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Fibroblasts maintained in 3 dimensions show a better differentiation state and higher sensitivity to estrogens

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ABSTRACT

Cell differentiation and response to hormonal signals were studied in a 3D environment on an in-house generated mouse fibroblast cell line expressing a reporter gene under the control of estrogen responsive sequences (EREs). 3D cell culture conditions were obtained in a Rotary Cell Culture System; (RCCS™), a microgravity based bioreactor that promotes the aggregation of cells into multicellular spheroids (MCS). In this bioreactor the cells maintained a better differentiated phenotype and more closely resembled in vivo tissue.
The RCCS™ cultured fibroblasts showed higher expression of genes regulating cell assembly, differentiation and hormonal functions. Microarray analysis showed that genes related to cell cycle, proliferation, cytoskeleton, migration, adhesion and motility were all down-regulated in 3D as compared to 2D conditions, as well as oncogene expression and inflammatory cytokines. Controlled remodeling of ECM, which is an essential aspect of cell organization, homeostasis and tissue was affected by the culture method as assessed by immunolocalization of β-tubulin. Markers of cell organization, homeostasis and tissue repair, metalloproteinase 2 (MMP2) and its physiological inhibitor (TIMP4) changed expression in association with the relative formation of cell aggregates.
The fibroblasts cultured in the RCCS™ maintain a better responsiveness to estrogens, measured as expression of ERα and regulation of an ERE-dependent reporter and of the endogenous target genes CBP, Rarb, MMP1 and Dbp. Our data highlight the interest of this 3D culture model for its potential application in the field of cell response to hormonal signals and the pharmaco-toxicological analyses of chemicals and natural molecules endowed of estrogenic potential.

Introduction

Whereas tissues and organs are three dimensionally organized (3D), the in vitro assessment of cell physiology, behavior and homeostasis and our ability to understand tissue formation, function and pathology has mostly depended on two dimensional (2D) cell culture systems (Yamada and Cukierman, 2007). Unfortunately, cell bi-dimensionality and microenvironment of conventional cultures often failed to mimic the dynamic of in vivo contexts, where cell behavior and homeostasis are regulated by systemic cues (Mazzoleni et al., 2009; Baker and Chen, 2012). As an intermediate model between cell cultures and human tissues animals have been so far used to allow the definition and understanding of specific and complex bioprocesses (Yamada and Cukierman, 2007; Sonneveld et al., 2006; Cook et al., 2012). However, the ethical limit of performing animal based-experimentations and the always more stressing regulatory rules have rendered the search of alternative methods a high priority issue. The extrapolation of results to humans is often difficult due to the fact that animal models may not accurately reproduce some human features of physiological and pathological conditions (Ferrarini et al., 2013a; Bracken, 2009; Hartung, 2008; Mazzoleni and Steimberg, 2012). In light of these considerations, in the least decades, there was an increasing need to create new model systems able to mimic in vitro the features and functionality of living organs (Elsdale and Bard, 1972); for a number of tissue/cells, in vitro 3D tissue models provided a fruitful strategic approach that bridged the gap between traditional cell cultures and animal models (Ferrarini et al., 2013b; Griffith and Swartz, 2006; Rangarajan et al., 2004; Pampaloni et al., 2007; Hirschhaeuser et al., 2010).

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When cultured on plastic dishes, cells face an artificial microenvironment where they are forced to grow in an abnormal bipolar organization. In such conditions, they dramatically differ in their morphology, cell–cell and cell–matrix interactions, behavior and differentiation, from those growing in more physiological 3D microenvironments as well as in tissue-specific context (Cukierman et al., 2002; Griffith and Swartz, 2006; Nelson and Bissell, 2006; Xu et al., 2009). The difference is not only in terms of cell morphology, adhesion and modality by which cells exert forces on their own surrounding extracellular matrix (ECM) and reciprocally the way by which ECM senses extrinsic forces and transmits them to the ECM-embedded cells (Pedersen and Swartz, 2005), but also in biological responses to microenvironmental stimuli such as biochemical, mechanical and biophysical factors (Silver et al., 2003; Huang et al., 2012). An appreciation is emerging for the extent to which the 3D environment governs the cell responses, particularly for cells naturally existing in 3D interstitial spaces like fibroblasts (Pedersen and Swartz, 2005). In fibroblasts, for example, whereas the 2D culture induces cell spreading with prominent cellular extensions, a 3D configuration favors a globular or bipolar dorsal–ventral or stellate shape according to the ECM characteristics, that governs the cell features (Beningo et al., 2004; Larsen et al., 2006) by affecting mechanotransduction of signals and specific intracellular signaling networks (Cukierman et al., 2002; Torres and Rosen, 2006).

In vitro, the microenvironment properties (composition of ECM, dimensionality, architecture, topography, compliance of the ECM) may directly modulate the overall cell morphology, cell–matrix adhesion, and cytoskeletal organization (Giretti and Simoncini, 2008; Ananthakrishnan and Ehrlicher, 2007) and as a consequence they regulate cell phenotype survival, proliferation rate (Boateng et al., 2003), inhibition of proliferation (Sarasa-Renedo and Chiquet, 2005), cellular migration, cellular homeostasis and gene expression (Birgersdotter et al., 2005; Cukierman et al., 2002; Pedersen and Swartz, 2005).

Thus, the maintenance of such 3D dynamic conditions is of importance when fundamental cell processes such as steroid hormone regulation are studied. Steroid hormones act as extranuclear signaling factors which regulate cell morphology (Naftolin and Malaspina, 2007) via rapid signaling to the actin cytoskeleton (Simoncini et al., 2006; Giretti et al., 2008; Sanchez et al., 2009; Louvet-Vallée, 2000; Tsukita and Yonemura, 1999). For instance, estrogens activate moesin in breast cancer, endothelial and neuronal cells through a rapid, extranuclear signaling cascade originated by the interaction of estrogen receptor alpha (ERα) with the G protein Ga_{13}. This process leads to the recruitment of RhoA and of Rho-associated kinase, ROCK-2 and the activation of moesin which leads to modification of the interaction with the extracellular matrix and nearby cells (Sanchez and Simoncini, 2010; Fu and Simoncini, 2008).

In this work we studied the role of the 3D microenvironment on fibroblast phenotype and more especially on a fundamental signaling process such as the response to estrogens. We maintained an in-house generated mouse fibroblast cell line expressing a reporter gene under the control of estrogen responsive sequences (EREs) in a dynamic 3D microenvironment where high mass transfer was allowed in the absence of significant shear stress. Such conditions were obtained in a Rotary Cell Culture System; (RCCS™), a microgravity based bioreactor that promotes the aggregation of cells into multicellular spheroids (MCS) by preventing cell adhesion to an artificial surface (Grun et al., 2009). This bioreactor has shown advantages over static and other dynamic tissue culture systems in that cells maintained in a laminar fluid state better express their differentiated phenotype and more closely resemble in vivo tissue equivalents (Ferrarini et al., 2013b; Hammond and Hammond, 2001; Unsworth and Lelkes, 1998; Mazzoleni and Steimberg, 2010; Mazzoleni et al., 2011; Steimberg et al., 2010). In parallel, the biological response of these cells to estrogen receptor activation was evaluated in the classical static 2D conditions. We here show that: a) the RCCS™ is suitable to maintain fibroblasts in a more differentiated state, demonstrated shown by the higher expression of genes involved in several pathways regulating cell differentiation and functions; b) the fibroblasts cultured in the RCCS™ maintain a better responsiveness to estrogens, thus suggesting that estrogen receptors and the estrogen signaling pathways are in a more functional situation than in 2D Petri dishes.

Our data highlight the opportunity to further investigate this 3D culture model for its potential application in the field of research on nuclear receptor functions and for the pharmaco-toxicological analyses of chemicals and natural molecules endowed of hormonal potential.

Material and methods

Fibroblast immortalization

Generation of immortalized embryonic mouse fibroblasts (MEFs) was performed on fibroblasts isolated from 13.5-day-old embryos from ERE-tK-Luciferase mice. Dermal fibroblasts were obtained from ventral embryo skin. The skin was cut and placed into Petri dishes maintaining the dorsal–ventral polarization. The tissue was maintained in DMEM (Lonza, Milan, Italy), which contained 20% fetal bovine serum (Invitrogen, Milan, Italy), 2 mM L-glutamine and antibiotic mix 1× (Sigma, Pomezia, Italy) (complete culture medium). After 10 days, confluent fibroblasts were transfected with the plasmid pSV40-Neo encoding for the SV40-large T antigen by mean of Lipofectamine 2000 (Invitrogen, Milan, Italy), as described by the manufacturer. Forty-eight hours after transfection, cells were selected for G418 resistance (0.4 mg/ml), this selection was performed over three weeks. Foci of resistant cells were isolated using cloning rings and further expanded. The characterization of clones was based on their capacity to respond to estrogens, in DMEM without phenol red (Invitrogen, Milan, Italy) supplemented with 2% of fetal bovine serum (Invitrogen, Milan, Italy), 2 mM L-glutamine, 1× antibiotic mix (Sigma, Pomezia, Italy; 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone) and 1 mM Na-pyruvate (Sigma, Pomezia, Italy) (treatment medium). 10^4 dermal fibroblasts were seeded onto 35 mm-diameter Petri dishes in complete culture medium. Cells were daily harvested and their number determined using a hemocytometer.

3 dimensional cell culture in the RCCS™

The selected clone was expanded, harvested, resuspended in 10 ml culture medium and introduced into the 10 ml-HARV culture vessel (RCCS™ bioreactor) at the final concentration of 1.5 million of cells/ml (http://science.nasa.gov/NEWHOME/br/bioreactor.htm) (Houston, USA). The culture medium was the same as the 2D culture. Cells were grown for 2 or 7 days in 3D and then harvested. Immortalized fibroblasts were amplified in monolayer (2D) and subsequently cultured in the RCCS™ device to characterize the influence of the 3D culture conditions on the specific cell viability, phenotype and functions. Cells were harvested at different times of culture (from T0 up to 192 h) and immunolocalization of specific proteins was performed.

Cell treatments

Immortalized fibroblasts maintained either in 2D or in 3D conditions were treated with increasing doses of 17β-estradiol (Sigma-Aldrich), genistein (Sigma-Aldrich) or Bisphenol A (Sigma-Aldrich) for 24 h, in phenol free culture medium. After 24 h cells were harvested for further studies.

Luciferase assay

Luciferase assay was performed using a Luciferase Assay kit (Promega, Milan, Italy). Cells were lysed using the reporter lysis buffer 1×, previously added to the cells. Cells were collected, maintained on ice for 30 min. Then, luciferase activity was estimated and protein