Abstract: This study was performed to determine the capability of embryonic stem (ES) cells at late passage number to form functional germ cells and to orientate the sexual development of chimeric mice produced from ES cell injection into embryos. When an R1 ES cell line was routinely cultured on mitotically inactivated feeder cells in culture medium supplemented with leukemia inhibitory factor (LIF), the colonies maintained an undifferentiated ideal ES cell morphology. When the ES cells were injected into 45 eight-cell embryos, 38 embryos (84%) combined with ES cells. Twenty-eight percent of the embryos transferred to recipients developed to term. Thirty percent of offspring born were chimeric. In these chimeric individuals the contribution of ES cells to the coat was about 70% and 99% in two male and one female chimeras, respectively. Furthermore, ES cells contributed to the germ line in the female chimera whereas they did not contribute to the germ line in two male chimeras. This study showed that female chimeras may result from injected embryos with the male ES cell and may transmit the ES cell genome to their offspring.

Key Words: Mouse, ES cells, chimera, sex conversion, germ cells.

Introduction

Murine embryonic stem cells are permanent cell lines that are derived from the inner cell mass (ICM) of a preimplantation mouse embryo (1,2). One distinct property of ES cells is that they remain diploid even after being cultured for many weeks. Generally this is in contrast to
other tissue culture cell lines that often do not remain diploid but spontaneously gain and lose chromosomes at a high rate. A second unique property of ES cells is that they remain totipotent and maintain the ability, like ICM cells, to form chimeras. These two properties, maintaining a normal karyotype and extensive contribution in chimeras, are both necessary for ES cells to form functional germ cells in chimeras (3,4). ESC-embryo chimeras are produced either by injection of cells into blastocysts (4-7) or morula (4,5,8), or by aggregation of cells with morula (4,5,9). Usually, the ES cells are male, whereas the recipient embryos can be either male or female. The injection of male ES cells into a female embryo frequently converts the developing embryo to a phenotypic male chimera, thereby reducing the number of female chimeras born (6,10). Morphological differentiation of the sex organs is caused by a localized effect and is dependent on the relative contributions of XX and XY cells to the fetal genital ridge. The pattern of integration of XY culture cells in a female embryo affects sexual differentiation and determines the fate of the two cell populations in terms of their ability to form functional germ cells. The ability of the injection culture-derived cells to form sperm is governed by, firstly, the sex of the host embryo and, secondly, the number and position of the colonizing cells in the early embryo (11).

Long term passage of ES cells in suitable culture conditions may cause some abnormality and this may affect the function of ES cells. This study was performed to determine the capability of ES cells at a late passage number to form functional germ cells and to orientate the sexual development of chimeric mice produced from the ES cell injection into embryos.

**Materials and Methods**

In this study, BALB/C inbred mouse strain was used as the donor for obtaining host embryos and CB6 F1(C57BL/6 X BALB/C) hybrid mouse strain was used as the pseudopregnant recipient. R1 ESC line (provided by A. Nagy of Mount Sinai Hospital, Samuel Lunenfeld Research Institute, Toronto, Canada) was used for injection into host embryos.

**Recovery of Embryos**

Donors were superovulated with intraperitoneal injection of 5 I.U. pregnant mare serum gonadotropin (PMSG-G-4877 Sigma) 48 hours before the injection of 5 I.U. human chorionic gonadotropin (HCG, Pregnyl, Organon) and were caged with fertile males. Morulae were recovered from these superovulated females on day 2.5 of pregnancy by flushing the oviducts with M2 medium (8, 12-14).

**Production of Embryonic Feeder Layers**

Primary mouse embryonic fibroblast (PMEF) cells were obtained from embryos of 14-16 day pregnant mice as described in the literature (4,8,15). The cells in Dulbecco’s modified Eagle’s medium (DMEM, 41966-029 Gibco) + 10% fetal calf serum (10106-075, Gibco) were split on tissue culture plates (150350, 150326 Nunc) and incubated at 37 °C, 5% CO₂ (4,5,8). The culture plates, to which the embryonic fibroblast was added at this stage, were pretreated
with a 0.1 % (w/v) gelatine solution (G-9382 Sigma) (4,8). When the cells formed a confluent monolayer, the medium was removed from the plates and the medium containing 10 μg/ml Mitomycin C (M-0503 Sigma) was added to the plates in order to permanently arrest PMEF proliferation. The PMEF plates were then incubated at 37 °C, 5% CO₂ for 2.5-3 hours. After 3 hours, the medium containing Mitomycin C was replaced with fresh culture medium (4,8,15).

Culture of ES cells

R1 ESC line was routinely cultured on an embryonic fibroblast feeder layer inactivated with Mitomycin C in DMEM supplemented with Cos-cell conditioned medium containing leukemia inhibitory factor (LIF), 0.1 mM non-essential amino acids (100x stock, 320-1140 AG Gibco), 1 mM sodium pyruvate (100x stock, 320-1360 AG Gibco), 0.1 Mm 2-β mercaptoethanol (00564ag Sigma) and 15% fetal bovine serum (lot 30F7043S Gibco) (15). A cos-cell-conditioned medium containing LIF was prepared and tested for convenient dilution as described in the literature (16,17). This medium was added to the culture medium in 1/1000 dilution.

One vial of frozen R1 cells at passage 18 was thawed in culture medium and plated on a 60 mm dish of PMEF. The medium was changed the next day. The ES cells were trypsinized (0152-13-1, Trypsin 1:250 Difco) on the second day and transferred to 100 mm dishes of PMEF (about 2 x 10⁶ R1 cells/100 mm dish). On the second day, confluent plates were trypsinized. Some cells were kept for injection as a single cell suspension and the remaining cells were frozen (8,15).

Production of Chimeric Mice

All injections were done in M2 medium under an inverted microscope. Morulae and the single cell suspension of ES cells were introduced into a drop of M2 medium. Approximately 5-6 small, round ES cells were injected into each morula. The tip of the injection pipette was slowly inserted through the zona pellucida and the cells were expelled into the perivitelline space (Figure 1) (4,8,15)

After injection, morulae were cultured to blastocysts for 24 hours. The next day, the blastocysts were transferred to the uteri of 2.5-day pseudopregnant mice (4,12,13,15). Since host embryos were from an albino strain and the ES cells were derived from a pigmented strain, chimeric offspring were identified by observing coat pigmentation. The contribution of the ES cells in individual chimeras was estimated from the proportion of coat with pigmentation (4,5,8,14,15).

Detection of transmission of ESC genome

Chimeric mice were mated with BALB/C mice. Since offspring derived from the host blastocyst gametes would have pink eyes and albino coat while ES-derived gametes would produce black eyes and pigmented coat, transmission of the ES cell genome was identified by coat pigmentation of offspring of chimeras (3,5).
Results

This study was performed to determine how the long term passage of ES cells might affect the contribution of ES cells in the formation of tissue and development of functional germ cells and sex in chimeric mice.

First, whether the ES cells used in this study show expected morphological characteristics was tested when maintained under defined conditions. Frozen R1 cells at passage 18 were plated on a 60 mm dish. After 24 hours of culture, ES cell colonies displayed normal morphology (Figure 2). After 48 hours culture, the plate was confluent. When the plate was transferred to 100 mm dishes, the colonies continued to grow and maintained undifferentiation ideal ES cell morphology. This suggested that the cells used were morphologically normal and suitable for injecting into host embryos.

To determine the ability of such ES cells to form chimeric mice forty-five eight-cell embryos were injected with ES cells. After 24 hours in culture, 38 embryos (84%) were at the blastocyst stage and combined with the ES cells as visualized under a microscope (Figure 3). The remaining embryos did not combine with ES cells and were not at the blastocyst stage. When 36 blastocysts were transferred to the uterus of 2.5-day pseudopregnant mice, 10 embryos developed to term. Three of the ten offspring (30%) born were chimeric. Although male ES cells usually produce phenotypic male chimeras, two of the chimeras obtained in this study were male and one was female. The rate of chimerism was estimated by observing the coat pigmentation in these individuals. The ES cells made up about 70% of the coat in the male chimeras and about
Figure 2. After 24 hours in culture, undifferentiated ES cell colonies. Magnification 200x.

Figure 3. After 24 hours in culture, chimeric embryos developed to blastocysts.
99% in the female chimera. All chimeras had black eyes (Figure 4). It was well established that the contribution rate of ES cells to the coat is similar to the contribution rate to other tissue, including the genital ridge (5). Therefore, it is expected that a chimera with a high percentage of coat pigmentation would normally be male. The fact that an individual produced in this study with 99% coat pigmentation was female was unexpected.

To determine the transmission rate of the ES cell genome from individual chimeras they were mated with albino mice. When two male chimeras were mated with albino mice, they produced albino offspring. Since the male mice were not germ-line chimeras they could not transmit the ES cell genome. When one female chimera was mated with an albino mouse, both pigmented and albino offspring were produced. This indicated that both ES cells and host blastocyst cells contributed to the germ line and produced gametes in this chimera.

Discussion

In this study, ES cells were grown in a defined culture medium supplemented with LIF and on primary embryonic feeder layer cells and maintained an undifferentiated ideal ES cell morphology as reported previously (8). This was expected because the pluripotent state of ES cells as undifferentiated cells can be maintained in vitro by culturing in this medium. It is known that when ES cells grow in the absence of a suitable culture condition, they will spontaneously differentiate into embryonic structures (4,6,15,16).
In the present study, 30% of offspring resulting from the injected embryos with ES cells were chimeric. This ratio was similar to the frequency of formation of chimeras reported previously for different strains (8,18,19). In this study the contribution levels of ES cells to the coats of female and male chimeras were about 99% and 70%, respectively. Similar ratios were reported previously for the contribution of ES cells to the coats of chimeras (8,18,19). A highly chimeric female (99%) transmitted the ES cell genome as determined by the occurrence of pigmented offspring. In general, the degree of coat colour chimerism of a particular animal correlates with the degree of germ-line contribution. If a clone consistently contributes to more than 50-60% of the coat in a series of chimeras, then it is quite likely that it will contribute to the germ line at a similar level (5). Normally, it is expected that 70% chimeric males can transmit the ES cell genome, but this did not occur in this study.

The chimeric female produced both pigmented and albino offspring in this study. This could be due to gametes that were derived from both host embryos and the ES cells which were possible female and male, respectively. Although the gametes produced by chimeras are expected to derive either from one or both embryos, breeding tests show that even in XX/XX and XY/XY chimeras the germ cells are frequently derived from only one of the two components (11,20).

It was surprising that the highly chimeric mouse derived from the male ES cells developed as a phenotypic female. This was unexpected as it is known that efficient and widespread incorporation of XY cells into XX host embryos causes phenotypic sex conversion to give male animals that can transmit only ES cell derived sperm in the germ line, due to the inability of XX cells to undergo spermatogenesis (5,10). This sex conversion presumably occurs when XY cells colonize sufficient portions of the various tissues which determine sex in the developing embryo and it is due to the dominant effect of the Y chromosome. Therefore, it is reasonable to suppose that the XX/XY females are those that, by chance, contain relatively few XY cells as embryos (5,21). How a 99% chimeric fertile female was derived from the male ES cell line is not known. However, there are two different hypotheses on female chimeras resulting from male ES cells and on the formation of functional germ cells from these XY cells. The first hypothesis is that XY germ cells can become functional oocytes and the developmental fate of the primordial XY germ cell is not irrevocably determined before its migration to the gonads is complete. Evidently, the sex of a germ cell is not an autonomous property but is determined by the nature of the gonad in which it finds itself. So Y and XY ova certainly can exist in female chimeras and can form viable XY, XXY and XYY animals after normal fertilization (21,22). The second hypothesis is that since XY cells do not normally undergo oogenesis in chimeras, this phenomena may be due to the loss of part or all of the Y chromosome, resulting in effectively XO cells which are capable of forming ova, as was shown in at least one case of transmission from a female chimera (5,6,23). In the present study although male ES cells contributed about 99% of the coat in one chimera, sex conversion did not occur. The other two male chimeras (70% chimeric) did not transmit the ES cell genome. Therefore, all the evidence supports the second hypothesis regarding the possible loss of the Y chromosome. Long term passage of ES cells may have caused the loss of the Y chromosome. Studies supporting this view showed that germ-line transmission and contribution to all cell lineages of cells decreased and chromosomal
abnormalities in ES cells occurred in normal culture conditions when the passage number of ES cells increased (24,25).

In conclusion, the results obtained in this study suggest that long term passage of ES cells might cause some abnormality when such cells are used to produce chimeric mice. In addition, although female chimeras are in general less valuable than the males and are not preferred, this study shows that they can transmit the ES cell genome to their offspring. Although female chimeras are clearly less able than male chimeras to lead to an ES cell-derived mouse line, the female chimeras raise some provocative questions concerning the reproductive biology of chimeric animals and the differences between male and female meiosis.

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References


