Mammalian preimplantation development is a process in which the union of two highly specialized gametes endows the resulting zygote with the potential to form undifferentiated cell types. This is followed by cell proliferation and the beginning of differentiation during the brief period leading up to implantation. In these processes, a number of cellular components and structures are regulated spatially and temporally, as seen in repeated cell division, cell cycle progression and genomic reprogramming via global epigenetic modifications [1]. Hence, many researchers have been attracted to these fundamental biological processes. However, the numbers of oocytes and embryos that can be collected are very limited, especially in mammals. Therefore, analysis of molecular mechanisms is hampered because of difficulties in conducting biochemical analyses on sufficient materials. Also, immunostaining methods requiring cell fixation are insufficient for understanding embryonic development because many of the events progress in time-dependent manners and are causally interconnected. Consequently, it is important to develop an experimental system that enables one to obtain live-cell time-lapse imaging, such as using fluorescent proteins [2–4]. Indeed, several studies have reported time-lapse observations of preimplantation development for analyzing the changes in localization of a certain molecule [5], the determinants of embryonic polarity [6–8] and the reprogramming process in cloned embryos [9, 10].

In addition to time-lapse analysis, another benefit of live-cell imaging in the study of preimplantation embryos can be raised. Molecular level phenomena observed in early stages of embryogenesis can be connected directly with further developmental potential by culturing and transferring the embryos to recipients after fluorescence observations, allowing both retrospective and prospective analyses. This point has been underemphasized as an advantage of imaging techniques to date, but this is the most important and innovative aspect in the study of early embryonic development. For example, the growth rates of embryos produced by reproductive technologies such as intracytoplasmic sperm injection (ICSI) and round spermatid injection (ROSI) are not as good as those produced by in vitro fertilization (IVF) [11]. In addition, the success rate of cloning by somatic cell nucleus transfer (SCNT) is extremely low (approximately 5%) [12, 13]. When such resulting embryos are immunostained, some abnormalities including cytogenetic and epigenetic aberrations can be detected, but it is impossible to ascertain the influence of such abnormalities on subsequent development. However, the live-cell imaging approach allows us to link a specific phenomenon observed directly at a certain point to each embryo’s developmental capacity by culturing and transplanting it to a recipient pseudopregnant mother.

We have developed a live-cell imaging technique for mammalian preimplantation embryos [14] in which embryos are microinjected with mRNA encoding fluorescent proteins and then...
observed by time-lapse microscopy. Using this technique, we analyzed global changes in DNA methylation status during preimplantation development in normal and cloned embryos [15, 9]. At that time, as we used a conventional fluorescent microscope, it was impossible to reconstruct the images three-dimensionally, and the embryos were damaged by repeated fluorescent observations. In fact, it is widely believed that embryonic development is hampered by exposure to fluorescent or even room light wave lengths [16, 17]. However, Squirrell et al. [18] reported that they succeeded in obtaining pups after fluorescent imaging of embryos using two-photon laser scanning microscopy in the hamster. Unfortunately, in their report, the imaging period was relatively short (up to 24 h), and it was impossible to construct the three-dimensional structure of embryos because only few images were acquired in the z-axis. Moreover, they failed to obtain pups after imaging using scanning-type confocal microscopy. Ross et al. [19] conducted a similar trial in mouse embryos using a spinning-disk confocal microscope. Although pups were obtained after a ‘snapshot’ image at one time point, they did not succeed in acquiring time-lapse images of preimplantation embryos without damage. In addition, both of these groups used only MitoTracker, a fluorescent agent for mitochondria imaging, as a marker, and so application appears to be restricted. If we could overcome these problems by developing a new live-cell imaging technique for preimplantation embryos, it would become a powerful tool for analysis of phenomena observed in early stages of development and for determining their impact on developmental potential by both retrospective and prospective analyses. In this report, we describe how our imaging technology was improved by modifying the imaging devices and optimizing the conditions for observation. This enabled embryos to withstand, survive and develop to full term normally, even after numerous repeated fluorescence observations following mRNA injection.

Materials and Methods

Animals

BDF1 strain (C57BL/6 × DBA/2) and ICR mice (Japan SLC) were used for preparation of gametes and for recipients, respectively. All animals were maintained in accordance with the Animal Experiment Handbook at the Kobe Center for Developmental Biology (RIKEN).

Gamete collection

Female BDF1 mice (7–12 weeks old) were superovulated by intrauterine injections of 5 IU pregnant mare serum gonadotropin (PMSG) and 5 IU human chorionic gonadotropin (hCG) (Teikoku Zoki, Tokyo, Japan) at 48 h intervals. Cumulus-intact oocytes were recovered 13–15 h after hCG injection. Sperm were collected from the cauda epididymis of BDF1 males (>12 weeks) in 0.2 ml drops of TYH medium [20] and capacitated by incubation for 2 h at 37 C under 5% CO2 in air.

In vitro fertilization (IVF)

Cumulus-intact oocytes were collected in 0.2 ml of TYH medium and inseminated with capacitated sperm (final concentration 100/μl). After 2 h incubation at 37 C under 5% CO2 in air, the cumulus cells were dispersed by brief treatment with hyaluronidase (Type-IS, 150 units/ml, Sigma-Aldrich, St Louis, MO, USA).

Synthesis of mRNA in vitro

After linearization of the template plasmid at the Xba I (EGFP-α-tubulin and H2B-mRFP1) or Xho I (EGFP-β-actin) sites, mRNA was synthesized using RiboMAX™ Large Scale RNA Production Systems-T7 (Promega, Madison, WI, USA). For efficient translation of the fusion proteins in embryos, the 5’ end of each mRNA was capped using Ribo m7G Cap Analog (Promega), according to the manufacturer’s protocol. To circumvent integration of template DNA into the embryo genome, reaction mixtures of in vitro transcription were treated with RQ-1 RNase-free DNase I (Promega). Synthesized RNAs were treated with phenol-chloroform followed by ethanol precipitation. After dissolution in RNase-free water, mRNAs were subjected to gel filtration using a MicroSpin™ G-25 column (Amersham Biosciences, Piscataway, NJ, USA) to remove unreacted substrates and then stored at –80 C until use.

Microinjection of mRNA

Each synthesized mRNA was diluted to an appropriate concentration using Milli-Q ultrapure water (Millipore, Madison, WI, USA), and an aliquot was placed on a micromanipulation chamber. Anaphase II/telephase II oocytes (approximately 2 h after insemination) were transferred to HEPESS-buffered Chatot-Ziomek-Bavister (CZB) medium [21] in the chamber and injected with mRNA using a piezo manipulator with a narrow glass pipette (1–3 μm diameter). Once the mRNA solution had been aspirated into the pipette, piezo pulses were applied to the oocyte to break the zona pellucida and plasma membrane. A few picoliters of solution was introduced into the oocyte, and the pipette was removed quickly. The mRNA-injected embryos were incubated at 37 C under 5% CO2 in air for at least 3 h to translate the mRNA sufficiently for imaging. More than 200 oocytes could be injected in 1 h, and the survival rate after mRNA injection was near 100%.

Live-cell imaging

The embryos were transferred to 5 μl drops of CZB medium on a glass-bottomed dish (12 embryos/drop) and placed in an incubation chamber (Tokai Hit, Shizuoka, Japan) set at 37 C on the microscope stage. A gas mixture of 5% CO2 and 95% air was introduced into the chamber (160 ml/min). An inverted microscope (IX-71; Olympus, Tokyo, Japan) was attached with a Nipkow disk confocal microscope (CSU10; Yokogawa Electric, Tokyo, Japan) [22, 23], electron multiplying charge coupled device (EM-CCD) camera (iXON BV-887; Andor Technology, Belfast, Northern Ireland) and filter wheel and z motor (Mac5000; Ludl Electronic Products, Hawthorne, NY, USA). An argon-krypton laser (CVI Melles Griot, Albuquerque, NM, USA) was used as a light source for the Nipkow disk confocal unit. For fluorescent imaging using a conventional microscope, a mercury or xenon lamp was used. Fifty-one images in the z-axis and two color (green and red) images were captured at regular time intervals. As our imaging device has an auto x-y stage (Sigma Koki, Tokyo, Japan) attached, multiple embryos can be monitored in one assay. A set of
the imaging system is placed in a dark room with the room temperature maintained at 30 °C. Device control and image analysis were performed with the MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). The system is shown in Fig. 2A.

**Embryo transfer**

Morulae and blastocysts were transferred to the oviducts or uteri of Day 0.5 or Day 2.5 pseudopregnant mothers, respectively.

**Plasmid construction**

The plasmids of mRNA for EGFP-α-tubulin [14] and histone H2B-mRFP1 [24] inserted in pcDNA3.1poly(A83) vector were as constructed previously. EGFP-β-actin was generated by fusion of the EGFP with the N-terminal of full-length cDNA encoding mouse β-actin. To isolate the β-actin cDNA, we performed PCR amplification using first-strand cDNAs from the mouse testis as templates and the following primer set: 5’-TGGATCCGGAGCTATGGAATGACGATATCGCTGCGT-3’ and 5’-AAGCGGCCGCTAGAAGCACTTGCGGTGCACGAT-3’. Amplified fragments were co-inserted with EGFP fragments to the EcoRI and NotI sites of the pcDNA3.1-poly(A83) vector.

**Genotyping**

Tail tips of pups were cut off and frozen immediately. They were put into lysis buffer (50 mM Tris/HCl [pH 7.6], 0.1 M NaCl, 20 mM EDTA, 1% SDS, 0.1 mg/ml of proteinase K and 0.08 mg/ml of RNase A) and incubated at 50 °C overnight. Extracted genomic DNA was treated with phenol-chloroform mixture twice and precipitated with ethanol. After dissolution with TE, DNA concentrations were adjusted to 0.3 μg/μl. One microliter of DNA solution was used for each 12.5 μl reaction mixture for PCR analysis (Ex Taq; TaKaRa, Kyoto, Japan). The PCR reaction was carried out using 35 cycles of 95 °C for 10 sec, 58 °C for 30 sec and 72 °C for 60 sec. The primer sets used were 5’-AATCTAGAATGGAGCTGACGGAGGCGAG-3’ and 5’-AATCTAGACTTGACTCGTCCATG-3’ for amplification of the open reading frame of EGFP and 5’-AAAAGCTGGGGCCTTTGACTCAGGA-3’ and 5’-GGAATTCAAGTCAGTGTACAGGCCAG-3’ for amplification of the β-actin gene as a PCR control. Genomic DNAs from B6D2F1 and GOF18-GFP transgenic mice [25] were used as negative and positive controls, respectively.

**Results**

**Impact of the amount of mRNA injected on full-term development**

To determine the optimal amount of mRNA for injection, embryos generated by in vitro fertilization (IVF) were microinjected with various concentrations of mRNAs, incubated until the morula/blastocyst stage and transferred to pseudopregnant females. A mixture of mRNAs encoding enhanced green fluorescent protein coupled with α-tubulin (EGFP-α-tubulin) [14] and monomeric red fluorescent protein 1 (mRFP1) fused with histone H2B (H2B-mRFP1) [24] was used. In contrast to the histone H4 fused with HcRed that we used previously [14], H2B-mRFP1 gave intense fluorescence even at the morula/blastocyst stage (Fig. 1). However, dose-dependent cellular toxicity was observed. When we injected 250 ng/μl of mRNA solution, both the preimplantation and full-term development rates were affected significantly (Table 1), although the maximum fluorescence intensities could be obtained (Fig. 1). In contrast, injections of 10 or 50 ng/μl of mRNA yielded weak signals but were sufficient for fluorescent detection even at later stages of development (Fig. 1) and did not affect full-term development (Table 1). Therefore, the concentration of 10 ng/μl (5 ng/μl each of mRNA) was used in subsequent assays.

**Table 1. Developmental capacity of embryos injected with various concentrations of mRNA**

<table>
<thead>
<tr>
<th>Conc. of mRNA injected (ng/μl)</th>
<th>No. of oocytes examined</th>
<th>No. (%) of morula/blastocyst stage embryos</th>
<th>No. of recipients</th>
<th>No. (%) of pups</th>
<th>No. of transgenic pups</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>70</td>
<td>63 (90.0)</td>
<td>4</td>
<td>20 (29.0)</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>71</td>
<td>66 (93.0)</td>
<td>4</td>
<td>32 (45.0)</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>80</td>
<td>74 (92.5)</td>
<td>5</td>
<td>28 (37.8)</td>
<td>0</td>
</tr>
<tr>
<td>250</td>
<td>69</td>
<td>24 (34.8)</td>
<td>4</td>
<td>2 (3)</td>
<td>0</td>
</tr>
</tbody>
</table>

* Anaphase II/telophase II IVF embryos were injected with the mRNA indicated and incubated for approximately 70 h. Fluorescent images taken just before embryo transfer are shown in Fig. 1. The mixture contained equivalent amounts of EGFP-α-tubulin and H2B-mRFP1 mRNAs. Compacted embryos with more than eight nuclei were judged as being at the morula/blastocyst stage. The embryos were transferred to the uteri of pseudopregnant females 2.5 days post coitum.
Establishment of the six-dimensional imaging system

We next modified the imaging devices (Fig. 2). The equipment used in the system is shown in Fig. 2A. To analyze the embryos three-dimensionally, images for 51 sections at 2-μm intervals (a total of 100 μm) were acquired in the z axis at each time point using a Nipkow disk confocal unit (Fig. 2B). In this confocal unit, unlike the laser scanning-type confocal microscope in which a single laser beam scans the specimen, many light points obtained through multiple pinholes in the spinning disk, named Nipkow disk, raster scans at the same time [22, 23]. The embryos were exposed to two different wavelengths of excitation (488 and 561 nm). Furthermore, to observe multiple embryos in a single assay for comparative and quantitative analyses, the system was equipped with an automatic x-y stage in which we could obtain images at multiple stage positions. Usually, 12 embryos were arranged in 5 μl drops of CZB medium, and images of each set were captured at regular times (Fig. 2C). Typically, we placed 14 drops in glass-bottomed dishes, and it took about 1 min to acquire an image of each drop. Therefore, up to 168 (12 embryos × 14 positions) embryos could be analyzed in one assay run when the time interval was set at 15 min (Fig. 2C). We term this imaging approach six-dimensional (6D; x, y and z axes, time-lapse, multicolor and multisample).

Impact of fluorescence microscopy on full-term development

Next, we optimized the conditions for fluorescence microscopy.
Embryos generated by IVF were injected with an mRNA mixture for EGFP-α-tubulin and H2B-mRFP1 and then imaged until the morula/blastocyst stage under various conditions (light source, optical intensity and time intervals). Developmental potential was tested by transferring the embryos to pseudopregnant recipients (Fig. 3A). Table 2 summarizes the relationships between the conditions used for observation and the capacity for full-term development. A typical image of time-lapse observations of nuclear morphology and spindle formation during preimplantation development is shown in Fig. 3B. Unexpectedly, normal pups were born even after capturing long-term, short-interval, 6D movies of preimplantation development (Fig. 3C and Table 2). These embryos were exposed to two different wavelengths of excitation (488 and 561 nm) with high laser power (set at 70 mW) at 7.5-min intervals for about 70 h, and 51 images were acquired in the z axis at each time point; thus, a total of 56,814 fluorescent images were taken (Supplementary Movie 1, Table 2). However, when the time interval was set at 3.75 min and a total of 114,750 fluorescent micrographs were taken, both the preimplantation and full-term development rates were impaired significantly (Table 2). When we used the mercury or xenon lamp as a light source and imaged the embryos under the same conditions (exposure time, numbers of images in the z axis and time intervals), all the embryos died prior to the 2-cell stage (Table 2). There was no difference in the outcome when the probe combination was changed from EGFP-α-tubulin to EGFP-β-actin (Supplementary Movie 2 and Table 2).

Normality of pups

The pups born after long-term 6D imaging were transferred to foster mothers for suckling, and postnatal development was assessed. Twenty-six, 30 and 22 pups generated from embryos exposed to three different intensities, 0, low or high intensity of excitation, respectively, at 7.5-min intervals were analyzed. Their body weights increased well, and the values were comparable among these three groups (Fig. 4A). All of the animals grew into normal adults. When we chose 12 pairs of male and female pups randomly and mated them, they were fertile (data not shown). Moreover, as we used mRNA but not DNA injection, none of the pups were transgenic for EGFP as is evident from their nonfluorescent appearance (Fig. 4B) and the absence of exogenous DNA integration (Fig. 4C and Table 2). Thus, we conclude that our imaging technology is safe for embryos and does not affect their genomes.
A major advancement in this study is that the embryos survived and developed to full term normally even after long-term 6D imaging of preimplantation development. It is well known that fluorescent observation causes not insignificant damage to cells, called phototoxicity. Experimentally, the incidence of toxicity appears to depend on the intensity and wavelength of the excitation light and exposure time. In addition, our data presented here revealed that the time interval between the observations is also important. When it was set at 3.75 min, the viability of the embryos dramatically decreased when compared with an interval of 7.5 min (Table 2). Moreover, continuous exposure had a more adverse effect on development than repetitive intermittent excitation even though the total exposure time was the same (data not shown). These data suggested that the embryos may have an ability to neutralize the toxicity. Although phototoxicity itself is still an ill-defined phenomenon at present, an accumulation of reactive oxygen species seems to be a candidate involved in it [18]. The mechanisms of production and neutralization of phototoxicity should be clarified by further study.

At any rate, it can be considered that our imaging technology established here could somehow avoid such deleterious effects in spite of the vast number of excitations. This was probably because of the use of the Nipkow disk confocal unit made by Yokogawa Electric Corp. [22, 23] for detection of fluorescence. Of course, the primary advantage of this unit is that three-dimensional cellular images can be taken by eliminating ‘out-of-focus blur’. However, as another advantage, the optical intensity projected from the tip of the lens is extremely low compared with those of mercury and xenon lamps (see Table 2, column of ‘optical intensity’). In fact, when we performed imaging using a mercury lamp fitted with a neutral density filter (ND6; 6% transmission), embryonic development improved significantly (data not shown). Laser scanning type confocal microscopy has failed to allow production of pups, probably due to cellular damage evoked by its extremely intense laser beam [18]. Moreover, Ross et al. also could not avoid damage to the embryo after imaging, even though they used a spinning-disk confocal microscope [19]. This is thought to result from the high intensity of excitation light. They estimated that approximately 8.4 mW of illumination was received by the sample, and this value is approximately 40–80 fold higher than our system (see Table 2, column of ‘optical intensity’). In the confocal system they used (CARV system, BD Biosciences), much more intensity of excitation light is needed to obtain a sufficient signal because the recovery efficiency of fluorescence is low when compared with Yokokawa’s spinning-disk confocal unit [23]. Taken together, these several lines of evidence suggest that there is a threshold for light intensity that affects survival of embryos. Therefore, for

### Table 2. Developmental capacity of embryos after long-term 6D imaging

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Light source</th>
<th>Optical intensity (nm, mW)b</th>
<th>Time intervals (min)</th>
<th>No. of images acquired</th>
<th>No. of embryos examinedd</th>
<th>No. of morula/blastocyst stage embryosd</th>
<th>No. (%) of recipientsd</th>
<th>No. of pupsd</th>
<th>No. (%) of transgenic pups</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2B</td>
<td>Kr/Ar Laser</td>
<td>Low 488, 0.082, 561, 0.092</td>
<td>30 13,158</td>
<td>84</td>
<td>77</td>
<td>(91.7)</td>
<td>5</td>
<td>43/77</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High 488, 0.281, 561, 0.225</td>
<td>3.75 144,750</td>
<td>36</td>
<td>20</td>
<td>(56.6)</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mercury</td>
<td>488, 49.02, 561, 111.8</td>
<td>15 23,526</td>
<td>95</td>
<td>0</td>
<td>(0)</td>
<td>–</td>
<td>–</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Xenon</td>
<td>488, 14.0, 561, 13.7</td>
<td>15 23,460</td>
<td>96</td>
<td>0</td>
<td>(0)</td>
<td>–</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>H2B</td>
<td>Actin</td>
<td>Kr/Ar Laser 488, 0.082, 561, 0.092</td>
<td>15 25,908</td>
<td>84</td>
<td>78</td>
<td>(92.9)</td>
<td>6</td>
<td>32/78</td>
<td>0</td>
</tr>
</tbody>
</table>

* IVF embryos were injected with a mixture of 5 ng/μl each of mRNAs for EGFP-α-tubulin and H2B-mRFP1, transferred to 5 μl drops of CZB medium on glass-bottomed dishes (12 embryos/drop) and imaged until the morula/blastocyst stage (approximately 70 h). Fifty-one images in the z axis and two color (green and red) images were captured at the time intervals indicated. b The optical intensities of each wavelength projected from the tip of the lens were monitored by optical power meter (TB200, Yokogawa Electric Corp.). c Just before embryo transfer, the embryos were observed once under fluorescence confocal microscopy. d Compacted embryos with more than eight nuclei were judged as being at the morula/blastocyst stage. e The embryos were transferred to the uteri or oviducts of pseudopregnant females at 2.5 or 0.5 days post coitum, respectively. f Percentages were calculated as the number of pups divided by the number of morula/blastocyst stage embryos transferred. n.d. means not determined.

### Discussion

**Safety of fluorescence microscopy for embryo development**

A major advancement in this study is that the embryos survived and developed to full term normally even after long-term 6D imaging of preimplantation development. It is well known that fluorescent observation causes not insignificant damage to cells, called phototoxicity. Experimentally, the incidence of toxicity appears to depend on the intensity and wavelength of the excitation light and exposure time. In addition, our data presented here revealed that the time interval between the observations is also important. When it was set at 3.75 min, the viability of the embryos dramatically decreased when compared with an interval of 7.5 min (Table 2). Moreover, continuous exposure had a more adverse effect on development than repetitive intermittent excitation even though the total exposure time was the same (data not shown). These data suggested that the embryos may have an ability to neutralize the toxicity. Although phototoxicity itself is still an ill-defined phenomenon at present, an accumulation of reactive oxygen species seems to be a candidate involved in it [18]. The mechanisms of production and neutralization of phototoxicity should be clarified by further study.

At any rate, it can be considered that our imaging technology established here could somehow avoid such deleterious effects in spite of the vast number of excitations. This was probably because of the use of the Nipkow disk confocal unit made by Yokogawa Electric Corp. [22, 23] for detection of fluorescence. Of course, the primary advantage of this unit is that three-dimensional cellular images can be taken by eliminating ‘out-of-focus blur’. However, as another advantage, the optical intensity projected from the tip of the lens is extremely low compared with those of mercury and xenon lamps (see Table 2, column of ‘optical intensity’). In fact, when we performed imaging using a mercury lamp fitted with a neutral density filter (ND6; 6% transmission), embryonic development improved significantly (data not shown). Laser scanning type confocal microscopy has failed to allow production of pups, probably due to cellular damage evoked by its extremely intense laser beam [18]. Moreover, Ross et al. also could not avoid damage to the embryo after imaging, even though they used a spinning-disk confocal microscope [19]. This is thought to result from the high intensity of excitation light. They estimated that approximately 8.4 mW of illumination was received by the sample, and this value is approximately 40–80 fold higher than our system (see Table 2, column of ‘optical intensity’). In the confocal system they used (CARV system, BD Biosciences), much more intensity of excitation light is needed to obtain a sufficient signal because the recovery efficiency of fluorescence is low when compared with Yokokawa’s spinning-disk confocal unit [23]. Taken together, these several lines of evidence suggest that there is a threshold for light intensity that affects survival of embryos. Therefore, for
longer and safer imaging, samples should be exposed to as low an intensity of light as possible, and instead, the fluorescent signals should be collected at the maximum level of efficiency using an ultrasensitive camera such as the electron multiplying charge coupled device (EM-CCD) camera we chose.

When we injected 250 ng/μl of mRNA solution, both the preimplantation and full-term development rates were affected significantly (Table 1), suggesting that an excess amount of mRNA is also a detrimental factor for the embryo. In our previous paper [14], even though mRNA encoding solely EGFP was injected at a concentration of 1 mg/ml, 36% of embryos could reach to morula stage. Moreover, although the design of the mRNA injected (coding sequences, UTRs and poly(A) tail) was different, other groups used 0.5 mg/ml of mRNA for injection, and it seems that morula/blastocyst development was not affected [5, 26]. Thus, the toxicity to embryonic development appears to depend on the types and amounts of exogenous proteins translated from the mRNA injected rather than the mRNA itself.

Difference in tolerance of embryos to imaging among mammalian species

To our knowledge, so far the embryos of the mouse [14, 19], hamster [18], rabbit [14] and bovine [19] have been examined by fluorescence imaging. Of them, mouse [14, 19] and hamster [18] embryos have been transferred to recipients after imaging, and indeed pups have been obtained. Unfortunately, however, the tolerance to fluorescence imaging cannot be uniformly compared among these species because the probes and imaging devices used in the assays are quite different. Only Ross et al. [19] have compared mouse and bovine embryos in a side-by-side experiment, and they found that bovine embryos were more tolerant of the imaging procedures than mouse embryos. They discussed that this difference may come from the different ability of antioxidants and/or scavengers for oxygen in the embryos. To apply our imaging system to another species in the future, we have to minimize the phototoxicity by optimizing the conditions in each species.

Applications

Our technique offers a novel assay system for developmental and reproductive research. In addition, as the pups born after imaging are healthy and are not transgenic, this technique might be applicable to assessment of embryonic quality in human assisted reproductive technologies (ARTs) and in production animal industries. Advancements in ARTs now enable infertile couples to have babies even using gametes that are not necessarily ‘normal’, as in the case of ICSI [11]. However, a significant number of ART pregnancies end up as early pregnancy losses mainly due to the poor quality of the embryos transferred [27]. In addition, single embryo transfer is now recommended routinely to reduce the risks of multiple gestations in human ART [28]. Therefore, selection of a ‘normal’ embryo before transfer is essential to establishing a healthy pregnancy. In fact, trials assessing the developmental potentials of human embryos by time-lapse imaging using light microscopy have already performed in clinics [29, 30].

Fig. 4. Normality of the pups after imaging. A) Chart depicting the changes in the body weights of the pups. The pups were generated from embryos exposed to three intensities (0, low or high) of fluorescent laser light at 7.5-min intervals for about 70 h (details are given in Table 2). The horizontal and vertical axes represent weeks after birth and body weight, respectively, and ‘n’ means number of mice analyzed. B) Fluorescent image of the pups. The asterisk indicates an EGFP-expressing transgenic mouse included as a positive control. C) Typical electrophoresis pattern of genotyping analysis of tail-tip DNA. Lanes 1–22 are from pups among the imaged embryos; lane N is from a normal BDF1 strain mouse as a negative control; P is from an EGFP-expressing mouse as a positive control; and M is a DNA size marker. The β-actin gene was amplified simultaneously as a PCR control.
cause of early pregnancy loss is thought to lie in chromosomal abnormality and aneuploidy of cleavage stage embryos [31, 32]. Therefore, imaging of the chromosome dynamics using H2B-mRFP1 as a probe may help to assess the chromosomal integrity of embryos and, furthermore, to eliminate the chromosomally ‘ill-fated’ embryos from transfer in ART. Moreover, if the fluorescent probe is changed, the other states of the embryos can be assessed. The low success rate of bovine SCNT embryos is now a major problem in the production animal industry [33]. This is probably partially due to the incomplete reprogramming process in epigenetic status such as global DNA methylation [9, 12]. We have previously developed a fluorescent probe, named EGFP-MBD-NLS, which can detect the methylated DNA in living embryos in which EGFP has fused to the methyl-CpG-binding domain and nuclear localization signal of human MBD1 [14, 15]. Thus, by using this probe for assessment of embryos, the birth rate might be improved in bovine cloning.

Although we used mRNA injection but not transgenesis for fluorescence expression in the mouse embryos, it might be difficult to apply our technology to human clinical use directly because of ethical objections and because it is unconventional. However, these problems may be relieved to some extent by the use of a non-RNA molecule, such as a protein, antibody or fluorochrome, as reporters. We believe that our safe 6D live-cell imaging technology will be broadly applicable to assessment of embryo quality at the molecular level.

Acknowledgements

We are grateful to Dr. E Mizutani, Mr. H Kami and Mr. H Tanaka for imaging assistance. We also thank Mrs. M Brahmajosyula for critical reading of the manuscript. This study was partly supported by Grants-in-Aid for Scientific Research on Priority Areas and for Young Scientists (B) from the Japan Society for the Promotion of Science (JSPS), the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) and Special Coordination Funds for Promoting Science and Technology from MEXT.

References