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# Spontaneous Redifferentiation of Dedifferentiated Human Articular Chondrocytes on Hydrogel Surfaces

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Chondrocytes rapidly dedifferentiate into a more fibroblastic phenotype on a two-dimensional polystyrene substratum. This impedes fundamental research on these cells as well as their clinical application. This study investigated the redifferentiation behavior of dedifferentiated chondrocytes on a hydrogel substratum. Dedifferentiated normal human articular chondrocyte-knee (NHAC-kn) cells were released from the sixth-passage monolayer cultured on a polystyrene surface. These cells were then subcultured on a chemically crosslinked copolymer hydrogel, that is, poly(NaAMPS-co-DMAAm), and the cells thus obtained were used as the seventh-passage cultivation. Copolymer gels were synthesized from a negatively charged monomer, the sodium salt of 2-acrylamido-2-methyl-1-propanesulfonic acid (NaAMPS), and a neutral monomer, *N,N*-dimethylacrylamide (DMAAm). These gels were of different compositions because the molar fraction ( $F$ ) of NaAMPS was varied ( $F = 0, 0.2, 0.4, 0.6, 0.8,$  and  $1.0$ ). The dedifferentiated NHAC-kn cells spontaneously redifferentiated to normal NHAC-kn cells on neutral ( $F = 0$ ) and poly(NaAMPS-co-DMAAm) hydrogels of low charge density ( $F = 0.2$ ). This was deduced from the cell morphology and expression of cartilage-specific genes and proteins. These results should enable us to establish a simple and efficient method for preparing large amounts of chondrocytes by cultivation on the surfaces of neutral and low-charge-density hydrogels.

## Introduction

CULTIVATION OF CELLS on a flat-sheet-shaped substratum is the most common method for cell proliferation *in vitro* in both fundamental research and tissue engineering applications.<sup>1</sup> However, chondrocytes quickly dedifferentiate to a more fibroblastic phenotype on a two-dimensional (2D) substratum and show low expression of cartilage-specific genes and proteins such as type II collagen and aggrecan.<sup>2-6</sup> Type II collagen disappears after three passages of 2D cultivation of chondrocytes on polystyrene (PS), the most frequently used substratum. The term "dedifferentiation" is used here only to emphasize the process by which chondrocytes lose their ability to express articular-cartilage-specific extracellular matrix (ECM); usage of this term does not imply conversion of cells to other cell types.<sup>2</sup>

This easy dedifferentiation of chondrocytes causes several problems. One is that fundamental research studies have to be performed using primary chondrocytes that are directly isolated from cartilage. Moreover, most studies are limited because chondrocytes from an animal source have to be used

instead of cells from humans due to shortage in the availability of donor biopsy cartilage.<sup>7-12</sup> This problem is even more pronounced in tissue engineering. Cartilage tissues have a very limited capacity to regenerate or self-repair due to their avascular characteristics.<sup>13,14</sup> Therefore, studies on articular cartilage repair have focused on the creation of cartilaginous tissue *in vitro*, which would be suitable for subsequent implantation and joint resurfacing in tissue engineering.<sup>15-19</sup> Thus, chondrocytes removed from patients have to be multiplied *in vitro* to obtain the required number of cells because the amount of donor biopsy cartilage available is limited. However, the easy dedifferentiation of chondrocytes is a challenge when generating large amounts of chondrocytes *in vitro*.

Some studies have shown that dedifferentiated chondrocytes that have been cultured on a 2D substratum can redifferentiate to chondrocytes by reexpressing the differentiated phenotype during three-dimensional (3D) cultivation. Such behavior has been observed when the cells were embedded in pellets, alginate beads, or polymer gels, even combined with pressure stimulation.<sup>20-25</sup> For tissue engineering

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applications, the redifferentiated cells have to be isolated and collected from the 3D scaffolds, which severely limits the choice of scaffolds. Biodegradable polymer materials are suitable 3D scaffolds. Another drawback of the 3D cultivation system is that it is difficult to carry out *in situ* observations of the culture process. On the other hand, the 2D cultivation system has certain advantages. First, these systems are simple to operate in terms of culture and harvest of cells from the surface. Second, unlike the 3D cultivation system, cell observation by microscopy is possible without interference. Accordingly, the development of a suitable 2D substratum that can maintain chondrocytes in their undifferentiated state or can allow these cells to redifferentiate from their dedifferentiated state would be of great use in the research and application of cartilage tissue engineering.

One obvious question that arises is why chondrocytes can redifferentiate in a 3D cultivation system but not in a 2D cultivation system. Many recent studies have shown that the behavior of cells on a substratum is strongly dependent on the competition between the cell–cell and cell–substratum interactions.<sup>26</sup> For cells that exist as monolayers *in vivo*, such as endothelial cells, a relatively strong adhesive substratum favors cell proliferation to the confluent state *in vitro*.<sup>27–29</sup> On the other hand, a weakly adhesive substratum favors cell aggregation and microtissue formation.<sup>26,30,31</sup> For example, chondrocytes seeded on a standard PS scaffold dedifferentiate and exhibit spindle morphology in a monolayer, whereas they form cartilage tissue analogs on PS substrates coated with agarose, which is a neutral polysaccharide that has relatively weak adhesion to cells.<sup>32</sup> Recently, spontaneous articular cartilage regeneration was observed *in vivo* on a novel double-network hydrogel embedded in a large osteochondral defect.<sup>33</sup> The double-network hydrogel surface was covered by a layer of the neutral polymer poly(*N,N*-dimethylacrylamide) (PDMAAm); the adherence of this polymer to the cells is probably weak yet suitable.<sup>34</sup> Considering that the chondrocytes are present in cartilages in a 3D manner, we assume that the dedifferentiation of chondrocytes to a fibroblastic phenotype on the 2D PS substratum is due to the very strong adhesion of the PS substratum to the cells. By tuning the interaction between the substratum and chondrocytes, we expect chondrocyte redifferentiation to occur on a 2D scaffold along with proper adhesion of the chondrocytes to the substratum.

There are many studies on the tuning of the cell–substrate interaction by coating specific proteins or polymer onto the surface of solid scaffolds.<sup>30,32</sup> Furthermore, the dynamic behavior of endothelial cells is regulated by the physical properties of synthetic hydrogels, without any modification of cell-adhesive proteins in terms of characteristics such as chemical structure, surface zeta potential, and elasticity.<sup>35–37</sup> In this study, we investigate the behavior of dedifferentiated chondrocytes on 2D hydrogels by varying the charge density of the hydrogels to regulate the cell–substratum interaction.

We seeded dedifferentiated normal human articular chondrocyte–knee (NHAC–kn) cells on chemically cross-linked poly(NaAMPS-*co*-DMAAm) copolymer hydrogels containing various molar fractions (*F*) of the charged moiety 2-acrylamido-2-methyl-propane sulfonic acid sodium salt (NaAMPS). The morphology and expression of the cartilage-specific ECM of NHAC–kn cells were investigated at both the gene and protein levels. The NHAC–kn cells were found to

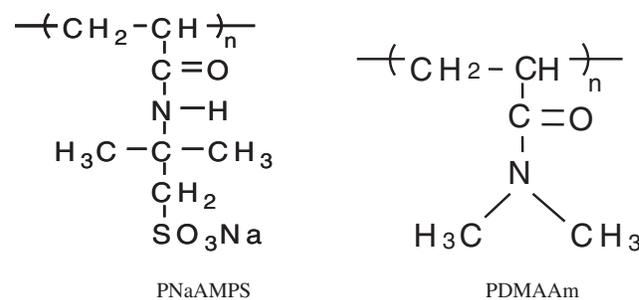
form cell colonies on neutral and low-charge-density hydrogels. Further, high gene expression of aggrecan, collagen II, and *sox9* was observed in the NHAC–kn cells grown on these hydrogels. These results demonstrated that dedifferentiated NHAC–kn cells can spontaneously reexpress cartilage-specific markers on neutral and low-charge-density hydrogels. This behavior may be due to weak yet sufficient adhesion of these hydrogels to the adhesive protein present in the cultivation solutions, which would prevent strong adhesion of the substrates to the cells. The results indicated that this may be a novel, less expensive, and efficient method for generating large amounts of redifferentiated chondrocytes. In future, the following procedure can be used. First, large amounts of dedifferentiated chondrocytes can be obtained by culturing a limited number of chondrocytes on the PS surface with high proliferation efficiency. Second, redifferentiated chondrocytes can be obtained by simply culturing the dedifferentiated chondrocytes on a sheet-shaped hydrogel substratum. Using this method, the required number of chondrocytes can be obtained from a limited number of chondrocytes isolated from patients. The excellent biocompatibility of the chemically crosslinked PDMAAm hydrogels used in this study has been recently confirmed by the authors of another study.<sup>33</sup> Therefore, this technically simple and economic method of inducing the formation of redifferentiated chondrocytes from dedifferentiated chondrocytes by 2D hydrogel cultivation should be of great use in fundamental medical research and tissue engineering for regeneration of articular cartilage.

## Materials and Methods

### Hydrogels

**Materials.** The NaAMPS monomer was obtained by neutralization of AMPS, kindly provided by Toagosei, with sodium hydroxide in ethanol; it was purified by recrystallization from acetone. The DMAAm monomer (Tokyo Kasei Kogyo) was distilled at a reduced pressure before use. *N,N'*-methylenebisacrylamide (Tokyo Kasei Kogyo) was purified by recrystallization from ethanol. 2-Oxoglutaric acid (Wako Pure Chemicals) was used as purchased. A 24-well PS tissue culture dish (Iwaki & Co., Ltd.) was also used as the control.

**Synthesis.** The chemical structures of PNaAMPS and PDMAAm are shown in Scheme 1. Poly(NaAMPS-*co*-DMAAm) hydrogels containing various molar fractions (*F*) of NaAMPS were synthesized by radical polymerization



**SCHEME 1.** Chemical structure of polymers used in this study.

from a precursor aqueous solution containing 1 M of monomer (a mixture of NaAMPS and DMAAm with various molar fractions of NaAMPS,  $F$ ), 4 mol% crosslinker ( $N,N'$ -methylenebisacrylamide), and 0.1 mol% initiator (2-oxoglutaric acid); the amounts of crosslinker and initiator were relative to the amount of monomer. The precursor solutions were added to the reaction cells. After purging with nitrogen gas for 30 min, the cells were irradiated with UV light (wavelength, 365 nm) to initiate polymerization (6 h). Gel sheets of 1 mm thickness were synthesized in the reaction cells. Details of the process have been described in our previous study.<sup>35</sup>

After polymerization, the gels were separated from the glass plates and immersed in a large amount of deionized water for 1 week. The water was changed twice daily to remove unreacted residual chemicals. The gels were then immersed in HEPES buffer solution (HEPES,  $5 \times 10^{-3}$  M; NaHCO<sub>3</sub>,  $1.55 \times 10^{-2}$  M; and NaCl, 0.14 M; pH 7.4). Phenol red ( $2.5 \times 10^{-3}$  g/L) was used as a visual indicator of the gel pH. After reaching equilibrium, the pH and ionic strength of the solution containing the gels were adjusted to 7.4 and  $\sim 0.15$  M, respectively. Gel disks were punched out of the gel plates using a hole punch of radius 7.5 mm. The chemically crosslinked hydrogels were then sterilized in an autoclave (120°C, 20 min). After sterilization, the gels were placed in a 24-well PS tissue culture dish (Iwaki & Co., Ltd.) for cell culture.

#### Cell culture

Second-passage NHAC-kn cells were purchased from Clonetics (Lonza Walkersville, Inc.) and cultured in accordance with the manufacturer's instructions.<sup>38,39</sup> The NHAC-kn cells cultured in the PS tissue culture flask as third-passage (3P) cells were used as control for gene expression in this study. The NHAC-kn cells were subcultured in the PS tissue culture flask until the sixth passage (6P). The 6P cells were separated from the PS substratum and loaded onto various types of hydrogels at a density of  $6 \times 10^4$  cells/cm<sup>2</sup> (Scheme 2). The NHAC-kn cell-loaded samples were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The medium was changed every 48 h without damaging the NHAC-kn cells or the gels.

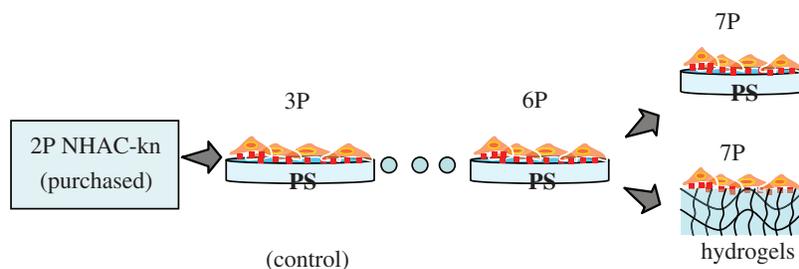
Cell adhesion and proliferation were quantified on the basis of the activity of the dehydrogenase in the seventh passage (7P) NHAC-kn cells at 6 h and 7 days using a cell counting kit, according to the manufacturer's protocol. Be-

fore each measurement, the culture medium was removed from the culture system to remove the floating cells. The cell density was calculated from a standard curve.

#### Expression of cartilage-specific markers of NHAC-kn cells on hydrogels

**Real-time polymerase chain reaction.** The NHAC-kn cells were cultured on the hydrogel and PS surface for 7 days. The scaffolds were then removed from the original 24-well culture wells and rinsed with phosphate-buffered saline (PBS). The NHAC-kn cells cultured on the hydrogels and PS surface were scraped off the hydrogel surface using a cell culture scraper and collected in a microcentrifuge tube. The cells cultured on these hydrogels should be removed from the gel surface before RNA isolation from the cells. Otherwise, the RNA concentration will decrease due to RNA adsorption to the charged gels by electrostatic interactions (data not shown). To isolate total RNA, all the cells were treated according to the RiboPure™ kit protocol. The RNA quality of each sample was confirmed on the basis of the A260/280 ratio. RNA was reverse transcribed into single-stranded complementary deoxyribonucleic acid (cDNA) using the PrimeScript™ RT reagent kit (TakaraBio). For first-strand cDNA synthesis, 125 ng RNA was used as the template in 10  $\mu$ L of reaction mixture in a microcentrifuge tube containing 125 ng total RNA, 2  $\mu$ L 5 $\times$  PrimeScript buffer, 0.5  $\mu$ L PrimeScript RT enzyme mix I, 0.5  $\mu$ L Oligo dT primer, and 0.5  $\mu$ L random 6-mers. RNase-free dH<sub>2</sub>O was added up to a total volume of 10  $\mu$ L, and the contents were mixed. The mixture was incubated at 37°C for 15 min and then heated to 85°C for 5 s to stop the reaction. cDNA was stored at  $-20^\circ\text{C}$  until required.

All primers used for real-time polymerase chain reaction (PCR) were designed by TakaraBio. Details of all the primer sequences are given in Table 1. All PCRs were performed with SYBR Premix Ex Taq™ II (TakaraBio) in standard 25- $\mu$ L reactions containing 2  $\mu$ L cDNA, 1  $\mu$ L PCR forward primer (10  $\mu$ M), 1  $\mu$ L PCR reverse primer (10  $\mu$ M), 12.5  $\mu$ L SYBR Premix Ex Taq II (2 $\times$ ), and 8.5  $\mu$ L sterile distilled water. All PCR data were calculated using the  $\Delta\Delta\text{Ct}$  method. In all samples, the data were normalized to the levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) ( $\Delta\text{Ct}_1 = \text{Ct}_1$  gene of interest  $- \text{Ct}_1$  *GAPDH*) and further normalized to the levels of the 3P NHAC-kn cells cultured on the PS surface ( $\Delta\text{Ct}_0 = \text{Ct}_0$  gene of interest  $- \text{Ct}_0$



**SCHEME 2.** Procedures used to investigate the redifferentiation behavior of dedifferentiated chondrocyte cells. The NHAC-kn cells were purchased and cultured on a PS surface until the 3P, and these cells were used as the positive control. The 6P cells were used to generate dedifferentiated cells. The 6P cells were removed from the PS surface and then loaded on hydrogels with various kinds of surfaces as well as on PS to obtain the 7P cells. NHAC-kn, normal human articular chondrocytes-knee; PS, polystyrene; 3P, third passage; 6P, sixth passage; 7P, seventh passage. Color images available online at [www.liebertonline.com/ten](http://www.liebertonline.com/ten).

TABLE 1. PRIMERS USED FOR REAL-TIME-POLYMERASE CHAIN REACTION

Gene	Forward	Reverse
GAPDH	GACCCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
Aggrecan	CGCTACGACGCCATCTGCTA	CTCCATGTCAGGCCAGGTCA
Collagen II	CCTGAAGGTGCTCAAGGTCCTC	GGAATTCCATCTGTTCCAGGGTTAC
SOX9	AACGCCGAGCTCAGCAAGA	CCGCGGTGGTACTTGTAAATC
Collagen I	CTGCTGGACGTCCTGGTGAA	ACGCTGTCCAGCAATACCTTGAG

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

GAPDH) under the same conditions. The data are therefore presented as an increase/decrease in the expression level of the sequence relative to that of 3P NHAC-kn cells in the PS control.

**Immunocytochemistry and alcian blue staining.** For fluorescence staining of collagen II, the cultured cells were washed with sterile PBS without calcium and magnesium [PBS(-)] and then fixed with 4% formaldehyde in PBS(-) for 20 min in an incubator (37°C, in a humidified atmosphere of 5% CO<sub>2</sub>). After washing with PBS(-) at 37°C for 5 min, the samples were permeabilized with 0.1% Triton X-100 in PBS(-) at 4°C for 5 min. The samples were again washed with PBS(-) at 37°C for 5 min, followed by pretreatment with 5% BSA in PBS(-) for 10 min in an incubator to block the nonspecific reaction sites. The samples were then washed with PBS(-) at 37°C for 5 min. The primary immunoreaction was carried out using rabbit polyclonal antibodies against collagen II (Abcam) in 0.1% Triton X-100 in PBS(-). After washing three times with 0.1% Tween 20 in PBS(-), the secondary immunoreaction was performed by adding fluorescein-isothiocyanate-conjugated goat polyclonal antibodies to rabbit IgG (Abcam) in 0.1% Triton X-100 in PBS(-) in the dark. This was followed by three rinses with 0.1% Tween 20 in PBS(-). To stain the cell nuclei, the cells were incubated in 2 µg/mL Hoechst 33258, followed by rinsing with PBS(-). The fluorescence images were recorded with a fluorescence microscope (Eclipse TE2000-E; Nikon).

To observe the accumulation of aggrecan, the NHAC-kn cells cultured on hydrogels were stained with alcian blue. The samples were rinsed with PBS(-), fixed with 4% formaldehyde in PBS(-) for 20 min in an incubator (37°C, in a humidified atmosphere of 5% CO<sub>2</sub>), stained with 1% alcian blue 8GS (Fluka) in 0.1 M HCl for 5 min at room temperature, and finally rinsed with distilled water. The images were recorded with an optical microscope (Olympus).

#### Statistical analysis

Data expressed as mean ± standard deviation are the average values of at least three samples. Statistical significance was evaluated using one-way analysis of variance. Values with  $p < 0.05$  were considered to be significant.

## Results

### Behavior of NHAC-kn cells on hydrogels of various charge densities

**Morphology.** Similar to 7P NHAC-kn cells, 6P cells removed from the PS surface were cultured on poly(NaAMPS-co-DMAAm) hydrogels of various charge densities. These

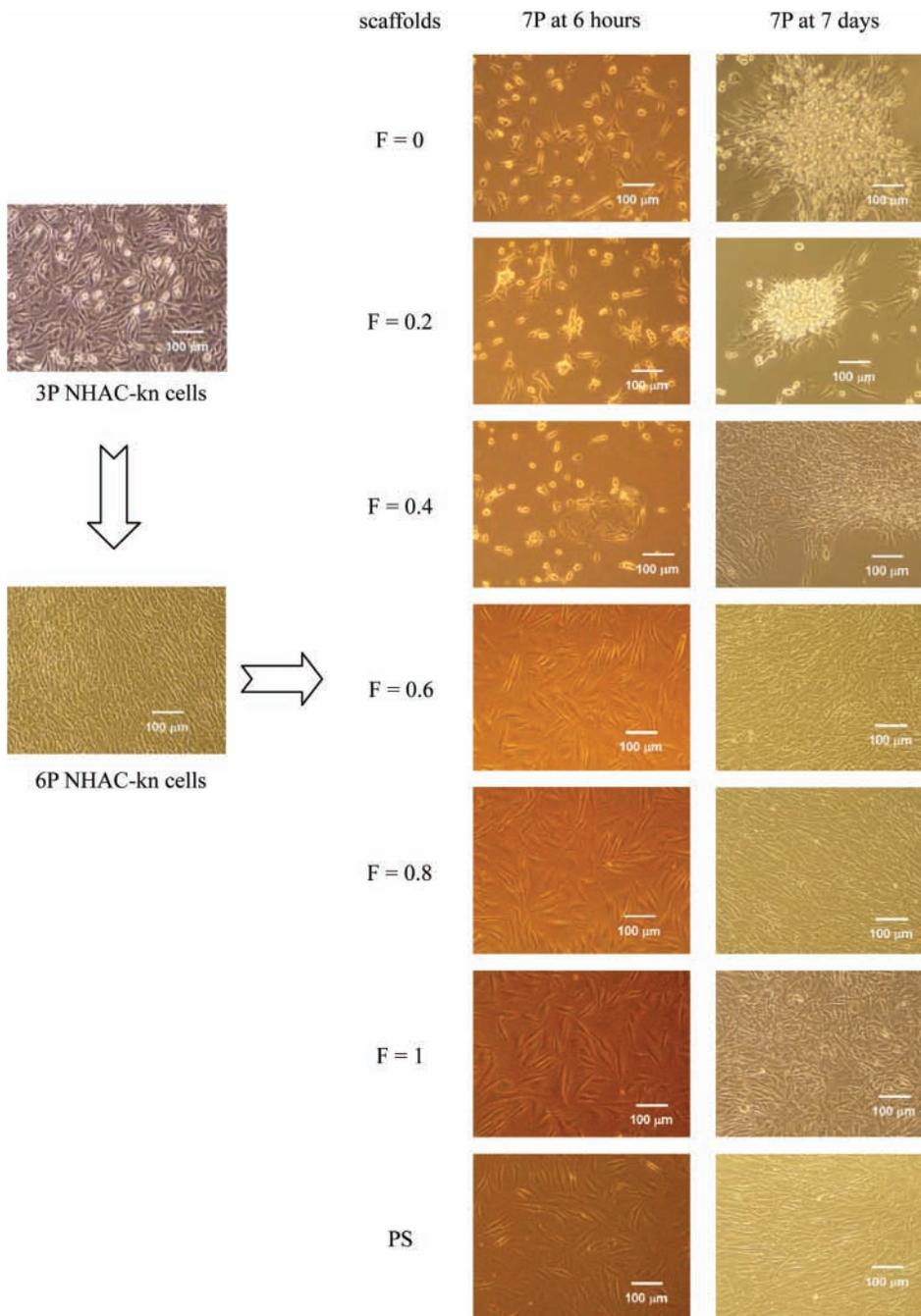
hydrogels were prepared by regulating the molar fraction [ $F = x/(x + y)$ ] of the anionic monomer NaAMPS ( $x$  M) and neutral monomer DMAAm ( $y$  M). The cells were also cultured on the PS surface. The latter is widely used as a scaffold for *in vitro* culture and was used as the control in this study. The morphologies of the 3P and 6P NHAC-kn cells cultured on the PS scaffold are shown in Figure 1. The 3P NHAC-kn cells showed a round and polygonal phenotype on the PS scaffold, whereas the 6P NHAC-kn cells showed an elongated fibroblastic phenotype on the PS scaffold.

Phase-contrast microscopy images of the typical morphology of 7P NHAC-kn cells cultured on hydrogels and PS at an initial stage of 6 h and over a long-term period of 7 days are shown in Figure 1. At 6 h, the cells could adhere to the surface of all the scaffolds, but their morphology was obviously different. The cells showed a round or polygonal phenotype on neutral and low-charge-density hydrogels ( $F = 0, 0.2, \text{ and } 0.4$ ). On the other hand, the cells showed an elongated fibroblast-like phenotype on high-charge-density hydrogels ( $F = 0.6\text{--}1.0$ ), which was similar to the morphology observed on the PS surface. At 7 days, the cells formed colonies on the hydrogels with  $F = 0$  and  $0.2$ , but not on the PS surface or on the hydrogels with  $F = 0.4\text{--}1.0$ . The cells showed an elongated fibroblastic phenotype on the PS surface and high-charge-density hydrogels, and the cells could reach confluence on these scaffolds at  $F = 0.6\text{--}1.0$ .

The morphology of NHAC-kn cells was inhomogeneous on hydrogels with  $F = 0$  and  $0.2$ , although all the cells on these gels could form colonies. As shown in Figure 1, the colonies formed on these hydrogels were composed of cells exhibiting two kinds of morphology: an elongated fibroblastic phenotype, which is the specific phenotype of fibroblasts, was observed at the bottom of the colonies; a round phenotype, which is the specific phenotype of chondrocytes, was observed at the top of the colonies.

Further, the size of the colonies formed on hydrogels with  $F = 0$  was larger than that of colonies formed on hydrogels with  $F = 0.2$ , as shown in Figure 1. The area of cell colonies on these hydrogels was measured using Image-Pro software, and the size distribution of cell colonies on these hydrogels is shown in Figure 2. The average area of cell colonies on hydrogels with  $F = 0$  was  $\sim 0.33 \text{ mm}^2$ , whereas that on hydrogels with  $F = 0.2$  was  $\sim 0.04 \text{ mm}^2$ .

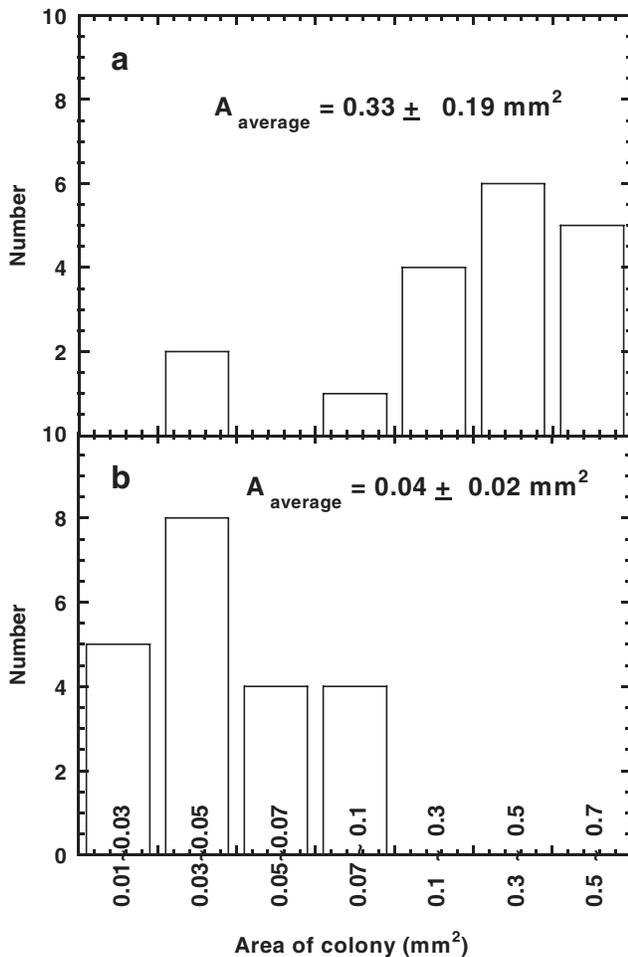
**Cell density and proliferation.** The cell density on the various hydrogels at 6 h and 7 days is shown in Figure 3. At 6 h, the cell densities were in the range of  $1.68\text{--}1.94 \times 10^4$  cells/cm<sup>2</sup> on hydrogels with  $F = 0, 0.2, \text{ and } 0.4$ , whereas the cell densities increased sharply to  $2.68\text{--}3.56 \times 10^4$  cells/cm<sup>2</sup> on hydrogels with  $F = 0.6, 0.8, \text{ and } 1$ . The value increased to



**FIG. 1.** Phase-contrast micrographs of 3P NHAC-kn cells cultured on the PS surface, 6P NHAC-kn cells cultured on the PS surface for 7 days, and 7P NHAC-kn cells cultured for 6 h and 7 days on poly (NaAMPS-co-DMAAm) hydrogels with various molar fractions of charge ( $F$ ). The images of 7P cells cultured on the PS surface for 6 h and 7 days are also shown. Scale bar: 100  $\mu\text{m}$ . NHAC-kn, normal human articular chondrocytes-knee; PS, polystyrene; 3P, third passage; 6P, sixth passage; 7P, seventh passage; NaAMPS, 2-acrylamido-2-methyl-propane sulfonic acid sodium salt; DMAAm, *N,N*-dimethylacrylamide. Color images available online at [www.liebertonline.com/ten](http://www.liebertonline.com/ten).

$6.26 \times 10^4$  cells/cm<sup>2</sup> on the PS surface. At 7 days, the cell densities were in the range  $3.20\text{--}5.77 \times 10^4$  cells/cm<sup>2</sup> on hydrogels with  $F = 0, 0.2,$  and  $0.4$ , whereas a higher cell density of  $\sim 10 \times 10^4$  cells/cm<sup>2</sup> was found on hydrogels with  $F = 0.6, 0.8,$  and  $1$ . The highest cell density of  $\sim 15 \times 10^4$  cells/cm<sup>2</sup> was observed on the PS surface. The results indicated that NHAC-kn cells can proliferate on all scaffolds, even on low-charge-density hydrogels. The low proliferative density on low-charge-density hydrogels was partly because the cells could easily be detached from these hydrogels and could be removed from the culture system due to their weak adhesion. However, even if this loss was disregarded, the harvest ratio (cells harvested/cells seeded) of cells on hydrogels with  $F = 0$  was  $\sim 100\%$ .

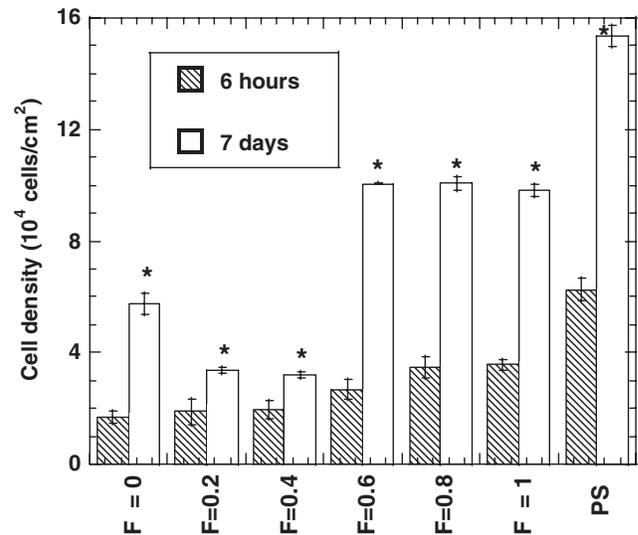
Analysis of gene expression of cartilage-specific markers by real-time PCR. The relative RNA levels of cartilage-specific markers expressed in NHAC-kn cells cultured on various surfaces are shown in Figure 4. The data are shown for aggrecan (Fig. 4a), which forms a major structural component of cartilage, particularly articular cartilage; collagen II (Fig. 4b), which is a cartilage-specific protein that accounts for 50% of all the proteins in the cartilage; SOX9 (Fig. 4c), which is a key transcription factor associated with chondrocyte differentiation; and collagen I (Fig. 4d), which is the most abundant collagen in the human body and is believed to be expressed in dedifferentiated chondrocytes. The results were normalized to those of 3P NHAC-kn cultured on the PS surface.



**FIG. 2.** Size distribution of the 7P NHAC-kn cell colonies on poly(NaAMPS-*co*-PDMAAm) hydrogels with  $F=0$  (a) and  $F=0.2$  (b). The average area of the cell colonies was measured using Image-Pro software with 15 samples. PDMAAm, poly(*N,N*-dimethylacrylamide).

First, we analyzed the PCR results for the cells on the PS surface. As shown in Figure 4b, the relative gene expression level of collagen II decreased with an increase in the number of passages of the NHAC-kn cells on the PS surface, and collagen II expression in 6P and 7P NHAC-kn cells almost disappeared. Instead, as shown in Figure 4d, the relative gene expression level of collagen I increased with an increase in the number of passages of the NHAC-kn cells on the PS surface. These results indicated that the dedifferentiation of NHAC-kn cells was enhanced by an increase in the number of passages of the NHAC-kn cells on the PS surface; similar observations have also been reported by other researchers.<sup>1-3,40</sup> These PCR results, together with those on the morphology of 6P NHAC-kn cells showing an elongated fibroblastic phenotype (Fig. 1), further confirmed that most of the 6P NHAC-kn cells were dedifferentiated cells in a heterogeneous population.

The PCR results were also validated by gel analysis. As shown in Figure 4a-d, it was surprising that 7P NHAC-kn cells cultured on neutral ( $F=0$ ) hydrogels showed very high relative gene expression of cartilage-specific markers, which was much higher than that of 3P NHAC-kn cells cultured on

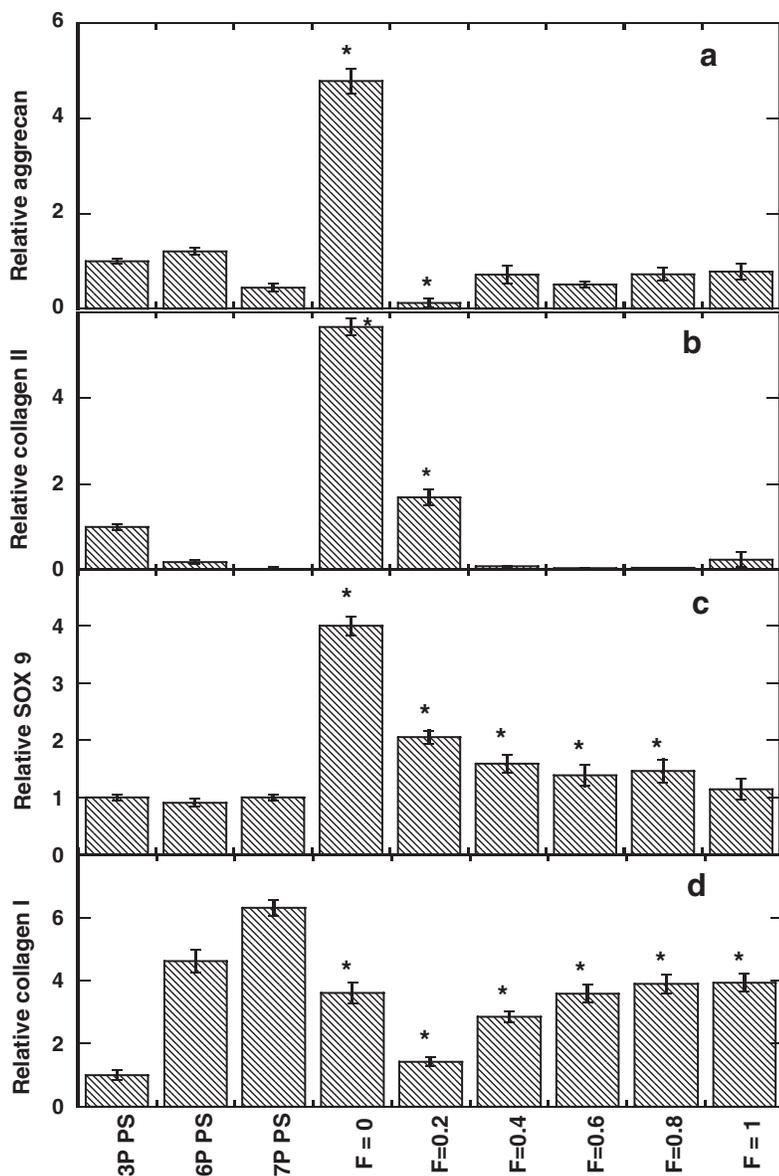


**FIG. 3.** The cell densities of the 7P NHAC-kn cells at 6 h and 7 days on poly(NaAMPS-*co*-PDMAAm) hydrogels versus the molar fraction of the charge ( $F$ ). The corresponding values for the cells cultured on the PS surface are also shown. The error ranges represent the standard deviation over three samples. \* $p < 0.05$  versus the cell densities at 6 h on these hydrogels, respectively.

the PS surface. Moreover, the relative collagen I expression level of 7P NHAC-kn cells on neutral ( $F=0$ ) hydrogels was lower than that of 6P NHAC-kn cells on PS. Since the 7P NHAC-kn cells cultured on hydrogels had proliferated from the 6P NHAC-kn cells cultured on the PS surface and had dedifferentiated, these results indicated that the dedifferentiated NHAC-kn cells spontaneously redifferentiated on the neutral hydrogels.

However, the spontaneous redifferentiation phenomenon was suppressed when charges were incorporated in the hydrogel, as observed in the case of high-charge-density hydrogels. As shown in Figure 4a-c, except for the hydrogel with  $F=0.2$ , gene expression of collagen II was very weak and that of *SOX9* decreased with an increase in the charge density of the hydrogel. Moreover, the relative collagen I expression level of NHAC-kn cells increased with an increase in the charge density of the hydrogel. Unexpectedly, collagen I expression of NHAC-kn cells on PDMAAm gel was even higher than that on the hydrogel with a charge density of  $F=0.2$ . The results suggested that NHAC-kn cells cultivated on hydrogels with charge densities of  $F=0$  and 0.2 were of the mixed cell type and were composed of redifferentiated and dedifferentiated cells; this was particularly true for the cells cultivated on the neutral PDMAAm gel. These results were in agreement with our observations on the morphology of NHAC-kn cells cultured on these hydrogels (shown in Fig. 1). The specific phenotypes of fibroblasts and chondrocytes<sup>2,3,40,41</sup> could be simultaneously observed on hydrogels with  $F=0$  and 0.2, particularly on hydrogels with  $F=0$ .

In this study, we used third-passage human chondrocytes as the control due to difficulties in obtaining human primary chondrocytes for the experiment. Partial dedifferentiation might occur even in third-passage chondrocytes on the PS surface; therefore, very high expression of chondrogenic genes in cells cultured on the neutral hydrogel compared to



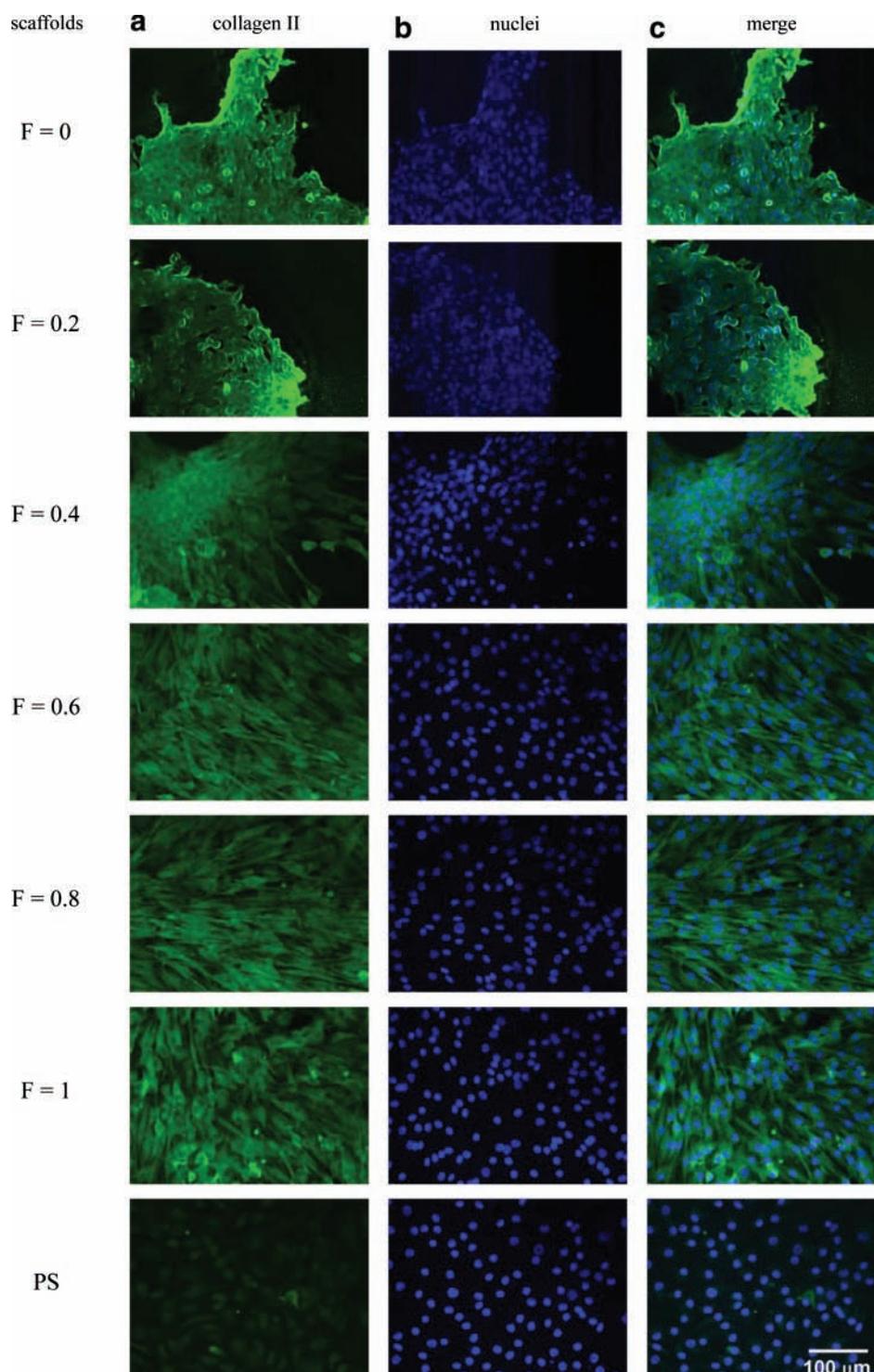
**FIG. 4.** The relative RNA levels of cartilage-specific markers expressed in NHAC-kn cells cultured on various surfaces. The 7P NHAC-kn cells were cultivated on poly(NaAMPS-*co*-PDMAAm) hydrogels ( $F=0, 0.2, 0.4, 0.6, 0.8,$  and  $1.0$ ). Data are shown for (a) aggrecan, (b) collagen II, (c) SOX9, and (d) collagen I. The error ranges represent the standard deviation over three samples. All the results were normalized to those of the 3P NHAC-kn cells on the PS surface. \* $p < 0.05$  versus the 7P NHAC-kn cells on the PS surface.

those in the control did not indicate that the cells cultured on neutral hydrogels overexpressed chondrogenic genes, which may occur during apoptosis. The proliferation of cells even on neutral hydrogels, as shown in Figure 3, also confirmed this observation. In a separate experiment, we used rabbit primary chondrocytes and also found dedifferentiation behavior on the PS surface and redifferentiation behavior on neutral and low-charge-density hydrogels; these results were quite similar to those obtained with human chondrocytes (data not shown).

Analysis of cartilage-specific protein expression by immunocytochemistry and alcian blue staining. The behavior of NHAC-kn cells cultured on poly(NaAMPS-*co*-DMAAm) hydrogels was further studied at the level of expression of cartilage-specific proteins for the two main specific proteins present in the ECM of chondrocytes, that is, collagen II and aggrecan. Immunocytochemistry and alcian blue staining, respectively, were used for this purpose (Figs. 5 and 6). For

collagen II staining, 7P NHAC-kn cells cultured for 7 days on poly(NaAMPS-*co*-DMAAm) hydrogels and the PS scaffold were treated with the antibody against collagen II (Fig. 5). For observing the accumulation of aggrecan, which is composed of sulfated glycosaminoglycan, 7P NHAC-kn cells cultured for 7 days on poly(NaAMPS-*co*-DMAAm) hydrogels and the PS scaffold were stained with alcian blue (Fig. 6).

Figures 5a and c show that collagen II was abundant in 7P NHAC-kn cells cultured on all hydrogels than that on the PS surface. The continuous pattern of collagen II expression on low-charge-density hydrogels ( $F=0$  and  $0.2$ ) indicates 3D microtissue formation. Figure 5b shows stained nuclei and indicates that the cell density on low-charge-density hydrogels ( $F=0$  and  $0.2$ ) was higher than that on high-charge-density hydrogels. Further, the cell morphology changed from 3D colonies to monolayer as the charge density of these hydrogels increased. The fluorescence intensity was similar for all the hydrogels; however, this does not indicate that similar amounts of collagen II were expressed in 7P



**FIG. 5.** Immunofluorescence staining images of (a) collagen II (green) and (b) nuclei (blue) in 7P NHAC-kn cells cultured for 7 days on poly(NaAMPS-co-PDMAAm) hydrogels ( $F = 0, 0.2, 0.4, 0.6, 0.8,$  and  $1.0$ ) and the PS scaffold. (c) Merged images. Scale bar:  $100\ \mu\text{m}$ . Color images available online at [www.liebertonline.com/ten](http://www.liebertonline.com/ten).

NHAC-kn cells cultured on these surfaces. This is because in this method, we observed only the fluorescence intensity derived from the surface of the 3D structural cell colonies on low-charge-density hydrogels ( $F = 0$  and  $0.2$ ).

The cells stained with alcian blue were clear bluish-green, as shown in Figure 6. This indicated that 7P NHAC-kn cells had abundant aggrecan expression and a 3D morphology on low-charge-density hydrogels ( $F = 0$  and  $0.2$ ). However, 7P NHAC-kn cells showed poor aggrecan expression on the PS

scaffold. It is difficult to compare the above data with the alcian blue staining data of 7P NHAC-kn cells on high-charge-density hydrogels ( $F = 0.4$ – $1.0$ ). This is because the copolymer hydrogels composed of PNaAMPS contain a sulfate ( $-\text{SO}_4^{2-}$ ) group that can also be stained by alcian blue as the charge density of the hydrogels increases, which would show up as a bluish-green background (data not shown).

The results of collagen II and aggrecan staining for 7P NHAC-kn cells on hydrogels did not correlate well with the

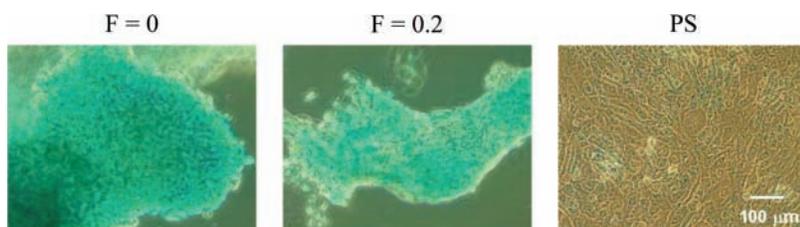


FIG. 6. Micrographs of 7P NHAC-kn cells cultured for 7 days on poly(NaAMPS-co-PDMAAm) hydrogels ( $F = 0, 0.2$ ) and the PS scaffold. Alcian blue staining was used. Scale bar: 100  $\mu\text{m}$ . Color images available online at [www.liebertonline.com/ten](http://www.liebertonline.com/ten).

results of real-time PCR. This could be due to several reasons. First, real-time PCR analysis is a tool for detection at the gene level, whereas ECM staining is used at the protein level. Second, real-time PCR is a quantitative technique, whereas ECM staining is a qualitative analysis. Further, all the real-time PCR results were relative to those of 3P NHAC-kn cells, whereas the ECM staining results represented absolute amounts. Third, gene expression is a dynamic process, and real-time PCR shows the results of a certain dynamic stage, whereas ECM staining shows the results of the accumulated amount of ECM produced over the entire dynamic process. Considering this, we concluded that despite the discrepancies in the real-time PCR and ECM staining data, the redifferentiation of chondrocytes occurred on low-charge-density hydrogels ( $F = 0$  and  $0.2$ ).

#### Behavior of NHAC-kn cells on hydrogels of various elastic modulus

The results indicated that chondrocytes can redifferentiate on low-charge-density hydrogels ( $F = 0$  and  $0.2$ ). However, as the charge density changed, the elastic modulus and degree of swelling of the poly(NaAMPS-co-DMAAm) hydrogels also changed, although the gels were prepared with the same amount of crosslinking agent in the feed. In the case of the poly(NaAMPS-co-DMAAm) gels prepared with 4 mol% crosslinker, when the molar fraction of the charged moiety was modulated over the range of  $F = 0$ – $1.0$ , the elastic modulus of the hydrogels changed in the range of 40–200 kPa, as shown in Figure 7, and the degree of swelling was in the range of 8–16.<sup>36</sup> The elastic modulus of hydrogels with  $F > 0.6$  decreased sharply because the crosslinking reaction decreased as the molar fraction of NaAMPS increased. This was because the latter has poor reactivity. Therefore, the behavior of NHAC-kn cells in hydrogels of various elastic modulus should be studied. We further studied the behavior of 7P NHAC-kn cells on PNaAMPS ( $F = 1$ ) with elastic modulus in the range of 40–656 kPa and on PDMAAm ( $F = 0$ ) hydrogels with elastic modulus in the range of 45–247 kPa. The elasticity and degree of swelling was modulated by altering the amount of crosslinker during gel polymerization. The phenotype specific to chondrocytes was observed on all PDMAAm hydrogels, and that specific to fibroblasts was seen on all PNaAMPS hydrogels, regardless of the change in the elastic modulus of the hydrogels (Fig. 8). Further, the real-time PCR results also showed that the redifferentiation behavior of chondrocytes could be observed on all PDMAAm hydrogels but not on all PNaAMPS hydrogels (data not shown). These results indicated that the redifferentiation behavior of chondrocytes observed in this study was regulated by the charge density of poly(NaAMPS-

co-DMAAm) hydrogels and not by changes in the elastic modulus and degree of swelling.

#### Discussion

##### Effects of the charge density of hydrogels on the redifferentiation of NHAC-kn cells

The redifferentiation of NHAC-kn cells is considered to be strongly affected by the charge densities of poly(NaAMPS-co-DMAAm) hydrogels, as shown by the morphology and expression of cartilage-specific markers at both the gene and protein levels (Figs. 1, 4, 5, and 6). The competition between the cell–cell and cell–substrate interactions, which is regulated by the charge density of poly(NaAMPS-co-DMAAm) hydrogels, is considered to play an important role in the redifferentiation of NHAC-kn cells cultured on such hydrogels. The cell–hydrogel interaction is mediated by the proteins adsorbed onto the hydrogels from the culture medium. Results from our previous studies have shown that the amount of proteins such as fibronectin adsorbed from the cell culture medium strongly depends on the surface charge density and/or surface zeta potential of the poly(NaAMPS-co-DMAAm) hydrogel. When the zeta potential of the gel was greater than  $-20$  mV (molar ratio of NaAMPS  $F < 0.4$ ), the concentration of adhesion proteins such as fibronectin adsorbed on the gels was low. In contrast, when the zeta

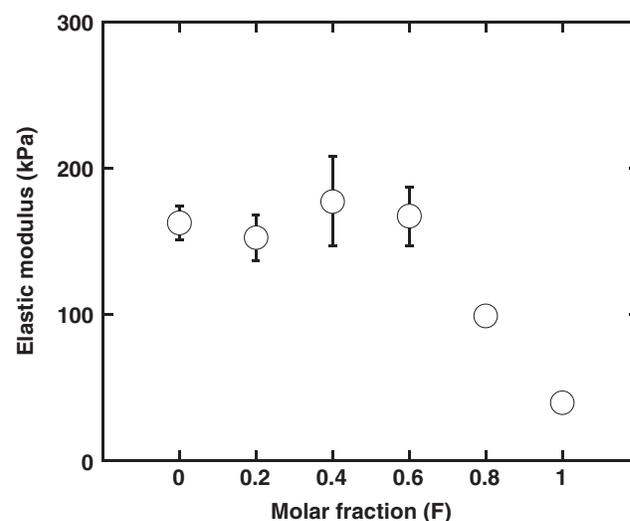
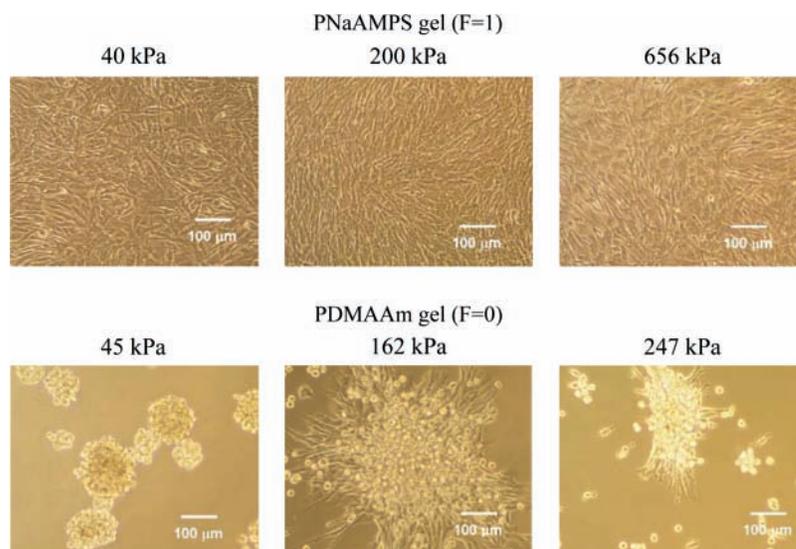


FIG. 7. Elastic modulus of poly(NaAMPS-co-DMAAm) hydrogels with various molar fractions of charge ( $F$ ). These were synthesized at a constant crosslinker concentration in the feed (4 mol%  $N,N'$ -methylenebisacrylamide). The error ranges represent the standard deviation over three samples.



**FIG. 8.** Phase-contrast micrographs of 7P NHAC-kn cells cultured for 7 days on PNaAMPS ( $F=1$ ) and PDMAAm ( $F=0$ ) hydrogels of various elastic modulus ( $E$ ). Scale bar: 100  $\mu\text{m}$ . Color images available online at [www.liebertonline.com/ten](http://www.liebertonline.com/ten).

potential of the gel was less than  $-20\text{mV}$  (molar ratio of NaAMPS  $F=0.4$  or higher), the concentration of the proteins adsorbed on the gels began to increase dramatically.<sup>36</sup> Our previous studies also showed that the behavior of cells cultured on these hydrogels also changed dramatically, consistent with this adhesive protein adsorption. On gels with  $F < 0.4$  (low charge density), endothelial cells had a small area, fast migration velocity, and showed less proliferation due to poor fibronectin adsorption, whereas on gels with  $F=0.4$  or higher (high charge density), endothelial cells had a large area and were less mobile, reaching confluence on the gel surface.<sup>34,36</sup> On the basis of these results, we also assumed that the fast migration of NHAC-kn cells on neutral and low-charge-density hydrogels was due to poor fibronectin adsorption, which enhances the collision frequency between NHAC-kn cells and favors the vertical stacking of cells. The weaker adhesion of the gel favors the 3D stacking of the cells, which is similar to the behavior *in vivo*, and this promotes the redifferentiation of the cells. It is known that chondrogenesis is promoted under hypoxic conditions.<sup>42,43</sup> The 3D stacking of cells on hydrogels with  $F=0$  and 0.2 might reduce the oxygen supply from the medium to the cells inside the microtissue, which would favor the redifferentiation of NHAC-kn cells on these hydrogel scaffolds. In contrast, on highly charged hydrogels that adsorb large amounts of adhesive protein, the adhesion of hydrogels to the cells was stronger than the cell-cell interaction. As a result, the dedifferentiated NHAC-kn cells formed 2D monolayers on the substrate and could not redifferentiate.

Cell behavior strongly depends on the hydrophilic/hydrophobic property of the scaffolds.<sup>44,45</sup> Hydrogels are considered to be very hydrophilic materials because these are composed of large amounts of water and small amounts of hydrophilic polymers. In particular, the copolymer hydrogels used in this study had a water content of  $\sim 88\text{--}94\text{wt}\%$ . These hydrogels showed good wetting to water (contact angle to water was close to zero). This indicated that the redifferentiation of chondrocytes on these hydrogel scaffolds could not be explained in terms of the hydrophilic/hydrophobic property of the scaffolds. This is because the protein-mediated interaction between scaffolds and cells

could be influenced not only by van der Waals interaction, which is strongly dependent on the hydrophilic/hydrophobic property of scaffolds, but also by electrostatic interaction between charged moieties that is dependent on the surface charges of the scaffolds. In the real *in vivo* system, besides the van der Waals interaction, the electrostatic interaction is considered to play an important role since both collagen and proteoglycan aggregates are with charged moieties. The present results indicate that adhesive interaction between the cell and the gel is weak in the case of the neutral polymer gel ( $F=0$ ) via the van der Waals interaction, whereas the interaction is greatly enhanced by the negative charges of the gel via the electrostatic interaction.

Since the interaction between the hydrogel and cells is always mediated by the adsorbing proteins, the effect of the charge of the hydrogel on chondrocyte behavior would strongly depend on the presence of active factors in the culture medium, such as insulin and other growth factors. In fact, opposite effects of the charge density on the differentiation of ATDC5 cells in the absence or presence of added insulin in the culture medium have recently been reported. ATDC5 is a chondrocytic cell line that is generally used to study the chondrogenetic process.<sup>46</sup> This culture-medium-dependent effect of the charge density might also explain the counter-intuitive considering that chondrocytes *in vivo* exist in a highly charged and hydrated microenvironment.

The cells showed inhomogeneous monolayer distribution on hydrogels with intermediate adhesive strength ( $F=0.4$ ) for cells (Figs. 1 and 5). Since the charge distribution on this copolymer hydrogel was homogeneous, as confirmed by the alcian blue staining of the gel ( $F=0.4$ ) (data not shown), the inhomogeneous cell distribution on the hydrogels ( $F=0.4$ ) was probably due to poor adhesion of the gels to the cells, similar to poor wetting of a liquid on a solid surface.<sup>47</sup>

## Conclusions

In this study, we investigated the behavior of dedifferentiated NHAC-kn cells cultured on poly(NaAMPS-co-DMAAm) copolymer hydrogels with different charge densities. The dedifferentiated chondrocytes on the PS surface

showed a cartilage-specific phenotype and gene expression after subculture on the surfaces of neutral and low-charge-density hydrogels. Further, the results of immunofluorescence staining for collagen II and alcian blue staining for aggrecan showed that the chondrocytes had rich collagen II and aggrecan expression on neutral and low-charge-density hydrogels. These results indicate that the dedifferentiated NHAC-kn cells spontaneously redifferentiate on neutral or low-charge-density hydrogels. On the other hand, the dedifferentiated chondrocytes retain their fibroblast-like phenotype on high-charge-density hydrogels and on the PS surface, showing a high proliferation rate and a harvest ratio. These results might aid in the establishment of a simple and efficient 2D cultivation method for preparing large amounts of chondrocytes by combining charged hydrogels (or PS scaffolds) and neutral hydrogels.

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### Disclosure Statement

No competing financial interests exist.

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