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Preparation of rabbit kidney immortalized cell culture

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SUMMARY
Preparation of immortalized cell lines obtained from organs and tissues of farm animals is an essential area of biotechnology. The paper presents results of continuous (immortalized) cell line preparation from a primary trypsinized cell culture of an adult rabbit kidney. Cytomorphologic analysis and karyotyping were performed during the process of subcultivation in the cell culture at passages 1, 3, 24, 31, 38, 56, 66, 75, 86, 101. Dynamics of spontaneous continuous cell line formation during long-term serial passaging was examined using standard nutrient media and fetal serum. Contrary to the known cell lines of rabbit origin (*Oryctolagus cuniculus* L.), immortalization was not accompanied with enhanced cell production and cell size reduction. The prepared continuous cell line in its adhesive phase was up to 200 μm in size and its productivity was about 7,000 cells/cm^2. Significant differences (compared to the known cell lines) in the karyotype were detected during passaging. The formed genotype was found to be near-tetraploid when the CCL cultural properties were stabilized at passages 66–101. The known cell lines – rabbit kidney (RK-13) and rabbit cornea (SIRC) – can be characterized as pseudotriploid basing on their karyotype. This culture demonstrated low sensitivity to viruses – causative agents of rabbit diseases and sensitivity to heterologous porcine and bovine viruses.

Key words: continuous cell line, primary trypsynized cell culture, immortalization, apoptosis, telomere.

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Получение иммортализованной культуры клеток почки кролика

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РЕЗЮМЕ
Получение иммортализованных клеточных линий, происходящих из органов и тканей сельскохозяйственных животных, является актуальным направлением биотехнологии. В статье представлены результаты получения постоянной (иммортализованной) клеточной линии из первично трипсинизированной культуры клеток почки взрослого кролика. В процессе субкультивирования проводили цитоморфологические и кариологические исследования на 1, 3, 24, 31, 38, 56, 66, 75, 86, 101-м пассажах. При проведении длительных последовательных пассажей была прослежена динамика спонтанного формирования постоянной клеточной линии с использованием стандартных питательных сред и эмбриональной сыворотки. В отличие от известных клеточных линий, произошедших от обыкновенного домашнего кролика (*Oryctolagus cuniculus* L.), было отмечено, что иммортализация не сопровождалась увеличением продуктивности клеток и уменьшением их размеров. Размеры клеток полученной перевиваемой линии достигали в адгезированном состоянии 200 микрометров, продуктивность составляла 7000 клеток на квадратный сантиметр. Значительные отличия (в сравнении с известными линиями) в процессе пассирования обнаруживались и в кариотипе. При стабилизации культуральных свойств постоянной линии клеток на 66–101-м пассажах генотип сформировался как околотетраплоидный. Известные клеточные линии – почка кролика (RK-13) и роговица глаза кролика (SIRC) – по кариотипу оказались псевдотриплоидные. Данная культура оказалась малочувствительной к вирусам – возбудителям болезней кроликов и чувствительной к гетерологичным вирусам свиней и крупного рогатого скота.

Ключевые слова: постоянная линия клеток, первично трипсинизированная культура, иммортализация, апоптоз, теломеры.

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INTRODUCTION
Immortalization of subcultured cells of mammalian organs can be considered as a shift away from terminal proliferation arrest which is eventually observed in all normal somatic cells. In early 60s of the last century L. Hayflick and P. S. Moorhead discovered that normal diploid human fibroblasts proliferate *in vitro* only a certain number of times and subsequently they stop dividing and start aging [1–3]. Given that the process of aging clearly differs from any form of a cell's death, for instance, apoptosis, it is essential to distinguish these statuses during successive passages. Studies, performed by researchers in 1990s demonstrated that immortalization is associated with activation of telomere maintenance mechanism via telomerase activity or other unknown process [2, 4]. Immortalization is a complex process associated with many genetic modifications a part of which may not be related to telomere maintenance [4]. There is evidence for existence of three basic compensatory mechanisms of maintaining viability and stimulating cell line proliferation in case of the genetic material (telomere) loss: polyploidization of the initial cell clone, oncogene amplification, and amplification of whole autosomes or their fragments.

Cell culture specialists know only a small amount of stable and high performance continuous cell lines (CCL) of rabbit origin (*Oryctolagus cuniculus* L.). The most widespread lines are: RK-13 (rabbit kidney, obtained in 1963), SIRC (rabbit cornea, obtained in 1965), RSK (rabbit skin). Methods of karyotyping used for identification of the RSC continuous cell line obtained from the VIEV revealed that it belonged to porcine cell lines [5]. The rest cell lines from the Catalogue of the Russian Cell Culture Collection (RCCC), split ratio 1:2, have the status of subcultures [6]. Preparation of non-malignant continuous (immortalized) mammalian cell lines, including those from rabbit organs, is not performed by stereotypical manipulations. Stable CCL develop spontaneously: through long-term passaging in stable conditions or through changing cultivation conditions – change of media, sera and other components.

Organs of newborn animals or their embryos are used for long-term passaging of mammalian cells. The status of these cells is indicative of their longer mitotic activity *in vitro*.

The aim of the research was preparation of the primary cell culture, sensitive to rabbit viruses, from a kidney of an adult animal, maintaining of the subculture during long-term successive passages, and studying the degree of the prepared cell line transformation.

MATERIALS AND METHODS
Kidneys of a 4–5 month old rabbit weighing 2.0–2.5 kg were taken for the test. The primary cell culture was prepared using a modified method of fractional trypsinization [7–9]. Following testing of primary and subcultured cells for sensitivity to rabbit viruses, the cells were subcultured on DMEM/F-12 (pH 7.1–7.2) with 0.1% lactalbumin hydrolysate, containing 5–10% bovine fetal serum [10]. Subsequently the subculture was adapted to the MEM-like medium.

Rabbit kidney cells were cultured in a thermostat according to the generally accepted method in glass and plastic vessels with different growth areas at (38.0 ± 0.5) °C.

Cell morphology tests. Morphology of native cells from the primary trypsinized culture was studied using a phase-contrast microscopy technique.

For cell culture identification P. S. Moorhead's method for formed metaphase plate karyotyping (1960) was used.

Lifetime cytochemical detection of mycoplasma contamination and cell morphology studies were performed using 0.001% acridine orange dye.

Comparative studies of two stable widespread cell lines of rabbit origin: RK-13 – rabbit kidney and SIRC – rabbit cornea cells, were performed.

RESULTS AND DISCUSSION
Any process of primary cell subcultivation includes long-term continuous passaging of the prepared populations [11]. In this respect monitoring of cytogenetic and cultural characteristics of cells derived from an adult rabbit kidney was performed. For this purpose subculturing was performed twice a week starting with passage 4, split ratio 1:2, 1:3. Herewith, the monolayer confluence was 100%, cells remained large – up to 50 μm in diameter [10]. Besides DMEM/F-12 MEM-like (1/3) was added to the culture medium starting with passage 4, and starting with passage 45 the culture was grown on MEM-like medium with 10% bovine fetal serum (Germany).

Cytomorphologic analysis and karyotyping were performed during the process of subcultivation in the cell culture at passages 1, 3, 24, 31, 38, 56, 66, 75, 86, 101. The cells were subject to cryopreservation at approximately the same passages and afterwards their ability to revive was observed. Vials with cells derived from an adult rabbit kidney (RK) concentrated to the density of (2.0–5.0) × 10^6 cells/ml in the fresh growth medium with 10% dimethylsulphoxide, were frozen using a programmable freezer and stored in liquid nitrogen. The cells were thawed by intense agitation in a water bath at 37–38 °C. The survival rate was 80–90%. The optimal level of the cell culture proliferative activity observed prior to freezing was achieved already at passage 2 after thawing (Fig. 1).

The level and dynamics of proliferation have become stable by passage 38. By the same passage population

polymorphism decline was observed. In contrast to the primary subcultured populations epithelioid and spindle-shaped cells began to prevail (Fig. 2). In case the culture was kept over three days pseudo-syncytia (Fig. 3) were formed which hindered examination and studying of cytoplasmic structure. Cells grown in culture vessels of different volume had an equal morphological status.

Micrometry of the RK confluent monolayer was performed at passage 101. Figure 4 shows a micrometer grid used for cell size measuring (minimal cell size – 10 μm). The average size of epithelioid cells was 30–50 μm. The size of spindle-shaped cells reached 100 μm. During pseudosyncytium formation the number of cells did not increase, the cells remained alive for up to ten days at 37 °C, pH did not demonstrate any critical change and was about 6.9–7.0.

As seen from the Table the productivity of the subculture and the forming continuous cell line was not very high. Growth rate on the flat surfaces (300 cm^2) reached 4.1 within four days and 1.7 – in rotating vessels (800 cm^2) within three days. When in 4 hours the seeding concentration was 40,000 cells/ml adherent cells covered the entire surface of the culture vessel. In 72 hours the monolayer became dense and the productivity increased up to 100,000 cells/ml.

Cell micrometry data correlated with the productivity of the prepared cell line which did not exceed 130,000/cm^2.

The prepared cell culture was used in different FGBI "ARRIAH" units for virological testing. It turned out to be sensitive to infectious bovine rhinotracheitis, classical swine fever, and Aujeszky's disease viruses. Cytopathic effect was detected in the RK cell culture after the first passage of the myxomatosis virus. The decrease of the virus cytopathic effect (CPE) was observed during subsequent passaging. CPE of the rabbit haemorrhagic disease virus was not observed in RK cell culture.

The main objective of the research was not achieved as the prepared cell line had low sensitivity to the viruses – agents of rabbit diseases. Although many heterologous viruses reproduced effectively with titer increase at passages. That is why studies of cytological and karyotypic changes in the new continuous cell line were continued.

Cytokaryotypic transformation. The studies of cytokaryotypic signs of the most common CCLs derived from rabbit organs and tissues – RK-13 (rabbit kidney) and SIRC (rabbit cornea) – revealed that both of these cultures have a near-triploid karyotype (Fig. 5, 6) and a distinct cell morphology with a predominance of epithelial-like cells in RK-13 (Fig. 7) and spindle-shaped cells in SIRC (Fig. 8).

In appropriate cultivation conditions the SIRC cell line produces extracellular matrix (presumably, of protein origin) which aggregates cells during trypsinization and monolayer formation. In this case one of the reasons for continuous cell line formation derived from different rabbit organs is gene amplification by 40–47% due to autosome extracopying. Probably this is the reason for telomerase activity increase and formation of stable cell line immortalization.

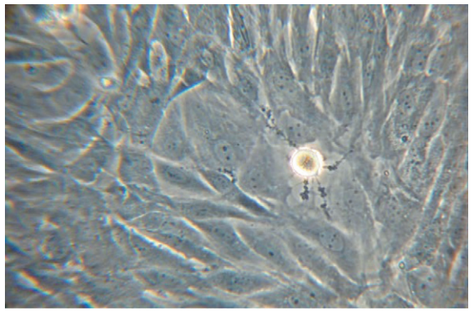

Fig. 1. Morphology of RK cells, passage 2
Рис. 1. Морфология клеток ПКр, 2-й пассаж

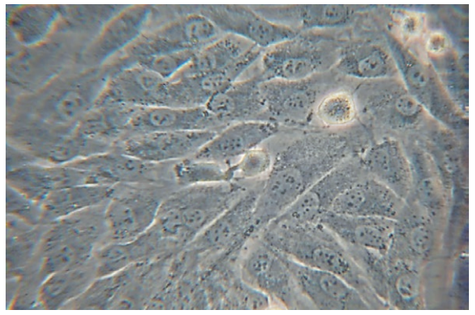

Fig. 2. Morphology of RK cells, passage 38–56
Рис. 2. Морфология клеток ПКр, 38–56-й пассаж

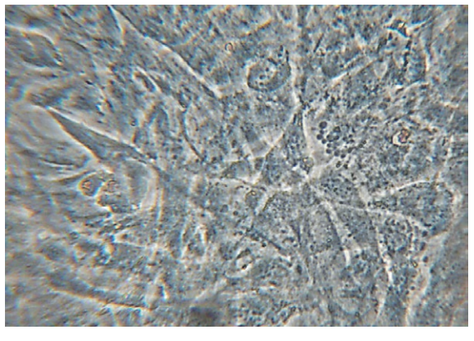

Fig. 3. RK pseudosyncytium
Рис. 3. Псевдосинцитий ПКр

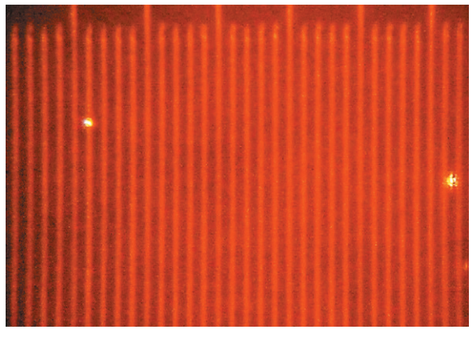

Fig. 4. Micrometer grid, cell size – 10 μm
Рис. 4. Микрометрическая сетка, размер одного деления 10 мкм

Table
Dynamics of the RK continuous cell line proliferation in different culture media

Таблица
Динамика пролиферации постоянной линии клеток ПКр в разных культуральных сосудах

Conditions for cell growth on a monolayer	Seeding concentration, mln/cm^3	Cultivation time, days	Cell productivity, mln	Cell productivity, 1 cm^2	Growth rate
Glass culture vessel (cultivation flask), volume 1,500 ml (300 cm^2)	0.02	4	25.0 ± 0.2	83,000	4.1
	0.04	3	30.0 ± 0.2	100,000	2.5
	0.05	3	40.0 ± 0.1	130,000	2.6
Rotating vessel, volume 3,000 ml (800 cm^2)	0.07	3	50.0 ± 0.2	62,000	1.7
	0.10	3	70.0 ± 0.2	87,000	1.7

Near-triploid chromosome set in comparison with normal, diploid karyotype is a consistent and specific sign of a long-term passaging of RK-13 and SIRC continuous cell lines. Split ratio of these "old" CCLs reaches 1:4, 1:8.

Immortalization of cell lines considerably changes cytomorphological and physiological properties of tissue-derived primary cells. This particularly affects the CCL sensitivity to viruses. Very often continuous cell lines become non-sensitive to homologous viruses and sensitive to heterologous viruses.

Thus, RK-13 cells are sensitive to African horse sickness, swine vesicular disease, cowpox and at the same time to myxomatosis and rabbit pox viruses. SIRC CCL is used for roseola virus reproduction.

As previously noted the prepared RK cell line exhibited immortalization signs starting from passage 30. Performed karyotyping analysis demonstrated the tendency for RK cell hyperdiploidization starting from passage 38 (Fig. 9). Starting from passage 56 near-tetraploid population prevalence was observed (Fig. 10). The performed karyotyping

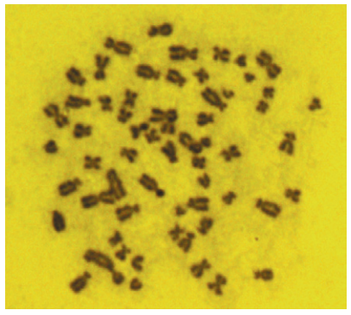

Fig. 5. RK-13 metaphase (62 chromosomes)
Рис. 5. Метафаза RK-13 (62 хромосомы)

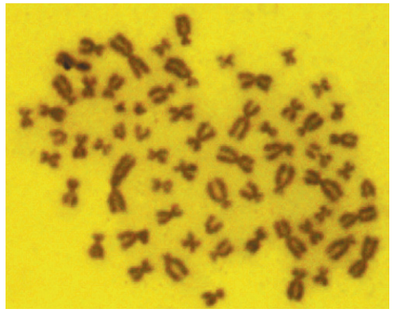

Fig. 6. SIRC metaphase (65 chromosomes)
Рис. 6. Метафаза SIRC (65 хромосом)

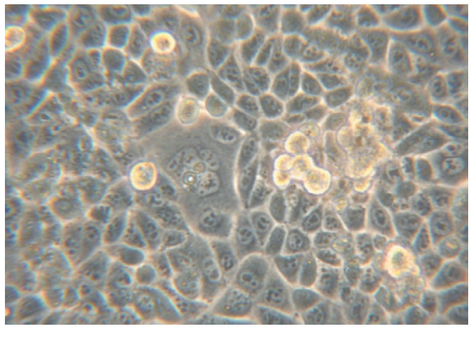

Fig. 7. RK-13 CCL morphology
Рис. 7. Морфология ПЛК RK-13

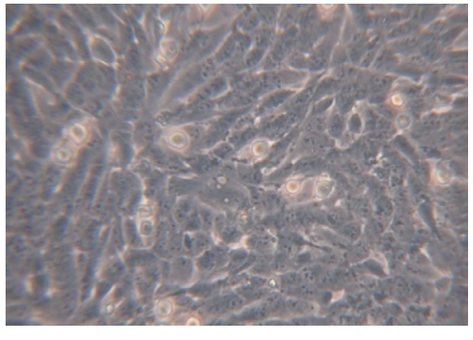

Fig. 8. SIRC CCL morphology
Рис. 8. Морфология ПЛК SIRC

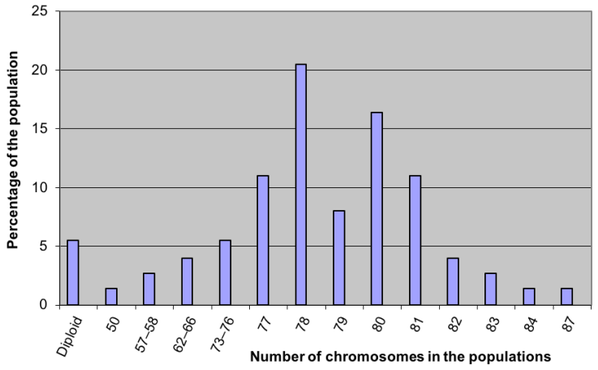

Fig. 9. RK cell line ideogram, passage 38

Рис. 9. Кариограмма клеточной линии ПКр, 38-й пассаж

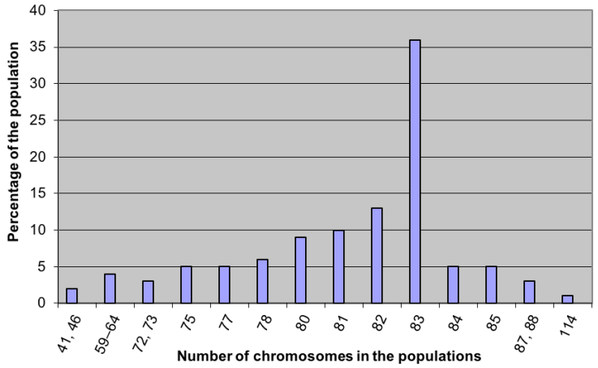

Fig. 10. RK cell line ideogram, passage 56

Рис. 10. Кариограмма клеточной линии ПКр, 56-й пассаж

at passages 66, 75, 86 and 101 demonstrated stability of near-tetraploid population (77–83 chromosomes) in the karyotype (Fig. 11).

Variability of the RK cell line populations basing on the amount of chromosomes was consistent with the variability of the immortalized cell line with stable cytomorphological and cultural characteristics. It should be noted that the number of polyploids at long-term passaging in standard optimal conditions and keeping for up to 10 days at 37 °C was 1%.

The cytochemical analysis of the CCL derived from an adult rabbit kidney demonstrated polymorphism both of cell and nucli (Fig. 12). This method did not detect mycoplasmas in the intercellular space and on the cell membranes. Absence of latent contamination allows performing long-term passaging of the CCL without changing split ratio, culture conditions, and without a cryo stage.

This culture turned out to be sensitive to viruses of infectious bovine rhinotracheitis, classical swine fever, and Aujeszky's disease viruses. Cytopathic changes detected in the RK culture were caused by the rabbit myxomatosis virus and the titer increase was not observed during passaging.

CONCLUSION

Immortalization of cell cultures derived from organs of farmed animals has several patterns which are the reason for rather a small amount of permanent cultures. There are, for instance, only five cell lines derived from a European rabbit (*Oryctolagus cuniculus* L.). It is well known that the probability of spontaneous formation of continuous cell lines from organs and embryos and newly-borns is higher than from organs of adult animals and such cell lines are more preferable as they contain a large amount of stem cells with an increased proliferation activity [3]. As demonstrated by E. L. Duncan and R. R. Reddel spontaneous immortalization of non-tumorigenic origin can have unpredictable ways of cell transformation *in vitro* [2, 11]. The results of the research for rabbit kidney CCL stabilization at the tetraploidy level bring new perspectives to use of adult animal organs. It was established that the prepared subculture was not highly productive but it can be used as a substrate for cultivation of porcine and bovine heterologous viruses.

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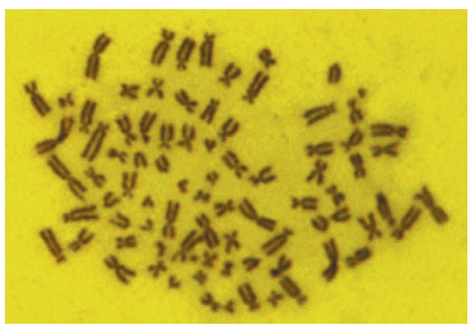

Fig. 11. Metaphase plate of the RK cell line, passage 56, 83 chromosomes

Рис. 11. Метафазная пластинка клеточной линии ПКр, 56-й пассаж, 83 хромосомы

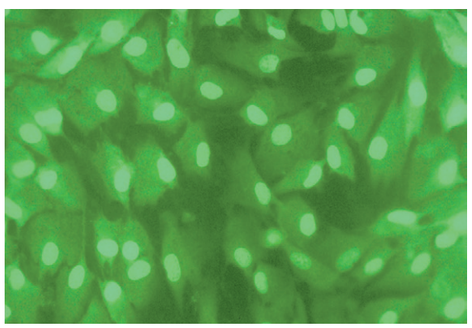

Fig. 12. RK cytochemical preparation. Staining with acridine orange, lens ×40

Рис. 12. Цитохимический препарат ПКр. Окраска акридиновым оранжевым, объектив ×40

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