Cell and Organ Transplantology. 2017; 5(1):62-66.
doi:10.22494/cot.v5i1.69

Morphological and functional characteristics of cell culture derived from the mouse nail unit

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ABSTRACT

Nail unit is a complex anatomical structure that is capable of rapid growth and regeneration throughout the life. Such significant reparative potential is associated with the presence different types of stem and progenitor cells, whose biology remains one of the fundamental issues today. Taking into account the active search for new stem cell sources for cell therapy, the view of the nail unit as a potential site for the localization of undifferentiated cells with stem potency is topical problem.

PURPOSE. *The study was conducted with an objective to establish the morphological, morphometric and proliferative characteristics of cultured cells isolated from the mouse nail unit.*

MATERIALS AND METHODS. *Primary cultures of cells were obtained from tissue sampling, which included areas of the proximal nail fold, nail matrix and onychodermis of the FVB mouse nail organ. Cells were cultured in DMEM:F12 medium with 15 % fetal bovine serum during 6 passages. We determined the colony-forming activity, the population growth rate and doubling time, measured the area of cells, nuclei, and calculated the nuclear-cytoplasmic ratio. For cell morphology analysis, we used staining with Bemer's hematoxylin and eosin, Heidenhain's iron hematoxylin and May-Grünwald stain.*

RESULTS. *According to the morphological analysis in vitro the cells from mouse nail unit are heterogeneous with high synthetic activity and a low nuclear-cytoplasmic ratio – the features characteristic of the low-differentiated cells. The population doubling time of the culture was 80 ± 6.5 hours on average, the fastest growing cells were at the 4th passage (63 ± 7 hours). The specific growth rate for cell culture is low (0.01 ± 0.0007).*

The colony forming efficiency at the 5th passage was only 4 %. A significant number of colonies was small with large poorly proliferative cells, which may indicate a production of large numbers of transitional progenitor cells.

CONCLUSION. *The obtained cell culture from the mouse nail unit according to the analysis of their morphology, morphometry and proliferative potential is heterogeneous and requires the further development of pure culture technologies for the detailed characterization of separate subpopulations of cells.*

KEYWORDS: *nail unit; nail matrix; onychodermis; cell culture; clonogenic assay*

Traumatic amputation of distal phalanges of fingers is the most common type of hand injury in humans [1]. Fingers phalanges of some mammals (including mice) may partially recover after injury, while regeneration of the entire limb in mammals does not occur [2]. It is assumed that the presence of the nail provides the growth of the lost distal part of the finger [3].

The nail organ is a complex structural functional unit [4] located on the dorsal side of the distal phalanx and composed of many types of cells, most of which are of epithelial origin – epitheliocytes (hyponychium, cuticle), keratinocytes (ventral nail matrix), melanocytes, Merkel cells, immune cells (Langerhans cells) [5], onychofibroblasts [6, 7], stem cells of the nail [8]. The protective function of the nail organ is ensured by the formation of a highly keratinized nail plate that grows throughout life. This continuous renewal is dependent by the activity of the stem cells of the nail, which biology is still poorly understood. The open questions remain their localization [11, 12, 13]) and origin.

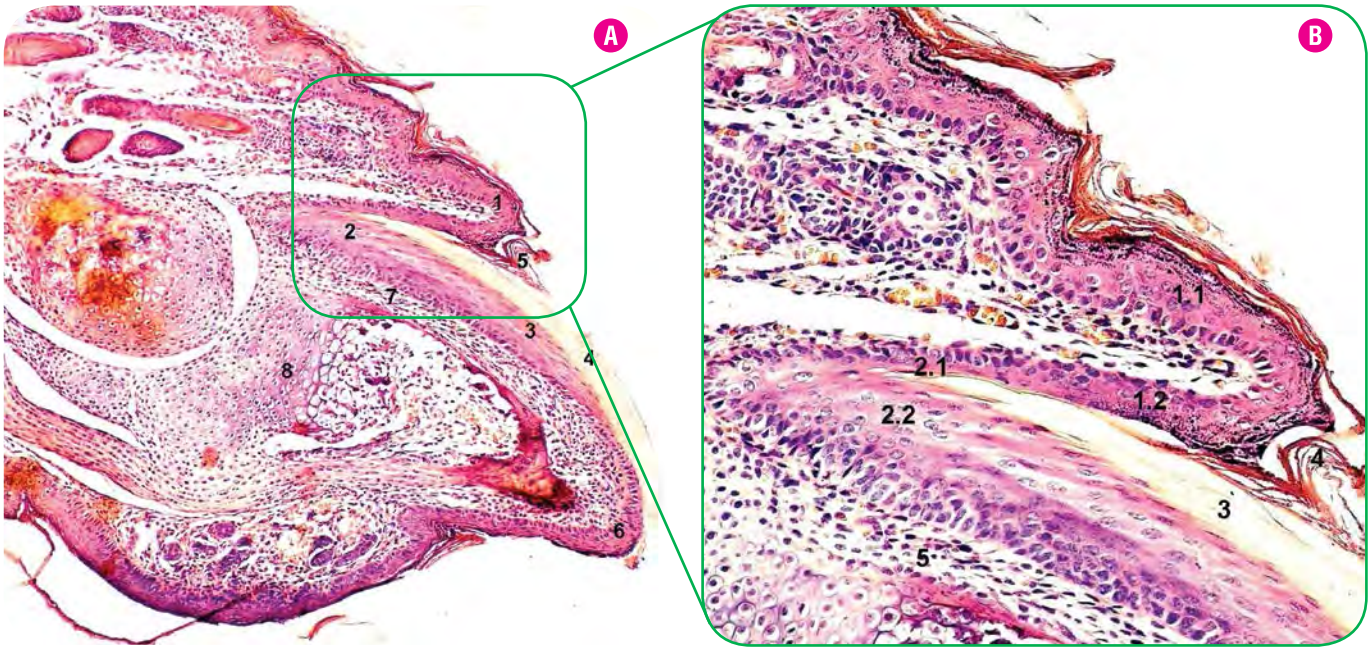

Fig. 1. Microphotography of the histological longitudinal sections of the adult mouse nail unit (hematoxylin-eosin staining, **A** – ×40, **B** – ×100).

A) 1 – proximal nail fold (PNF); 2 – nail matrix (NM); 3 – nail bed; 4 – nail plate; 5 – cuticle; 6 – hyponychium; 7 – onychodermis; 8 – bone of distal phalanx.

B) 1.1 – dorsal part of PNF; 1.2 – ventral part of PNF; 2.1 – dorsal part of NM; 2.2 – ventral part of NM; 3 – nail plate; 4 – cuticle; 5 – onychodermis.

As you know, ectodermal skin appendages in mammals (hair, nails, hoof, horns and teeth [14]) develop from epithelium and neural crest (derivatives of ectoderm [15, 16]) or mesenchyme (derivatives of mesoderm [17]). The presence of undifferentiated cells with high proliferative potential is demonstrated in the distal and proximal parts of the nail matrix [9, 10], ventral proximal nail bed [8] and onychodermis [11, 12, 13]. Among skin appendages, the most similar in structure to the nail unit is a hair follicle. Similar in their functions are hair follicles and nail matrix, hair shaft and nail plate [18]. In the hair follicle, the location of a pool of undifferentiated stem cells (bulge) that are derivatives of the neural crest [19, 20] is revealed, but the search for such a site in the nail organ is still ongoing.

The study of nail stem cells is very important both in the fundamental and in the applied aspect, since the search for new sources of stem cells for cell therapy is relevant today. In addition, the study of regulatory pathways for the interaction of different types of cells (keratinocytes, melanocytes, epitheliocytes, low-differentiated progenitors) of the nail unit will allow more detailed disclosure of the mechanisms of the pathogenesis of such diseases as vitiligo [21], psoriasis [22, 23]. It is known that the manifestations of these diseases are often associated with changes in nails, which may have important diagnostic value, and will allow the development of new therapies due to effects on the signaling cascade of cells.

The study was conducted with an objective to establish morphology, morphometry, self-renewal ability and proliferative potential of cultured cells derived from the mouse nail unit.

MATERIALS AND METHODS

All experiments on animals are carried out in compliance with the international principles of the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (European Convention, Strasburg, 1986), Article 26 of the Law of Ukraine «On the Protection of Animals from Cruelty» (No. 3447-IV, February 21, 2006), as well as all norms of bioethics and biological safety.

The studies were performed on cell cultures obtained from FGV-C-Tg(GFPU)5Nagy/J mice transgenic for green fluorescent protein (GFP) gene, aged 6-7 days. Mice were kept under the standard conditions in the vivarium of the State Institute of Genetic and Regenerative Medicine (Kyiv, Ukraine), with ad libitum access to water and food.

Obtaining and cultivation of cells from the murine nail unit. The culture of the nail unit cells (NUCs) was obtained from tissue explants, which included the ventral part of the proximal nail fold (**Fig. 1B-1.2**), onychodermis (**Fig. 1A-7, 1B-5**), dorsal (**Fig. 1B-2.1**) and ventral (**Fig. 1B-2.2**) parts of the nail matrix (**Fig. 1**). This zone was isolated from the nail unit under sterile conditions under the binocular stereoscopic microscope MBS-10. The implants were cultured in a 35 mm Petri dishes in DMEM:F12 (*Sigma*, USA) supplemented with 15 % fetal bovine serum (*Sigma*, USA) and 40 μg/ml penicillin/streptomycin in a CO_2 incubator Thermo 150 (*Thermo* , USA) at 37 °C, 95 % humidity and 5 % CO_2. Passaging of the primary NUCs culture was carried out on the 14th day of cultivation, further subcultivation was carried out upon reaching the confluence.

Staining and morphometric analysis. After removing the culture medium, the cells were washed with phosphate buffered saline (PBS) and fixed in 96 % ethanol for 20 minutes. For morphological analysis, the cells were stained with May-Grünwald azur-eosin stain for 20 minutes.

To characterize the NUCs processes and cytoskeleton, the culture was stained with Heidenhain's iron hematoxylin. After fixation, the samples were washed with PBS, treated with 2.5 % ammonium iron (III) sulfate for 10 minutes at 37 °C in a thermostat, after which they were stained with Heidenhain's iron hematoxylin for 10 minutes at 37 °C in a thermostat and washed in PBS and in water.

For morphometric analysis (counting the number of nuclei, area of nucleus and cells, as well nuclear-cytoplasmic ratio), staining with Bemer's hematoxylin and eosin was used, which gives a bright contrast and clearly distinguishes the cytoplasm from the nucleus. Fixed and dried NUCs were stained with Bemer's hematoxylin for 5-10 min, and then kept for 10-15 minutes in water, after that they were stained with eosin for 3 min.

Vital microscopy and cytological analysis were performed using an inverted fluorescence microscope IX 71 (*Olympus*, Japan), with a digital camera DP-20 and QucikPHOTO MICRO software (*Promicra*, Czech Republic). Morphometric analysis was performed on digital microphotographs using ImageJ v. 1.45 software (*National Institutes of Health*, USA).

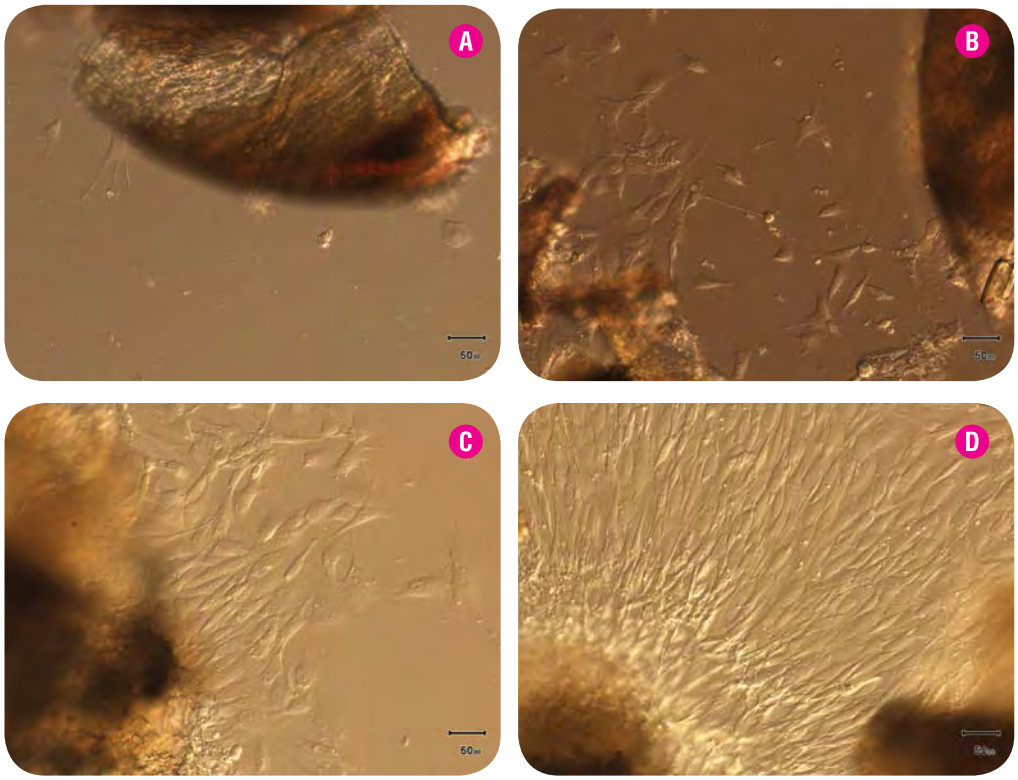

Fig. 2. Microphotographs of cell culture from mouse nail unit (×40, phase-contrast microscopy):
A – the 3rd day of cultivation;
B – the 6th day of cultivation;
C – the 9th day of cultivation;
D – the 12th day of cultivation.

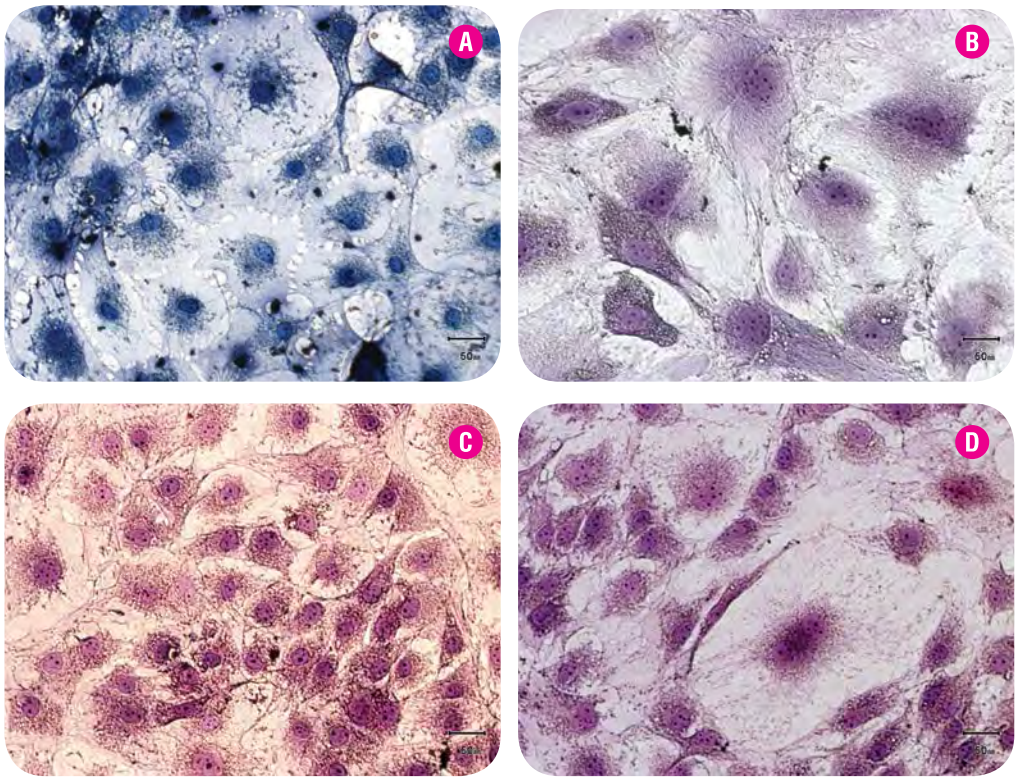

Fig. 3. Micrographs of the mouse nail unit cell culture (×100):
A – Heidenhain's iron hematoxylin staining (passage 2);
B – May-Grünwald staining (passage 2);
C – Bemer's hematoxylin and eosin staining (passage 1);
D – Bemer's hematoxylin and eosin staining (passage 4).

Clonogenic assay and self-renewal ability. The NUCs, starting from the 2nd passage, were seeded in a 35 mm Petri dishes, $12 \cdot 10^4$ per one dish ($12.5 \cdot 10^3$ cells per cm^2), and cultivated in the medium DMEM:F12 at 5 % CO_2 for a further 7 days. The medium was changed every 48 hours. The cells were taken with 0.25 % trypsin solution with EDTA and were counted in Goryaev chamber. We calculated the population doubling time and the growth rate according to the standard formulas [24].

For the clonogenic assay (counting of colony-forming units), a serial dilution method in a 96-well plate with an initial concentration of $2 \cdot 10^4$ cells/ml or $4 \cdot 10^3$ cells in the first well (200 µl) was used, which was ultimately brought to dilution in 1 cell per well [25]. The final largest number of NUCs in the well was $1 \cdot 10^3$, which decreased in geometric progression to 1. After 10 days in culture, cells were fixed with 4 % paraformaldehyde solution and stained with May-Grünwald stain.

Fig. 4. Dynamics of the population doubling time (**A**) and population growth rate (**B**) of the mouse nail unit cell culture at 2, 3, 4 and 5th passages.

Statistical analysis. The statistical analysis of the obtained results was carried out using the Statistica 10 (*StatSoft,* USA) and MS Excel (*Microsoft*, USA) software. Morphometry was performed using ImageJ v. 1.45 software. The results are presented as M ± m (mean ± standard error of the mean).

RESULTS AND DISCUSSION

On the 3-4th day of culture, migration and active proliferation of fibroblast-like cells began from the explants of the nail unit (**Fig. 2**). Obtained mouse NUCs reached confluent monolayer on the 14th day of cultivation on the average. Most cells had fibroblast-like morphology with bipolar processes, but there were widespread cells of polygonal form without processes, as well as rounded, not attached cells.

STAINING AND MORPHOMETRIC ANALYSIS.

The NUCs were characterized by the nucleus of the correct round form at the presence of several clearly visible nucleoli (**Fig. 3**). After staining by various methods in the perinuclear cytoplasm of many cells, there are fine dispersed grains – a sign of abundant granular endoplasmic reticulum and active protein synthesis. By the form, the NUCs are round, polygonal and fibroblast-like with long bipolar processes. Such heterogeneity of cells form was also noted in other previous studies in the cultivation of human nail matrix (polygonal and spindle-shaped cell morphology). [26] All cells have numerous short and thin processes, most of which form contact with surrounding cells.

The obtained morphometric data (**Table 1**) showed an increase in the area of NUCs in 4.5 times and nucleus in 4 times at subsequent passages (comparison was made on 1 and 4 passages). However, this increase is not proportional, since the nuclear-cytoplasmic ratio (NCR) on these passages did not change and remained stable low and averaged 0.071 ± 0.01. Moreover, such data regarding the low value of NCR are corresponding with the results of another experiment on the cultivation of human nail matrix [27]: besides low NCR, a high level of euchromatin to heterochromatin was shown by electronic microscopy, which indicates the high activity of the synthesis processes in the nucleus. In our work, the increased number of nucleoli (an average of 4 per nucleus versus 1-2 generally) evidenced the significant level of synthesis activation.

Table 1. Data of the morphometric analysis of the cell culture from mouse nail unit.

PASSAGE	CELL'S AREA (μm²)	NUCLEUS AREA (μm²)	NUCLEAR-CYTOPLASMIC RATION	NUMBER OF NUCLEOLI
P1	1032 ± 61	62 ± 3	0.078 ± 0.004	4 ± 0.2
P4	5235 ± 595	239 ± 13.5	0.065 ± 0.003	3 ± 0.2

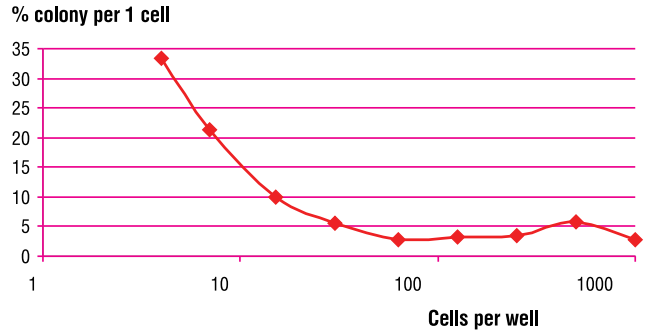

Fig. 5. Clonogenic assay of the mouse nail unit cell culture at the 5th passage.

PROLIFERATIVE POTENTIAL AND SELF-RENEWAL ABILITY.

The population doubling time of the NUCs culture (**Fig. 4A**) averaged 80 ± 6.5 hours, with the fastest cells growing at the 4th passage (63 ± 7 hours). The growth rate of the cell culture (**Fig. 4B**) is generally low (0.01 ± 0.0007), which may be due to the culture of the NUCs at once from several zones of the probable location of the low-differentiated cells, as well as with their small number in comparison with the amount of other cells.

Clonogenic assay was performed at the 5th passage, which could have had an effect on the colony forming efficiency, since it was only 4 % (**Fig. 5**), the highest frequency of colonies (33 ± 3.6 %) per cell was observed at a cell concentration of 4 cells per well. A significant number of colonies was small with large poorly proliferative cells, which may indicate a production of large numbers of transitional progenitor cells.

In general, the data presented in the morphological and morphometric analysis of primary cultures of cells from the murine nail unit are only the initial stage of further detailed and thorough study of this type of cells. Heterogeneity of these cell population *in vitro* confirms the complexity of the histological structure of the nail unit and the presence of many types of cells in it, which may differ in morphological characteristics, proliferative activity, surface and intracellular markers, responsible for intercellular interaction and the realization of specific functions, including reparative potentials [29, 30]. Therefore, the issue of obtaining pure cultures of cells derived from specific anatomical areas of the nail unit, in particular, from the proximal nail fold, nail matrix and onychodermis, remains actual.

CONCLUSIONS

The obtained cell culture from the mouse nail unit according to the analysis of their morphology, proliferative potential and the results of morphometry is heterogeneous and requires the further development of pure culture technologies for the detailed characterization of separate subpopulations of cells.

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The authors indicate no potential conflicts of interest.

Received: April 14, 2017
Accepted: May 22, 2017