that are on the verge of extinction. Thus, preservation of somatic cells provides an alternative method aimed at both maintenance of genetic resources for endangered mammalian species and the *in situ* or *ex situ* conservation of their biodiversity.

The development and optimization of somatic cell cloning technology in domesticated animal species and the establishment of somatic cell banking for the purposes of recovery of endangered mammalian breeds and species threatened with extinction appear to be inevitable (Samiec, 2005 a; Gómez and Pope, 2015; Moulavi et al., 2017; Wani et al., 2017). The variability of animal genetic resources is a pivotal determinant of perpetuating the biodiversity in domesticated and free-living mammalian species. On the one hand, if these genetic resources are not protected from the extinction, they will be lost forever. On the other hand, research focused on the thorough explanation of biological mechanisms underlying proliferative activity, genetic stability, replicative senescence, physiological aging, programmed cell death (apoptosis) of cultured nuclear donor somatic cells and the subsequent epigenetic reprogramming of their cell nuclei both in the oocytes reconstructed by SCNT and in the resultant cloned embryos will have not been completed (Miyamoto et al., 2007; Gómez et al., 2008; Samiec and Skrzyszowska, 2010 a, 2012 a, 2013; Wang et al., 2017; Agrawal et al., 2018; Zhang et al., 2018).

The efficiency of intra- and interspecies somatic cell cloning of mammals (including domesticated and wild felids) is dependent, to a high degree, on not only the type/origin of nuclear donor cells but also morphological, cytological and molecular quality of frozen/thawed and *ex vivo* expanded nuclear donor cell lines that encompass genetic resources derived from different animal species or breeds (Lee et al., 2010; Samiec and Skrzyszowska, 2010 a, 2012 a; Samiec et al., 2013 a, b, 2015; Opiela et al., 2013, 2017; Veraguas et al., 2017; Gouko et al., 2018). This efficiency is also affected both by epigenetic reprogrammability of nuclear donor cell-inherited genome (Samiec and Skrzyszowska, 2005, 2012 b, 2018 a, b; Imsoonthornruksa et al., 2010; Gómez et al., 2012; Jin et al. 2018; Wang et al., 2018) and by nuclear-cytoplasmic intergenomic communication that takes place before and after artificial activation of mammalian nuclear-transferred oocytes and following onset of developmental program of resultant cloned embryos (Samiec, 2005 a, b; Samiec et al., 2012; Samiec and Skrzyszowska, 2010 b, 2014; Imsoonthornruksa et al., 2012; Wittayarat et al., 2013 a). Moreover, it is beyond any doubt that fetal or adult cutaneous fibroblast cell lines provide the most often used source of nuclear donor cells intended for SCNT in mammals, including domesticated animal species and their threatened, vulnerable or

endangered wild living counterparts (Yin et al., 2008 a, b; Imsoonthornruksa et al., 2010, 2012; Samiec and Skrzyszowska, 2010 a, 2012 a; Do et al., 2016).

Available reference materials fail to provide uniform culture protocols for dermal fibroblasts obtained from the domestic cat. Similar to other mammalian species, these cells are usually cultured in DMEM (Skrzyszowska et al., 2002; de Barros et al., 2010; Song et al., 2007; Guan et al., 2010; Wittayarat et al., 2013 a, b; Moro et al., 2015) or DMEM/F12 media (Wen et al., 2003; Song et al., 2007; Moulavi et al., 2017; Veraguas et al., 2017), typically supplemented with serum (10% or 20%) and antibiotics (1%).

Basic fibroblast growth factor (bFGF or FGF-2) belongs to the FGF family and is involved in wound healing, angiogenesis and cell differentiation. It is a well known mitogen that affects growth, proliferation and migration of various types of cells, including fibroblasts (Gospodarowicz and Moran, 1975; Gospodarowicz et al. 1976; Kanazawa et al., 2010; Makino et al., 2010). It was assumed that supplementation of culture media with bFGF would exert a positive effect on proliferation of domestic cat dermal fibroblasts and thus contribute to increasing the efficiency of cell culture. Currently, there are no detailed data concerning the impact of bFGF on feline fibroblasts under *in vitro* conditions. Therefore, the aim of the study was to optimize the cell culture protocol for cat dermal fibroblasts by assessing the influence of different culture media and different doses of bFGF on the proliferation and viability of dermal fibroblasts in terms of cell banking and somatic cell cloning of felids. We used domestic cat dermal fibroblasts as a model for those from wild felids.

Material and methods

Chemicals

All chemical reagents were purchased from Sigma-Aldrich Poznań, Poland, unless otherwise indicated.

Skin biopsy and ethical approval

Skin biopsies were obtained according to guidelines issued by ethical committees (Kraków, Wrocław) and consent from the owners of 7 domestic cats (*Felis catus*) of different breeds (8-year-old Ragdoll male, 7-year-old Siamese female, 2-year-old Devon Rex female, 6-year-old Norwegian Forest female, 4-year-old British shorthair female, 3-year-old Maine coon female and a dead newborn Neva Masquerade male). Tissues (0.25–0.4 cm^2) were collected from the inguinal area following anesthesia of the animal. Immediately after biopsy, skin pieces were submerged individually in phosphate-buffered saline (PBS; Polfa, Lublin, Poland) supplemented with 1% antibiotics (AAS: Antibiotic-Antimycotic-Solution) and transported on ice to the laboratory.

Cat dermal fibroblast cultures

The skin samples were rinsed in 70% ethanol and then 3 times in PBS supplemented with AAS, cut into small (~1 mm^2) pieces, seeded on the bottom surface

of 25-cm^2 tissue culture flasks (T25) and cultured in medium DMEM (D) and/or DMEM/F12 (F), both supplemented with 5 ng/ml bFGF (D-5, F-5, respectively; see subsection Experimental design). The skin samples from each animal were cultured individually at 37°C in a humid atmosphere containing 5% CO_2. When the cells reached 70–80% confluency around the explants, they were rinsed in PBS, routinely trypsinized (2 min) in 0.25% trypsin-EDTA solution, suspended in medium without bFGF (D-0 or F-0) and centrifuged (467 × g; 7.5 min). The cell pellets obtained were resuspended in 1–2 ml of D-0 or F-0 medium, and the cells were counted using a hematocytometer. After estimation of their viability (using 0.4% trypan blue), the cells were seeded (passage I) in equal numbers in new T-flasks in culture media with (D-5, F-5) or without bFGF (D-0, F-0) and cultured. The media were changed every 2–3 days. After reaching ~70–80% of confluency, the cells were trypsinized, counted, evaluated in terms of viability and passaged 3–4 times or stored in freezing media. The cells were frozen in DMEM or DMEM/F12, supplemented with 10% dimethyl-sulfoxide (DMSO) and 10% FBS, or in two commercial media: CellBanker 2 (Takara Clontech Laboratories, USA) or CryoDeffend-Cell lines (R&D Systems, USA).

Cell counts and growth curves at different doses of bFGF

Secondary fibroblasts obtained from skin explants as described above were inoculated in 24-well plates (at a density of 20000 cells/well) in 0.5 ml of medium D (DMEM) supplemented with 0 (D-0), 2.5 (D-2.5), 5 (D-5) or 10 (D-10) ng/ml bFGF and cultured for 24, 48, 6–7, 8–9, 10–11 or 13–14 days. On designated days, the cells were harvested from two wells for each bFGF treatment and counted. The viability of cells was also estimated. The mean cell count/well was recorded and growth curves were plotted against culture time. The population doubling time (PDT) was calculated based on the curves using a Doubling Time Cell Culture Calculator (http://www.doubling-time.com/compute.php; Roth, 2006). The experiment was performed in duplicate on two different lines of fibroblasts.

Experimental design

Experiment I. In the first experiment, the influence of two different culture media supplemented with or without bFGF (5 ng/ml) on cat dermal fibroblast proliferation and viability was tested. The culture media used in the experiment were: I) DMEM (Dulbecco's Modified Eagle's Medium), supplemented with 1% Hepes (1M Hepes buffered saline) and 1% NEAA (100x Non-essential Amino Acid mixture) and II) DMEM/F12 (Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham's Medium). Both media were supplemented with 10% FBS (Fetal Bovine Serum), 1% AAS and a) either with 5 ng/ml bFGF (D-5, F-5) or b) without bFGF (D-0, F-0).

Experiment II was performed based on the results obtained from the first experiment. In order to determine the optimal doses of bFGF, secondary fibroblasts were cultured in 24-well plates in medium DMEM supplemented with 0, 2.5, 5 or 10 ng/ml of bFGF for 2–14 days. On the designated days (see above) the viability and the number of cells per well were estimated.

Statistical analysis

The effect of DMEM and DMEM/F12 supplemented with/or without bFGF on feline fibroblasts was analyzed using Student's t-test. The influence of different doses of bFGF on viability of fibroblasts and their ability to proliferate was analyzed using ANOVA followed by Tukey's test. The results are presented as means ± SEM. Differences between means were considered statistically significant at $P<0.05$.

Results

The effect of different culture media and bFGF on feline dermal fibroblast proliferation and viability

In both culture media (DMEM and DMEM/F12) the first fibroblast-like cells (Figure 1 a, d) near the skin explants obtained from living individuals were observed after 5–6 days of culture, while it took 2–3 days longer than this for them to appear around the explants obtained from the dead newborn Neva Masquerade. After 12–14 days, the cells cultured in D-5 reached ~80% of confluency and the first passages were performed. For cells cultured in F-5, the first passages were carried out after 12–21 days of culture. During passage I, the mean cell count/flask was similar for both media (Figure 2). In subsequent passages in all media an increased cell number per flask was noted, especially for fibroblasts cultured in D-5, as compared to D-0, F-5 and F-0. For cells cultured in D-5, passages II, III and IV were performed 8–9, 5–7 and 6–8 days after the previous passages, respectively. For fibroblasts incubated in D-0, slower proliferation was observed and subsequent passages were carried out after 8–12, 6–16 and 8–11 days of culture. In medium DMEM/F12, no significant effect of bFGF on the proliferation of cultured fibroblasts was observed and the results were similar to those obtained for D-0 (Figure 2). For cells cultured in F-5 and F-0, passages II and III were made after 8–15 days of culture. Due to poor proliferation in F-0, passage IV was carried out only for cells cultured in F-5. No effect of media on cell viability was observed (Figure 3). In all media, the cells exhibited fibroblast-like morphology, however in media without bFGF the cells were spindle-shaped (fusiform) and more elongated than in the media with bFGF. In the latter, fibroblasts were smaller, slightly more convex, bipolar, triangular or polygonal and had 2–3 short processes (Figure 1).

The effect of different doses of bFGF on feline fibroblast proliferation and viability

A dose-dependent effect of bFGF on the proliferation of domestic cat dermal fibroblasts was found. In media with bFGF (D-5 and D-10), feline fibroblasts exhibited higher ($P<0.05$) proliferation compared with D-0 (Figure 4) and the growth curves resembled an "S-like" shape with latent, exponential and steady phases.

After a lag phase lasting approximately 48 h, cells reached the logarithmic growth phase in all culture media. On day 6.5, the mean cell count per well in D-10, D-5, D-2.5 and D-0 was 9.5, 8.6, 6.8 and 4.9 times higher, respectively, compared

to day 0. In the following days the cells tended to proliferate faster in D-5 and D-10 than in D-2.5, while the lowest proliferation rate was observed in D-0. This pattern continued during the entire period of cell culture. Around day 8, cells in medium D-0 reached the plateau phase, while those cultured in media with bFGF were still dividing and reached that phase approximately 48 h later. The population doubling time at the exponential phase was 36.2–46.8 h and 40.2–45.3 h for cells cultured in D-5 and D-10, respectively; 50.9–51.6 h for cells cultured in D-2.5 and 39.5–76.6 h for fibroblasts incubated in D-0. No effect of supplementation of culture media with different doses of bFGF on the viability of domestic cat dermal fibroblasts was found (Figure 5). As in the previous experiment, cells cultured with bFGF were smaller, polygonal and less elongated than those cultured in D-0.

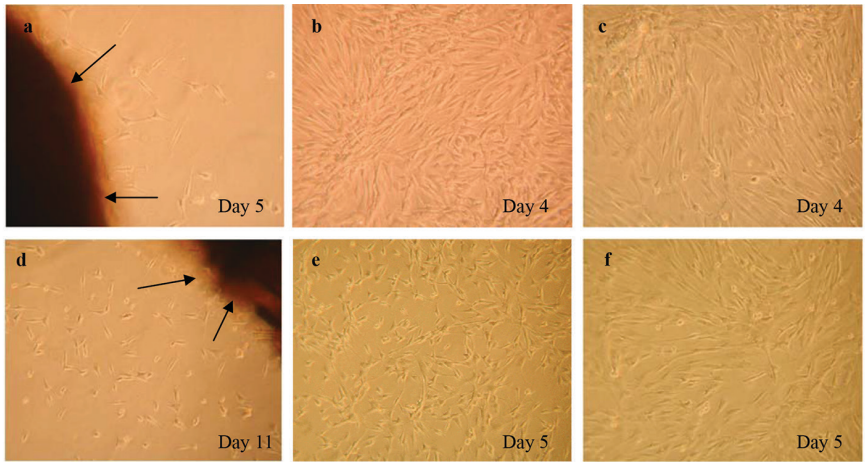

Figure 1. Representative microphotographs of cultured cat dermal fibroblasts in medium DMEM (a–c) and DMEM/F12 (d–f) either with (a, b, d, e) or without (c, f) bFGF. Near skin explants (arrows) the first fibroblasts are seen; secondary fibroblasts on the 4th (b–c) and 5th (e–f) days of culture after the first passage. In medium D-5 (b), more cells are seen than in medium D-0 (c), F-5 (e) and F-0 (f)

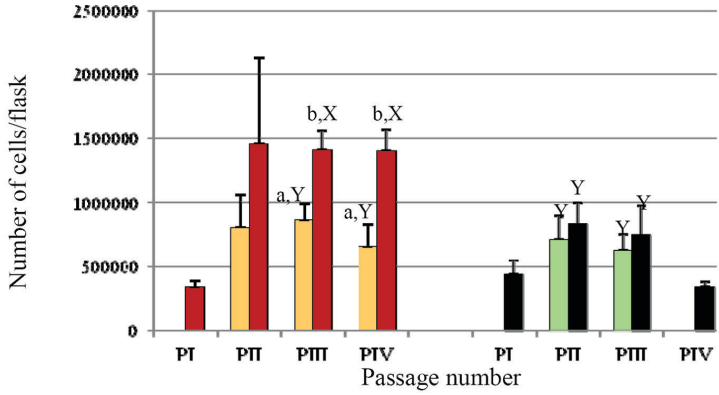

Figure 2. Mean (± SEM) cell count per flask in subsequent passages, obtained in DMEM and DMEM/F12 either with (D-5; F-5) or without (D-0; F-0) 5 ng/ml bFGF. a, b = P<0.5 – for comparison within a given passage number between media (D-5 vs. D-0; F-5 vs. F-0); X, Y = P<0.05 – for comparison between different media and different passages

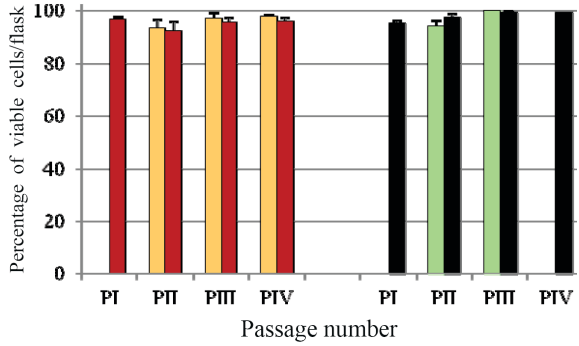

Figure 3. Percentage (mean ± SEM) of viable cells in subsequent passages obtained in DMEM and DMEM/F12 either with (D-5; F-5) or without (D-0; F-0) 5 ng/ml bFGF

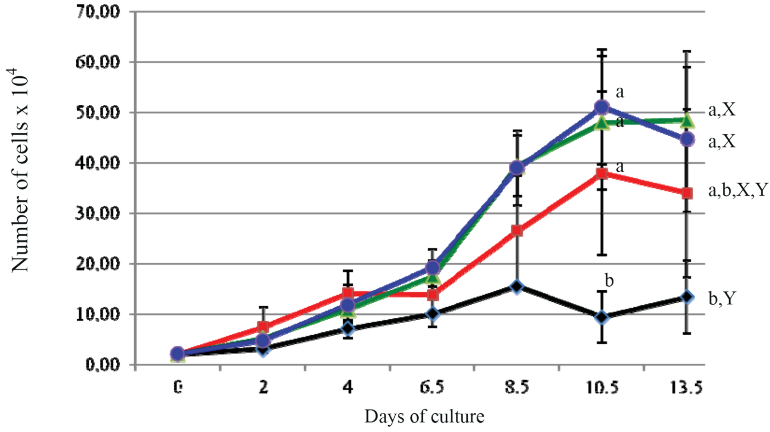

Figure 4. Mean (± SEM) cell count/well obtained for cat dermal fibroblasts on successive days of culture in medium DMEM with 0 (D-0), 2.5 (D-2.5), 5 (D-5) and 10 (D-10) ng/ml bFGF; a, b; X, Y= P<0.05

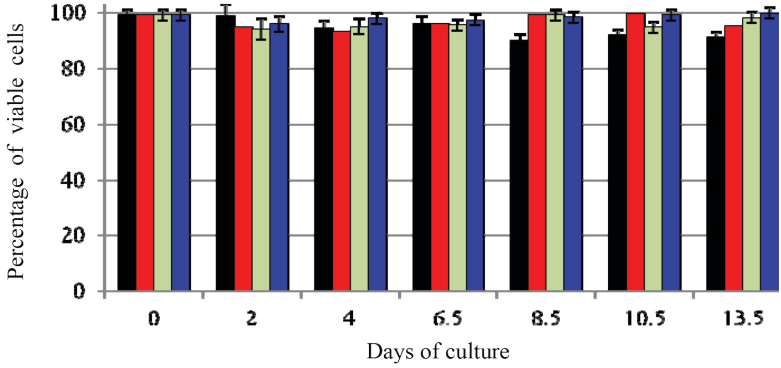

Figure 5. Percentage (mean ± SEM) of viable cells on successive days of culture obtained in DMEM supplemented with 0 (D-0), 2.5 (D-2.5), 5 (D-5) and 10 (D-10) ng/ml bFGF

Discussion

In this study we have demonstrated a significant effect of culture media as well as bFGF on the ability of domestic cat dermal fibroblasts to proliferate under *in vitro* conditions. It has been recognized that culture conditions, including the type of media used, may change gene expression in the *ex vivo* expanded somatic cells and the ability of the somatic cell nuclei to be epigenetically remodeled/reprogrammed after their transfer into a cytoplasm of enucleated oocytes, and thus affect the overall efficiency of somatic cell cloning in different mammalian species (Powell et al., 2004; Giraldo et al., 2008; Imsoonthornruksa et al., 2010; Gómez et al., 2012; Kim et al., 2015; Samiec and Skrzyszowska, 2018 a, b). In our study, explants were cultured in DMEM and DMEM/F12 media, which are routinely used for feline fibroblasts as well as those from other mammalian species. Both media were supplemented with 5 ng/ml bFGF (D-5, F-5). In both media, the first fibroblast-like cells appeared after 5–6 days of establishing the culture or after 8 days at the latest around the skin explants originating from a dead newborn Neva Masquerade. These observations are in agreement with the findings of other authors, who have also reported the presence of the first fibroblast-like cells around domestic cat skin explants incubated in DMEM/F12 within 1 week of culture (Moulavi et al., 2017). On the other hand, in cultures established from skin explants from the Siberian tiger (Song et al., 2007) and Bengal tiger (Guan et al., 2010) and cultured in DMEM, the first fibroblast-like cells were noted after 5 and 5–12 days of culture, respectively. Generally, research indicates that in most mammalian species the first fibroblast-like cells usually migrate from skin explants between the 4th and 12th days of culture (cattle: Liu et al., 2008; goat: Singh and Sharma, 2011; Mehrabani et al., 2016; Duroc pig: Xiong et al., 2014; *Pecari tajacu:* Santos et al., 2016; Asian elephant: Siengdee et al., 2018).

As far as dead animals are concerned, Moulavi et al. (2017) did not obtain live cells from tissues (skin and muscles) collected from a female Asiatic cheetah (*Acinonyx jubatus venaticus*) that were frozen without a cryoprotectant and stored for 10 days at -20°C. However, it is worth mentioning that after reconstruction of domestic cat (*Felis silvestris catus*) enucleated oocytes by intracytoplasmic microinjection of the cell nuclei isolated from non-viable dermal Asiatic cheetah somatic cells, the inter-genus and inter-species (i.e., Asiatic cheetah→domestic cat) cloned embryos generated were able to undergo cleavage divisions and almost 6% of them developed *in vitro* to the morula stage (Moulavi et al., 2017). Aoued and Singh (2015) obtained goat fibroblasts from skin explants stored at 4°C for 0 (control) to 160 days after death of their donor. In control samples, the first fibroblast cells were found after 4 days, while it took 10–12 days following establishment of the culture for explants stored at 4°C. In our studies (Młodawska, unpublished data) we obtained (and froze) fibroblasts from the skin of a dead newborn tiger (*Panthera tigris*, female, that probably lived for only a few hours after birth). Unfortunately, we could not obtain fibroblasts from skin explants harvested from another dead tiger (male, stillborn baby tiger, approximately 60th day of pregnancy), despite maintaining the explants in culture for 21 days. The research mentioned above shows that cryoconservation of tissues and cells from dead individuals, especially in the case of endangered species,

is worth the effort. Preserved genetic resources may be used in the future not only for the purpose of assisted reproduction but also as a unique source of cells for various types of phylogenetic and evolutionary research.

Research suggests that culture conditions (such as, e.g., culture media, growth factors, seeding density, oxygen tension) may exert a significant impact on the proliferative lifespan of cultured mammalian somatic cells (Balin et al., 2002; Miyamoto et al., 2007; Gómez et al., 2008; Samiec and Skrzyszowska, 2012 a; Samiec et al., 2013 a, b; Gouko et al., 2018; Zhang et al., 2018). In the study presented here, in medium D-5 the cells proliferated faster and formed monolayers earlier than in DMEM without bFGF (D-0) or DMEM/F12 (F-5, F-0). These results suggest that DMEM supplemented with bFGF is more useful for culturing domestic cat dermal fibroblasts than DMEM/F12. This effect may be the result of different media composition (e.g., different glucose, vitamin or amino-acid content) as well as individual characteristics of the animal from which the cells were obtained (e.g., breed, sex, age). For instance, in the case of explants from the male Ragdoll (8 years) and a dead newborn Neva Masquerade male that were cultured in D-5 and F-5, the first passages were made after 13 and 14 days of culture. As for cells from the female Norwegian Forest cat (6 years), they proliferated at a faster rate in D-5 than F-5 and the first passages were performed after 14 and 21 days in culture, respectively. The effect of three different media on goat dermal fibroblast proliferation rates was shown by Singh and Sharma (2011). Kim et al. (2015) conducted experiments involving primary cell cultures obtained from dog skin explants grown in DMEM and reported the presence of a monolayer after 15 days in culture, while in RCMEp (medium for adipose-derived stem cells; including among other components, 10 ng/ml FGF) as early as 7 days after establishing the culture. The available literature does not contain information on the impact of bFGF and different types of media on the proliferation of domestic cat and wild felid fibroblasts.

To determine the optimal dose of bFGF for domestic cat dermal fibroblasts, in experiment II the cells were cultured in DMEM with 0, 2.5, 5 and 10 ng bFGF/ml. A dose-dependent effect of bFGF on the proliferation ability of these fibroblasts was found. The cells cultured in DMEM supplemented with 5 and 10 ng bFGF/ml (D-5, D-10) displayed similar and at the same time higher proliferative abilities compared to cells cultured in medium without bFGF (D-0). In medium with 2.5 ng bFGF/ml (D-2.5) the cells exhibited almost the same ability to proliferate as in D-5 and D-10. However, we did not find significant difference between proliferation of cells in D-2.5 and D-0. These results suggest that supplementation of the culture media with 2.5 ng/ml bFGF may be insufficient to increase proliferation rate of the domestic cat dermal fibroblasts *in vitro*. Similar results were reported by Makino et al. (2010) who demonstrated that bFGF stimulates proliferation of human dermal fibroblasts in a dose- and time-dependent manner and that this effect is mediated via the ERK1/2 and JNK signaling pathways. A stimulatory effect of bFGF on *in vitro* proliferation of human dermal fibroblasts has also been reported by Abdian et al. (2015). Song et al. (2007) were successful in culturing skin fibroblasts from the Siberian tiger (*Panthera tigris altaica*) in DMEM/F12 supplemented with 10 ng/ml EGF (epidermal growth factor). However, these authors did not culture cells in a medium without

EGF and thus, it is not possible to establish a causal effect of this growth factor on the proliferative ability of skin fibroblasts from the Siberian tiger.

Our results show that different types of media and diverse bFGF doses do not affect cell viability. However, the influence of bFGF on fibroblast morphology was noted. A similar impact of bFGF on the morphology of different cell types, including fibroblasts, was reported by other authors (Gospodarowicz and Moran, 1975; Gospodarowicz et al. 1976; Silvério et al., 2007; Hu et al., 2013). Cells cultured without bFGF were spindle-shaped and more elongated, were sometimes slightly flattened and exhibited the lowest proliferation ability. Addition of bFGF supported cultures with greater cell counts, and smaller, slightly more convex, polygonal cells with short processes. It can be supposed that such morphology is the result of faster cell proliferation. It is thought that the rounded cells are poorly adherent to the surface, or this type of morphology may indicate that the cells have just undergone mitotic divisions (Gamal et al., 1998; Silvério et al., 2007). A flattened morphology and increased cytoplasm volume is observed when cells stop dividing and enter the senescent state or under stressful culture conditions (Zainuddin et al., 2010).

In conclusion, medium DMEM supplemented with bFGF provides better culture results for domestic cat dermal fibroblast cells than DMEM/F12. Supplementation with bFGF has a beneficial effect on *in vitro* proliferation of domestic cat fibroblasts and could be recommended for addition to culture media. The results of the current study provide the biological and biotechnological foundations that can contribute not only to the restoration and maintenance of genetic resources deposited in the form of neonatal/adult dermal fibroblast cell lines derived from domestic cat breeds or endangered wild living felids, but also to the *in situ* and/or *ex situ* conservation of biodiversity by applying intra- or interspecies somatic cell nuclear transfer (SCNT) within the *Felidae* family.

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References

Abdian N., Ghasemi-Dehkordi P., Hashemzadeh-Chaleshtori M., Ganji-Arjenaki M., Doosti A., Amiri B. (2015). Comparison of human dermal fibroblasts (HDFs) growth rate in culture media supplemented with or without basic fibroblast growth factor (bFGF). Cell Tissue Bank, 16: 487–495.

Agrawal H., Selokar N.L., Saini M., Singh M.K., Chauhan M.S., Palta P., Singla S.K., Manik R.S. (2018). *m*-carboxycinnamic acid bishydroxamide improves developmental competence, reduces apoptosis and alters epigenetic status and gene expression pattern in cloned buffalo (*Bubalus bubalis*) embryos. Reprod. Domest. Anim., 53: 986–996.

Aoued H.S., Singh M. (2015). Recovery of fibroblast-like cells after 160 days of postmortem storage of goat skin tissues in refrigerated media. J. Veterinar. Sci. Technol., 6: 236.

Balin A.K., Fisher A.J., Anzelone M., Leong I., Allen R.G. (2002). Effects of establishing cell cultures and cell culture conditions on the proliferative life span of human fibroblasts isolated from different tissues and donors of different ages. Exp. Cell Res., 274: 275–287.

Barros F.R. de, Goissis M.D., Caetano H.V., Paula-Lopes F.F., Peres M.A., Assumpção M.E., Visintin J.A. (2010). Serum starvation and full confluency for cell cycle synchronization of domestic cat (*Felis catus*) foetal fibroblasts. Reprod. Domest. Anim., 45: 38–41.

Do L., Wittayarat M., Terazono T., Sato Y., Taniguchi M., Tanihara F., Takemoto T., Kazuki Y., Kazuki K., Oshimura M., Otoi T. (2016). Effects of duration of electric pulse on *in vitro* development of cloned cat embryos with human artificial chromosome vector. Reprod. Domest. Anim., 51: 1039–1043

Gamal A.Y., Mailhot J.M., Garnick J.J., Newhouse R., Sharawy M.M. (1998). Human periodontal ligament fibroblast response to PDGF-BB and IGF-1 application on tetracycline HCI conditioned root surfaces. J. Clin Periodontol., 25: 404–412.

Giraldo A.M., Hylan D.A., Ballard C.B., Purpera M.N., Vaught T.D., Lynn J.W., Godke R.A., Bondioli K.R. (2008). Effect of epigenetic modifications of donor somatic cells on the subsequent chromatin remodeling of cloned bovine embryos. Biol. Reprod., 78: 832–840.

Gómez M.C., Pope C.E. (2015). Cloning endangered felids by interspecies somatic cell nuclear transfer. Methods Mol. Biol., 1330: 133–152.

Gómez M.C., Pope C.E., Kutner R.H., Ricks D.M., Lyons L.A., Ruhe M., Dumas C., Lyons J., López M., Dresser B.L., Reiser J. (2008). Nuclear transfer of sand cat cells into enucleated domestic cat oocytes is affected by cryopreservation of donor cells. Cloning Stem Cells, 10: 469–483.

Gómez M.C., Biancardi M.N., Jenkins J.A., Dumas C., Galiguis J., Wang G., Earle Pope C. (2012). Scriptaid and 5-aza-2'deoxycytidine enhanced expression of pluripotent genes and *in vitro* developmental competence in interspecies black-footed cat cloned embryos. Reprod. Domest. Anim., 47 (Suppl 6): 130–135.

Gospodarowicz D., Moran J.S. (1975). Mitogenic effect of fibroblast growth factor on early passage cultures of human and murine fibroblasts. J. Cell Biol., 66: 451–457.

Gospodarowicz D., Weseman J., Moran J.S., Lindstrom J. (1976). Effect of fibroblast growth factor on the division and fusion of bovine myoblasts. J. Cell Biol., 70: 395–405.

Gouko R., Onuma M., Eitsuka T., Katayama M., Takahashi K., Nakagawa K., Inoue-Murayama M., Kiyono T., Fukuda T. (2018). Efficient immortalization of cells derived from critically endangered Tsushima leopard cat (*Prionailurus bengalensis euptilurus*) with expression of mutant CDK4, Cyclin D1, and telomerase reverse transcriptase. Cytotechnology, 70: 1619–1630.

Guan W.J., Liu C.Q., Li C.Y., Liu D., Zhang W.X., Ma Y.H. (2010). Establishment and cryopreservation of a fibroblast cell line derived from Bengal tiger (*Panthera tigris tigris*). Cryo Letters, 31: 130–138.

Hu F., Wang X., Liang G., Lv L., Zhu Y., Sun B., Xiao Z. (2013). Effects of epidermal growth factor and basic fibroblast growth factor on the proliferation and osteogenic and neural differentiation of adipose-derived stem cells. Cell Reprogram., 15: 224–232.

Imsoonthornruksa S., Lorthongpanich C., Sangmalee A., Srirattana K., Laowtammathron C., Tunwattana W., Somsa W., Ketudat-Cairns M., Parnpai R. (2010). Abnormalities in the transcription of reprogramming genes related to global epigenetic events of cloned endangered felid embryos. Reprod. Fert. Develop., 22: 613–624.

Imsoonthornruksa S., Sangmalee A., Srirattana K., Parnpai R., Ketudat--Cairns M. (2012). Development of intergeneric and intrageneric somatic cell nuclear transfer (SCNT) cat embryos and the determination of telomere length in cloned offspring. Cell. Reprogram., 14: 79–87.

Ishii T. (2014). Human iPS cell-derived germ cells: current status and clinical potential. J. Clin. Med., 3:1064–1083.

Jin L., Guo Q., Zhang G.L., Xing X.X., Xuan M.F., Luo Q.R., Luo Z.B., Wang J.X., Yin X.J., Kang J.D. (2018). The histone deacetylase inhibitor, CI994, improves nuclear reprogramming and *in vitro* developmental potential of cloned pig embryos. Cell. Reprogram., 20: 205–213.

Kanazawa S., Fujiwara T., Matsuzaki S., Shingaki K., Taniguchi M., Miyata S., Tohyama M., Sakai Y., Yano K., Hosokawa K., Kubo T. (2010). bFGF regulates PI3-kinase-Rac1-JNK pathway and promotes fibroblast migration in wound healing. PLoS One, 5(8): e12228.

Kim G.A., Oh H.J., Kim M.J., Jo Y.K., Choi J., Kim J.W., Lee T.H., Lee B.C. (2015). Effect of primary culture medium type for culture of canine fibroblasts on production of cloned dogs. Theriogenology, 84: 524–530.

Lee H.S., Yu X.F., Bang J.I., Cho S.J., Deb G.K., Kim B.W., Kong I.K. (2010). Enhanced histone acetylation in somatic cells induced by a histone deacetylase inhibitor improved inter-generic cloned leopard cat blastocysts. Theriogenology, 74: 1439–1449.

Liu C., Guo Y., Guan W., Ma Y., Zhang H.H., Tang X. (2008). Establishment and biological characteristics of Luxi cattle fibroblast bank. Tissue Cell, 40: 417–424.

Makino T., Jinnin M., Muchemwa F.C., Fukushima S., Kogushi-Nishi H., Moriya C., Igata T., Fujisawa A., Johno T., Ihn H. (2010). Basic fibroblast growth factor stimulates the proliferation of human dermal fibroblasts via the ERK1/2 and JNK pathways. Br. J. Dermatol., 162: 717–723.

Mehrabani D., Tajedini M., Tamadon A., Dianatpour M., Parvin F., Zare S., Rahmanifar F. (2016). Establishment, characterization and cryopreservation of Fars native goat fetal fibroblast cell lines. Asian Pacific J. Reprod., 5: 247–252.

Miyamoto K., Hoshino Y., Minami N., Yamada M., Imai H. (2007). Effects of synchronization of donor cell cycle on embryonic development and DNA synthesis in porcine nuclear transfer embryos. J. Reprod. Dev., 53: 237–246.

Moro L.N., Jarazo J., Buemo C., Hiriart M.I., Sestelo A., Salamone D.F. (2015). Tiger, Bengal and domestic cat embryos produced by homospecific and interspecific zona-free nuclear transfer. Reprod. Domest. Anim., 50: 849–857.

Moulavi F., Hosseini S.M., Tanhaie-Vash N., Ostadhosseini S., Hosseini S.H., Hajinasrollah M., Asghari M.H., Gourabi H., Shahverdi A., Vosough A.D., Nasr-Esfahani M.H. (2017). Interspecies somatic cell nuclear transfer in Asiatic cheetah using nuclei derived from post-mortem frozen tissue in absence of cryo-protectant and *in vitro* matured domestic cat oocytes. Theriogenology, 90: 197–203.

Opiela J., Samiec M., Bochenek M., Lipiński D., Romanek J., Wilczek P. (2013). DNA aneuploidy in porcine bone marrow-derived mesenchymal stem cells undergoing osteogenic and adipogenic *in vitro* differentiation. Cell. Reprogram., 15: 425–434.

Opiela J., Samiec M., Romanek J. (2017). *In vitro* development and cytological quality of inter-species (porcine→bovine) cloned embryos are affected by trichostatin A-dependent epigenomic modulation of adult mesenchymal stem cells. Theriogenology, 97: 27–33.

Powell A.M., Talbot N.C., Wells K.D., Kerr D.E., Pursel V.G., Wall R.J. (2004). Cell donor influences success of producing cattle by somatic cell nuclear transfer. Biol. Reprod., 71: 210–216.

Roth V. (2006). Doubling Time Computing. Avaible from: http://www.doubling-time.com/compute.php

Samiec M. (2005 a). The effect of mitochondrial genome on architectural remodeling and epigenetic reprogramming of donor cell nuclei in mammalian nuclear transfer-derived embryos. J. Anim. Feed Sci., 14: 393–422.

Samiec M. (2005 b). The role of mitochondrial genome (mtDNA) in somatic and embryo cloning of mammals. A review. J. Anim. Feed Sci., 14: 213–233.

Samiec M., Opiela J., Lipiński D., Romanek J. (2015). Trichostatin A-mediated epigenetic transformation of adult bone marrow-derived mesenchymal stem cells biases the *in vitro* developmental capability, quality, and pluripotency extent of porcine cloned embryos. Biomed Res. Int., 2015: 814686.

Samiec M., Skrzyszowska M. (2005). Molecular conditions of the cell nucleus remodelling/reprogramming process and nuclear-transferred embryo development in the intraooplasmic karyoplast injection technique: a review. Czech J. Anim. Sci., 50: 185–195.

Samiec M., Skrzyszowska M. (2010 a). Preimplantation developmental capability of cloned pig embryos derived from different types of nuclear donor somatic cells. Ann. Anim. Sci., 10: 385–398.

Samiec M., Skrzyszowska M. (2010 b). The use of different methods of oocyte activation for generation of porcine fibroblast cell nuclear-transferred embryos. Ann. Anim. Sci. 10: 399–411.

Samiec M., Skrzyszowska M. (2012 a). Roscovitine is a novel agent that can be used for the

activation of porcine oocytes reconstructed with adult cutaneous or fetal fibroblast cell nuclei. Theriogenology, 78: 1855–1867.

Samiec M., Skrzyszowska M. (2012 b). High developmental capability of porcine cloned embryos following trichostatin A-dependent epigenomic transformation during *in vitro* maturation of oocytes pre-exposed to *R*-roscovitine. Anim. Sci. Pap. Rep., 30: 383–393.

Samiec M., Skrzyszowska M. (2013). Assessment of *in vitro* developmental capacity of porcine nuclear-transferred embryos reconstituted with cumulus oophorus cells undergoing vital diagnostics for apoptosis detection. Ann. Anim. Sci., 13: 513–529.

Samiec M., Skrzyszowska M. (2014). Biological transcomplementary activation as a novel and effective strategy applied to the generation of porcine somatic cell cloned embryos. Reprod. Biol., 14: 128–139.

Samiec M., Skrzyszowska M. (2018 a). Intrinsic and extrinsic molecular determinants or modulators for epigenetic remodeling and reprogramming of somatic cell-derived genome in mammalian nuclear-transferred oocytes and resultant embryos. Pol. J. Vet. Sci., 21: 217–227.

Samiec M., Skrzyszowska M. (2018 b). Can reprogramming of overall epigenetic memory and specific parental genomic imprinting memory within donor cell-inherited nuclear genome be a major hindrance for the somatic cell cloning of mammals? – a review. Ann. Anim. Sci., 18: 623–638.

Samiec M., Skrzyszowska M., Lipiński D. (2012). Pseudophysiological transcomplementary activation of reconstructed oocytes as a highly efficient method used for producing nuclear-transferred pig embryos originating from transgenic foetal fibroblast cells. Pol. J. Vet. Sci., 15: 509–516.

Samiec M., Skrzyszowska M., Bochenek M. (2013 a). *In vitro* development of porcine nuclear-transferred embryos derived from fibroblast cells analysed cytometrically for apoptosis incidence and accuracy of cell cycle synchronization at the G0/G1 stages. Ann. Anim. Sci., 13: 735–752.

Samiec M., Skrzyszowska M., Opiela J. (2013 b). Creation of cloned pig embryos using contact-inhibited or serum-starved fibroblast cells analysed *intra vitam* for apoptosis occurrence. Ann. Anim. Sci., 13: 275–293.

Santos M.L.T., Borges A.A., Queiroz Neta L.B., Santos M.V.O., Oliveira M.F., Silva A.R., Pereira A.F. (2016). *In vitro* culture of somatic cells derived from ear tissue of collared peccary (*Pecari tajacu* Linnaeus, 1758) in medium with different requirements. Pesq. Vet. Bras., 36: 1194–1202.

Siengdee P., Klinhom S., Thitaram C., Nganvongpanit K. (2018). Isolation and culture of primary adult skin fibroblasts from the Asian elephant (*Elephas maximus*). Peer J., 6: e4302. doi: 10.7717/peerj.4302.

Silvério K.G., Martinez A.E., Rossa C. Jr. (2007). Effects of basic fibroblast growth factor on density and morphology of fibroblasts grown on root surfaces with or without conditioning with tetracycline or EDTA. J. Oral Sci., 49: 213–220.

Singh M., Sharma A.K. (2011). Outgrowth of fibroblast cells from goat skin explants in three different culture media and the establishment of cell lines. In Vitro Cell Dev. Biol. Anim., 47: 83–88.

Skrzyszowska M., Kątska L., Ryńska B., Kania G., Smorag Z., Pieńkowski M. (2002). *In vitro* developmental competence of domestic cat embryos after somatic cloning: a preliminary report. Theriogenology, 58: 1615–1621.

Song J., Hua S., Song K., Zhang Y. (2007). Culture, characteristics and chromosome complement of Siberian tiger fibroblasts for nuclear transfer. In Vitro Cell Dev. Biol. Anim., 43: 203–209.

Veraguas D., Gallegos P.F., Castro F.O., Rodriguez-Alvarez L. (2017). Cell cycle synchronization and analysis of apoptosis-related gene in skin fibroblasts from domestic cat (*Felis silvestris catus*) and kodkod (*Leopardus guigna*). Reprod. Domest. Anim., 52: 881–889.

Wang H., Cui W., Meng C., Zhang J., Li Y., Qian Y., Xing G., Zhao D., Cao S. (2018). MC1568 enhances histone acetylation during oocyte meiosis and improves development of somatic cell nuclear transfer embryos in pig. Cell. Reprogram., 20: 55–65.

Wang M., Gao Y., Qu P., Qing S., Qiao F., Zhang Y., Mager J., Wang Y. (2017). Sperm-borne miR-449b influences cleavage, epigenetic reprogramming and apoptosis of SCNT embryos in bovine. Sci. Rep., 7: 13403.

Wani N.A., Vettical B.S., Hong S.B. (2017). First cloned Bactrian camel (*Camelus bactrianus*) calf produced by interspecies somatic cell nuclear transfer: A step towards preserving the critically endangered wild Bactrian camels. PLoS One, 12 (5): e0177800.

Wen D.C., Yang C.X., Cheng Y., Li J.S., Liu Z.H., Sun Q.Y., Zhang J.X., Lei L., Wu Y.Q., Kou Z.H., Chen D.Y. (2003). Comparison of developmental capacity for intra- and interspecies cloned cat (*Felis catus*) embryos. Mol. Reprod. Dev., 66: 38–45.

Wittayarat M., Sato Y., Do L.T., Morita Y., Chatdarong K., Techakumphu M., Taniguchi M., Otoi T. (2013 a). Histone deacetylase inhibitor improves the development and acetylation levels of cat-cow interspecies cloned embryos. Cell. Reprogram., 15: 301–308.

Wittayarat M., Thongphakdee A., Saikhun K., Chatdarong K., Otoi T., Techakumphu M. (2013 b). Cell cycle synchronization of skin fibroblast cells in four species of family Felidae. Reprod. Domest. Anim., 48: 305–310.

Xiong H., He X., Zhang W., Li C., Li M., Guan W., Ma Y. (2014). Establishment and characterization of a fibroblast line from Duroc. Pakistan J. Zool., 46: 363–369.

Yin X.J., Lee H.S., Yu X.F., Choi E., Koo B.C., Kwon M.S., Lee Y.S., Cho S.J., Jin G.Z., Kim L.H., Shin H.D., Kim T., Kim N.H., Kong I.K. (2008 a). Generation of cloned transgenic cats expressing red fluorescence protein. Biol. Reprod., 78: 425–431.

Yin X.J., Lee H.S., Yu X.F., Kim L.H., Shin H.D., Cho S.J., Choi E.G., Kong I.K. (2008 b). Production of second-generation cloned cats by somatic cell nuclear transfer. Theriogenology, 69: 1001–1006.

Zainuddin A., Chua K.H., Abdul Rahim N., Makpol S. (2010). Effect of experimental treatment on GAPDH mRNA expression as a housekeeping gene in human diploid fibroblasts. BMC Mol. Biol., 11: 59.

Zhang Y., Qu P., Ma X., Qiao F., Ma Y., Qing S., Zhang Y., Wang Y., Cui W. (2018). Tauroursodeoxycholic acid (TUDCA) alleviates endoplasmic reticulum stress of nuclear donor cells under serum starvation. PLoS One, 13(5): e0196785.

Zhu Y., Hu H.L., Li P., Yang S., Zhang W., Ding H., Tian R.H., Ning Y., Zhang L.L., Guo X.Z., Shi Z.P., Li Z., He Z. (2012). Generation of male germ cells from induced pluripotent stem cells (iPS cells): an *in vitro* and *in vivo* study. Asian J. Androl., 14: 574–589.

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