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MSC^{TRAIL}-mediated HepG2 Cell Death in Direct and Indirect Co-cultures

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Abstract. *Background: Mesenchymal stem cells (MSCs) have attracted great interest in cancer therapy since the discovery of their tumor tropism. This study was performed to investigate the effects of TNF-related apoptosis-inducing ligand (TRAIL)-engineered MSCs on hepatocellular carcinoma (HCC) cells (HepG2) under different culture conditions. Materials and Methods: MSCs engineered with non-secreting TRAIL (MSC$^{TRAIL-GFP}$) (GFP, green fluorescence protein) and secreting TRAIL (MSC^{stTRAIL}) were used for the direct co-cultures, and conditioned media (CM) from corresponding cultures were applied to HepG2 as indirect co-cultures. Immunoblotting, ELISA and FACS analysis were used to detect the expression of TRAIL and TRAIL receptors. Cell death was assessed using live/dead assay. Results: Death receptor (DR) 5 was identified on the HepG2 cells. The expression of TRAIL was confirmed in the cell lysates (MSC$^{TRAIL-GFP}$ >MSC^{stTRAIL}) and the conditioned media (MSC^{stTRAIL} >MSC$^{TRAIL-GFP}$). Higher cell death was observed in high MSC/HepG2 ratio co-cultures. HepG2 cell death was proportionally related to CM from MSC$^{TRAIL-GFP}$ and MSC^{stTRAIL}. Conclusion: MSCs exhibit intrinsic inhibition of HepG2 which is potentiated by TRAIL-transfection.*

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer death in the world (the second in men and the sixth in women) (1), and the second leading cause of cancer death in China (2). Only 20% of HCCs are amenable to curative treatment (3). Surgical resection and liver transplantation are the only curative options available, with 5-year survival rates of 36-70% and 60-70% respectively (4-6). Systemic chemotherapy can increase the median survival of patients with unresectable HCC to approximately 14 months (7). If untreated, median survival is 6-12 months (8, 9) with few surviving beyond 3 years. Of the fatalities, the majority of HCC patients die from the recurrence of metastasis or therapy-related life-threatening complications. To a significant extent, current conventional cancer therapies are symptomatic and passive in nature. The major obstacle limiting the effectiveness of conventional therapies for cancer is their tumor specificity. Thus, it is critical to explore efficient remedy strategies specifically targeting tumor tissue.

Since the discovery of the tumor-oriented homing capacity of mesenchymal stem cells (MSCs), the application of specific anticancer gene-engineered MSCs has held great potential for cancer therapies. Tumor-directed migration and incorporation of MSCs have been demonstrated in a number of pre-clinical studies *in vitro* using transwell migration assays and *in vivo* using animal tumor models. The homing capacity of MSCs was demonstrated with almost all tested human cancer cell lines, such as lung cancer (10), malignant glioma (11-13), Kaposi's sarcomas (14), breast cancer (15, 16), colon carcinoma (17), pancreatic cancer (18, 19), melanoma (20) and ovarian cancer (15). Engineered with tumor-specific anticancer genes, MSCs are capable of producing anticancer agents locally and constantly. TNF-related apoptosis-inducing ligand (TRAIL, also known as Apo2L) is a member of the TNF super-family. It was originally identified and cloned on the basis of its sequence homology to the extracellular domain of CD95 ligand (CD95L) and TNF (21, 22). TRAIL is one of few anticancer proteins which selectively cause apoptosis of transformed or tumor cells through the activation of death receptors (DR), with no effects on healthy cells (23). Five TRAIL receptors are known, TRAIL receptor 1 (DR4); 2 (DR5); 3 (decoy receptor 1, DcR1); 4 (DcR2) and a soluble receptor, osteoprotegerin (OPG) (24). As a component part of developing a dual-targeted therapeutic strategy, this study was performed to detect the sensitivity of human HCC cells (HepG2) to TRAIL-engineered MSCs under various culture conditions.

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Materials and Methods

Cells and culture conditions. MSCs were isolated from human pancreas and *ex vivo* expanded as previously described (25). Human pancreases were obtained (with consent) from adult heart-beating cadaver organ donors through the organ procurement program of the British Columbia Transplant Society (BCTS, Vancouver, Canada). Pancreatic dutal tissue taken from the Ricordi chamber during islet isolation was utilized as the starting material. Primary culture was initiated by seeding chopped ductal tissue onto 100 mm culture dishes (CellBind, Corning, Acton, MA, USA). Subculture was performed once newly grown cells reached sub-confluence. Based on the minimal criteria for defining human MSCs established by the International Society of Cellular Therapy (ISCT), these MSCs were verified by both membrane biomarker determination and functional differentiation. They fulfilled the characteristics of human MSCs, exhibiting positive expression of $CD44^+$, $CD73^+$, $CD95^+$, $CD105^+$ and negative of $CD34^-$. The results of adipogenic and osteogenic differentiation also met the requirements. The MSCs were cultured in MEM with 10% FCS, 2 mM L-glutamine and 1% penicillin-streptomycin solution (all from Invitrogen, Carlsbad, CA, USA) and incubated at 37˚C in a humidified, 5% CO_2 atmosphere. The cells used in this study were limited within passages 5 to 7.

The human liver HCC cell line (HepG2, HB-8065) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The HepG2 cells were maintained as suggested by ATCC and their culture condition was kept consistent with the MSCs. The cells used in the present study were limited to within 3 passages.

Flow cytometric analysis. The expression of TRAIL receptors on the HepG2 cells was detected by flow cytometry. Sub-confluent HepG2 cells were detached with 0.25% trypsin and washed with PBS. A total of $1\text{-}5\times10^5$ cells were resuspended in 200 μl PBS for each reaction, and then 10 μl of primary antibody solution (25 μg/ml) (antibodies to human DR4, DR5, DcR1, DcR2 or isotype IgG; R&D Systems, Minneapolis, MN, USA) was added to each eppendorf tube and incubated at 4˚C for 30 min. The cells were washed 3 times with PBS and re-suspended in 200 μl PBS. The phycoerythrin-conjugated secondary antibody (R&D Systems) was then added to each reaction and the cells were incubated for 30 min at 4˚C in the dark. After being washed 3 times with PBS, the cells were resuspended with 500 μl PBS in FACS analysis tubes for flow cytometric analysis (FACSCalibur, Becton Dickinson, Heidelberg, Germany). The mean fluorescence intensity of each receptor was assessed on the live cell population. The reference gating location was determined by both isotype IgG and secondary antibody controls with the aid of CellQuest software.

TRAIL-bearing vector transfection of MSCs. TRAIL-bearing expression plasmids were used for the transfection, avoiding viral vectors with a view to potential clinical studies. Two types of plasmids were used, non-secreting TRAIL (TRAIL-GFP, Addgene plasmid 10953) and secreting TRAIL (stTRAIL; Advanced Protein Technologies, Richmond, Canada). The transfections were performed with TransIT-2020 (Mirus, Madison, WI, USA) as suggested by the manufacturer. Briefly, the MSCs were plated at 6×10^5 per well in six-well plates in 3 ml of MEM medium and dated as day 0. On day 1, the cells were transfected with 2.5 μg of TRAIL-GFP or stTRAIL DNA (each well) respectively. The control cells were treated with TransIT-2020 reagent without plasmid DNA. For the direct co-culture experiments, the transfected MSCs were harvested with 0.25% trypsin-EDTA (Invitrogen) on day 2. For the purpose of indirect co-culture and the assessment of TRAIL expression, conditioned media and transfected MSCs were collected on day 3.

Immunoblotting and ELISA analysis. Immunoblotting analysis was used to detect the cellular expression of TRAIL in the MSCs. The MSCs transfected with TRAIL-GFP or stTRAIL or mock infected were harvested in lysis solution. Whole-cell lysates (50 μg) were separated through 12% denaturing SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated overnight with rabbit anti-TRAIL antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). This was followed by a 1 h incubation with goat-anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (1:2500). The blot was developed using enhanced chemiluminescence detection (Amersham Bioscience, Baie d'Urfe, Quebec, Canada). For the sequential reprobing of the same blot, the membrane was stripped of the initial primary and secondary antibodies with stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) at 55˚C for 30 min and followed by sequential incubations with anti-GFP antibody (1:1000; Abcam, Cambridge, MA, USA) and secondary antibody respectively.

The soluble TRAIL in the culture supernatants was measured using enzyme-linked immunosorbent assay (ELISA) as per the manufacturer's instruction (Santa Cruz). Conditioned media collected from the corresponding cultures were equally concentrated using 10,000 MWCO (cat # 42406; Millipore, Millerica, MA, USA) and protein concentrations were determined using a Lowry based method (DC assay; Bio-Rad, Mississauga, ON, USA). All the samples were studied together in duplicate. The protein samples (4.8 μg each in dH_2O) were added into 384-well ELISA plates, and the covered plates were incubated for 5 h at 37˚C. The wells were then blocked with 5% milk in Tris-buffered saline (TBS: 10 mM Tris-HCl, 140 mM NaCl, pH 7.4) for 1 h at room temperature. After washing with wash buffer (0.05% Tween-20 in TBS), 20 μl rabbit anti-TRAIL antibody (1:100, Santa Cruz, sc-7897) was added to each well. After overnight incubation at 4˚C, the wells were washed 5 times with wash buffer. Secondary antibody (20 μl goat-anti-rabbit IgG-HRP, 1:1000; Jackson Immunolabs, West Grove, PA, USA) was added and incubated for 1 h at room temperature. After washing 5 times, 20 μl ABTS (2,2'-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid)) was added into each well and incubated for 30 min at room temperature. Absorbance was measured at 405 nm using an ELISA reader. A qualitative comparison was made with corresponding controls.

Fluorescence microscopy. The cell viability was detected using a live/dead Viability/Cytotoxicity Assay Kit (Invitrogen) as per the manufacturer's instruction with a slight modification. Briefly, a total of 1×10^5 HepG2 or HepG2 and MSC cells were plated onto 24-well plates in 500 μl of MEM medium on day 0. For the indirect co-cultures the media were replaced with 50 or 100% conditioned media on day 1. On day 3 for the direct and day 4 for the indirect co-cultures, the cultures were washed twice with PBS. Freshly prepared working solution (250 μl per well on the 24-well plates, containing 1 μM calcein AM and 2 μM EthD-1) was then added directly to the cultures and incubated at room temperature for 10 min in the dark. The images were taken using a fluorescence

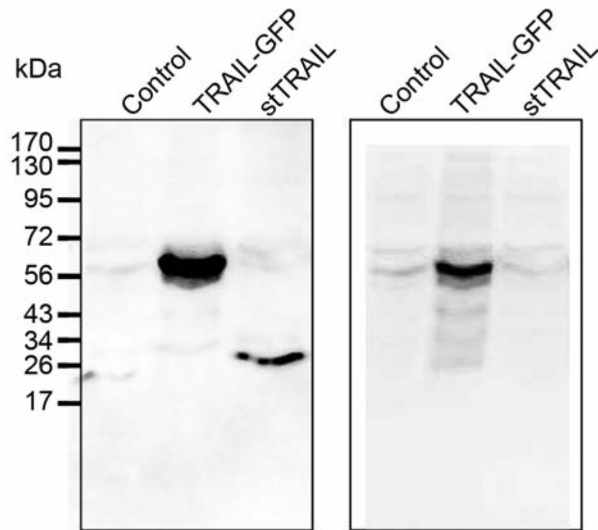

Figure 1. *Immunoblotting analysis of TRAIL in MSC cell lysates. Cells were harvested 2 days after transfection with different TRAIL-bearing plasmids. Left panel: signals with anti-TRAIL antibody; right panel: signals with anti-GFP antibody on the same blotting membrane and control: mock infected. The protein size markers are shown on the left.*

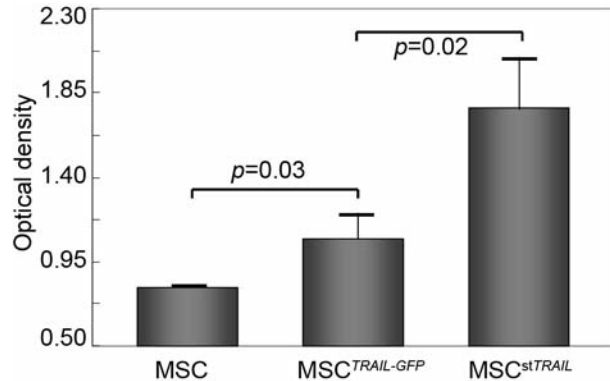

Figure 2. *ELISA analysis of TRAIL in MSC culture media. Conditioned media from MSC, MSC$^{TRAIL\text{-}GFP}$ and MSCst^{TRAIL} collected two days after transfection. Mean±SEM of four independent experiments.*

microscope (I×71; Olympus, Markham, Ontario, Canada) and the related analysis was performed through ImageJ (provided online by the National Institute of Health of USA).

Statistical analysis. Numerical data were expressed as mean±standard error. Statistical differences between the means for the different groups were evaluated with Prism 4.0 (GraphPad software, La Jolla, CA, USA) using the Student's *t*-test with the level of significance at $p<0.05$.

Results

TRAIL expression in engineered MSCs. Figure 1 demonstrates the TRAIL expression in the cell lysates by immunoblotting with a specific anti-TRAIL antibody. The blotting results with anti-GFP antibody distinguished non-secreting TRAIL from the secreting form. ELISA analysis confirmed the presence of soluble TRAIL in the culture supernatants (Figure 2). The TRAIL content in the supernatants from the MSC^{stTRAIL} was significantly higher than that from the MSC$^{TRAIL\text{-}GFP}$ ($p<0.05$).

TRAIL receptor expression in HepG2 cells. As shown in Figure 3, almost all the tested HepG2 cells expressed DR5 receptor and a small percentage of HepG2 cells had DcR2 receptor.

MSC^{TRAIL}-mediated HepG2 cell death in direct co-cultures. Figure 4 displays the MSC-mediated cell death in direct mixed co-culture under different conditions. A summary is presented in Figure 5. The percentages of dead cells were much higher in the high MSC/HepG2 ratio co-cultures than in the low MSC/HepG2 ratio co-cultures for all three types of MSCs (*i.e.* native MSCs, MSC$^{TRAIL\text{-}GFP}$ and MSC^{stTRAIL}). TRAIL-induced additional cell death was only observed in the high MSC/HepG2 ratio co-cultures in the order of MSC$^{TRAIL\text{-}GFP}$ > MSC^{stTRAIL}>native MSCs.

MSC^{TRAIL}-mediated HepG2 cell death in indirect co-cultures. As shown in Figure 6 and Figure 7, HepG2 cell death was proportionally related to CM from secreting and non-secreting TRAIL transfected MSCs. Marked cell death was not detected with CM from native MSCs under the current experimental conditions.

Discussion

A death domain in the intracellular region of DR4 and DR5 can recruit death-inducing signaling complex (DISC) upon TRAIL stimulation, and therefore, activate downstream caspase cascade leading to cell death by apoptosis. No intact death domain is present in the intracellular region of DcR1, DcR2 and OPG, so they are unable to induce apoptosis, even though they were able to compete with DR4 or DR5 for binding with TRAIL (26) and overexpression of DcR1 and/or DcR2 blocks TRAIL-mediated apoptosis in some cell types (27).

TRAIL and its receptors are important components of the extrinsic pathway of apoptosis. Recent studies have demonstrated TRAIL-induced apoptosis in HCC cells (28, 29) and increasing interest has been focused on MSCs as therapeutic tools and gene carriers for the treatment of HCC (30). To make use of MSCs as anticancer agent vehicles, MSCs must be appropriately transfected with selected anticancer genes. The presence of death receptors in cancer

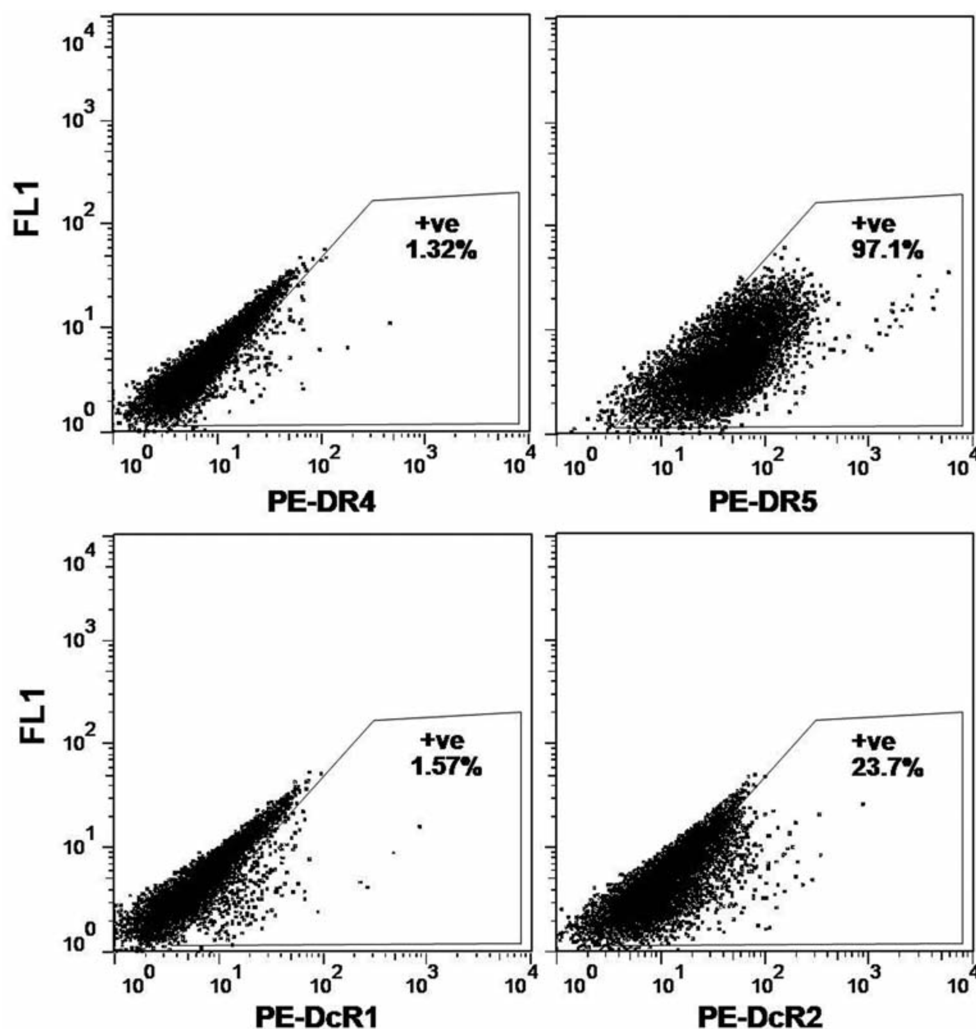

Figure 3. *FACS analysis of TRAIL receptors on HepG2 cells. The reference gate was set according to isotype IgG and secondary antibody controls. The graph represents one of three parallel assessments.*

cells is a prerequisite determinant for TRAIL to induce apoptosis in targeted cancer cells (31). In the present study, HepG2 cells were verified as DR5 dominant death receptors; the expression of TRAIL-GFP and stTRAIL was confirmed by immunoblotting and ELISA analysis. However, the presence of DR5 does not necessarily indicate the sensitivity of HepG2 cells to TRAIL and functional assessments are needed toward developing targeted therapeutic strategies.

In the light of ultimate application for therapeutic purposes, it is essential to understand any possible factors related to the interactions between MSCs and tumor cells. These factors include the forms of transfected anticancer genes, required MSC numbers (ratio), direct and/or indirect effects, as well as the effects of native MSCs. As shown in Figures 4 and 5, moderate cell death independent of TRAIL transfection was observed in the low MSC/HepG2 ratio co-cultures, while remarkable cell death was exhibited in the high MSC/HepG2 ratio co-cultures and TRAIL transfection induced additional cell death. The results indicated that MSCs alone play a major role in this MSC-mediated HepG2 cell death, although the cell type of the dead cells was not identified in the current study. In addition to the expression of the transfected anticancer genes, native MSCs secret a number of cytokines, which may contribute to their intrinsic anticancer effects (32). The innate antitumor effects of MSCs were previously recognized in experimental Kaposi's sarcoma (14), hepatic and pancreatic cancer studies (18, 33). The direct effects of MSCs on tumor cell viability are mainly attributed to their intrinsic antitumor properties. However, it is worth noting that MSCs possess both pro- and anti

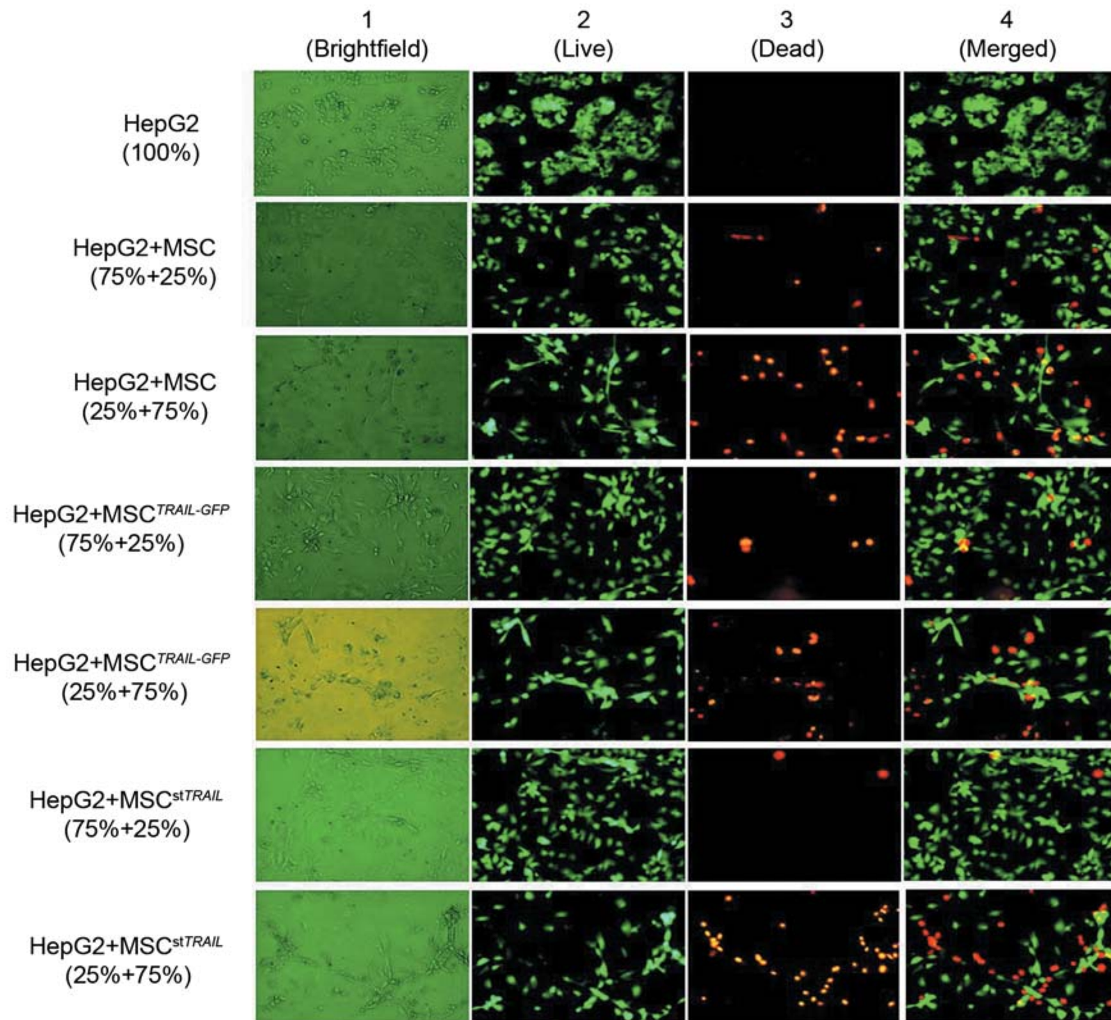

Figure 4. *Cell viability of direct co-cultures. Assessment on day 3 after plating on day 0. Column 1 (brightfield): whole population of cells which were still attached to the surface; column 2: live cells stained with calcein are green; column 3: dead cells stained with EthD-1 show red; column 4: merged images. Original magnification, ×400.*

tumorigenic effects (32, 34). Direct co-culture is capable of determining their dominant effect on particular cancer cells on a cellular level. The present results lay a basic foundation for the development of MSC-based strategies to treat HCC.

Theoretically, MSCs transfected with the secreting form of TRAIL may be more efficient at inhibiting tumor cell growth than the MSCs transfected with the non-secreting form of TRAIL. This viewpoint was not fully supported by the current co-culture study, even though the higher percentage of dead cells was displayed in the indirect co-culture with conditioned media from MSCst^{TRAIL}. It is likely that the presence of MSCs play a major part in MSC-mediated HepG2 cell death. Mueller *et al*.'s recent study on colorectal carcinoma demonstrated that TRAIL-transfected MSCs overcome TRAIL resistance in selected cell lines (35). Further *in vivo* studies are required to investigate the therapeutic potential of engineered MSCs in a real HCC microenvironment.

The live/dead assay has the advantage of being straightforward and reflecting the intact status of detected cells at any given time-point. It is also possible to exhibit cell-to-cell interactions under co-culture conditions. In the present study, a tendency was noticed that more HepG2 cell died in the MSC concentrated area, even though this trend was not quantitatively evaluated. However, the drawback of the live/dead assay is that it only applies to the cells which remain on the culture surface during the staining. The detached cells, most of which are dead cells, are not included in the assessment. Multiple assessments and *in vivo* experiments are required in further studies.

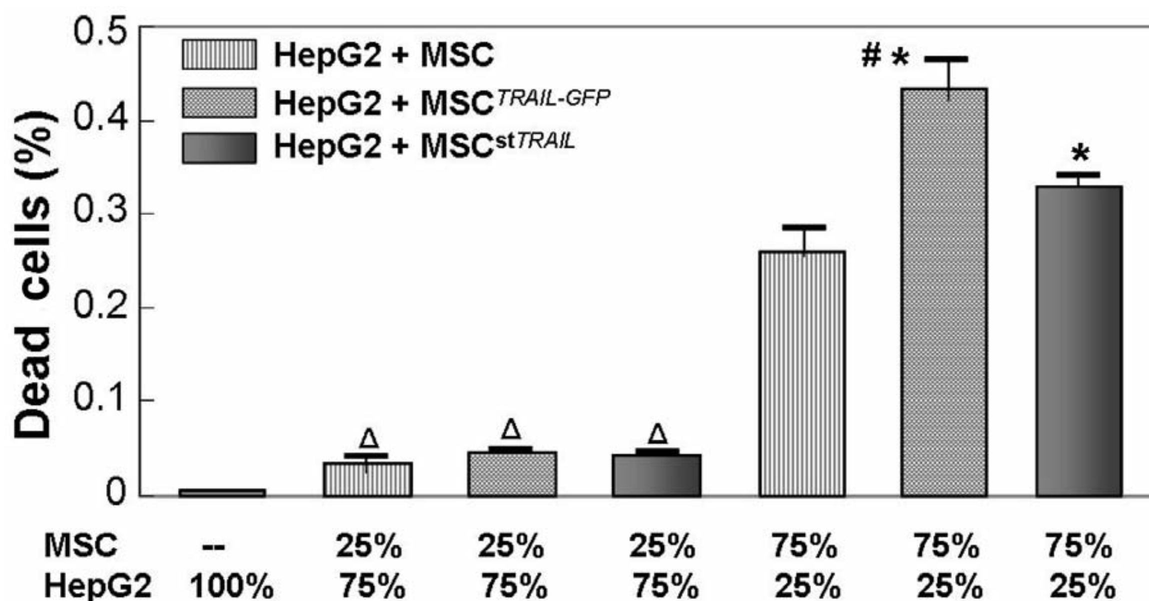

Figure 5. *Summary of cell viability of direct co-cultures. Mean±SEM for three independent experiments. Δ $p<0.05$ vs. control (100% HepG2); *$p<0.05$ vs. 75% MSC/25% HepG2; #$p<0.05$ vs. 75% MSC^{stTRAIL}/25% HepG2.*

In conclusion, the intrinsic properties of MSCs play an important role in the induction of HepG2 cell death under co-culture conditions. MSC-mediated HepG2 cell death is potentiated by TRAIL gene transfection. This study provides additional information on a cellular level contributing to the development of MSC-based strategies to treat hepatocellular carcinoma.

Acknowledgements

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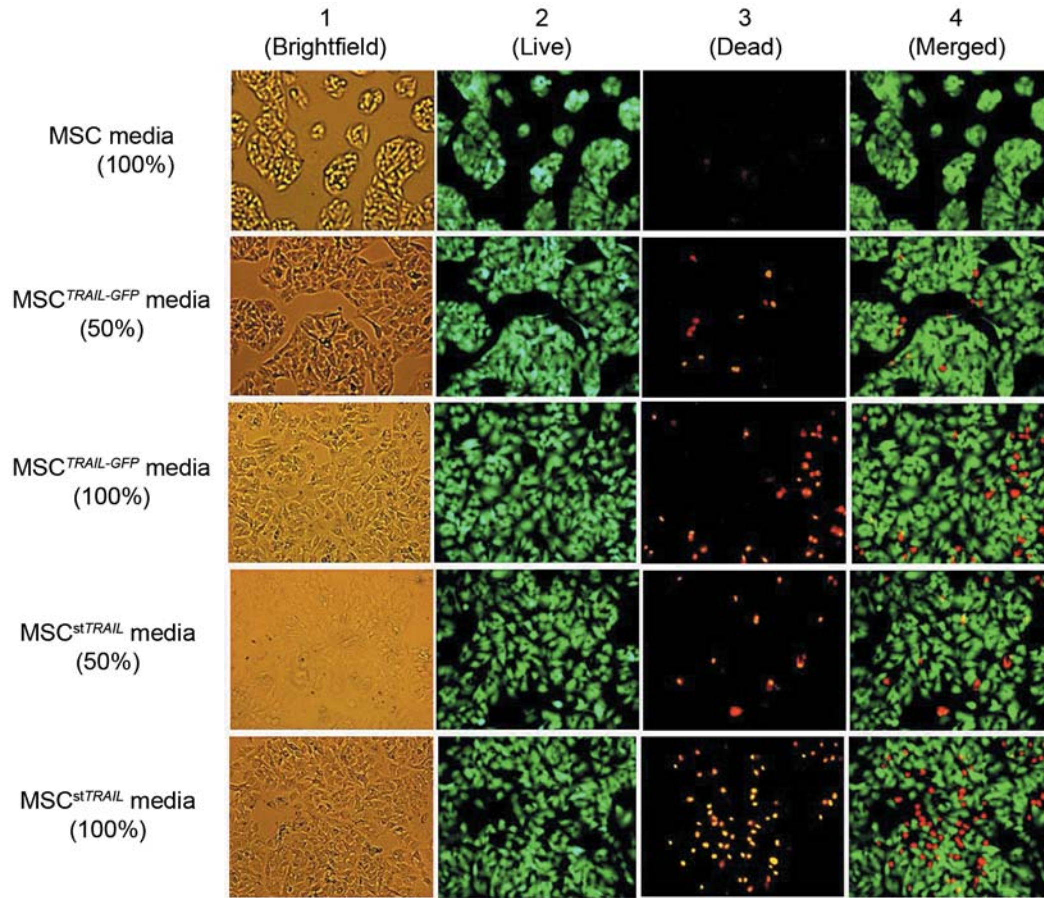

Figure 6. *HepG2 cell viability of indirect co-cultures. HepG2 cells incubated in conditioned media from native MSC, MSC$^{TRAIL-GFP}$ or MSC^{stTRAIL} for 3 days. Medium type and percentage were indicated on the left side of the graph. Images description as described in Figure 4. Original magnification, ×400.*

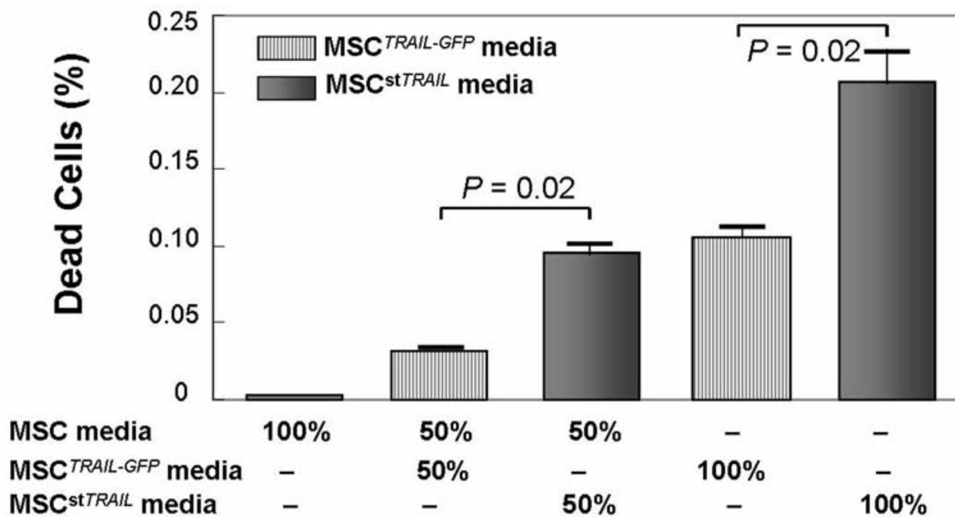

Figure 7. *Summary of HepG2 cell viability of indirect co-cultures. Mean±SEM for three independent experiments.*

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