ISSN 0012-4966, Doklady Biological Sciences, 2017, Vol. 475, pp. 175–179. © Pleiades Publishing, Ltd., 2017.
Original Russian Text © Y.Y. Khrunyk, I.V. Vyalykh, A.V. Korelin, S.V. Belikov, M.S. Karabanalov, S.B. Rakitin, R.V. Kamalov, A.A. Popov, 2017, published in Doklady Akademii Nauk, 2017, Vol. 475, No. 2, pp. 226–230.

CELL BIOLOGY

Isolation of Primary Osteoblast Cell Lines from Adult Rat and Rat Embryos and Their Use as Models for in Vitro Biocompatibility Tests of Nanostructured Titanium-Based Implants[1]

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Presented by Academician O. N. Chupakhin January 19, 2017

Received February 6, 2017

Abstract—Methods for obtaining osteoblast cultures from the calvaria of adult Wistar rats and 12-day-old embryos of these rats have been adapted for studying the biocompatibility and ossointegration of titanium-based implants. The osteoblast morphology and their differentiation into osteocytes on a titanium matrix with specially treated surface have been studied. It has been confirmed that two cultures of diploid rat cells obtained in the study can serve as efficient models for preclinical in vitro testing of nanostructured titanium implants for biocompatibility and osseointegration.

DOI: 10.1134/S0012496617040032

Nowadays titanium is the most preferable biocompatible metal for implants manufacturing. It is resistant to corrosion, has good mechanic characteristics and osseointegrative properties. During the last decade the research in this field was focused on various structural modifications of titanium constructions, aimed to foster implants biocompatibility [1]. The studies of the effect of titanium surface nanostructuring on the implants osseointegrative properties became crucial in this field. Implants, covered with three-dimensional surface layer of nanoparticles of a defined/undefined geometry, have unique properties, which need to be extensively researched.

In vitro analyses of titanium and its alloys were conducted employing different cell lines including rat bone mesenchymal stem cells [2], Sprague Dawley rat osteoblast cultures [3], human osteoblasts [4], human neural crest-derived stem cells [5], endothelial cell line GM 7373, harvested from the aorta of a bovine calf and osteoblasts from mice, carrying green fluorescent protein [6], mice embryonic fibroblasts [7] and many others.

Since implants are intended to be used for bone defect sites, osteoblast-like cell cultures serve as ideal in vitro model systems for biocompatibility and osseointegration tests. Osteoblasts are the cells responsible for the formation of bone; they are found on areas of growing or remodeling bone, where they synthesize almost all of the constituents of the bone matrix and direct its subsequent mineralization [8]. As a result of matrix deposition, osteoblasts become embedded in the matrix, cease matrix synthesis, and differentiate into osteocytes [9]. Osteocytes are the most abundant cells in bone. Individual osteocytes are buried in an isolated position within the bone matrix, however, they maintain contact with other osteocytes and with cells on the bone surface by long cell processes that run via small channels, the canaliculi. Gap junctions provide intracellular contact, where the cell processes of two osteocytes meet in a shared canaliculus [10]. Except for being used as a tool to run biocompatibility and osseointegration tests of implants, osteoblast cell cultures may be employed in a wide array of applications including the studies of bone formation mechanisms, screening for potential therapeutic agents that affect bone formation and bone disease research.

Our study aimed to obtain and characterize osteoblast cell lines from rat adults and embryos, and to test

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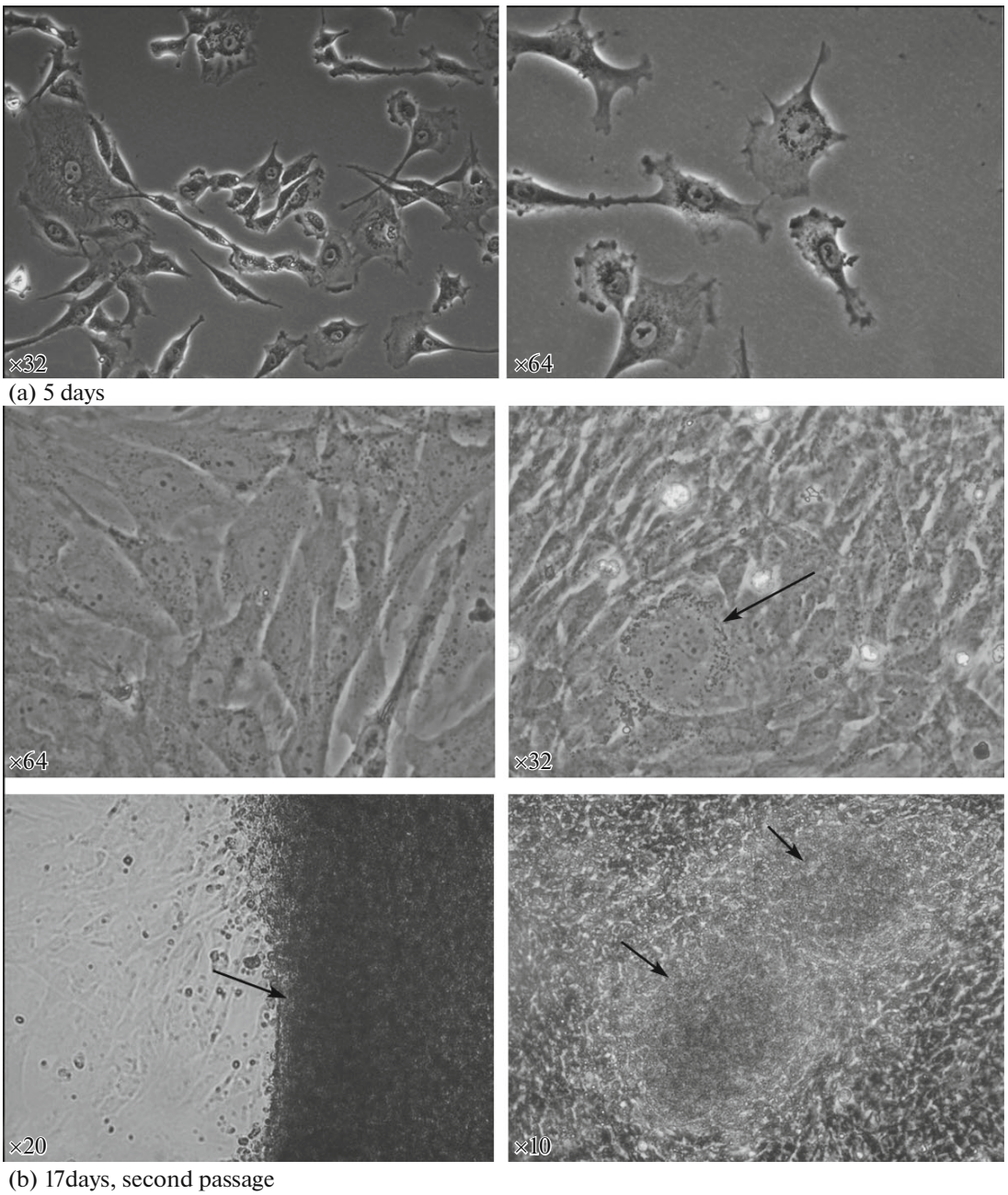

Fig. 1. Phase contrast microscopy of primary cell culture from Wistar rat embryos heads, REM-C-02, at different stages of cultivation. (a) Appearance of primary cell culture; (b) cell culture is heterogeneous: the most of observed cells are characterized by the osteoblastic morphology (×64), rare cells are large and contain three nuclei (×32 marked with a white arrow), which is a morphological phenotype of osteoclasts, besides, abundant mineralization matrix foci were formed (×20, ×10, labeled with black arrows).

their suitability for biocompatibility tests of titanium-based implants.

To obtain primary cell cultures, we used a modified method based on several osteoblast isolation protocols [4, 8]. For RAD-C-01 (Rattus norvegicus adult calvaria) and REM-C-02 (Rattus norvegicus embryo calvaria) cell cultures, calvaria of 4 month old Wistar female rat and calvaria of 12 days old Wistar rat embryos were used, respectively. After the enzymatic treatment, the obtained bone explants (for RAD-C-01) and grinded calvaria tissues (for REM-C-02), were cultured in culture flasks in a humidified 5% CO_2 atmosphere.

Mineralization assay was conducted employing titanium discs VT1-0 and cultural plastic (which served as a positive control). Cells were cultured in standard and osteogenic media (α-MEM containing 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin-

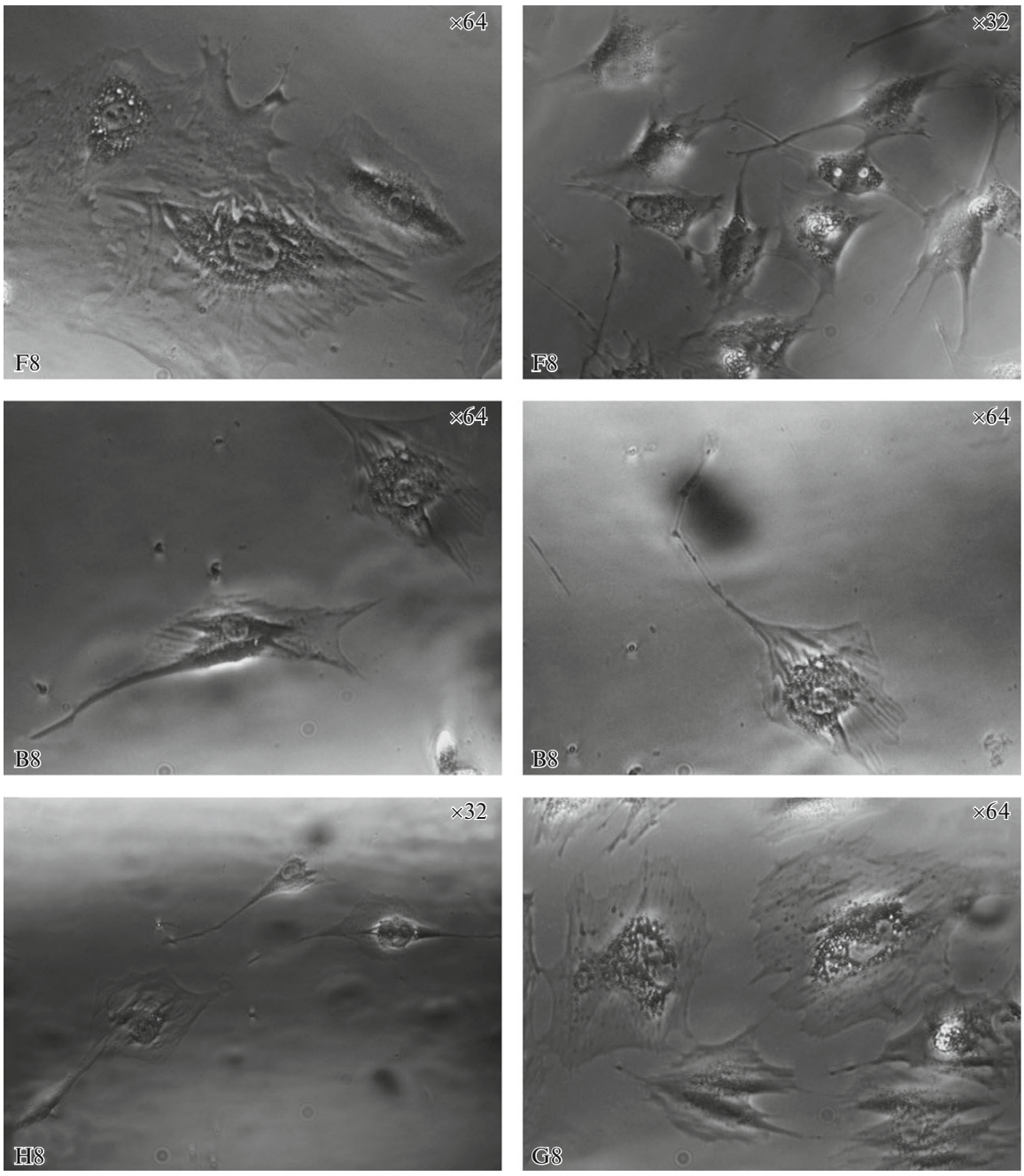

Fig. 2. Cloning of Wistar rat embryos heads cell culture, REM-C-02, using subsequent dilutions within 96-well plate, day 10, names of wells with diluted cell culture are shown in the lower left corners. Some wells (F8 and G8) demonstrate a mixed population of osteoblastic cells (polygonal in shape, have a well-developed rough endoplasmic reticulum), osteocytes (characteristic stellate shape, smaller cell body and long dendritic processes) and/or osteoblasts on their way to differentiate into osteocytes. In several wells (B8, H8) we obtained clones consisting solely of osteocytes.

streptomycin, 50 μg/mL L-ascorbic acid, 4 mM β-glycerophosphate, 0.1 mM non-essential amino acids, 1000 nM dexamethasone, 6.25 μg/mL insulin). Cells were cultured for 28 days, stained with Alizarin Red S (2%, Sigma) and visualized via phase microscopy using an inverted miscroscope Axio Observer A1 (Carl Zeiss, Germany).

After anodizing, titanium discs (d = 10 mm) were covered with amorphous oxide TiO_2 layer with pores of different size ranging from 20 to 120 nm. Such discs were seeded with osteoblast cell cultures at the exponential growth phase. We did not use standard fixation method employing toxic osmium tetroxide and investigated alternative ways of fixation. Within the same

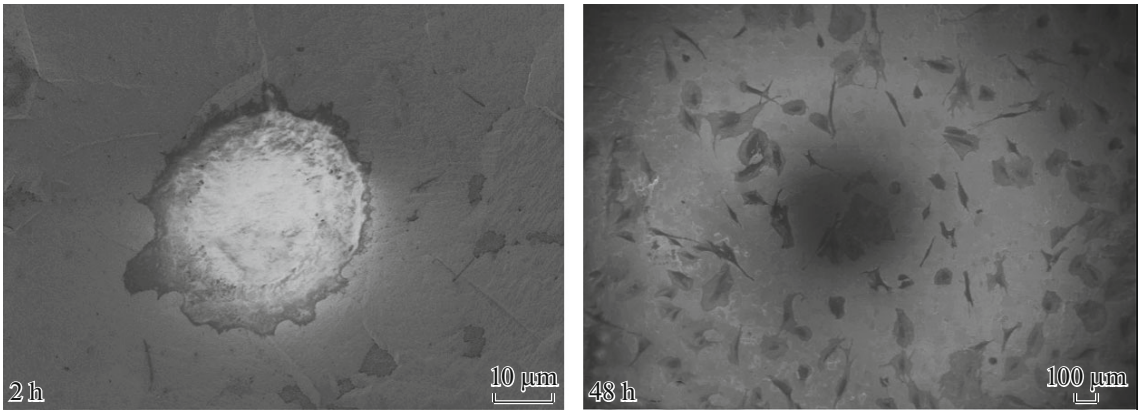

Fig. 3. SEM images of RAD-C-01 cells, cultivated on titanium discs and fixed at different time points since seeding.

six-slot cell culture plate, each slot of which contained a titanium disc seeded with osteoblast cell culture, we added a different solution to each two slots: (1) formalin, (2) chilled Karnua, and (3) glutaraldehyde solutions, and incubated cells for 30 min. Then samples were washed with ddH2O several times, dehydrated by washing in increasing ethanol concentrations (50% for 5 min, 70% for 5 min, 96% for 5 min), and air-dried. Samples were investigated with scanning electron microscope (SEM) Carl Zeiss Auriga Cross Beam employing secondary electrons image mode and backscatters electrons image mode at the accelerating voltage of 0.5–1 kV.

RAD-C-01

Adult rat calvaria bone explants were cultured for 12 days to allow the cells within the bone migrate out and adhere onto the surface of plastic flasks (Fig 1). RAD-C-01 cell culture reached confluence after 2 weeks; when the explants were removed, cells were trypsinized and replated. The obtained cell population contained mostly cells with a polygonal shape and abundant rough endoplasmic reticulum, typical for osteoblasts.

REM-C-02

Primary cell culture, obtained from enzymatically-treated embryonic head tissues (Fig. 1a), was trypsinized and replated. After second passage the cells were morphologically analyzed (Fig. 1b).

Although the cell culture, obtained from rat embryonic head tissues, was characterized by abundant osteoblastic cells, which was further confirmed by the appearance of numerous mineralization foci, it was heterogeneous, i.e. we observed multinucleated cells, presumably osteoclasts (Fig. 1b). To obtain pure colonies from single cells we conducted serial dilutions in the complete medium (see Materials and Methods) within 96-well plate.

Figure 2 presents microphotographs of cells from REM-C-02 mixed population, 10 days after cloning. While in some wells we observed cell colonies, consisting solely of osteocytes, indicated by their stellate cell shape, small cell body and long processes, the other wells contained both osteoblasts (with polygonal shape and well developed rough endoplasmic reticulum) and osteocytes or the third type of wells comprised osteoblasts, osteocytes and osteoblasts on their differentiation way. After several consequent subclonings we obtained a stable REM-C-02 cell line, derived from a single osteoblast-like cell.

To analyze osseogenic differentiation of RAD-C-01 and REM-C-02 cells, staining with Alizarin Red S was employed at day 28. For cells, grown on plastic and titanium discs in standard medium, such test was negative. However, for RAD-C-01 and REM-C-02 cell cultures, grown in osseogenic medium, Alizarin Red S staining turned to be positive for both, plastic and titanium substrates.

Both cell lines, RAD-C-01 and REM-C-02, showed a normal karyotype of *Rattus norvegicus* ($2n = 42$; NF = 64) in 80% metaphase chromosome spreads.

SEM images were captured at different time points after seeding the cells on titanium implants (Fig. 3). Based on the results of our comparative assay, which included three different methods to fix cell cultures prior to the SEM investigation (with formalin, Karnua solution and glutaraldehyde) we revealed that glutaraldehyde and Karnua solutions could be used to fix cells, while formalin caused serious damage to the cell shape.

SEM of RAD-C-01 and REM-C-02 cells, seeded onto titanium discs, was conducted at two different time points: 2 and 48 h (Fig. 3). After 2 h, we observed a round cell morphology, which can be explained by the initial stage of cell adhesion. However, cells, cultured for two days onto titanium discs, exhibited mostly spindle-like and branched morphology, which is a sign of a successful adhesion and proliferation. Hence, while conducting SEM of osteoblasts, seeded

onto titanium discs, we observed cell adhesion and cell morphology changes, which points to suitability of obtained diploid osteoblast cell cultures from R. norvegicus for in vitro biocompatibility and osseointegration tests of titanium-based implants.

Thus, our study demonstrated the effective use of two diploid rat osteoblast cultures, RAD-C-01 and REM-C-02, in research involving assays on osteoblasts. In this study, we have adapted methods to obtain osteoblasts cell cultures from calvaria of adult rats and 12-days old embryos and run primary biocompatibility tests. Our data appear promising in further studying of new generation titanium implants employing RAD-C-01 and REM-C-02 as models for pre-clinical biocompatibility and osseointegration assays.

SEM was conducted in the Laboratory of Structural Methods of Analysis and Properties of Materials and Nanomaterials of the Center of Collective Use of the Ural Federal University.

ACKNOWLEDGMENTS

We hereby acknowledge the support of the Ministry of Science and Education of the Russian Federation, in accordance to the decree of the Government of April 9, 2010, no. 218, project number 02.G25.31.0234.

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