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Three dimensional cellular microarray platform for human neural stem cell differentiation and toxicology

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Abstract We developed a three-dimensional (3D) cellular microarray platform for the high-throughput (HT) analysis of human neural stem cell (hNSC) growth and differentiation. The growth of an immortalized hNSC line, ReNcell VM, was evaluated on a miniaturized cell culture chip consisting of 60 nl spots of cells encapsulated in alginate, and compared to standard 2D well plate culture conditions. Using a live/dead cell viability assay, we demonstrated that the hNSCs are able to expand on-chip, albeit with lower proliferation rates and viabilities than in conventional 2D culture platforms. Using an in-cell, on-chip immunofluorescence assay, which provides quantitative information on cellular levels of proteins involved in neural fate, we demonstrated that ReNcell VM can preserve its multipotent state during on-chip expansion. Moreover, differentiation of the hNSCs into glial progeny was achieved both off- and on-chip six days after growth factor removal, accompanied by a decrease in the neural progenitor markers. The versatility of the platform was further demonstrated by complementing the cell culture chip with a chamber system that allowed us to screen for differential toxicity of small molecules to hNSCs. Using this approach, we showed differential toxicity when evaluating three neurotoxic compounds and one antiproliferative compound, and the null effect of a non-toxic compound at relevant concentrations. Thus, our 3D high-throughput microarray platform may help predict, in vitro, which compounds pose an increased threat to neural development and should therefore be prioritized for further screening and evaluation.
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Introduction

Neurotoxicity detection is a major challenge due to the complexity of the central and peripheral nervous systems. The majority of commercial chemicals have not been evaluated for developmental neurotoxicity, at least partly due to the high cost of animal testing (National Research Council, 2007). Regulatory agencies have traditionally used in vivo methods for adult and developmental neurotoxicity testing, including neurobehavioral evaluation of cognitive, sensory and motor functions accompanied by neuropathological studies, with no specific studies of the underlying cell biology (Bal-Price et al., 2010). There is also a need to test large sets of compounds to comply with specific regulatory requirements (Breier et al., 2010; Andersen and Krewski, 2009). To this end, there is pressure to develop alternative test strategies, which are rapid, economical, and, most critically, highly predictive (Breier et al., 2010).

An often overlooked aspect of neurotoxicity is the impact of chemicals, as well as drugs and drug candidates, on neural stem cells and their terminally differentiated lineages. Stem cells have been shown to exhibit differential sensitivities to both non-toxic (e.g., serum) and toxic compounds, as compared to terminally differentiated cells (Trosko and Chang, 2010; Dietrich et al., 2006). Broad knowledge of the toxicity of such compounds to stem cells in comparison to other cell types in a given tissue can provide fundamental information critical for assessing the safety of new drug candidates and the health effects of environmental agents. Thus, the development of new high-throughput screening tools that enable the study of these differential effects on stem cells and their differentiated progeny, should not only encompass endpoints that assess chemical toxicity, but also allow us to determine stem cell fate. This is generally achieved by following protein markers of multipotency and differentiation.

With this in mind, we have developed a three-dimensional (3D) cellular microarray platform for the high throughput analysis of hNSC differentiation and toxicity screening (Fig. S1). Our system has the ability to expand our knowledge of neurotoxicity by discriminating between toxic and nontoxic compounds. It can also detect differentiation stage-specific toxicities. Knowledge of differences in molecular toxicity to stem cells in comparison to other cell types is critical for assessing safety of new drug candidates and health effects of environmental agents (Laustriat et al., 2010). We demonstrated herein the differentiation of the ReNcell VM hNSC line into glial progeny on a 3D cellular microarray platform. This platform was then used to screen dose-dependent toxicity of a number of neurotoxic compounds, leading to identification of compounds with differential toxicity to hNSCs in relation to the differentiated glial progeny.

Materials and methods

Cell culture

ReNcell VM (Millipore) is an immortalized neural progenitor cell line derived from the ventral mesencephalon region of a 10-week human fetal brain. All cells used in this investigation were from passage 31 or lower; previous work (Donato et al., 2007) has shown that these cells maintain a stable karyotype past 45 passages. Cells were cultured according to the manufacturer's instructions. Briefly, the ReNcell VM cells were expanded in expansion medium (ReNcell NSC Maintenance Medium (Millipore) supplemented with 20 ng/ml of epidermal growth factor (EGF, Millipore) and 20 ng/ml of basic fibroblast growth factor (bFGF, Millipore)) on laminin-coated (1.7 $\mu g/cm^2$) TC-treated culture flasks at 37 °C in a 5% CO_2 humidifier incubator. The medium was renewed every two days during proliferation, and the cells subcultured approximately every five days (90% confluence) by detaching them with Accutase® (Millipore). After each passage, cell concentration and viability were determined by counting with a hemocytometer (Hauser Scientific) using the trypan blue dye (Invitrogen) exclusion test, and the cells were once again seeded at 5×10^4 cells/ml in freshly coated flasks. Differentiation of the cells was accomplished by adding fresh differentiation medium (ReNcell NSC Maintenance Medium without growth factors) to confluent monolayers of cells. Unless otherwise indicated, cells were incubated for 6 days in differentiation medium, replenishing the medium every 2 days.

Preparation of 3D microarray cultures

ReNcell VM cells were cultured in three-dimensional (3D) microarrays by embedding them in alginate, and printing the gel mixture in 60 nl spots on hydrophobic glass slides. Briefly, a poly-L-lysine (PLL)–$BaCl_2$ mixture was made by mixing a sterile 0.1 M $BaCl_2$ solution in deionized water with a 0.01% (w/v) sterile PLL solution (Sigma) in a 1:2 volume ratio. The PLL–Ba^{2+} mixture was spotted onto poly(styrene-co-maleic anhydride) (PSMA) coated glass slides using a MicrosSys 5100-4SQ microcontact microarray spotter (Genomics Solutions), making a 6 × 8 × 8 patterned array of 60 nl spots on the chip and allowing them to dry. Each spot had a diameter of ca. 600 μm and a height of ca. 70 μm. A ReNcell VM cell suspension in media was then mixed with a low viscosity alginic acid (Sigma) solution in deionized sterile water such that the final alginate concentration was 1% (w/v), and the concentration of cells in suspension was 5×10^6 cells/ml, unless otherwise indicated. Subsequently, the cell-alginate mixture was printed (60 nl/spot, 300 cells/spot) on top of the dried PLL–Ba^{2+} spots, which allowed the nearly instantaneous gelation of the alginate matrix. During the printing process, the humidity in the microarray chamber was maintained above 90% to prevent water evaporation of the spots. Following printing, the slide was fitted with a commercially available 8-well polystyrene medium chamber coated with a biocompatible adhesive (Lab-Tek II, Nunc) that allowed firm attachment of the chip to the chamber, and physically separated groups of spots within the slide. Each of the wells, containing 48 cell culture microarray spots, was filled with 250 μl of ReNcell expansion medium. Following expansion, differentiation, exposure to drugs and/or assaying the chambers could be easily removed with a slide separator.

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