NIBB INTERNSHIP REPORT

Distribution of diploids and tetraploids in a tetraploid↔diploid aggregation chimera of fluorescently labeled *Mus musculus* embryos

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INTRODUCTION

Polyploidy indicates a cell that contains more than two haploid (n) sets of chromosomes (**Figure 1**). It is commonly observed among plants and might also be observed in some "lower" animals; however, it is less frequently seen in most of the animals because of their complexity. For instance, polyploidy is rare in humans, yet it is observed in human skeletal muscle cells.

Polyploid mammals can be experimentally produced in a laboratory environment. Polyploid embryos can be used to provide evidence for further investigations of cell size regulation, cell number, and rate of cell cleavage. Tetraploidy is more widely used than other forms polyploidy in various studies. Although tetraploid–diploid (4n:2n) chimeras often survive the gestation period (Eakin and Behringer, 2003), tetraploid cells are under-represented in the embryo proper or the inner cell mass (ICM) because of selective disadvantage of diploid cells (Tarkowski *et al.*, 1977). However, the mechanism of under-representation and restriction of tetraploid cells remains unclear.

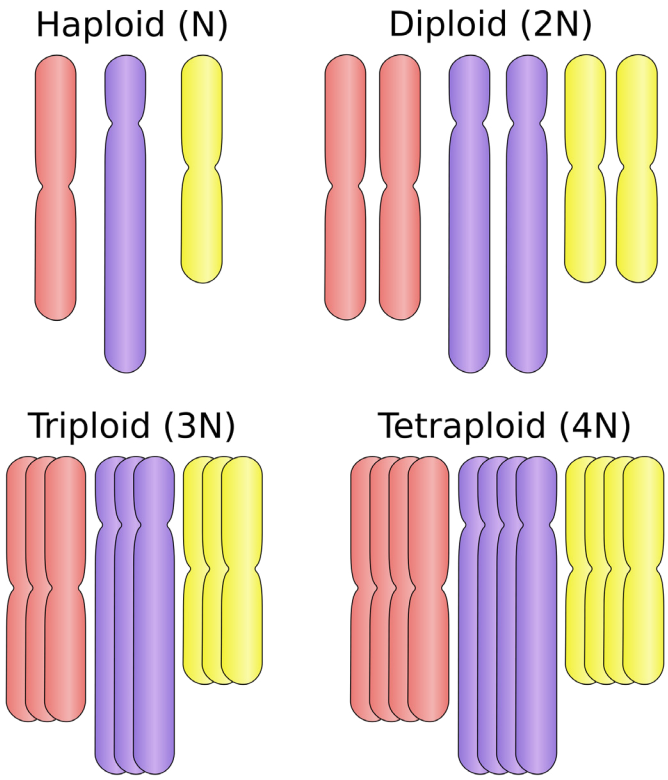

FIGURE 1. HAPLOID, DIPLOID, AND POLYPLOIDS
Visualizing the polyploidy. Triploids and tetraploids are considered as the polyploids; yet, tetraploidy is much rare than triploidy.
SOURCE: WIKIPEDIA COMMONS

Conventionally, chimeras can be created by two different techniques: blastocyst injection and aggregation. Each method has its advantages and disadvantages, and methods may vary depending on the procedure that is followed; however, the aggregation method is preferred for tetraploid chimera production in research because of its capability of mass production and ease (Wood *et al.*, 1993). Tarkowski *et al.* successfully created the first 4n:2n mosaic mouse by exposing 2- to 4-celled embryos to cytochalasin B. Surprisingly, less than 4% tetraploid cells were observed in embryo proper whereas approximately 50% tetraploid cells were present in the extraembryonic tissues (Tarkowski *et al.*, 1977). Another study showed that only 3% tetraploid cell contribution in the bone marrow of a 4n:2n chimera (Lu and Markert, 1980).

To assess the contribution level of the embryonic tetraploid and diploid cells in a chimera, ICR (Institute of Cancer Research) female *Mus musculus* were crossed with two different male strains: red fluorescent H2B-mCherry (Abe *et al.*, 2011) and green fluorescent H2B-GFP (Kurotaki *et al.*, 2007). GFP, a green fluorescent protein, was first discovered by Shimomura *et al.* in 1962; it has become an important tool to track and visualize the proteins of interest because of its ability to binding to the C-terminus of histone H2B (Kanda *et al.*, 1998). mCherry is a GFP derivative that emits red fluorescent.

This experiment aimed to investigate the contribution level of tetraploid cells in a chimeric embryo. The diploid blastomeres were electrofused to form tetraploids, and then they were aggregated with the diploid embryonic cells to form chimeras. After 24 hours, one 4n:2n aggregate chimera was produced, and it was used for time–lapse analysis.

MATERIALS AND METHODS

The experiments were performed in accordance with the following procedures described in the experimental manual with several modifications (Nagy *et al.*, 2003).

Flushing and Collecting Embryos

Before flushing the embryos, prepare a culturing medium using KSOM. Place several KSOM drops on a small dish and cover them with mineral oil. Place the medium in 37°C and 5% CO2 incubator beforehand.

After isolating a pair of fallopian tubes from a mouse, M2 medium (preferably pre-warmed to 37°C) was used to temporally soak the tubes and embryos during the process. Terumo's® 1 mL Tuberculin syringe containing M2 was used with a number 30 needle.

A stereomicroscope (Leica® MZ-16) was used to locate the infundibulum of uterine tube that is situated at the end of the fallopian tube. The infundibulum was held using No. 5 tweezers, the needle was inserted into it, and then embryos were flushed out from the uterus. These embryos were collected with capillary pipettes and then washed twice in M2 medium. Finally, they were gently placed in the KSOM media plate and incubated in 37°C.

Electrofusion of the Blastomeres

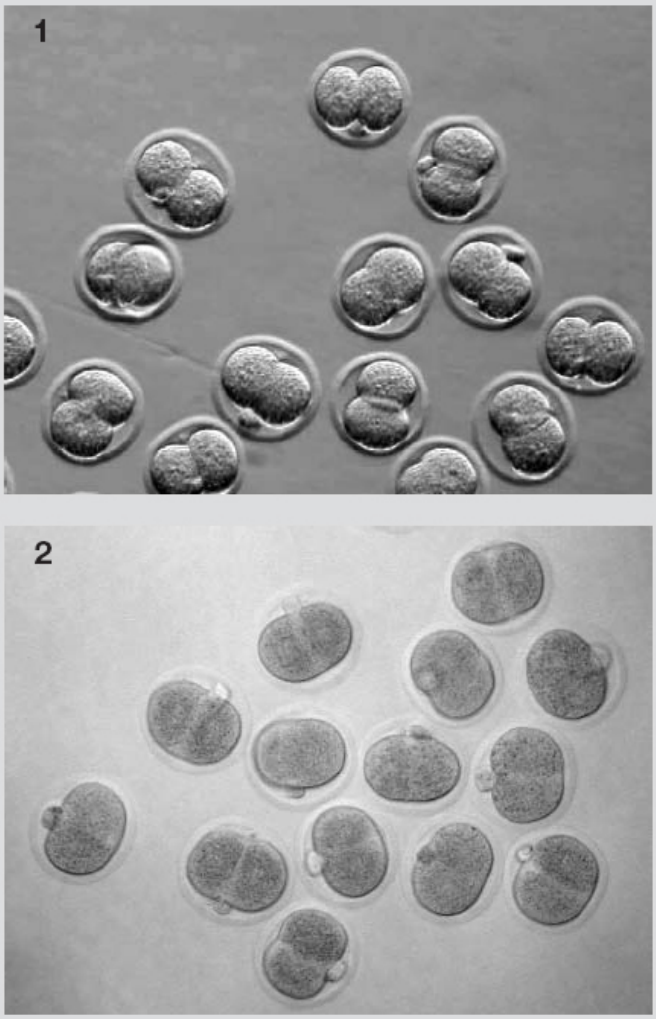

FIGURE 2. PROGRESS OF ELECTROFUSION
The image on the top shows the 2-celled embryos before the electrofusion and the one on the bottom shows the fused embryos 20 minutes after the electrofusion fusion.

SOURCE: EPPENDORF

Sufficient amount of mannitol (0.3 M) was placed in the slit of the electrode chamber, and the chamber was connected with the pulse generator (Soniel™ Electroporator CUY21 EDIT). Approximately 10 embryos (at once) were placed in the slit slowly and their blastomeres were carefully positioned perpendicularly to the electrodes. After operating the electrical pulse (100V), the embryos were collected and washed with KSOM before being incubated. The blastomere fusion can be observed whthin one hour (**Figure 2**).

Formation of Aggregation Chimeras

Multiple KSOM drops were placed on a 60-mm plastic dish plate. At the centre of each drop, a small depression was created with a 1-mm round-tip pin (preferably a Japanese "machi-bari"). Finally, all drops were covered with liquid paraffin. Several embryos were placed in acid tyrode to dissolve their zona pellucidae and immediately placed in M2 solution (with HEPES buffer) to neutralize acid. The embryos were then washed several times in KSOM solution. Each tetraploid embryo and diploid embryo was assigned to the same depression to form an aggregation. Finally, the aggregated chimeras were incubated (37°C/5% CO2) for 24 hours.

Time-lapse Imaging of the Development of Chimeras

Yokogawa's time-lapse image capturing device Cell Voyager™ was used for capturing images of 500 time points at 10-minute intervals. The images obtained from this process were then modified into movie clips and colored images to visualize and analyze tetraploids (GFP) and diploids (mCherry).

RESULTS

Dissecting Mus musculus *and flushing out embryos from fallopian tubes:*

On July 13 (group 1), the first two female mice were dissected approximately 1.5 days post coitum (dpc), and another two female mice were dissected after fertilization. Twenty-eight embryos were collected from the first two mice; three embryos were collected from the latter two.

On July 15 (group 2), 23 embryos were collected from two H2-mCherry (mCherry)-crossed mice and 27 embryos were collected from H2B-GFP (GFP)-crossed mice around noon. Approximately four hours later, 26 embryos were collected from the other two mCherry and 25 were collected from two GFP mice (**Table 1**).

On July 21 (group 3), embryos were collected from seven mice (three mCherry and four GFP). From the 84 embryos that were collected, 42 embryos each were collected from mCherry and GFP mice.

On July 27 (group 4), 33 embryos were collected from two mCherry mice and 52 embryos were collected from three GFP mice. Of the 33 mCherry embryos, 26 were 2-celled, six were 1-celled, and one embryo was abnormal, whereas all 52 GFP embryos were 2-celled.

Table 1. Amount and condition of embryos at about 1.5 dpc.
Most embryos demonstrated their 2-cell stages. 1-cell embryos possibly indicate the failure of fertilization. Only one 3-cell embryo was found between 1.5 to 2.0 dpc.

	Condition of Embryos			
Mice	1-Cell	2-Cell	3-Cell	Total
July 13, 2011 (Out of 4 Mice)				
WT	N/A	30	1	31
July 15, 2011 (Out of 8 Mice)				
mCherry	7	42	N/A	49
GFP	N/A	52	N/A	52
July 21, 2011 (Out of 7 Mice)				
mCherry	10	32	N/A	42
GFP	11	31	N/A	42
July 27, 2011				
mCherry	6	26	1	33
GFP	N/A	52	N/A	52
August 3, 2011				
mCherry	14	33	N/A	47
GFP	10	31	N/A	41
August 10, 2011 (Out of 7 Mice)				
mCherry	7	29	2	38
GFP	16	30	N/A	46

On August 3 (group 5), 47 mCherry embryos were collected of which 33 were 2-celled and 14 were 1-celled; 41 GFP embryos were collected of which 10 were 1-celled and 31 were 2-celled.

On August 10 (group 6), 38 mCherry embryos and 46 GFP embryos were collected. The significances of each strain are also described in **Table 1**.

Development of Diploid (2n) Embryos:

All embryos were cultured in vitro: KSOM was used as the medium and a water-jacket incubator was set to maintain constant temperature (37°C) and CO2 level (5%).

Group 1 (July 13) embryos were again observed under a microscope at approximately 2.5–3.0 dpc. In total, there were thirty-one embryos: 12 embryos in their compaction stage, one embryo in 4-celled stage, and the remaining in multi-celled (5-to 8-celled) stage.

In group 2 (July 15), only few embryos were observed at 2.5 dpc because 10 mCherry and twenty GFP embryos were used for time-lapse analyses. At 2.5 dpc, among 25 diploid (2n) mCherry embryos, four embryos exhibited compaction, 20 were in multi-celled stage, and one embryo was found dead. Meanwhile, all 16 2n GFP embryos were in multi-celled stage. Since the 2n GFP embryos were controls, they were not carefully observed throughout the experiment; however, they were examined at 4.5 dpc, and it was confirmed that the embryos had progressed towards the blastocyst stage. Because of inefficient chimera aggregation, no 2n mCherry was remained.

The group 5 (August 3) embryos did not fully grow due to high concentration of the growth medium (KSOM); however, the embryos in group 6 (August 10) grew uneventfully and 30 healthy GFP embryos were ready to be electrofused.

Health Conditions of Electrofused Embryos:

Fourteen 2-cell mCherry embryos and 15 2-cell GFP embryos were selected to undergo electrofusion to produce tetraploids. At 2.5 dpc, one 2-cell, four 3-cell, six 4-cell, and three multi-celled mCherry tetraploid embryos were observed; five 2-cell, four 3-cell, three 4-cell, and three multi-celled embryos were observed in GFP tetraploids.

In group 2, 30 mCherry embryos were electrofused and incubated in vitro. Approximately 3 hours later, the blastomere of only one embryo was verified to be fused because it was completely 1-cell whereas other artificially-fused embryos were 2-cell stage.

Out of 30 GFP embryos, only 16 of them were found fully fused after one hour from electrofusion. As for the unfused embryos, the electrofusion procedure was repeated and then they were cultured overnight.

Significance of Aggregated Chimera:

We attempted to produce nine aggregation chimeras in group 3; however, only one set of embryos was seemed to be successfully aggregated. Fluorescent microscopy showed that the aggregated embryos emitted high intensity green light and low intensity red light. Therefore, one chimera had surely been produced. Despite the successful aggregation of one set, all the embryos (including the chimeric one) did not seem to grow. The embryos in the control group (2n GFP embryos) had already progressed to the blastocyst stage, whereas the embryos of the aggregated chimeras were still at the 4- to 8-cell stage.

In group 6, 14 healthy tetraploid GFP embryos (out of 16) were used to produce aggregation chimeras with diploid mCherry. 13 successfully aggregated 4n:2n chimeras were obtained consequently.

Visualizing a 4n:2n Chimera (Group 6 Exclusive):

Twelve aggregated 4n:2n chimeras were selected to be visualized by time-lapse. The time-lapse image showed the distribution of tetraploids (green) and diploids (red) as shown in **Figure 3**. This image was taken at t = 210 (1.46 days after the first time-lapse image was taken), and it appeared that although the tetraploids and diploids had aggregated, they had not completely mixed up. The size of the nuclei of tetraploids seemed to be almost double that of the diploids, which would be reasonable considering that the tetraploids contained twice as much as chromosome pairs than the diploids.

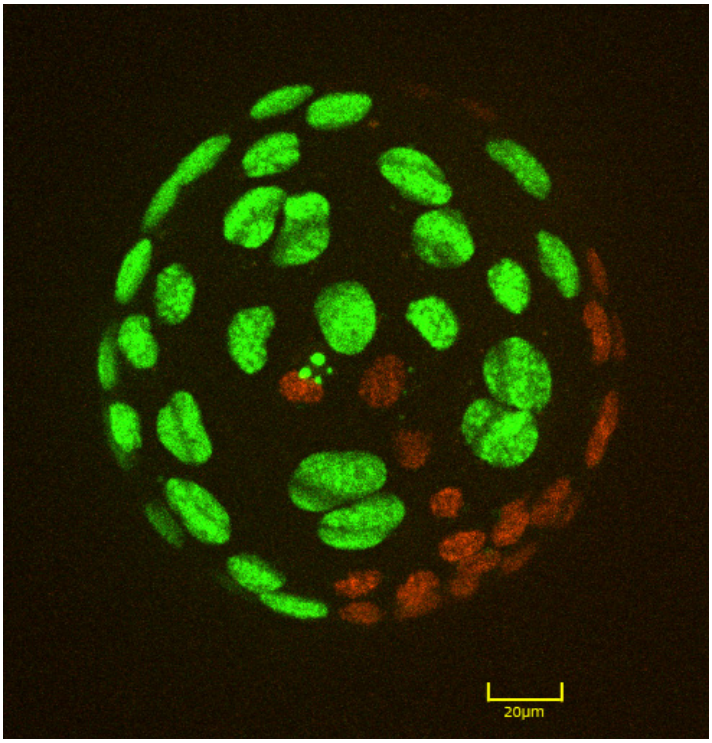

FIGURE 3. DIPLOIDS AND TETRAPLOIDS IN AN EMBRYO
The green cells (GEP) represent tetraploids whereas the red cells (mCherry) represents diploids. The image was captured at 2100 minutes after the time-lapse image was taken

BRIEF DISCUSSION

Analysis of time-lapse images of six of twelve chimeras has revealed several noteworthy phenomena.

In this experiment, the visualization and analysis of the time-lapse images were slightly challenging because of uncertainties regarding the inner cell mass and the trophectoderm regions. Therefore, dyeing the trophectoderm cells (possibly with Cdx2) to differentiate them from the ICM cells would prove effective in further studies.

The distribution and contributions of the tetraploids and diploids in embryos have a particular pattern. The movement of tetraploids in the embryo is intriguing and might provide explain the studies conducted by Tarkowski *et al.* (1977) and Lu and Markert (1980).

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