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Disruption of myoblast alignment by highly motile rhabdomyosarcoma cell in tissue structure

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Rhabdomyosarcoma (RMS) is a highly malignant tumor type of skeletal muscle origin, hallmarked by local invasion. Interaction between invasive tumor cells and normal cells plays a major role in tumor invasion and metastasis. Culturing tumor cells in a three-dimensional (3D) model can translate tumor malignancy relevant cell–cell interaction. To mimic tumor heterogeneity *in vitro*, a co-culture system consisting of a malignant embryonal rhabdomyosarcoma (ERMS) cell line RD and a normal human skeletal muscle myoblast (HSMM) cell line was established by cell sheet technology. Various ratios of RDs to HSMMs were employed to understand the quantitative effect on intercellular interactions. Disruption of sheet structure was observed in heterogeneous cell sheets having a low ratio of RDs to HSMMs, whereas homogeneous HSMM or RD sheets maintained intact structure. Deeper exploration of dynamic tumor cell behavior inside HSMM sheets revealed that HSMM cell alignment was disrupted by highly motile RDs. This study demonstrated that RMS cells are capable of compromising their surrounding environment through induced decay of HSMMs alignment in a cell-based 3D system. This suggests that muscle disruption might be a major consequence of RMS cell invasion into muscles, which could be a promising target to preventing tumor invasion.

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[**Key words:** 3D culture; Tumor microenvironment; Cell–cell interaction; Time-lapse movie; Rhabdomyosarcoma; Myoblast alignment]

Rhabdomyosarcoma (RMS), a type of cancer in which tumor cells are thought to arise from skeletal muscle, is the most common soft tissue sarcoma found in children (1). Of the two main subtypes of RMS, embryonal rhabdomyosarcoma (ERMS) is the prevalent type comprising 80% of RMS, while alveolar rhabdomyosarcoma (ARMS) is more aggressive with a worse overall outcome (2,3). This disease is characterized by its potential to undergo local invasion and metastasis. Approximately one in five RMS patients are diagnosed initially with metastatic diseases (4–6). The microenvironment a tumor faces consists of the extracellular matrix (ECM), growth factors, and vasculature in addition to the cells that beget them, such as fibroblasts and host tissue cells (7). Cell–cell interaction, especially which between invasive tumor cells and normal cells, plays a vital role in tumor invasion and metastasis (8).

Conventionally, heterotypic cellular interaction has been investigated using 2D substrates or 3D ECM systems with or without direct contact. However, the playground to support cell migration and interaction *in vivo* is always through use of other cells (9–11). Intravital microscopy associated with multiphoton microscopy benefits real-time tracking of fluorescent human tumor cell motility in model animals; however, a gap still remains between animal models and real conditions in human body (12,13). Human cells cultured as spheroid aggregates closely resemble *in vivo* solid tumors in terms of cellular heterogeneity, nutrient and oxygen gradients, and cell–cell connections (14,15). Studies in which normal and malignant epithelial cells were co-cultured in spheroids demonstrated that malignant epithelial cells engulf normal epithelial spheres (16). However, individual cell behavior inside a spheroid cannot be monitored in real time simultaneously with the bulk properties of the spheroid, thereby presenting a challenge in understanding cell migration and cellular interactions.

Cell sheet technology has been emerged as a powerful tool not only in tissue engineering, but also in modeling individual cell behavior on a 3D scale (17,18). In this technology, intact monolayer cell sheets containing ECM are harvested and layered to construct a multilayered cell sheet. This system consists of both cells and ECM, thereby closely resembling *in vivo* conditions. Quantitative analysis can be easily divided into components of the X–Y plane and the Z-axis. Unlike spheroid aggregates, the dynamic status of overall cell sheets can be estimated in terms of sheet fluidity (19). In our previous works, a five-layered human skeletal muscle myoblast (HSMM) sheet was fabricated as a 3D cell-based scaffold to study the target cell behavior that occurred within the structure. Localization of human skeletal muscle fibroblasts (HSMFs) and human umbilical vein endothelial cells (HUVECs) within a five-layered HSMM sheet was previously investigated (20–22).

Although cell sheet technology constitutes a novel technique to use in tissue engineering and *in vitro* model research, there have been few applications of this technique in the cancer research field. To the best of our knowledge, the only cancer-related instance of cell sheet use was studies in which monolayer or multilayer of cancer cells were harvested and used to induce tumors to mice

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in vivo (23,24). This study aims to reveal the interaction between RMS tumor cells (RDs) and non-malignant myoblast cells using a 3D cell sheet culture. Multilayered heterogeneous ERMS sheets were constructed by co-culturing HSMMs and RDs. Time-lapse observations were used to reveal the dynamics of tumor cells in the HSMM sheets that result in disruption of the heterogeneous cell sheet structure. This study revealed heterotypic interaction of cancer cells with normal myoblast cells in a 3D cell sheet model, and highlighted the value of using 3D cultures to analyze the dynamics of cancer cells.

MATERIALS AND METHODS

Cell preparation Human skeletal muscle myoblasts (HSMMs, Lot. No. 4F1619; Lonza Walkersville Inc., Walkersville, MD, USA) and a human ERMS cell line (RD, Cat. No. EC85111502, American Type Culture Collection, VA, USA) were used in this study. According to procedures described by Chowdhury et al. (25), subcultures of HSMMs on laminin-coated surfaces were conducted at 37°C in an atmosphere of 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM; Sigma–Aldrich, MO, USA) containing 10% fetal bovine serum (FBS; ThermoFisher Scientific, MA, USA) and antibiotics (100 U/cm^3 penicillin G, 0.1 mg/cm^3 streptomycin, and 0.25 mg/cm^3 amphotericin B; Invitrogen, CA, USA). RDs were grown in the same medium as the HSMMs without laminin coating. The medium depth was set to 2 mm throughout the experiments. All cells were harvested until 70–80% confluency.

Fabrication of heterogeneous tumor cell sheet HSMMs and RDs harvested from subcultures were stained with a fluorescent reagent (CellTracker Orange and Green, Invitrogen) to obtain different fluorescence according to the commercial instruction. Heterogeneous monolayers were prepared by mixing different ratios of RDs (0%, 10%, 25%, 50%, 75% and 100%) with HSMMs to make final seeding density of 3.5×10^5 cells/cm^2 in each well of 24-well Upcell plates (CellSeed, Tokyo, Japan) with a temperature-responsive poly-*N*-isopropylacrylamide (PNIPAAm) grafted surface and incubated for 24 h at 37°C in a 5% CO_2 atmosphere to form the monolayer sheet. Five-layered heterogeneous sheets were fabricated according to the method previously reported (19) and cultured at 37°C in a 5% CO_2 atmosphere. Cell sheet morphology was observed by an imaging system (IN Cell Analyzer 2000, GE Healthcare, BUX, UK) with a 2× objective lens at 0 and 96 h of incubation time. The samples were fixed at 96 h and observed using a confocal laser scanning microscope (FV1000, Olympus, Tokyo, Japan) with a 60× objective lens.

Evaluation of sheet fluidity in HSMM and RD sheet Evaluation of sheet fluidity in the HSMM and RD sheets was based on a previously reported method (19). Briefly, a five-layered cell sheet with a basal layer (stained by CellTracker Green) and upper layers (stained using CellTracker Orange) was prepared to observe the spatial distribution of the basal target layer using a confocal laser scanning microscope (FV1000 for spatial distribution and FV10i for time lapse, Olympus) with a 60× objective lens at 9 random positions in each sample. Original 8-bit images (256 × 256 pixels) of both colors in each slice were converted into binary images after identifying intensity threshold values. The ratio of green pixels to sum of green pixels in each slice was normalized to determine the distribution of green pixels by dividing into five layers. The normalized distribution of green pixels was assumed to be equivalent to the green cell distribution in the sheet, which was considered to be the frequency of green cells, f_G, in each layer. To quantify sheet fluidity, the *diffusivity* (D, μm^2/h) was applied to measure vertical distribution of target cells based on Fick's second law, $\frac{\partial f_G}{\partial t} = D\frac{\partial^2 f_G}{\partial h^2}$, in which f_G, t and h represent the green cell frequency, incubation time, and sheet thickness, respectively. The Crank–Nicolson finite difference method and least squares method were applied to calculate the diffusivity using a customized software designed by LabVIEW (National Instruments, Austin, TX, USA). Time-lapse observation for the dynamic demonstration of sheet fluidity was conducted by obtaining images every 1 h at several positions using a confocal laser scanning microscope.

Spatial distribution of target cells in five-layered HSMM and RD sheets To determine the vertical distribution of target cells (HSMMs and RDs) inside the HSMM and RD sheets, localization of the green target cells (HSMMs or RDs labeled green) within the red HSMM or RD sheets was observed by a confocal laser scanning microscope at 60× magnification. Quantitative analysis of the frequency of green target cells and diffusivity is same as described above. Time-lapse observation were conducted to assess the dynamic behavior of individual cells by obtaining images every 1 h at several positions using a confocal laser scanning microscope.

Statistical analysis Data presented in this study were obtained from three independent cultures and expressed as means with standard deviations (SDs). Student's *t*-test was used to determine the statistical significance of differences among data sets, and values of $P < 0.05$ were considered significant.

RESULTS

Effect of RD ratio on sheet morphology disruption and segregation To understand the effect of the RD ratio on the overall sheet structure, heterogeneous five-layered cell sheets were created by stacking monolayer sheets with different ratios of RDs ranging from 0% to 100%. Time-course observations of the overall sheet shape were conducted every 24 h. To visualize different cell types, RDs and HSMMs were stained green and red on captured images, respectively. Fig. 1A depicts the morphologies of different heterogeneous cell sheets at the initial ($t = 0$) and final ($t = 96$ h) of the culture. At $t = 0$, all sheets were constructed with an intactly round shape, thereby demonstrating success in sheet fabrication. At $t = 96$ h, the sheets with low ratio of RDs (10% to 50%) experienced destruction of overall sheet structure, while the

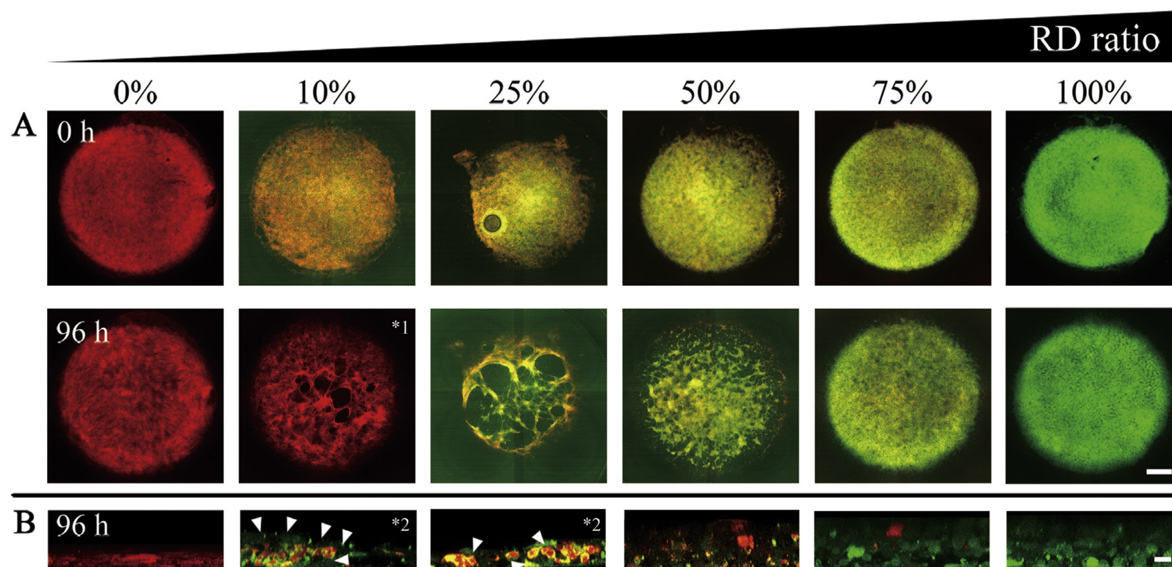

FIG. 1. Effect of RD ratio on sheet structure deformation. (A) Time course of sheet morphology at $t = 0$ and 96 h. Scale bar: 2 mm. (B) Localization of RDs (green) and HSMMs (red) in heterogeneous sheets at 96 h. Arrows: RDs localized outside of HSMMs aggregate. Scale bar: 20 μm. *1: Green channel was not combined due to weak fluorescence; *2: Contrast of green channel was enhanced to visualize RDs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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