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COCULTURING EMBRYONIC STEM CELLS WITH DAMAGED HEPATOCYTES LEADS TO RESTORATION OF DAMAGE AND HIGH FREQUENCY OF FUSION

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Abstract – Controversy surrounds issue of cell fusion as a repair mechanism whereby stem cells regenerate. To identify the ratio of fusion happens between stem cells and damaged cells, hepatic cells were damaged with 200μM H_2O_2 for 2 hr. Then, mouse ESCs were cocultured with damaged human hepatocytes. Fusion was detected directly by karyotyping after 48hr coculture as well as by Oct4 promoter drove GFP signal. Results showed that average ratio of fusion in undamaged control group was 0.031‰ while ratio of fusion in damaged group was 0.357‰, which was 10 times higher than fusion happened in the control group. Meanwhile, GFP signal indicated that fusion induced hepatic cells' Oct-4 reactivation. Fusion derived hybrid cells contained chromosomes from both parental cells. Most of the chromosomes were from damaged human hepatic cells. Activity of damage-related enzymes LDH, SGOT and SGPT were significantly lower at 48hr coculture than at 12hr coculture. Expression of albumin in co-culture system was up-regulated after coculture, which indicated the reparation of damage after coculturing. Also, by applying RT-PCR and immunocytochemistry differentiation status of ES cells were evaluated. It was shown that ES cells differentiated to hepatic lineage cells and expressed hepatic genes and proteins.

Key words: Hybrid cells, coculture; karyotype, differentiation.

INTRODUCTION

Cervical cancer is still one of the leading Fusion of heterotypic cells is important in development, tissue repair, and pathogenesis. Fusion occurs spontaneously in vivo and in vitro after transplantation and coculturing, respectively (1, 2). In particular, fusion occurs under selective pressures for example, cell damage (1, 3, 4). The idea of cell fusion was first reported by Barski et al. (5) and confirmed by Wang et al. (6) and Vassilopoulos et al. (7). It was clear that fusion, but not transdifferentiation, could explain liver regeneration in $FAH^{-/-}$ (fumarylacetoacetate hydrolase-deficient) mice, which is a model for liver regeneration (8). Later, Alvarez-Dolado et al. (9) confirmed cell fusion as the principal mechanism underlying the presence of bone-marrow-derived genomic materials in mature hepatocytes. Previous reports already had shown that cell fusion contributed to tissue repair (10-13). Thus, controversy was sparked in determining if regeneration of liver or other tissues proceeds through the fusion of stem cells with residual differentiated cells (14-16). An important basis for these controversies lies in the fact that cell fusion occurs at similarly low ratios among different tissues (17-21).

To clarify whether fusion contribute to tissue regeneration, it is necessary to determine the actual frequency of fusion because the frequency may be underestimated both in vivo and in vitro (22, 23) when using different analytical methods. For example, if we analyze fusion through FISH (fluorescent in situ hybridization), extra chromosomes, such as the Y chromosome, could be missed during tissue

Abbreviations: AFP, anti-alpha-fetoprotein; **BSA**, bovine serum albumin; **DAPI**, 4', 6-diamidino-2-phenylindole; **ESCs**, embryonic stem cells; **$FAH^{-/-}$**, fumarylacetoacetate hydrolase-deficient; **FISH**, fluorescent in situ hybridization; **SSEA-3**, Stage-specific embryonic antigens 3; **MEF**, mouse embryonic fibroblast; **MTT**, 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazohum bromide; **LDH**, Lactate dehydrogenase; **LIF**, leukemia inhibiting factor; **MDA**, malondialdehyde; **MSCs**, marrow stromal cells; **NSCs**, neural stem cells; **PI**, propidium iodide; **SGOT**, serum glutamic oxaloacetic transaminase; **SGPT**, serum glutamic pyruvic transaminase

sectioning. Moreover, if nuclei fuse, aberrant chromosome segregation will occur, and some chromosomes would be eliminated. Consequently, selected markers linked to the eliminated chromosomes may be lost after undergoing the various analytical processes. Therefore, the contribution to tissue repair from fusion may be underestimated in tissues, even when determined through advanced techniques. In addition, our knowledge of cell fusion is lacking, and the mechanism whereby it occurs is poorly understood, especially when fusion occurs under conditions of injury.

In the present study, we cocultured mouse ESCs (embryonic stem cells) with damaged human hepatic cells to determine the frequency of fusion under conditions of cell injury (supplement Fig. 1). Through direct karyotypic analysis of cocultured cells, we found that the frequency of fusion was actually higher than previously estimated. Chromosomes of damaged parental somatic cells took dominant positions in hybrid cells. Hybrid cells were GFP positive and expressed human stem cells markers Oct-4, Nanog and SSEA-3 (Stage-specific embryonic antigens 3), which means hybrid cells were totally or partially reprogrammed to stem cell-like cells. Results also showed that the damage was repaired following the coculturing process. In addition, after coculturing with damaged human liver cells, mouse ES cells differentiated to L-02-like cells and expressed liver-specific and functional genes. This outcome implies a potential method for differentiating ES cells directly to certain cell types.

Adult stem cells are rare in tissues about one adult stem cell occurs in 10^5 somatic cells but they maintain tissue functionality. Hybrid cells of the same or even higher ratio also can contribute to restoration and regeneration if hybrid cells are totally or partially reprogrammed. Future studies should aim to detect fusion with more scientific and full-scale protocols: for example, using markers linked to certain conditions or genes related to damage/repair of DNA or tissue.

MATERIALS AND METHODS

Cell culture

Mouse D3-ES cell line

The mouse D3-ES cell line was used as pluripotent partner cells in a cell coculture experiment. The D3-ES cells are undifferentiated pluripotent cells with a stable and normal karyotype (chromosome number, 40). This cell line was donated by Professor Huizhen Sheng (Shanghai Second Medical University). The cells were grown on the top of MEF (mouse embryonic fibroblast) feeder cells that had been inactivated with 0.01 mg mL-1 mitomycin C (MMC) in a standard ES cell media of advanced high-glucose DMEM (Dulbecco's Modified Eagle Media) (Gibco BRL, Grand Island, NY, USA). This high-glucose DMEM contained 15% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA); 1×penicillin/streptomycin (sigma); 1×nonessential amino acids (sigma); 0.1 mM 2-mercaptoethanol (sigma), and 1000 $U \cdot mL^{-1}$ leukemia inhibitory factor (LIF; ESGRO, Chemicon, CA, USA).

Human L-02 cell line

The L-02 cell line, an immortal cell line derived from embryonic human liver kindly provided by Professor Qinglong Guo (Department of Physiology, China Pharmaceutical University), was used as the other coculture partner. The L-02 cells are normal, non-tumorigenic hepatic cells. These cells were maintained in high glucose DMEM and supplemented with 15% newborn bovine serum (NBS, Hyclone, Logan, UT, USA) and 1×penicillin-streptomycin (sigma). The number of chromosomes in normal human L-02 cells was 46 while there was 1 non-diploid cell in 10^6 normal L-02 cells. Cells were transfected using GOF18, which carries EGFP under the control of the Oct-4 (Pou5f1) promoter. The Oct-4 promoter is active only in pluripotent and germline cells, and this transgene can be exploited as a convenient indicator in the acquisition of pluripotence. A stable transfected cell line was established after one month of G418 selection in the experiments described in the following paragraphs.

Establishing a model for cell damage

The L-02 cells were exposed to four concentrations (C1=600 μM, C2=400 μM, C3=200 μM, C4=100 μM, C5=50 μM) of the oxidative agent H_2O_2 for 0.5 hr, 1 h, 1.5 hr, 2 hr, and 4 hr, respectively. MTT (3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazohum bromide) assay was employed to detect cell survival rate. LDH (Lactate dehydrogenase), SGOT (serum glutamic oxaloacetic transaminase), and SGPT (serum glutamic pyruvic transaminase) activity in the supernatant were measured by spectrophotometry. A spectrophotometric assay kit (Jiancheng Nanjing) was used to measure the production of MDA (malondialdehyde). Total DNA of treated cells was isolated for apoptosis analysis. All assays were repeated three times.

Coculture

First, 10^5 L-02 cells were plated on 60 mm dishes 12 hr prior to treatment with H_2O_2 or H_2O (negative control) under optimum conditions and washed 3 times with phosphate-buffered saline (PBS) 2 hr after treatments. Then, 5×10^3 ES cells were seeded on top of L-02 cells. The cells were cocultured for 48 hr, and then all cells were trypsinized and fixed for counting chromosomes and undergoing karyotypic analysis. Meanwhile, the supernatant was collected for analysis of LDH, SGOT, and SGPT activity at 2 hr, 12 hr, and 48 hr, respectively. There were five repeats (dishes) in each group.

Karyotype

Karyotype analyzing was done as described previously (25). Cell number and non-diploid chromosomes were recorded from 10 slides prepared from each dish.

Detecting hepatic-specific genes by RT-PCR

Total RNA was extracted from cells using TRIzol (Invitrogen, USA). One microgram of RNA was used to synthesize cDNA by PCRamplification. The PCR conditions were as follows: initial denaturation at 94 ℃ (10 min) followed by 34 cycles (94 ℃ for 45 s, 56.7 ℃ for 45 s and 72 ℃ for 45 s) and an extension at 72 ℃ for 10 min. GAPDH (Eurogentec) was used as an endogenous control to normalize the mRNA level. Primers for PCR amplification were: hAlbumin (human specific primer 342 bp); Forward: 5'-GATGTCTTCCTGGGCATGTT-3'; Reverse: 5'-ACATTTGCTGCCCACTTTTC-3'; mAlbumin (mouse-specific primer 718 bp); Forward: 5'-TGAACTGGCTGACTGCTGTG3-3'; Reverse: 5'-CATCCTTGGCCTCAGCATAG-3'; mAFP (mouse-specific primer 609 bp); Forward: 5'-CCACCCTTCCAGTTTCCAG-3'; Reverse: 5'-GGGCTTTCCTCGTGTAACC-3'. GAPDH Forward:GATGCCCCCATGTTTGTGAT; Reverse: TTGCTGACAATCTTGAGTGAGTTGT. Primers were chosen from previously published sequences (26, 27).

Detecting hepatic-specific proteins by immunocytochemistry

Embryonic stem cells differentiated for 3 days were washed with PBS 3 times. Then, the cells were fixed with pre-colded 4% paraformaldehyde (freshly prepared) for 20 min. After aspirating the fixative, cells were washed 3 times for 10 min each time with PBS. Non-specific binding was blocked with PBS containing 5% BSA (bovine serum albumin) in phosphate buffered saline tween 20 (PBST) for 1 hr at room temperature. The cells were then incubated with primary antibody (AFP [anti-alpha-fetoprotein], Roche, Germany 1:100; CK-8&18, Chemicon, USA, 1:1000) in 1% BSA overnight at 4°C. Next, the cells were washed with 1×PBST 3 times for 10 min each time on a rocker. The cells were then incubated with a secondary antibody (anti-mouse-FITC for AFP (1:64) and CK8&18 (1:64) at room temperature for 2 h away from light. After 3 washes (5 min each) with PBST, the cells were exposed to 1 μg·mL^{-1} DAPI (4', 6-diamidino-2-phenylindole) or 20 μg·mL-1 PI (propidium iodide) solution for 10 min. After washing the cells 2 times for 5 min each time with 1×PBST, they were mounted with immunoXuore mountant (Sigma, USA). Negative control, omitting the primary antibody, also was carried out (data not shown). Images were captured using a Nikon DXM-1200F microscope.

RESULTS

Construction and evaluation of cell damage model (in supplement)

By treating hepatocytes with various concentrations of the oxidative agent H_2O_2 at different times, we analyzed the survival rate of cells, damage-related secretion, and DNA damage. The data showed that treatment in 2 hr with the C3 concentration caused the largest degree of damage to the hepatocytes, and the proportion of survival cells was greater than 50%. If the proportion of survival cells would have been less than 50%, the proportion of dead cells would have been high after a prolonged culture and would have been harmful to the cocultured cells. Thus, we considered the 2 hr treatment with the C3 concentration as the optimal condition for this system. Oxidative stress is a common method used to cause acute damage, and it was easy to control and evaluate. This model was feasible for the experiments described in the following paragraphs.

Hybrid cells in coculture system

To estimate the frequency of fusion, we counted chromosomes number of non-diploid cells (chromosome number of these cells was neither 40 nor 46) from all slides of both damaged group and non-damaged control group. The average ratio of non-diploid cells in the damaged group was 0.357‰ while the ratio in the control group was 0.031‰ (Table 1), which was almost 12 times greater than in the control group. To determine if the difference in cell numbers for each dish could affect the ratio of fusion, we counted the total cell number in all dishes and slides. The total cell number in the damaged group was less than in the control group ($p<0.05$), but no differences appeared among the 5 dishes in each group (Table 2). The difference in total cell number between the two groups likely was due to cell damage. All data indicated that frequency of non-diploid cells was much higher under the condition of damage.

To determine if non-diploid cells were hybrid cells, we analyzed the number and constitution of chromosomes in these cells. The number of chromosomes in non-diploid cells ranged from 30 to 107. The highest proportion of chromosomes in non-diploid cells was 57, and these cells were 25.71% of total non-diploid cells. The next highest proportion was 54, which was 20% of total non-diploid cells (Table 3).The number of chromosomes in non-diploid cells was larger than in each parental cell (40 or 46) but less than the summation number in both parental cells (86). To determine if these non-diploid cells derived from ES/ES hybrid, ES/L-02 hybrid, or L-02/L-02 hybrid, we paired and typed the chromosomes of non-diploid cells (Fig. 1) based on the length of the chromosome, centromere index, and differences in phenotype (mouse chromosomes are all telocentric), using the ISIS 5.0 karyotyping software and a digital imaging system (Metasystem, Altlussheim, Germany). Results showed that the non-diploid cells contained chromosomes from both parental cells. Most were human chromosomes while mouse chromosomes comprised only a small fraction of the total. This observation indicated that cell

Table 1. Cell and chromosome numbers of non-diploid cells in each group

Dish number (total number of non-diploid cells)	Damaged group					Control group				
	NO.1 (8)	NO.2 (6)	NO.3 (7)	NO.4 (7)	NO.5 (6)	NO.1 (0)	NO.2 (2)	NO.3 (0)	NO.4 (0)	NO.5 (1)
Chromosome number of each non-diploid cell	57 52 57 56 30 51 54 49	57 54 57 94 58 51	57 35 51 102 54 56 54	57 57 51 51 107 54 53	57 57 54 51 53 54		100 105			111
Total cell number on all slides in each dish	18,818	19,156	18,973	19,081	19,355	18,974	19,218	19,360	19,253	19,787
Ratio of no-diploid cells	0.425‰	0.313‰	0.369‰	0.367‰	0.309‰	0‰	0.104‰	0‰	0‰	0.051‰
Average	0.357‰					0.031‰				

Table 2. Cell numbers for each group in every dish

Damaged group					Control group				
NO.1	NO.2	NO.3	NO.4	NO.5	NO.1	NO.2	NO.3	NO.4	NO.5
1.15×106	8.5×105	1.27×106	8.2×105	9.1×105	1.81×106	1.05×106	1.93×106	1.64×106	1.37×106
Total: 5×106					Total: 7.8×106				

Table 3. Proportion and number of chromosomes in non-diploid cells

Damaged group			Control group		
Chromosome number	Total number (35)	proportion	Chromosome number	Total number (3)	proportion
30	1	2.86%	100	1	33.33%
35	1	2.86%	105	1	33.33%
49	1	2.86%	111	1	33.33%
51	6	17.14%			
52	1	2.86%			
53	2	5.71%			
54	7	20.00%			
56	3	8.57%			
57	9	25.71%			
58	1	2.86%			
94	1	2.86%			
102	1	2.86%			
107	1	2.86%			

fusion had occurred between two parental cells, and the dominant position of chromosomes derived from injured parental cells. In addition, L-02 cells carried a GFP transgene under the control of the Oct4 promoter. If fusion occurs, there would be GFP positive cells in the coculture system. It is important to note that GFP fluorescence was detected at 60 hr in the coculturing process (Fig. 2C).

Then we picked GFP positive hybrid cell clones and passaged them on MEF feeder layer. Hybrid cells grew like ES clones and they were still GFP positive (Fig. 2D). Meanwhile, they were positive for human stem cell markers Oct-4, Nanog and SSEA-1 (Fig. 3). Again, this observation demonstrated cell fusion and, potentially, either a total or partial reprogramming of hepatic cells by ES cells.

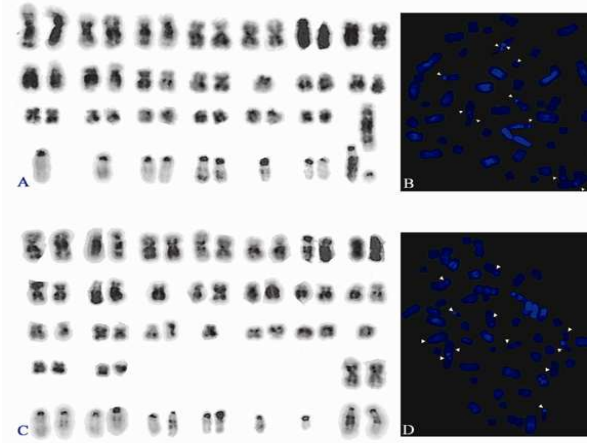

Figure 1. Karyotypes of hybrid cells. Karyotype of one hybrid cell with 51 chromosomes (A, B). Karyotype of another hybrid cell with 57 chromosomes (C, D). It is clear that there are two kinds of chromosomes in one hybrid cell. The larger portion of chromosomes is from human, and the smaller portion is from mouse. White triangle indicate mouse chromosome.

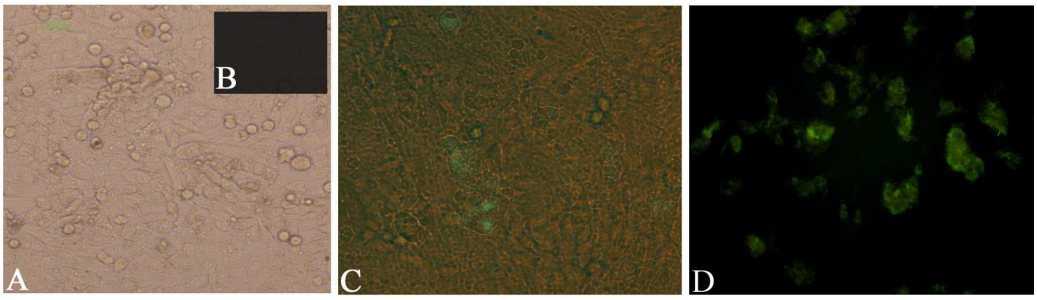

Figure 2. Oct-4 reactivation in cocultured cells. (A) Oct-4 does not re-express before 48 hr as seen under fluorescent excited light (B). (C) GFP is detected at 60 hr of coculture (×400). (D) GFP positive passage 3 hybrid cells.

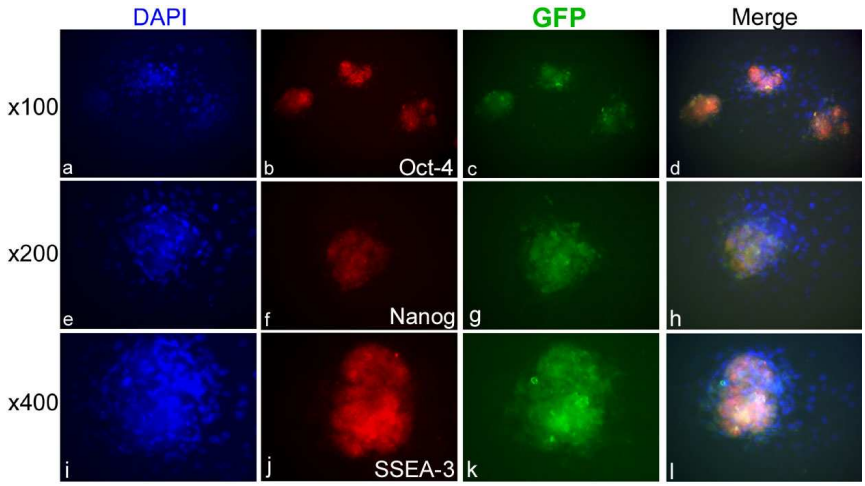

Figure 3. IF staining of reprogrammed hybrid cells of passage 4. Hybrid cells on passage 3 still GFP positive (c,g and k). Hybrid cells express pluripotent stem cells markers Oct-4, Nanog and human stem cell marker SSEA-3. DAPI staining of hybrid cell nuclei (blue) (a, e and i). The stem cell markers Oct-4 (b), Nanog (f) and SSEA-3 (j) are a hallmark of pluripotent stem cells and are expressed in the hybrid cells, which means hybrid cells are reprogrammed stem cell-like cells. Primary antibodies to Oct-4, nanog and SSEA-1 were utilized and PE labeled secondary antibodies were used to visualize genes' expression in the hybrid cells (red). Merged images are shown in d, h and l.

Behavior of ES cells in the coculture system

Twelve hours after the coculturing process began, mES clones showed typical ES cell clone morphology on the damaged L-02 cell layer (Fig. 4A, B). However, after the 40 h of coculturing, the ES cell clones became flat, and their morphology as clone disappeared. ES cells differentiated and resembled cocultured L-O2 cells (Fig. 4C, D). To study the fate of ES cells, ES cell clones were picked up 24 hr after coculturing and replated onto gelatin-coated dishes in conditioned DMEM (15% FCS [fetal cattle serum]+30% supernatant derived from the coculture system) without LIF (leukemia inhibiting factor). After another 24 hr sub-culture, ES cells differentiated into L-O2-like cells (Fig. 5) and expressed the liver-specific gene AFP, as well as albumin (Fig. 6). Results of immunofluorescence analysis showed immunoreactivity for the epithelial marker CK8&18 (Fig. 7 A) and the hepatic-specific marker AFP (Fig. 7 B) in differentiated ES cells.

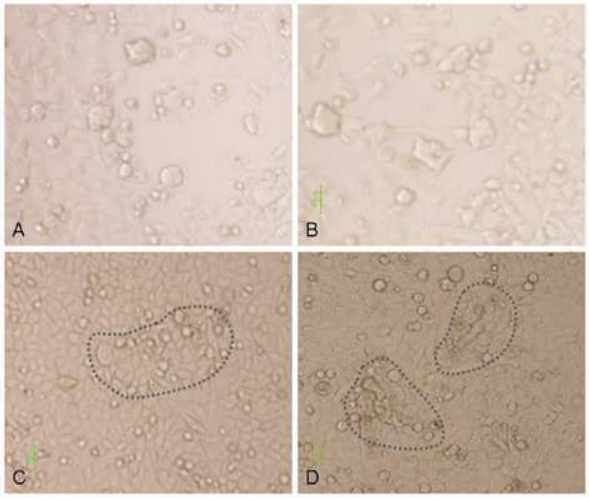

Figure 4. Behaviors of ES cells in coculture system. (A, B) 12 hr after coculture. Clones still can be seen on top of the L-02 cell layer, but they are not typical ES cell clones. (C, D) 48 hr after coculture. ES cells are enclosed in the dashed lines (×100).

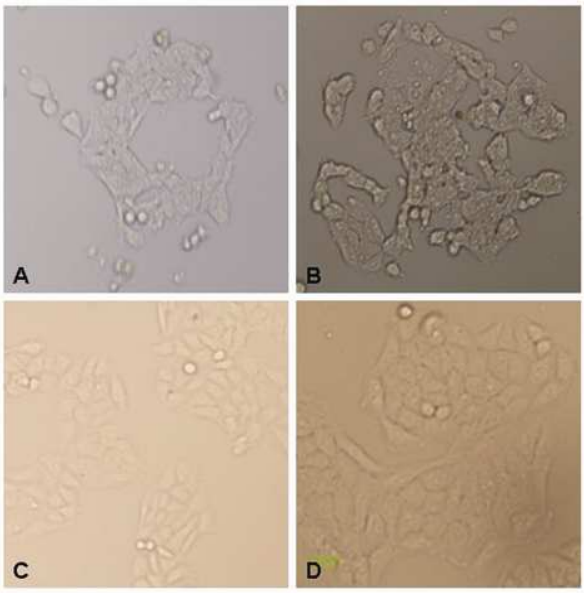

Figure 5. Phenotype of differentiated ES cells which are picked up from the coculture system. (A, B) L-O2-resembled, differentiated ES cell clones (×200). 24 hr after coculture, ES cell clones are picked up (using the same method of picking up ES cells from feeder cells) and replanted on dishes in conditioned DMEM (15% FCS+30% supernatant derived from the coculturing system) without LIF. After 24 hr sub-culture, ES cell clones differentiate to normal L-02-like cells. (C, D) Normal L-02 cells.

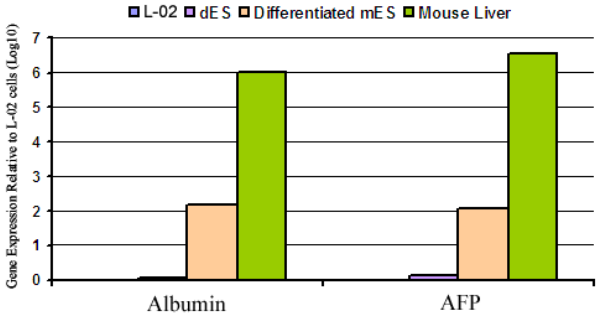

Figure 6. RT-PCR analysis of mouse hepatic-specific gene AFP and mouse hepatic functional gene albumin of differentiated ES cells. ES cells are picked up from the coculturing system at 24 hr and differentiate during another 24 hr. They express hepatic-specific gene AFP and the hepatic-functional gene albumin. Adult mouse liver was used as the positive control. Human L-02 cells and dES (differentiated ES cells at 48 hr without coculture) were used as the negative control.

Repair of damage

To determine if L-02 cells' damage was repaired in this system, we tested LDH activity at 2 hr and 48 hr and for SGOT and SGPT activity in co-culture system at 12 hr and 48 hr, respectively. We compared the expression of albumin between cocultured L-02 cells and normal L-02 cells at 48 hr. Injured L-02 cells (with H_2O_2) without coculture were used as negative control. Both SGOT and SGPT activity progressively decreased during coculture. Until 48 h, the activity of both enzymes was not significantly different from the control group ($P<0.05$) (Fig. 8A, B). Activity of LDH was significantly higher than in the control group at 2 hr ($P<0.01$), but there was no difference at 48 hr; LDH activity in negative control was significant higher than both cocultured group ($P<0.01$) (Fig. 8C).

Moreover, expression of albumin was upregulated during coculture (Fig. 9). In summary, all results suggested that injury of L-02 cells in coculture system had decreased.

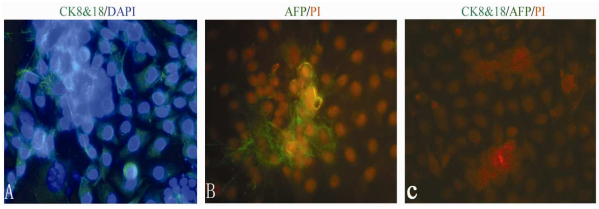

Figure 7. Immunofluorescence analysis of differentiated ES cells using mouse epithelium-specific marker CK8&18 (A) and mouse hepatic specific marker AFP (B). Immunofluorescence analysis of differentiated ES cells using human antibodies is used as the negative control (C). ES cells were picked up from the coculture system and replated on gelatin-coated dishes in conditioned DMEM (15% FCS+30% supernatant derived from coculuture system) without LIF for 2 days.

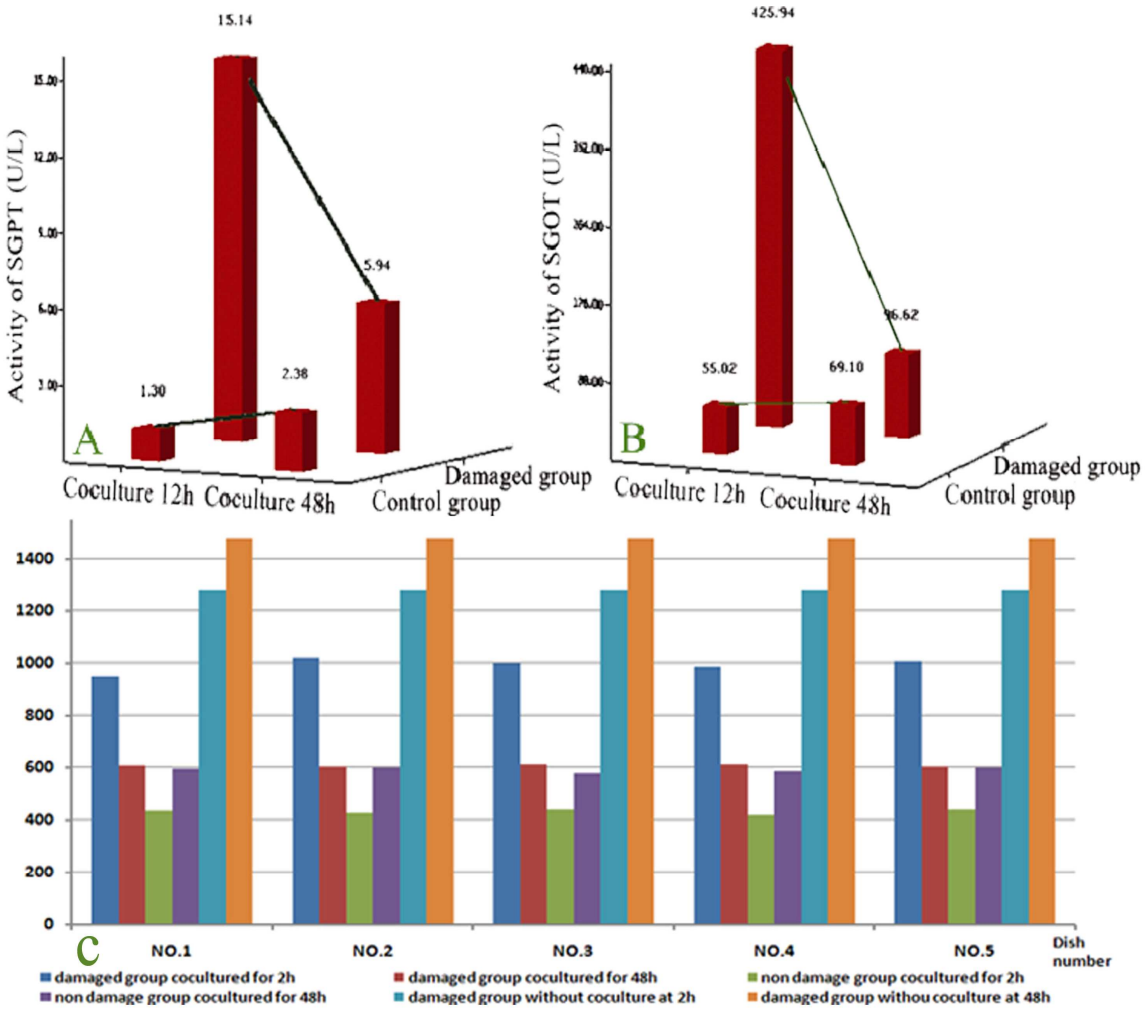

Figure 8. Release of damage-related enzymes detected by spectrophotometry assay after coculturing. Data are mean±SD (n=5). Activity of SGPT (A) and SGOT (B). In damaged group, activity of SGPT and SGOT progressively decreases during coculture. At 48 hr, their activity is not significantly different from the control group ($P<0.05$). Release of LDH (C) was significantly different at 2 hr between the two groups in all 5 dishes, but no difference was observed at 48 hr between the two groups. It appears that the injured L-02 cells have recovered to normal L-02 cells after 48 hr of coculturing.

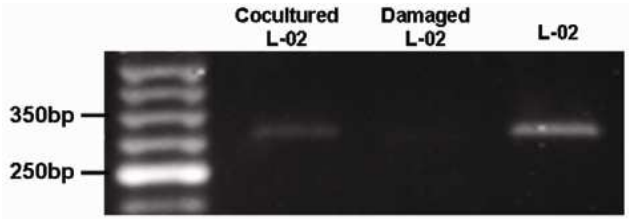

Figure 9. Analysis of albumin expression in cocultured L-02 cells by RT-PCR at the end of the coculturing process. Normal L-02 cells were used as the positive control; injured L-02 cells were used as the negative control. It is suggested that the expression of albumin is upregulated by coculturing with ES cells.

Supplement data

MTT

Data showed that cells treated with C3, C4 and C5 (Supplement Fig. 2) could survive over 50%. The survival rate of C5 was more than 100% (low concentration of H_2O_2 can improve cell proliferation), so C3 and C4 were chosen for the following experiment.

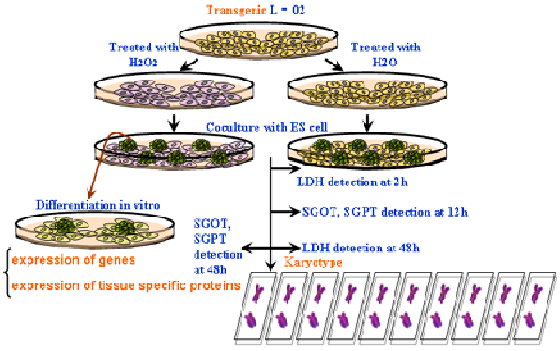

Figure 1. Schematic illustration about this study

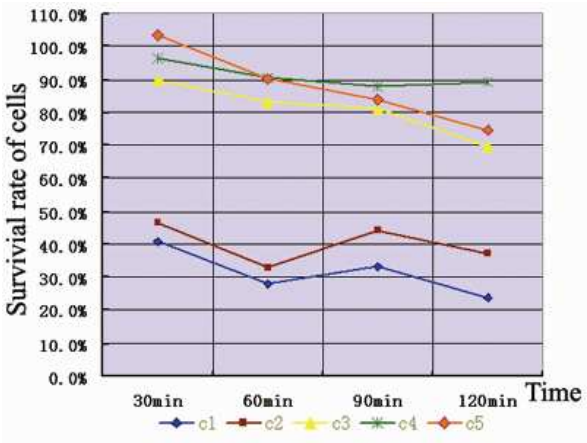

Figure 2. Cell survival rate after H_2O_2 treatment detected by MTT assay. Data are Mean±SD (n=5). C1=600μM,C2=400μM,C3=200μM, C4=100μM, C5=50μM. Survival rate of cells treated with C1, C2, C3 are more than 70%, survival rate of ells treated with C4, C5 are less than 50% which is not suitable for following experiments.

MDA content

MDA content was detected in the supernatant by spectrophotometry using assay kit.. The level of MDA is often used as an indication of oxidative damage and as a marker for free radicals-induced lipid peroxidation. MDA can be condensed with thiobarbituric acid (TBA) to produce red production. Damage extent can be detected by using colorimetric analysis. The result demonstrated that MDA content was treat time- and H_2O_2 dose-dependent (Supplement Fig. 3). MDA content of C3 group was significantly higher than C4 group at all four time points. In addition, MDA content of C3 at 1.5h was significantly different with MDA content at 2h. It showed that C3 was more suitable than C4 in this experiment.

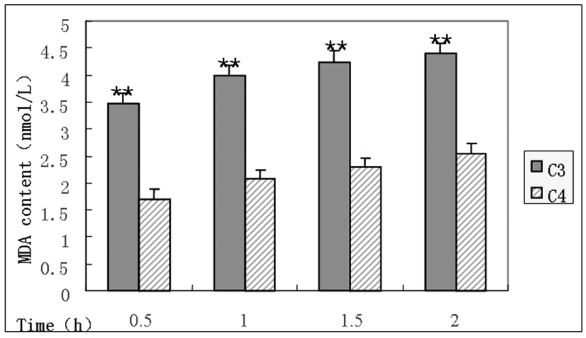

Figure 3. Effects of H_20_2 on the production of MDA detected by spectrophotometry assay. Data are mean±SD (n=3). **p<0.01 vs C4 group. MDA content of C3 group is significant different with C4 group at all four time point. In addition, MDA content of C3 at 1.5h is significant different with MDA content at 2h. It is indicated that C3 is more suitable than C4 in this experiment.

LDH, SGOT and SGPT activity

LDH is an enzyme that catalyzes the conversion of lactate to pyruvate. SGOT and SGPT are enzymes that indicate damage of liver. Many different types of cells contain these enzymes. Heart, kidney, liver, and muscle are relatively rich in LDH, SGOT and SGPT. LDH, as markers, are measured to evaluate the presence of tissue damage. LDH activity was also treat time- and H_2O_2 dose-dependently increase (Supplement Fig. 4), same with the trend of MDA content. LDH activity of C3 group was significant different from C4 group except at 1.5h. LDH release was significantly different at 1.5h and 2h, after treatment with C3. Activity of SGOT and SGPT increased in time-dependant and dose-dependent style (Supplement Fig. 5).

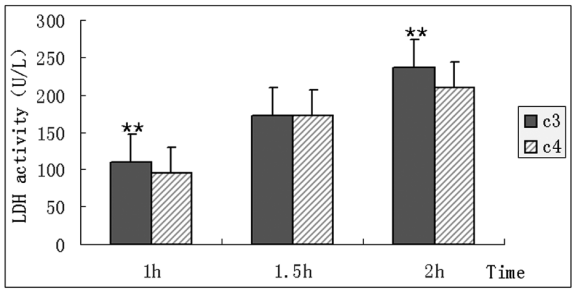

Figure 4. LDH release detected by spectrophotometry assay. Data are mean±SD (n=3). **p<0.01 vs. C4 group. LDH activity of C3 group is significant different with C4 group except at 1.5h. LDH release is significant different at 1.5h and 2h, after treatment with C3.

In short, damage of L-02 was time- and dose- dependent. Cells got the worst damage after treatment of C3 at 2h and more than half of total cells survived. So the optimum condition of treatment in this model was C3 for 2h.

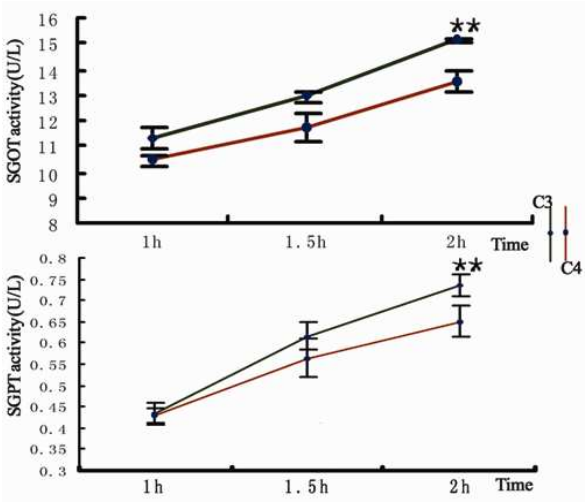

Figure 5. Activity of SGOT and SGPT. Data are mean±SD (n=3).
Activity of SGOT and SGPT increase in time-dependant and dose-dependant style. SGOT and SGPT activity of C3 group are significant higher than C4 group at 2h ($P<0.01$),
**p<0.01 vs. C3 group at 1.5h.

Apoptosis after treatment

DNA damage (Supplement Fig. 6) presented that late apoptosis happened but not as severe as damage occurred in control group □.

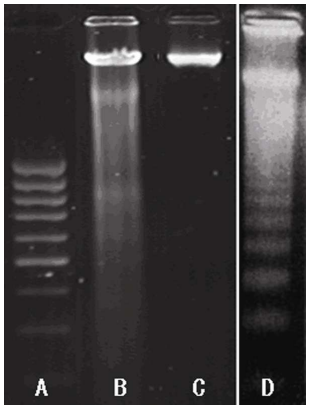

Figure 6. DNA damage of LO2 treated with the optimum time and dose. A: marker (100bp) B: 2h after treatment with C3, DNA ladder shows that apoptosis occurs after treatment. C: control□ (no treatment) D: control□ (late apoptosis after heat-shocked damage

DISCUSSION

High frequency of hybrid cells in coculturing system

Forty-eight hours after coculturing, we counted the cells and the chromosomes for non-diploid cells and calculated the percentage of non-diploid cells on each slide. No significant difference was observed in the number of cells among all five dishes or on all slides in each group. However, the proportion of non-diploid cells in the coculture system (coculturing ES cells with damaged cells) was at least 10 times the proportion of spontaneous cell fusions occurring in the normal coculturing system (coculturing ES cells with normal cells). It was reported that when ES cells were cocultured with normal cells in vitro, generally, the frequency of spontaneous fusion was 1 cell in 105 to 106 plated cells (1, 2, 28, 29). In addition, the highest frequency of spontaneous fusion was 4 cells in 470,000 plated cells occurring in a hepatocyte-ES-cell coculturing system (29). When MSCs (marrow stromal cells) were cocultured with heat-shocked cells in vitro, the ratio of cell fusions was 1 cell per 105 cells (4). Recently, Jessberger S et al. (2007) reported that approximately 0.2‰ of rat and mouse NSCs (neural stem cells) fused in a coculturing system, but fused cells in the system did not proliferate and could not be propagated (30). However, in this coculturing system, when ES cells were cocultured with damaged hepatocytes, the ratio of fusion ranged from 0.309–0.425‰, which equated to at least 3 fused cells in 10^4 cocultured cells.

These differences are due to three reasons. The first reason for the differences predicates on the coculturing microenvironment. In previous studies, parental cells, generally, were normal cells; however, here it is suggested that when ES cells are cocultured with normal cells, the ratio of spontaneous fusion is lower than the ratio under conditions of damage (1, 2, 28, 29). In addition, it has been reported that fusion is inclined to occur under conditions of selective stress, such as drug selection, damage, inflammation, and induction of inflammatory cytokines (31, 32). Spees et al. (2003) cocultured ES cells with heat-shocked cells while in the present study, an oxidative agent was used to induce injury.

The second reason is the effects of different methods used to detect fusion. Generally, as previously mentioned, fusion is detected by specific markers of parental cells, such as the Y

chromosome (17, 18, 20, 33). However, re-segregation and elimination of chromosomes occur easily during the fusion process; therefore, if chromosomes are missing, the markers may be lost, and hybrid cells may be missed. By analyzing normal mouse ES/ES hybrid cells or normal mouse ES/somatic hybrid cells, Matveeva et al. (32) found that ES/ES hybrid cells demonstrated stable tetraploid cells during in vitro cultivation for both selective and nonselective conditions. Over 90% of the cells contained 75–85 chromosomes, and 60–80% contained 80 chromosomes. However, chromosome segregation occurred after fusion of ES cells with somatic cells in both selective conditions. Over 80% of the cells contained from 60–70 chromosomes with a mean 64–65 chromosomes, although there were a few cells that contained less than 50 chromosomes. There were two types of clones in inter-hybrid cells: (a) bilateral loss of chromosomes for both ES cells and somatic partners and (b) unilateral segregation of chromosomes in the somatic partner. All demonstrated loss of chromosomes in hybrid cells.

The third reason predicates on the type of parental cells. It has been reported that pluripotent cells fuse more efficiently with pluripotent cells than with somatic cells (34), and epithelial cells fuse more easily than other types of somatic cells (22). Many researchers have demonstrated that certain factors associated with karyoplasts (35), cytoplasts (36), or cybrids (37) in pluriportent cells can contribute to the fusion process (38). The pattern of fusion also could affect the fusion event. Therefore, there are numerous factors that can influence the formation fusion cells. Karyotyping is the most simple but direct way to evaluate fusion. Through large scale statistical procedures, we were able to detect fusion directly by counting and karyotyping chromosomes. This procedure showed that fusion occurred with the ratio of fusion being higher than previously know. In addition, EGFP, whose expression is under the control of Oct-4 from hepatic cells, was detected after the coculturing process. The EGFP signal proved fusion and potential reprogramming.

Due to the presence of non-diploid cells, heterokaryons (hybrid cells) can undergo mitosis and cell division as monocytes and binucleate or multinucleate units if the constituent nuclei enter hybridization at a similar stage in the mitotic cycle. As reported by Brenda M Ogle et al. (2004)(28), heterokaryons act as intermediates during the fusion process. If cell sorting and selective loss of chromosomes occur under conditions where the cell remains viable or if an intact nucleus is shed, then what we detect are still “normal cells” synkaryons with only one nucleolus. In addition, if we cannot detect loss or re-segregation of chromosomes, we are not able to determine the proportion of hybrid cells. For example, human bone-marrow-derived cells were injected into fetal pigs. Several months after birth, a study of the peripheral blood from pigs showed that more than 60% of the cells contained DNA from both humans and pigs in a single nucleus (synkaryon) (28). This synkaryon could explain in part the inconsistencies observed during a fusion event. In the present study, the greatest number of chromosomes in non-diploid cells was less than 86 (40+46), which indicate that chromosomes in one or both parental cells are missing or not all chromosomes were involved in the fusion event. The biggest question is how chromosomes are sorted and lost or reorganized. Ogle et al. (2005) has stated that, in synkaryons, chromosomes may be shed by reduction division, as in somatic meiosis (39) or multipolar mitosis (the formation of multipolar spindles in mitosis). For example, reduction division of fused cells has been offered as one explanation for the regeneration of liver tissue (6).

Therefore, based on the length of the chromosome, centromere index, and differences in karyotype, we coupled and typed chromosomes of cells using ISIS 5.0 software from Metasystems. We found that in hybrid cells, chromosomes from damaged cells were dominant; Hybrid cells lost most part of the chromosome from non-damaged parental cells mouse ES cells after 48 hr coculture. However, it was reported that when fusion occurred between normal human cells and mouse cells, hybrids lost most human chromosomes. Matveeva et al. (2005) found in a study where microsatellite analysis and in situ hybridization with labeled species-specific probes were utilized that preferential elimination of chromosomes in the somatic partner was a characteristic of inter-specific hybrid cells. Our experiment differed from the Matveeva et al. (2005) study in that ES cells were cocultured with damaged hepatocytes rather than normal somatic cells. In addition, results from the Matveeva et al. (2005) study were based on the condition of selection and evaluated after several cell passages while our study was based on cocultured cells that did not

undergo either selection or passaging. Perhaps our result was a phenomenon in the cell-damage model thus requires further research to confirm. It is important to note that the function of hybrid cells is not determined by the number of chromosomes even if hybrid cells lose chromosomes derived from pluripotent or somatic cells. Further, it has been reported that in intra-specific and inter-specific hybrid cells, that the contribution from the somatic partner varied from a single chromosome to a complete complement (32, 40). After evaluating the pluripotency of ES cells, the researchers demonstrated that pluripotency is manifested as a dominant trait in the ES hybrid cells and does not depend substantially on the number of somatic chromosomes. These results suggest that the developmental potential derived from ES cells is maintained in ES-somatic cell hybrids by the cis manner, and this developmental potential is rather resistant to trans-acting factors emitted from the somatic cell (40).

Moreover, ES cells differentiated to L-02-like cells cultured either in a coculture system or alone on gelatin-coated dishes. The differentiated ES cells expressed AFP and albumin, which indicated that the coculturing system not only promoted fusion but also promoted differentiation of ES cells to functional hepatic cell types. We assumed before that under coculturing condition, ES cells would fuse spontaneously with damaged hepatocytes and supply damaged cells with the chromosomes which needed for repair. However, in this study, ES cells actually adopted the phenotype of damaged cells and expressed hepatic specific genes, with both capable of contributing to the repair process. In addition, results indicated that under condition of damage, pluripotent cells could become one potential participator of fusion.

Repairing damaged hepatocytes

We have analyzed the contribution of the coculture system to restoring damaged hepatocytes. We detected activity of three hepatic cell-damage enzymes, LDH, SGOT, and SGPT, at 2 hr and 48 hr after coculturing. Activity of the three enzymes in the damaged group was significantly higher than in the normal control groups at 12 hr ($P<0.01$); however, at 48 hr of coculture, there were no significantly differences between the two groups ($P<0.05$). The SGOT and SGPT enzymes are special and sensitive indicators of liver injury. In the control group where all cells were normal cells, the activity of the three enzymes was higher after coculturing. It was a normal biochemical and physiological phenomenon when cells were cocultured in vitro for a long time, especially when cells were in a high confluence. However, the activity of the three enzymes in the damaged group was dramatically lower, which demonstrated injury repair. In addition, expression of albumin was upregulated after coculturing damaged L-02 cells with ES cells. Differentiated ES cells and not hybrid cells may contribute to these effects because the coculturing process was not long enough for hybrid cells to become functional. However, the relationship between fusion and repair must be distinguished. Until now, our studies have concentrated on the relationship between fusion and repair. Despite experimental limitations, this study suggests that the injury of damaged hepatocytes was reversed after coculturing with ES cells.

In conclusion, hybrid cells appeared after coculturing ES cells with damaged hepatocytes; the damaged hepatocytes seemed to be repaired; and the ratio of fusion was far higher than previously reported. These results show that fusion to some extent plays an important role in cell damage repair. The problem is how to detect and analyze fusion events. Markers and powerful techniques such as FISH can be used to detect fusion, but they are limited by a high “missing” rate. Karyotyping is a simple but more explicit method. Although techniques for whole-genome FISH are available, they are costly and not convenient for wide use, especially in researches in which fusion will be analyzed in large-scale cultured engineered cells. Of course, karyotyping also can cause errors; for example, we will not “find” fused cells by karyotyping during fusion if the number of chromosomes is the same as the parental cells after re-segregation. The methods described in this study are suitable for detecting fusion in vitro, but inadequate for detecting fusion in vivo. However, our study minimally has demonstrated that during the damage repair process, the proportion of fused cells is much higher than previously believed. We should improve our methods for detecting and analyzing cell fusion. One promising protocol relies on choosing markers based on certain conditions; for example, choosing markers that are related to the damage. We are researching and validating this protocol currently.

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