AN INDUCED-FLOW KIDNEY-BREAST CANCER *IN VITRO* MODEL FOR STUDY OF CANCER SUPPRESSION BY VITAMIN D₃ DERIVATIVES

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ABSTRACT

This paper reports an *in vitro* platform that demonstrates the suppression of cancer cells by vitamin D₃ derivatives on a single system, by simultaneously enabling (1) co-culture of multiple cells; (2) induced fluidic flows among cells; and (3) cancer suppression by the vitamin D₃ derivatives. This *in vitro* model mainly consists of three wells, each containing kidney cells, breast cancer cells, and final transferred media. The wells are connected by porous membranes that allow fluidic flows by hydrostatic pressure. The developed model successfully (1) co-cultured kidney cells (HKC8) and breast cancer cells (MCF7) to the confluency of >1,800cells/mm² under the same media; and (2) showed the suppression of cancer cells by the vitamin D₃ derivatives: 25-hydroxyvitamin D₃ (25(OH)D₃) and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃); and (3) demonstrated fluidic flow effects on cancer suppression.

INTRODUCTION

Breast cancer has been one of the top five causes for mortality in the US in recent years [1]. In 2007 it was reported that 125 people in every 100,000 women had breast cancer and 23 people died of the breast cancer [1]. To lower the fatalities, numerous efforts have been reported covering from drug-treatment [2] to X-ray based chemotherapy [1], resulting in notable reduction of fatalities in recent years. However, the treatment processes have been often criticized for accompanied harsh side effects of weight loss, abnormal hormone discretion, and even mental instability [3,4,5].

Breast cancer has recently been proven to be suppressed by the vitamin D₃ derivatives, 1,25(OH)₂D₃ [6]. As illustrated in Fig. 1, the vitamin D₃, gained by food intake or by sun exposure [7], produces subsequent derivatives: first, 25(OH)D₃ in the liver and then, 1,25(OH)₂D₃ from the 25(OH)D₃ in the kidney [8]. The 1,25(OH)₂D₃, when transferred to the breast cancer cells, suppresses cyclin D-dependent kinases activity and delays G1/S phase of cell cycle, ultimately inhibiting further growth of breast cancer cells [6]. Previous literature reported that the growth of breast cancer cells (MCF7) started to slow down after day 2 of introducing 10⁻⁷M of 1,25(OH)₂D₃ in *in-vitro* 96 wells and at day 7, approximately 10× decreased in cell number was observed [6]. However, detailed studies of cancer suppression by vitamin D₃, such as translation of

individual derivatives among organs and their cross-effects [9], have been much limited mainly due to the lack of low-cost, high-throughput co-culture model. Conventional *in vivo* models, such as animals, present the issues of cost, throughput, repeatability, controllability as well as ethics, while *in vitro* 96-well models failed to provide co-culture environments and fluidic flows among cell groups, which is critical to study translational effects that have not been studied yet [10].

To resolve such limitations, we report the development of a low-cost, high-throughput, and co-culture model where the suppression of cancer cells by vitamin D₃ derivatives has been demonstrated. Particularly, the fabrication, testing, and measurement results of the developed kidney-breast cancer model are reported.

STRUCTURE AND FABRICATION

Structure

The induced-flow *in vitro* model consists of three wells, each containing kidney cells, breast cancer cells and final transferred media (Fig. 2), which was described in details in a previous publication [11]. The three wells were fluidically connected through cellulose membranes with either 1.2, 5.0 or 8.0μm pore size, and induced fluidic flow from the first well through the second to the third well by fluidic potential difference. The fluidic potential difference was applied by maintaining the media volumes as 320μl, 170μl, and 20μl at the kidney, breast cancer, and drain wells, respectively. The media volume was re-adjusted according to the pore size used. The diameter of each well was 6.5mm and the height was 10.5mm. Cellulose membranes provided the fluidic path among wells. The footprint of the membrane was 1mm×3.5mm, while their thicknesses were between 105 and 140μm.

Fabrication

The fabrication of the platform was previously described [11]. Briefly, each set of three holes of a standard flat bottom 96-well holey (bottomless) microplate (Corning Inc., Corning, NY) are linearly connected using two cellulose membranes (1mm×3.5mm) with either 1.2, 5 or 8μm pore size. A polystyrene film, which was taken from a Corning® HYPERStack™ Cell Culture Vessel (Corning Inc., Corning, NY) was adhered to the bottom of the standard flat bottom 96-well holey microplate using a double-sided

Figure 1. Motivation: Vitamin D₃ is converted into its subsequent derivatives through different organs, ultimately being capable of suppressing breast cancer cells.

Figure 2. Proposed model that combines multi-cell co-culture, induced-flow, and cancer suppression into a single *in vitro* platform.

pressure sensitive adhesive sheet (110mm×75mm) where holes and channels are cut out for the wells and the cellulose membranes, respectively (Fig.2) [11].

Cell culture

Both HKC8 (ATCC, Manassas, VA) and MCF7 (ATCC, Manassas, VA) cell lines, each representing the kidney and the breast cancers, were cultured in Dulbecco's modified Eagle's medium: Ham's F12 (Cassison Labs, North Logan, UT) supplemented with 10% fetal bovine serum (Fisher Scientific, Pittsburgh, PA), 1% amphotericin B (Corning Inc., Manassas, VA) and 1% penicillin/streptomycin (Lonza, Walkersville, MD). These cell lines were cultured until they reached 80% confluency in a monolayer in a T75 flask (Corning Inc., Corning, NY). The culture conditions of 37°C in a humidified atmosphere containing 5% CO₂ were utilized in the incubator (NU-4750, Nuair, Plymouth, MN). The media was changed every 3~4 days.

Vitamin D₃ derivatives, 1,25(OH)₂D₃ and 25(OH)D₃, were diluted in pure ethanol into various ranges of concentrations (10⁻⁵~10⁻¹⁰ M) and stored in freezer at -20°C. The concentrations of 1,25(OH)₂D₃ and 25(OH)D₃ were finally adjusted again in the media right before the use. Note that the concentration of ethanol dilution did not exceed 0.8% to avoid any toxicity effects on cell growth. The utilized chemical compounds were purchased from EMD Millipore (EMD Chemicals Inc., San Diego, CA).

TESTING METHODOLOGY

Cell viability measurement

In order to establish a cell growth reference, the fluorescence intensity of the incubated cells was monitored every 24 hours over 9 days by utilizing a fluorescence spectrometry (Synergy HY, BioTEK Instrument, Winooski, VT). When the HKC8 kidney cells and MCF7 breast cancer cells were cultured to a confluency of 80%, they were harvested by washing twice with PBS and adding 4ml Trypsin EDTA (Corning Inc., Manassas, VA). These harvested cells were then seeded in triplicate in the standard 96-well microplate (Corning Inc., Corning, NY). The initial cell numbers ranged between 5,000 and 100,000 cells/well. After 1 day, a cell viability reagent, PrestoBlue (Invitrogen, Frederick, MD), was applied to stain the cells for fluorescence measurement. The mixture ratio was 10μl of PrestoBlue and 90μl of media. After incubation for 30 minutes at 37°C and 5% CO₂, the fluorescence intensity of the incubated cells was measured utilizing the light wavelengths at 530 and 590nm.

Cancer suppression measurement

By 1,25(OH)₂D₃: To confirm the cancer suppression effect of 1,25(OH)₂D₃, the proliferation of the MCF7 cancer cells was

monitored every 24 hours over 9 days utilizing fluorescence spectrometry under direct supply of 1,25(OH)₂D₃. MCF7 breast cancer cells were seeded in the standard 96-well microplate in a concentration of approximately 5,000 cells/well in triplicate. They were supplied with different dosing concentrations between 10⁻⁹ and 10⁻⁶M every 24 hours over 7 days. The cell viabilities were measured every 24 hours by the spectrometer. The optical images of the cells were also collected for comparison purposes.

By 25(OH)D₃: The cancer suppression effect of 25(OH)D₃ on MCF7 breast cancer cells have been additionally examined by utilizing the identical method described above. In this case, different concentrations of 25(OH)D₃ between 10⁻⁵ and 10⁻⁸M were applied. Note that to the best of our knowledge, cancer suppression effects of 25(OH)D₃ on MCF7 breast cancer cells has not been reported previously. However, the hypothesis that mammary cells express CYP27B1 and locally produce 1α,25(OH)₂D₃, which acts in an autocrine manner to regulate cell turnover had been studied previously [12]. CYP27B1 is a cytochrome P450-containing hydroxylase expressed in kidney and other tissues that generates 1α,25(OH)₂D₃. In Bland *et al.*'s study, CYP27B1 mRNA was detected in both MCF7 and HKC8 cells lines, but expression was higher in MCF7 cultures compared with HKC8 cells [12], which were characterized previously as an *in vitro* model of vitamin D metabolism [9]. Thus we suspected that MCF7 cells may also convert 25(OH)D₃ into 1,25(OH)₂D₃ by itself.

By indirectly produced 1,25(OH)₂D₃: In comparison to direct applications of 25(OH)D₃ and 1,25(OH)₂D₃ onto the cancer cells (MCF7), the application of 25(OH)D₃ onto the kidney cells (HKC8) was examined. HKC8 cell has been known to produce 1,25(OH)₂D₃ from 25(OH)D₃ [9]. Thus, HKC8 cells were cultured at the upstream well, while MCF7 cells were cultured at the downstream well. In this case, HKC8 and MCF7 cells were separately cultured in the wells of a standard 96-well microplate. Every 24 hours, 25(OH)D₃ was added to the HKC8 cells and after 5 hours of adding 25(OH)D₃ to the HKC8 cells, the media in the HKC8 cell well was exchanged with fresh media without 25(OH)D₃ while the "used" HKC8 media was exchanged with the media in the MCF7 cell well. The proliferation was measured every 24 hours over 9 days utilizing fluorescence spectrometry. The concentrations of the supplied 25(OH)D₃ ranged between 10⁻⁵ and 10⁻⁷M.

Measurement of flow effects on cancer suppression

In order to measure the flow rate effects on the cancer suppression, the proliferation of the MCF7 cancer cells in the *in vitro* platform were monitored using cellulose membranes with different pore sizes (1.2, 5.0 or 8.0 μm) (Fig. 2). The average flow rates were previously measured as 1.3, 3.3, and 6.7μl/hr for the

membrane pore sizes of 1.2, 5.0, and 8.0 μ m, respectively [11]. In the first well HKC8 kidney cells were seeded, while MCF7 breast cancer cells were seeded in the second well (Fig.2). The initial cell numbers were approximately 5,000 cells/well. Depending on the induced flow rates (pore sizes of cellulose membranes), the applied media with or without 25(OH) D_3 was adjusted in the first HKC8 cell well and the third drain well every 1, 2 or 3 days, respectively. After 5 days, the cell viabilities were measured by the same protocol as the previous tests.

Figure 3: (Top) Measured reference curve from breast cancer (MCF7) cells. PrestoBlue in DMEM media (Reagent:Media=10 μ l:90 μ l) was utilized to stain the cells for optical spectrometry for proliferation measurement (Synergy HT - BioTEK with fluorescence 560/590nm). (Bottom) Photos of the cell growth

RESULT

Cell viability: MCF7 breast cancer cells

The fluorescence measurement showed that the relative fluorescence unit (RFU) monotonously increased from 56 to 504 with the increasing number of MCF7 cells from 5,000 to 60,000 cells/well (Fig. 3-(top)). A positive linear correlation was observed with a slope of 0.0089 RFU/cells, indicating the validity of the curve as a cell growth reference. In the case of 5,000 cells/well, the surface of the well was covered with sparsely-distributed cells, while the well with 60,000 cells were nearly fully packed with the cells (Fig. 3-(bottom)).

Breast cancer suppression by 1,25(OH) $_2D_3$

The measurement results showed that 1,25(OH) $_2D_3$ with concentrations of $10^{-6}M$ and $10^{-7}M$ suppressed the proliferation of MCF7 cells by 36% in comparison to lower concentrations ($10^{-8}M$ and $10^{-9}M$) and control (0M) on day 7, as shown in Fig.4-(top). The suppression was clearly distinguished from day 4, and the proliferation difference further increased with the time. The suppression differences were measured as 22, 30, 33% at day 4, 5, and 6, respectively. The concentrations of $10^{-8}M$ and $10^{-9}M$ did not show any noticeable suppression in comparison to the control (0M). The optical images of $10^{-7}M$ (suppression) and $10^{-9}M$ (no suppression) present the clear differences in cancer cell proliferation (Fig.4-(bottom)). After day 7, MCF7 cells in the control and 10^{-8} , $10^{-9}M$ wells, started peeling off from the wall due to extremely high cell density from cell growth.

Breast cancer suppression by 25(OH) D_3

The suppression data indicated that concentrations of 25(OH) D_3 above $10^{-6}M$ were capable of suppressing the

proliferation of MCF7 cells by 54% on day 7, while the lower concentrations ($10^{-7}M$ and $10^{-8}M$) showed little suppression, as shown in Fig.5-(top). To the best of our knowledge, this is the first experimental results that showed the cancer cell suppression by 25(OH) D_3 . The cancer suppression effects become distinct from day 4, and the proliferation difference further increased with time. The suppression differences were measured as 32, 44, 46% at day 4, 5, and 6, respectively. The optical images of $10^{-5}M$ (suppression) and $10^{-8}M$ (little suppression) presented the clear differences in cancer cell proliferation (Fig.5-(bottom)).

Figure 4: (Top) Measured MCF7 suppression in fluorescence intensities over time under different dosing concentrations by 1,25(OH) $_2D_3$, indicating the effects of concentrations. (Bottom) Photos of breast cancer cells showing the suppression effects at higher concentrations of $>10^{-7}M$.

Figure 5: (Top) Measured MCF7 suppression in fluorescence intensities over time under different dosing concentrations by 25(OH) D_3 . (Bottom) Photos of breast cancer cell suppression over time, demonstrating the effects of dosing concentrations ($>10^{-8}M$).

Breast cancer suppression by converted 1,25(OH) $_2D_3$

The normalized cell proliferation data showed that MCF7

cancer cells, located at the downstream, were suppressed under the application of 25(OH) D_3 onto the HKC8 kidney cells upstream. The suppression was up to 48% in comparison to the control (0M) samples after 7 days (Fig. 6-(top)), which was close to the results from the direct application of 25(OH) D_3 onto the MCF7 breast cancer cells (Fig. 5-(top)). Although this small difference (48% compared with 54%) was within our experimental error, more experiments are needed in order to isolate the path of MCF7 suppression to whether 25(OH) D_3 was indeed converted into 1,25(OH) D_3 by the HKC8 kidney and/or MCF7 cells. Further study is needed to explain the exact causes.

Figure 6: (Top) Measured MCF7 suppression in fluorescence intensities over time under different dosing conditions of 25(OH) D_3 : (top) control, (middle) onto HKC8 kidney cells, and (bottom) onto MCF7 cells. (Bottom) Photos of breast cancer cell suppression over time.

Figure 7: (Top) Measured MCF7 suppression with $10^{-5}M$ of 25(OH) D_3 added to the HKC8 cell wells upstream by measuring the fluorescence intensities in the induced flow in vitro model after 5

days culture. (Bottom) Photos of cell suppression under different flow rates of 1.3, 3.3, and 6.7ul/hr.

Breast cancer suppression at different flow rates

The measurement data demonstrated that different flow rates of 1.3, 3.3 and 6.7ul/hr resulted in different levels of cancer cell suppression of 21.1, 19.4 and 28.9% with $10^{-5}M$ of 25(OH) D_3 added to the HKC8 cell well, as illustrated in Fig.7-(top). The maximum suppression of 28.9% was observed at the highest flow rate of 6.7ul/hr. We are still trying to understand the relatively lower suppression compared with previous cases (Fig. 4-6-(top)). The optical images also supported the trend: different levels of MCF7 suppression under different flow rates.

DISCUSSIONS AND CONCLUSIONS

This paper presents an *in vitro* platform that simultaneously enables (1) co-culture of multiple cells in one system; (2) induced fluidic flows by different pressure; (3) cancer suppression by the derivatives of vitamin D_3 . The cancer suppression distinctly appeared at the concentrations of 1,25(OH) D_3 above $10^{-7}M$ after 96 and 72 hours for each cases and of 25(OH) D_3 above $10^{-6}M$ after 96 hours, while the suppression differences, in comparison to control (0M), were measured as 36%, 54% and 48%, respectively. The measurement data also showed that the cancer suppression reaches 28.9, 21.1, 19.4% with $10^{-5}M$ of 25(OH) D_3 at the flow rates of 6.7, 1.3 and 3.3ul/hr, respectively.

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