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Original Articles

Non-CSCs nourish CSCs through interleukin-17E-mediated activation of NF-κB and JAK/STAT3 signaling in human hepatocellular carcinoma

Yongli Luo [a,1], Zhi Yang [a,1], Li Su [a], Juanjuan Shan [a], Huailong Xu [a], Yanmin Xu [a], Limei Liu [a], Wei Zhu [a], Xuejiao Chen [a], Chungang Liu [a], Jun Chen [a], Chao Yao [a], Feifei Cheng [b], Chengcheng Zhang [a], Qinghua Ma [a], Junjie Shen [a,*], Cheng Qian [a,**]

[a] Institute of Pathology and Southwest Cancer Center, Southwest Hospital, Third Military Medical University, Chongqing, 400038, China
[b] School of Life Science, Zhejiang Sci-Tech University, Hangzhou, 310018, China

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ABSTRACT

Within the cancer stem cell (CSC) niche, non-CSCs play an indispensable role in facilitating a microenvironment capable of maintaining CSC properties. Non-CSCs contribute to not only the structure and topology of the tumor microenvironment but also the maintenance of the dynamic state of CSCs. Interleukin-17E (IL-17E/IL-25) is important in allergic inflammation and protection against parasitic infection. Moreover, it has also been demonstrated that IL-17E takes part in different cancers recently. Here, for the first time we demonstrate that discrepant expression of IL-17E and the IL-17 receptor B (IL-17RB) exists in Nanog positive ($Nanog^{Pos}$) CSCs and Nanog negative ($Nanog^{Neg}$) non-CSCs in hepatocellular carcinoma (HCC). Moreover, we further demonstrate that IL-17E binding to IL-17RB activates NF-κB and JAK/Stat3 pathways to promote proliferation and sustain self-renewal of CSCs in HCC. Meanwhile, the beneficial effect of IL-17E on $Nanog^{Pos}$ CSCs could be blocked by specific inhibitors of JAK and NF-κB signaling. All the findings indicated that non-CSC-derived secreted IL-17E binds IL-17RB on CSCs to signal via JAK/Stat3 and NF-κB pathways to mediate crosstalk between CSCs and non-CSCs. Therefore, IL-17E/IL-17RB signaling represents a potential therapeutic target for treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) remains a major cause of cancer-related mortality globally. Like other solid tumors, HCC is thought to be hierarchically organized with growth driven by small subpopulation of undifferentiated cancer stem cells (CSCs) or termed tumor-initiating cells (TICs) [1,2]. CSCs are characterized by their self-renewal capacity, multi-lineage differentiation properties and highly oncogenic potential [3,4]. While CSCs reside in cancer stem cell niche (CSC-niche) and its fate is largely determined by extrinsic signals stemming from the tumor-microenvironment by means of cell–cell contact or secreted factors [5,6]. The concept of CSCs entitles cancer stem cells to the core position for maintaining tumor growth. However, the role of Non-CSCs in sustaining the tumor fate, especially for the CSCs fate, remains largely unexplored.

Recent studies have revealed that interactions between different subclones of tumor cells help maintain the tumor heterogeneity [7]. Although some studies have explored the supporting roles of Non-CSCs for CSCs [8], the details of interactions between CSCs and Non-CSCs, especially in HCC, have not been well described. Our previously-established Nanog promoter fluorescence reporter strategy can easily isolate and trace both Nanog positive ($Nanog^{Pos}$) CSCs and Nanog negative ($Nanog^{Neg}$) non-CSCs, which facilitates study of the relationship between CSCs and non-CSCs in HCC [9].

Here we describe the crosstalk between $Nanog^{Pos}$ CSCs and $Nanog^{Neg}$ non-CSCs in HCC. Moreover, the interleukin-17 (IL-17) family of cytokines has emerged as a critical factor in inflammatory diseases and some cancers. In this study, we demonstrated that $Nanog^{Neg}$ non-CSCs maintain the properties of $Nanog^{Pos}$ CSCs by promoting Interleukin-17E (IL-17E)/Interleukin-17 receptor B (IL-17RB) signaling through both the NF-κB and JAK/Stat3 pathways. Our data underscore the non-CSCs back-feeding role in tumorigenicity and provide new prospect on the effects and underlying mechanisms of IL-17E on the hepatocellular carcinoma CSCs.

Abbreviations: CSCs, cancer stem cells; non-CSCs, non- cancer stem cells; IL, interleukin; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; JAK, Janus kinases; STAT, signal transducer and activator of transcription; NF-kB, nuclear factor-k-gene binding; shRNA, short hairpin RNA; TICs, tumor-initiating cells; RT-PCR, reverse-transcriptase polymerase chain reaction; SCID, severe combined immunodeficiency; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

* Corresponding author. Tel.: 86 23 68765461; fax: 86 23 68752247.
E-mail address: junjiesh@gmail.com (J. Shen).
** Corresponding author. Tel.: 86 23 68765957; fax: 86 23 68752247.
E-mail address: cqian8634@gmail.com (C. Qian).
[1] These authors contributed equally to this work.

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Materials and methods

Cell culture and cell lines

All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO-BRL) at 37 °C under a 5% CO_2 atmosphere. Human HCC cell lines Huh7 and PLC/PRF/5 were purchased from the Shanghai Cell Collection (Shanghai, China). Patient-derived primary HCC cells T1115 and T1224 were obtained from patient tumor specimens with informed consent according to protocols approved by the Institutional Review Board of the Southwest Hospital, Third Military University (Chongqing, China), as previously reported [9].

Cytokines array analysis

Approximately 5×10^6 isolated $Nanog^{Pos}$ or $Nanog^{Neg}$ cells derived from the HCC cell line Huh7 were seeded into a 10 cm dish and incubated for 4 h with DMEM + 2% FBS, followed by a change to 5 mL serum-free DMEM and further incubation for 24 h. Supernatant was collected for a cytokines array test performance (RayBiotech, Inc.Cat#:AAH-BLM-1-2(2-2)).

Clone formation assay

For clone formation efficiency assay, 10 cells were sorted by FACS and seeded per well in 96-well plates. After 14 days culture, clones were fixed by methanol and dye with Giemsa (Sigma-Aldrich) and clone (>50 cells) numbers were assessed microscopically. Fresh media was added every 3 days.

Sphere formation assay

For sphere formation efficiency assay, single $Nanog^{Pos}$ and $Nanog^{Neg}$ cell were sorted and plated into ultra-low attachment 96-well plates (Costar, Corning Inc., Corning, NY). Each well was seeded with 10 cells. Cells were cultured in DMEM/F12 media (Sigma-Aldrich, St. Louis, MO) with B27 supplement (GIBCO), antibiotics, 20 ng/mL epidermal growth factor (PeproTech, Rocky Hill, NJ), 20 ng/mL basic fibroblast growth factor (PeproTech), and 10 ng/mL of hepatocyte growth factor (PeproTech). Next, 1% methylcellulose (Sigma-Aldrich) was added to prevent cell aggregation, and individual spheres derived from a single cell were confirmed. After 4–5 days, equal fresh media was added. Cells were incubated for 2 weeks, and spheres of diameter >75 μm were counted.

Cell proliferation assays

Cells were harvested and suspended for cell counting using TC10™ Trypan Blue Dye, and a Cell Titer-Blue Cell proliferation assay was performed according to the manufacturer's instructions (Promega). Briefly, 96-well plates were seeded with 1000 or 3000 cells/well. Every 24 h–48 h, reagent was added to each well and absorbance at 450 nm measured.

Tumor formation assay

Male non-obese diabetic mice with severe combined immunodeficiency (NOD-SCID) mice at 4–6 weeks of age were maintained in pathogen-free conditions at the animal facility of the Third Military Medical University and received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences. $Nanog^{Pos}$ and $Nanog^{Neg}$ cells were sorted and counted by FACS. Approximately 1×10^4 cells were then resuspended in serum-free medium and mixed with Matrigel at ratio of 1:1. Cells were injected subcutaneously into NOD-SCID mice. Tumor formation was evaluated regularly after injection by palpation of injection sites.

Statistical analysis

All data are presented as mean ± standard deviation. When two groups were compared, Student's *t*-test was used. A *P*-value < 0.05 was considered significant statistically and is marked with an asterisk. A *P*-value < 0.01 was considered highly significant statistically and is marked with a double asterisk.

Results

Crosstalk between CSCs and non-CSCs promotes and maintains tumor malignancy

To investigate whether an interaction between CSCs and non-CSCs exists in HCC, a Nanog promoter-driven green fluorescent protein (GFP) reporter system was used [9]. Firstly, according to the GFP expression intensity, $Nanog^{Pos}$ CSCs and $Nanog^{Neg}$ Non-CSCs were sorted from Huh7 cells by fluorescence activated cell sorting (FACS). Then, the cells were divided into serial combinations: $Nanog^{Pos}$, $Nanog^{Neg}$ and their mixture with different proportions ($Nanog^{Neg}$: $Nanog^{Pos}$ = 1:49, 1:19, 1:9 and 1:1). All groups were cultured for 9 days *in vitro* to analyze their proliferation rate. Comparing with the $Nanog^{Pos}$ alone group, co-cultured $Nanog^{Neg}$ non-CSCs and $Nanog^{Pos}$ CSCs groups increased cell proliferation rates by 0.7%, 13.9%, 43.5% and 26.4% at ratio of 1:49, 1:19, 1:9 and 1:1, respectively (Fig. 1A). Interestingly, we found that mixing $Nanog^{Pos}$ cells with a small amount of $Nanog^{Neg}$ cells could significantly promote the global cells growth rate, although non-CSCs generally proliferate significantly slower than CSCs counterparts do, including these $Nanog^{Neg}$ non-CSCs and $Nanog^{Pos}$ CSCs cells [10]. Importantly, the cell proportion of 1:9 ($Nanog^{Neg}$:$Nanog^{Pos}$ = 1:9) displayed the greatest difference among all these groups ($P < 0.01$). We also noticed that adding more $Nanog^{Neg}$ cells into $Nanog^{Pos}$ cells as the ratio of 1:1($Nanog^{Neg/Pos=1:1}$) decreased the global cells proliferation compared with $Nanog^{Neg/Pos=1:9}$ group. That might be caused by increasing proportion of slow-growth $Nanog^{Neg}$ cells reduced the whole group proliferation. Additionally, adding $Nanog^{Pos}$ cells into $Nanog^{Neg}$ cells group as the ratio of 1:9 ($Nanog^{Pos/Neg=1:9}$) also raised the global cells proliferation (data not shown). All these findings suggested that the cell proportion ratio of 1:9 represent a reasonable rate within all these tested groups, which is used for the rest of this study.

Then, the mixture of $Nanog^{Neg/Pos=1:9}$ and $Nanog^{Pos/Neg=1:9}$ as well as their respective counterparts $Nanog^{Pos}$ and $Nanog^{Neg}$ cells were cultured *in vitro* for 11 days in Huh7 and PLC/PRF/5 cells. The data showed that co-culture of $Nanog^{Pos}$ and $Nanog^{Neg}$ cells grow much faster than the corresponding control cells. Moreover, the proliferation rates of co-cultured groups were significant higher than the theoretical value. This value was calculated by the ratio of $Nanog^{Pos}$ and $Nanog^{Neg}$ multiplied respective proliferation rate (Fig. 1B and Supplementary Fig. S1). Subsequently, 1×10^4 cells of these groups were subcutaneously implanted into non-obese diabetic with severe combined immunodeficiency (NOD-SCID) mice to investigate their tumor-initiating capacity *in vivo*. Tumor volume and weight were measured regularly and animals were sacrificed after 36 days. As shown in Fig. 1C, tumors derived from $Nanog^{Neg/Pos=1:9}$ or $Nanog^{Pos/Neg=1:9}$ groups were significantly larger and heavier than $Nanog^{Pos}$ or $Nanog^{Neg}$ groups, respectively ($P < 0.01$; Fig. 1C). Noticeably, the tumor derived from $Nanog^{Neg/Pos=1:9}$ group was initiated the earliest (17 days after injection) among these groups. Taken together, our data suggest that, indeed, $Nanog^{Neg}$ non-CSCs and $Nanog^{Pos}$ CSCs interact, which results in more aggressive tumor behavior both *in vitro* and *in vivo*.

IL-17E is a potential candidate for mediating the crosstalk between $Nanog^{Pos}$ CSCs and $Nanog^{Neg}$ non-CSCs in HCC

To investigate whether the interaction between $Nanog^{Neg}$ non-CSCs and $Nanog^{Pos}$ CSCs was mediated by means of a cell–cell direct or an indirect manner. $Nanog^{Pos}$ cells were indirectly co-cultured with $Nanog^{Neg}$ cell, separated by Millicell™ Culture Plate Inserts for 7 days (Fig. 2A), or cultured with supplied of $Nanog^{Neg}$ cells condition medium (CM) for 9 days (Fig. 2B). Then the proliferation rates were measured. We interestingly found that under both conditions, $Nanog^{Pos}$ CSCs and $Nanog^{Neg}$ non-CSCs cultured within mixed supernatant significantly improved the proliferation compared with both the control groups and the theoretical value (Indicated by "△"). These findings suggested that the indirect communication plays a crucial role in mediating the crosstalk between $Nanog^{Pos}$ and $Nanog^{Neg}$ cells.

We next searched for the mediator of the $Nanog^{Pos}$ CSC–$Nanog^{Neg}$ non-CSC interaction by a cytokines-array analysis on supernatant from a 24-h culture of $Nanog^{Pos}$ CSCs and $Nanog^{Neg}$ non-CSCs. As a result, TGF-β2, IL-17E (also known as IL-25) and MMP-13