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Research article

HYBRID CELLS DIFFERENTIATE TO HEPATIC LINEAGE CELLS AND REPAIR OXIDATIVE DAMAGE

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Abstract: Hybrid cells derived from stem cells play an important role in organogenesis, tissue regeneration and cancer formation. However, the fate of hybrid cells and their range of function are poorly understood. Fusing stem cells and somatic cells induces somatic cell reprogramming, and the resulting hybrid cells are embryonic stem cell-like cells. Therefore, we hypothesize that fusion-induced hybrid cells may behave like ES cells in certain microenvironments. In this study, human hepatic cells were induced to apoptosis with H_2O_2, and then co-cultured with hybrid cells that had been derived from mouse ES cells and human hepatic cells using a transwell. After co-culturing, the degree of apoptosis was evaluated using Annexin-V/PI double-staining analysis, flow cytometry and Western-blot. We observed that H_2O_2-induced cell apoptosis was inhibited by co-culture. In addition, the activity of injury-related enzymes (GSH-Px, LDH and SOD) and the level of albumin release in the co-culture system trended toward the level of normal undamaged hepatic cells. The stably increased levels of secretion of ALB in the co-culture system also confirmed that co-culture with hybrid cells helped in recovery from injury. The fate of the hybrid cells was studied by analyzing their gene expression and protein expression profiles.

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Abbreviations used: AAT – alpha-1 antitrypsin; CPSASE1 – carbamoyl-phosphate synthetase I; GSH-Px – glutathione peroxidase; HNF3 – hepatocyte nuclear factor 3; LDH – lactate dehydrogenase; LST1 – liver-specific organic anion transporter; MEF – mouse embryonic fibroblast; MSCs – mesenchymal stem cell; PEPCK – phosphoenolpyruvate carboxykinase; TDO – tryptophan 2,3-dioxygenase; TTF – tail tip fibroblast; TTR – transthyretin; SOD – superoxide dismutase

The results of RT-PCR indicated that during co-culturing, like ES cells, hybrid cells differentiated into hepatic lineage cells. Hybrid cells transcripted genes from both parental cell genomes. Via immunocytochemical analysis, hepatic directional differentiation of the hybrid cells was also confirmed. After injecting the hybrid cells into the mouse liver, the GFP-labeled transplanted cells were distributed in the hepatic lobules and engrafted into the liver structure. This research expands the knowledge of fusion-related events and the possible function of hybrid cells. Moreover, it could indicate a new route of differentiation from pluripotent cells to tissue-specific cells via conditional co-culture.

Key words: Hybrid cells, Regeneration, Embryonic stem cell, Differentiation, Oxidative damage, Apoptosis

INTRODUCTION

Cell-cell fusion is a highly regulated cellular event that plays a role in tissue regeneration. The concept of cell fusion was first reported by Barski *et al.* in 1960 [1] and it was confirmed by Wang *et al*. [2] and Vassilopoulos *et al*. [3]. Since then, a number of studies have indicated the importance of cell fusion in tissue regeneration [4-11]. It was clear that fusion, but not transdifferentiation, could explain liver regeneration in $FAH^{-/-}$ mice, which is a model for liver regeneration [12]. Later, Alvarez-Dolado *et al.* [13] confirmed cell fusion as the principal mechanism underlying the presence of bone marrow-derived genomic materials in mature hepatocytes using a unique Cre/lox model system. However, little was known about the fate and function of hybrid cells, how they contribute to tissue regeneration, and how they change during the process of regeneration.
Fusing somatic cells with stem cells allows the resulting hybrid cells to be reprogrammed as stem cell-like cells. Recently, hybrid cells derived from stem cells have been proven to be reprogrammable into ES cell-like cells *in vitro* [14-20]. They are pluripotent and are capable of contributing to cells from all three germ lines. This indicates that reprogrammed hybrid cells might act like stem cells. For example, they can differentiate directly into tissue-specific cells in certain microenvironments. Thus, one potential function of hybrid cells is to contribute to tissue regeneration in a similar manner to adult stem cells. Under certain conditions such as co-culturing, hybrid cells have the capacity to differentiate into tissue-specific cells, in a process like the directional differentiation of pluripotent stem cells.
It has also been reported that a proper co-culture system promotes the differentiation of stem cells such as embryonic stem cells and MSCs (mesenchymal stem cell) [21-26]. Moreover, a number of groups have presented evidence that co-culturing stem cells with somatic cells could affect the fate and biochemical functions of the latter [27-31]. In this study, we co-cultured injured liver cells with hybrid cells using transwells to elucidate the fate of hybrid cells under conditions of injury, and to investigate how they contribute to the healing of injury. In this, we assume that hybrid cells act like stem cells.

In our previous study, we developed a H_2O_2-induced cell apoptosis model. We co-cultured ES cells with damaged L-02 cells and found that a high ratio of fusion occurred. Based on these results, we decided to fuse human hepatic cells (L-02 cells) with mouse ES cells to obtain reprogrammed hybrid cells. Then we devised a strategy for co-culturing hybrid cells with apoptotic L-02 cells using a transwell protocol in order to study the fate of hybrid cells and injured cells in the same microenvironment. We found that, like ES cells, hybrid cells were able to differentiate into hepatic lineage cells. On the other hand, damage to co-cultured hepatic cells was repaired by inhibiting apoptosis. Taken together, these observations suggest that, like stem cells, hybrid cells could differentiate into functional hepatic lineage cells and help repair injury of damaged cells by inhibiting their apoptosis after co-culturing. We hypothesize that in the injured liver, hybrid cells (derived from MSC, HSC or liver stem cells) differentiate and proliferate quickly into tissue-specific functional cells in order to ensure regeneration. Meanwhile, via cell-cell contact or via specific protein cytokines and epigenetic factors that they secrete, the apoptosis of all the cells in injured tissue is inhibited. Cells will then proliferate normally and the injury will be healed.

MATERIALS AND METHODS

Cell culture

Mouse cell line D3-ES. The D3-ES cell line (ATCC number: CRL-11632™), donated by Professor Huizhen Sheng (Shanghai Second Medical University), was grown on mouse embryonic fibroblast (MEF) feeder cells that had been inactivated with 10 μg ml^{-1} mitomycin C (MMC) in standard ES cell medium: advanced-high glucose DMEM (Gibco BRL, Grand Island, NY, USA) containing 15% heat-inactivated fetal bovine serum (FBS, Hyclone, USA); 1 × penicillin/streptomycin (P7539-100 ml, Sigma); 1 × non-essential amino acids (M7145-100 ml, Sigma); 0.1 mM 2-mercaptoethanol (M7522-100 ml, Sigma) and 1000 U ml^{-1} leukaemia inhibitory factor (LIF, ESGRO, Chemicon, CA, USA).

Human cell line L-02. L-02 cell line (HL-7702) immortal cells derived from embryonic human liver were provided by Professor Qinglong Guo, Jiangsu Key Laboratory of Carcinogenesis and Intervention (China Pharmaceutical University). L-02 cells are non-tumorigenic normal hepatic cells. The cells were maintained in high glucose DMEM (Gibco BRL, Grand Island, NY, USA) supplemented with 15% new-born bovine serum (NBS, Hyclone, USA), and 1 × penicillin/streptomycin.

Transfection. L-02 cells were transfected with phOct4-EGFP using Lipofectamine 2000 (cat. no. 11668-019, Invitrogen). The stably transfected L-02 cells were named Oct4-L-02. Oct-4 can be exploited as a convenient indicator of the acquisition of pluripotency [32]. After 12 days of selection using 350 μg ml^{-1} G418, the surviving colonies were trypsined and subcloned to

harvest stable transgenic Oct4-L-02 cells. Stably transfected cells were established after one month of G418 selection for the subsequent experiments.

Hybrid cells. Hybrid cells were generated from the human transgenic immortal hepatic cell line Oct4-L-02 and the mouse embryonic stem cell line D3-ES. Fusion was induced with polyethylene glycol 1500 (Molecular Biochemicals Roche) according to the manufacturer's instruction. After fusion, cells were selected with 350 μg ml^{-1} G418 for 12 days to harvest GFP-positive ES-like colonies. The culture system of hybrid cells was the same as that for ES. The hybrid cells used in the experiments were from passages 3 through 5. Details of the identification of hybrid cells can be found in the supplementary material (http://dx.doi.org/10.2478/s11658-010-0018-0).

Co-culture

The analysis of the L-02 cells. Ten thousand L-02 cells were plated in a 24-well plate. After 12 h, the cells were damaged with 200 μM H_2O_2 for 2 h. After the cells had been washed three times with PBS, hybrid cells from a 2-day culture in a transwell (Cat. No. 3470, 0.4 μm, Corning, NY) were inserted into wells with injured L-02 cells. The media contained advanced-high glucose DMEM with 15% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 1 × penicillin/streptomycin; 1 × non-essential amino acids; 0.1 mM 2-mercapto-ethanol and 1000 U ml^{-1} LIF.

The analysis of the hybrid cells. Injured L-02 cells (200 μM H_2O_2, 2 h) were seeded in the lower compartment of the 24-well plate and changed every day. One thousand hybrid cells were plated in transwells (cat. no. 3472, 3.0 μm, Corning, NY) in a medium containing: KSR DMEM (cat. no. 10829 GIBCO-Invitrogen, Carlsbad, CA, USA) with 15% KSR (cat. no. 10828 GIBCO-Invitrogen, Carlsbad, CA, USA); 1 × penicillin/streptomycin; 1 × non-essential amino acids; and 0.1 mM 2-mercaptoethanol, without LIF.

Cell morphological assessment

Co-cultured L-02 cells were stained with a dye mixture containing 100 mg l^{-1} acridine orange (AO) and 100 mg l^{-1} ethidium bromide (EB) in PBS at 24, 48 or 60 h. The cells were observed immediately under a fluorescence microscope (Olympus IX51, Japan) with a peak excitation wavelength of 490 nm.

Annexin-V/PI double-staining assay and flow cytometry

Early apoptotic cells were labeled with Annexin-V^+/PI^-. Cells at each time point (24, 48 and 60 h) were harvested, and 1×10^6 cells were washed and re-suspended in PBS. The apoptotic cells were identified by double supravital staining with recombinant FITC-conjugated Annexin-V and PI, using the Annexin V-FITC Apoptosis Detection kit (BioVision, USA) according to the manufacturer's instructions. Flow cytometric analysis was performed immediately after supravital staining. Data acquisition and analysis were

performed in a Becton-Dickinson FACSCalibur flow cytometer using CellQuest software.

Western-blot analysis for p53, bcl-2, bax, bcl and caspase-3 proteins

After co-culturing with hybrid cells, the proteins of the L-02 cells were isolated with a lysis buffer (100 mM Tris-Cl, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol, 200 mM 2-mercaptoethanol, 1 mM PMSF, and 1 g ml^{-1} aprotinin) and measured using the Bradford assay with a BioPhotometer (BioPhotometer 6131 GB/HK, Eppendorf) at 595 nm at 24, 48 or 60 h. The protein samples were separated with 15% SDS-polyacrylamide gel (SDS-PAGE) and transferred onto PVDF membranes (Millipore). The immune complexes were formed by incubating the proteins with primary antibodies (rabbit anti-Bax, rabbit anti-Bcl-2, rabbit anti-caspase-3, and goat anti-Actin) overnight at 4ºC. The blots were washed and incubated for 1 h with IRDye 800-conjugated anti-goat and anti-rabbit secondary antibodies. All of the antibodies were obtained from Santa Cruz Biotechnology, CA, USA. The immunoreactive protein bands were detected with an Odyssey Scanning System (LI-COR Inc., USA).

Detection of liver-specific proteins via immunocytochemistry

Differentiated hybrid cells in the transwells were washed with PBS three times each on days 3, 6 and 10. Then the cells were fixed with pre-cold 4% paraformaldehyde (freshly prepared) for 20 min. After aspirating the fixative, the cells were washed three times for 10 min each time with PBS. Non-specific binding was blocked with PBS containing 5% BSA in PBST for 1 h at room temperature. The cells were then incubated with the primary antibody (AFP, Roche, German 1:100; CK-8&18 Chemicon, USA, 1:1000; albumin Santacruz, USA, 1:1000) in 1% BSA overnight at 4ºC. Next, the cells were washed with 1 × PBST. The cells were then incubated with the secondary antibody (anti-mouse-FITC for AFP, 1:64; and CK8&18, 1:64, and anti-mouse-Rhodamine for ALB, 1:500) at room temperature for 2 h in darkness. After the three washes, the cells were exposed to 1 μg ml^{-1} DAPI or 20 μg ml^{-1} PI solution for 10 min. After washing the cells, they were mounted with immunoXuore mounting agent (Sigma, USA). Images were captured using a Nikon DXM-1200F microscope.

Detection of liver-specific genes by real-time-PCR

cDNA was prepared by reverse transcription of 1 μg of RNA from differentiated hybrid cells using a High Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Eurogentec) and β-actin were used as an endogenous control. Primer sequences of characteristic liver cell and housekeeping genes are shown in Tab. 1. The analysis was performed using the SDS 7500 FAST software v1.4.

Tab. 1. The primer sequences of characteristic differentiated cells and housekeeping genes.

Gene name	Forward	Reverse
Carbamoyl-phosphate synthetase I (CPSase I)	ATGACGAGGATTTTGACAGC	CTTCACAGAAAGGAGCCTGA
Phosphoenolpyruvate carboxykinase (PEPCK)	TCTGCCAAGGTCATCCAGG	GTTTTGGGGATGGGCACTG
Transthyretin (TTR)	AGTCCTGGATGCTGTCCGAG	TTCCTGAGCTGCTAACACGG
Albumin	TGAACTGGCTGACTGCTGTG	CATCCTTGGCCTCAGCATAG
Alpha-fetoprotein (AFP)	CCACCCTTCCAGTTTCCAG	GGGCTTTCCTCGTGTAACC
Alpha-1 antitrypsin (AAT)	TGGGGTCTACTGCTTCTGG	TCATGGGCACCTTCACCGT
Tryptophan 2,3-dioxygenase (TDO)	AGAGCCAGCAAAGGAGGAC	CTGTCTGCTCCTGCTCTGAT
Hepatocyte nuclear factor 3 (HNF3)	TATTGGCTGCAGCTAAGCGG	GACTCGGACTCAGGTGAGGT
Liver-specific organic anion transporter-1 (LST-1)	AGCTACACCGACCAAAGCTG	GTTGGCCTGCGATGCTGTC
hAlbumin (human-specific primer)	GATGTCTTCCTGGGCATGTT	ACATTTGCTGCCCACTTTTC
hAFP (human-specific primer)	TGCCAACTCAGTGAGGACAA	TCCAACAGGCCTGAGAAATC
hCps1 (human-specific primer)	CAGCCACACCAAGGAATCTT	GCCATTGAAAAGGTGAAGGA
mAlbumin (mouse-specific primer)	TGAACTGGCTGACTGCTGTG	CATCCTTGGCCTCAGCATAG
mAFP (mouse-specific primer)	CCACCCTTCCAGTTTCCAG	GGGCTTTCCTCGTGTAACC
mCK18 (mouse-specific primer)	CGATACAAGGCACAGATGGA	CTTCTCCATCCTCCAGCAAG
Bax (human-specific primer)	ATGGACGGGTCCGGGGAG	ATCCAGCCCAACAGCCGC
Oct-3/4	AGGTGTGGGGGATTCCCCCAT	GCGATGTGGCTGATCTGCTGC
GAPDH	GATGCCCCCATGTTTGTGAT	TTGCTGACAATCTTGAGTGAGTTGT

Measurement of enzymes and ALB release in the co-culture system

At the end of each time point (12, 24, 36, 48 and 60 h), the supernatant was collected to measure the secretion of ALB and the levels of the enzymes: glutathione peroxidase (GSH-Px), super oxide dismutase (SOD) and lactate dehydrogenase (LDH). LDH activity in the supernatant was measured via spectrophotometry. A spectrophotometry assay kit (Jiancheng Nanjing) was used to measure the production of SOD, GSH-Px and ALB.

Animal injections

Hybrid cells were pre-labeled with GFP prior to *in vivo* injection, as described previously [33]. The GFP lentivirus was prepared as described [34]. Hybrid cells were infected with the GFP lentivirus overnight in the presence of 8 μg ml^{-1} Polybrene. 48 h post-infection, the hybrid cells were collected by trypsinization, suspended into single cells and injected into the SCID/NOD mouse liver. All of the animal studies were done according to National Institute of Health Guidelines following approval by the Institutional Animal Care and Use Committee. SCID/NOD mice, 6-8 weeks of age, were anesthetized with 0.5 mg g^{-1}

Avertin intraperitonally prior to transplantation. Each mouse was injected with 2 × 106 hybrid cells via percutaneous injection into the liver with a 27-gauge needle. Brief pressure was applied to the injection sites to ensure proper hemostasis. The animals were killed and processed for microscopy or immunostaining. GFP+ cells were identified in the liver sections. The mouse anti-GFP (1:50) obtained from Chemicon, Billerica, was used to counter-stain GFP-labeled cells in the liver sections to establish the specificity of GFP labeling.

RESULTS

Damaged L-02 cells in the co-culture system

Apoptosis of the L-02 cells. In our previous study, we selected an optimum level of damage that could cause significant apoptosis of L-02 cells. To investigate the effect of hybrid cells in co-culture with damaged cells, we first assessed the degree of apoptosis of damaged cells during the co-culturing process (Fig. 1). Via Annexin-V/PI double staining, apoptotic cells could be defined by flow cytometry as early stage apoptotic cells (Q4), which were able to recover from damage, and late stage apoptotic cells (Q2), which were not able to recover from damage (Fig. 1B). Data showed that after co-culturing, the percentages of late stage apoptotic cells declined dramatically at each time point compared to control group without co-culture (4.3% vs. 15.8%, 3.1% vs. 17.4%, and 2.1% vs. 13.4% at 24, 48 and 60h, respectively). Also in comparison to the non-co-cultured control group, the ratios of apoptotic cells in the total cell population were lower than in the control group (20.2% vs. 32.1%, and 8.8% vs. 25.1% at 48 and 60 h, respectively; Fig. 1B). This decrease occurred from the point 24 h after co-culture. The decrease in the number of apoptotic cells was also confirmed by the observed increase in the number of viable cells in the total population, as shown in Fig 1C, a, b. Moreover, together with the data obtained from Fig. 1A, the proliferation and viability of the L-02 cells in the co-culture group were more activated than those of the cells in the control group (Fig. 1A, C). This data implied that apoptosis of damaged cells was inhibited after co-culturing with hybrid cells.

Changes in the p53 apoptosis pathway during co-culture. We tested if there are protein expression changes in one of cell apoptosis pathways, specifically in the p53 pathway, during the co-culture process, in order to verify the inhibition of cell apoptosis. The data showed that both procaspase-3 (32 kDa) and one activated caspase-3 (17 kDa) degraded in the process of co-culture (Fig. 2A), which suggested early stage apoptosis was inhibited during co-culturing. The expression of p53 increasingly decreased (Fig. 2D) after co-culturing for 24 h. The level of proapoptotic Bax protein was correspondingly down-regulated during co-culturing (Fig. 2B). The ratio of Bax/Bcl-2 expression was the determining factor for the induction of apoptosis, and we found that the ratio of

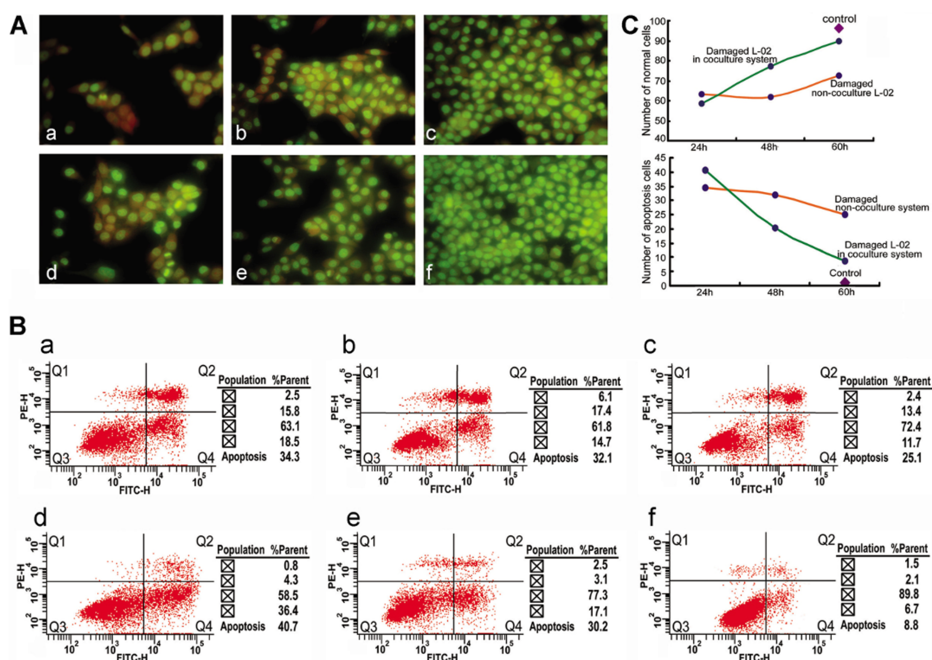

Fig. 1. Co-culture inhibits the apoptosis of L-02 cells in the culture period from 24 h to 60 h. A – AO/EB staining of L-02 cells by fluorescence microscope detection (x 200). The cells stained red with EB are dead cells, and the cells stained orange are apoptotic cells. a-c – Control group: apoptosis induced by H_2O_2 without co-culture. a – 24 h after damage; b – 48 h after damage; c – 60 h after damage. d-f – Apoptosis induced by H_2O_2 in the co-culture system. d – co-culture for 24 h; e – co-culture for 48 h; f – co-culture for 60 h. The apoptotic cell population in the co-culture group is smaller than that in control group. B – Flow cytometric analysis for Annexin-V and PI staining. a-c – Control group. Apoptosis induced by H_2O_2 without co-culture. a – 24 h after damage; b – 48 h after damage; c – 60 h after damage. d-f – Apoptosis induced by H_2O_2 in the co-culture system: d – co-culture for 24 h; e – co-culture for 48 h; f – co-culture for 60 h. Q2: necrotic cells and late apoptotic cells labeled with PI and Annexin V-FITC. Q3: fully viable cells. Q4: early apoptotic cells labeled with Annexin V-FITC but not with PI. C – Trendline of the percentage of apoptotic cells in the co-culture and non-co-culture groups, corresponding with B. L-02 cells without injury cultured alone at 60 h were used as the control. Taken together, after co-culture, the apoptosis of cells was inhibited and the cell proliferate ratio of co-cultured L-02 cells was higher than the proliferation of cells in the control groups.

Bax/Bcl-2 protein expression significantly decreased in a time-dependent manner after co-culturing (Fig. 2B, C). As shown in Fig. 2, the downregulation of Bax was most obvious in the p53 pathway; thus, to determine whether the downregulatory effect was endogenous or exogenous, we analyzed the transcription of Bax. The proapoptotic gene Bax showed a significant decrease

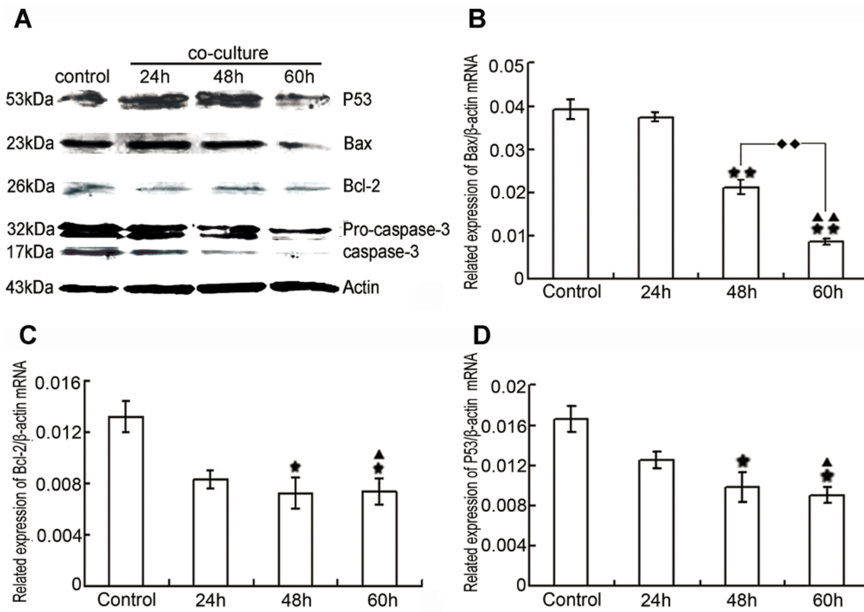

Fig. 2. The expression of the proteins p53, Bcl-2, Bax and caspase-3 in the injured L-02 cells. Proteins were extracted from damaged L-02 cells after co-culture with hybrid cells at 24, 48 and 60 h. Injured L-02 cells cultured alone for 60 h were used as the control. Representative immunoblots are shown in (A). The results are expressed as means ± SD (n = 3). *P < 0.05, **P < 0.01, compared with "24 h" (B-D); ▲P < 0.05, ▲▲P < 0.01, compared with control (B-D); ◆◆P < 0.01 compared with "48 h" (B). The protein expression profile shows that the apoptosis of L-02 cells was inhibited during co-culturing with hybrid cells.

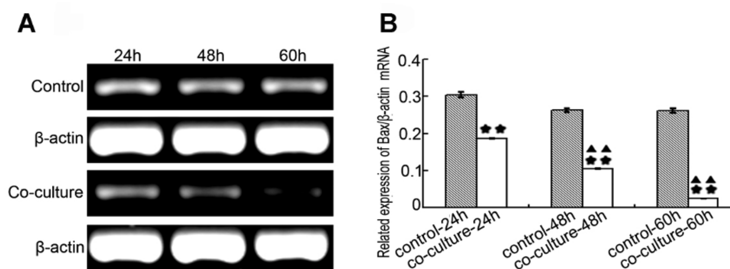

Fig. 3. The mRNA expression of Bax in injured L-02 cells. mRNA was extracted from damaged L-02 cells of both the non-co-culture and co-culture groups at 24, 48 and 60 h. Representative RT-PCR results are shown in A. Injured L-02 cells cultured alone were used as the control group. The results are expressed as means ± SD (n = 4). **P < 0.01, compared with the control group (B); ▲▲P < 0.01, compared with "Co-culture 24 h"; β-actin was used as an internal housekeeping gene.

in mRNA expression (Fig. 3A), while no significant change was observed for Bcl-2 mRNA expression. The ratios of Bax/β-actin of the co-cultured groups constantly decreased, and were significantly different from the control groups at each time point (Fig. 3B). Altogether, the data demonstrated again that apoptosis was inhibited by co-culturing with hybrid cells.

Repair of the damage. In addition to demonstrating the hybrid cells' inhibition of the cell apoptosis function in the co-culture system, we tested damage-related enzymes (GSH-Px, SOD and LDH) and the release of albumin into the supernatant. The secretions of GSH-Px (Fig. 4A) and SOD (Fig. 4B) were observed to increase with time, and the secretion of LDH (Fig. 4D) decreased correspondingly, while the secretion of albumin increased during co-culturing (Fig. 4C). For the control group without damage (positive control group), the

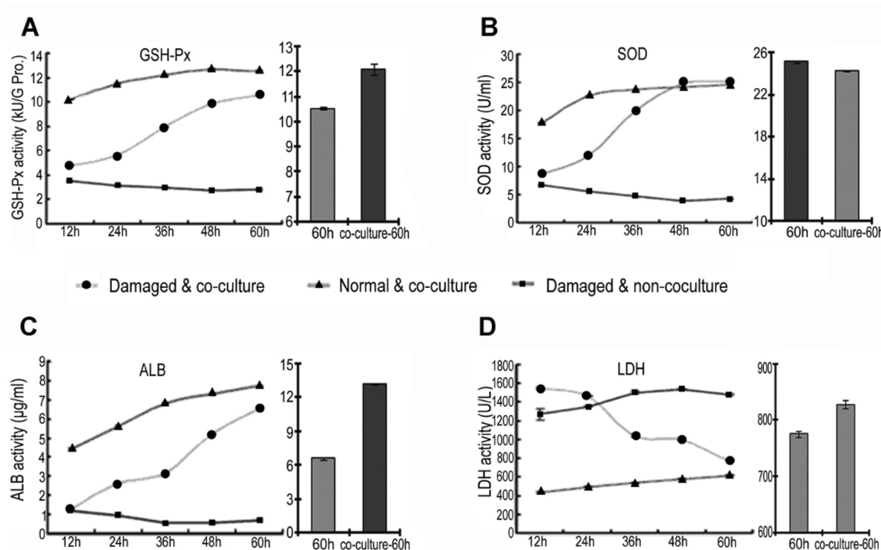

Fig. 4. The expression of damage-related enzymes and the secretion of albumin into the supernatant. There was a time-dependant increase in the levels of: A – GSH-Px; B – SOD; and C – ALB. D – There was a time-dependant decrease in the level of LDH. During co-culturing, the levels of the damage-related enzymes GSH-Px and SOD increased while that of LDH decreased. Meanwhile, the secretion of ALB increased during co-culture. Moreover, compared with normal L-02 cells without a co-culture system at 60 h, the expression of damage-related enzymes was not significantly different in the supernatant of the damaged L-02 cells and the co-culture system, which means damage in the co-culture system is repaired. *p < 0.05 compared with "control-60 h". The results are expressed as the means ± SD (n = 4). Normal L-02 cells co-cultured with hybrid cells were used as the positive control group. Damaged L-02 cells without co-culture were used as the negative control group. After co-culturing with hybrid cells, the activity of these enzymes trended toward the level of normal L-02 cells.

secretion levels of GSH-Px, SOD and LDH were no different to those for each corresponding co-cultured group at 60 h (Fig. 4A, B, D). By contrast, for the damaged cells without co-culture (negative control group), the secretion of GSH-Px and SOD and the release of albumin into the supernatant were all significantly higher, while the activity of LDH was significantly lower ($P < 0.01$). These results confirmed again that the damaged L-02 cells recovered after co-culturing with hybrid cells.

Hybrid cells in the co-culture system

Morphologies of the hybrid cells in transwells. Fig. 5A shows the morphology of the hybrid cells in the transwells on day 2. The hybrid cells displayed a typical ES cell clone phenotype, and were tightly packed. The entire colony had depth with a refractive ring around it. Most of the hybrid cells were GFP positive, which was driven by the Oct-4 promoter (Supplement Figs 1 and 2). The karyotype of the hybrid cells showed that the hybrid cells were derived from cells of human and mouse origin (Supplement Fig. 3). After co-culturing with damaged L-02 cells in media without LIF, the clones became flat and began to differentiate (Fig. 5B) and the GFP-positive cell number decreased (Supplement Fig. 2). Initially, differentiated cells formed the clusters, increasing in both size and number, and then, differentiated cells formed the characteristic cuboidal shapes (Fig. 5C).

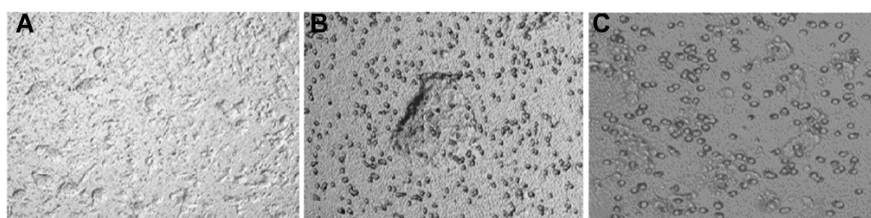

Fig. 5. The morphology of hybrid cells on a 0.4-µm transwell under phase contract microscopy. A – Hybrid cells cultured in an optimized ES culture medium on day 2 (x 100). B – A single hybrid cell clone on the transwell on day 3 of co-culture. The clones were not typical, and had begun to differentiate (x 200). The little black dots are wells on the PET membrane. C – Differentiated hybrid cells on day 6 (x 100). The cuboidal morphology typical of L-02 cells is seen. Hybrid cells differentiate to hepatocyte-like cells. The little dots are wells on the PET membrane.

The gene expression profile of the hybrid cells during the co-culture. Like the undifferentiated ES cells, undifferentiated hybrid cells expressed the pluripotency marker Oct-4. However, after three days of co-culture (five days after differentiation), Oct-4 was down-regulated and eventually silenced. Instead, the hepatic-specific genes AFP and ALB, epithelium marker CK18, and the hepatic-specific functional genes Cps1 were all expressed in differentiated hybrid cells from day 3 of co-culturing onwards. The expression of these tissue-

specific genes upregulated with time. These results implied that hybrid cells differentiated into hepatic lineage cells. It is interesting and important that these hepatic genes were not expressed from only mouse ES cells or human L-02 cells, but from both species. This demonstrated the bidirectional function of hybrid cells. In this co-culture system, ES cells also differentiated to express the mouse hepatic-specific genes AFP, ALB and CK18 (Fig. 6).

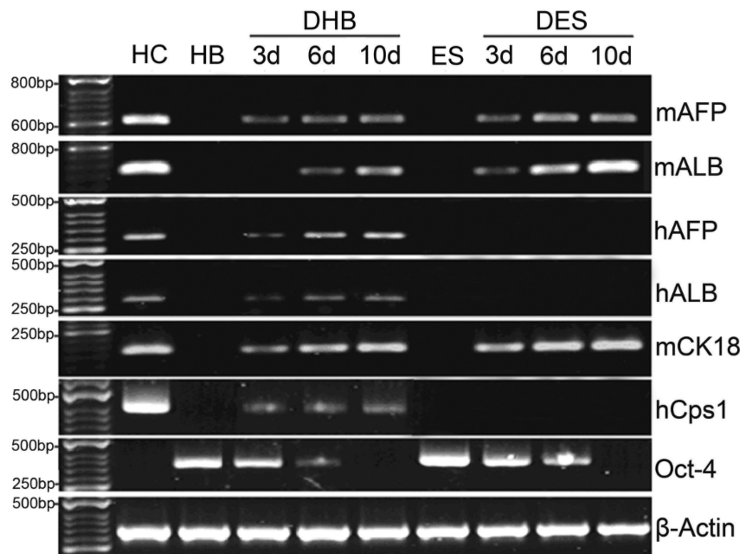

Fig. 6. The gene expression profile during the differentiation of fused cells (HB) and ES cells assessed via RT-PCR. Differentiated fusion cells (DHB) and differentiated ES cells (DES) were analyzed at various time points during co-culture (Days 3-10) via RT-PCR. Undifferentiated hybrid cells and ES cells both expressed the pluripotent marker Oct-4. Differentiated hybrid cells express both human- and mouse-specific hepatic markers, specifically mAFP, mALB, hAFP, hALB, mCK18 and hCps1, without expressing the pluripotent marker Oct-4. When ES cells were co-cultured in the same co-culture system with damaged L-02 cells, ES cells also differentiated to express the mouse hepatic-specific markers mAFP, mALB and mCK18. Adult mouse liver and L-02 cells were used as positive control (HC). ES cells were used as a control of pluripotent cells and β-actin is used as an internal housekeeping gene. The expression profile shows that fused cells have a bidirectional differentiating ability: genes from both parental cells are transcripted in fused cells.

Cellular characterization of differentiated hybrid cells. From day 3 (Fig. 7A-C) until day 10 (Fig. 7D-F) of co-culturing, the epithelial marker CK8&18 could be detected in the differentiated hybrid cells, which indicated that differentiated hybrid cells show a differentiation trend to epithelium. The liver-specific markers ALB (Fig. 8A-C) and AFP (Fig. 8D-I) were both detectable from day 3 of co-culture; this result corroborated the data obtained from the gene expression profile of the differentiated hybrid cells. As shown in Fig. 9, on day 3, the

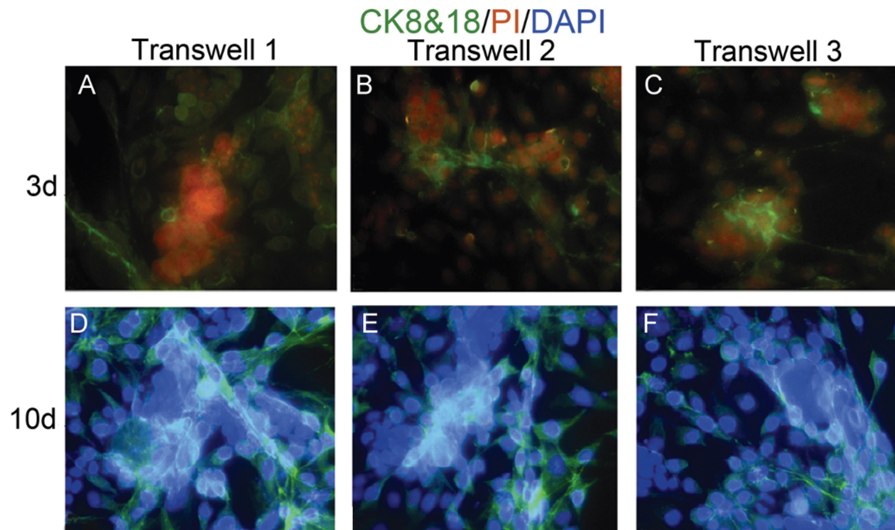

Fig. 7. Cellular characterization of the differentiated hybrid cells on days 3 and 10. The immunofluorescence of differentiated hybrid cells is analyzed using the epithelium-specific marker CK8&18 in three different dishes. A-C: Hybrid cells in three different transwells on day 3. D-F: Hybrid cells in three different transwells on day 10.

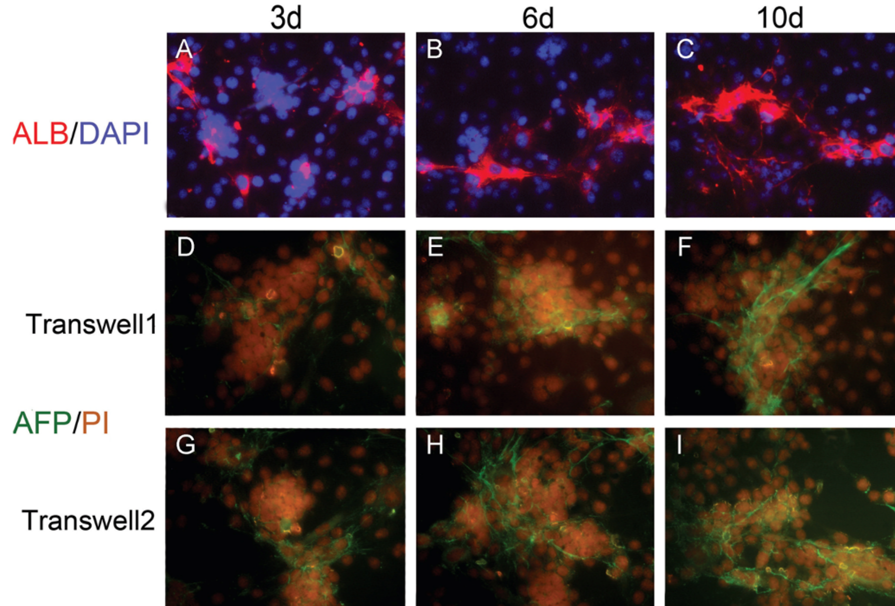

Fig. 8. Cellular and functional characterization of the differentiated hybrid cells. The immunofluorescence of the differentiated hybrid cells is analyzed using the hepatic-specific markers ALB and AFP on days 3, 6 and 10 in two parallel groups. A-C: ALB-positive hybrid cells on days 3, 6 and10 respectively. D-F: AFP-positive hybrid cells in the group of “transwerll1” on days 3, 6 and 10, respectively. G-I: AFP-positive hybrid cells in the group of “transwerll2” on days 3, 6 and 10, respectively.

differentiated hybrid cells began to express the hepatic-specific gene AFP, and albumin, alpha-1 antitrypsin (AAT), carbamoyl-phosphate synthetase I (CPSASE1), hepatocyte nuclear factor 3(HNF3), liver-specific organic anion transporter (LST1), phosphoenolpyruvate carboxykinase (PEPCK), tryptophan 2,3-dioxygenase (TDO) and transthyretin (TTR). On day 12, the expression of these genes was much higher than on day 3 except for that of AFP. The ES cells differentiated via co-culturing with damaged L-02 cells were used as a positive control, and the gene expression profile of the differentiated hybrid cells was the same as that of the differentiated ES cells. Meanwhile, the control mouse tail tip fibroblasts (TTF) did not express the mRNAs of all the tested markers. Therefore, hybrid cells had the same ability as ES cells to differentiate into hepatic lineage cells. Due to this co-culturing with injured L-02 cells, hybrid cells had the capacity to differentiate into hepatic epithelial cells.

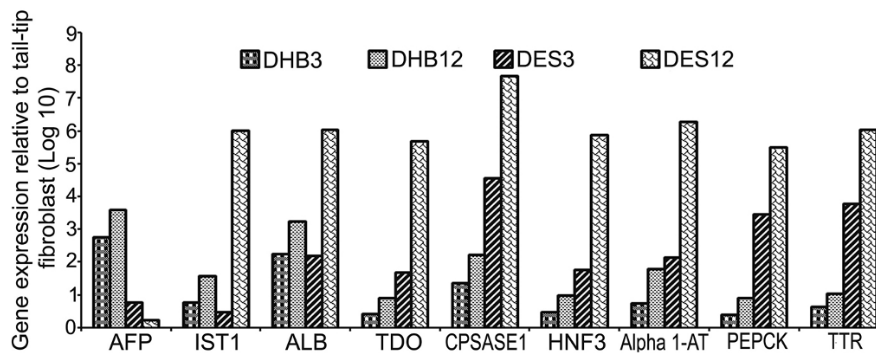

Fig. 9. Hepatic-specific gene expression assessed via RT-PCR analysis. RNA was isolated from mouse tail tip fibroblasts (TTF), differentiated ES cells, and differentiated hybrid cells on days 3 and 12. Like ES cells differentiated by co-culturing with damaged L-02 cells, differentiated hybrid cells express the hepatic-specific gene markers AFP, albumin, AAT, CPSASE1, HNF3, LST1, PEPCK, TDO and TTR. However, TTF cells do not express the mRNAs of all of the tested markers. Therefore, differentiated hybrid cells have the same ability to differentiate to hepatic lineage cells. DHB3: differentiated hybrid cells on day 3; DHB12: differentiated hybrid cells on day 12; DES3: differentiated mouse ES cells on day 3; DES12: differentiated mouse ES cells on day 12. The control TTF did not express all those genes.

The engraftment and structural integrity of the transplanted hybrid cells. To assess the fate of the hybrid cells *in vivo*, GFP-labeled 2 × 10^6 cells were injected directly into the liver parenchyma to examine the liver distribution of GFP-labeled transplanted cells by immunofluroscent staining of liver tissue with antibodies against GFP (Fig. 10). 20 days after transplantation, the GFP-labeled transplanted cells were distributed in the hepatic lobules and engrafted in the liver structure. Three months after cell transplantation, there was no evidence of terotoma formation.

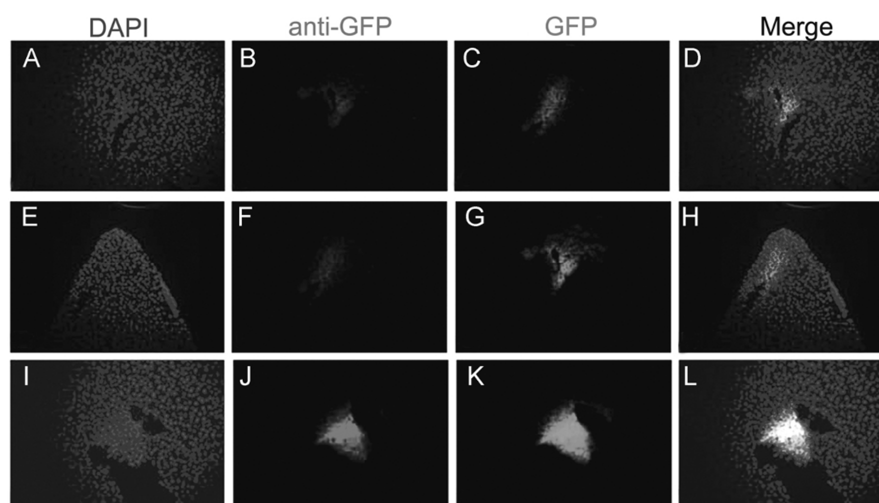

Fig. 10. The distribution of hybrid cells in the mouse liver. GFP-labeled hybrid cell-derived cells are distributed throughout liver tissue after transplantation. The GFP-labeled cells are visible in liver tissue (C, G and K). In order to make sure that the GFP signal was not due to autofluorecensce, anti-GFP antibody was utilized to mark hybrid cell-derived cells (B, F and J). DAPI staining revealed numerous hybrid cell-derived cells in the liver tissue sample (A, E and I). The merged images are shown as D, H and L.

DISCUSSION

Fusion has been demonstrated as a possible mechanism for tissue regeneration [8, 12-13]. Modified hybrid cells will be one suitable cell type for transplantation and regeneration. However, little is known about the fate and function of hybrid cells under conditions of injury. After fusing cells of embryonic origin with somatic cells, the genome of the somatic cells can be reprogrammed such that the resulting hybrid cells show pluripotency, an identical characteristic to ES cells [8, 12-13, 35-44]. Thus, we hypothesize that hybrid cells could contribute to regeneration in the same way as pluripotent cells. In this study, we co-cultured human hepatic L-02 cells with H_2O_2-induced damage with hybrid cells derived from mouse embryonic stem cells and L-02 cells to study the fates of the two co-culturing partners. Our results showed three things.

1. The apoptosis of the L-02 cells was inhibited after co-culturing. The ratio of proliferated cells was higher than the ratio for the control group without co-culture.
2. The other co-culture partner, hybrid cells, differentiated into hepatic lineage cells.
3. After the hybrid cells had been transplanted to the mouse liver, they were able to engraft to the liver structure.

We suggest one possible function for hybrid cells: they help inhibit the apoptosis of damaged tissue, and they could differentiate to tissue-specific lineage cells and participate in tissue regeneration.

Co-culturing with hybrid cells inhibits the apoptosis of total L-02 cells

We tested several aspects of apoptosis in damaged L-02 cells by analyzing the phenotype, proteins related to the apoptosis signaling pathway and transcription. Our results indicate that the apoptosis of L-02 cells was significantly inhibited compared to the control group without co-culturing. The number of proliferating L-02 cells was far higher than in the control group. Koh *et al.* co-cultured MSCs with CD34+ cells to study the effect of MSCs upon the expansion of HSCs driven by aza-D and TSA, which can cause cell death of HSCs. Their results suggest that a co-culture of CD34+ cells with the MSCs might not only simply deliver the proliferation signals, but also stemness and survival signals, and overlap with the action of epigenetic regulators [29]. In agreement with their research, our results show that the ratio of apoptosis of L-02 cells in co-culture was lower than for the control group, while the percentage of survival for cells is higher than that for the group without co-culture. In most previous studies [5, 29, 45], different kinds of cells were co-cultured together directly. By contrast, in our co-culture system, the cells were co-cultured indirectly using transwells. However, the promising key factors and/or signal factors can pass through the PET (polyester) wells onto the transwell. However, because all the enzymes and albumin were secreted into the supernatant, we cannot tell whether all the damage-related enzymes and albumim were from recovered hepatic cells or from differentiated hybrid cells in this co-culture system. There are three possibilities. One is that after the co-culture, the damaged L02 cells recovered and started to secrete normal levels of all these enzymes. The second is that the differentiated hybrid cells secreted all the enzymes. Finally, both may have occurred at the same time. We can see from Fig. 6 that the differentiated hybrid cells expressed both mouse and human original albumim, so we assumed that both the recovered hepatic cells and differentiated hybrid cells contributed to the enzyme changes. Consequently, we suggest that in this co-culture system, the epigenetic regulators and the microenvironments but not the cell-cell contact in the selective press played a decisive role in inhibiting apoptosis.

In the p53-related apoptosis signaling pathway, apoptosis stimulators down-regulate in a cascading fashion (p53, Bax, caspase-3). Among them, the change in Bax levels is the most obvious one. Via RT-PCR, we found that the change in the endogenous Bax levels corresponded to the inhibition of apoptosis. Changes in the other factors in this signaling pathway were not significantly different from the results for the control group. This implies an important function for Bax in inhibiting apoptosis in our co-culture system. Taken together, this indicates that the hybrid cells efficiently inhibit the apoptosis of damaged L-02 cells through a Bax-related signaling pathway. In further studies, specific protein

cytokine and epigenetic factor arrays [45] should be used to identify the key factors in the co-culture microenvironment.

Hybrid cells differentiated into functional hepatic lineage cells when co-cultured with injured L-02 cells

Numerous reports have demonstrated that the co-culture system promotes the differentiation of stem cells in a tissue-specific direction [27, 29, 45]. In these studies, stem cells were co-cultured with somatic cells or progenitor cells directly, using specific conditioned media, or stem cells were differentiated directly. In this study, hybrid cells differentiated into hepatic lineage cells. The differentiated hybrid cells were positive for AFP, ALB and CK8&18 antibodies and they expressed liver-specific genes. Meanwhile, like the differentiated ES cells, the differentiated hybrid cells express the hepatic-specific gene markers AFP, Albumin, AAT, CPSASE1, HNF3, LST1, PEPCK, TDO and TTR. Liver-specific genes are not only re-expressed from human L-02 cells, but also from mouse embryonic stem cells. This indicates a bidirectional function for hybrid cells. Fusing somatic cells with stem cells allows hybrid cells to be stem cell-like cells [14] and to be reprogrammed to ES cell-like cells *in vitro* [14-19]. Therefore, differentiation from stem cell-like cells to tissue-specific cells might be one functional pattern of hybrid cells under conditions of injury. Under certain conditions (co-culturing), reprogrammed hybrid cells have the capacity to differentiate into tissue-specific cells, just like the directional differentiation of ES cells. However, hybrid cells are not exactly like embryonic stem cells. Hybrid cells may have epigenetic memory from their parental cell line. We performed fusion using human fibroblast BJ cells and mouse ES cells. We were able to generate hybrid cells, but after co-culturing those fibroblast-derived hybrid cells with damaged L02 cells, there were no significant differences between the hybrid cell group and the positive control group (data not shown). After that, we fused mouse ES cells directly with L02 cells. Induced pluripotent cells (iPS) derived from different cell types have different memories from their parental cells [46]. Those epigenetic memories make hybrid cells unique in certain tissues meaning that different sources of hybrid cells may have different functions. Our karyotype data also shows that in hybrid cells, most of the chromosomes are from a human hepatic parental cell, and partially of mouse origin. Therefore, in our study, hybrid cells derived from hepatic cells have a particular function to inhibit the apoptosis of hepatic cells.

It was reported that fusion occurs, under selective pressure, such as under conditions of damage [47-50]. Fusion occurred spontaneously *in vivo* after transplantation and *in vitro* after co-culturing [26, 50]. It was suggested that the fusion that occurs in the damaged liver might be derived from liver cells with HSC, MSC or LSC [47]. Thus, fusion by stem cells could be very important for the regeneration of the injured liver. However little was shown about the fate and function of fused cells. In this study, we show that, like other embryonic or adult stem cells, hybrid cells derived from somatic liver cells and embryonic

stem cells differentiated into hepatic lineage cells. Moreover, for the first time, we evaluated the function and fate of both injured cells and fused cells. This data may help uncover the function of hybrid cells in injured tissue.

In the co-culture system, thanks to the use of transwells, hybrid cells and injured L-02 cells do not make direct contact with one another. Hence, the cell stress (injury) and the microenvironment play an important role in connecting the two kinds of cells. In other words, some factors and/or proteins secreted by the two kinds of cell play a key role in this system. This is an important aspect that we should evaluate in detail. Fusion occurred under selective pressure such as under conditions of damage [47, 49-50]. However, only by irradiation did human cord blood CD34+ cells still develop into hepatocytes in the liver [48]. It is notable that CD34 is a 110-kDa transmembrane glycoprotein. This is one type of adhesion molecule found in hematogenic primitive progenitor cells, endothelial cells of small blood vessels and embryonic fibroblasts. Together with CD44, CD34 promotes cell fusion in injured tissue [51]. Therefore, whether damage exists or not, the secretion of some factors and/or proteins should be decisively important for determining the fate of fused cells and for tissue regeneration.

It is exciting that we can harvest autologous cells for transplantation therapies from differentiated hybrid cells without oocytes and embryos. Research into cell fusion-based reprogramming is still in its infancy, and critical progress has yet to be made. Serious obstacles have to be overcome before we can eliminate the pluripotent genome, for example. A lot remains to be done before we will be able to use this promising cell type for practical applications.

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