Introduction

Changes in the gravitational field have significant effects on the development of plants and animals [1]. Therefore, the potential effect of a microgravity (μG) environment on reproduction has been a major biological theme in the age of space exploration. So far, several experiments on reproduction in such environments have been reported using sea urchins, fish, amphibians or birds. Here, we studied the possibility of mammalian fertilization and preimplantation development under microgravity (μG) conditions using a three-dimensional (3D) clinostat, which faithfully simulates $10^{-3}$ G using 3D rotation. Fertilization occurred normally in vitro under μG. However, although we obtained 75 healthy offspring from μG-fertilized and -cultured embryos after transfer to recipient females, the birth rate was lower than among the 1G controls. Immunostaining demonstrated that in vitro culture under μG caused slower development and fewer trophectoderm cells than in 1G controls but did not affect polarization of the blastocyst. These results suggest for the first time that fertilization can occur normally under μG environment in a mammal, but normal preimplantation embryo development might require 1G.
gametes for IVF in space, but oocyte freezing is still imperfect [19]. Moreover, IVF is a difficult biological procedure, and fertilization can fail even in a ground-based laboratory if the experimentalist is inexperienced.

Methods for simulating μG on Earth have been investigated as alternatives to expensive space flight. A microgravity condition can be produced either by a space flight or by a free fall. However, the duration of a microgravity condition produced by a free fall is usually too short to alter cell growth and differentiation. Because of limited access to space flight, many efforts have been made to establish alternative methods for simulating microgravity on Earth, and one of these—the ‘clinostat’—is considered to be a suitable device. The clinostat mimics μG by ‘nullifying the gravitational vector’ through continuous averaging of G forces using three-dimensional (3D) rotation [see Movie S1] [20]. Moreover, this device can subject the specimen to μG conditions immediately, without exposure to the hypergravity and strong vibrations of a space launch. This allows the investigator to test the effects of μG conditions in isolation from other physical factors.

In earlier studies, the clinostat was rotated only in a single dimension (1D), such as horizontal rotation [21] or vertical rotation [22,23], which generated extra centrifugal forces and made it difficult to nullify the gravitation vector fully. Although the USA’s National Aeronautics and Space Administration (NASA) has improved this system [24], the results of some papers have been contradictory [22,25]. Recently, a 3D clinostat has been developed to generate a multidirectional G force, resulting in a more accurate environment averaging 10⁻³ G [25–27], which is the same as inside a space shuttle (Movie S1). Several studies of cultured cells have been published using a 3D clinostat [28], and the results were similar to true space experiments [29], which validated its effectiveness for simulation [30]. Therefore, 3D clinostat experiments will help in predicting and planning real space experiments, which can reduce costs and effort.

Interestingly, one recent study discovered that the μG condition generated by a 3D clinostat inhibits the differentiation of stem cells [25]. Thus, a 3D clinostat might have clinical applicability in regenerative medicine to help proliferate stem cells for patients. However, if this effect is also true for undifferentiated early embryos, they might fail to differentiate or develop to offspring.

In the present study, we examined in vitro fertilization and preimplantation development in the mouse under μG using a 3D clinostat. To evaluate the embryos, some were examined for quality and cell numbers by immunostaining, and others were transferred into pseudopregnant recipient females to test their potential for development to full term.

Results

Effects of μG on in vitro fertilization (IVF)

At 6 h after IVF, the clinostat was stopped, and zygotes were collected from the culture flasks. Control studies at 1G were performed using the same batch of preincubated spermatozoa and the same incubator but outside the clinostat (Fig. 1a, arrow). The fertilization rate, judged by second polar body extrusion and the presence of 2 PN: similar to 1G controls (80%; Table 1). Therefore, μG appears to have no harmful effects on mouse gametes—at least in terms of fertilization in vitro.

Effects of μG on development in vivo

To demonstrate the normality of μG-fertilized and cultured embryos, the strongest evidence is to show the potential to develop to full-term offspring. Therefore, we transferred μG-generated embryos at either the 2-cell stage (24 h culture) or the blastocyst stage (96 h culture) to oviduct of 0.5 dpc or uteri of 2.5 dpc recipient pseudopregnant females, respectively. In this experiment, to mimic the μG condition exactly, embryos were cultured continuously in the clinostat from IVF to the time of embryo transfer. Therefore, we could not determine the exact fertilization or embryo development rates in each flask. However, control 1G experiments were performed under exactly the same conditions (Fig. 1a) except that they were outside the clinostat.

As shown in Table 2, when embryos were collected from μG-cultured flasks at 24 h, more than half of the embryos had developed to the 2-cell stage (Fig. 2c), without any difference from controls. However, after transfer to recipient females, the rate of production of offspring from μG-cultured embryos (35%) was significantly less than with the 1G controls (63%). When embryos were collected from the μG culture flask at 96 h, the rate of development to the blastocyst stage (30%; Fig. 2d) was also significantly lower than control cultures (57%). It should be noted that these control results are poorer than with conventional IVF methodology [16–18,33–37]. As discussed above, the use of a flask and a large volume of medium was essential in using the 3D clinostat system, and our preliminary experiments showed that these had negative effects on embryo development. However, the μG experiments and control studies were done at exactly the same time and under the same conditions, except for the use of the 3D clinostat (Fig. 1a, arrow). Therefore, we believe that our results are valid.

After transferring blastocysts to recipient females (see Table 3), the rate of producing live offspring from μG-cultured embryos (16%; Fig. 2c) was also significantly lower than with the 1G controls (37%). Although fewer offspring resulted from μG culture than in the 1G controls, their body and placental weights were within normal range, and the mice grew to adulthood. Three male and three female mice derived from the μG-cultured blastocyst transfers were selected at random and paired for mating. All pairs delivered litters a few months later (Fig. 2f), demonstrating that the fertility of μG-generated offspring was normal.

Effect of μG for cell differentiation and polarization of blastocyst

As shown in Table 2 and Table 3, 96 h culture in μG conditions caused a reduced rate of development to the blastocyst stage, as well as reduced full-term development following transfer. To examine the reason for this low rate of development in μG conditions, we examined the quality of blastocysts based on cell number (Table 4), cell differentiation and polarity, using immunostaining (Fig. 3). In mice, the transcription factor Oct4 is expressed throughout preimplantation development and is restricted to the inner cell mass (ICM) at the blastocyst stage [38–43]. Oct4 is required for the maintenance of ICM fate and the pluripotency of ES cells. Trophoderm (TE) differentiation begins before the downregulation of Oct4 in the outer cells and is marked by Cdx2 expression. Therefore, only Cdx2-positive cells were judged as differentiated, and only Oct4 positive cells were judged as undifferentiated. Oct4/Cdx2 double-positive cells were
judged as transition stage from undifferentiated to differentiated. Nonreactive cells that were DAPI positive (nuclear staining) were judged to be in metaphase.

We examined 21 of the μG-cultured blastocysts and 26 control 1G blastocysts. The mean ICM cell number in μG blastocysts (8.0 cells) was almost same as in the 1G controls (8.7 cells; Fig. 3d, i; red). However, downregulation of Oct4 was incomplete in the μG blastocysts, and the numbers of cells that were doubly positive for Oct4 and Cdx2 were increased significantly (Fig. 3d, i; yellow). There were fewer completely differentiated TE cells in μG-cultured blastocysts (5.9 cells) than in 1G controls (17.0 cells) (Fig. 3d, i; green). In addition to cell number, we also observed the localization of the ICM in blastocysts using a 3D imaging system (Fig. 3k and 1 and Movie S2 and S3). If ground-level gravity is essential for polarity in the blastocyst, the localization of the ICM in μG-cultured blastocysts might be impaired, and this could allow Oct4-positive cells to disperse into the blastocoel. However, the ICM was located normally in all the μG-cultured blastocysts. These results suggest that μG culture conditions impaired both the rate of embryo growth to the blastocyst and differentiation into the TE lineage. Nevertheless, polarization of the ICM and TE components was unaffected.

Discussion

In this study, we produced healthy offspring from embryos produced under μG conditions including sperm preincubation, IVF and in vitro culture to the blastocyst stage. IVF occurred
normally under μG conditions. However, the quality of blastocysts grown under μG conditions was reduced.

The production of healthy offspring is the strongest evidence of the normality of μG-fertilized embryos. At 24 h after IVF, although live offspring were born after embryo transfer into recipient females, the overall rate of production from the μG embryos was significantly lower than in the 1G controls (p<0.05). Moreover, when culture periods were extended to 96 h in μG, the live birth rate was significantly lower than in controls (5% vs. 21%). Kojima et al. reported similar results using a 1D clinostat [44]. However, they showed no difference in offspring production rates between μG and 1G. Possibly this reflects differences between the 1D and 3D clinostat...
environments. Another possibility for the discrepancy is that they transferred zygotes (6 h after IVF) into recipient females, whereas we transferred 2-cell embryos (24 h after IVF), which reinforces the idea that prolonged μG culture impairs embryo quality.

These results strongly suggest that although fertilization had occurred normally, μG conditions had a harmful effect on embryo development even after one day of exposure. If we could remove the negative effects of a large volume of medium, for example by using a completely self-contained microfluidics chamber, this could facilitate study on the real effects of μG.

In this study, 61 and 14 pups were obtained from embryos cultured for 24 h or 96 h under μG conditions, respectively. All these offspring appeared normal, and randomly selected animals were later proven fertile by natural mating. This suggests that, although embryo growth was impaired under μG conditions, some of the treated embryos maintained a normal potential for development. However, obviously the recipient females could not be kept in the clinostat, so implantation and gestation in vivo was at 1G. The μG-fertilized and -cultured embryos would thus have spent about 2 days (2-cell embryo transfer) or half a day (blastocyst transfer) in female body under 1G conditions before implantation. It is possible that even if all the μG-fertilized and -cultured embryos were abnormal, this was corrected in some embryos before implantation.

### Table 1. In vitro fertilization of mouse gametes under microgravity.

| Category | Collected oocytes | Fertilized (total) | Parthenogenetic (%) | Polyspermic (%) | Normal (%) |
|----------|------------------|--------------------|---------------------|-----------------|------------|
| 1G       | 203              | 184                | 33 (18)             | 2 (1)           | 149 (81)   |
| μG       | 229              | 207                | 8 (4)               | 25 (12)         | 174 (84)   |
| p-Value  | >0.01            | >0.01              | <0.01               |                 |            |

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### Table 2. In vitro development of embryos under microgravity.

| Culture period | Category | p-Value | Collected embryos | In vitro development (%) |
|----------------|----------|---------|-------------------|--------------------------|
|                |          |         |                   | No. abnormal | No. 2-cell | No. blastocysts |
| 24 h           | 1G       | <0.05   | 51                | 24 | 27 (53) | – |
|                | μG       |         | 494               | 230 | 264 (53) | – |
| 96 h           | 1G       | >0.05   | 42                | 18 | – | 24 (57) |
|                | μG       |         | 282               | 196 | – | 86 (30) |

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### Table 3. Production of offspring from embryos fertilized and cultured under microgravity.

| Culture period | Category | p-Value | Embryo transfer | No. pups (%) | Weight (g)±SD |
|----------------|----------|---------|-----------------|--------------|----------------|
|                |          |         |                 |              | Pups | Placenta |
| 24 h (2-cell)  | 1G       | >0.05   | 27              | 17 (63)      | 1.49±0.13 | 0.11±0.02 |
|                | μG       |         | 172             | 61 (35)      | 1.40±0.11 | 0.11±0.02 |
| 96 h (Blast)   | 1G       | >0.05   | 24              | 9 (38)       | 1.46±0.15 | 0.13±0.02 |
|                | μG       |         | 86              | 14 (16)      | 1.41±0.12 | 0.12±0.03 |

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### Table 4. Cell numbers and populations in microgravity-fertilized and cultured blastocysts.

| Category | Total cell number | No. TE cells (%) | No. transition (%) | No. ICM cells (%) | No. M phase cells (%) |
|----------|------------------|------------------|-------------------|------------------|-----------------------|
|          |                  | (Cdx2 positive)  | (double positive) | (Oct4 positive)  | (DAPI only)           |
| 1G       | 32.0±10.4        | 17.0±10.6 (53)   | 3.5±2.7 (11)      | 8.7±3.4 (27)     | 2.8±2.0 (9)           |
| μG       | 22.3±9.3         | 5.9±5.6 (26)     | 5.3±2.5 (24)      | 8.0±3.0 (36)     | 3.2±3.2 (14)          |
| p-Value  | >0.05            | >0.05            | >0.05             | <0.05            | <0.05                 |

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Figure 3. Blastocyst quality after culture under microgravity. The trophectoderm (TE) and inner cell mass (ICM) cell numbers were counted by immunostaining for Cdx2 (a and f, green) or Oct4 (b and g, red), respectively. DNA was stained with DAPI (c and h, blue). Cdx2/Oct4 doubly positive cells were counted on merged images (d and i). Metaphase cells (DAPI staining only) were counted on triple-merged images (e and j). The ICM localization was examined using a three-dimensional (3D) viewer (k and l, and Movie S2 and S3). As we acquired 51 focal planes in the z-axis, we could determine the 3D structure of all embryos. (m) Rates of development to the 2-cell and blastocyst stages in 1G and μG culture systems. (n) Cell numbers and cell types compared between 1G- and μG-cultured blastocysts.

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To test the effect of prolonged μG culture, we examined the quality of blastocysts by immunostaining at 96 h after IVF. The TE cell numbers in μG-cultured blastocysts were significantly lower than in the 1G controls, whereas the numbers of other cell types were unaffected (Fig. 3n). There are two possible explanations. One is that μG conditions slow embryo growth rates, another is that they affect the potential for cell differentiation. It was reported that the μG conditions generated by a 3D clinostat inhibited stem cell differentiation [25]. However, it is unclear whether this also affects mammalian embryos. However, if so, μG conditions appear to inhibit the differentiation of TE cells from totipotent stemores, rather than slowing embryo growth. On the other hand, the localization of the ICM in μG-cultured blastocysts appeared normal. In amphibians, in which the zygotes show strong polarity during early cell division, μG had no harmful effect on embryo development [45]. Thus, the effects of μG might be limited to embryonic cell growth rate or differentiation, but μG does not appear to impair cell localization within the blastocyst. In other words, the polarization of preimplantation embryos was independent of gravity in this model.

Unlike studies on early development, far more space research has focused on the latter half of pregnancy, during which many of the early, sensitive phases of development including placentation and early organogenesis normally occur at 1G. Thus far, female rats at several different stages of pregnancy have been launched into space. These data provide clear evidence that rats kept in space at several different stages of pregnancy have been launched into space.

In the conventional IVF protocol at 1G, mouse sperm are capacitated in IVF medium (TYH; Mitsubishi Kagaku, Tokyo) [16], insemination was performed in embryo culture medium (CZB) [53] rather than TYH medium, because the zygotes needed to be cultured continuously under μG conditions without a wash step. However, the fertilization rate with this system was almost the same as with conventional IVF media (91% and 88%, respectively; repeated three times). This result suggests that IVF can be achieved without any problem in embryo culture medium rather than IVF medium, and that the large amount of medium in the μG flask had no effect on this outcome.

In terms of extended culture, we have compared the rate of blastocyst development in the flask culture method with the standard protocol outlined above. At 96 h after IVF, the rate of blastocyst development was significantly decreased in flask cultures compared with routine dish cultures (63% vs 97%, respectively; repeated three times). Although a low embryo-to-culture volume ratio is critical for the optimal development of embryos in vitro [54–56] it would be impossible to produce sufficient numbers of embryos to compensate for the 1000-fold greater volume of flasks compared with microdrops in dishes. This suggests that a decrease in the rate of blastocyst development is inevitable when using a large flask. However, even if the large volume of medium affects embryo development, in this study the 1G controls and μG experiments were performed in exactly the same conditions, except for the use of the 3D clinostat (see Fig. 1 arrow). Therefore, this appropriate control enabled us to identify the exact effects of μG during IVF and embryo development.

In terms of embryo transfer, in the standard protocol, in vitro-produced/manipulated embryos are transferred into 1-day-delayed pseudopregnant females to compensate for the timing of development. Therefore, 2-cell-stage embryos at 24 h after insemination (day 2), or morulae/blastocysts at 72 h after insemination (day 4) are normally transferred into the oviduct (day 1) or uterus (day 3), respectively. However, we noticed that when embryos were cultured in flasks, blastocyst development was retarded and the embryos required 5 days to develop to the expanded stage. In addition, it was known that the developmental potential of offspring was unaltered when embryos were cultured in vitro for 4 or 5 days. However, when embryos were cultured for 6 days, the implantation rates of transferred embryos decreased significantly [57–59]. Therefore, in this study, 2-cell embryos were transferred into 1-day-delayed recipient females as usual. However, day 5 blastocysts were transferred into 2-day-delayed (day 3) recipient females. Although the rate of producing offspring after embryo transfer was lower than with the ordinary method (29% vs 57%, respectively; repeated three times), this modified method was entirely reproducible.
Sperm and oocyte preparation

Mouse IVF medium (TYH) was added to the flask one day before experiments and equilibrated in a 37°C, 5% CO₂ incubator. Next day, epididymides were collected from two euthanized male mice. A dense sperm mass was squeezed out from the cut epididymis and placed into the flask. TYH medium was added to fill the flask without any bubbles. The flask was capped and placed in the 3D clinostat, which was then started. During sperm preincubation, oocyte cumulus complex (COCs) were collected from the oviducts of two or three females, and transferred into a Falcon flask filled with CZB medium that had been equilibrated for one day before use and then placed in the incubator until starting the experiment. Because COCs were used to enable high fertilization rates, we could not count the number of oocytes in each flask. However, after the experiments, we found that each flask had 60–100 oocytes.

In vitro fertilization

After finish the sperm preincubation, the flasks were removed from the 3D clinostat, and 5 ml aliquots of sperm suspension were introduced into the flasks containing the oocytes. The average final sperm concentration of this method was about 10⁶/ml. CZB medium was added to fill the flask without any bubbles. The 3D clinostat was then started immediately. It took less than 20 min from collecting the sperm suspension to restart the 3D clinostat for IVF. In control experiments, IVF was performed under exactly the same conditions without using the 3D clinostat, and gametes were cultured in the same incubator at the same time (Fig. 1).

Examination of μG-fertilized zygotes

At 6 h after IVF, all oocytes were collected from the flasks and the fertilization rates were estimated, based on second polar body extrusion. Then all zygotes were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 min, washed twice with 1% bovine serum albumin (BSA) in PBS, transferred into 1% BSA–PBS containing 0.1% Triton X-100 (Nacalai Tesque Inc., Kyoto, Japan) and incubated overnight at 4°C. All zygotes were washed, and their DNA was stained with 6-diamidino-2-phenylindole (DAPI) (2 μg/mL; Molecular Probes Inc., Eugene, OR, USA). Some zygotes were incubated with a rabbit polyclonal antibody directed against histo H3 trimethyl K9 (ChIP grade; ab60986, Abcam plc, Cambridge, USA) at room temperature for 2 h before DNA staining. After the embryos had been washed twice in 1% BSA–PBS for 15 min each, they were incubated for 1 h with the fluorochrome-conjugated secondary antibody, Alexa Fluor 488-labeled chicken anti-rabbit IgG (Molecular Probes Inc.). This staining protocol is specific for the female PN. All the embryos were observed using a confocal scanning laser microscope (FV1000, Olympus, Tokyo, Japan).

Embryo transfer

At 24 h or 96 h after IVF, all embryos or oocytes were collected from the flasks. The rates of 2-cell or blastocyst development were calculated from all collected oocytes, including abnormally ovulated ones (these cannot be distinguished from unfertilized oocytes at this stage). Two-cell stage embryos were transferred to oviduct of pseudopregnant ICR mice at 0.5 days post cohabitation (dpc). These had been mated with a vasectomized ICR male the night before transfer. Blastocyst-stage embryos were transferred to the uteri of pseudopregnant mice at 2.5 dpc. Six to 10 embryos were transferred into each oviduct or uterus, respectively [17,33,37]. At days 18.5 to 19.5 dpc, the offspring were delivered naturally or by cesarean section, and we recorded the body and placental weights and sex. All offspring were fostered to other females to allow them to grow to adulthood. When they matured sexually, randomly selected males and females (three each) were paired and mated to confirm their fertility.

Note that the 3D clinostat culture was carried out in Hiroshima, and the animals were maintained in Kobe. Therefore, after finishing each experiment, the 1G control and μG culture flasks were moved from Hiroshima to Kobe, which takes 3 h, but the flasks were kept at 37°C in a warming box. As shown in the 1G control experiments, this short travel did not affect embryo development.

Assessment of blastocyst quality by immunostaining

In preliminary experiments, we found that embryo development was slightly slower than normal in both μG and control 1G conditions when this modified IVF system was used. Therefore, in this study, we examined the quality of blastocysts at day 4.5 of culture (96 h after IVF).

In the 96 h experiments, all the embryos were fixed immediately after stopping the clinostat. The total cell numbers of blastocysts were counted, and localizations of the ICM and TE cells were observed by immunostaining. Briefly, blastocysts were fixed in 4% paraformaldehyde in PBS for 40 min at room temperature, washed twice with PBS containing 0.5% polyvinyl-pyrollidone (Sigma-Aldrich, St Louis, MO, USA) and permeabilized with PBS containing 0.25% Triton X-100 (Nacalai Tesque, Inc.) for 20 min at room temperature (RT). After blocking with 1% BSA/PBS containing 0.1% Tween-20 (Sigma-Aldrich) for 1 h, the blastocysts were further incubated with the primary antibody diluted in the blocking solution at RT for 2 h. The antibodies used were anti-POU5F1/Oct3/4 rabbit polyclonal antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for detecting the ICM, and anti-CDX2 mouse monoclonal antibody (1:200; BioGenex, Inc., San Ramon, CA, USA) for detecting the TE. After the embryos had been washed twice in the blocking solution, they were incubated for 1 h with fluorochrome-conjugated secondary antibodies: Alexa Fluor 488-labeled goat anti-rabbit IgG (Molecular Probes, Inc.) or Alexa Fluor 568-labeled goat anti-mouse IgG (Molecular Probes, Inc.). The embryos were washed again, and their DNA was stained with 4’,6-diamidino-2-phenylindole (DAPI) (2 μg/mL; Molecular Probes, Inc.).

To analyze the embryos three-dimensionally, the embryos were transferred to 5 μl drops of 1% BSA–PBS in a glass-bottomed dish and observed under an inverted fluorescence microscope (IX-71, Olympus) equipped with a Nipkow disk confocal unit [17,37], exposed to three different wavelengths of excitation (405, 488 and 561 nm). Images sectioned optically at 2 μm intervals (a total of 100 μm) were acquired in the z-axis, and three color images (blue, green and red) were captured. Device control and image analysis were performed using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

Outcomes were evaluated using χ² tests, and p<0.05 was regarded as statistically significant.

Supporting Information

Movie S1 3D clinostat inside CO₂ incubator
Found at: doi:10.1371/journal.pone.0006753.s001 (5.38 MB MOV)

Movie S2 The localization of the ICM in blastocysts using a 3D imaging system (Control 1G)
Found at: doi:10.1371/journal.pone.0006753.s002 (9.52 MB MOV)

**Movie S3** The localization of the ICM in blastocysts using a 3D imaging system (µG)
Found at: doi:10.1371/journal.pone.0006753.s003 (8.36 MB MOV)

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**Author Contributions**

Conceived and designed the experiments: SW LY TW. Performed the experiments: SW YK CL KY TW. Analyzed the data: YK LY TW. Contributed reagents/materials/analysis tools: TW. Wrote the paper: LY TW.

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