1 Clusters of neural stem/progenitor cells cultured on a soft poly(vinyl alcohol)
2 hydrogel crosslinked by gamma irradiation

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19 Running title: Neural stem/progenitor cells on PVA hydrogels

ABSTRACT

Neural stem/progenitor cells (NSPCs) in the central nervous system (CNS) have the capacity to self-renew by proliferation and are multipotent, giving rise to neurons, astrocytes, and oligodendrocytes. NSPCs can be amplified in neurosphere suspension cultures for cell transplantation therapy to treat CNS diseases as well as for in vitro pharmacological/toxicological assays; however, these suspension cultures have certain limitations, including the inconvenience of changing the culture medium as well as difficulty of live imaging. In the present study, we prepared a gamma-crosslinked poly(vinyl alcohol) (PVA) hydrogel and assessed its suitability as a substrate for adherent NSPC cultures. Differentiation was determined by evaluating the expression of the markers nestin (progenitors), β III tubulin (neurons), and glial fibrillary acidic protein and S100 β (glia) by immunocytochemistry and quantitative reverse transcriptase PCR. The levels of the marker genes were similar between the two types of culture; although some variability was observed, there were no fold differences in expression. NSPCs adhered to the PVA gel as clusters and grew without differentiating into neurons and glia. The proliferation rate of cells grown on the soft PVA gel (3.75-7.5% (w/v) PVA) was approximately 70% of that of neurospheres in suspension. We conclude that gamma-crosslinked PVA hydrogels can function as a novel scaffold for maintaining adherent NSPCs in an undifferentiated state.

Key words: poly(vinyl alcohol); neural stem cell; neurosphere; cell adhesion, gamma ray; hydrogel

INTRODUCTION

45

46 Poly(vinyl alcohol) (PVA) is a biologically inert, biocompatible, hydrophilic
47 polymer (1–9) that can be made into a hydrogel by physically crosslinking molecules
48 via a hydrogen bond (5) or by chemical crosslinking via a covalent bond. An elastic,
49 transparent PVA hydrogel can be formed by inducing the covalent crosslinking of
50 PVA molecules in aqueous solution via a radical reaction with a high dose of gamma
51 rays (2–4, 6). The PVA hydrogel is hydrophilic, nontoxic to many cell types, and is
52 non-adhesive to cells except when conjugated with peptides mimicking
53 integrin-binding sites in the extracellular matrix (ECM) (10).

54 Neural stem cells (NSCs) derived from central nervous system (CNS) are a
55 promising tool for transplantation therapy and assays used to evaluate drug efficacy
56 and toxicity (11), since neurons are terminally differentiated and therefore do not
57 proliferate. NSCs can normally be amplified in neurosphere cultures (12, 13) in which
58 undifferentiated immature cells adhere to each other and proliferate, forming spherical,
59 heterogeneous cell clusters suspended in serum-free medium. Cells generated in
60 neurosphere cultures are referred to as neural stem/progenitor cells (NSPCs) owing to
61 the difficulty in distinguishing neuronal and glial progenitor cells from the original
62 stem cells. Neurosphere cultures are considered as the gold standard method for
63 amplifying NSPCs despite limitations such as the time required to obtain high cell
64 densities and the inconvenience of changing the medium in which cells are always
65 suspended.

66 Another method for culturing NSPCs is adherent monolayer culture on a
67 laminin-coated substrate at low cell densities (14, 15). NSPCs derived from fetuses
68 (16, 17) and adults (18) have been established using this method, which is more
69 convenient for continuous monitoring and cell manipulation.

70 Monolayer and neurosphere cultures fundamentally differ in terms of the
71 microenvironment to which NSPCs are exposed. NSCs originate from ectodermal
72 neuroepithelial cells present in the neural tube at early stages of CNS development
73 and remain only in a restricted region of the adult brain (19). Neurospheres cultured in
74 vitro partially mimic fetal brain tissue as organoids consisting of closely packed
75 NSPCs (20, 21) that maintain their undifferentiated state via Notch signaling while
76 possessing a capacity for motility and active migration within the neurosphere (20).
77 Thus, the neurosphere microenvironment is suitable for NSPC proliferation, much
78 like a stem cell niche (19–21).

79 Monitoring the location of a single cell in a neurosphere and tracing its motility
80 can provide important information on cell-cell interactions and morphogenesis during
81 neural development. Neurosphere cultures were recently used as a three-dimensional
82 (3D) model for developmental neurotoxicity screening (22–24), which would not be
83 possible with a monolayer NSPC culture. On the other hand, adherent cultures are
84 more suitable for stable and reliable cell monitoring, imaging, and controlled
85 amplification of NSPCs and are also more amenable to automation for large scale
86 NSPC generation.

87 Artificial CNS tissue has potential applications for cell transplantation therapy in
88 the treatment of intractable CNS diseases (13, 25) as well as
89 pharmacological/toxicological assays to evaluate the efficacy and toxicity of chemical
90 substances (11). The objective of the present study was to establish a technique for
91 culturing NSPC clusters attached to a soft substrate that combines the advantages of
92 the neurosphere stem cell niche and the ease of handling of adherent cultures. After
93 testing various substrate materials, we found that NSPCs could be amplified and
94 maintained in an undifferentiated state on soft gamma ray-crosslinked PVA hydrogels.

95 The characterization of the PVA hydrogel as a scaffold for amplifying NSPCs is
96 described in this report.

97

98 MATERIALS AND METHODS

99

100 **Materials.** The following reagents were used: PVA (Nacalai Tesque, Kyoto,
101 Japan), recombinant human epidermal growth factor (EGF) (Pepro Tech, Rocky Hill,
102 NJ, USA), recombinant human basic fibroblast growth factor (b-FGF) (Pepro Tech),
103 B27 supplement (Life Technologies, Grand Island, NY, USA, USA), Dulbecco's
104 modified Eagle's medium nutrient mixture (DMEM/F-12), antibiotic-antimycotic
105 solution (Nacalai Tesque), 0.05% (w/v) trypsin/EDTA solution (Nacalai Tesque),
106 O.C.T. compound (Sakura Finetek, Tokyo, Japan), normal goat serum (Vector
107 Laboratories, Burlingame, CA, USA, USA), Sepasol-RNA I Super G (Nacalai
108 Tesque), SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan), Prime Script RT reagent
109 kit (Takara Bio), mouse monoclonal anti-tubulin β III antibody (MMS-435P; Covance,
110 Emeryville, CA, USA), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP)
111 antibody (G9269; Sigma-Aldrich, St. Louis, MO, USA), mouse monoclonal
112 anti-nestin antibody (MAB353; Merck Millipore, Darmstadt, Germany), rabbit
113 monoclonal anti-Ki67 antibody (RM-9106-S0; Thermo Fisher Scientific, Fremont,
114 CA, USA), Alexa Fluor 488-conjugated goat anti-mouse antibody (Life Technologies),
115 Alexa Fluor 568-conjugated goat anti-rabbit antibody (Life Technologies), and 4',
116 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Dojindo, Mashikimachi, Japan).

117 The following materials and instruments were also used: cell culture dishes with
118 diameters of 6 and 9 cm (Nunc, Roskilde, Denmark), 24-well microplates (Nunc),
119 glass slides (S9443; Matsunami, Kishiwada, Japan), an epifluorescence microscope

120 (Optiphot-2; Nikon, Tokyo, Japan) equipped with a digital camera (DS-5Mc; Nikon),
121 a cryostat microtome (CM1850; Leica Microsystems, Wetzlar, Germany), a
122 compressive testing machine (FGP-FGS; Nidec-Shimpo, Kyoto, Japan), and a Fourier
123 transform infrared (FTIR) spectrometer (IRAffinity-1; Shimadzu, Kyoto, Japan).

124 **Preparation of the PVA hydrogel.** An aqueous solution of PVA [1.5 ml at
125 concentrations of 3.75% (w/v), 7.50 % (w/v), or 15.0 % (w/v)] in a 6-ml sample tube
126 (0203-22; Maruemu, Osaka, Japan) was irradiated with various doses of gamma ray
127 (10, 20, or 40 kGy) at a dose rate of 4.08–4.45 kGy/h at room temperature (25°C) at
128 the cobalt 60 (^{60}Co) gamma ray facility of the Radiation Research Center of Osaka
129 Prefecture University. Samples of 3.75% (w/v), 7.50% (w/v), and 15.0% PVA
130 irradiated at 10, 20, and 40 kGy, respectively, were used for rheological measurements
131 in the swelling test.

132 **Measurement of FTIR spectra.** PVA gels impregnated with distilled water
133 for 15 days were dried in a vacuum, crushed into powder, and placed on the sample
134 folder for the measurement of FTIR spectra in attenuated total reflection mode.
135 Lyophilized PVA solution without gamma irradiation was used as a control sample.
136 The scan was repeated 40 times to obtain average data at 700–4000 cm^{-1} with a
137 resolution of 2.0 cm^{-1} .

138 **Swelling test.** The wet weight of the PVA hydrogel prepared as described
139 above was measured under sterile conditions before swelling in solution. Gels were
140 then incubated for the swelling test in either distilled water or culture medium
141 (DMEM/F-12 with antibiotic-antimycotic solution) at room temperature (25°C). The
142 wet weight of the gels was measured again at 1, 2, 6, and 12 h, and 1, 2, 4, 6, 8, 10, 12,
143 and 14 days.

144 The swelling ratio, Q_w , of the gel was calculated from the wet weight at n days

145 after swelling (W_{wn}) and before swelling (W_{w0}), according to the following formula.

146 $Q_w = W_{wn} / W_{w0}$ (1)

147 **Rheological measurements.** The compression test was carried out to
148 determine the Young's modulus, E , of PVA gels. The surface of the PVA gel was
149 compressed using the flat circular disk plate (12 mm in diameter) of the compressive
150 testing machine at a speed of 20 mm/min, and the load value and thickness of the gel
151 were recorded. E was obtained by calculating the relationship (from a stress-strain
152 graph) between stress (σ) and strain (ε) converted from the load value P and the
153 thickness deformation of PVA gels, as described by the equation:

154 $E = \sigma/\varepsilon = (P/A)/(dL/L)$ (2)

155 where A , L , and dL are the area of the compressing disk plate, original thickness of the
156 gel, and the thickness of the gel under compression subtracted from L , respectively.
157 The stress value at 10% strain was used to calculate E .

158 **NSPC neurosphere cultures.** NSPCs were isolated from the fetal brain of an
159 imprinting control region mouse (embryonic day 14) and resuspended in DMEM/F-12
160 medium supplemented with EGF (20 ng/ml), b-FGF (20 ng/ml), and B-27 supplement,
161 as previously described (25, 26). Cells (1.0×10^5 /ml in 10 ml medium) were cultured
162 in dishes at 37°C and 5% (v/v) CO₂ to induce neurosphere formation and were
163 expanded by dissociation with trypsin and reseeding every 7 days. All animal
164 experiments were conducted in accordance with institutional guidelines and national
165 standards with approval from the Animal Experiment Committee of Osaka Prefecture
166 University. NSPCs amplified for several passages as described above were used for
167 analysis on a PVA gel.

168 **NSPC culture on PVA hydrogels.** Three different types of PVA gel [3.75%,
169 7.50%, and 15.0% (w/v) PVA irradiated at 10, 20, and 40 kGy, respectively] in a

24-well plate (0.5 ml/well) were incubated with 0.5 ml of sterile distilled water for 30 min, washed twice to removed unreacted polymer, and incubated with 0.5 ml DMEM/F-12 medium for 2 days. Samples were incubated for an additional 5 days before cell culture in order to completely equilibrate the culture medium. NSPCs resuspended in medium (0.5 ml of 1.0×10^5 cells/ml) were seeded on PVA gels placed in each well of the 24-well plate and cultured at 37°C and 5% (v/v) CO₂. After 3 days of culture, 0.5 ml of medium was added to each well. Cells were analyzed after 7 days of culture. To evaluate growth, cells were dissociated with trypsin/EDTA, stained with Trypan Blue dye to distinguish live from dead cells, and counted under the microscope.

Immunocytochemistry and imaging. Frozen sections of NSPCs grown on PVA gels were prepared for immunocytochemistry. Cells were not detached from the surface of the PVA hydrogel, which was removed from the culture medium and fixed with 4% (w/v) paraformaldehyde in Dulbecco's phosphate buffered saline (PBS) for 30 min at 4°C. Samples were rinsed three times with PBS for 10 min each, then incubated in PBS containing 20% (w/v) sucrose overnight at 4°C. The samples were transferred to a mold and mounted in O.C.T. compound, flash-frozen on dry ice, and stored at -80°C. The frozen sample was sectioned at a thickness of 12 µm on a cryostat microtome; sections were collected on glass slides, which were washed twice in PBS for 5 min. Samples were permealized by incubating for 5 min in PBS containing 0.3% (w/v) Triton X-100. After blocking for 1 h in PBS containing 10% (v/v) normal goat serum and 0.01% (w/v) Triton X-100, samples were incubated overnight at 4°C with primary antibodies against the following proteins diluted in PBS containing 10% normal serum and 0.01% (w/v) Triton X-100: tubulin βIII (1:500), GFAP (1:100), nestin (1:500), and Ki67 (1:500). After washing three times

for 5 min with PBS, samples were incubated with Alexa Fluor 488-labeled goat anti-mouse (1:500) or Alexa 568-labeled goat anti-rabbit (1:500) IgG diluted in PBS containing 10% normal serum and 0.01% (w/v) Triton X-100 for 1 h at room temperature in a humid chamber. Nuclei were labeled by staining for 5 min with DAPI (1 µg/ml) diluted in the same solution. After washing three times with PBS, samples were mounted with PermaFluor mounting medium (Thermo Fisher Scientific, Waltham, MA, USA) and protected with a coverslip in the dark.

Quantitative reverse transcriptase (qRT-) PCR. The mRNA expression of five marker genes (tubulin βIII, *Tubb3*; GFAP, *Gfap*; S100β, *S100b*; nestin, *Nes*; sex-determining region Y-box 2, *Sox2*) in NSPCs cultured for 7 days was analyzed by qRT-PCR according to a previously described protocol (27). Total RNA was extracted from NSPCs with Sepasol-RNA I Super G solution and complementary DNA was synthesized with the Prime Script RT reagent kit; qRT-PCR was performed with SYBR Premix Ex Taq II on an Opticon real-time PCR thermal cycler (Bio-Rad Laboratories, Hercules, CA) with the following gene-specific forward and reverse primer sets: mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), 5'-CAT GGC CTT CCG TGT TCC T-3' and 5'-TGC CTG CTT CAC CAC CTT CT-3'; mouse *Gfap*, 5'-GAG AAA GGT TGA ATC GCT GGA-3' and 5'-GCT TGG CCA CAT CCA TCT C-3'; mouse *Nes*, 5'-TGG AAG GTG GGC AGC AA-3' and 5'-GGG GTC AGG AAA GCC AAG A-3'; mouse *Tubb3*, 5'-CCC CAG CGG CAA CAT GTA-3' and 5'-AAC CTG GGC TGT CTC TCT GG-3'; mouse *S100b*, 5'-ACT GAA GGA GCT TAT CAA CAA CGA-3' and 5'-ACA CTC CCC ATC CCC ATC T-3'; and mouse *Sox2*, 5'-ACA TGG CCC AGC ACT ACC A-3' and 5'-AAA ATC TCT CCC CTT CTC CAG TTC-3'. Relative mRNA expression levels were normalized to that of *Gapdh* by calculating the comparative cycle time relative to the control group and is expressed

220 as a fold change ($2^{-\Delta\Delta CT}$).

221 **Statistical analysis.** Data are represented as mean $\pm$ standard deviation.
222 Statistical analysis was performed by statistical software (Excel and JSTAT) using
223 one-way ANOVA with multiple comparison post hoc tests (William's multiple
224 comparison test was used for statistical significance of concentration-dependent
225 increasing of Young's modulus of PVA gels in Fig. 3B, and Dunnett's multiple
226 comparison test for comparisons of cell number and the expression levels of marker
227 genes in NSPCs on each PVA gel with control groups in Fig. 5 and 7). N.S. was
228 presented if significance was not obtained by one-way ANOVA. The data in Table 1
229 and 2 were tested by Kruskal-Wallis test with Tukey's multiple comparison test,
230 because the homoscedasticity were rejected by Bartlett's test.

231

232 RESULTS

233

234 A transparent PVA hydrogel was formed from viscous PVA aqueous solution upon
235 strong gamma irradiation (Fig. 1A). PVA molecules are covalently crosslinked via
236 gamma ray-induced radical reactions (2, 6). The peaks in the FTIR spectrum of the
237 dried PVA hydrogel were similar to those that were previously reported (4, 7, 28); a
238 slight difference between the spectra of the three different PVA hydrogels was seen in
239 the regions at around 1,600–1,800 and 2,400 cm^{-1} . We did not consider these minor
240 differences to be of critical significance in terms of our results. Minor differences at
241 1,700 cm^{-1} were partly attributable to stretching vibrations of carbonyl in the residual
242 carboxyl present in PVA after its industrial production by saponification of polyvinyl
243 acetate, as well as to bending vibrations of -OH groups in water molecules (H_2O).
244 CO_2 has a strong stretching vibration absorption peak at 2,400 cm^{-1} in the IR

spectrum. Therefore, CO₂ and H₂O from the air could affect the FTIR spectrum (compare the IR spectra of CO₂, H₂O, and CH₃COOH in the NIST database: <http://webbook.nist.gov/chemistry/name-ser.html>). We conclude that the molecular structure of PVA itself was not drastically altered by gamma radiation-induced crosslinking.

Deformation of the hydrogel by swelling must be minimized for its application as a cellular scaffold. The swelling test was carried out for PVA hydrogels to determine suitable conditions for scaffold preparation. Both PVA concentration and gamma ray dose affected swelling (Table 1); a balance between these two parameters was essential for reproducible hydrogel preparation. Three experimental conditions were tested: 3.75% (w/v) PVA/10 kGy, 7.5% (w/v) PVA/20 kGy, and 15% (w/v) PVA/40 kGy. Deformation of the PVA hydrogel by swelling was negligible under these conditions (Fig. 2).

The PVA hydrogel was subjected to the compression test after swelling in culture medium and stress-strain graphs were generated (Fig. 3A). The Young's modulus of each gel was calculated from the slope of each curve (Fig. 3B). Similar graphs were obtained for gels impregnated with distilled water (data not shown). Young's modulus values calculated from the linear region of each curve are summarized in Table 2. Slightly larger values were obtained for gels incubated in culture medium as compared to distilled water.

Phase-contrast micrographs of cultured NSPCs are shown in Figure 4. Cells proliferated in suspension in the culture medium to form neurospheres in the uncoated polystyrene culture dish (Fig. 4A–C). The cells weakly interacted with the surface of the dish via pseudopodia but never adhered, which was confirmed by gently agitating the culture dish under the microscope. In contrast, cells attached to each other and

270 proliferated to form clusters that firmly adhered to the surface of the PVA hydrogel
271 (Fig. 4D–L) and had hemisphere- or mound-like shapes (Fig. 4F, I, L). Attached cells
272 tended to spread out from the center of cell clusters to the periphery on the hydrogel
273 surface at higher Young's modulus values (i.e., greater stiffness).

274 NSPC proliferation on day 7 was only slightly lower on the PVA hydrogel than in
275 neurosphere cultures (Fig. 5A). Cells grown on the hydrogel (Fig. 5C–E) and in
276 neurosphere cultures (Fig. 5B) similarly expressed Ki67. These results indicate that
277 there is no difference in proliferative capacity of NSPCs grown on the two substrates.
278 Therefore, soft PVA hydrogels are useful for amplifying NSPCs in vitro at a rate and
279 efficiency that are similar to neurosphere cultures.

280 The capacity for cells grown on the PVA hydrogel to differentiate was evaluated
281 by immunocytochemical labeling of cell type-specific markers (Fig. 6). Nestin (Fig.
282 6D, I, N, S) and Sox2 (Fig. 6E, J, O, T), both markers of immature NSPCs, were
283 highly expressed. In contrast, the astrocyte marker GFAP (Fig. 6B, G, L, Q) was only
284 weakly expressed, whereas no immunoreactivity was observed for tubulin β III, an
285 early neuronal marker. There were no differences in marker expression between
286 neurospheres in suspension and cells grown on the PVA hydrogel (Table 3). A
287 qRT-PCR analysis confirmed that the levels of the marker genes were similar between
288 the two types of culture; although some variability was observed, there were no fold
289 differences in expression (Fig. 7). These results indicate that NSPCs grown on the
290 PVA hydrogel and in neurosphere cultures both maintain a progenitor cell identity.

291

292 **DISCUSSION**

293

294 We have demonstrated that gamma ray-crosslinked PVA hydrogels can function as

scaffolds for adherent NSPC cultures. Clusters of NSPCs attached to the gel surface and proliferated without differentiating into neurons and glia. NSPC amplification is useful for pharmacological/toxicological assays and cell transplantation. We previously reported that human NSPCs can be expanded for almost 200 days in a neurosphere cultures without loss of multipotency or proliferative activity (13). We have not yet attempted a similar long-term culture with the PVA hydrogel to determine the stability of NSPCs; however, we conjecture that long-term culture conditions can be improved by reducing the mechanical/oxidative stress on NSPCs during passaging—for instance, by minimizing repeated pipetting and centrifugation—since immature NSPCs should be amplified under static conditions with minimal agitation of the culture medium.

One advantage of an adherent culture is its suitability for automation combined with a monitoring system enclosed in a sterile apparatus for transplantation therapy. If a neurosphere suspension culture were to be automated, the size of the spheres must be standardized to avoid clogging the flow channel, and culture medium changes would require the physical trapping of spheres in the vicinity of the inlet/outlet parts of the liquid flow system. In contrast, a simple constant flow system without agitation of the medium is possible with adherent cells. Cell processing work by an operator in an aseptic area equipped with an air filtration system is costly but unavoidable for clinical stem cell applications. Automating the culture system will reduce costs associated with cultivation, the size of the system, and risk of bacterial, viral, and fungal contamination.

Maintaining cell clusters in a fixed position on the substrate enables the continuous monitoring of cells migrating within the cluster by time-lapse imaging and analysis of the 3D location of each cell. Information obtained from images of NSPCs

in the 3D cluster can be useful for pharmacological/toxicological assays in investigations of CNS development (21–24, 29). Recent studies have shown that spherical cell clusters or spheroids are often incorporated into toxicity- or drug-testing microdevices to serve as multicellular biomimetic tissue models (30–32). A means of restraining the spheroids on the microdevice during required medium changes and application of test substances is needed. A nontoxic, biologically inert, chemically stable, mechanically compressible hydrogel with high water content such as our PVA hydrogel can be an effective coating material for microdevices upon which live cells can be cultured.

We initially tested gamma ray-crosslinked polyethylene glycol (PEG) and calcium alginate hydrogels as well as a gamma ray-crosslinked nonfibrillar collagen gel (33) as alternative substrates (data not shown). NSPCs did not adhere to the surface of the PEG or alginate hydrogels. Some adhesion was observed with the collagen gel, but cells started to differentiate into neurons and astrocytes. For this reason, we chose PVA as the base material for the hydrogel. Unirradiated PVA solution is viscous and hydrogel formation can be induced by gamma irradiation, which crosslinks linear PVA molecules via hydrogen (3, 5, 8) or covalent (1–4, 28) bonds. Our PVA hydrogel is inert, nondegradable, and safe, as it does not employ a cytotoxic chemical crosslinker such as glutaraldehyde (28).

We tested three types of PVA hydrogel prepared at different PVA concentrations, gamma ray doses, and stiffness. The 3.75% (w/v) PVA gel irradiated at 10 kGy gave the best results in terms of generating NSPC clusters similar to those in neurosphere cultures and maintained cells in an undifferentiated state while showing very little deformation upon impregnation (Fig. 2). We therefore conclude that the PVA gel is the best substrate among those tested for adherent NSPC cultures.

345 The growth, differentiation, and spreading of NSPCs in adherent cultures depend
346 in part on the stiffness of the substrate (34–36). In this study, NSPCs grown on softer
347 PVA hydrogels—e.g., 3.75% (w/v) PVA irradiated at 10 kGy—spread very little and
348 spontaneously formed neurosphere-like hemispherical cell clusters.

349 We used the PVA hydrogel to culture mouse NSPCs, but the same strategy can be
350 applied to human NSPCs isolated from fetal or adult tissues or differentiated from
351 induced pluripotent or embryonic stem cells (iPS and ES cells, respectively), although
352 this has not yet been attempted. It was recently reported that a soft gelatin-conjugated
353 polyacrylamide hydrogel induced the upregulation of immature stem cell markers in
354 iPS cells derived from embryonic fibroblasts (37); moreover, a soft substrate is
355 required to maintain the stemness of somatic and ES cells. Our soft PVA hydrogel can
356 be applied not only to the culture but also to the shipping and storage of stem cells.

357 The molecular basis for the interaction between NSPCs and our PVA hydrogel
358 remains unclear; however, ECM components such as tenascin C and chondroitin
359 sulfate proteoglycans maintain the stemness of NSPCs in neurospheres (38–40), and
360 may be secreted from NSPCs and adsorbed onto the surface of the PVA hydrogel to
361 promote cell adhesion. Future studies will focus on identifying the ECM proteins that
362 mediate NSPC adhesion to PVA gels.

363 In conclusion, our results demonstrate that soft PVA hydrogels prepared by
364 gamma irradiation can serve as an effective substrate for the culture of adherent NSPC
365 clusters. The hydrogel can be used in pharmacological/toxicological studies
366 investigating CNS development as well as for NSPC amplification for cell
367 transplantation therapies.

368

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370

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503

504 **FIGURE LEGENDS**

505

506 Figure 1. (A) PVA hydrogels prepared under three different conditions and (B) their
507 FTIR spectra after freeze-drying.

508

509 Figure 2. Time course of swelling of PVA hydrogels in DMEM/F-12 culture medium.
510 Data are shown as the average swelling ratio, Q_w of the hydrogels ($n = 3$).

511

512 Figure 3. (A) Stress-strain curve in the compression test and (B) Young's modulus
513 values calculated from measurements ($n = 6$ in each condition). Error bars represent
514 the standard deviation. Difference was statistically significant. * $p < 0.05$ (William's
515 multiple comparison test).

516

517 Figure 4. Micrographs of NSPCs in culture. Phase-contrast images of NSPCs grown
518 in (A–C) a culture dish or on (D–F) 3.75%, (G–I) 7.5%, or (J–L) 15% PVA gels on
519 day 0 (A, D, G, and J), 3 (B, E, H, and K), and 7 (C, F, I, and L). Scale bar = 200 μm .

520

521 Figure 5. Cell proliferation in each culture condition assessed by (A) counting cell
522 number and (B–E) evaluating other evidence of cell proliferation, such as
523 immunocytochemical labeling of marker proteins. Cell numbers were counted on day
524 7 in four different cultures ($n = 4$) in (A). Error bars represent the standard deviation
525 (S.D.). Difference was statistically significant. ** $p < 0.01$ (Dunnett's multiple
526 comparison test). Micrographs of frozen sections of PVA hydrogels with cultured
527 NSPCs on day 7. Proliferating cells were labeled with anti-Ki67 antibody (red) and
528 nuclei were stained with DAPI (blue) in (B), (C), (D), and (E). Scale bar = 100 μm .

529

530 Figure 6. Micrographs of frozen sections of PVA hydrogels with cultured NSPCs on
531 day 7 as analyzed by immunocytochemistry. Nuclei were stained with DAPI (blue) in
532 (B), (D), (E), (G), (I), (J), (L), (N), (O), (Q), (S), and (T). Cells positive for the
533 neuronal marker tubulin β III (green) and glial marker GFAP (red) are shown in (B),
534 (G), (L), and (Q); cells positive for the immature NSPC marker nestin (green) are
535 shown in (D), (I), (N), and (S); and cells positive for the immature NSPC marker Sox2
536 (green) are shown in (E), (J), (O), and (T). Scale bar = 100 μm . Images of the same
537 sample acquired in bright field mode are shown in (A), (C), (F), (H), (K), (M), (P),

538 and (R).

539

540 Figure 7. Relative expression levels of marker genes as analyzed by qRT-PCR.

541 Expression levels were normalized to that of Gapdh and are shown as fold changes

542 relative to control samples (n = 3). Error bars represent the standard deviation.

543 Difference was statistically significant. $*0.01 \leq p < 0.05$, $** p < 0.01$ (Dunnett's

544 multiple comparison test); *S100b*, *Sox2*, N.S.

545

1 **TABLE 1.** Swelling of PVA hydrogels.

Concentration of PVA	Swelling ratio (Q_w) of PVA hydrogels ^a		
	Dose of γ ray (kGy)		
	10	20	40
3.75%(w/v)	1.27±0.02	0.74±0.01 ^c	0.76±0.03
7.5%(w/v)	2.63±0.13	1.31±0.04	0.87±0.04
15%(w/v)	— ^b	3.05±0.15 ^d	1.66±0.04

2 ^a Values are Q_w of PVA hydrogels immersed in distilled water for 14 days. Values were shown as
3 mean $\pm$ S.D. ($n=3$).

4 ^b Hydrogel has dissolved in distilled water.

5 ^c There was a significant difference between the sample and the 15% PVA hydrogels prepared by
6 20 and 40 kGy gamma radiation, $p < 0.01$ (Tukey's test).

7 ^d There was a significant difference between the sample and the 3.75% PVA hydrogels prepared
8 by 20 and 40 kGy gamma radiation, $p < 0.01$ (Tukey's test).

9

TABLE 2. Young's modulus of PVA hydrogels after swelling in either distilled water or culture medium.

Concentration of PVA	Young's modulus (<i>E</i>) of PVA hydrogels ^a (kPa)		
	Dose of γ ray (kGy)		
	10	20	40
After swelling in distilled water			
3.75%(w/v)	4.54±0.35 ^c	9.06±0.15	10.2±2.26
7.5%(w/v)	5.95±0.19	8.76±0.11	20.7±2.21
15%(w/v)	— ^b	4.10±0.52 ^c	20.2±0.80
After swelling in culture medium			
3.75%(w/v)	4.31±0.28 ^d	6.81±0.06	12.3±2.82
7.5%(w/v)	7.32±0.91	7.63±0.55	20.0±0.50
15%(w/v)	— ^b	4.24±0.17 ^c	16.4±2.54

^a Values were shown as mean $\pm$ S.D. ($n=3$).

^b Hydrogel has dissolved in distilled water and culture medium.

^c There was a significant difference between the sample and the 7.5% and 15% PVA hydrogels prepared by 40 kGy gamma radiation, $p < 0.01$ (Tukey's test).

^d There was a significant difference between the sample and the 7.5% PVA hydrogel prepared by 40 kGy gamma radiation, $p < 0.01$ (Tukey's test).

1 **TABLE 3.** Summary in markers for immature / differentiated cells derived from NSPCs
2 observed by immune-fluorescent microscopy.

Marker	Neurospheres	Cell clusters on a PVA hydrogel		
	on a culture	3.75%(w/v)	7.5% (w/v)	15% (w/v)
	dish (control)	PVA , 10 kGy	PVA , 20 kGy	PVA, 40 kGy
βIII tubulin (neuron)	–	–	–	–
GFAP (astrocyte)	+	+	+	+
S100β (astrocyte)	+	+	+	+
Nestin (NSPC)	++	++	++	++
Sox 2 (NSPC)	++	++	++	++
Ki 67 (proliferative cell)	++	++	++	++

3 –: signal was not significantly detected or very rarely detected.

4 +: signal was weakly detected.

5 ++: signal was clearly detected.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7