Reciprocal Reprogramming of Cancer Cells and Associated Mesenchymal Stem Cells in Gastric Cancer

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Key Words. Tumor microenvironment • Tumor associated MSC • Reprogramming of MSC • Tumor-stromal cell interactions • Cancer stem cells • Organotypic culture • Gastric cancer

ABSTRACT

The interactions of cancer stem cells (CSCs) within the tumor microenvironment (TME), contribute to the overall phenomenon of intratumoral heterogeneity, which also involve CSC interactions with noncancer stromal cells. Comprehensive understanding of the tumorigenesis process requires elucidating the coordinated gene expression between cancer and tumor stromal cells for each tumor. We show that human gastric cancer cells (GSC1) subvert gene expression and cytokine production by mesenchymal stem cells (GSC-MSC), thus promoting tumor progression. Using mixed composition of human tumor xenografts, organotypic culture, and in vitro assays, we demonstrate GSC1-mediated specific reprogramming of “naïve” MSC into specialized tumor associated MSC equipped with a tumor-promoting phenotype. Although paracrine effect of GSC-MSC or primed-MSC is sufficient to enable 2D growth of GSC1, cell–cell interaction with GSC-MSC is necessary for 3D growth and in vivo tumor formation. At both the transcriptional and at the protein level, RNA-Seq and proteome analyses, respectively, revealed increased R-spondin expression in primed-MSC, and paracrine and juxtacrine mediated elevation of Lgr5 expression in GSC1, suggesting GSC-MSC-mediated support of cancer stemness in GSC1. CSC properties are sustained in vivo through the interplay between GSC1 and GSC-MSC, activating the R-spondin/LGR5 axis and WNT/β-catenin signaling pathway. β-catenin+ cell clusters show β-catenin nuclear localization, indicating the activation of the WNT/β-catenin signaling pathway in these cells. The β-catenin+ cluster of cells overlap the Lgr5+ cells, however, not all Lgr5+ cells express β-catenin.

A predominant means to sustain the CSC contribution to tumor progression appears to be the reprogramming of MSC in the TME by cancer cells. STEM CELLS 2019;37:176–189

SIGNIFICANCE STATEMENT

This article describes the utilization of patient gastric carcinoma-derived cancer cells (GSC1) to demonstrate subversion of naïve MSC from adjacent tissue, which are reprogrammed to express a tumor-promoting phenotype, whose cardinal manifestation is to sustain CSC. Paracrine effects of such primed-MSC are sufficient to enable 2D growth of GSC1, while cell–cell interactions are necessary for 3D growth or in vivo tumor formation. Increased expression of R-spondin in primed-MSC mediated elevation of LGR5 expression in GSC1, activation of the WNT/β-catenin signaling pathway and β-catenin nuclear translocation. Subversion of MSC by cancer cells appears to be a prominent means to sustain the CSC underpinning of tumor progression.

INTRODUCTION

Recruitment of tumor supporting stromal cells coupled with extensive remodeling of adjacent tissues are essential for providing a tumor microenvironment (TME) which supports cancer cell proliferation, invasion, metastasis, and chemoresistance [1–7]. Tumor-supporting stromal cells include cancer associated fibroblasts, mesenchymal stem cells (MSC), endothelial cells, and immune cells that interact both with the tumor cells, as well as with each other to drive tumor progression and drug resistance. However, despite accumulating evidence for stromal effects on cancer cells, little is known about the transcriptional regulators that are responsible for tumor-supporting stromal reprogramming, more specifically with respect to MSC in the tumor stroma. MSC are recruited by the cancer cells into the tumor site from either the bone marrow or...
from adjacent tissues, and become an integral cellular component of the TME where they modulate tumor progression [8–11]. MSC tropism into tumor sites is mediated by a combination of extrinsic signals together with secretion of chemokines, cytokines and growth factors secreted by cells of the TME [3–7]. The intercellular communication among tumor cancer cells and recruited MSC can be executed either through cell-to-cell contact, or through paracrine interactions mediated by secreted signaling molecules and/or through microvesicles (MV) constitutively shed from cancer cells and MSC [4, 5, 7, 12–14].

A single tumor mass comprises heterogeneous cancer cell populations, each displaying diverse cellular morphology, phenotypic expression, tumor initiation capacities and inherent or acquired resistance to anticancer drugs. This complex intratumoral heterogeneity contributes to the “nefarious ingenuity” of human cancers and challenges their eradication. Accumulating evidence shows that both the “cancer stem cells” (CSCs) and the “clonal evolution” models, can explain the intratumoral heterogeneity, as CSCs undergo clonal evolution [15–22]. The continuous accumulation of mutations generates heterogeneity of cells within a solid tumor and its metastases, causing certain subsets of tumor cells become more aggressive during tumor progression. A crucial insight relates to understanding that the self-renewal capacity of CSC is not a durable state, but rather a dynamic and niche-dependent process [18, 19]. Tumor stroma interaction signals also regulate epithelial cancer cell plasticity via epithelial to mesenchymal transition programs. This facilitates invasion and metastasis of cancer cells, enables non-CSCs to convert into CSCs, and may influence the TME by converting cancer cells into tumor supportive stroma [23–26]. The complexity and plasticity of solid tumors is enabled by a TME that provides a compatible network of interactions between the heterogeneous cancer cells and non-neoplastic tumor-supporting cells [19, 27–29]. Therefore, attempts to eradicate a single stable self-renewing subpopulation within any given tumor are often futile.

In gastric carcinoma, malignant cells reside within a TME niche comprised of a rich milieu of different types of stromal cells and ECM which contribute to tumorigenesis and resistance to therapy [3, 30]. Several studies have described various roles of the TME, but none have investigated its relevance to clinical drug responsiveness [31–33]. In addition, these and other studies have led to important advances in characterization of gastric CSC subpopulations with properties of self-renewal, sphere forming, drug resistance and in vivo tumorigenicity, which are able to drive tumor growth, recurrence, and metastasis. Gastric CSC express CD44, CD133, CD24, EpCAM, Lgr5, OCT4, Nanog, Sox2, ABG2, and ALDH1 [34–37].

We have previously reported that the proliferation capacity in vitro and tumorigenesis in vivo of gastric cancer cells (GSC1) extracted from a patient gastric carcinoma, critically depends on the presence of their counterpart GSC-MSC or conditioned medium (CM) derived therefrom [38]. GSC1 cancer cell growth and migration are mediated through activation of the hepatic growth factor (HGF)/c-MET signaling pathway by HGF secreted exclusively from the GSC-MSC. Accordingly, our previous observation revealed the following: (a) HGF levels secreted by GSC-MSC are elevated in the presence of GSC1 CM; (b) significantly increased tumor volume and recapitulation of the original patient tumor phenotype in vivo were observed only when GSC1 cancer cells were injected to SCID/Beige mice together with GSC-MSC; (c) GSC-MSC express extremely high levels of the ABCB1 transporter, which is responsible for decreased drug accumulation in multidrug-resistant cells; and (d) GSC1 cancer cells specifically recruit MSC, which in turn are programmed to secrete HGF [38, 39].

In the current study, we used this experimental platform to examine the alterations in MSC gene expression following in vivo reprogramming by the GSC1 carcinoma cells, which in turn, are potentially available to confer and sustain stemness properties in this malignancy.

**MATERIALS AND METHODS**

**Primary Culture of Cells Derived from Patient Tumor Tissues**

Anonymous patient tumor tissue samples were obtained from the Pathology Department of the Rambam Medical Center in accordance with Institutional ethics Review Board (IRB) approval (IRB approval 920110624). The tissue samples were processed to achieve single cell suspensions as described in [38]. Tumor-derived cells were separated into GSC1 cancer cells and GSC-MSC stromal cells which were identified as MSC [38]. Cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 20% fetal bovine serum, 1% penicillin, and streptomycin. Cell cultures were repeatedly initiated from frozen stocks every 3–4 months, so that the cells durably and consistently maintain the “bona fide” gastric cancer characteristics and xenograft tumor histological phenotype.

**Mice and Tumor Formation**

Severe combined immune deficiency (SCID)/beige were purchased from Harlan Laboratories Ltd., Jerusalem, Israel. The mice were housed and maintained under specific pathogen-free conditions according to the instructions of the Committee for Oversight of Animal Experimentation at the Technion—Israel Institute of Technology, Haifa, Israel. This was approved by the Institutional Animal Care and Use Committee of the Technion (Protocol #IL-0550516). For tumor formation, 4 × 10^5 cancer cells were injected into the mice hindlimb musculature and harvested at 60–64 days following injection. Mice were examined for tumor formation once a week and sacrificed by CO2 inhalation.

**In Vivo Reprogramming of MSC**

T-MSC were co-injected with GSC1 into the hindlimb musculature of immunodeficient mice. Tumors were harvested, and dissociated into a single cell suspension, as described in detail in Supporting Information [38]. The epithelial cancer cells were separated using human anti-CD326 (EpCAM) microbeads and the fibroblast-like stromal cells were separated using human antifibroblast microbeads with magnetic activated cell sorting. The human origin of the stromal cells was identified using the antihuman mitochondria antibody. The stromal cells were also identified as MSC by flow cytometry positive expression of CD73, CD105, CD90, CD44, and CD29, and negative expression of the hematopoietic stem cell markers CD45, and endothelial progenitor cell marker CD34 as described in detail in Supporting Information. Supporting Information includes additional detailed materials and methods.
RESULTS

Reprogramming of “Naïve” MSC by GSC1 Cancer Cells

In a recent publication [38], we described the synergistic relationship between GSC1 cancer cells and GSC-MSC which may be stronger in some cancers than others, but can fundamentally affect both tumor growth and treatment and should be an important focus for cancer research. To understand the mechanisms underlying the synergistic relationship between GSC1 cancer cells and GSC-MSC tumor stromal cells, we developed an in vivo recruitment/reprogramming process as described in Figure 1A. As previously described for this experimental model [38], we injected GSC1 cancer cells-derived MV encapsulated within matrigel plug, into human embryonic stem cell-derived teratomas generated within immunodeficient mice. MSC recruited from the teratoma tissue into the matrigel plug were extracted, identified as MSC of human origin, and designated as teratoma-derived MSC (T-MSC) that were specifically recruited by GSC1 cancer cells. These GSC1-tropic T-MSC are normal “naïve” cells with a limited population

Figure 1. MSC recruitment and reprogramming by gastric cancer cell (GSC1) cancer cells. (A): Schematic presentation of the recruitment and reprogramming process. (B): Stromal cells extracted from tumors generated by coinjection of GSC1 cells and T-mesenchymal stem cell (MSC) were examined for phenotype (upper panel; scale bars = 100 μm) and for their human origin using antihuman mitochondria (hMit) antibody (lower panel; scale bars = 20 μm). (C): The expression of MSC specific cell surface markers in RP-31 and RP-32 MSC in comparison with GSC-MSC and recruited T-MSC, was examined by flow cytometry analysis using a triple staining reaction where CD73 BV421 antibody was used as a reference in each reaction. Red graphs indicate unstained cells. Blue graphs indicate the percentage of cells expressing each specific cell surface antigen. (D): The Reprogrammed (RP) MSC RP-31 and RP-32 display a multipotent differentiation capacity into adipocytes, osteoblasts, and chondrocytes (scale bars = 50 μm for adipogenesis, 20 μm for chondrogenesis, 50 μm and 20 μm for osteogenesis of RP-31 and RP-32, respectively).
doubling capacity that were attracted into the matrigel plugs by the GSC1-derived MV and had no prior direct interaction with GSC1 cancer cells [38]. For the reprogramming process, T-MSC were coinjected with GSC1 into the hindlimb musculature of immunodeficient mice as described in Figure 1A. Tumors were harvested and processed as described in [38]. The stromal cells of human origin were identified as MSC by their differentiation capacity and by flow cytometry using previously demonstrated MSC biomarkers [38, 40] (Fig. 1B, 1D). This procedure yielded three different types of MSC: (a) GSC-MSC which are the original patient tumor-derived stromal cells; (b) T-MSC which are “naïve” teratoma-derived MSC specifically recruited by GSC1; and (c) reprogrammed (RP) MSC, RP-31 and RP-32 which were primed by GSC1 cancer cells. Notably, all three types of MSC express the CD146—melanoma cell adhesion molecule on the cell surface which is associated with multipotency [41].

GSC1 Cancer Cell Growth Is Supported by RP MSC In Vitro

The capacity of medium conditioned by the various types of MSC to support the growth of GSC1 in vitro, was examined using the cell proliferation assay. Greater GSC1 proliferation capacity was observed when the cells were grown in GSC-MSC CM as compared with T-MSC CM. However, following reprogramming, GSC1 growth support by RP-31 and RP-32 reprogrammed MSC CM was elevated to the level of GSC-MSC CM (Fig. 2A). Since GSC-MSC elicit their effect on GSC1 growth capacity through a paracrine effect, specifically by secreted HGF [38], we examined whether priming of T-MSC by GSC1 results in increased HGF expression and secretion levels. qRT-PCR analysis, showed increased HGF expression levels in RP-31, RP-32 reprogrammed MSC as compared with the low level of HGF expressed in “naïve” T-MSC (Fig. 2B). As shown in Figure 2C, recruited “naïve” T-MSC secreted low levels of HGF, as previously reported [38]. However, following in vivo priming of T-MSC by GSC1 cancer cells, the HGF secretion level was markedly elevated to levels equivalent those of GSC-MSC. In addition, the levels of HGF encapsulated within MVs derived from “naïve” T-MSC before and after in vivo priming with GSC1 cancer cells was markedly elevated in MV derived from RP-31 and RP-32 reprogrammed MSC (Fig. 2D).

RP MSC Support the Growth of GSC1 Cancer Cells In Vivo

Previous in vivo tumorigenic analyses reported that significantly higher tumor volumes were observed when GSC1 cancer cells were grown in GSC-MSC CM and coinjected with their tumor specific GSC-MSC [38]. Therefore, GSC1 were coinjected with “naïve” T-MSC or with RP-31 and RP-32 reprogrammed MSC into the hindlimb musculature of immunodeficient mice to examine their capacity to support GSC1 in vivo growth and tumorigenicity. Generated tumors demonstrated higher tumor volume as compared with tumors generated by GSC1 grown in regular medium (Fig. 2E, 2F). However, only tumors generated by RP MSC revealed histological phenotypes similar to the phenotype observed in tumors generated by GSC1 coinjected with GSC-MSC, which also recapitulate the original patient-derived tumor phenotype (Fig. 2G). These tumors are characterized by a high degree of differentiation as indicated by the presence of tubular structures with fibroblast-like cells lining the spaces among the microscopic substructures of the tumor, with no observable necrosis within the tumor tissue.

In Vitro Organotypic Culture Model

To examine the role of GSC-MSC in supporting the growth of GSC1 cells, we established an in vitro organotypic culture model in which GSC1 and GSC-MSC are grown in a 3-dimensional (3D) culture. GSC1 by themselves did not sustain 3D culture growth beyond days 4–5. However, in the presence of GSC-MSC or RP MSC, the formation of organoid-like structures was observed, wherein the epithelial cancer cells were observed to line the margins (Fig. 3A). In order to delineate the composition of the 3D organoid-like structures, we transduced GSC1 with green fluorescent protein (GFP) and GSC-MSC with red fluorescent protein (RFP-tdTomato) expressing lentiviral vectors. Naïve T-MSC, and RP MSC RP-31 and RP-32 were also transduced with the tdTomato expressing vector. For generating organotypic culture, GFP-expressing GSC1 cells were grown together with tdTomato—expressing GSC-MSC. As shown in Figure 3B, during the first stages of organoid formation, GSC-MSC (in red) form spheroids. As early as 7 days, homing of GSC1 (in green) to these spheroids was observed. At later stages, GSC1 cells generated an epithelial structure that surround the MSC-derived spheroids (Fig. 3C). Organoids comprising GSC1 and GSC-MSC demonstrated high take rates when injected into the hindlimb musculature of immunodeficient mice by readily generating significantly larger tumors (~2.27 cm³ at 4 weeks) in comparison with GSC1-derived spheroids (~0.3 cm³ at 4 weeks; Fig. 3D). Interestingly, lung carcinoma-derived stromal cells (LC-MSC), which could not support the growth of GSC1 in vitro in a 2D culture or in vivo [38], also formed spheroids. However, GSC1 did not home in on them as efficiently as on GSC-MSC-derived spheroids (Fig. 3B). On the other hand, GSC1-mediated reprogrammed RP-31 and RP-32 MSC recapitulated the GSC1 organotypic 3D growth in the presence of GSC-MSC in contrast to T-MSC (Fig. 3E). These results further emphasize the tumor specific interaction between cancer cells and stromal cells and point to GSC1-mediated reprogrammed RP-31 and RP-32 MSC as GSC-MSC analogs in supporting GSC1 growth in vitro and in vivo.

Transcriptomic Profiling of GSC1-Mediated RP Cells

To decipher the mechanisms responsible for generating a tumor specific reactive stroma, we used the GSC1-RP T-MSC as a model to identify the alterations that GSC-MSC undergo within the tumor niche. For this purpose, gene expression profiles and transcriptome changes were compared between T-MSC, RP-31 and RP-32 MSC and GSC-MSC using RNA-Seq analyses. Changes detected in gene expression between T-MSC and their GSC-RP MSC counterparts were validated using Real-Time Quantitative Reverse Transcription polymerase chain reaction (qRT-PCR) and then examined in GSC-MSC. Since GSC-MSC and T-MSC are cells of different lineage backgrounds, we referred only to differentially expressed genes (DEGs) between T-MSC and RP-31 and RP-32 MSCs and validated their expression in GSC-MSC. DEGs were identified based on fold change (>2) and corresponding statistically significant p-values (<.05). GSC1 were used as a “readout” to examine the effect of changes in GSC-MSC on this tumor growth via cancer cell-stroma interaction. Principal component analysis was performed to assess the relative hierarchical contribution of
differences in gene expression among the samples (Supporting Information Fig. S1A). Distinct clustering of GSC-MSC, T-MSC, RP-31, and RP-32 samples was observed. A clustering relationship is evident between RP-31 and RP-32 samples (although originated from different mice) and T-MSC samples. Hierarchical cluster analysis of each sample in triplicate confirmed these findings (Supporting Information Fig. S1B). Expression levels for 29,997 genes in all four populations were obtained. The data was analyzed to identify genes that are most consistent with two patterns of interest—higher and lower expression levels in the RP populations (RP-31, RP-32, and GSC-MSC) compared with the T-MSC population. These two types of changes were termed reprogramming overexpression and reprogramming underexpression, respectively. We defined and calculated a single reprogramming factor for every gene (RepF[g]), as described in the “Materials and Methods” section. The resulting distribution of RepF[g] is provided in Supporting Information Figure S1C. Genes with RepF[g] significantly greater than

Figure 2. Reprogramming-mediated alteration of mesenchymal stem cell (MSC) supports gastric cancer cell (GSC1) growth. (A): The proliferation capacity of GSC1 cells grown in vitro in RPMI or in medium conditioned by GSC-MSC, T-MSC, reprogrammed (RP)-31 and RP-32 MSC as indicated. (B): RNA samples extracted from GSC-MSC, T-MSC, RP-31, and RP-32 MSC were subjected to qRT-PCR to examine the level of hepatic growth factor (HGF) gene expression. Expression levels were compared with T-MSC HGF expression level. Analyses were performed in triplicates using HPRT1 as an internal control. Bars and error bars represent means and SEM calculated for all samples in each group over two independent experiments. (C): The level of secreted HGF in GSC-MSC, T-MSC, RP-31, and RP-32 MSC was examined using enzyme-linked immunosorbent assay (ELISA). (D): The level of HGF captured in microvesicles (MV) derived from GSC-MSC, T-MSC, RP-31, and RP-32 MSC was examined using ELISA. (E, F): Tumorigenic capacity of GSC1 cancer cells was examined in vivo following injection of GSC1 cancer cells without or with GSC-MSC, T-MSC, RP-31, and RP-32 MSC, as indicated. Tumors were harvested at day 60 from injection. (G): The histological phenotype of the tumors presented in (E) was examined and compared with the original patient tumor histological phenotype. The blood observed in the tumor of the RP-31 group was identified as a hemorrhagic cyst, and histological examination did not reveal necrosis. Scale bars = 200 μm.
Figure 3. In vitro three-dimensional (3D) organotypic growth capacity of gastric cancer cell (GSC1) cancer cells. (A): GSC1 cells were grown in a 3D culture with GSC-mesenchymal stem cell (MSC), T-MSC, and RP-31 and RP-32 MSC. For comparison, GSC1 or GSC-MSC were grown in suspension as indicated. Scale bars = 100 μm. (B): Organoid formation in 3D growth. eGFP-expressing GSC1 (green) were grown together with tdTomato-expressing GSC-MSC (red) in suspension. First, GSC-MSC cells form spheroids and then GSC1 home on them to generate epithelial structures. Organoids were monitored at 7, 14, and 22 days as indicated. Organoid formation of GSC1 in the presence of lung carcinoma-derived MSC (LC-MSC) was monitored at day 14. Scale bar = 100 μm for all images. Using LSM 510 inverted microscope. (C): Generation of epithelial-like structures by GSC1 following homing on GSC-MSC derived spheroid in a 3D organotypic growth scale bar = 100 μm. (D): Tumors generated following injection of 5 days old GSC1-derived spheroids in comparison with 10 days old organoids comprised of GSC1 and GSC-MSC. Tumors were harvested at day 30 from injection. (E): Organoid formation in 3D growth of GSC1 together with naïve T-MSC and RP-31 and RP-32 as indicated. Scale bar = 100 μm for all images.
10 have higher expression in the RP populations compared with T-MSC expression (designated as 10), and those with values significantly smaller than 10 have relatively lower expression levels. Supporting Information Table S1 depicts the 200 genes with the highest reprogramming over expression and under expression values, respectively. This table also presents examples of the measured absolute expression levels for several genes from each of the categories—reprogramming overexpressed and reprogramming underexpressed. Furthermore, we validated these findings by qRT-PCR for several of these genes in comparison with their secretion levels measured by Human Cytokine Antibody Array as demonstrated in Supporting Information Figure S2. Of note, HGF which has been demonstrated to play a key role in GSC1 growth and proliferation in vitro and in vivo presents a high RepF score of 12.36. FGF7 which demonstrates an expression pattern similar to that of HGF following reprogramming, also has a high RepF score of 13.91. These genes are ranked 70 and 5 out of 29,996 genes, respectively. Notably, the mechanism of action for FGF7 did not involve support of GSC1 proliferation (data not shown). Another gene which presents a high RepF score of 14.0 is the ABCC3 multidrug-resistance transporter (ranked number 2).

Reciprocal Reprogramming Between GSC-MSC and GSC1 Cancer Cells Leads to Increased Expression of R-Spondin in GSC-MSC and Increased Expression of Lgr5 in GSC1 Cancer Cells

The RNA-Seq analyses of recruited “naive” T-MSC and RP MSC RP-31 and RP-32 showed elevated expression levels of the genes RSPO1 and RSPO2 following reprogramming by GSC1 cancer cells, and this finding was validated by qRT-PCR (Fig. 4A). RSPO1 and RSPO2 genes encode for the R-spondin proteins, secreted agonists of the canonical Wnt/β-catenin signaling pathway [42]. Although R-spondins are unable to initiate Wnt signaling, they are necessary and sufficient for Wnt signal potentiation and enhancement following binding to the Lgr5 receptor [42]. Although RSPO3 and RSPO4 expression levels were also elevated following reprogramming as compared with T-MSC, GSC-MSC exhibited low expression levels of these genes (Fig. 4A). None of these cells expresses the LGR5 gene as was observed also in the RNA-Seq analysis and in the validation studies (Fig. 4A). To investigate the role of GSC-MSC secreted R-spondin in the tumor, we used GSC1 for readout of the changes related to activation of the R-spondin/Lgr5 signaling pathway. To this end, we took advantage of the conducted RNA-Seq analyses on GSC1 cancer cells grown in RPMI and in GSC-MSC CM (data not shown) to detect changes in the expression profiles. Interestingly, GSC1 express no or negligible levels of RSPO1-4. However, elevated expression levels of Lgr5, the receptor which binds R-spondin, were observed when GSC1 were grown in GSC-MSC CM (Fig. 4A). Lgr5 homologs, Lgr4 and Lgr6 which also bind R-spondin are expressed at a higher level in GSC1 (330, 1,900, and 3,348 reads, respectively). However, only Lgr5 expression levels were elevated when GSC1 grew in GSC-MSC CM (Fig. 4B). Elevated Lgr5 expression was also observed at the protein level in GSC1 grown in CM in a 2D culture as indicated by Western blot analysis (Fig. 4C). In a 3D culture, organoids comprising GSC-MSC and GSC1 demonstrate even higher (≈sixfold) Lgr5 protein levels as compared with GSC1 spheroids grown in GSC-MSC CM. This contrasts with GSC-MSC grown without or with GSC1 CM, which express significantly lower levels of Lgr5 protein (as was also observed in RNA-Seq analyses, data not shown; Fig. 4B). These results point to a GSC-MSC-mediated paracrine R-spondin/Lgr5 signaling pathway in GSC1. Immunofluorescence analysis using anti-Lgr5 antibody indicated localization of Lgr5 protein to the cytoplasm, specifically around the nucleus, in all GSC1 grown in a 2D culture with or without GSC-MSC CM (Fig. 4D). A similar pattern of Lgr5 protein was observed in GSC1 grown together with GSC-MSC as organoids in 3D culture (Fig. 4E). Expression of Lgr5 was also observed in GSC-MSC when grown as spheroids in 3D culture (Fig. 4E). Since Lgr5 was previously associated with stemness both in adult stem cells and in CSC of the gastrointestinal tract in mice and in human [43–45], these results suggest that GSC1 might express their CSC potential in the presence of GSC-MSC.

Expression of Gastric Tumor CSC Specific Markers Lgr5 and CD133 In Vivo

Accumulating evidence suggests that CSC in the gastrointestinal tract express EpCAM, CD44, CD133 in addition to Lgr5 [46–49]. In agreement, FACS analyses of GSC1 cancer cells indicated expression of EpCAM, CD44 and CD133 in 100%, 97.8%, and 37.3%, respectively, when grown both in RPMI and in GSC-MSC CM (Supporting Information Fig. S3). On the other hand, qRT-PCR analysis to assess the expression levels of Oct4, Nanog, Sox2, and lin 28 stemness markers, indicated no or extremely low levels of expression of these genes in GSC1 cancer cells (data not shown). These results were confirmed by the RNA-Seq analyses, and are in agreement with a previous observation [41]. To examine whether GSC1 cancer cells express Lgr5 and CD133 in vivo, serial paraffin sections of tumors generated following intramuscular injection of GSC1 with and without GSC-MSC were examined by IHC using antihuman Lgr5 and CD133 antibodies. CD133, also known as prominin-1, is a transmembrane glycoprotein considered as a biomarker for CSC that have a critical role in recurrence of various malignancies including gastrointestinal tumors [50]. In tumors generated by GSC1 together with GSC-MSC, the results of these analyses exhibit clusters of Lgr5 and CD133 positively stained large tumor cells with large nuclei and a low cytoplasm/nucleus ratio. The negatively stained tumor cells contain smaller nuclei and a high cytoplasm/nucleus ratio, and they are organized in well-differentiated phenotypic structures (Fig. 4F). In tumors generated only by GSC1, the cancer cells were slightly and homogeneously stained for anti-Lgr5 antibody but were negatively stained for anti-CD133 antibody (Fig. 4F). Lgr5 and CD133 positively stained cells were also identified in paraffin sections of the original patient tumor (Fig. 4F). The negatively stained cells in these tumors were also organized into well differentiated structures. These results suggest that GSC-MSC might confer stemness properties and support gastric CSC in tumors generated in the presence of GSC-MSC.

GSC-MSC Mediated Activation of Wnt/β-Catenin Pathway and β-Catenin Localization In Vitro

Accumulating evidence supports the observation that RSPO binding to Lgr5 stimulates the canonical Wnt/β-catenin pathway through enhancing phosphorylation of the low-density lipoprotein receptor-related protein 5/6 coreceptors (LRP5/6) [51]. RSPO can also interact with the cell surface E3-ligases
Figure 4. Expression of R-spondin (RSPO) in gastric cancer cell-derived mesenchymal stem cell (GSC-MSC) and Lgr5 in GSC1 cancer cells. 
(A): RNA samples extracted from GSC1 grown in RPMI or in GSC-MSC CM, GSC-MSC, recruited “naïve” T-MSC and RP-31 and RP-32 MSC, were subjected to qRT-PCR with specific primers for RSPO1-4 and Lgr5 genes. Expression of the indicated genes in the various RNA samples are demonstrated relative to naïve T-MSC (T-MSC expression level = 1). Analyses were performed in triplicate using HPRT1 as an internal control. Bars and error bars represent means and SEM calculated for all samples in each group over two independent experiments. 
(B): The influence of GSC1 and GSC-MSC interaction on Lgr4, Lgr5, and Lgr6 gene expression was monitored by using qRT-PCR analysis in GSC1 grown in RPMI and in GSC-MSC CM and on GSC-MSC grown in RPMI and in GSC1 CM. Expression of the indicated genes (Figure legend continues on next page.)
cells were selected by FACS according to mCherry expression, pathway induces also expression of eGFP in the cells. Infected
(rescence, while activation of the Wnt/\(\beta\)-catenin signaling pathway in GSC1 cells was assessed using a
sette [58]. Cells infected with the 7TGC virus display red
with the 7TGC lentiviral vector which carries an SV40-mCherry
transcription-based reporter construct. GSC1 were transduced
mation Fig. S4). Next, the activation of Wnt/\(\beta\)-catenin signaling pathway leads to the disruption of the
-\(\beta\)-catenin degradation complex, accumulation of \(\beta\)-catenin in the
cytoplasm and translocation of \(\beta\)-catenin to the nucleus, where it activates the transcription of canonical Wnt target
genes [42, 55–57]. Therefore, we sought to determine whether stemness of GSC1 epithelial cancer cells might be governed by the
MSC component of the TME. First, we observed differential
expression of Lgr5 when GSC1 cells were grown in GSC-
MSC CM as compared with RPMI (Fig. 5A). This contrasts with
no difference in expression of \(\beta\)-catenin and its target genes
Axin2 and RNF43. We sought to examine whether GSC-MSC-
mediated induction of Lgr5 in GSC1 coactivates the Wnt/\(\beta\)-
catenin signaling pathway as a possible mechanism to support
gastric CSC. For this purpose, anti-\(\beta\)-catenin antibody was used for
immunofluorescence analysis of the expression and localization of \(\beta\)-catenin in GSC1 grown in vitro in a 2D culture with or
without GSC-MSC CM. In both culture conditions, distinct
expression of \(\beta\)-catenin can be observed in the cell membrane.
Screening examination of the entire culture plate did not indic-
ate \(\beta\)-catenin localization to the cell nucleus under these
growth conditions (Fig. 5B). Similar analysis performed in orga-
noids comprising GSC1 grown together with GSC-MSC cells in
a 3D culture, revealed low numbers of GSC1 cells which
expressed \(\beta\)-catenin in the cell nucleus, suggesting that despite
Lgr5 expression across all GSC1 cells, only few activate the
Wnt/\(\beta\)-catenin signaling pathway (Fig. 5C and Supporting Infor-
Fig. 4F). Tumors generated from
the tumor sections described in Fig. 4F). Tumors generated from
only GSC1 exhibit a homogeneous histological phenotype in
which high intensity staining for anti-\(\beta\)-catenin was identified in the
cells localized in the tumor margins. \(\beta\)-Catenin positive stain-
ing progressively diminished toward the center of the tumor.
Localization of \(\beta\)-catenin to the cell membrane suggests the
involvement of \(\beta\)-catenin in adhesion of these cells. Surprisingly,
tumors generated by injection of GSC1 together with GSC-MSC
revealed a complete change in the \(\beta\)-catenin localization pattern
in the tumor. In this case, \(\beta\)-catenin positively stained cells
appeared in clusters in which \(\beta\)-catenin is localized to the cell
nucleus (Fig. 6C). Importantly, the clusters of \(\beta\)-catenin positive
cells overlap the clusters of Lgr5 positive cells shown in
Figure 4F. However, not all the Lgr5 positive cells express
\(\beta\)-catenin (Fig. 6D). Taken together, these results suggest that
GSC-MSC induce stemness properties in Lgr5-positive GSC1
through secretion of R-spondin and activation of the Wnt/\(\beta\)-
catenin signaling pathway which eventually leads to translocation

(Figure legend continued from previous page.)
in the various RNA samples are demonstrated relative to GSC1 grown in RPMI (expression level = 1). Analyses were performed in tripli-
cates using HPRT1 as an internal control. Bars and error bars represent means and SEM calculated for all samples in each group over two
independent experiments. (C): Lgr5 protein level in GSC1 and GSC-MSC grown in vitro. Plasma membrane proteins were extracted from
GSC1 cancer cells grown in vitro in two-dimensional (2D) and 3D culture in various conditions as indicated. Lgr5 protein level was
assessed by Western blot analysis with antihuman Lgr5 antibody (left panel). Lgr5 protein level was measured using the Totallab quantifi-
cation program as presented in the right panel. Anti-\(\alpha\)-tubulin antibody was used for protein sample quantification. (D): Expression of
Lgr5 in GSC1 grown in a 2D culture in RPMI (upper panel) or in GSC-MSC CM (middle panel) were subjected to immunofluorescence anal-
ysis using anti-Lgr5 antibodies (green). Phalloidin was used for staining F-actin in the cell membrane (red) and DAPI was used for nuclei
staining (blue). Lower panel represents no primary antibody control. Bar = 50 \(\mu\)m for all images, using the LSM700 microscope. (E): Expression of Lgr5 in in vitro grown organoids. GSC1 and GSC-MSC grown in a 3D culture were subjected to immunofluorescence analysis
(upper panel) using anti-Lgr5 antibodies (green). Phalloidin was used for staining F-actin in the cell membrane (red) and DAPI was used for nuclei
staining (blue). Middle panel represents no primary antibody control. Lower panels demonstrate staining of a GSC-MSC derived
spheroid. Bar = 50 \(\mu\)m for all images, using the LSM700 microscope. (F): Expression of gastric cancer stem cell markers LGR5 and CD133 in
vivo. Paraffin sections of tumors generated by GSC1 injected with or without GSC-MSC into the hindlimb musculature of SCID/beige
mice were stained with anti-LGR5 and anti-CD133, as indicated. Lgr5 and CD133 positive cells were also detected in paraffin sections of the
original patient tumor. The depicted areas in the upper and middle panels are enlarged in the right panels. Arrows indicate clusters of
cells positively stained with anti-LGR5 and CD133. Bars = 100 \(\mu\)m for the left panels and 50 \(\mu\)m for the enlarged panels.
Figure 5. Gastric cancer cell-derived mesenchymal stem cell (GSC-MSC) mediated activation of Wnt/β-catenin pathway and β-catenin localization in vitro. (A): The effect of GSC1 and GSC-MSC interaction on β-catenin and Wnt targeted genes Axin 2, Lgr5 and RNF43 was monitored using qRT-PCR analyses with specific primers. RNA was extracted from GSC1 cancer cells grown in RPMI and in GSC-MSC conditioned medium (CM) and from GSC-MSC grown in RPMI and in GSC1 CM. Expression of the indicated genes in the various RNA samples are demonstrated relative to GSC1 grown in RPMI (expression level = 1). Analyses were performed in triplicates using HPRT1 as an internal control. Bars and error bars represent mean and SEM calculated for all samples in each group over two independent experiments. (B): Expression of β-catenin in GSC1 grown in a two-dimensional culture in RPMI (upper panel) or in GSC-MSC CM (middle panel) were subjected to immunofluorescence analysis using anti-β-catenin antibody (green). Phalloidin was used for staining F-actin in the cell membrane (red) and DAPI was used for nuclei staining (blue). Lower panel represents no primary antibody control. Bar = 50 μm for all images, using the LSM700 microscope. (C): Expression of β-catenin in in vitro grown organoids. GSC1 and GSC-MSC grown in a 3D culture were subjected to immunofluorescence analysis (upper panel) using anti-β-catenin antibody (green). Phalloidin was used for staining F-actin in the cell membrane (red) and DAPI was used for nuclei staining (blue). Lower panel represents no primary antibody control. Bar = 200 μm for all images, using the LSM700 microscope. (D): mCherry (green) and eGFP (red) labeling in the same organoid. Yellow arrow indicates the location of the two markers.
of β-catenin into the cancer cells nuclei for transcripational activation of downstream targets.

**DISCUSSION**

In the process of tumor formation, the specification and proper arrangements of heterogeneous cell types occur through the coordinated regulation of gene expression and interactions between cancer cells and neighboring stromal cells which are recruited into the tumor niche to support the tumor cells. An important goal is to identify the signaling pathways that coordinate dynamic changes in gene expression which mutually influence properties of heterogeneous cancer cells including CSC and their supporting stromal cells within the TME [18, 19]. The signaling pathways responsible for these functions are induced by extracellular ligands and their receptors, and modify cancer cell properties, such as stemness, survival, proliferation, adhesion and migration. In the current study, we demonstrate an extreme, but illustrative example in which the “seeds” of tumor cancer cells subvert the “soil” in a way that shapes the nature of the tumor MSC component to serve tumor progression. Increasing evidence indicates that CSC comprise a distinct self-renewing subpopulation with reduced replicative potency that can initiate tumors and mediate recurrence and metastasis. Most anticancer cell cycle active drugs kill the bulk of cancer cell populations and skip CSC as they divide rarely and can escape chemotherapy by drug expulsion [59]. The Wnt signaling pathway is a powerful regulator of cell proliferation and differentiation, which involves proteins that directly participate in both gene transcription and cell adhesion. The central player of this pathway is β-catenin, which can act as a transcription cofactor and a structural adaptor protein linking cadherins to the actin cytoskeleton to achieve cell–cell adhesion [60, 61]. Misguided interactions of β-catenin with adhesion molecules in the cell membrane, with a destruction complex in the cytoplasm or with a transcription complex in the nucleus can lead to tumor formation [60, 62]. Lgr5 is a Wnt target gene that marks adult stem cells with self-renewal activity in the intestinal crypt and CSC in gastrointestinal tumors [54]. The fact that Lgr5 acts as receptor for R-spondin, reinforces the linkage between Wnt signaling and activation of stem cells in normal developmental and in tumorigenic processes [56]. In the normal intestinal crypt, Lgr5 stem cells receive essential niche support from their own specialized progeny Paneth cells which provide them with Wnt signals to maintain their stemness properties [54]. Lgr5 is also associated with stemness properties in CSC in gastrointestinal tumors in mice and in human [44, 45]. Recently, we reported on the specific contribution of HGF-secreting tumor-derived MSC to in vivo tumorigenic capacity in gastric carcinoma. Moreover, we described the “basic skills” of the GSCs to recruit specific competent HGF-secreting “naive” MSC from the adjacent tissue in the process of tumorigenesis and to educate/repurpose them in a tumor type specific manner to support tumor progression [38]. In the current study, we took advantage of this gastric tumor, in which the GSC1 cells critically depend on their GSC-MSC tumor counterpart, as a model to examine the interplay between these two components that leads to generation of tumor reactive stroma. A marked elevation of HGF expression and secretion levels was identified in primed RP-31 and RP-32 reprogrammed MSC as compared with GSC-MSC, pointing to the changes in naïve T-MSC undergo to become qualified to support GSC1 proliferation capacity. Similar to what we describe here, it has been reported that HGF secreted by myofibroblasts in colon tumors exhibited an enhanced Wnt signaling pathway, activation of β-catenin-dependent transcription and subsequently enhancement of CSC clonogenicity [63].

Significantly increased expression of ABCB3, a multidrug-resistance family member, was also observed in primed RP-31 and RP-31 and RP-32 reprogrammed MSC, which follows the same line of elevated expression levels for the ABCB1 gene in GSC-MSC [38]. The ABC transporters are responsible for decreased drug accumulation in drug-resistant cells and often mediate cancer cell survival despite anticancer therapy reagents [64]. The extremely high expression level of the ABCB1 gene in GSC-MSC stromal cells enables them to avoid eradication by cytotoxic effects, thereby providing a continuous support for their cancer cell counterpart in the tumor. The patient, from whom the GSC1 cancer cells were derived, was treated with chemotherapeutic agents which are substrates for those transporters. This also reminds us that reprogramming affects a process wherein rare MSC have been recruited and evoke such an effect. Those which remain, have been selected to support tumor growth. In this regard, it will be important to investigate the detailed mechanism(s) underlying the reprogramming of MSC by GSC1 cancer cells, which leads to generation of a more powerfully tumor supportive niche.

Primed RP-31 and RP-32 reprogrammed MSC resemble GSC-MSC also in supporting in vitro 3D growth of GSC1, which do not form spheroids by themselves. GSC1 cells can home specifically to GSC-MSC or primed MSC-derived spheroid and generate epithelial structures in organotypic growth in contrast to T-MSC and LC-MSC. In addition, primed MSC exhibited GSC1 growth support in vivo by their capacity to recapitulate both the tumor volume and the phenotypic histological appearance observed in the case of GSC-MSC grown with GSC1 cells and the original tumor. It is important to note that T-MSC which were specifically recruited by GSC1 cancer cells from the adjacent tissue also support GSC1 in vivo growth but the histological phenotype of the generated tumors indicate that they are not fully equipped with tumor promoting capacity as observed for RP MSC. These results indicate that the paracrine effect of GSC-MSC is sufficient for a 2D growth of GSC1, however, cell–cell interaction with the tumor specific
stromal cells GSC-MSC or their homolog primed RP-31 and RP-32 MSC are necessary for 3D growth and for in vivo tumor formation. Paracrine and juxtacrine mediated elevation of Lgr5 expression at both the transcription and the protein levels suggest GSC-MSC mediated stemness support in GSC1 cancer cells in vitro. However, GSC1 cells do not express the RSPO1 and two genes which encode for the Lgr5 receptor ligand R-spondin, as compared with GSC-MSC. The interplay between GSC1 cancer cells and GSC-MSC observed for the HGF/cMET signaling pathway [38], is augmented in the case of the R-spondin/Lgr5 axis, consistent with WNT/β-catenin signaling pathway involvement in maintaining CSC properties. Although Lgr5 expression is observed in all GSC1 cancer cells grown in vitro in a 2D culture in CM, and in 3D cultures together with GSC-MSC, only ~1.2% of this Lgr5+ GSC1 cell population exhibit the activation of WNT/β-catenin signaling pathway and translocation of β-catenin into the nucleus. Only these cells are capable of spheroid formation when grown by themselves in a 3D culture.

Figure 6. Gastric cancer cell-derived mesenchymal stem cell (GSC-MSC)-induced β-catenin nuclear localization in organoids and in gastric tumors in vivo. (A): Expression and localization of β-catenin in organoids comprised of GSC1 cancer cells and GSC-MSC. Transduced GSC1 used for this organotopic growth display constitutive mCherry red fluorescence and β-catenin/TCF transcription induction of eGFP. Bar = 50 μm, using the LSM880 microscope. (B): Transduced GSC1 cells which express both mCherry and eGFP form spheroids in a three-dimensional (3D) culture in CM (upper panel), and organoids together with nonstained nontransformed GSC-MSC (middle panel). Lower panel exhibit 3D images prepared using the Imaris software. Bar = 50 μm for all images, using the LSM880 microscope. (C): Expression and localization of β-catenin in gastric tumors in vivo. Paraffin sections of tumors generated by GSC1 injected with or without GSC-MSC into the hindlimb musculature of SCID/beige mice were stained with anti-β-catenin antibodies as indicated. Serial sections are referring to the paraffin sections described in Figure 4F. The depicted areas in the left panels are enlarged in the right panels. Arrows indicate clusters of cells positively stained with anti-β-catenin. Bars = 100 μm for the left panels and 50 μm for the enlarged right panels.

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culture, results which are consistent with CSC properties. Future studies will be needed to verify conclusively that these are CSC by using in vivo serial passages.

The RP MSC effect on GSC1 cancer cell stemness is most strikingly evident in vivo. In this case, injection of Lgr5+ GSC1 cells together with GSC-MSC results in clusters of large Lgr5+ cells that can both self-renew and differentiate into smaller nonstem Lgr5− cells. Thus, GSC-MSC confer stemness properties to Lgr5+ GSC1 cancer cells, as demonstrated by the appearance of β-catenin-positive clusters of cells, in which β-catenin expression is localized to the nucleus. The latter indicate the activation of the WNT/β-catenin signaling pathway. There is evident overlap of β-catenin positive clusters of cells with the Lgr5 positive cluster of cells. However, not all Lgr5+ cells express β-catenin. These results suggest that Lgr5 is necessary but not sufficient to maintain Wnt/β-catenin activated CSC in this gastric tumor. These observations suggest that potential Lgr5+ CSC exist in GSC1 derived tumors, however, their CSC potential requires the presence of the RP MSC component of the TME within the entire tumor milieu. In future studies it will be of particular interest to examine the involvement of the R-spondin/Lgr5 interaction and wnt/β-catenin signaling pathways in GSC1 and GSC-MSC reciprocal reprogramming.

**CONCLUSION**

The results of the current study provide a basis for elucidating the signaling interactions between cancer cells and the stromal cells which are recruited into the tumor niche. The approach described herein succeeded in uncovering the subversion of gene expression and cytokine production by MSC recruited into the TME, and the prominent contribution of this process in sustaining the cancer stem cell underpinnings of tumor progression.

**ACKNOWLEDGMENTS**

We thank Gal Akiri for helpful discussions. We thank Sara Selig and Ayala Ofir for critical reading of the manuscript, and Irena Reiter and Nisreen Hassan for excellent technical assistance. We thank Liat Linde, Ronit Modai-Hod, and Nili Avidan for excellent bioinformatics assistance. We also thank Maya Holdengreber Edith Suss-Toby and Melia Gurewitz for excellent bioimaging assistance. This research was supported by grants from the Daniel M. Soref Charitable Trust, the Skirball Foundation, and the ATS-Cell Stem Cell Research Foundation.

**AUTHOR CONTRIBUTIONS**

Y.S.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; D.C.A.: data analysis and interpretation, final approval of manuscript; Z.Y.: data analysis and interpretation, final approval of manuscript; K.S.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript; M.T.: conception and design, financial support, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.

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