Tissue Engineering Human Placenta Trophoblast Cells in 3-D Fibrous Matrix: Spatial Effects on Cell Proliferation and Function

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Nonwoven polyethylene terephthalate (PET) fabrics with different porosities and knitted fabric were used as support matrices to grow human trophoblast cells to study the spatial effects of fibrous matrix on cell adhesion, spatial organization, proliferation, and metabolic functions. In general, cells grown on 2-D surface and knitted fabric had faster metabolic rates and also showed higher proliferation activities as detected by cyclin B assay. For nonwoven PET fibers, matrix porosity had profound effects on cell morphology, spatial organization, and proliferation. Cells grown in a low-porosity fibrous matrix formed small aggregates (~100 cells per aggregate), whereas cells grown in high-porosity matrix formed big aggregates (~1000 cells per aggregate). This was attributed to the difference in pore volume or averaged fiber distance, which dictated a cell’s ability to cross over and form a bridge between adjacent fibers. The high-porosity matrix had a relatively poor surface accessibility for cells to attach and spread, which are essential for cell proliferation. Dual staining with PI and BrdU showed that 60% of cells in the small aggregates found in the low-porosity matrix were proliferating, while only 18% of cells in the large aggregates found in the high-porosity matrix were proliferating. These results suggest that spatial characteristics of fibrous matrix are important to cell proliferation and function and should be considered in tissue-engineering human cells.

Introduction

The human placenta is the organ responsible for the transport of all substances between a mother and a developing fetus. In human placenta, chorionic villi are the major functional parts that project into the maternal intervillous space and provide a vast contacting surface area for maternal–fetal exchange (1, 2). On the outermost layers of the chorionic villous, multinucleate syncytiotrophoblast, differentiated by cytotrophoblast fusion, lines the entire placenta by late first-trimester and forms a selective maternal–fetal barrier which provides the transport interface between maternal and fetal compartments. Syncytiotrophoblast secretes many hormones and plays an important role in maintaining the well-being of the developing fetus. Secretion of functional hormones, such as 17β-estradiol, human placental lactogen (hPL), and human chorionic gonadotropin (hCG), is often used as a functional index of human trophoblast cells (3).

Drug administration is often needed due to many medical complications during pregnancy. Understanding drug transport and metabolism in placenta under both normal and pathological conditions is thus important to the development of therapeutic agents for use during pregnancy. Because of the increasing awareness of animal welfare in medical research and discrepancy between human and animal species, in vitro culture systems that can faithfully reproduce important aspects of trophoblast in vivo conditions are highly desirable. The goal of this work is to develop a novel tissue-engineered human trophoblast culture system as an in vitro human placenta model for drug testing.

We have developed a bioreactor system for long-term culturing of human trophoblasts in a nonwoven PET fibrous matrix (4). Fibrous material is among the most popular material forms used in various tissue-engineering applications because its high specific surface area, excellent mechanical properties, high void volume, and 3-D matrix structure are desirable for high-density cell and tissue cultures. Many synthetic polymers can be fabricated into fibrous forms for tissue-engineering applications. Nonwoven polyglycolic acid (PGA) and polylactic acid (PLA) have been widely used for tissue engineering of chondrocytes (5–7), smooth muscle cells (8), epithelial cells (9, 10), keratinocytes and fibroblasts (11–14), nerve cells (15), and cardiac muscle (16). Chemical compositions (5), spatial patterning of biochemical ligands (15), cell seeding method and density (8), and scaffold thickness (7) were found to have significant effects on the properties of cell–polymer composites. It has also been found that the surface texture of a biomaterial controls cell adhesion, shape, proliferation, and function (15, 17–21). The effects of scaffold surface...
are also discussed in this paper.

The effects of porosity of the fibrous matrix on the spatial cell organization and aggregate functions of cells have been investigated for endothelial cells (15, 17, 18), nerve cells (15), hepatocytes (19, 22), and skin fibroblast (20). However, very little is known about the spatial effects of the three-dimensional fibrous matrix on tissue engineering, although the 3-D structure has profound effects on cell morphology, proliferation, migration, differentiation, and function (23).

Fibrous materials offer a wide range of suprastructures by changing fiber diameter, orientation, porosity, and woven and knitting characteristics. Nonwoven fabrics are manufactured by entangling fibers or filaments to form an isotropic 3-D matrix structure, leaving a vast empty space with a typical porosity in the range of 80–99% (24). Knitted fabrics can offer well-defined textures in two or three dimensions, and nonwoven ones are ideal candidates for forming three-dimensional structures. In this work, the spatial effects of nonwoven and knitted polyester fabrics on cell aggregate formation, proliferation, and function of human trophoblast ED27 cells (25) were studied. Metabolic, hormone secretion, and proliferation activities of cells cultivating in nonwoven fibrous matrixes, in knitted fabrics, and on 2-D surface were studied. The spatial cell organization and aggregate formation in various fibrous matrixes were also studied. The effects of porosity of the fibrous matrix on the apparent pore volume or distance between adjacent fibers, which in turn may affect cell adhesion and stretching on fiber surfaces and thus cell proliferation, are also discussed in this paper.

### Table 1. Physical Parameters of Nonwoven and Knitted PET Fabrics

<table>
<thead>
<tr>
<th>fabric type</th>
<th>matrix thickness before compression (mm)</th>
<th>matrix thickness after compression (mm)</th>
<th>disk diameter (mm)</th>
<th>weight (g)</th>
<th>solid fraction</th>
<th>porosity</th>
<th>specific surface area (cm²/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nonwoven fabrics with thermal compression</td>
<td>LP 1.670 ± 0.025</td>
<td>1.029 ± 0.025</td>
<td>26.0 ± 0.5</td>
<td>0.115 ± 0.001</td>
<td>0.156 ± 0.002</td>
<td>0.844 ± 0.002</td>
<td>284 ± 8</td>
</tr>
<tr>
<td></td>
<td>MP 1.511 ± 0.025</td>
<td>1.012 ± 0.025</td>
<td>26.0 ± 0.5</td>
<td>0.096 ± 0.001</td>
<td>0.132 ± 0.001</td>
<td>0.868 ± 0.001</td>
<td>241 ± 7</td>
</tr>
<tr>
<td></td>
<td>HP 1.422 ± 0.025</td>
<td>1.003 ± 0.025</td>
<td>26.0 ± 0.5</td>
<td>0.075 ± 0.005</td>
<td>0.103 ± 0.007</td>
<td>0.897 ± 0.007</td>
<td>190 ± 5</td>
</tr>
<tr>
<td>nonwoven fabrics without thermal compression</td>
<td>HLP 1.634 ± 0.025</td>
<td>26.5 ± 0.5</td>
<td>0.127 ± 0.001</td>
<td>0.105 ± 0.001</td>
<td>0.895 ± 0.001</td>
<td>190 ± 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HHp 1.782 ± 0.025</td>
<td>26.5 ± 0.5</td>
<td>0.096 ± 0.005</td>
<td>0.072 ± 0.003</td>
<td>0.928 ± 0.003</td>
<td>132 ± 4</td>
<td></td>
</tr>
<tr>
<td>knitted fabric without thermal compression</td>
<td>Kf 0.631 ± 0.025</td>
<td>26.0 ± 0.5</td>
<td>0.076 ± 0.002</td>
<td>0.168 ± 0.002</td>
<td>0.832 ± 0.002</td>
<td>190 ± 5</td>
<td></td>
</tr>
</tbody>
</table>

The data indicate a significant difference (ANOVA, \( P < 0.05 \)) in porosity between LP, MP, and HP and a significant difference (t test, \( P < 0.005 \)) in porosity between HLP and HHP. The data also indicate no statistically significant difference in thickness between LP, MP, and HP. For knitted fabric, the porosity here is bulk porosity.

### Microstructure

Microstructure on modulating the spatial organization and functions of cells have been investigated for endothelial cells (15, 17, 18), nerve cells (15), hepatocytes (19, 22), and skin fibroblast (20). However, very little is known about the spatial effects of the three-dimensional fibrous matrix on tissue engineering, although the 3-D structure has profound effects on cell morphology, proliferation, migration, differentiation, and function (23).

Fibrous materials offer a wide range of suprastructures by changing fiber diameter, orientation, porosity, and woven and knitting characteristics. Nonwoven fabrics are manufactured by entangling fibers or filaments to form an isotropic 3-D matrix structure, leaving a vast empty space with a typical porosity in the range of 80–99% (24). Knitted fabrics can offer well-defined textures in two or three dimensions, and nonwoven ones are ideal candidates for forming three-dimensional structures. In this work, the spatial effects of nonwoven and knitted polyester fabrics on cell aggregate formation, proliferation, and function of human trophoblast ED27 cells (25) were studied. Metabolic, hormone secretion, and proliferation activities of cells cultivating in nonwoven fibrous matrixes, in knitted fabrics, and on 2-D surface were studied. The spatial cell organization and aggregate formation in various fibrous matrixes were also studied. The effects of porosity of the fibrous matrix on the apparent pore volume or distance between adjacent fibers, which in turn may affect cell adhesion and stretching on fiber surfaces and thus cell proliferation, are also discussed in this paper.

### Materials and Methods

#### Culture and Medium

The trophoblast cells (known as ED27 cells), obtained from human first-trimester chorionic villi (25), were used in this study. The cultures have been maintained by continuously subculturing in T-flask since 1990 without apparent phenotypic drift (25). The stock, frozen cultures were thawed in room temperature and then transferred to phenol-red free Ham’s F12/Dulbecco’s modified Eagle’s medium (F12/DMEM, 1:1) (Gibco, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS) (Upstate Biotechnology, Lake Placid, NY), 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 µg/mL gentamicin sulfate.

#### Fibrous Matrix

Both knitted and nonwoven polyethylene teraphthalate (PET) fabrics (fiber diameter, ~20 µm; fiber density, 1.35 g/cm³) were used as cell culture matrixes. The PET fabrics were first treated with 1% NaOH solution at boiling temperature for 1 h to reduce surface hydrophobicity and to increase biocompatibility by partially hydrolyzing PET to create carboxyl and hydroxyl groups on fiber surfaces (4, 26). For nonwoven fabrics, some were thermally compressed in a 121 °C chamber by weight for 90 min to permanently reduce the thickness of the fibrous matrix to ~1 mm and to create fabrics with different matrix densities (solid fractions) or porosities (Table 1). The compressing process also yielded a relatively uniform thickness of the nonwoven fabrics. These fabrics were then cut into small circular patches (diameter: 2.6 cm). The total fiber volume in each fabric patch was determined by the weight of the patch divided by the PET density of 1.35 g/cm³ (27). The volume fraction of the PET fiber in the fabric matrix, or solid fraction, and porosity of the fibrous matrix were then determined from the fiber volume and total matrix volume, which was calculated from the thickness and diameter of the fabric patch. The nonwoven fabrics were isotropic and had a random but relatively uniform fiber spatial arrangement within the matrix. The knitted fabric, however, was not isotropic, with relatively large pores (~300 µm) and open areas among yarns (diameter: ~400 µm).

#### Cell Culture Experiments

Unless otherwise noted, all cell culture experiments were conducted in 6-well plates and each well contained one fabric patch. The PET fabrics were first autoclaved, and each patch was then placed in one well and incubated with 4 mL of the medium in a CO₂ incubator overnight. Seeding cultures were prepared by trypsinizing ED27 cells grown in a T-flask to make a cell suspension containing (0.1–1) × 10⁶ cells/mL. Each well containing the PET fabric was inoculated with a known amount of cells (~8.5 × 10⁶) and then incubated in a CO₂ incubator for ~60 min to allow for cell attachment to the fibrous matrix. The matrix with cells was then transferred to a new plate and washed with media to remove cells that were unattached but stayed in the interstitial space in the matrix. Thus, the cells retained inside the matrix were cells attached to the fiber surface by adhesion or interception. The PET matrixes containing attached cells were then transferred to a new plate (each well contained 6 mL of the medium) to begin the cell culture kinetic study. This seeding procedure ensured that all growth was attributed to cells attached to the PET fiber, instead of the well surface. The number of cells attached to the PET matrix was estimated from the total seeding cell number minus the number of unattached cells left in the old well. Similar seeding efficiency, ~30%, was attained for various fibrous matrixes studied by this seeding procedure (Table 2).

The cultures were incubated in a humidified CO₂ incubator (37 °C) for about 54 h. Liquid samples (200 µL each) were taken at various time intervals for glucose, lactate, and estradiol assays to monitor the metabolic
activities of cells grown in various fibrous matrixes. Samples were stored in a −85 °C freezer. Parallel control experiments with cultures grown in wells without any fabrics were also conducted to study the effect of growth on a 2-D surface, in comparison with growth in a 3-D matrix. Triplicate fabrics were prepared for each condition, and duplicate samples were used for each data point. All quantitative results were expressed as the mean ±sd. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Fisher’s least-squares difference (LSD) procedure for multiple comparisons using a commercial software package (Minitab Inc., State College, PA). Student’s t test was used to compare the difference between selected groups. Differences were considered as statistically significant when \( P < 0.05 \).

To study cell proliferation activities in various fibrous matrixes, we took culture samples at 24 h for BrdU assay and Cyclin B assay described latter. At the end of the culturing period, the total cell number was determined by measuring the total DNA content using a fluorometric DNA assay, and fibrous matrix samples were taken for determination of Glucose, Lactate, and Estradiol concentrations. Glucose and lactate concentrations in the medium samples were analyzed using YSI Biochemistry Select Analyzer (Yellow Spring, OH). Estradiol was assayed by a specific EIA kit (Cayman Chemical Co., Ann Arbor, MI) as outlined by the manufacturer. Each assay more times in the blocking buffer, samples were incubated with a secondary antibody of anti-mouse IgG-FITC (Jackson ImmunoResearch Laboratories, Inc., West Groce, PA) for 30 min. The samples were then completely rinsed in PBS.

To determine the fraction of total cell population in the matrix that was proliferating or nonproliferating, we also stained the entire cell population with propidium iodide (PI), a nuclic acid dye that would stain all of the cell nuclei regardless of the cell cycle stage (28). Briefly, after incubation with FITC-conjugated secondary antibody, the culture samples were then stained with PI at a concentration of 20 \( \mu \text{g/mL} \) for 5 min at room temperature. Then, the samples were examined under a confocal laser scanning microscope (CLSM) (BioRad MRC-600, Hercules, CA) using dual excitation filters, 488 nm for FITC labeling (BrdU-containing cells) and 568 nm for PI labeling (total cell population). The cell images of the same sample obtained at these two different wavelengths would allow one to identify proliferating and nonproliferating cells in the cell–fiber matrix.

Negative controls were prepared for cultures grown without BrdU, following the same fixation procedures and stained with the secondary antibody only. Completely negative pictures were obtained for the negative controls, suggesting the high specificity of the antibody used in this assay. Negative controls were also prepared for cells only stained by PI, following the same fixation procedures. Completely negative images were observed at 488 nm for cells only stained by PI, indicating that there was no false BrdU-cell image from PI staining.

**Cyclin B Assay.** Cyclin B is a protein found in replicating cells, and its cellular level can be used as an index for cell proliferation (29). Detection of cyclin B in cell samples from 2-D cultures, nonwoven fabrics, and knitted fabric followed the method given by Perkins et al. (30) with some modifications. Briefly, 24 h culture samples were rinsed with PBS before lysis (1% Triton X-100, 120 mM sodium chloride, 25 mM HEPES, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 10 \( \mu \text{g/mL} \) leupeptin, 10 \( \mu \text{g/mL} \) aprotinin, and 10 \( \mu \text{g/mL} \) antipain). Cell extracts were incubated at 4 °C for 30 min and centrifuged at 12,000 rpm for 10 min. An appropriate amount of supernatant, determined by protein assay, was solubilized in 5× SDS–PAGE buffer (1× SDS–PAGE buffer) and loaded for 5 min. Protein samples (15 \( \mu \text{g/mL} \)) were fractionated on a 10% SDS–polyacrylamide gel, transferred to nitrocellulose filters, and incubated in Tris-buffered saline, 0.2% Tween-20, and 5% nonfat milk (BLOTTO). Nitrocellulose filters were then incubated with an anti-cyclin B monoclonal antibody (1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Secondary antibody incubations with peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Groce, PA) were in BLOTTO for 1 h. Immunoreactive proteins were visualized by development in LumiGLO (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and exposure to X-ray films. Densitometric analysis was performed using Personal Densitometer SI and ImageQualNT (Molecular Dynamics Inc., Sunnyvale, CA).

**Determination of Glucose, Lactate, and Estradiol Concentrations.** Glucose and lactate concentrations in the medium samples were analyzed using YSI Biochemistry Select Analyzer (Yellow Spring, OH). Estradiol was assayed by a specific EIA kit (Cayman Chemical Co., Ann Arbor, MI) as outlined by the manufacturer. Each assay

### Table 2. Seeding Efficiency and Growth of Trophoblast Cells in Nonwoven and Knitted Fabrics and in 2-D Culture

<table>
<thead>
<tr>
<th>Fabric Type</th>
<th>Initial Cell Number a (10^6)</th>
<th>Percent Cell Attachment (%)</th>
<th>Final Cell Number b (10^6)</th>
<th>Percent Cell Increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP</td>
<td>2.60 ± 0.02</td>
<td>30.6 ± 0.2</td>
<td>4.38 ± 0.14</td>
<td>68.4 ± 4.5</td>
</tr>
<tr>
<td>MP</td>
<td>2.55 ± 0.12</td>
<td>30.0 ± 1.4</td>
<td>4.37 ± 0.17</td>
<td>73.2 ± 4.4</td>
</tr>
<tr>
<td>HP</td>
<td>2.40 ± 0.17</td>
<td>28.2 ± 2.0</td>
<td>3.45 ± 0.12</td>
<td>44.2 ± 5.3</td>
</tr>
<tr>
<td>HLP</td>
<td>2.72 ± 0.16</td>
<td>30.8 ± 1.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HHP</td>
<td>2.46 ± 0.30</td>
<td>27.8 ± 3.4</td>
<td>3.99 ± 0.20</td>
<td>124 ± 11.1</td>
</tr>
<tr>
<td>Kf</td>
<td>1.78 ± 0.08</td>
<td>20.9 ± 0.9</td>
<td>5.90 ± 0.50</td>
<td>70.0 ± 14.5</td>
</tr>
<tr>
<td>2-D</td>
<td>3.47 ± 0.69</td>
<td>5.50 ± 0.10</td>
<td>120 ± 3.9</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.84 ± 0.31</td>
<td>5.62 ± 0.05</td>
<td>569 ± 8.4</td>
<td>-</td>
</tr>
</tbody>
</table>

a The inoculation cell number for LP, MP, HP, and Kf was 8.5 × 10^6. The inoculation cell number for HLP and HHP was 8.84 × 10^6. The data indicate no significant difference between initial cell numbers in various nonwoven fabrics (ANOVA, \( P > 0.05 \)). The data also indicate a significant different initial cell number in Kf from various nonwoven fabrics (ANOVA, \( P < 0.05 \)). b The final cell number was measured at 54 h, except for the 2-D was at 64 h, after inoculation. c The data indicate a significant different cell number increase in HP from LP and MP (ANOVA, \( P < 0.05 \)). The data also indicate a significant different cell number increase in Kf from various nonwoven fabrics (ANOVA, \( P < 0.05 \)). For 2-D culture, the cell number increase depends on initial seeding density.
was run in duplicate with a standard curve and measured at 405 nm. Detailed procedures can be found elsewhere (4).

**DNA Assay.** The amount of DNA was assayed and used to determine the cell number in the culture matrices. DNA digestion procedure followed the standard method (31). Cell–polymer matrixes were washed with PBS and then digested in a buffer solution (pH 8) containing 0.1 mg/mL proteinase K, 5 mg/mL SDS, 10 mM Tris-HCl, 25 mM EDTA, and 0.1 M NaCl at 50 °C for 12 h with occasional mixing. Standards were prepared by dissolving salmon testes DNA (Sigma, St. Louis, MO) in digestion solution and were calibrated using a spectrophotometer at 260 nm wavelength. The enzyme-digested samples (10 μL) were aliquoted into a 96-well plate and mixed with a dye solution (100 μL; composition: 10 mM Tris-base, pH 8, 0.1 M NaCl, 0.4 μg/mL Hoechst 33258 dye). The plate was then read in a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The average DNA content of ED77 trophoblast cells was found to be 8.6 pg/cell.

**Scanning Electron Microscopy (SEM).** Fibrous matrix samples were fixed with 1.6% glutaraldehyde in a phosphate buffer (pH 7.0). After osmication in 1% OsO4 and dehydration in ethanol, the samples were dried in a critical point dryer. After being sputter coated with gold/palladium, the samples were examined using a scanning electron microscope (Philips XL 30). Details have been given elsewhere (4).

**Results**

**Modifications of PET Matrix.** PET is a viscoelastic material; PET matrix can be compressed to result in a permanent deformation. The compressing process is accelerated at elevated temperatures. To study the spatial effects of PET matrix on cell morphology, proliferation, and function, we thermally compressed several nonwoven PET fabrics to yield PET fibrous matrixes with various matrix solid fractions (0.072–0.156), porosities (0.844–0.928), and specific surface areas (132–284 cm²/cm³) (see Table 1). As can be seen in Table 1, thermal compression reduced the matrix thickness and volume by 30–40%, depending on the initial thickness. Therefore, compression also reduced the pore volume and distance between fibers. The glass transition temperature (Tg) for PET is ~80 °C, and the melting temperature (Tm) is above 238 °C (27, 32). The temperature (122 °C) used in this study thus should not alter or compromise the chemical and physical properties, such as surface composition and mechanical strength, of the PET fiber. The diameter of the PET fibers in all matrixes studied was the same, ~20 μm. Any effects observed for cultures grown in various matrixes thus could be attributed to the spatial effects arising from differences in available surface area, surface topology, and pore volume (or distance between adjacent fibers).

**Cell Seeding.** The static seeding method used in this study gave approximately the same inoculation efficiency (~30%, P > 0.10) for all nonwoven fabrics with various porosities (see Table 2), indicating that thermal compression did not significantly change the projected opening areas on the X–Y plane of the fibrous matrix. Previous study has suggested that compression reduced the pore size of nonwoven fabric proportionally regardless of the original pore size (33). The knitted fabric had a low seeding efficiency (~21%) because of its large pore size (~300 μm) and percent open area. The relatively low seeding efficiency could be due to poor cell contacts with fibers (due to high porosity), which can be improved by mixing or agitation as used in a dynamic seeding method (8), and low cell viability in the seeding culture (nonviable and apoptotic cells are nonadhesive).

**Cell Culture Kinetics and Metabolic Rates.** Glucose consumption, lactate formation, and estradiol production were monitored for cultures grown in various matrix environments. Typical kinetics for cultures grown in 2-D (no fiber), knitted fabric, and nonwoven fabrics with high and low porosities are shown in Figure 1. In general, the rates for glucose consumption and lactate formation were relatively constant for the first 50 h, indicating no oxygen limitation. In contrast, estradiol production was low at the beginning but then increased significantly at ~20 h. As shown in Figure 2, there was no significant difference in lactate yield or estradiol production from glucose for cells grown in various matrix environments. Also, the lactate yield from glucose was constant, ~1.4 mol/mol of glucose, which was well within the range of aerobic metabolism.

Although the kinetics of lactate and estradiol production from glucose appeared to be similar for all matrixes studied, there were significant differences in cell growth and metabolic rates. It is noted that cells cultured in the knitted fabric had significantly higher growth and metabolic rates than those with nonwoven fabrics. As shown in Table 2, the cell number increased by more than 120% in knitted fabric, but only ~70% increase was obtained in nonwoven fabrics. Growth was faster for cells grown in the knitted fabric because the fiber surfaces were more accessible for cells to attach and spread, which were essential for cell proliferation. On the other hand, nonwoven fibers were separated with a greater distance, which made cell spreading to neighboring fibers more difficult. This effect was especially prominent for nonwoven fabrics with a high porosity and high pore volume (sample HP), which gave only 44% increase in cell number.

As also shown in Table 2, growth on 2-D surface was highly dependent on the seeding density. Regardless of the initial cell density, the final total cell number in the 2-D culture at 60 h was about the same, ~5.6 × 10⁵ cells, indicating confluence on the well surface. Consequently, the apparent growth rate decreased by 8-fold as the seeding number increased from 0.84 × 10⁵ to 3.47 × 10⁶ cells. The results clearly indicated that there was more available surface for cells to spread and grow at a lower seeding density and the better surface availability yielded a faster cell growth. Contact inhibition also might have contributed to the decreased growth rate at the high seeding density. At a comparable initial seeding cell number, 2-D culture had a much faster growth rate than those from the nonwoven PET matrixes.

The specific glucose consumption rate was estimated from the initial glucose consumption rate divided by the initial cell number. As shown in Figure 3, Kf and 2-D cultures with higher growth rates also showed higher specific glucose consumption rates than those for growth in the nonwoven matrixes (LP, MP, and HP), and the difference was statistically significant based on ANOVA (P < 0.05).

**Cell Proliferation.** Cyclin B is one component of the mitotic promoting factor in all cells; the intracellular level of this protein must reach a threshold level for cells to enter mitosis (29). Therefore, cyclin B can be used as indicative of cell proliferation activity. Figure 4 shows intracellular cyclin B expression levels for 24 h cell
samples from various matrixes studied. As expected, the cyclin B level for cells from knitted fabric was 2.6 times higher than that from nonwoven fibers. It is noted that the 2-D culture had the highest cyclin B expression level among all cultures studied, indicating that cells grown on a 2-D surface are highly proliferating. This is consistent with the fact that cells grown on a 2-D surface would have a greater accessibility to available surface area for adhesion, stretching, and spreading, which are considered to be stimulating factors for cell proliferation (18, 19, 34, 35). Trophoblast cells from a full-term delivered placenta were also tested for the cyclin B level as a negative control. As expected, a negligible amount of cyclin B was detected for trophoblast cells in term placenta (Figure 4), as they were fully differentiated and nonproliferating cells.

Spatial Effects on Cell Adhesion and Aggregation. The observed differences in cell proliferation and metabolic rates from cultures in various matrix environments might be attributed to the spatial effects on cell adhesion and spreading on solid surfaces and formation

![Figure 1](image1.png) Kinetics of trophoblast cell cultures grown in various support matrixes: LP, low-porosity nonwoven PET fabric; HP, high-porosity nonwoven PET fabric; Kf, knitted PET fabric; 2D, 2-dimensional culture without any fabric.

![Figure 2](image2.png) Lactate and estradiol production from glucose in trophoblast cultures grown in various support matrixes: LP, low-porosity nonwoven PET fabric; HP, high-porosity nonwoven PET fabric; Kf, knitted PET fabric; 2D, 2-dimensional culture without any fabric.

![Figure 3](image3.png) Comparison of specific glucose consumption rates in trophoblast cultures grown in various support matrixes: LP, low-porosity nonwoven PET fabric; MP, medium-porosity nonwoven PET fabric; HP, high-porosity nonwoven PET fabric; Kf, knitted PET fabric; 2D, 2-dimensional culture without any fabric.
of cell aggregates in the 3-D matrix structure (36). We examined the spatial organization of cells in fibrous matrixes using both SEM and CLSM. In general, four distinctive cell organizations in the fibrous matrixes were observed: (1) cells spread and attached on fiber surface (Figure 5a); (2) cells attached and bridged between two fiber surfaces (Figure 5b); (3) small cell aggregates, with a cell number less than 10^2, formed between adjacent fibers (Figures 5c and 6a); and (4) large cell aggregates, with a cell number larger than 10^3, formed around several intersecting fibers (Figures 5d and 6b).

It is noted, however, that cell spatial organizations in different fibrous matrixes were quite different. For culture grown in the knitted fabric, most cells were well dispersed on the fiber surfaces or embedded in the space between fibers on the surface of a yarn. In contrast, large percentages of cells were present in the form of cell aggregates in nonwoven fabrics, with large cell aggregates (~10^3 cells/aggregate) as the dominant cell formation in the high-porosity fibrous matrix (HP) and small cell aggregates (with cell number less than 10^2) in the low-porosity matrix (LP).

The different spatial cell organizations can be explained by the difference in the accessible surface area for cell attachment in different fabrics. The importance of accessible surface area for cell adhesion and proliferation is well-known (35). However, the effects of available surface area are not only determined by the available surface area within the matrix support but also controlled by the surface accessibility to cells, which is characterized by the distance between adjacent fibers in the 3-D space. The pore volume and average distance between fibers in an isotropic nonwoven matrix should increase with increasing porosity when the fiber diameter is the same. When the distance separating two adjacent fibers is not too big, cells attached on one fiber should be able to reach out to the neighboring fiber, as was observed in the low-porosity matrix (LP) (Figure 5b). Thus, smaller aggregates were formed in lower-porosity matrixes (Figure 6a). On the other hand, when the distance is too big to allow cells to cross over to form a bridge between two fibers, such as in the high-porosity matrixes (HP), large cell aggregates were formed (Figure 6b).

Localization of Proliferating Cells in Fibrous Matrix. The different spatial organizations of cells in the fibrous matrixes seemed to be closely related to the
observed different cell proliferation activities and metabolic rates. BrdU assay was thus used to study and identify proliferating cells that were in the DNA synthesis stage. A dual-staining technique (with both BrdU and PI) was also developed to differentiate proliferating cells from nonproliferating cells within the same cell–fiber matrix. The percentage of proliferating cells in the total cell population can be estimated by comparing the same cell image observed under two different wavelengths for different stains. Figure 7 shows the CLSM images of cells stained with BrdU. The same CLSM images for cells stained with PI are shown in Figure 5. It is apparent that the majority (~64%) of cells on the knitted fabric surface were in proliferation (Figure 7a). Most cells (~75%) that were adhered on the fiber surface or bridged between adjacent fibers were BrdU-incorporated cells (Figure 7b). About 60% of cells in the small cell aggregates formed between close adjacent fibers also had high proliferating activity (Figure 7c). However, most cells in the large cell aggregates found in the high-porosity fibrous matrix were not proliferating; BrdU-incorporating cells were found mostly at the surface of the aggregate, not in the center, and the percentage of BrdU-incorporating cells was less than 20% (Figure 7d). The results from this study are consistent with findings from cyclin B assay and cell culture kinetics discussed earlier.

In tissue engineering, cells need first to attach to the matrix scaffold and then to rearrange themselves as they grow into high density. Cell density in the PET fibrous matrix reached more than $10^8$ cells/mL in a bioreactor environment with controlled pH and dissolved oxygen (4). Figure 8 shows that there were extensive extracellular matrix (ECM) proteins on the fiber surface and intimate
cell–fiber and cell–cell contacts, suggesting that the PET matrix was biocompatible for trophoblast cells.

Discussion

In tissue engineering, the organization of three-dimensional structure is vital for functions and for the tissue development in vitro. Cell proliferation in a fibrous matrix scaffold is a critical step in engineering human cells in vitro. Extensive research has been conducted to modify the biocompatibility of surface condition for cell attachment and proliferation. Recently, the role of scaffolds is increasingly thought to be beyond just providing a biocompatible surface for cell attachment. In this study, PET fibrous matrix, as scaffold, architecture was found to be an important factor in regulating cell–fiber and cell–cell contacts, and as a result, in determining the overall cell proliferation and functions.

Spatial Effects on Cell Proliferation and Differentiation.

It is clear that the spatial properties of fibrous matrix have profound effects on cell adhesion, stretching, and aggregate formation on fiber surface and in the 3-D space within the fibrous matrix, which in turn may affect cell shape, proliferation, differentiation, and function (35). In this study, we found that morphological variations and spatial distribution of cells in nonwoven fabrics with different porosities contributed to the differences in cell proliferation and metabolic activities. The formation of large cell aggregates lowered cell proliferation activities, especially for cells in the center of cell aggregates. This phenomenon has also been reported for cells grown on aggregates on microcarrier beads (36). In contrast, cells grown on 2-D smooth surface or on the surfaces of closely bundled fibers in a yarn of knitted fabric usually do not form aggregates, especially when the cell density in the matrix is not high, and have high proliferation activities. Nonwoven fibrous matrix yielded different spatial organizations of cells and sizes of cell aggregates, depending on matrix porosity. Therefore, porosity of the fabric matrix should be optimized to allow fast cell growth and proliferation to reach a high cell density before the formation of large aggregates, which inevitably reduces proliferation due to the cell–cell contact triggering growth inhibitory signal (35). The portion of quiescent, nonproliferating cells usually increases toward the center of the cell aggregates, and a central necrosis could occur in multicell spheroids exceeding a diameter of 300–400 μm due to nutrient limitation (36).

The porosity of the fibrous matrix may also affect cell differentiation since cell–cell contact and aggregation usually may also lead into cell differentiation (35), which is another important aspect in engineering functional tissues. Nontransformed cells cultivated on 2-D surfaces usually grow into confluent monolayer cells, which often then leave cell cycle and enter apoptosis, instead of differentiation. In contrast, multilayer cell growth or aggregation is usually found in the 3-D culture matrix. It is thus important to also consider the spatial effects on cell differentiation. The PET fibrous matrix used in this work supported good cell adhesion, stretching, proliferation, aggregation, and differentiation (4). As shown in Figure 8, intimate cell–cell contact was found in cell aggregates, which set the stage for cell fusion into multi-nucleate syncytiotrophoblast, a morphological differentiation essential to placenta trophoblast cell function (2). Further study on the design of fibrous matrix to

Figure 7. CLSM BrdU-stained cell images of trophoblast grown in various fibrous matrixes (200×): A, ~64% cells on knitted fiber surfaces were BrdU stained cells; B, ~75% cells on fibers and bridging between two fibers in LP nonwoven fabric were BrdU-stained (arrows pointing to some cells not stained with BrdU and were nonproliferating); C, ~60% cells in the small aggregates on fibers and between two adjacent fibers in LP nonwoven fabric were BrdU stained; D, ~18% cells in the large cell aggregate formed around intersecting fibers in HP nonwoven fabric were BrdU stained, most of them were on the surface of the aggregate (arrows pointing to some cells stained with BrdU). The confocal images for each sample were taken at 488 nm excitation wavelength with 5 μm increment: in A and B, each picture is the projection of a total of 9 slices; C is the projection of a total of 14 slices; D is the sixth slice of a total of 9 slices.
optimize both proliferation and differentiation is underway in our laboratory.

**Accessible Surface Area.** The more important spatial effect may be attributed to the accessible surface area controlled by porosity or pore volume of the 3-D matrix. The results from this study support the hypothesis of accessible surface area regulating trophoblast proliferation activity. Chen et al. (18) reported a correlation between cell proliferation and accessible surface area and found that cells adhered on a large surface area, survived better, and proliferated faster than cells with a more rounded shape adhered to a smaller surface area. Singhvi et al. (19) also presented a similar relationship between cell shape and DNA synthesis. By using spongelleike collagen matrix, Nehrer et al. (37) showed that pore size influenced canine chondrocytes morphology and DNA content. However, most previous studies were conducted in a 2-D environment or in spongelleike materials with a continuous solid surface for cell attachment; little has been done with a 3-D synthetic fibrous matrix with discrete surfaces in space. Because of the unique 3-D structural characteristics offered by synthetic fibrous materials, understanding and characterizing the spatial effects from a fibrous matrix are important to tissue engineering.

There appears to be a critical porosity or fiber distance that controls cell spreading (bridging) and aggregation. The significant differences in cell spatial organization, metabolic rates, and proliferation activity were observed only between MP (porosity: 0.868) and HP (porosity: 0.897); no significant differences were found for matrix samples in the groups with porosities higher than 0.895 (HLP and HHP) or lower than 0.868 (LP and MP). This indicates that, when the fiber distance is bigger than a critical distance, no cell bridging can occur and only large cell aggregates can be formed when cells proliferate because of limited surface accessibility. As the fiber distance reduces to below the critical level, the effects also disappear because cells have similar accessibility to all fiber surfaces and can bridge and spread well. On the basis of the microscopic images of the cell—fiber matrix (Figures 5 and 6) and the calculation of pore volume using the known porosity and fiber diameter, the critical distance for cell bridging to occur should be in the range 35–60 μm, which is 4–6 times the length of the cell diameter (~10 μm). The determination of pore volume and pore size distribution in nonwoven fabrics also can be done with several published methods (33, 38).

**Dual Staining for Cell Proliferation Study.** Cell proliferation in 3-D culture systems has been studied by a variety of techniques. For example, DNA assay was correlated to the cell number and used as an indicator of cell density and cell proliferation (8, 39–41). Recently, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used as a marker of biochemical activity (20). Flow cytometry was employed to investigate the cell cycle stages of human skin fibroblasts in grooved silicone surface (20). However, these methods cannot identify or localize the proliferating cells in the cell matrix. Because of the strong coupling between scaffold microstructure and cell cycle stages, it is necessary to localize the cells that are in a specific stage. The dual staining CSLM method developed in this work proved to be a useful technique to evaluate the relationship of cell proliferation to its surrounding architecture.

**Conclusion**

As an important component of cellular composite, scaffold architecture plays a critical role in organizing cells into functional units and therefore regulating cell proliferation and functions. This phenomenon is receiving increasing attention in scaffold structure design for tissue-engineering applications. In this study, we found that the porosity, an important structural parameter of nonwoven fabric, is a determining factor in regulating the distribution of cells in the matrix and aggregate formation, which in turn affected cell proliferation activity. In general, increasing surface accessibility with lowered matrix porosity would decrease aggregate size and increase proliferation and cell metabolic rates. Thus, the spatial biocompatibility has important implications in applying fibrous matrices as a tissue-engineering scaffold.

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**References and Notes**
