Activated hepatic stellate cells promote hepatocellular carcinoma development in immunocompetent mice

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Activated hepatic stellate cells (HSCs) play a central role in the hepatic fibrosis and cirrhosis. Recently, HSCs were reported to have strong immune modulatory activities. However, the role of HSCs in hepatocellular carcinoma (HCC) remains unclear. In this study, we showed that HSCs could promote HCC growth both in vitro and in vivo. We examined the HSC-mediated inhibition of T-cell proliferation and the ability of conditioned medium from activated HSCs to promote the growth of murine HCC cell lines in vitro. We also assessed the immune suppression by HSCs during the development of HCC in immunocompetent mice. Cotransplantation of HSCs promoted HCC growth and progression by enhancing tumor angiogenesis and tumor cell proliferation and by creating an immunosuppressed microenvironment. Cotransplanted HSCs inhibited the lymphocyte infiltration in tumors and the spleens of mice bearing tumors, induced apoptosis of infiltrating mononuclear cells, and enhanced the expression of B7H1 and CD4+ CD25+ Treg cells. The immune modulation by HSCs seemed to be systemic. In conclusion, our data provide new information to support an integral role for HSCs in promoting HCC progression in part via their immune regulatory activities, and suggest that HSCs may serve as a therapeutic target in HCC.

Hepatocellular carcinoma (HCC), a common malignant tumor worldwide, is a severe public health concern. Carcinogenesis of HCC is a multifactor, multistep and complex process. Recently, many studies have demonstrated that hepatic fibrosis and liver cirrhosis, which are the ultimate results of chronic liver disease, are closely associated with HCC. Liver fibrosis, cirrhosis and HCC all arise from liver parenchymal cells and mesenchymal cells.

Genetic and cellular biological studies on malignant tumors have shown that the interaction between parenchymal and stromal cells are important for tumor formation and development. Stromal cells include myofibroblasts and endothelial cells, both of which interact with parenchymal cells via (1) the secretion of cytokines and other chemical factors, (2) extra cellular matrix (ECM)-mediated interaction and (3) direct cell-to-cell contingency. Stromal cells are closely related to angiogenesis, cancer desmoplasia and tumor immunity and are therefore important players in the progression, growth and spread of tumors. Furthermore, several studies have shown that tumor cells can further induce the expression of tumorigenic factors in tumor-associated stromal cells, indicating a mutual interaction between cancer cells and stromal cells. Thus, studies on the role of tumor stroma in the development and progression of cancer will help to clarify the mechanisms of carcinogenic lesions and lead to more effective therapeutic approaches.

Hepatic stellate cells (HSCs) are the main collagen-producing cells in the injured liver. Following a chronic liver injury, HSCs play important roles during the development of...
cachectic and cirrhotic animal models. HSCs undergo morphologic and functional transdifferentiation. Recent studies demonstrate that activated HSCs can strongly suppress T-cell responses in vitro and in vivo. Up-regulation of B7H1 (Human B7 homolog 1, also known as programmed death ligand-1, PD-L1), an inhibitory molecule of the B7 family, on activated HSCs was found to contribute to this immune modulatory activity. However, the effect of the immune regulation by HSCs in the HCC microenvironment remains unknown. To study the role of HSCs in HCC progression, we first studied the immune inhibitory activity of HSCs and examined HCC-cell proliferation upon treatment with HSC-conditioned media in vitro. We further employed a cellular transplantation model in immunocompetent mice to analyze the immune modulation of activated HSCs by crosstalk with murine HCC.

In this study, we investigated whether the immune regulatory activities of HSCs could promote HCC growth, especially by inhibiting T-cell infiltration and creating an immunosuppressed tumor microenvironment. Our findings demonstrate that HSCs are key modulators of the immunosuppressed HCC microenvironment, and that this modulation is associated with decreased T-cell infiltration, increased apoptosis of tumor infiltrating mononuclear cells and up-regulation of B7H1 and Treg cells expression.

Material and Methods
Isolation and culture of HSCs
HSCs were isolated from BALB/c mouse livers as previously described. Briefly, the liver was perfused via the portal vein with 10 ml PBS in situ at a rate of 10 ml/min, followed by perfusion with 1 ml 0.01% collagenase IV (Gibco). The whole liver was removed and homogenized and then digested for 30 min at 37°C using 0.01% collagenase. Enzyme activity was neutralized using RPMI 1640 (HyClone) containing 10% fetal bovine serum (FBS). The mixture was then filtered through a 300 μm nylon mesh and purified via Percoll (Sigma) gradient centrifugation. The isolated HSCs were cultured in uncoated plastic plates with RPMI 1640 supplemented with 10% FBS and incubated overnight at 37°C and 5% CO2. After incubation, the plates were washed extensively with PBS to remove residual nonadherent cells. The viability of HSCs was determined via trypan blue exclusion. The medium was changed every 3 days, and the cells were split when they reached 70% confluence. HSCs were used at passages 3–10 for the experiments.

Cell lines
The murine hepatic cancer cell lines Hepa-1-6 and H22 were purchased from Shanghai Cell Bank, Chinese Academy of Sciences. The Hepa-1-6 cell line was maintained in Dulbecco’s Modified Eagle Medium (DMEM, HyClone) supplemented with 10% FBS. H22 was maintained in RPMI 1640 containing 10% FBS.

Animals
C57BL/6 (H-2b, haplotype) and BALB/c (H-2d, haplotype) mice were purchased from the National Rodent Laboratory Animal Resources, Shanghai branch. Mice were maintained in pathogen-free conditions and cared for according to the Laboratory Animal Care guidelines. They were given radiation-sterilized food pellets and distilled water. Adult male animals aged 8 to 12 weeks were used.

Mixed lymphocyte reaction
The MLR was performed in 96-well microtiter plates using RPMI 1640 medium supplemented with 10% FBS. Nylon wool-elicited BALB/c T cells were plated at 2 × 105 cells per well with 2 × 104 25 Gy γ-irradiated DCs from C57BL/6 mice in complete culture medium. To examine the effect of HSCs on T-cell proliferation, 50 Gy γ-irradiated HSCs were added to the MLR at 2,000, 4,000 or 20,000 cells per well at the start of culture. Triplicate cultures were set up for each treatment. After 72 hr in culture, cell proliferation was measured using a BrdU Cell Proliferation Assay (Chemicon). Subsequently, cells were incubated with 20 μl/well fresh medium containing BrdU at a 1:500 dilution for 4 h at 37°C. After removal of the culture medium, cells were fixed and then incubated with 100 μl/well anti-BrdU monoclonal antibody (1:200 dilution) for 1 hr at room temperature. After washing, 100 μl/well peroxidase-conjugated goat anti-mouse IgG was added, and the cells were incubated for 30 min at room temperature. The cells were then washed and incubated with 100 μl/well TMB Peroxidase Substrate for 30 min at room temperature in the dark. After the 30-min incubation, the reaction was stopped by addition of 100 μl/well acid Stop Solution. The developed color was read at 450 nm using a microtiter plate reader (Bio-rad). The percentage of suppression was calculated using the following formula: percentage suppression = (1 – [Test cell + MLR OD value/MLR OD value]) × 100%.

Measurement of cell proliferation
Conditioned medium was harvested from cultured activated HSCs (HSC-CM) after incubation in serum-free RPMI 1640 for 24 hr. At the end of the incubation period, the medium was filtered and stored at 80°C until use. To determine the effect of the HSC-CM, the cancer cells were cultured in triplicate at 4 × 103 cells/well in 96-well plates and allowed to adhere overnight; the culture medium was then replaced with conditioned medium containing 10% FBS for 3 days. Cell proliferation was measured using the BrdU Cell Proliferation Assay (Chemicon) as described for the mixed lymphocyte reaction assay. The absorbance values directly correlated with the amount of DNA synthesis and thus with the number of proliferating cells in culture.

Cytokine Assay and VEGF ELISA
The conditioned medium was harvested and analyzed for the presence of cytokines using a mouse cytokine array panel.
Int. J. Cancer: were examined and the five areas with the most intense neo-
vascularization (the hot spots) were selected. Microvessels (composed of CD31-positive endothelial cells) in these areas were counted at high-power fields (HPFs) (×200). The mean microvessel count of the five most vascular areas was taken as the MVD, which was expressed as the absolute number of microvessels per HPF (×200).

Western blot analysis
Tissue samples were homogenized using an ultra-turrax T10 B (IKA) and centrifuged to remove debris. Tissue extracts were resolved on 12% SDS polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked in 5% nonfat dried milk for 1 hr. Blots were then incubated with an anti-VEGF (Beyotime) overnight at 4°C. An HRP-conjugated secondary antibody was added and the blots were incubated for 1 hr at room temperature. The immunoreactive products were then detected using Enhanced Chemiluminescence kit (Pierce Thermo Scientific) and quantified by densitometry. β-Actin (Sigma) was used as a loading control. Densitometric analyses of the bands were performed using Gel Pro Analyzer 4.0.

Flow cytometry
Splenocytes were isolated from the spleens of mice bearing tumors by disaggregation into 10 ml RPMI 1640 complete culture medium. Erythrocytes were lysed in Red Blood Cell Lysis Buffer (Beyotime) and subsequently washed three times in RPMI 1640 containing 10% FBS. The cells were counted, and 10^6 splenocytes were incubated with FITC-CD3, PE-CD4 and PE-CD8. The fluorescently labeled cells were analyzed on a flow cytometer (Beckman) using CELIQuest Pro software. The appropriate isotype control antibodies were also used.

TUNEL assay
Apoptotic cells were evaluated using a TUNEL assay, the FragEL DNA Fragmentation Detection Kit (Calbiochem), according to the manufacturer’s instructions. Briefly, tumor slides were deparaffinized and permeabilized with proteinase K (20 μg/ml) for 10 min and treated with 3% H_2O_2 to inactivate endogenous peroxidase. Slides were then incubated with terminal deoxynucleotidyl transferase (TdT) enzyme in a humidified chamber at 37°C. After incubation in blocking buffer, the sections were incubated with the peroxidase streptavidin and 3,3’diaminobenzidine staining. Slides were counterstained with a hematoxylin before microscopic examination (Leica, DM2500). The apoptotic index was calculated by counting the number of positively stained cells in 10 randomly selected HPFs.

Statistical analysis
Data were analyzed using SPSS software (version 13.0). Results are expressed as means ± SD. Statistical analyses were performed using a one-way ANOVA and Student’s t-test. The significance level was set at 0.05.
Results

HSC isolation and activation
HSCs were obtained by culturing BALB/c mouse liver non-parenchymal cells. Cells were selected in uncoated plastic flasks to remove hematopoietic cells. HSCs exhibited a star-like morphology in the first week after isolation, and then gradually developed into fibroblast-like cells (Fig. 1a).

Desmin and \( \alpha \)-SMA are specific markers of HSCs. The former is expressed in quiescent and activated HSCs, while the latter is only expressed in activated HSCs. To confirm the identity of the HSCs in our study, the cells were stained with an antibody against desmin. As shown in Figure 1b, more than 95% of cells were positive for desmin (Fig. 1b, upper panel). After 14 days in culture, the isolated HSCs were intensely activated with strong \( \alpha \)-SMA expression throughout the cells (Fig. 1b, lower panel).

HSCs inhibit T cell proliferation
To test the immunosuppressive functions of activated HSCs, we performed a mixed lymphocyte reaction (MLR) assay. HSCs were added into the MLR assay culture as the third-party (regulatory) cells. The results showed that with the increases in the HSC/T cell ratio, the inhibitory effect of the HSCs on T cells increased from 30 to 80% \((p < 0.001)\), indicating that HSCs suppressed T-cell proliferation in a dose-dependent manner (Fig. 2).

Stimulation of HCC cell proliferation by conditioned media from activated HSCs
We then investigated whether HSCs could induce proliferation of HCC cells despite inhibiting T lymphocyte cell proliferation \textit{in vitro}. When two murine HCC cell lines (H22 and Hepa 1-6) were cultured with the conditioned media from activated HSCs, the proliferation of these cells obviously increased \((p < 0.05); \text{Fig. 3a})\). This indicated that some stimulatory factors secreted by HSCs might be present in the conditioned media. To determine the profile of these potential stimulatory factors, the HSC-CM was analyzed using a mouse cytokine panel assay (R&D Systems). As shown in Figure 3b, high levels of the following cytokines were identified: granulocyte colony-stimulating factor (G-CSF), soluble intercellular adhesion molecule-1 (sICAM)/CD54, interleukin-1 receptor antagonist (IL-1ra), interleukin-6 (IL-6), CXCL10, KC, monocyte chemotactic protein-1 (MCP-1)/CCL2, MCP-5/CCL12, macrophage-inflammatory protein-2 (MIP-2), MIP1\( \alpha \)/CCL3, CCL5/ regulated on activation, normal T expressed and secreted (RANTES) and tissue inhibitor of metalloproteinase-1.
(TIMP-1). Moderately high levels of granulocyte macrophage colony-stimulating factor (GM-CSF), I309/CCL1, interferon-gamma (IFN-γ), IL-1α, macrophage colony-stimulating factor (M-CSF), MIP1β/CCL4 and stromal cell-derived factor-1 (SDF-1)/CXCL12 were also detected in the conditioned media. These factors are potentially responsible for immune modulation and cell growth in the tumor microenvironment.

**Cotransplantation with HSCs promotes HCC growth in vivo**

We then further examined the effect of HSCs on HCC growth in vivo. H22 cells (5 × 10^5 cells/mouse) originating from BALB/c mice HCC lines were implanted subcutaneously, either alone or in combination with activated HSCs (2 × 10^5 or 5 × 10^5 cells/mouse), into BALB/c mice. All of the mice developed tumors at the site of implantation. The mice implanted with HSCs in addition to H22 cells developed much larger tumors than the mice implanted with H22 cells alone, and the size of the tumors was HSC dose dependent (Fig. 4a). Cotransplantation with activated HSCs also significantly promoted tumor growth compared with injection of H22 cells alone. As shown in Figure 4b, at 12 days after cell implantation, the volume of tumors derived from H22 cells plus HSCs (H22: HSC ratio = 10:1) was twofold greater than that derived from H22 cells alone (p < 0.05). This difference in tumor volume was even more distinct on day 20 postinjection (p < 0.01). Activated HSCs (2 × 10^5 cells/mouse) alone, which were implanted as a negative control, did not result in any tumor formation within 3 months.

To verify the presence of residual HSCs in the tumor, HSCs were stained with CFSE before injection, and the tumors were cryosectioned and photographed. As shown in Figure 4c, CFSE-labeled HSCs were found in the tumors, implying that HSCs were present in the tumor tissue.

We next investigated the effect of HSCs on HCC tumor cell proliferation in vivo. Immunohistochemistry was used to examine tumor samples for the expression of nuclear Ki67, a cellular proliferation marker. As shown in Figure 4d, the number of Ki67-positive cells significantly increased in the...
H22 plus HSC condition compared with the H22 alone condition \( (p < 0.001) \). These results demonstrated that cotransplantation of HSCs significantly promoted tumor cell proliferation in vivo.

**Effect of cotransplantation with HSCs on tumor microvessel density and VEGF expression**

Rapid and adequate tumor revascularization is another crucial factor for tumor growth. To assess whether HSC implantation-induced tumor growth and development is correlated with HSCs’ in vivo pro-angiogenic effect, we performed immunohistochemistry stain on tumor samples for CD31, a specific endothelial marker widely used for microvessel quantification.\(^{17}\) As shown in Figure 5a, numerous CD31-positive cells were detected in HSC cotransplantation tumors, whereas only a few CD31-positive cells were present in H22 transplantation tumors. Microvessel quantification also indicated that cotransplantation of HSCs significantly unregulated angiogenesis \( (p < 0.001) \).

VEGF, a secreted protein, is the most critical molecular for angiogenesis; therefore, we assessed VEGF expression in tumors. For this purpose, an ELISA assay was carried out to examine the expression level of VEGF in the HSCs. The data showed that the level of VEGF in serum-free conditioned medium was 138 ± 14 pg/ml, while the control RPMI 1640 medium did not contain detectable VEGF (Fig. 5b). We also analyzed the expression level of VEGF in tumor samples using immunohistochemistry. Tumors from the HSC cotransplantation group exhibited more intensive VEGF staining than the H22 alone control tumors (Fig. 5c), and the integral optical density (IOD) was significantly increased in the HSC/H22 cotransplantation group, over the H22 alone transplantation group \( (p < 0.05) \). Moreover, western blot analysis of VEGF also indicated that the expression of VEGF was up-regulated in the HSC/H22 cotransplantation group, and quantitative analysis by densitometry further confirmed the significance of this increase in VEGF expression \( (p < 0.05; \text{Fig. 5d}) \). Thus, the data above suggest
that cotransplantation of HSCs significantly enhances tumor angiogenesis.

**Cotransplantation of HSCs creates an immunosuppressive microenvironment**

HSCs are known to be involved in immune modulation, and we showed that they could inhibit T-cell proliferation in vitro (Fig. 2). To determine whether HSCs could attenuate T-cell infiltration in tumors, we detected T-cell infiltration in the tumor samples using immunostaining. As shown in Figure 6a, upon cotransplantation with HSCs, the tumors contained fewer CD3-, CD4- and CD8-positive cells, than tumors formed from H22 cells alone ($p < 0.05$). These results indicate that HSCs play a role in attenuating T-cell infiltration. Meanwhile, we also examined the expression of subtypes of T cells in the spleens of the mice by flow cytometric analysis. As shown in Figure 6b, the number of CD3$^+$ T cells was significantly decreased in the spleens of HSC cotransplantation mice ($p < 0.001$). Importantly, both CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T-cell subpopulations were decreased in the HSC
cotransplantation group ($p < 0.05$). These in vivo data suggest that HSCs may take part in the maintenance of immune tolerance, which in turn contributes to the tumor growth.

To investigate whether HSC cotransplantation results in the creation of an immune inhibitory microenvironment, we examined apoptosis of infiltrating mononuclear cells in the tumor tissue using a TUNEL assay. Representative immunohistochemical results and its quantification data are presented in Figure 6c. The infiltrating mononuclear cells were identified based on their characteristic morphology. The results
demonstrated that there were many more apoptotic mononuclear cells (indicated by arrowheads), which possess smaller nuclei than tumor cells (arrows), in the HSC cotransplanted tumors than the H22-transplanted tumors. Quantification of the number of TUNEL-positive mononuclear cells showed that cotransplantation with HSCs results in a approximately twofold ($p < 0.01$) increase in apoptotic rate compared with transplantation with H22 cells alone ($13.2 \pm 4.8$ TUNEL-positive cells per HPF in the H22 group vs. $25 \pm 6.2$ in the HSC cotransplantation group; Fig. 6c).

B7H1 is expressed within the tumor microenvironment. It appears that upregulation of B7-H1 is a mechanism that cancers employ to evade the host immune system. Therefore, we examined B7H1 expression in the tumor samples. The results showed that cotransplantation of HSCs obviously increased B7H1 expression (Fig. 6d). It has been reported that regulatory T (Treg) cells can inhibit antigen-specific immune responses and thus play an important role in tumor immune tolerance. We, therefore, evaluated the presence of Treg cells in the tumors. The results demonstrated that the percentage of CD4$^+$CD25$^+$ Treg cells of the infiltrated CD4$^+$ T cells in HSC/H22 cotransplanted tumors was much higher than that in the H22 alone transplanted tumors ($p < 0.05$; Fig. 6e). Thus, the results above indicate that HSCs can create a microenvironment that inhibits the host immune response.

Discussion

In this study, HSCs isolated from BALB/c mice and activated by culture in vitro were cotransplanted with H22 hepatoma cells into immunocompetent BALB/c mice to examine tumor growth. The results showed that cotransplantation of HSCs significantly increased tumor volume, indicating that HSCs may assist in HCC growth in vivo.

Multiple mechanisms may be involved in the HSC-induced hepatic tumor growth. However, it is currently unknown whether the immune modulation activity of HSCs plays a role in this process. Most previous experiments addressing this topic were performed in immunodeficient mice, and therefore the role of the immune function of HSCs in tumorigenesis was not investigated. By using immunocompetent mice and murine hepatic tumor cells, we were able to demonstrate that HSCs promoted HCC tumor growth by acting as key immune modulators. Additionally, we showed that HSCs promoted tumor angiogenesis and HCC cell proliferation both in vitro and in vivo. Our study provides new information to support the growing evidence that HSCs play an integral role in promoting HCC progression and reinforces the importance of HSCs as a therapeutic target for HCC.

Angiogenesis is required for tumor growth and plays a key role in cancer progression. In our study, we found that the number of neovessels and the level of VEGF expression were both significantly increased in the tumors derived from HSC cotransplanted mice (Fig. 5a), indicating that these tumors have a higher vascular density and a better blood supply than control tumors. A pro-angiogenic role of HSCs in experimental melanoma metastasis has also been reported by Olaso et al.

The architecture of a malignant solid tumor consists of a mixture of cancer cells and host cells in variable proportions. The interactions of cancer cells with stromal cells and especially with cytokines, chemokines and growth factors derived from stromal cells, are important for the cancer development and are currently under investigation. The stromal cells and their secreted factors constitute a microenvironment that promotes cancer growth. In our study, conditioned medium from activated murine HSCs significantly increased the proliferation of murine HCC cells (H22 and Hepa1-6 lines, specifically). This finding is consistent with other reports that conditioned medium from human HSCs can induce the growth of human HCC cell lines.

We found that HSCs could suppress T-cell proliferation stimulated by allogeneic DC in vitro. This finding is in line with previous reports in which activated HSCs were shown to have important immune regulatory roles in vitro and in vivo. However, the mechanism by which HSCs suppress the proliferation of T cells remains unclear. It has been reported that direct contact of HSCs with responder T cells may not be necessary for immunosuppression; similar results were reported for mesenchymal stem cells. Therefore, soluble factors secreted by activated HSCs, such as growth factors, chemokines and cytokines may be responsible for their immunosuppressive effect. As shown in the Figure 3b, conditioned medium from HSCs contained high concentrations of sICAM-1 and IL-6. ICAM-1 belongs to the immunoglobulin superfamily with the function of cellular adhesion and transmigration of leukocytes. It is also an immunosuppressant factor, because local release of ICAM-1 appears to promote local immune tolerance and cancer cell immune escape. IL-6, a pro-inflammatory and tumor-promoting cytokine, is involved in hepatic inflammation and activation of the oncogenic transcription factor STAT3. The secretion of IL-6 by mesenchymal stem cells has been reported to inhibit the differentiation of monocytes into DCs and thus decrease the ability of monocytes to stimulate T cells. However, neutralizing antibodies against CD54 and IL6 did not efficiently reverse the suppressive effect of HSCs on T-cell proliferation (data not shown). This suggests that factors secreted by HSCs may provide synergistic signals and that one factor alone may be insufficient to mediate immunosuppression. Thus, HSC-mediated immune modulation and promotion of cellular proliferation may be a result of the cumulative actions of several factors.

In addition, the high level expression of B7H1 on HSCs is another potential mechanism for the immune regulatory function of these cells. HSC-induced T-cell hyporesponsiveness is associated with enhanced T-cell apoptosis via B7-H1. In our study, we found that cotransplantation of HSCs resulted in increases in apoptosis of infiltrating mononuclear cells and B7H1 expression. Apoptosis of infiltrating mononuclear cells is considered as a mechanism through which colorectal cancer cells escape attacks from the immune system.
system and subsequently grow without immune control. B7H1 is abundant in various cancer cells, and overexpression of B7H1 is involved in immune evasion of tumor cells.\(^6\) Blockade of B7H1 improves the immune response against murine hepatocarcinoma H22.\(^3,^2\) B7H1 has emerged as an important mediator of host immune suppression. Additionally, the number of Treg cells was also increased in HSC cotransplantation tumors. An early study demonstrated that IFN-\(\gamma\)-activated HSCs could act to expand the population of Treg cells with strong immune regulatory activities \textit{in vivo} and \textit{in vitro}, thereby inducing T-cell hyporesponsiveness \textit{in vivo}.\(^3,^3\) The role of Treg cells in the control of immune responsiveness has been clearly identified in the inhibition of innate immune responses to tumors.\(^3,^4\) These observations point all to the underlying regulatory mechanisms by which HSCs create an immunosuppressed microenvironment.

Interestingly, the immunosuppressive activity of HSCs in our model seemed to have a systemic effect to some extent. Cotransplantation of HSCs was able to not only decrease the numbers of CD4\(^+\)T and CD8\(^+\) T cells in tumors, but also decrease the total and subset of T lymphocyte populations of the splenocytes isolated from tumor-bearing mice. This result is in contrast to a previous report suggesting that the cotransplanted HSCs have only a local effect, \textit{i.e.}, they were able to protect islet allografts from rejection, but they could not protect islet allografts implanted into the contralateral kidney.\(^12\) This phenomenon further supports the notion that factors secreted by HSCs indeed play an important role in immune modulation. It is likely that the inhibition of the local and systemic immune response and the creation of a local immunosuppressive microenvironment by activated HSCs, which are known to enhance carcinoma progression, resulted in acceleration of HCC development \textit{in vivo}.

In summary, we demonstrated an inhibitory effect of HSCs on immune responses via regulation of T cells in the HCC microenvironment. Our present study is the first to assess the immune suppressive function of HSCs during HCC development using a cell transplantation model in immunocompetent mice. Our results demonstrate that HSCs can promote the progression of HCC by the following mechanisms: (1) creating an immunosuppressive microenvironment, (2) promoting angiogenesis, or (3) inducing proliferation of the tumor cells. Because HSCs play such an important role in HCC progression, activated HSCs may be a suitable target for HCC therapy. Importantly, targeting these stromal cells rather than the HCC cells may result in a greater response, because the stromal cells appear to have a more normal and relatively stable genetic landscape when compared to the heterogeneous and genetically unstable HCC cells.\(^23\)

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