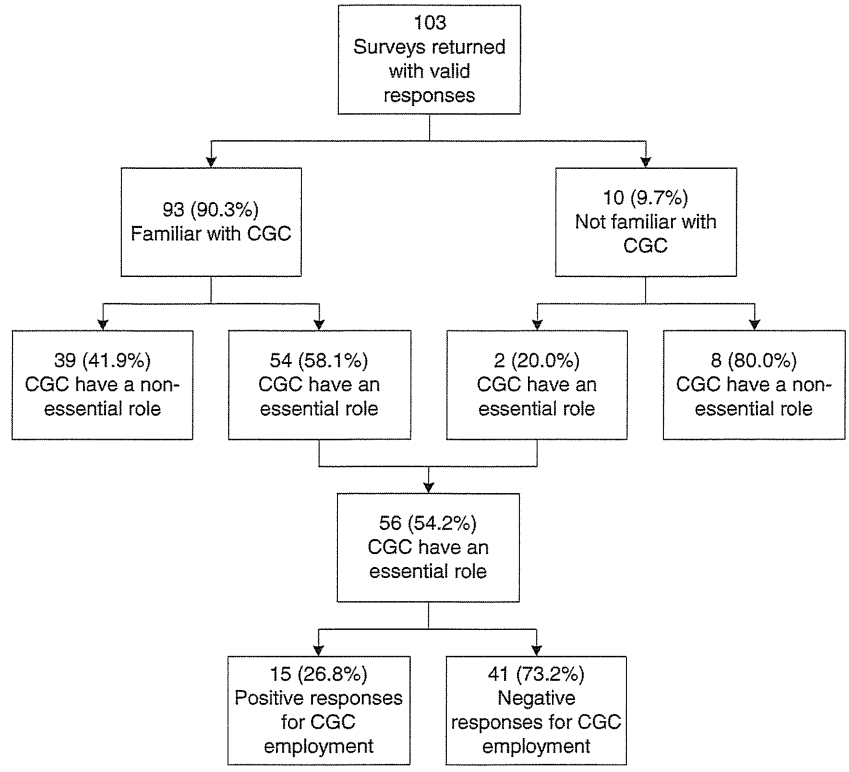

Fig. 1 Familiarity with CGC and employment opportunities at clinical practices offering amniocentesis for prenatal diagnosis

The variations in the amount and quality of genetic counseling could be due to unequal knowledge about the importance of genetic counseling in obstetric practice. Interestingly, for genetic counseling regarding normal fetal chromosome results, understanding of the JSOG guideline for prenatal testing, and having more experience providing prenatal chromosome results were significantly correlated with respondents who spent more time in genetic counseling. This suggests that these respondents recognize the importance of genetic counseling for normal results may be providing information regarding the limitations of chromosome analysis based upon their understanding of the guidelines and their clinical experience. Additionally, an understanding of the JSOG guidelines was one of the significant correlating factors regarding spending more time in genetic counseling for abnormal results. These data suggest that these respondents understand

Table 6 Correlation factors with the respondents who answered that CGC have an essential role in clinical practices offering amniocentesis for prenatal diagnosis of fetal chromosome abnormalities

Factor	Essential role (n=56)		Non-Essential role (n=47)		Change	P value	OR
	n	%	n	%			
Age: < 50	34	60.7 %	14	29.8 %	2.038	0.002	3.643
Experience: < 15 years	26	46.4 %	16	34.0 %	1.364	0.203	1.679
Private clinic	23	41.1 %	26	55.3 %	0.742	0.149	0.563
University hospital	12	21.4 %	5	10.6 %	2.014	0.142	2.291
# of patient visits: < 30 daily	31	55.4 %	33	70.2 %	0.788	0.089	0.488
MD geneticist	11	19.6 %	5	10.6 %	1.846	0.209	2.053
Amniocentesis: ≥30 annually	18	32.1 %	9	19.1 %	1.679	0.135	2.000
Amniocentesis: ≥10 annually	34	60.7 %	17	36.2 %	1.679	0.013	2.727
Experience with abnormal results	48	85.7 %	39	83.0 %	1.033	0.703	1.231

Table 7 Correlation factors with the respondents who indicated a positive attitude toward employing CGC at the clinical practice offering amniocentesis for prenatal diagnosis of fetal chromosome abnormalities

Factor	Want to employ (n=15)		Do not want to employ (n=41)		Change	P value	OR
	n	%	n	%			
Age: < 50	10	66.7 %	24	58.5 %	1.139	0.581	1.417
Experience: < 15 years	7	46.7 %	19	46.3 %	1.007	0.983	1.013
Private clinic	3	20.0 %	20	48.8 %	0.410	0.053	0.263
University hospital	9	60.0 %	3	7.3 %	8.200	<0.0001	19
# of patient visits: < 30 daily	7	46.7 %	24	58.5 %	0.797	0.429	0.62
MD geneticist	6	40.0 %	5	12.2 %	3.280	0.020	4.8
Amniocentesis: ≥30 annually	12	80.0 %	6	14.6 %	5.467	<0.0001	23.333
Amniocentesis: ≥10 annually	15	100.0 %	19	46.3 %	2.158	<0.0001	34.74
Experience with abnormal results	15	100.0 %	33	80.5 %	1.242	0.065	7.27

the importance of the interaction with pregnant women discussing the issues of abnormal results. Therefore, information discussed with pregnant women should be further explored to support these assumptions. Such exploration might show that education of obstetricians in Japan regarding prenatal diagnosis, as listed in the JSOG guideline, could promote the understanding of the importance of genetic counseling in the clinical practice of medicine. It is conceivable that offering amniocentesis could be recognized as a genetic service, not an obstetric service.

Our study found that few CGC were involved in all genetic counseling situations for fetal chromosome analysis, 0.0 % for pre-amniocentesis, 3.4 % of abnormal results and 1.0 % of normal results. These data reveal that most of the CGC's in Japan are not involved with prenatal genetic testing. The lack of the recognition of the skills and the role of CGC could be one of the reasons why there are few opportunities for CGC to make a significant contribution in obstetric practice. In fact, our study showed that although the vast majority of respondents in this study were familiar with CGC, 40 % of them indicated that CGC do not have an essential role in their clinical practice. CGC possess the skills that would allow them to provide information about prenatal testing and to support informed decision making (Farrelly et al. 2012). Additionally, comprehensive genetic risk assessment by CGC improves the detection of identifiable genetic risk factors that may indicate the fetus is at risk for a genetic disorder (Cutillo et al. 2002; Koscica et al. 2001). Thus, CGC are genetic professionals serving a significant role in prenatal genetic counseling. Therefore, reasons why some obstetricians would not support CGC as an integral part of their service should be further explored to consider the appropriate involvement of CGC at a clinical practice offering prenatal genetic testing in Japan.

Another reason why there is little involvement of CGC in prenatal genetic testing in Japan might be attributed to obstetricians who may recognize the essential role of CGC but do not employ CGC. In this study, over 70 % of respondents who consider CGC to have an essential role did not employ CGC due to their small amniocentesis procedure volumes and few abnormal results. In addition, they often had access to refer patients to a facility with an MD geneticist and/or CGC, as needed. These findings reflect that amniocentesis procedures are performed at various practice settings with varying numbers of procedures, from less than 10 to greater than 100 in a year. Additionally, some obstetricians answered that they did not employ CGC due to financial considerations, although they recognized the need for CGC in clinical practice. The reason why they have financial concerns could be due to the healthcare system in Japan, universal health insurance coverage. Although this system provides healthcare services with Japanese patients accepting responsibility for 30 % of these costs while the government pays the remaining 70 %, genetic counseling is not incorporated into this healthcare system. Therefore, the hospitals request private compensation for genetic counseling for their patients. If genetic counseling is incorporated into the universal health insurance coverage, it might allow the hospitals which have financial responsibility for the employment of CGC to have CGC in their clinical practice. This might lead to the establishment of the appropriate involvement of CGC in prenatal genetic counseling and reconstruct the utilization of prenatal diagnosis from a part of obstetric medicine to an indispensable part of genetic medicine.

Some obstetricians indicated that CGC have an essential role in the obstetric practices offering amniocentesis. Data analysis in this study found that this attitude was statistically significantly correlated with obstetricians less than 50 years old with over ten amniocentesis procedures in a year. These data suggest that the role of CGC is more likely to be well recognized and accepted by younger obstetricians. Interestingly, hospitals with over 30 specimens submitted annually were not

significantly correlated. In these hospitals, there were more than two MD geneticists, more than three full-time obstetricians, and the respondent was an MD geneticist. This finding suggests that they might have more time to spend with pregnant women and have high skill-sets obtained through their experiences. Therefore, they might determine that they can deal with all the issues related to genetic counseling by themselves without utilizing CGC's and don't consider CGC have an essential role at their practice. However, respondents at hospitals submitting over 30 specimens annually had a statistically significant correlation to employ a CGC. These findings suggest that although they don't consider CGC to have an essential role, they need the help of CGC at their clinical practice to reduce the burden of their work or to improve the quality of genetic counseling services for pregnant women. Other significant factors which correlated with a positive response for CGC employment were respondents working at a university hospital, to be an MD geneticist, and perform over ten amniocentesis procedures in a year. The employment of CGC at large facilities performing more amniocentesis procedures would provide opportunities for CGC to work with MD geneticists and thereby expand access to professionals with the appropriate skills set in obstetric practice.

Study Limitations

One of the limitations of the present study involved extrapolating the study findings to the general population. Since the survey was only sent to clients of LabCorp Japan, the results may not be representative of all hospitals providing amniocentesis in Japan. Based on the number of amniotic fluid specimens that were received in 2009 and the Sasaki et al. (2011) reported volume of 13,000 women who had amniocentesis in 2008, our study population accounted for about a third of all specimens in Japan. Additionally, since the surveys were anonymous, we were unable to recognize who did or did not return the questionnaire to us, and therefore, follow-up contact was not performed. As a result, only 37.2 % (103) of the LabCorp Japan clients returned the surveys. Since the surveys were anonymous, it was not possible to estimate the total number of amniotic fluid specimens submitted by the 103 clients who responded to the survey. Nonetheless, we believe this is the first Japanese study to explore the provision of pre- and post-amniocentesis genetic counseling to pregnant women. Therefore, our findings provide a helpful description of the current practice.

Although statistically significant differences were noted regarding the amount of time spent in genetic counseling, another limitation of the current study involved the questionnaire. This instrument was not designed to examine the specific information provided to the pregnant women or the context of discussions in each genetic counseling setting. However, the amount of time spent in genetic counseling might indicate a recognition of the importance of providing information to the pregnant women.

This study did not evaluate individual pregnant women's decisions or their understanding of the information provided during genetic counseling. Because genetic counseling should help pregnant women understand the testing and facilitate informed decision making, future studies should evaluate a pregnant woman's comprehension following genetic counseling in order to explore the appropriate information that should be provided during genetic counseling. Moreover, the practice of genetic counseling targets the decision-making process, not decision outcome (Farrelly et al. 2012). Therefore, future studies should also evaluate the pregnant woman's satisfaction with the delivery of information in helping her to make an informed decision.

Conclusion

In order for pregnant women to make informed decisions regarding amniocentesis for fetal chromosome analysis, they should be provided with accurate and clear information about the risks, benefits and limitations of testing. While this study showed that obstetrician alone in Japan currently provide pregnant women with information regarding prenatal genetic testing, they spend limited time in genetic counseling and are more likely to refer pregnant women with abnormal fetal chromosome results to genetics professionals. The limited genetic counseling available in Japan creates potential opportunities for expanding the use of CGC.

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REPRODUCTION
RESEARCH

Live births from isolated primary/early secondary follicles following a multistep culture without organ culture in mice

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Abstract

Although the ovary has a large store of germ cells, most of them do not reach mature stages. If a culture system could be developed from early growing follicles to mature oocytes, it would be useful for biological research as well as for reproductive medicine. This study was conducted to establish a multistep culture system from isolated early growing follicles to mature oocytes using a mouse model. Early growing follicles with diameters of 60–95 μm corresponding to primary and early secondary follicles were isolated from 6-day-old mice and classified into three groups by diameter. These follicles contained oocytes with diameters of ~45 μm and one or a few layered granulosa cells on the basal lamina. Embedding in collagen gel was followed by first-step culture. After 9-day culture, the growing follicles were transferred onto collagen-coated membrane in the second step. At day 17 of the culture series, the oocyte–granulosa cell complexes were subjected to *in vitro* maturation. Around 90% of the oocytes in follicles surviving at day 17 resumed second meiosis (metaphase II oocytes: 49.0–58.7%), regardless of the size when the follicle culture started. To assess developmental competence to live birth, the eggs were used for IVF and implantation in pseudopregnant mice. We successfully obtained two live offspring that produced next generations after puberty. We thus conclude that the culture system reported here was able to induce the growth of small follicles and the resultant mature oocytes were able to develop into normal mice.

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Introduction

In mammalian ovaries, there are numerous follicles at various stages of growth. Especially, gonadotropin-independent small follicles are abundant but most of them do not reach ovulation. If a whole culture system from small follicles to preovulatory follicles can be established, it would be useful for follicular biology and future infertility therapy. Such a culture system provides a valuable model to study critical interactions between oocyte and follicular cells and factors regulating follicle development at each developmental stage. Eventually, follicle culture techniques could be applied to fertility preservation.

Through advances in aggressive chemotherapy and/or radiotherapy as well as abdominal surgery, survival rates from cancers have significantly increased. However, such therapies also damage normal cells including gametes, resulting in cancer survivors becoming infertile due to ovarian failure (Meirow & Nugent 2001, Lutchman Singh *et al.* 2005, Anderson *et al.* 2008). Women with cancer may cryopreserve their own mature eggs before starting aggressive cancer therapy, but the numbers that can be used for infertility therapy are very small. Ovarian tissue cryopreservation is an option to recover fertility. For prepubertal girls, ovarian tissue cryopreservation is the only way to preserve their future fertility. Recently, ovarian tissue autografting after cryopreservation has been applied to a young patient who had recovered from Hodgkin's lymphoma and it resulted in a healthy baby being born (Donnez *et al.* 2004). Subsequent successful cases have been reported (Meirow *et al.* 2005, Demeestere *et al.* 2007, Andersen *et al.* 2008). However, autografting carries a risk of reintroduction of malignant cells in patients who have recovered from cancer (Shaw *et al.* 1996, Meirow *et al.* 2008). The technologies of *in vitro* growth (IVG) of follicles or oocyte–granulosa cell complexes (OGCs), *in vitro* maturation (IVM) of oocytes, and IVF are feasible methods for such patients.

Many researchers have reported about IVG of immature oocytes in mice (Eppig & Schroeder 1989, Nayudu & Osborn 1992, Cortvrindt *et al.* 1996, Eppig & O'Brien 1996, Lenie *et al.* 2004). Induction and maintenance of follicle growth are more difficult in earlier stages of preantral follicles, which require longer culture periods to reach mature stages (Smitz & Cortvrindt 2002, Hirao & Miyano 2008). It is well

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established that folliculogenesis and meiotic maturation of the oocyte are strictly timed processes. To reach fully grown stages, intrinsic culture periods are necessary depending on follicular diameters at the start of the culture. For recruitment of dormant primordial follicles in culture, a multistep culture system including organ culture is necessary before IVG of isolated OGCs or isolated follicles (Eppig & O'Brien 1996, Telfer *et al.* 2008, Jin *et al.* 2010).

In the ovary, the recruited primary follicles start to grow in the preantral stages until antrum formation. Preantral folliculogenesis consists of several further stages as follows: i) oocytes and one-layered granulosa cells are present, ii) granulosa cell proliferation from one layer to two to three layers, iii) granulosa cell layers increase from a few layers to four to six layers, and iv) fully grown preantral follicles having seven to eight granulosa cell layers capable of forming an antrum. In this article, follicles at each stage of preantral folliculogenesis from the above-mentioned stages are designated as primary, early secondary, middle secondary, and late secondary follicles respectively. Primary/early secondary follicles are presumed to be gonadotropin independent and middle/late secondary follicles are considered to be gonadotropin dependent. Development of optimal culture systems for different follicle stages is necessary for application to reproductive technology because mammalian reproductive ovaries contain follicles at various growth stages. In animal experiments, middle/late secondary follicles have been reported to grow in culture achieving live births, while primary and early secondary follicles are still very difficult to be grown in culture. The immature granulosa cells at these stages do not organize normal follicle shape under culture conditions.

To keep the adequate follicle structure during culture, different approaches have been attempted. All of them stated that keeping three-dimensional structure of follicles was important for successful growth of follicles. First, polyvinylpyrrolidone (PVP) was applied to the culture medium. PVP gave viscosity to the culture medium and prevented dispersal of growth factors released by follicular cells (Hirao *et al.* 2004). Secondly, an inverted drop method was reported. This method prevents follicular cells from attachment and spread on the bottom of the culture dish (Wycherley *et al.* 2004). Thirdly, embedding follicles in biomaterial gels such as collagen (Torrance *et al.* 1989, Carroll *et al.* 1991), alginate (Pangas *et al.* 2003), and matrigels (Hwang *et al.* 2000, Scott *et al.* 2004) was adopted for the culture. More recently, alginate prepared from brown algae has been shown to give more successful results in various animal species including mice (Kreeger *et al.* 2006, Xu *et al.* 2006), monkeys (Xu *et al.* 2009*b*), and humans (Xu *et al.* 2009*a*).

Maintaining three-dimensional structure of follicles is important for keeping interactions between the cells themselves and the extracellular matrix to achieve mature stages. Cross-linked biomaterials should retain the growth factors around the oocyte and help the formation of local gap junctions between oocytes and granulosa cells. Naturally occurring extracellular matrices such as fibronectin, laminin, and collagen are deposited in ovarian follicles during follicle development. These matrices have important functions in a stage-specific manner (Berkholtz *et al.* 2006). In this study, we focused on collagen gel, because collagen has been reported to stimulate cell growth and development in various mammalian cell culture systems (Wicha *et al.* 1979, Yang *et al.* 1980, Yang & Nandi 1983). Not only that, the protein is also found throughout the animal world and mainly constitutes connective tissues. It is believed to have a physical function such as maintaining morphology and strength of organs. Collagen is also an essential factor for ovarian folliculogenesis. The culture systems using collagen, therefore, may keep normal ovarian functions and support cell–cell communications, regulation of cell development, and biological signaling pathways from the extracellular environment.

In 1989, Eppig's group reported the use of collagen-coated membrane for a culture method of OGCs (Eppig & Schroeder 1989). This group also succeeded in obtaining live births from neonatal mouse ovary by organ culture followed by subsequent culture of OGCs that were isolated from 8-day-old ovaries chronologically (Eppig & O'Brien 1996, O'Brien *et al.* 2003). Similarly, in our own research, preantral follicles isolated from cryopreserved ovaries of 16-day-old mice grew and reached mature stages in culture, and the oocytes were fertilized and resulted in live births (Hasegawa *et al.* 2006). However, primary/early secondary follicles having diameters of <100 μm with one to three layers of granulosa cells did not grow in these culture conditions. Oocyte developmental competence is defined as the oocyte's potential to undergo maturation, fertilization, development into blastocyst, and as a final outcome to give rise to live offspring. To our knowledge, live birth has not been achieved from such small follicles. This study was designed to establish an effective culture method for mouse primary/early secondary follicles with diameters of <95 μm. For this purpose, we used 6-day-old mouse ovaries that do not contain middle secondary follicles with more than four layers of granulose cells. We also assessed the competence of the derived oocytes to achieve live births.

Materials and methods

Animals and materials

The mice used in this study were BDF1 females derived from matings between DBA/2 males and C57BL/6 females. ICR male mice (18–20 weeks old) and ICR pseudopregnant female mice (10–13 weeks old) were used for IVF and embryo transfer respectively.

Animals were purchased (Japan SLC, Inc., Shizuoka, Japan; CLEA Japan, Inc., Tokyo, Japan) and housed in a temperature- and light-controlled environment on a 12 h light:12 h darkness photoperiod and were provided with food and water *ad libitum*. The animal experiments in this study were approved by the Committee on Animal Experimentation of Hyogo College of Medicine. Unless otherwise noted, all chemicals were purchased from Sigma–Aldrich.

Follicle culture

Late secondary follicles with diameters of 125–140 μm were collected from 16-day-old mice for IVG, as described in the previous report (Hasegawa *et al.* 2006). Primary/early secondary follicles surrounded by one or a few layers of somatic cells were mechanically dissected using 30 G needles from 6-day-old BDF1 mouse ovaries. The follicles were isolated in L-15 medium (Invitrogen) supplemented with 20 mg/ml BSA and antibiotic antimycotic solution (penicillin, 10 IU/ml; streptomycin, 10 μg/ml; and amphotericin B, 25 ng/ml). The follicles corresponding to classes 3a and 3b (Pedersen & Peters 1968) were collected. The follicle classes were also confirmed by the number of granulosa cell layers in serial sections of whole ovaries stained with hematoxylin and eosin. We also calculated the number of granulosa cells per follicle. The isolated follicles were separated into oocytes and granulosa cells by treatments with collagenase and trypsin followed by repeated pipetting. Numbers of oocytes and granulosa cells were counted by a hematocytometer to determine the average number of granulosa cells surrounding an oocyte.

The collected follicles were divided into three groups based on the follicle diameters: group A, 80–95 μm, two or three partial layers of granulosa cells; group B, 70–80 μm, granulosa cell layers similar to those in group A but diameters are smaller than those of group A; and group C, 60–70 μm, one or two partial layers of granulosa cells. The isolated and grouped follicles were cultured in collagen gels for 9 days, which were designated IVGf-1. The follicles were transferred to a second culture with collagen-coated membrane for 8 days, which were designated IVGf-2. The details are as follows.

In vitro growth of follicles-1

Collagen gel (Cellmatrix; Nitta Gelatin, Inc., Osaka, Japan) was prepared according to the manufacturer's instructions. Briefly, 10 μl of reconstituted collagen solution were poured into a 60 mm dish (FALCON 351007 Petri Dish: Becton Dickinson Labware, Franklin Lakes, NJ, USA) in the form of droplets as a base layer and allowed to gel for 30 min at 37 °C. Those base layers were used to prevent the follicles from attaching directly to the culture dish and growing there. The follicles in each group were washed three times in growth medium and ten follicles were put separately onto collagen gel base layer. Immediately after this, an additional collagen solution was poured onto the base layer to cover the follicles. Follicles were embedded in collagen gel. After the top layer was fixed, 100 μl of growth medium were overlaid onto the follicle-containing gel to make microdroplets and then the microdroplets were covered with mineral oil. The medium used for IVGf-1 was α-minimum essential medium (MEM) supplemented with 5% FCS, ITS (insulin, 10 μg/ml; transferrin, 5.5 μg/ml; and sodium selenite, 5 ng/ml), antibiotic antimycotic solution (penicillin, 10 IU/ml; streptomycin, 10 μg/ml; and amphotericin B, 25 ng/ml), 1 mIU/ml of recombinant human FSH (Follistim: Organon, The Netherlands), and 1 ng/ml of mouse epidermal growth factor (mEGF). Follicles were cultured at 37 °C in 5% CO_2, 5% O_2, and 90% N_2 for 9 days. Half of the medium was changed every other day. On the first and ninth days of culture, follicle and zona pellucida diameter (excluding oocyte diameter) were determined by measuring two different axes (length and width) using an inverted microscope equipped with a micrometer. Oocyte-enclosing cell clusters having a diameter >110 μm were regarded as growing follicles.

In vitro growth of follicles-2

At day 9 of IVGf-1, growing follicles were removed from the collagen gel by treatment with 100 IU/ml collagenase (COLLAGENASE L: Nitta Gelatin, Inc.) for 20 min at 37 °C. Follicles were washed with IVGf-2 medium composed of α-MEM, 5% FCS, ITS, antibiotic solution (penicillin, 10 IU/ml; and streptomycin, 10 μg/ml), and 100 mIU/ml of recombinant human FSH and transferred to the 12-well plate (2 ml/well) equipped with a collagen-coated membrane insert (Transwell-COL: Corning Incorporated Life Sciences, Tewksbury, MA, USA) and cultured in IVGf-2 medium for 8 days at 37 °C in 5% CO_2 in air. Half of the medium was changed every other day. A follicle comprising an oocyte and granulosa cells attached to the collagen-coated membrane was considered to be a surviving follicle.

IVM, IVF, and embryo transfer

At day 17 of the whole culture series (IVGf-1 and IVGf-2), the surviving follicles were dislodged from the Transwell-COL membrane by pipetting and transferred to the maturation medium. As the maturation medium, IVGf-2 medium supplemented with 2.5 IU/ml hCG (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) and 10 ng/ml mEGF was used. After 19 h of incubation, *in vitro* ovulation was observed. Most follicles released mucified COCs. Those COCs were collected to estimate the diameters of the oocytes and to assess oocyte nuclear maturation. The mature oocytes that underwent GVBD

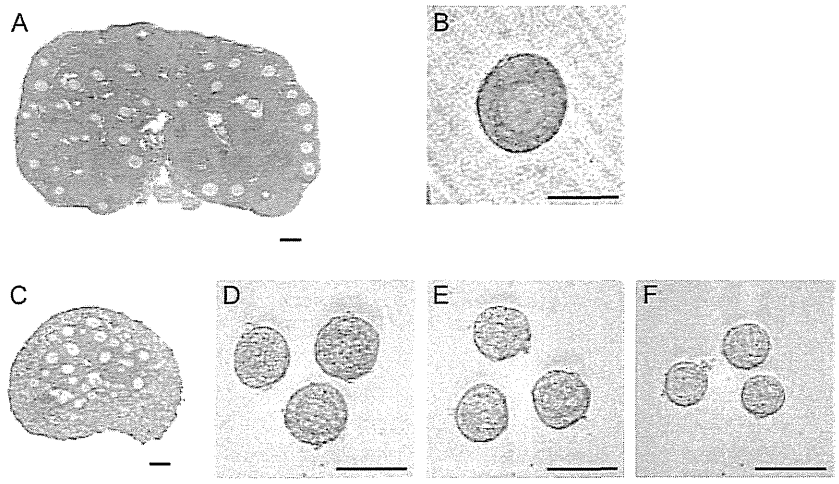

Figure 1 Comparison of 6- and 16-day-old mouse ovaries and follicles. (A) Hematoxylin–eosin staining of 16-day-old mouse ovarian section. There are many preantral and early antral follicles. (B) A typical OGC derived from preantral follicles isolated from 16-day-old mouse ovaries. The follicles grew on collagen-coated membrane without collagen gel culture (IVGf-1). (C) Hematoxylin–eosin staining of 6-day-old mouse ovarian section. There are many primordial follicles in the periphery region. Early secondary follicles are mostly found in the medullar region of the ovary. (D, E, and F) Follicles isolated from 6-day-old mouse ovaries were classified by their diameters. (D) Group A: follicle diameter is 80–95 μm. (E) Group B: follicle diameter is 70–80 μm. (F) Group C: follicle diameter is 60–70 μm. Scale bar=100 μm.

or reached MII were transferred to modified HTF medium (zenith HTF for Mouse IVF: IVFonline.com, LLC, Guilford, CT, USA) for IVF. Sperm were collected from ICR mouse epididymis and used for insemination at 0.8×10^6 sperm/ml. After 6 h, oocytes were examined in fresh modified HTF medium. Fertilized zygote, MII stage oocyte, and GVBD oocyte were assessed by the presence of two pronuclei, by the extruded first polar body, and by no GV membrane respectively. They were further cultured overnight in the same medium and the resultant two-cell-stage embryos were cultured in modified KSOM (KSOMaa Evolve: IVFonline.com, LLC) for 96 h to examine their competence for development into blastocysts.

In the experiment for obtaining live offspring, two-cell-stage embryos were vitrified by a minimum volume cooling method to collect sufficient number of embryos. Vitrification and warming were performed using vitrification/thawing kits (VT101; VT102: KITAZATO Co., Ltd., Shizuoka, Japan). Surviving embryos after warming were cultured in modified KSOM (KSOMaa Evolve: IVFonline.com, LLC) for 15 h and developing embryos at the four-cell stage were transferred into the oviducts of pseudopregnant ICR female mice (0.5 days postcoitum). Five to ten embryos were transferred to each uterine horn in a minimal volume of culture medium. Cesarean section was performed to deliver live offspring at 19.5 days postcoitum.

Statistical analysis

The results of follicle culture and embryo development were shown as mean percentages of multiple independent experiments. Differences among the three classified groups were examined using contingency tables and the χ^2 test. One-way ANOVA followed by Tukey's multiple comparison test was used for statistical analysis of follicle and oocyte diameter. Differences were considered to be significant at $P<0.05$.

Results

IVG of early secondary follicles compared with preantral follicles

Preantral and early secondary follicles were collected from 16- and 6-day-old mouse ovaries respectively (Fig. 1A and C). The preantral follicles were covered with five to six layers of granulosa cells and their diameters were 125–140 μm (Fig. 1B), while the early secondary follicles were covered with two to three layers of granulosa cells and their diameters were <100 μm (Fig. 1D). 95.5% of the preantral follicles grew in collagen-coated membrane culture after 6 days and the grown follicles were matured in IVM (Table 1). The resultant mature oocytes were then fertilized and the presumed embryos were cleaved. However, the early secondary follicles did not grow under these culture conditions (Table 1).

Table 1 Comparison of follicle growth between preantral stage and early secondary stage by collagen-coated membrane culture system.

	Used follicles	Grown follicles after 6 days	Mature oocytes (metaphase II) after IVM	Fertilized eggs (2PN)	Cleaved embryos
Preantral follicles (125–140 μm)	134	128/134 (95.5%)	80/134 (59.7%)	47/80 (58.8%)	42/47 (89.4%)
Early secondary follicles (<100 μm)	67	0/67 (0%)	NA	NA	NA

PN, pronuclei; NA, not applicable.

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).

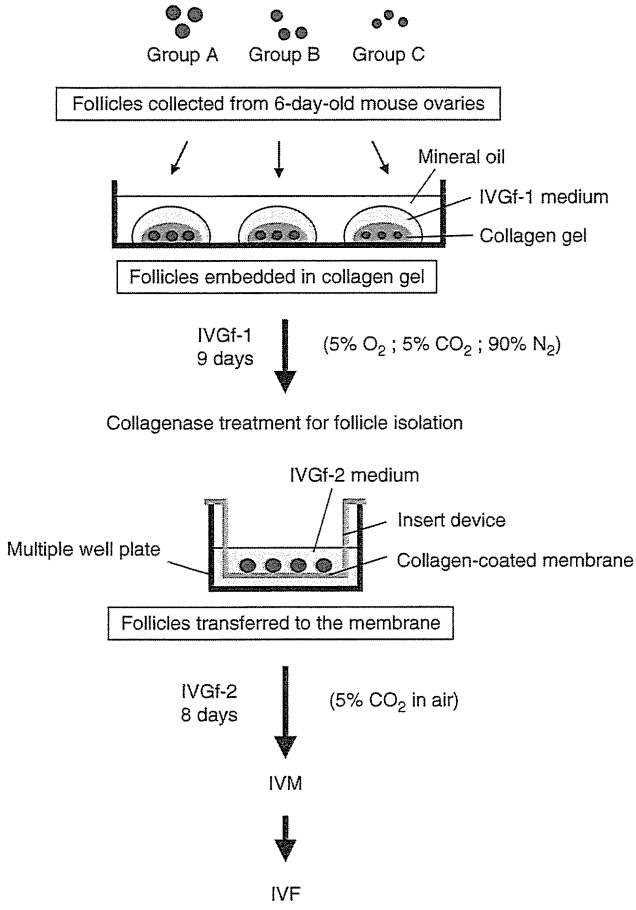

Figure 2 Schematic illustrating the two-step culture method. Intact follicles were mechanically isolated from 6-day-old mouse ovaries and classified into three groups, groups A, B, and C, based on their diameters. Group A: 80–95 μm, group B: 70–80 μm, and group C: 60–70 μm. Ten follicles in each group 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

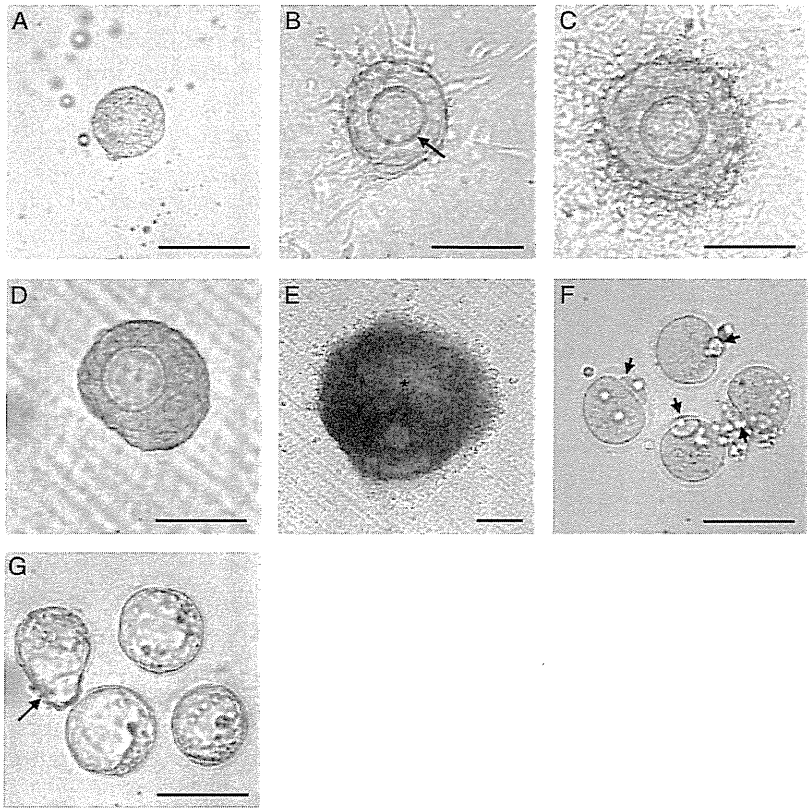

Figure 3 Morphological observation of follicle growth and embryo development in *in vitro* growth of follicles-1 (IVGf-1), *in vitro* growth of follicles-2 (IVGf-2), and *in vitro* maturation (IVM) followed by IVF. (A, B, and C) Follicle development from 6-day-old mouse ovaries during 9 days in IVGf-1. (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.

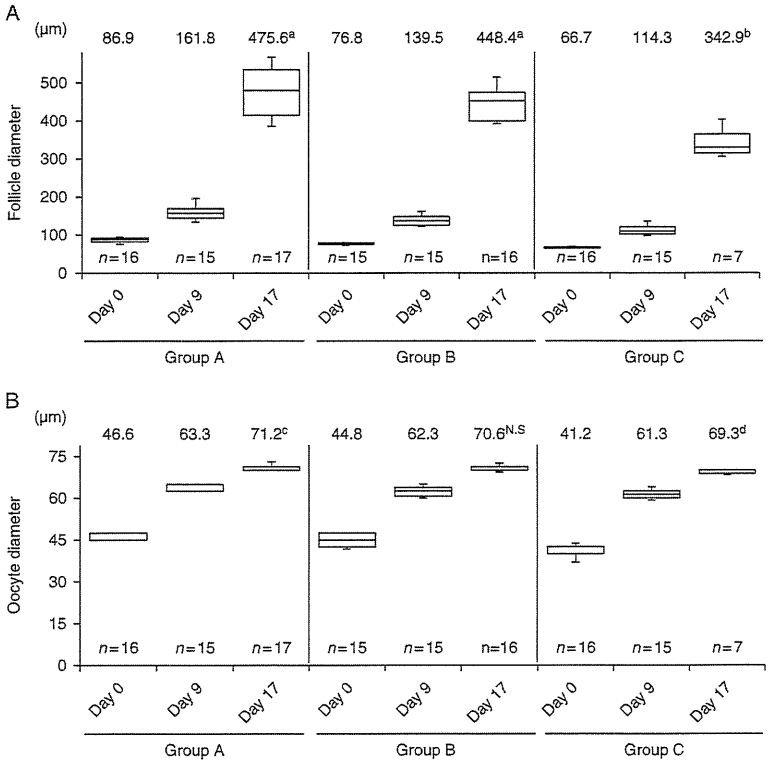

Figure 4 Increase in follicle and oocyte diameters in the series of two-step culture system. *In vitro* growth culture started at day 0 to day 9 (IVGf-1) and subsequent culture was performed from day 10 to day 17 (IVGf-2). (A) Follicle diameter. (B) Oocyte diameter. Box-and-whiskers plot demonstrating 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.

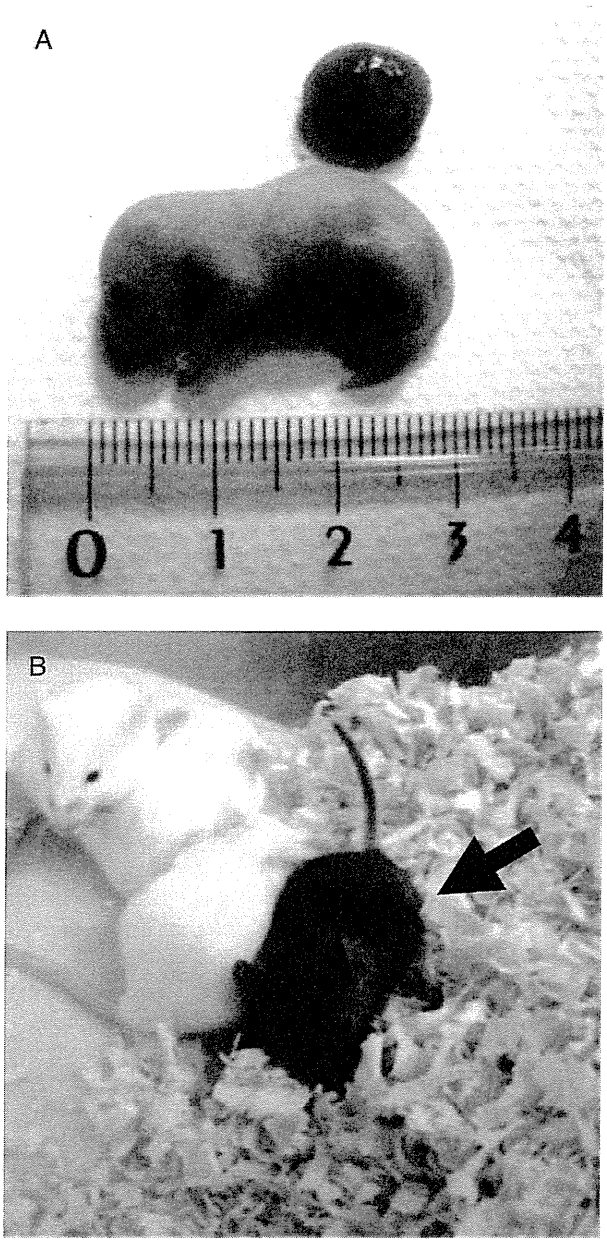

Figure 5 Live offspring derived from primary/early secondary follicles cultured by the series of two-step *in vitro* growth of follicles (IVGf), *in vitro* maturation (IVM), IVF followed by embryo transfer.
(A) Live offspring delivered by Cesarean section and its placenta.
(B) The growing mouse at 15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

A large seminoma occurring 20 years after diagnosis of complete androgen insensitivity syndrome: A case report ☆

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Introduction

Complete androgen insensitivity syndrome (AIS) is a rare X-linked disease with an estimated prevalence of 1 in 20,000 and is characterized by a 46,XY karyotype and a female external phenotype (Wisniewski et al., 2000). AIS is caused by mutations in the androgen receptor gene, located on chromosome Xq11-12, which results in impaired embryonic sexual differentiation. The risk of malignancy is considerably lower in complete AIS than in partial AIS or other intersex disorders and occurs at a later age (Cheikhelard et al., 2008). Morris reported a 22% incidence of malignant gonadal tumors in patients over 30 years of age (Morris, 1953); however, because 50% of the patients studied had already undergone a previous gonadectomy, the risk of malignancy was underestimated. Current recommendations for patients with AIS suggest that cryptorchid testes be retained through puberty in order to receive the benefits from their hormone production, enhance bone maturation, and allow completion of secondary sexual development (Alvarez et al., 2005). Currently, limited data is available on individuals who have not had their testes removed (Deans et al., 2012). We report a case of a seminoma that developed in the testes 20 years after diagnosis of AIS.

Case report

This report presents the case of a 36-year-old female who, at the age of 16, presented at a gynecologic clinic with primary amenorrhea. After thorough examination, the patient was diagnosed with AIS and a 46,XY karyotype but either did not return to the clinic or was lost to follow-up. Twenty years later, the same patient presented to our hospital with abdominal swelling and a large abdominal mass. The patient was 156 cm in height, 76 kg in weight, and of a female external phenotype with well-developed breasts (Tanner IV), a blind-ending vagina, and no axillary or pubic hair. A tumor the size of 23 cm in diameter was detected in the abdomen by palpation and was determined to be solid upon examination by computed tomography (CT) and magnetic resonance imaging (MRI) images (Fig. 1A). Results of laboratory tests showed elevated serum levels of cancer antigen (CA)-125 (325 IU/mL), lactate dehydrogenase (LD; 485 IU/mL), and beta-human chorionic gonadotropin (beta-hCG; 8.5 ng/mL). Serum levels of alpha-fetoprotein, testosterone, free testosterone, estradiol, luteinizing hormone, and follicle-stimulating hormone were 2.1 ng/mL, 3.9 ng/mL, 7.8 pg/mL, 33 pg/mL, 13.3 mIU/mL, and 10.6 mIU/mL, respectively. Abdominal biopsy of a tumor on the left ovary showed that the tumor was a seminoma (Fig. 2A). Because of the size of the tumor, 3 courses of neoadjuvant chemotherapy (NAC) (BEP: bleomycin [20 mg/m^2] on days 1, 8, and 15; etoposide [100 mg/m^2] on days 1–5; and cisplatin [20 mg/m^2] on days 1–5, repeated every 3 weeks) were administered. Following NAC, the tumor shrank to the size of 3.5 cm in diameter (Fig. 1B), and serum levels of CA-125, LD, and beta-hCG decreased to within the normal levels. Abdominal surgery was performed to remove the tumors derived from the left and right gonads (Fig. 3), and pathological examination revealed that no residual tumor remained in the obtained specimen (Fig. 2B). The serum levels of testosterone, free testosterone, and estradiol were maintained after NAC but declined to 0.25 ng/mL, 0.9 pg/mL, and 15 pg/mL, respectively, after the removal of the right and left gonads. Genetic analysis determined that the patient had a de novo 2-base deletion in the androgen receptor gene, which introduced a stop codon at position 500 of exon 1. The patient was treated with estrogen and had no evidence of recurrence in the 52 months following surgery.

Discussion

Complete AIS is typically diagnosed at puberty after an individual presents with primary amenorrhea or an inguinal hernia. Several long-term

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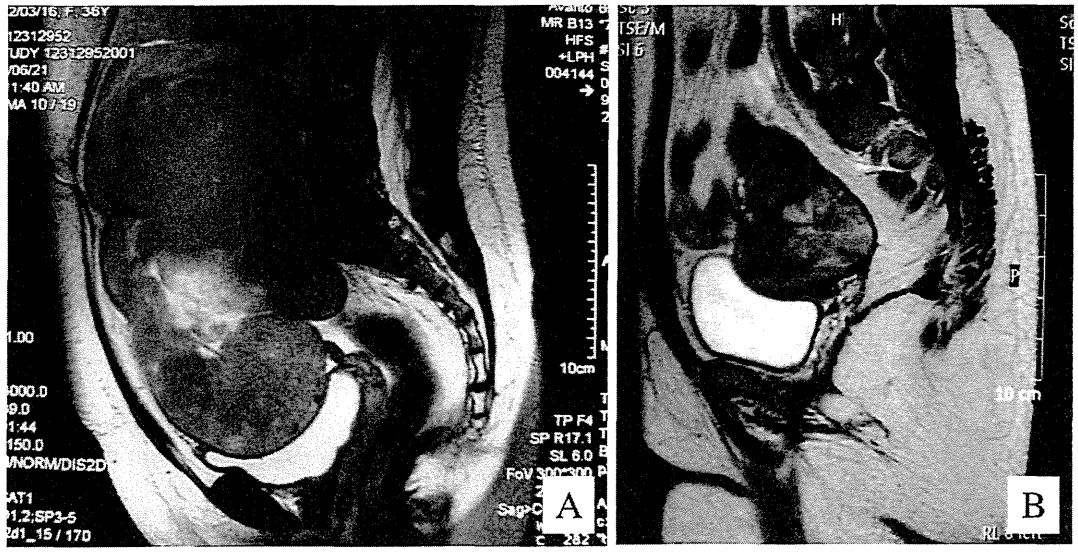

Fig. 1. Magnetic resonance imaging of a tumor developing from the left testis in a patient with androgen insensitivity syndrome. (A) Before chemotherapy, a T2-weighted image showed that the internal intensity was slightly high. (B) Marked shrinkage of the tumor occurred after 3 cycles of neoadjuvant chemotherapy (NAC).

follow-up studies have shown that in women with complete AIS, gonadectomy can be delayed until completion of sexual maturation because puberty will cause the spontaneous conversion of testosterone to estradiol (Cheikhelard et al., 2008; Chantilis et al., 1994). Although there are no standard or reliable screening tools for early detection of malignant changes in patients with AIS, routine ultrasound examination is usually conducted. Once puberty is complete, prophylactic gonadectomy is recommended (Purves et al., 2008), and continual estrogen replacement is required for maintaining bone health and general good health.

For patients who exhibit malignant transformation, adjuvant therapy must be considered. If the tumor is contained within the testis, the standard adjuvant treatment is postoperative irradiation of the paraaortic lymph nodes with or without irradiation of the ipsilateral pelvic lymph nodes. Because the present patient had such a large tumor, primary en bloc resection was thought to be too harmful to the surrounding organs (Rescorla et al., 2003). A pathologically complete response was observed after 3 cycles of NAC with BEP.

In most cases, complete AIS is an X-linked recessive disease. However, the present patient had a de novo mutation of the androgen receptor gene; her mother was not a carrier of this mutation. When the patient first visited a clinic, she and her family did not receive adequate genetic or psychological counseling. Neither close follow-up nor prophylactic gonadectomy was conducted, and the patient exhibited malignant transformation. Although the patient survived without recurrence at 52 months after surgery, this case reaffirms the importance of appropriate genetic counseling for patients with AIS (Purves et al., 2008).

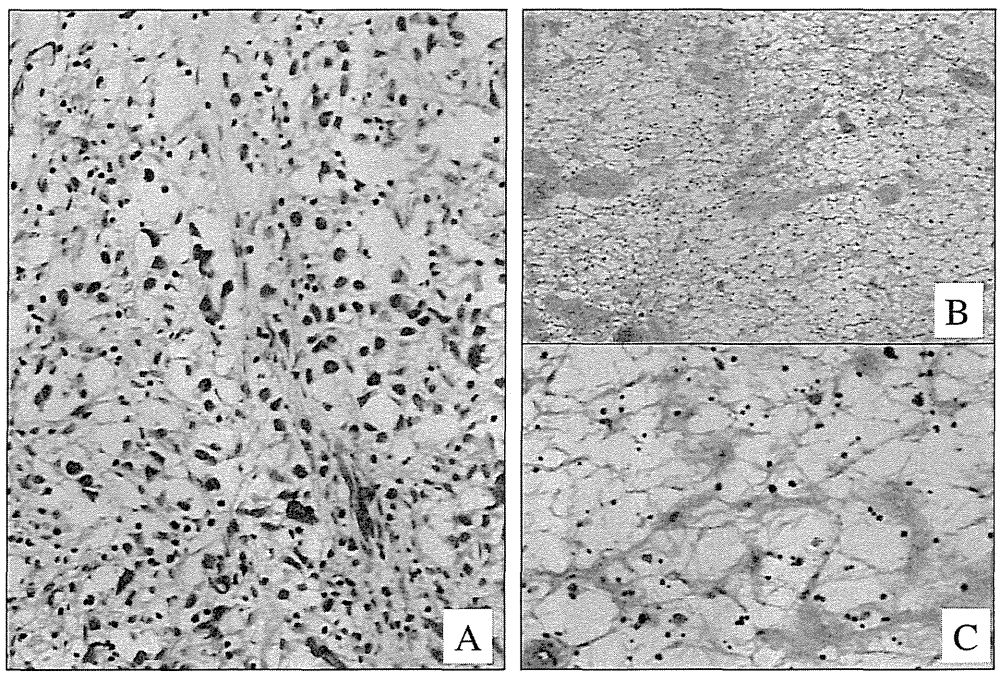

Fig. 2. (A) A biopsy specimen obtained before chemotherapy showed evenly spaced and relatively large uniform tumor cells with distinct cell borders (magnification × 200). (B and C) Infiltration of lymphocytes was noticed, but tumor cells were not seen after NAC (B, magnification × 40; C, magnification × 200).

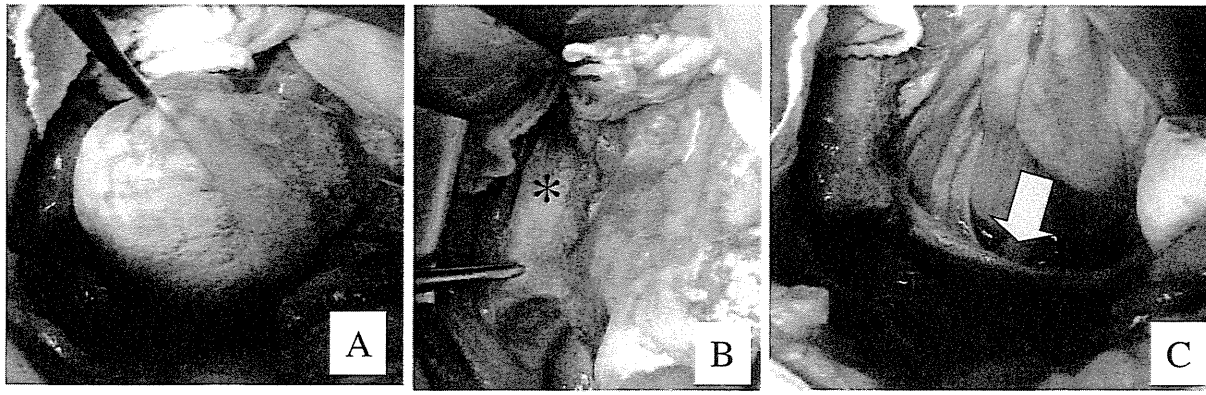

Fig. 3. Laparotomy after NAC. (A) The tumor shrank following NAC. (B) The right testis is indicated by an asterisk. (C) The uterine streak is indicated by arrows.

Conflict of interest statement

The authors have no financial conflicts of interest to disclose.

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特集　遺伝子検査による早期診断

疾患

骨系統疾患

澤井 英明

骨系統疾患とは

骨系統疾患とは，軟骨・骨の発生・成長の異常により骨格の形態や構造に系統的な異常をきたす疾患である[1]。骨系統疾患はInternational Skeletal Dysplasia Societyにより定期的に分類と疾患の見直しが行われており，2010年の最新版では，40グループ456疾患にわたっている[2]。なお国際分類2010年版の疾患名の和訳(日本語の疾患名を決定)した論文が日本整形外科学会から報告されている[3]。この最新の和訳において，従来の致死性骨異形成症がタナトフォリック骨異形成症に，また窒息性胸郭異形成症が呼吸不全性胸郭異形成症に疾患名が改められた。骨系統疾患は，疾患頻度は数万人に1人程度と少なくても，疾患数が多いため，出生1万人中2～3人の頻度であると推定されている[4]。従来の分類は骨変化の臨床所見，特にX線診断に基づく類似性をもとに疾患グループとしてまとめられてきたが，近年の分子遺伝学的研究の進歩により，原因遺伝子が次々と判明したことで，疾患グループは原因遺伝子に基づいて再編されつつある。そのため従来は同一疾患グループの亜型とされていたものが，全く異なる原因遺伝子であることが判明して，疾患によっては病名としては同一の疾患の亜型が別疾患グループに分類されるという状況も生じている。

遺伝子診断の対象疾患と実施可能性

各疾患の遺伝子変異が同定されているかどうかは，2010年国際分類に示されている[2,3]。これらのうち日常的に遭遇する可能性のある疾患とその原因遺伝子を表に示す。しかし，遺伝子変異が報告されていて，それが原因であるとしても，臨床的に遺伝子診断が可能かどうかは一概にいえない。その判断の根拠の一つは児の臨床情報がどの程度得られるかであり，診断しようとする時期が出生前か出生後かによっても異なる。一般には出生前に児の臨床症状を詳しく入手することが困難であるため，直接児を観察できる出生後の遺伝子診断に比べて実施できる疾患に制限が多い。また原因遺伝子の変異が特定の部位に集中しているかどうかも重要で，軟骨無形成症のようにFGFR3遺伝子の特定の部位に変異が集中している場合は比較的容易であるが，骨形成不全症のようにⅠ型コラーゲンが原因ではあっても，特定の部位に変異が集中していない場合には，遺伝子の全領域を検査する必要があり，前者よりも難しくなる。

出生前遺伝子診断[5]

1．家系内に特定の遺伝性の骨系統疾患があり，妊娠前または妊娠初期から，夫婦が出生前診断を希望している場合

例えば夫婦のいずれかが軟骨無形成症や骨形成不全症の患者であるようなケースや前回妊娠で低フォスファターゼ症の児を出産して夫婦がいずれも保因者であるとわかっているケースである。こうした場合は，事前に罹患者や保因者の遺伝子変異が同定されていれば，実施可能である。ただし，出生前診断は羊水や絨毛が検体となるため，成人の遺伝子検査が可能であっても出生前診断が可能であるとは限らない。実施の可能性については，

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表　代表的な骨系統疾患と原因遺伝子

疾患グループ名 疾患名	遺伝形式	原因遺伝子(略語)
1．FGFR3軟骨異形成症グループ(FGFR3 chondrodysplasia group)		
タナトフォリック骨異形成症(Thanatophoric dysplasia type 1：TD1)	AD	Fibroblast growth factor receptor 3 (FGFR3)
タナトフォリック骨異形成症(Thanatophoric dysplasia type 2：TD2)	AD	同上
軟骨無形成症(Achondroplasia)	AD	同上
軟骨低形成症(Hypochondroplasia)	AD	同上
2．2型コラーゲングループ(Type 2 collagen group)		
軟骨無発生症2型(Achondrogenesis type 2：ACG2)	AD	Type 2 collagen (COL2A1)
軟骨低発生症(Hypochondrogenesis)	AD	同上
先天性脊椎骨端異形成症(Spondyloepiphyseal dysplasia congenita)	AD	同上
Kniest骨形成症(Kniest dysplasia)	AD	同上
4．硫酸化障害グループ(Sulphation disorder group)		
軟骨無発生症1B型(Achondrogenesis type 1b：ACG1B)	AR	SLC26A2 sulfate transporter
捻曲性骨異形成症(Diastrophic dysplasia)	AR	同上
5．Perlecanグループ(Perlecan group)		
分節異常骨異形成症(Dyssegmental dysplasia)	AR	PLC (HSPG2)
9．短肋骨異形成症(多指を伴う/伴わない)グループ [Short-rib dysplasia (with or without polydactyly) group]		
軟骨外胚葉性異形成症(Ellis-van Creveld)	AR	EVC1 or EVC2
短肋骨多指症候群1/3型(Short-rib polydactyly syndrome：SRP)	AR	DYNC2H1 or IFT80 or不明
呼吸不全性胸郭異形成症(Asphyxiating thoracic dysplasia：ATD)	AR	同上
18．彎曲骨異形成症(Bent bone dysplasias)		
屈曲肢異形成症(Campomelic dysplasia)	AD	SOX9
21．点状軟骨異形成症(CDP)グループ		
点状軟骨異形成症，X染色体優性(Conradi-Hünermann型：CDPX2)	XLD	Emopamil-binding protein (EBP)
点状軟骨異形成症，X染色体劣性，末節骨短縮型(CDPX1)	XLR	Arylsulfatase E (ARSE)
25．骨形成不全症グループ(Osteogenesis imprefecta group)		
骨形成不全症，非変形型(Osteogenesis imperfect：OI 1型)	AD	COL1A1，COL1A2
骨形成不全症，周産期致死型(OI 2型)	AD，AR	COL1A1，COL1A2，CRTAP，LEPRE1，PPIB
骨形成不全症，変形進行型(OI 3型)	AD，AR	COL1A1，COL1A2，CRTAP，LEPRE1，PPIB，FKBP10，SERPINH1
骨形成不全症，中等症型(OI 4型)	AD，AR	COL1A1，COL1A2，CRTAP，FKBP10，SP7
【COL1A1：Collagen 1 alpha-1 chain, COL1A2：Collagen 1 alpha-2 chain, CRTAP：Cartilage-Associated Protein, LEPRE1：leucine proline-enriched proteoglycan (leprecan) 1, PPIB：peptidylprolyl isomerase B (cyclophilin B), FKBP10：FK506 binding protein 10, SERPINH：serpin peptidase inhibitor clade H1, SP7：SP7 transctiption factor (Osterix)】		
26．異常骨石灰化グループ		
低フォスファターゼ症，周産期致死型・乳児型 (Hypophosphatasia, Perinetal lethal and infantile forms)	AR	Alkaline phosphatase, tissue non-specific (TNSALP)
低フォスファターゼ症，成人型(Hypophosptatasia, Adult forms)	AD	同上

各グループの先頭の数字は，2010年国際分類のグループ番号を記載している。
AD：常染色体優性遺伝，AR：常染色体劣性遺伝，XLR：常染色体劣性遺伝，XLD：常染色体優性遺伝
重症骨系統疾患ではADであっても，事実上は突然変異による発症に限られる。