# Mouse Hepatocytes Retain the Expression of the Main Differentiation Markers during Culturing on Collagen-Chitosan Matrices

**A. V. Eremeev[1,2], A. V. Svetlakov[1,2], I. N. Bolshakov[2], U. I. Sheina[1,3], O. D. Nekhlyudova[2], and N. A. Setkov[4,5]**

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 151, No. 5, pp. 581-584, May, 2011
Original article submitted January 28, 2010

Mouse hepatocytes cultured on artificial 3D collagen-chitosan biopolymer matrices retained the expression of hepatocyte markers for 14 days.

**Key Words:** *primary hepatocyte culture; cell matrix; hepatocyte markers; collagen-chitosan complex*

Artificial materials intended for substitute therapy for human diseases involving loss of functionally competent cells have been created in recent years. Normal tissues are characterized by complex ultrastructure, including, in addition to various specialized cells, the extracellular matrix which serves as not only mechanical support for cells and blood vessels, but also provides delivery of proliferative and differentiation signals to them. Acknowledgment of the absolute importance of the matrix for the formation of the natural cellular microenvironment led to a wide-scale search for its artificial substitutes and creation on their basis of constructions providing more accurate reproduction of cell-cell interactions characteristic of intact tissues. It is assumed that 3D biopolymeric microenvironment promotes normal orientation of the cell cytoskeleton and enables cell growth and migration in the matrix, with proliferative activity and differentiation status of these cells retained. Natural biopolymers (collagen, fibronectin, polyoxyalcanates, alginates, and hydrogels with different bases) and synthetic materials (lactide or glycolide biodegraded polyesters, *etc.*) are now used. Biopolymers on the basis of chitosan and its derivatives are also interesting in this respect. We have previously shown the possibility of long-term culturing of human and animal fibroblasts and human fetal stem cells on collagen-chitosan matrix (CCM) [2-4], and scientists in St. Petersburg created chitosan films for culturing of human skin fibroblast [6]. On the other hand, there is a pressing need in the creation of artificial 3D matrices supporting and preserving for a long time the activity of differentiated functionally intact cells, for example, liver cells. These matrices can be fit for not only transplantation, but also for short-term support of a defective organ.

Polysaccharide biopolymers possess unique characteristics, are suitable for preparing cell matrices, and are biocompatible with human tissues [2-4], which suggests that CCM could maintain viability of hepatocytes during long-term culturing without changing the differentiation level and do not exhibit cytotoxicity. The aim of this study was to verify this hypothesis.

## MATERIALS AND METHODS

The study was carried out on newborn ICR rats. The parental population was obtained from vivarium of Institute of Cytology and Genetics (Novosibirsk).

[1]Center of Reproductive Medicine, Krasnoyarsk; [2]Department of Operative Surgery with Topographic Anatomy, Laboratory of Cell Technologies, V. F. Voino-Yasenetsky State Medical University, Ministry of Health and Social Development, Krasnoyarsk; [3]Laboratory of Genetic Bases of Cell Technologies, N. I. Vavilov Institute of General Genetics, Russian Academy of Sciences, Moscow; [4]Krasnoyarsk Research Center, Siberian Division of the Russian Academy of Sciences; [5]Siberian Federal University, Krasnoyarsk, Russia. ***Address for correspondence:*** artkingf@rambler.ru. N. A. Setkov

0007-4888/11/1515-0642 

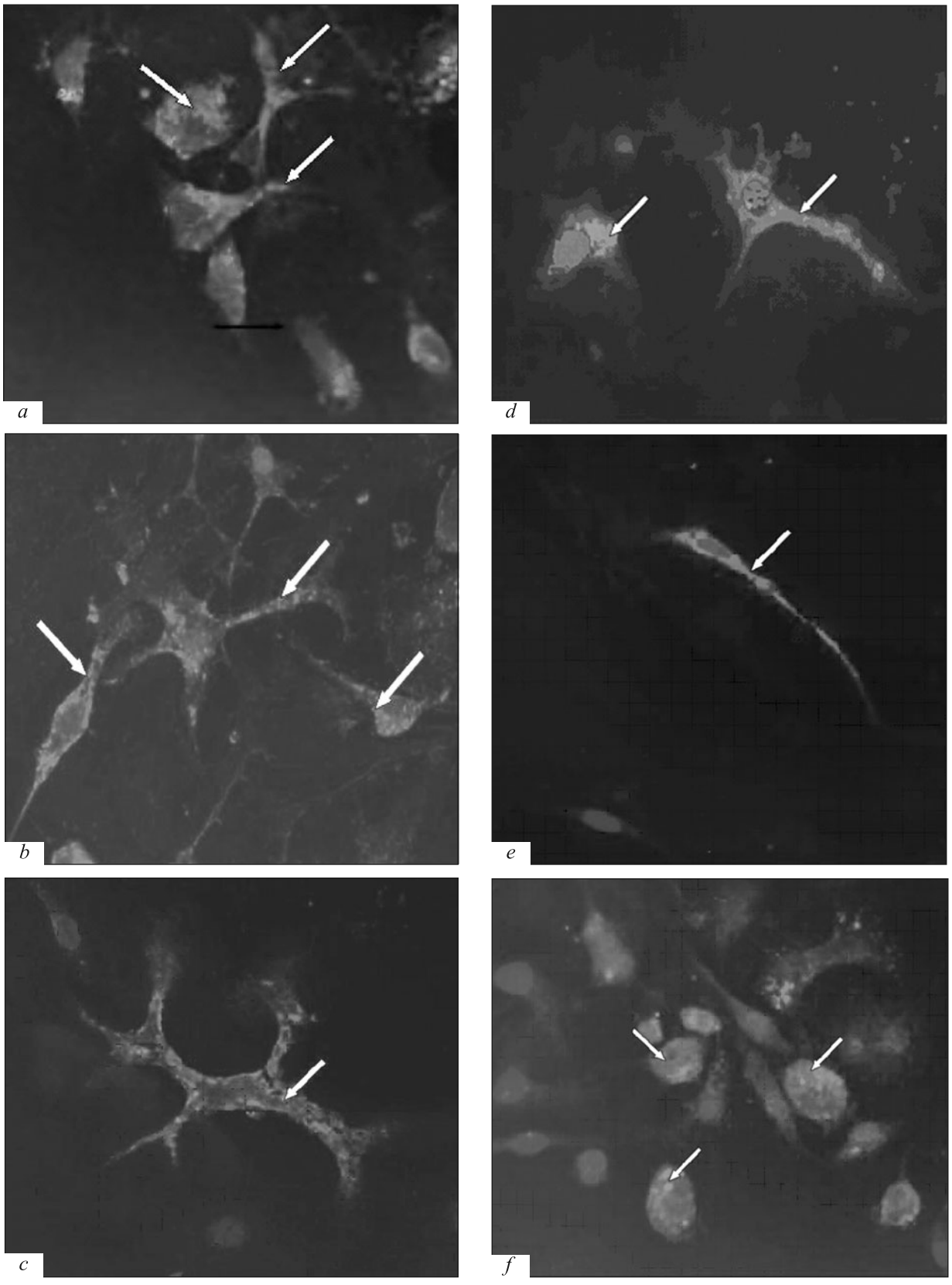


**Fig. 1.** Immunocytochemical visualization of differentiation markers on the surface of hepatocytes cultured on gelatin (*a*, *c*, *e*) or on CCM (*b*, *d*, *f*). Cells fixed in 1% formaldehyde in phosphate buffer and treated with antibodies to α-fetoprotein (*a*, *b*), hepatocytic marker (*c*, *d*), or antitrypsin (*e*, *f*) with subsequent labeling with second antibodies and fluorescence detection (shown by arrows).

**TABLE 1.** Mitotic Activity (Number of Metaphase Plates) in Mouse Liver Hepatocytes Cultured on Gelatin or CCM (*M*±*m*)

| Culturing medium | Day of culturing | | | | |
|---|---|---|---|---|---|
| | 1 | 3 | 6 | 9 | 14 |
| Gelatin | 59.7±5.6 | 24.3±2.1 | 7.2±0.3 | 11.2±1.4 | 0.16±0.04 |
| CCM | 67.4±2.0 | 30.9±2.0 | 6.9±0.1 | 11.7±0.4 | 0.18±0.06 |

Collagen-chitosan matrices for cell culturing were prepared as described previously [1] with subsequent lyophilization of the mixture, containing 2% collagen acetate solution, 2% solution of >95% deacetylated chitosan ascorbate (100-700 kDa), containing 1.8 g ascorbic acid per 1 g dry chitosan, 5-100 mg chondroitinsulfuric acid, 10-100 mg D-glucuronic acid, and 2.5-5 mg heparin.

In order to reduce acidity of the resultant spongy matrices, they were plunged in sterile bicarbonate buffer before culturing. After this treatment, the matrices were washed 3 times in Dulbecco's sterile phosphate buffer, placed into culture flasks, and cell suspension in medium containing all components needed for hepatocyte culturing was carefully layered.

Primary hepatocyte culture was obtained from the livers of 5-7-day-old mice [5]. Complete resection of the liver was carried out under ether narcosis. The livers were carefully fragmented with ophthalmic scissors in 0.5% Hanks saline with collagenase from *Clostridium histiliticum*, 0.8 U/ml (Serva). The cells were then filtered through a sterile Nylon filter with 50-μ pores. The resultant cell suspension was washed 3 times from collagenase in pure F12 medium (Sigma-Aldrich) and the cells were inoculated in flasks coated with 0.1% gelatin or on CCM.

The cells were cultured at 37°C in DMEM/F12 mixture (Sigma-Aldrich) with 10% serum substitute (SR; Sigma-Aldrich), 100 μg/ml kanamycin sulfate, 1 mM essential amino acids (Sigma-Aldrich), 0.2 mM L-glutamine, 0.5 mg/ml BSA, 0.1% glucose, 0.5 μg/ml insulin, 0.2 μg/ml glucagon, 50 ng/ml dexamethasone, 0.02 mM β-mercaptoethanol, 10 mM HEPES, and 10 ng/ml epidermal growth factor (Biolot; complete medium). All manipulations were carried out under sterile conditions. The status of the culture was evaluated visually under an Olympus BX-51 microscope; at least 100 fields of view were examined.

In order to detect hepatocyte markers, every 3 days the cells were fixed in formalin with subsequent immunocytochemical detection using antibodies (Abcam) to hepatocyte-specific antigen (hepatocyte marker), antitrypsin, and α-fetoprotein. The markers were detected by the method suggested in the instruction of antibody manufacturer. Cell nuclei were stained with DAPI (0.1 μg/ml) for 10 min. The images were analyzed in an Olympus BX-51 fluorescent microscope using Applied Spectral Imaging software. Each cell marker was detected repeatedly: 3 times in 3 flasks with cells, 6 zones in each of them chosen at random for immunocytochemical studies; 30 visual fields in each zone were examined under a microscope.

Demecolcine (0.01 mg/ml) was added to culture flasks 4 h before cell fixation. The cells for cytogenetic studies were fixed by the standard method using 0.6% sodium citrate solution as the hypotonic reagent with the fixative (methanol:glacial acetic acid 3:1). Chromosome preparations were stained with 4% Giemsa stain after pretreatment in 0.1% trypsin in phosphate buffer. Metaphase plates were counted in 100 visual fields under an Olympus BX-51 microscope.

## RESULTS

Culturing of mouse hepatocytes on CCM did not change the cell differentiation status (Fig. 1). The cells expressed hepatocyte markers after 14 days of culturing similarly as the cells cultured by the traditional method on gelatin.

Mitotic activity of hepatocytes evaluated by the number of metaphase plates was virtually the same for hepatocytes cultured on gelatin and CCM (Table 1).

Reduction of mitotic activity over 14 days was presumably due to increase in cell density (density-dependent growth suppression), characteristic of normal nontransformed cell cultures, or was caused by culture aging.

Hence, the results indicate that hepatocytes retain their morphology and phenotype, expressing specific differentiation markers, after culturing on CCM.

## REFERENCES

1. I. N. Bolshakov, S. M. Nasibov, A. V. Eremeev, *et al.*, *Patent of the Russian Federation No. 2252787*, *A61 L 15/28, 15/32. 27/60*, *Byull. Izobr.* No. 15 of 27.05.2005.
2. A. V. Eremeev, A. S. Zamai, N. V. Zotova, *et al.*, *Tsitologiya*, **9**, 804 (2008).

3. A. V. Eremeev, A. V. Svetlakov, I. N. Bolshakov, and Yu. I. Sheina, *Sib. Med. Obozreniye*, No. 5, 41-46 (2009).
4. A. V. Eremeev, A. V. Svetlakov, I. N. Bolshakov, *et al.*, *Klet. Transpl. Tkan. Inzh.*, **4**, No. 2, 55-62 (2009).
5. A. V. Eremeev, O. A. Timofeyeva, V. I. Kaledin, and N. A. Setkov, *Tsitologiya*, **46**, No. 4, 301-311 (2004).
6. E. F. Panarin, P. A. Nud'ga, V. A. Petrova, *et al.*, *Ibid.*, No. 3, 42-46.