IVG of primary and early secondary follicles collected from 6-day-old mouse ovaries

To establish a novel culture system for primary/early secondary follicles, collagen gel culture (IVG of follicles-1 (IVGf-1)) was adopted before collagen-coated membrane culture (IVG of follicles-2 (IVGf-2)). The procedure is illustrated in Fig. 2. Follicles were isolated from 6-day-old mouse ovaries and classified into three groups based on the diameters (Fig. 1D, E, and F). In preliminary experiments of IVGf-1, we checked gas conditions and found that growth rates of follicles in 5% CO_2, 5% O_2, and 90% N_2 were better than those in 5% CO_2 in air. Under a condition of 5% CO_2 in air, some follicles from group A grew 25.0% (4/16) but none grew from group B or group C, 0% (0/16) or 0% (0/22) respectively. However, under 5% CO_2, 5% O_2, and 90% N_2 conditions, the follicles from groups A, B, and C grew at 71.4% (15/21), 52.2% (12/23), and 20.0% (4/20) respectively. Therefore, we chose the gas conditions of 5% CO_2, 5% O_2, and 90% N_2. After 9-day culture of IVGf-1, the follicles in groups A, B, and C were successfully growing at 78.7, 48.6, and 18.0% respectively (Table 2). Larger follicle groups resulted in higher growth rates. When the follicles isolated from the gel were transferred to IVGf-2 and cultured for 8 days, they grew at 61.6, 33.5, and 9.9% in each group. The growth rates were also correlated with the follicle size at the beginning of culture. The oocytes in the fully grown follicles became fertilizable mature eggs at germinal vesicle breakdown (GVBD) or metaphase II (MII) stage after IVM culture. Typical morphology is shown in Fig. 3. Figure 3A shows a collected early secondary follicle embedded in the collagen gel at day 0 of the culture. After 5-day culture of IVGf-1, the follicle was growing, which could be recognized by increased oocyte diameter and significantly thicker granulosa cell layers than at day 0 (Fig. 3B). Fibrous cells located around the edge region of the follicle spread outward. Zona pellucida was clearly observed as shown by the arrow. By day 9 of the IVGf-1 culture, the follicle had grown noticeably (Fig. 3C). The growing follicle was removed from the gel and transferred to the collagen-coated membrane for IVGf-2 (Fig. 3D). After a further 8-day culture, the follicle formed an antral-like cavity indicated by an asterisk (*) in Fig. 3E. The follicle were mechanically removed from the membrane and transferred to the IVM culture. The matured eggs were fertilized by IVF and developed into blastocysts (Fig. 3F and G).

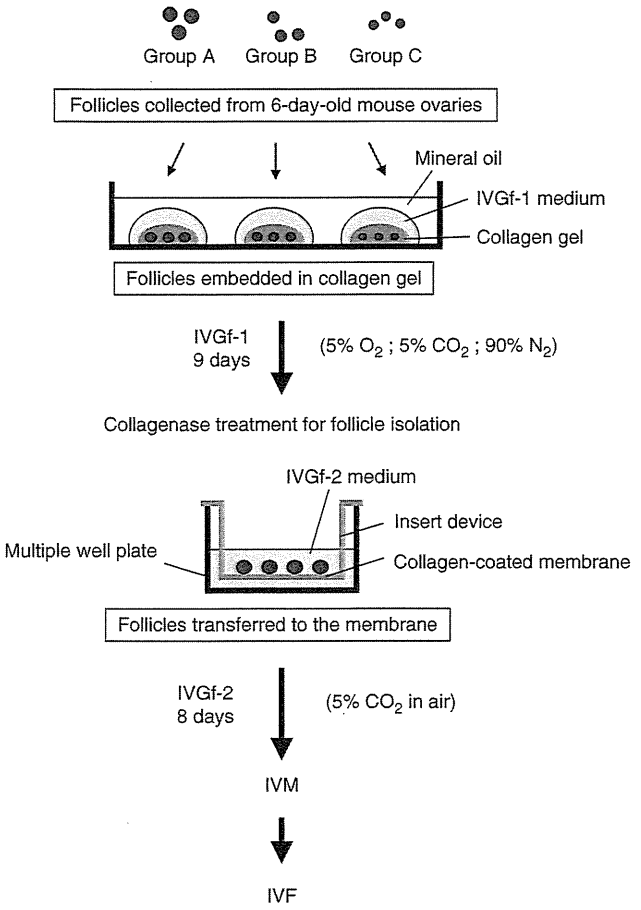

Figure 2 Schematic illustrating the two-step culture method. Intact follicles were mechanically isolated from 6-day-old mouse ovaries and classified into three groups, groups A, B, and C, based on their diameters. Group A: 80–95 μm, group B: 70–80 μm, and group C: 60–70 μm. Ten follicles in each group were embedded in a collagen gel microdroplet and cultured for 9 days (IVGf-1). Growing follicles were isolated by collagenase treatment to transfer to the collagen-coated membrane for a further culture (IVGf-2). About 30 follicles were cultured in a membrane insert. Medium components of IVGf-1 and IVGf-2 are described in the Materials and methods section. After 8 days, the resultant grown follicles were examined for maturation potential by *in vitro* maturation (IVM). Nuclear maturation and fertilizing ability of the oocyte were analyzed by IVF.

Comparison of follicle and oocyte growth in groups A, B, and C

Figure 4 shows the increase in follicle and oocyte diameters during a whole culture series (IVGf-1 and IVGf-2). The diameters of follicles and oocytes increased depending on their original sizes. The follicle diameter increased more during IVGf-2 than during IVGf-1 in the three groups. Distribution of the diameters in the grown follicles was larger at day 17 (Fig. 4A). On the other hand, oocyte diameters increased in IVGf-1 culture rather than in IVGf-2 culture (Fig. 4B). The follicle diameters seemed to be determined by granulosa cell proliferation. At the end of the whole culture series, the differences in oocyte diameters were smaller than those of follicle diameters in each group. Physical oocyte growth mainly occurred in IVGf-1, and functional processes such as nuclear/cytoplasmic maturation occurred in IVGf-2.

Table 2 Acquisition of competent oocytes after *in vitro* growth and maturation of primary/early secondary growing follicles.

Group (follicle diameter)	Follicles used for culture	No. of growing follicles after IVGf-1 (at day 9)	No. of growing follicles after IVGf-1+IVGf-2 (at day 17)	Nuclear status after IVGf-1+IVGf-2+IVM	
A (80–95 μm)	315	248 (78.7%)*	194 (61.6%)*	MII	95 (49.0%)
				GVBD	91 (46.9%)
				GV	8 (4.1%)
B (70–80 μm)	325	158 (48.6%)†	109 (33.5%)†	MII	64 (58.7%)
				GVBD	34 (31.2%)
				GV	11 (10.1%)
C (60–70 μm)	323	58 (18.0%)‡	32 (9.9%)‡	MII	16 (50.0%)
				GVBD	12 (37.5%)
				GV	4 (12.5%)

Significant differences of follicle growth rate are compared with three groups (*,†$P<0.001$, †,‡$P<0.001$). No significant differences of nuclear status are detected among three groups. IVGf, *in vitro* growth of follicles; IVM, *in vitro* maturation; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MII, metaphase II.

IVM and IVF of oocytes derived from two-step IVGf

The growing follicles having an antral-like cavity at day 17 were transferred to the IVM medium to induce maturation. After 19 h, most follicles released mucified cumulus–oocyte complexes (COCs) in the three groups. There were no significant differences in the nuclear maturation rates among them (Table 2). The terminal follicle size can be quite different among the three groups (groups A and B vs group C) but this does not appear to have any significant bearing on the ultimate meiotic and developmental competence of the egg. When the mucified COCs that included oocytes with GVBD or MII stage were subjected to IVF, fertilization rates in groups A, B, and C were shown to be 39.2% (49/125), 51.0% (25/49), and 50.0% (7/14) respectively. The development rates of the zygotes to blastocysts in groups A, B, and C were 32.7% (16/49), 28.0% (7/25), and 28.6% (2/7) respectively. There were no significant differences in fertilization rates and blastocyst development rates among the three groups.

Live births from embryos derived from the two-step IVGf (IVGf-1 and IVGf-2), IVM, and IVF

The two-cell-stage embryos obtained from the two-step IVGf, IVM, and IVF were vitrified to collect sufficient

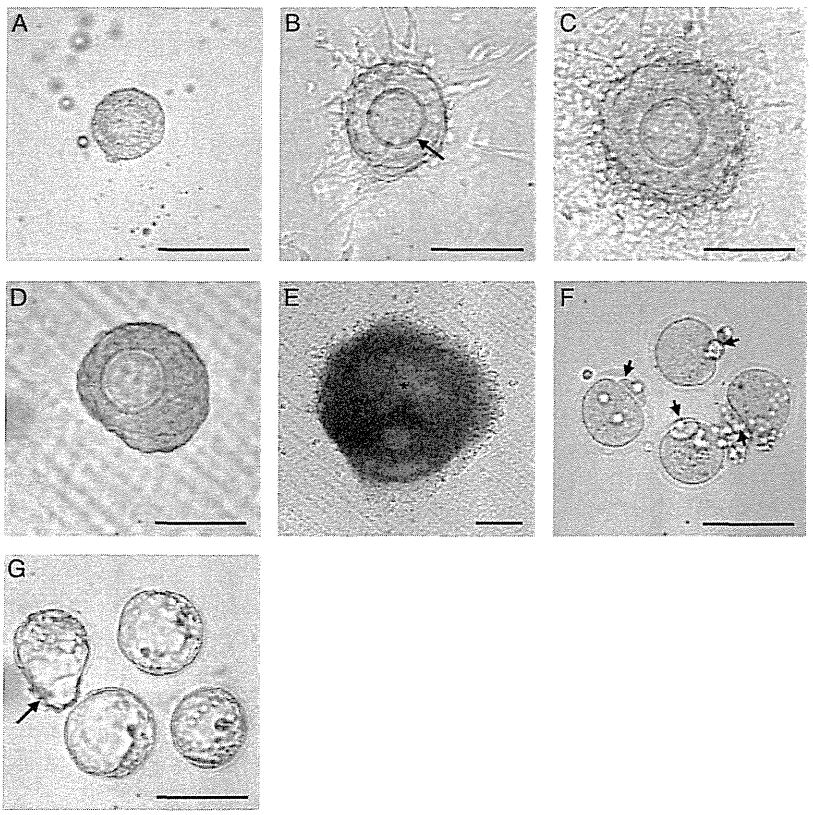

Figure 3 Morphological observation of follicle growth and embryo development in *in vitro* growth of follicles-1 (IVGf-1), *in vitro* growth of follicles-2 (IVGf-2), and *in vitro* maturation (IVM) followed by IVF. (A, B, and C) Follicle development from 6-day-old mouse ovaries during 9 days in IVGf-1. (A) At day 0 of the culture, an early secondary follicle with a centrally located oocyte embedded in collagen gel. (B) At day 5 of the culture, growing follicle with growing oocyte. Zona pellucida can be clearly seen (arrow). (C) At day 9, further growing follicle in IVGf-1. Granulosa cells proliferated significantly compared with those shown in (B). (D and E) Subsequent follicle development in IVGf-2. (D) At day 9, the follicle was isolated from collagen gel and transferred to collagen-coated membrane. (E) At day 15, the follicle formed an antral-like cavity (asterisk). (F) Mature eggs derived from the series of two-step IVGf followed by IVM. They resumed meiosis and extruded first polar body (arrows). (G) Blastocysts after *in vitro* culture at 96 h from IVF. One is under hatching from zona pellucida (arrow). Scale bar=100 μm.

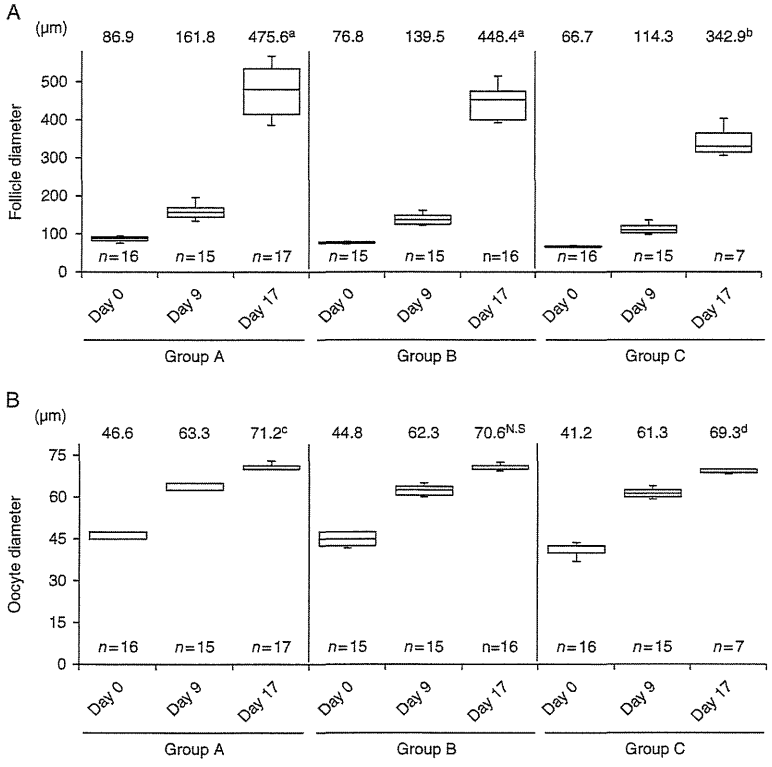

Figure 4 Increase in follicle and oocyte diameters in the series of two-step culture system. *In vitro* growth culture started at day 0 to day 9 (IVGf-1) and subsequent culture was performed from day 10 to day 17 (IVGf-2). (A) Follicle diameter. (B) Oocyte diameter. Box-and-whiskers plot demonstrating association between diameter and days of culture. Box contains values between 25th and 75th percentiles of diameter (central line, median). Vertical lines represent 10th and 90th percentiles. Numbers above the box plot indicate mean diameters (μm), and different letters beside the mean diameters indicate significant differences among the different groups at day 17 of the culture ($P<0.05$).

numbers for embryo transfer. One hundred and thirty-one embryos at the two-cell stage from three-run experiments were cryopreserved. After warming, 96.2% (126/131) survived. Fifty-three embryos that developed into the four-cell stage after overnight culture were selected and combined from the three groups for transfer to four pseudopregnant mice; therefore, which follicle group the live offspring were derived from could not be distinguished. The remaining embryos were cultured in modified KSOM for 60 h to examine developmental competence to blastocyst *in vitro*. Three mice became pregnant and two offspring were born by Cesarean section from two pregnant mice (Fig. 5 and Table 3). Their body/placenta appearance and weights were normal, and abnormality was not observed at birth. However, in the uteri, there were several dead embryos at mid stage of gestation. Also there was a fetus that was considered to have been alive to just before full term in the uterus of the other pregnant mouse. The two live offspring were females and started breathing spontaneously. They grew up and matured sexually to show normal reproductive ability.

Discussion

Developing a culture system of small ovarian follicles is important, because the ability to culture oocytes from the relatively abundant primary stage would provide a research tool to study the complex process of follicle development. A mouse model is useful for this purpose. Several culture methods were applied to preantral mouse follicles with diameters of 110–140 μm (Cortvrindt & Smitz 1998, Kreeger *et al.* 2005, Wang *et al.* 2011). However, these methods are still insufficient for earlier preantral stages. The study presented here was designed to establish an IVG system to induce the growth of earlier stage follicles such as primary/early secondary stages with diameters <95 μm. We assumed that physical maintenance of follicle structure was important to give the granulosa cells the opportunity to increase in number of layers. To verify this assumption, we used collagen gel culture (IVGf-1) before a collagen-coated membrane method (IVGf-2) for growth induction of primary/early secondary follicles. By embedding such small follicles in collagen gel, suppression of rapid migration of follicular cell occurred and three-dimensional follicle structure was maintained. It suggests that the two-step culture method for primary/early secondary follicles is very successful if organ culture is not applied to the culture system.

A number of researchers have reported that the covering of oocytes with granulosa cells and the keeping of junctional contacts among adjacent granulosa cells are essential for normal follicle growth. Autocrine/paracrine systems in the follicle seemed not to be retained in plain coculture systems of oocytes and granulosa cells. Oocytes in primary/early secondary follicles did not form functional mature oocytes having fertilizing ability in the coculture system, either (Eppig 1979, Eppig & Schroeder 1989). Therefore, maintaining normal follicle structure was considered to be crucial for successful follicle growth in culture.

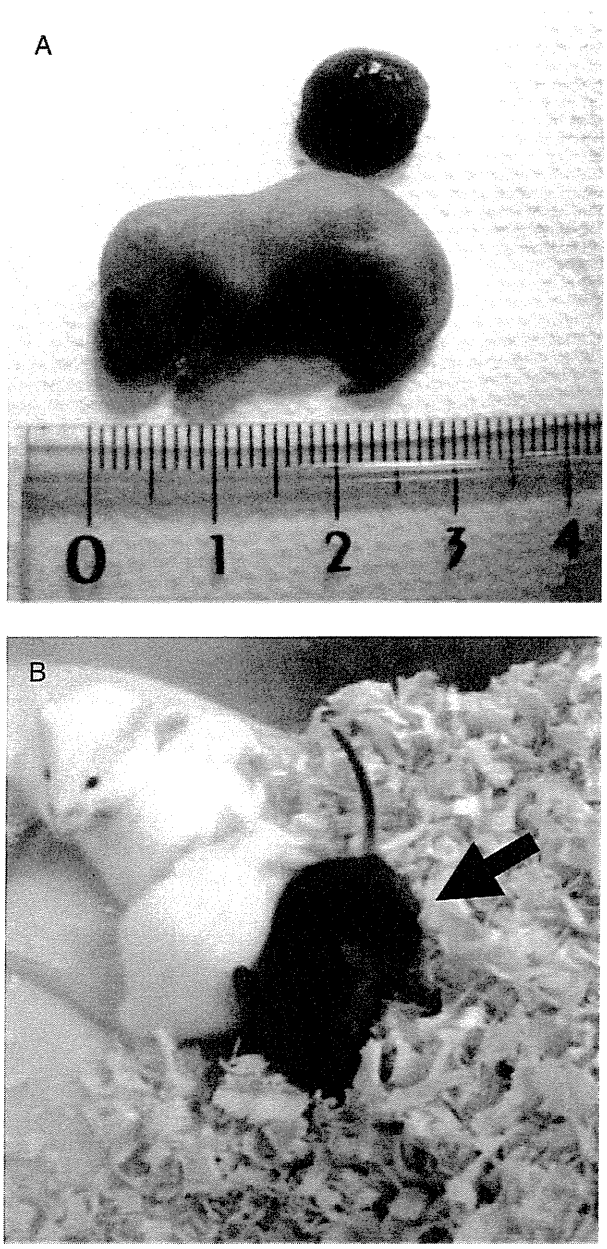

Figure 5 Live offspring derived from primary/early secondary follicles cultured by the series of two-step *in vitro* growth of follicles (IVGf), *in vitro* maturation (IVM), IVF followed by embryo transfer.
(A) Live offspring delivered by Cesarean section and its placenta.
(B) The growing mouse at 15 days after birth (arrow).

In this study, we used collagen gel, an extracellular matrix, for the culture substratum. This matrix probably gives growth signals to the follicles as well as mechanical stability. Actually, collagen has been reported to regulate ovarian functions (Matsumura *et al.* 2009). Under IVGf-1 culture, we succeeded in the induction of small follicle growth having diameters of 60–70 μm designated group C. These oocytes reached mature stages after subsequent culture of IVGf-2 and IVM. The oocytes were fertilized by IVF and the derived zygotes developed into blastocysts.

Transfer of growing follicles to IVGf-2 from IVGf-1 is essential for sufficient growth of the follicles. If IVGf-1 continues for 12 days, the follicles degenerate in the collagen gels (data not shown). Similar observations have been reported in collagen gel culture (Torrance *et al.* 1989). The reason why the follicles stop growing in the continuous collagen gel culture is not clear, but it is possible that too much follicular cell proliferation results in a shortage of necessary nutritional and/or growth factors. As another explanation, toxic metabolites may be released from follicular cells and their local densities may easily increase due to the viscosity of the gel. Recently, a novel hypothesis has been proposed (Woodruff & Shea 2011); follicle growth is dependent on the physical environment of the ovary in addition to well-established hormone control. During follicle development, early growing follicles move from the cortex, a collagen-rich zone, to perimedullar region of ovary and move again to the ovarian surface prior to ovulation. This study suggests that the change in the physical environment from rigid gel (IVGf-1) to a less rigid liquid culture (IVGf-2) may recreate the situation of follicle growth in the ovary. Large mammalian species such as human follicles may have diameters >20 mm at final stage but they are still small at primary/early secondary stages. Therefore, IVGf-1 using a small amount of the gel followed by IVGf-2 using a large amount of liquid medium will support the follicle growth of large mammalian species.

Gas conditions in culture are also important. When gas conditions for IVGf-1 were checked, a condition of 5% CO_2, 5% O_2, and 90% N_2 showed a higher follicle growth than that of 5% CO_2 in air. In the latter condition, the follicles degenerated with higher rates. A few follicles from group A grew, but the diameters were smaller compared with those observed in the former condition. The follicles from groups B and C were denuded and degenerated within 24 h in the latter condition. This suggests that 5% O_2 conditions are suitable for primary/early secondary follicles during early growth phase. It is known that angiogenesis in physiological conditions occurs markedly during progress of ovarian development (Fraser 2006). Several groups reported that modulating oxygen concentration was effective in follicle culture (Heise *et al.* 2009, Hirao *et al.* 2012).

Table 3 Live offspring from early growing follicles after *in vitro* culture followed by ET.

Recipient ID	No. of embryos transferred	No. of IUFD	No. of live offspring
1	11	2	1 (female, bw: 1.990, pw: 0.232)
2	9	2	1 (female, bw: 2.044, pw: 0.243)

IUFD, intrauterine fetal death; bw, body weight (g); pw, placenta weight (g). Embryos were developed from 6-day-old mouse ovarian follicles after two-step IVGf, IVM, and IVF.

Taken together, O_2 supply seems to be necessary for the later stages of folliculogenesis. In our own findings, the change of gas conditions from IVGf-1 to IVGf-2 was also important to imitate physiological conditions to achieve high success rates of follicle growth.

Although we succeeded in inducing primary follicles to develop into fertilizable mature eggs, the growth rate of group C was lower than that of the other groups during IVGf-1. Less-developed granulosa cells of primary/early secondary follicles very often adhered to the collagen-coated membrane or even the collagen gel, probably due to the granulosa cells' nature and/or immature communications between oocyte and granulosa cells such as poor gap junction formation.

When comparing the fertilization rates and blastocyst-achieving rates from two pronuclei-formed zygotes, there were no significant differences among the three groups classified by follicular diameters. However, the fertilization and blastocyst-achieving rates from any group were lower than those of *in vivo* grown oocytes (data is not shown). In the study presented here, we demonstrated the production of two live offspring (3.7%) from transferred embryos derived from oocytes grown *in vitro* from primary/early secondary follicles without organ culture. The success rate of live births was still low similar to the previous studies (Eppig & Schroeder 1989, Spears *et al.* 1994, Eppig & O'Brien 1996, O'Brien *et al.* 2003, Xu *et al.* 2006). Further efforts are needed to improve culture conditions.

Lenie reported a multistep culture system that changed concentrations of FCS in the medium in order to develop isolated ovarian follicles smaller than 100 μm but they did not examine developmental competence to birth (Lenie *et al.* 2004). Currently, different culture methods have been reported that support the growth and survival of isolated primary/early secondary follicles. These methods have demonstrated that co-culturing early-stage follicles encapsulated in alginate with mouse embryonic fibroblasts or ovarian stromal cells improves follicle growth and survival (Tingen *et al.* 2011, Tagler *et al.* 2012). Furthermore, group-cultured primary follicles have been reported to survive and effectively grow when compared with those in individual culture (Hornick *et al.* 2013). It seems that follicles themselves can exert a beneficial coculture effect. These results suggest that early-stage follicles may require unidentified factors responsible for promoting their growth and survival. We should also develop components of the culture medium as well as culture devices.

Recently, the activation mechanism of dormant primordial follicles has been reported in mice (Reddy *et al.* 2008). However, the molecular mechanisms underlying follicle selection and development from primary stage to preovulatory stage remain poorly understood. It is important that follicle culture methods, not culture of ovarian tissues, can provide the opportunity to study regulatory mechanisms of folliculogenesis. We would be able to study molecular mechanisms of follicular development, selection, and atresia using uniform follicles that were categorized by oocyte diameters and the number of granulosa cell layers, as opposed to a mixture of follicles at various developmental stages in ovarian tissue. The somatic component of stromal tissue associated with intraovarian regulatory process is not known. To clarify this, we can design experiments without systemic influences, as follicles in ovarian tissue would be inevitably affected by around environment.

It has been recognized that the efficiency of assisted reproductive technology could be enhanced by using oocytes from immature follicles for IVG and subsequent IVM. However, adjustment of optimal culture conditions is difficult for follicles at different stages because human follicles isolated from ovarian tissue vary in number and growth stage. Consequently, the technologies of IVG from various stage follicles would offer new applications for fertility preservation. This culture system provides the first encouraging step toward achieving full IVG of human small follicles, although we should make further effort to develop a more effective culture system. Also, we demonstrated that vitrification can be applicable to adequate embryo transfer. A combination of IVGf and vitrification may become a feasible method for future infertility treatment, because the vitrification technique has already been widely applied for clinical use (Gosden 2011, Herrero *et al.* 2011).

In conclusion, this study presents a two-step IVGf system for primary/early secondary follicles comprising small oocytes (diameters of ~45 μm), with one to three granulosa cell layers and less-developed theca cells. The oocytes in the grown follicles from this system resumed meiosis and fertilized with high success rates. The presumed embryos developed to live offspring by embryo transfer after vitrification and warming at four-cell stage. To our knowledge, this is the first report of offspring born after culture of individual follicles (diameters of <100 μm) followed by IVGf, IVM, and IVF-ET.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Ⅳ. 実施アンケート

2012年9月吉日

「重症骨系統疾患診療の現状把握のためのアンケート調査」ご協力のお願い

産科・小児科・新生児科
御担当医先生　各位

厚生労働科学研究費補助金　難治性疾患克服研究事業
「重症骨系統疾患の予後改善に向けての集学的研究」班
研究代表者：大阪大学 小児科　大薗恵一
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岡山大学 小児科　長谷川高誠
大阪府立母子保健総合医療センター　道上敏美
長野県立こども病院　中村友彦

謹啓

初秋の候、皆様には益々ご清栄のこととお慶び申し上げます。

突然のお手紙にて失礼致しますが、「重症骨系統疾患診療の現状把握のためのアンケート調査」にご協力頂きたく、ご依頼申し上げます。

骨系統疾患には、これまで、治療法がなく、予後が不良であった疾患が多く含まれますが、その状況は変化しつつあります。例えば、最近、低ホスファターゼ症に対する酵素補充療法が実用化され、近くわが国にも導入されます。また、FGFR3関連性疾患に対するCNP治療も臨床応用にむけて研究が進行しております。こうした状況から、私達は、今年度より厚生労働科学研究費　難治性疾患研究事業の助成を受けまして、「重症骨系統疾患の予後改善に向けての集学的研究」班を組織致しました。本研究班におきましては、骨系統疾患の予後改善をめざし、新規治療法の開発や診療ガイドラインの作成などを目的としております。今回、そのための第一歩と致しまして、わが国における重症骨系統疾患診療の現状把握のため、実際に臨床の場で骨系統疾患に遭遇される診療施設を対象に、全国アンケート調査を実施することとなりました。

つきましては、ご多用中のところ恐縮でございますが、添付のアンケート調査にご協力頂きたく、お願い申し上げる次第です。アンケートは産科用、小児科・新生児科用の２種類作成しており、いずれかを同封させていただいております。貴診療科で依頼が重複した場合には、代表者の方がご回答ください。また、恐縮でございますが、ご回答は10月9日（火）までにご返信いただければ幸甚です。

なにとぞ、骨系統疾患児及び家族の希望を拓くという本研究班の趣旨をご理解いただき、ご協力賜りますよう、お願い申し上げます。また、ご不明な点がございましたら、お手数でございますが、下記事務局までお問い合わせ下さいますよう、お願い致します。

謹白

「重症骨系統疾患の予後改善に向けての集学的研究」班　事務局
大阪大学大学院医学系研究科　小児科学
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重症骨系統疾患診療の現状把握のためのアンケート調査
（小児科・新生児科用）

この度はアンケート調査にご協力頂きありがとうございます。
以下の質問にご記入、あるいは該当する回答に○をつけてください。

Q1：貴施設名、回答者名を記載してください。

貴施設名：
回答者名：

Q2：貴施設では、2003年～2012年の10年間に骨系統疾患の診療経験がありますか？

はい　　いいえ

「はい」の方は以下の質問にもご回答ください。いずれも、2003年～2012年の10年間の御経験について記載してください。

Q3：Thanatopholic Dysplasia(致死性四肢短縮症)あるいはSADDAN症候群の御経験についておうかがいします。

総数および現時点での生存数：
呼吸管理を要した人数：
看取りの医療を選択した人数：
遺伝子診断を施行した人数：
出生前診断されていた人数：

Q4：骨形成不全症の御経験についておうかがいします。

総数および現時点での生存数：
呼吸管理を要した人数：
看取りの医療を選択した人数：
ビスフォスフォネート治療を施行した人数：
遺伝子診断を施行した人数：
出生前診断されていた人数：

Q5：低ホスファターゼ症の御経験についておうかがいします。

呼吸管理を要した人数：
けいれんを認めた人数：

(裏面もご記入お願いします)

高カルシウム血症を呈した人数：
看取りの医療を選択した人数：
2012年9月現在、生存している人数：
遺伝子診断を施行した人数：
出生前診断されていた人数：

Q6：乳児大理石骨病の御経験についておうかがいします。

総数および現時点での生存数：
呼吸管理を要した人数：
看取りの医療を選択した人数：
骨髄移植を施行した人数：
遺伝子診断を施行した人数：
出生前診断されていた人数：

Q7：上記以外の骨系統疾患症例の病名及び症例数を記載してください。

病名：　　症例数および現時点での生存数：
病名：　　症例数および現時点での生存数：
病名：　　症例数および現時点での生存数：

また、そのうち呼吸管理を行った症例の病名及び症例数を記載してください。

病名：　　症例数：
病名：　　症例数：
病名：　　症例数：

また、そのうち出生前診断されていた症例の病名及び症例数を記載してください。

病名：　　症例数：
病名：　　症例数：
病名：　　症例数：

ご協力どうもありがとうございました。

重症骨系統疾患診療の現状把握のためのアンケート調査
（産科用）

この度はアンケート調査にご協力頂きありがとうございます。
以下の質問にご記入、あるいは該当する回答に○をつけてください。

Q1：貴施設名、回答者名を記載してください。

貴施設名：
回答者名：

Q2：貴施設では、2003 年～2012 年の 10 年間に児が骨系統疾患であった妊娠／分娩の経験がありますか？

はい　　いいえ

「はい」の方は以下の質問にもご回答ください。いずれも、2003 年～2012 年の 10 年間の御経験について記載してください。

Q3：児が Thanatopholic Dysplasia(致死性四肢短縮症)あるいは SADDAN 症候群であった妊娠／分娩の御経験についておうかがいします。

総数および現時点での生存数：
児が呼吸管理を要した人数：
看取りの医療を選択した人数：
妊娠中絶を選択した人数：
遺伝子診断を施行した人数：
出生前診断されていた人数：

Q4：児が骨形成不全症であった妊娠／分娩の御経験についておうかがいします。

総数および現時点での生存数：
児が呼吸管理を要した人数：
看取りの医療を選択した人数：
妊娠中絶を選択した人数：
遺伝子診断を施行した人数：
出生前診断されていた人数：

(裏面もご記入お願いします)

Q5：児が低ホスファターゼ症であった妊娠／分娩の御経験についておうかがいします。

総数および現時点での生存数：
児が呼吸管理を要した人数：
看取りの医療を選択した人数：
妊娠中絶を選択した人数：
遺伝子診断を施行した人数：
出生前診断されていた人数：

Q6：児が上記以外の骨系統疾患であった妊娠／分娩についておうかがいします。

病名および現時点での生存数：
児が呼吸管理を要した人数：
看取りの医療を選択した人数：
妊娠中絶を選択した人数：
遺伝子診断を施行した人数：
出生前診断されていた人数：

Q7：上記以外の骨系統疾患症例の病名及び症例数を記載してください。

病名：　　症例数および現時点での生存数：
病名：　　症例数および現時点での生存数：
病名：　　症例数および現時点での生存数：

また、そのうち呼吸管理を行った症例の病名及び症例数を記載してください。

病名：　　症例数：
病名：　　症例数：
病名：　　症例数：

また、そのうち出生前診断されていた症例の病名及び症例数を記載してください。

病名：　　症例数：
病名：　　症例数：
病名：　　症例数：

ご協力どうもありがとうございました。

2013年9月吉日

「重症骨系統疾患診療の現状把握のためのアンケート調査：二次調査」ご協力のお願い

産科・小児科・新生児科

御担当医先生　各位

厚生労働科学研究費補助金　難治性疾患克服研究事業

「重症骨系統疾患の予後改善に向けての集学的研究」班

研究代表者：大阪大学 小児科　大薗恵一

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京都大学 内分泌代謝内科　八十田明宏

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長野県立こども病院　中村友彦

謹啓

初秋の候、皆様には益々ご清栄のこととお慶び申し上げます。

さて、昨年、当研究班が行いました「重症骨系統疾患診療の現状把握のためのアンケート調査（一次調査）」におきまして、ご多用中にもかかわらず、ご回答を頂きまして誠にありがとうございました。おかげさまで、産科191施設、小児科／新生児科281施設からご回答をいただき、本邦における重症骨系統疾患診療の現状の概要について、非常に有意義な調査結果を得ることができました。これも諸先生方の御協力の賜物であり、班員一同、深謝申し上げます。

本年度は、一次調査の結果を踏まえまして、個々の症例の詳細な情報を把握することを目的に、二次調査を計画致しました。この「二次調査御協力のお願い」は、昨年度の一次調査において「症例あり」とご回答頂いた施設に送付させていただいております。それぞれの疾患について個別の調査用紙を作成し、同封致しております。診療の御経験のある疾患に関して御記入を頂き、御返送頂ければ幸いです。ご多用中のところ、貴重なお時間を頂き誠に恐縮ではございますが、何卒、骨系統疾患児及び家族の希望を拓くという本研究班の趣旨をご理解いただき、ご協力賜りますようお願い申し上げます。また、恐縮ではございますが、10月31日（木）までにご返信いただければ幸甚です。ご回答を頂きました先生には、些少ではございますが、謝礼と致しまして2000円分のQUOカードを郵送にて謹呈させていただきます。なお、本調査は大阪大学医学部附属病院臨床研究倫理審査委員会において承認されております（承認番号13150）。ご不明な点がございましたら、お手数ではございますが、下記事務局までお問い合わせ下さいますようお願い致します。

謹白

「重症骨系統疾患の予後改善に向けての集学的研究」班　事務局

大阪大学大学院医学系研究科　小児科学

大薗恵一、難波範行、窪田拓生

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骨形成不全症に関するアンケート（小児科）

厚生労働科学研究費補助金　難治性疾患等克服研究事業
重症骨系統疾患の予後改善に向けての集学的研究班　作成

貴施設名・所属先
回答者名

Q１．貴施設における、2003年～2012年の10年間の骨形成不全症の症例数をご記入ください。　　生存中：　　例　（男　　例、女　　例）
死亡：　　例　（男　　例、女　　例）

Q２．臨床病型とその人数をご記入ください。
Ⅰ型（　　）名　Ⅱ型（　　）名　Ⅲ型（　　）名　Ⅳ型（　　）名
Ⅴ型（　　）名　Ⅵ型（　　）名　その他（　　）名

Q３．出生前診断されていた症例はありますか？
あり（　　）名
なし（　　）名
上記で「あり」の場合、その方法は？（複数回答可）
（　　）超音波検査
（　　）胎児CT
（　　）その他（具体的に：　　）

Q４．人工呼吸管理をされた例はありますか？
あり（　　）名　　なし（　　）名

Q５．ビスフォスフォネートで治療中または過去に治療した症例はありますか？
あり（　　）名　　なし（　　）名

Q６．骨形成不全症の治療に使用したことのある薬剤に○を付して下さい。

［注射薬］

（　　）パミドロネート（アレディア®）

（　　）ゾレドロネート（ゾメタ®）

（　　）アレンドロネート（ボナロン注®）

（　　）その他（　　）

Q６（つづき）

［経口薬］

（　　）エチドロネート（ダイドロネル®）

（　　）アレンドロネート（フォサマック®、ボナロン®）

（　　）リセドロネート（アクトネル®、ベネット®）

（　　）ミノドロネート（ボノテオ®、リカルボン®）

（　　）その他（　　）

Q７．遺伝子診断を施行されましたか？

はい（　　）　　いいえ（　　）

Q８．責任遺伝子が判明したものを挙げて下さい。

COL1A1（　　）名　　*COL1A2*（　　）名

CRTAP（　　）名　　*LEPRE1*（　　）名

IFITM5（　　）名　　*SERPINF1*（　　）名

その他　（　　）名

Q９．遺伝子診断をされた場合、遺伝カウンセリングはされていましたでしょうか？

されていた　　（　　）名

されていなかった（　　）名

上記で「されていた」場合、カウンセリングを担当したのは？

（　　）産科医

（　　）小児科医

（　　）遺伝専門医（産科医、小児科医以外）

ご協力いただきありがとうございました。

致死性骨異形成症(タナトフォリック骨異形成症)に関するアンケート（小児科）

厚生労働科学研究費補助金　難治性疾患等克服研究事業
重症骨系統疾患の予後改善に向けての集学的研究班　作成

貴施設名・所属先
回答者名

Q１．貴施設における、2003年～2012年の10年間の致死性骨異形成症(タナトフォリック骨異形成症)の症例数をご記入ください。

生存中：　　例　(男　　例、女　　例)
死亡：　　例　(男　　例、女　　例)

Q２．臨床病型とその人数をご記入ください。

致死性骨異形成症　　type Ⅰ　(　　)例　type Ⅱ(　　)例

以下は症例ごとにお書きください

【症例番号】(　　)(各科各疾患に１から通し番号を付けて下さい)

Q３．現在の年齢(死亡例の場合、死亡時年齢)をご記入ください
また性別、生存例か死亡例かに○をおつけください。

(　　歳　　ヶ月)　　男　　女
生存　　死亡

Q４．出生前診断はされていましたでしょうか？

・されていた(方法:　　診断時妊娠週数:　　週　　日)
・されていなかった（診断時の年齢、月齢、日齢：　　歳　　ヶ月　　日）

Q５．遺伝カウンセリングはされていましたでしょうか？

（該当箇所に○をおつけください）

・されていた

施行者：産科医　　小児科主治医　　遺伝専門医　　遺伝カウンセラー

・されていなかった

Q６．呼吸器を使用した場合にご記入ください。

呼吸器補助開始年齢　（　　歳　　ヶ月）、終了年齢（　　歳　　ヶ月）

その間中断はありましたでしょうか

あり　（計　　ヶ月　中断）、　　なし

Q７．FGFR3（線維芽細胞増殖因子受容体３型；Fibrobrast Growth Factor Receptor Type 3）遺伝子診断を施行されましたか？

・はい　　その変異をご記入ください（　　）

・いいえ

Q８．生存例の場合にお聞きします。（該当箇所に○をおつけください）

現在の合併症：　てんかん　水頭症　四肢麻痺　その他（　　）

現在の状況：

入院中　　呼吸管理あり　　なし

在宅　　呼吸管理あり　　なし

発達状況：おすわり可など自由記載でお願いします。

（　　）

Q９．死亡例の場合にお聞きします。

死亡原因　（　　）

ご協力いただきありがとうございました。

低ホスファターゼ症に関するアンケート（小児科）

厚生労働科学研究費補助金　難治性疾患等克服研究事業
重症骨系統疾患の予後改善に向けての集学的研究班　作成

貴施設名・所属先
回答者名

Q１．貴施設における、2003 年～2012 年の 10 年間の低ホスファターゼ症の症例数をご記入ください。　生存中：　　例　（男　　例、女　　例）
死亡：　　例　（男　　例、女　　例）

Q２．臨床病型とその人数をご記入ください。
周産期型（　　）名　周産期良性型（　　）名　乳児型（　　）名
小児型（　　）名　成人型（　　）名　歯限局型（　　）名
不明（　　）名

以下は症例ごとにお書きください。

【症例番号】（　　）（各科各疾患に１から通し番号を付けて下さい）

Q３．性別と現在の年齢
（　　）歳（ヶ月）　性別　（　男、　女　）

Q４．初発時年齢とその症状をご記入ください。
（　　）歳（ヶ月）　症状　（　　）

Q５．出生前診断はされていましたでしょうか？
されていた　（方法：　　）
されていなかった

Q６．遺伝カウンセリングはされていましたでしょうか？
されていた

カウンセラー：産科医　　小児科主治医　　遺伝専門医
されていなかった

Q７．呼吸器を使用した場合にご記入ください。
呼吸器補助開始年齢(　　)、終了年齢(　　)
転帰　生存　死亡
その間中断はありましたでしょうか
あり　(計　　ヶ月　中断)、　なし

Q８．遺伝子診断を施行されましたか？
はい（　　）例　その変異をご記入ください（　　）
いいえ（　　）例

Q９．生存例の場合にお聞きします。(該当箇所に○をおつけください)
現在の合併症：呼吸障害　成長障害　骨変形
発達遅延　乳歯早期脱落　頭蓋骨早期癒合
なし
現在の状況：
入院中　　呼吸管理あり　　なし
在宅　　呼吸管理あり　　なし
発達状況：
おすわり　可、　つかまり立ち　可、　独歩　可
単語　可、　２語文　可
その他　発達状況（　　）

Q10. 死亡例の場合にお聞きします。
死亡原因　(　　)、死亡時年齢　(　　)

Q11. 本症に対する酵素補充療法の治験が始まっていることをご存知ですか？
知っていた（　　）　　知らなかった（　　）

ご協力いただきありがとうございました。