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SHORT COMMUNICATION

Culture of preantral follicles in poly(ethylene) glycol-based, three-dimensional hydrogel: a relationship between swelling ratio and follicular developments

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Abstract

This study was undertaken to examine how the softness of poly(ethylene) glycol (PEG)-based hydrogels, creating a three-dimensional (3D) microenvironment, influences the *in vitro* growth of mouse ovarian follicles. Early secondary, preantral follicles of 2 week-old mice were cultured in a crosslinked four-arm PEG hydrogel. The hydrogel swelling ratio, which relates to softness, was modified within the range 25.7–15.5 by increasing the reactive PEG concentration in the precursor solution from 5% to 15% w/v, but it did not influence follicular growth to form the pseudoantrum (60–80%; $p = 0.76$). Significant ($p < 0.04$) model effects, however, were detected in the maturation and developmental competence of the follicle-derived oocytes. A swelling ratio of > 21.4 yielded better oocyte maturation than other levels, while the highest competence to develop pronuclear and blastocyst formation was detected at 20.6. In conclusion, gel softness, as reflected in swelling ratio, was one of the essential factors for supporting folliculogenesis *in vivo* within a hydrogel-based, 3D microenvironment. © 2014 The Authors. *Journal of Tissue Engineering and Regenerative Medicine* published by John Wiley & Sons, Ltd.

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On the basis of niche theory regarding the control of cellular function by the extracellular matrix, new culture systems have been sought for generating clinically useful biomaterials containing therapeutic cells and tissues and for establishing novel models for the regulation of cellular function. Hydrogels that create a three-dimensional (3D) space can be used for mimicking *in vivo*-like environments under chemically defined conditions (Brandl *et al*., 2007), and various types of hydrogels have been suggested for the 3D culture of different cells and tissues (Discher *et al*., 2005). In our previous study, we applied a poly(ethylene) glycol (PEG)-based hydrogel formed from vinyl sulphone (VS)-functionalized PEG conjugated with a crosslinker (Lutolf and Hubbell, 2003) for the culture of murine (Lee *et al*., 2010, 2012) and human (Jang *et al*., 2013) stem cells. Based on our promising results in the culture of pluripotent stem cells in both mouse and human (Lee *et al*., 2010, 2012; Jang *et al*., 2013), we further attempted to apply this scaffold system for culturing ovarian preantral follicles. By employing the 3D system, we were able to reduce zona hardening (De Meestere *et al*., 1997), abnormal cytoskeleton distribution (Contvrindt *et al*., 1996), low developmental outcome and the decreased or altered activity of

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granulosa and theca cells for supporting oogenesis (Nayudu and Osborn, 1992) that has frequently been seen after two-dimensional (2D) culture. As the first step to apply the 3D system for follicle culture, we optimized the mechanical properties of the hydrogel matrix, and the final goal is to develop a 3D follicle culture system for deriving developmentally competent oocytes.

Consequently, we attempted to optimize a physical environment for developing a hydrogel-mediated 3D system for the culture of ovarian preantral (early secondary) follicles. We modified the softness of the hydrogel by changing the reactive PEG concentration of the hydrogel precursor and thus the swelling ratio of the formed hydrogel without preantral follicles. As shown in Table 1, changing the reactive PEG-VS concentration (w/v) from 5% to 15% yielded significantly ($p < 0.001$) reduced swelling ratios, from 25.7 to 15.5.

The hydrogel used for the follicle culture was designed with VS-functionalized, four-arm PEG-crosslinked via a Michael-type conjugate addition reaction with a crosslinker formed from a matrix metalloproteinase (MMP)-sensitive peptide (Ac-GCRD-GPQG↓IWGQ-DRCG-NH_2), as described previously (Lee *et al*., 2010); the thiol side-chain on the flanking cysteine (C) residues form the crosslinking sites, and the protease cleavage site is indicated by the down arrow (↓). The swelling ratio was determined as the mass ratio of the network at swelling equilibrium in the dry state after freeze-drying (Lutolf and Hubbell, 2003).

Preantral follicles were isolated from the ovaries of 2 week-old B6CBAF1 prepubertal mice, and each individual follicle was encapsulated into a hydrogel of 15.5–25.7 swelling ratio. All procedures for animal management followed the standard protocols of Seoul National University, Korea, and the Institutional Animal Care and Use Committee (IACUC) at Seoul National University approved the relevant experimental procedure (Approval No. SNU070423-4). Retrieval of the follicles was conducted by a non-enzymatic, mechanical method (Lee *et al*., 2007), and follicles classified as early secondary stage, 100–125 μm diameter (Pedersen and Peters, 1968; Smitz and Cortvrindt, 2002), were immediately seeded in a 6 μl liquid PEG gel precursor drop; crosslinking into a solid occurred in the presence of the follicle. Solidified gels containing early secondary follicles were transferred into 96-well tissue culture plates containing 200 μl follicle culture medium, which consisted of α-modified Eagle's medium–glutaMAX (Gibco Invitrogen, Paisley, UK) supplemented with 5% v/v fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA), 1% v/v insulin, transferrin and selenium liquid medium (Gibco Invitrogen), 100 mIU/ml recombinant human FSH (Organon, Oss, The Netherlands) and antibiotics (Smitz and Cortvrindt, 2002). To trigger maturation of follicle-derived oocytes, encapsulated follicles in the hydrogel were transferred into culture dishes and 2.5 IU/ml hCG (Pregnyl™; Organon) and 5 ng/ml epidermal growth factor (EGF; Sigma-Aldrich, St. Louis, MO, USA) were added to the medium 208 h after culture (Lee *et al*., 2008). Follicle growth to form a pseudoantrum was monitored 216 h after culture. We employed a standard operation protocol for the culture of follicles in the 2D system (Lee *et al*., 2008).

Mature oocytes were chemically activated with 10 mM $SrCl_2$ and 5 μg/ml cytochalasin B to monitor developmental competence, 240 h after culture. Activated oocytes were subsequently cultured in 5 μl droplets of modified Chatot, Ziomek and Bavister (CZB) medium, consisting of 81.6 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.7 mM $CaCl_2 \cdot 2H_2O$, 25.1 mM $NaHCO_3$, 31.3 mM sodium

Table 1. Developmental competence of mature oocytes generated by the culture of early secondary follicles in the 3D culture system using poly(ethylene) glycol (PEG)-based hydrogel of different softnesses or a conventional 2D system

Types of culture	% (w/v) of PEG-based hydrogel	Swelling ratio (mean ± SE)	No. of follicles cultured	No. (%)[b] of COCs collected	No. (%)[c] of oocytes matured[d]	No. of activated oocytes[e] developing to: Pronuclear stage (%)[f]	Two-cell embryo (%)[g]	Blastocyst (%)[h]
3D	5	25.69 ± 0.34[i]	69	25 (36)[i]	17 (68)[i]	5 (29)[i]	4 (80)	0 (0)[ij]
	7.5	21.44 ± 1.03[j]	88	37 (42)[i]	26 (70)[ik]	13 (50)[i]	13 (100)	0 (0)[i]
	10	20.61 ± 0.79[j]	70	32 (46)[i]	12 (38)[j]	11 (92)[j]	9 (82)	3 (27)[j]
	12.5	18.64 ± 0.68[k]	74	28 (38)[i]	11 (39)[j]	4 (36)[i]	4 (100)	0 (0)[ij]
	15	15.48 ± 0.32[l]	74	26 (35)[i]	12 (46)[jk]	6 (50)[i]	6 (100)	0 (0)[ij]
2D	–	–	181	139 (77)[j]	95 (68)[i]	89 (93)[j]	85 (96)	67 (79)[k]

Oocytes matured, oocytes developed to the metaphase II stage; COCs, cumulus–oocyte complexes.
Model effects of treatment on softness and number of COCs collected and oocytes matured, and number of activated oocytes developing to the pronuclear, two-cell embryo and blastocyst stages, which are indicated as *p* value, were < 0.0001, 0.0001, 0.0010, 0.0001, 0.2267 and 0.0001, respectively.
[a]Softness was indicated by measuring the swelling ratio of the PEG-based hydrogel matrix with four-arm crosslinker under 0.9 stoichiometric ratio.
[b]Percentage of number of follicles cultured.
[c]Percentage of number of COCs collected.
[d]Evaluated 16 h after the addition of human chorionic gonadotrophin and epidermal growth factor.
[e]Activated with $SrCl_2$ and cytochalasin B.
[f]Percentage of number of oocytes matured.
[g]Percentage of number of activated oocytes developing to the pronuclear stage.
[h]Percentage of number of activated oocytes developing to a two-cell embryo.
[ijkl]Different superscripts within the same column indicate statistical significance ($p < 0.05$).

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lactate, 0.3 mM sodium pyruvate, 1 mM glutamine, 0.1 mM EDTA, antibiotics and 5 mg/ml BSA. Maturation of the follicle-derived oocytes and pronuclear formation, cleavage and blastocyst formation after oocyte activation were evaluated accumulatively (Lee *et al*., 2008). The generalized linear model (PROC-GLM) in Statistical Analysis software (SAS) was employed for statistical analysis.

As shown in Table 1, a total of 556 follicles were cultured in different systems. When compared the swelling ratio within the 3D culture, pseudoantrum formation was detected at all levels without any significant difference ($p = 0.76$). However, a decreased ratio as low as 20.6 significantly (model effect = 0.014) inhibited oocyte maturation (68–70% to 38–46%). The largest number of oocytes that formed a blastocyst (three vs zero oocytes, $p = 0.038$) was detected at 20.6. No significant difference in the number of oocytes collected (35–46%, $p = 0.67$) and the number of activated oocytes cleaved after activation (80–100%, $p = 0.35$) was detected among the levels. On the other hand, overall retardation of development after 3D culture was detected when compared with 2D culture. Fewer ($p < 0.0001$) oocytes were retrieved (35–46% vs 77%) and developed into the pronuclear (29–50% vs 93%) and blastocyst (0–27% vs 79%) stages collected. However, there was no difference in maturity between oocytes retrieved from 2D and those retrieved from 3D systems (68% vs 38–70%).

Based on these results, hydrogel softness, being altered by PEG concentration in the hydrogel precursor and thus swelling ratio in the formed hydrogel, influenced the developmental competence of oocytes developed from *in vitro*-cultured, preantral murine follicles. Follicular growth *in vitro*, however, was not affected by the softness within the ranges tested in this study (Figure 1). The PEG-based hydrogel forming a swelling ratio of 20.6 was optimal for acquiring follicle-derived, developmentally competent oocytes. Such materials thus appear to be useful for the culture and maturation of murine preantral follicles.

An apparent decrease in the developmental competence of oocytes grown in a 3D system was detected when compared with that of oocytes grown in a 2D system. This retardation was apparently to retrieve oocytes from the culture system, to activate mature oocytes and to develop into the blastocyst stage. This implies that an improved strategy for oocyte retrieval from a 3D culture system and support of cytoplasmic maturation under 3D culture are urgently required. In contrast, nuclear maturation can effectively be induced by simply adjusting the swelling ratio, which is one of the parameters for gel softness.

A previous study has shown that decreasing the concentration of the solids leads to enhancing follicle growth and blastocyst development in the follicle culture, using alginate gels (Xu *et al*., 2006). Compared with the PEG hydrogel-based 3D culture, 2D culture induced the attachment of follicular (granulosa) cells to the bottom of the culture dish. These may alter cellular communication between the intrafollicular oocyte and its surrounding granulosa cells (Figure 2f, g). The

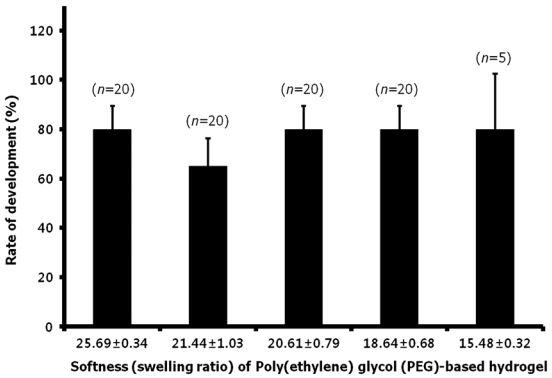

Figure 1. Growth of early secondary follicles in a 3D PEG-based hydrogel of different softnesses (as represented by swelling ratio). The follicles were cultured for 9 days in αMEM-based follicle culture medium and their growth was determined by the percentage of the follicles that formed pseudoantrum. No significant ($p = 0.76$) model effect was detected. Data are indicated as mean ± SE

hydrogel provides space for expansion of the follicle, while it restricts to maximal expansion without cell attachment to the bottom of the dish. Such a change modifies cell–cell communication through gap junction and paracrine signalling (Contvrindt *et al*., 1996).

This decreased development in 3D culture demonstrates an apparent lack of critical components for oocyte development in the system. The 3D system used in this study did not contain critical embryotrophic factors for a certain stage of oocyte activation. At least, it might be important to mechanically optimize the active PEG concentration (softness, density or surface tension) for regulating the growth and development of oocytes, as well as recruiting chemical regulators, such as regulating the diffusion rate of soluble factors in the medium.

As shown in Figure 2, an apparent difference in morphology was noticed and there must be different regulatory mechanisms for growth and development. Nevertheless, the 3D environment may be more similar to the *in vivo* condition than the 2D environment and, to develop the model 3D system using PEG-VS hydrogel, we first attempted to discover the optimal motifs for supporting oocyte maturation. Considering that a variety of cellular networks within the 3D microenvironment yielded unclear results, a 3D-based, model system for elucidating the cellular or acellular network under defined conditions is a critical factor for elucidating the mechanism of oogenesis and folliculogenesis.

In this study, we did not undertake qualitative assessment of the blastocysts derived from the 3D culture, due to small number. Under microscopic observation, one expanded while the other blastocysts remained at an early stage. Several important parameters, such as the number of inner cell mass cells and trophectodermal cells, the activity of specific enzymes showing differentiation and embryonic gene activation, and the degree to transform the inner cell mass cells into embryonic stem cell-like colonies, were additionally analysed. Those data clearly demonstrate the feasibility of the 3D culture system for clinical applications, and

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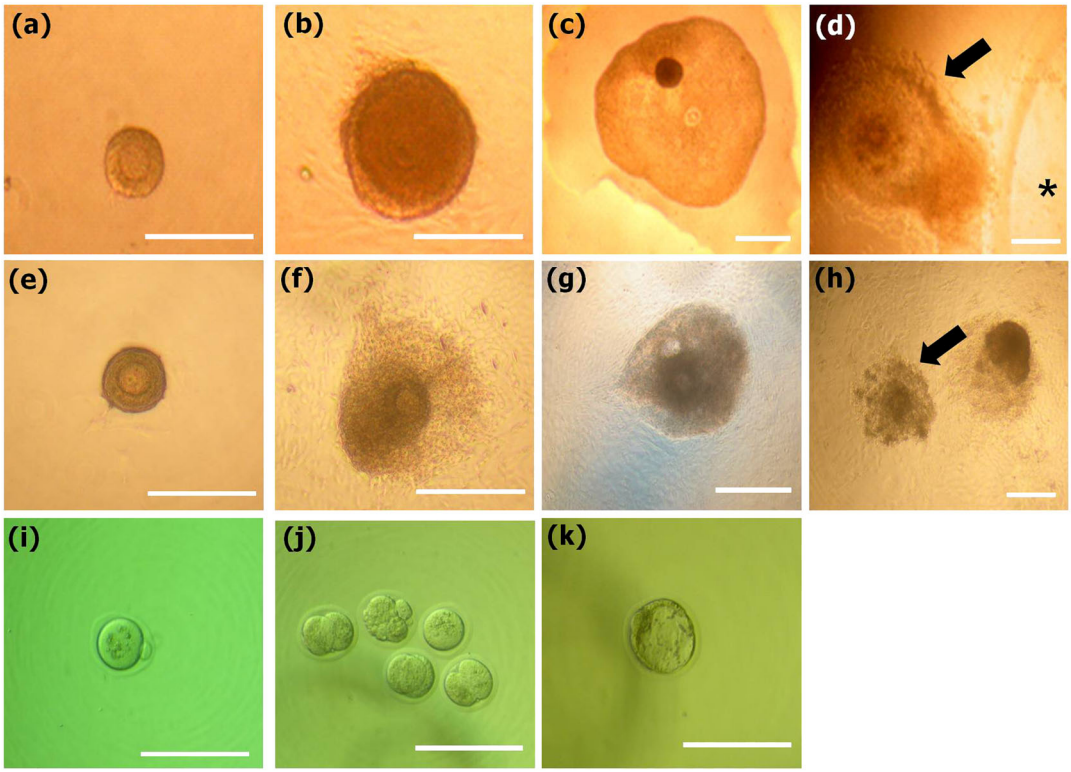

Figure 2. Morphological change of early secondary follicles growing in 3D PEG-based hydrogel and development of the follicle-derived oocytes after chemical activation. The preantral follicles embedded into the hydrogel and plated on culture dish were cultured for 9 days in αMEM-based follicle culture medium and matured by treatment with human chorionic gonadotrophin and epidermal growth factor. (a–d) Development of preantral follicles in the 3D PEG-based hydrogel: (a) the follicular stage of the follicle at 2 days after culture; (b) the diffuse stage of the follicle at 5 days after culture; (c) the pseudoantral stage of the follicle at 9 days after culture; (d) extrusion of the cumulus–oocyte complexes (arrow) in the hydrogel (*) was notable in the pseudoantral follicle. (e–h) Development of preantral follicles in the conventional, 2D culture system; (e) day 2 after culture; (f) day 5 after culture; (g) the pseudoantral stage of the follicle 9 days after culture; and (h) cumulus-oocyte-complexes (arrow). Overall, granulosa cells of the preantral follicle cultured in the 2D system showed further expansion, compared with those of the follicles cultured in the 3D system. (i) Derivation of the mature oocyte: (j) development to two- to four-cell embryos, 28 h after oocyte activation; and (k) development to the blastocyst stage, 124 h after oocyte activation. Scale bar = 200 μm

further study on the development of 3D culture will include such information.

A PEG hydrogel formed from 10% PEG–VS (20.6 swelling ratio), which yielded the best derivation of developmentally competent oocytes, did support oocyte maturation. The study to optimize the 3D system contributes to elucidating the niche-mediated regulation of oogenesis and folliculogenesis. Results from the 3D culture for preantral follicles will also be useful for deriving mature oocytes of good quality, which leads to increasing efficiency for establishing gamete-derived pluripotent stem cells (Choi *et al.*, 2011) and for developing novel assisted reproductive technologies for infertility or cancer patients. Extensive effort should be made, however, to apply this novel technique in both the industrial and clinical fields.

Conflict of interest

The authors have declared that there is no conflict of interest.

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References

Brandl F, Sommer F, Goepferich A. 2007; Rational design of hydrogels for tissue engineering: impact of physical factors on cell behavior. *Biomaterials* **28**: 134–146.

Discher DE, Janmey P, Wang PL. 2005; Tissue cells feel and response to the stiffness of their substrate. *Science* **310**: 1139–1143.

Lutolf MP, Hubbell JA. 2003; Synthesis and physicochemical characterization of end-linked poly(ethylene glycol)-co-peptide hydrogels formed by Michael-type addition. *Biomacromolecules* **4**: 713–722.

Lee ST, Yun JI, Jo YS *et al.* 2010; Engineering integrin signaling for promoting

embryonic stem cell self-renewal in a precisely defined niche. *Biomaterials* **31**: 1219–1226.

Lee ST, Yun JI, van der Vlies AJ *et al*. 2012; Long-term maintenance of mouse embryonic stem cell pluripotency by manipulating integrin signaling within 3D scaffolds without active Stat3. *Biomaterials* **33**: 8934–8942.

Jang M, Lee ST, Kim JW *et al*. 2013; A feeder-free, defined three-dimensional polyethylene glycol-based extracellular matrix niche for culture of human embryonic stem cells. *Biomaterials* **34**: 3571–3580.

De Meestere I, Barlow P, Leroy F. 1997; Hardening of zona pellucida of mouse oocytes and embryos *in vivo* and *in vitro*. *Int J Fertil Womens Med* **42**: 219–222

Contvrindt R, Smitz J, Van Steirteghem A. 1996; *In vitro* maturation, fertilization and embryo development of immature oocytes from early prenatal follicles form pre-pubertal mice in simplified culture system. *Hum Reprod* **11**: 2656–2666.

Nayudu PL, Osborn SM. 1992; Factors influencing the rate of preantral and antral growth of mouse ovarian follicles *in vitro*. *J Reprod Fertil* **95**: 349–362.

Lee ST, Choi MH, Gong SP *et al*. 2007; Establishment of a basic method for manipulating preantral follicles: effects of retrieval method on *in vitro* growth of preantral follicles and intrafollicular oocytes. *Zygote* **15**: 109–116.

Pedersen T, Peters H. 1968; Proposal for a classification of oocytes and follicles in the mouse ovary. *J Reprod Fertil* **17**: 555–557.

Smitz JE, Cortvrindt RG. 2002; The earliest stage of folliculogenesis *in vitro*. *Reproduction* **123**: 185–202.

Lee ST, Choi MH, Lee EJ *et al*. 2008; Establishment of autologous embryonic stem cells derived from preantral follicle culture and oocyte parthenogenesis. *Fertil Steril* **90**: 1910–1920.

Xu M, West E, Shea LD *et al*. 2006; Identification of a stage-specific permissive *in vitro* culture environment for follicle growth and oocyte development. *Biol Reprod* **75**: 916–923.

Choi JH, Kim GA, Park JH *et al*. 2011; Generation of viable embryos and embryonic stem cells from cultured primary follicles in mice. *Biol Reprod* **85**: 744–754.

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