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Perspective

GPR56 and TG2

Possible Roles in Suppression of Tumor Growth by the Microenvironment

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ABSTRACT

Metastasis is a complex process that involves multiple levels of cell-cell interaction. Among these interactions, tumor-stroma interactions are being actively investigated. Metastatic cells are hypothesized to show gene expression changes that contribute to their survival and growth at the distant site. Such changes could contribute either to enhancement of growth or to evasion of growth inhibition by the normal tissue environment thus allowing growth as metastases. Our recent report that tumors from highly metastatic melanoma derivatives express low levels of a suppressor of tumor progression, GPR56, is consistent with such a model. GPR56 associates in a complex with Gαq and the tetraspanin protein CD81. We further identified a ligand that interacts with GPR56 in the extracellular matrix (ECM) as TG2, a major crosslinking enzyme in the matrix. TG2 also binds to fibronectin and integrins and affects their cell adhesion functions. TG2 itself has been implicated in suppression of tumor progression; therefore TG2 might serve as a host defense against the invading metastatic cells. The highly metastatic cells may escape from this inhibition by down-regulation of GPR56. Much future work will be needed to test this hypothesis and further our understanding of metastasis in general.

INTRODUCTION

Metastasis is the dispersal of cancer cells from the primary tumor to distant organs and is the major cause of death in cancer patients. However, despite intense research efforts, the underlying mechanisms of metastasis have remained elusive. Metastasis is generally considered a multi-step process[1]: tumor cells first detach from the primary tumor mass, then enter the circulation through blood vessels or lymphatics (intravasation), arrest in the capillary bed of a distant organ, transmigrate through the blood vessels (extravasation) and survive and grow as metastases. A cancer cell must complete all of these steps to form clinically relevant metastases.

The survival and growth of metastases in distant organs are believed to be rate-limiting steps during metastasis. Clinicians have noticed that, despite a large number of cancer cells shed into the blood stream of human cancer patients, metastases were either rarely detected or only detected in selected organs. Such an example came from a case study by Tarin and colleagues.[2] When the ascitic fluids of patients with abdominal metastases were delivered directly into their jugular veins to alleviate the pain, very few metastases were detected in the lungs of these patients after a long period of time, even though millions of cancer cells had been delivered. This result strongly suggests that the survival and growth of metastases is an inefficient process and therefore a rate-limiting step during metastasis. This concept has also been supported by experimental data. When radiolabeled or fluorescently labeled tumor cells were injected into immunodeficient mice, they seeded in organs throughout the body, but formed detectable metastases only in selected organs or tissues.[3,4]

Stephen Paget, a pathologist in the late 1800s, proposed a "Seed and Soil" hypothesis to explain this selectivity of metastasis.[5] He hypothesized that tumor cells (seeds) needed an amenable environment (soil) to survive and proliferate. A "friendly" tissue might provide growth factors to support the survival and growth of circulating tumor cells. A "hostile" tissue, on the other hand, might express factors to inhibit it. Such inhibition could be one reason why metastasis is a relatively rare event: highly metastatic cells could somehow evade this defense (via epigenetic or genetic alterations) leading to their survival and growth as metastases. The "Seed and Soil" hypothesis stresses the importance of the interactions between tumor cells and their microenvironment during metastasis. These interactions have been actively studied in recent decades and many results support this hypothesis.

Several reviews have been written on this subject,[6-9] therefore below we will only summarize our current understanding of these interactions in the context of tumor progression and metastasis.

A major component of the tumor microenvironment is the stroma, which comprises stromal cells (such as fibroblast cells, immune cells, and endothelial cells) and acellular components, such as proteins and carbohydrates of the extracellular matrix (ECM).[6,10] In normal tissues, the stroma is quiescent and establishes or maintains the polarity and function of adjacent epithelial cells. When tissues are injured or wounded, however, their stroma becomes "activated".[6,11] Fibrin clots are formed and provide a temporary matrix for the migration of fibroblasts and other cells. The fibroblasts produce a collagen-rich matrix to replace the existing matrix, which is degraded by matrix metalloproteinases (MMPs). Cells in the fibrin clots also secrete chemokines to recruit immune cells and release growth factors, such as TGFβ, PDGF, VEGF and bFGF, to stimulate angiogenesis and initiate tissue repair. Subsequently, fibroblasts become myofibroblast cells and contract the wound for its closure. The wound is finally healed when tissue matrix is remodeled by crosslinking of matrix proteins, especially collagens, and scar tissue forms after the clearance of the stromal cells by apoptosis.

Tumors have been considered wounds that do not heal.[12] Consistent with this idea, recent microarray analyses have shown that a wound response is often associated with cancer malignancy.[13] Many factors important for wound healing were also found to be involved in tumor progression. For example, molecules involved in thrombosis and fibrinolysis, such as fibrinogen, thrombin and its receptors, and plasminogen activator inhibitors (PAIs), are frequently dysregulated in cancer, and numerous studies have suggested their roles in tumor progression and metastasis.[14-17] Similarly, MMPs have been found to be important for both wound healing and tumor progression.[18] Another important molecule that plays central roles in both wound healing and cancer is TGFβ. TGFβ can serve dual roles in tumor progression.[19] Its direct effect on various cell types is to inhibit their growth. TGFβ signaling components are frequently disrupted in human cancers,[20] and mice deficient in TGFβ showed enhanced tumorigenesis.[21,22] However, the TGFβ pathway has been shown to function as an enhancer for metastasis. Mice expressing an activated form of TGFβ receptor, when crossed into an MMTV-neu breast cancer model, showed decreased Neu-induced mammary tumorigenesis but increased pulmonary metastasis.[23] TGFβ stimulates the secretion of matrix proteins, such as fibronectin or collagen, in stromal cells and facilitates the formation of a wound-responsive matrix that supports metastasis formation.[24] TGFβ also promotes the proliferation of stromal cells and enhances angiogenesis.[20] All these functions of TGFβ contribute to its role in wound healing. Finally, tumors contain similar ECM as do wounds. Both often have elevated levels of tenascin,[25] collagens,[10] and specific alternatively spliced forms of fibronectin,[26,27] which are either not detectable or present at low levels in normal tissues.

Tumor cells and tumor stroma communicate with each other and affect each other's properties.[6] Tumor cells often secrete MMPs themselves or produce cytokines to enhance MMP secretion from stromal cells. They also produce their own ECM proteins that contribute to the tumor matrix. The expression of chemokine receptors or adhesion receptors, such as integrins, are also dysregulated on tumor cells.[28,29] These receptors may interact with tumor stroma to affect the migration/invasion or growth of tumor cells during metastasis.

Recently a combination of animal metastasis models and microarray analyses have greatly accelerated the identification of players in metastasis and thus improved our understanding of the mechanisms of metastasis.[30-33] A commonly used animal metastasis model is to inject a pool of poorly metastatic human cancer cells into the blood stream of immunodeficient mice and collect the rare metastases (most often in lung) for in vitro culturing and expansion.[34] The derived cells, when injected, usually form more metastases than the parental line and therefore are considered more metastatic. Several rounds of such selections can be applied and cells with increasing metastatic potentials are obtained. The genes that are differentially expressed in the metastatic cells compared with the parental line can then be identified by microarray analyses. Their roles in metastasis can be studied by overexpression or down-regulation experiments in the derived cell lines.

Using the above approach, we recently reported that an atypical G protein-coupled receptor (GPCR), GPR56, is down-regulated in tumors from highly metastatic melanoma cells, and that elevated expression of GPR56 in those same cells reduced their metastatic ability.[33] This suppressive effect of GPR56 is, at least in part, due to the inhibition of tumor growth, since melanoma cells with high levels of GPR56 grew more slowly when injected subcutaneously than did control cells with low GPR56. Furthermore, GPR56 was found to interact with a ubiquitously expressed crosslinking enzyme, tissue transglutaminase (TG2), in the ECM. TG2 itself has been shown to play suppressive roles in tumor progression[35] and therefore it might contribute to GPR56-mediated suppression of metastasis. In fact, the growth inhibition by GPR56 only occurs in vivo, but not in vitro, suggesting that the function of GPR56 involves a factor in the tumor microenvironment. In light of these results, we hypothesize that metastatic melanoma cells with downregulated expression of GPR56 are able to evade the suppression from the invaded tissue, which might lead to establishment of a favorable microenvironment for metastases to form. The possible implications of our findings and hypotheses are discussed below.

GPR56 AND TG2 INTERACTIONS IN METASTASIS

GPR56. GPCRs are seven-transmembrane receptors that signal through small heterotrimeric GTP-binding proteins and activate multiple signaling pathways through second messengers (such as cAMP, Ca^{2+}, IP3).[36] GPR56 belongs to a recently described family of GPCRs known as Long N-terminal class B 7-TransMembrane proteins (LNB-7TM).[37-39] This family comprises over 30 members all classified as class B secretin-like GPCRs based on the sequences of their seven-transmembrane domains. They also contain features that differ from those of other class B GPCRs. As their name indicates, they all have long N-terminal segments, which often contain domains found in adhesion proteins, such as cadherin, lectin, laminin G, immunoglobulin, and thrombospondin domains. They are thus hypothesized to be adhesion molecules that signal through G proteins. In addition, they all contain a GPCR Proteolytic cleavage Site (GPS) N-terminal to the 7-transmembrane domain. Many LNB-7TM proteins, including GPR56 based on our work, are cleaved to form two-subunit receptors during maturation.[33,40] The cleavage is believed to be important for the localization of the receptors onto the cell surface, since mutations in the GPS of Latrophilin, an LNB-7TM protein, were found to cause retention of the receptor in the ER.[40] The enzyme(s) responsible for this cleavage is not known.

LNB-7TM proteins have been implicated in diverse biological processes including exocytosis (Latrophilin),[41] leukocyte trafficking

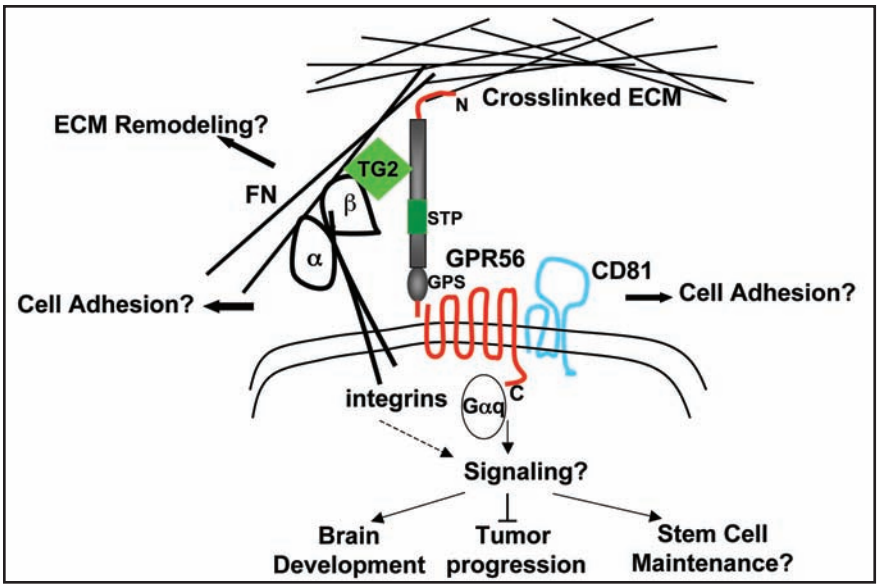

Figure 1. A model of GPR56 functions. GPR56 is involved in metastasis suppression, brain development and possibly stem cell maintenance. GPR56 protein is cleaved, presumably in the GPS domain, into two fragments that remain non-covalently associated. The N-terminal fragment contains a Serine-Threonine-Proline (STP) -rich region. The C-terminal fragment contains the seven transmembrane segments. GPR56 associates with CD81 and Gαq in a complex and may be involved in cell adhesion. The N-terminus of GPR56 interacts with TG2, a transglutaminase, which crosslinks the ECM proteins and interacts with fibronectin and integrins at the cell surface. These functions of GPR56 and TG2 may lead to ECM remodeling and cell adhesion. The intracellular signaling events that may mediate the functions of GPR56 are not known.

(CD97),[42] angiogenesis (BAI1),[43] and planar cell polarity (Celsr1/Flamingo).[44] Mutations in the N-terminus of GPR56 were found to associate with a type of brain malformation (called BFPP: bilateral frontoparietal polymicrogyria) in human patients.[45] These patients have abnormally numerous and small gyri in their cerebral cortex and are mentally retarded. How GPR56 functions during brain development is unknown. GPR56 has been speculated to play roles in stem cell maintenance, because several groups have shown that its mRNA is upregulated in neuronal progenitor cells and hematopoietic stem cells.[45-47] Such a function might also be applicable to its role during metastasis: it might control the proliferation of melanoma cells and therefore inhibit their metastatic growth. Besides GPR56, other members of the LNB-7TM family have also been implicated in cancer progression. CD97, one of the most studied members of this family, was found to be overexpressed in various cancer types.[48-50] In contrast, BAI1 (Brain Angiogenesis Inhibitor 1) exhibited decreased expression levels in glioblastoma compared with the adjacent non-neoplastic brain.[51] Overexpression of BAI1 in glioblastoma cell lines reduced angiogenesis and growth of human tumors in immunodeficient mice.[52] Whether GPR56 functions similarly to BAI1 awaits further investigation.

The role of GPR56 in metastasis could also be related to its putative role in cell adhesion. GPR56 is reported to complex with CD81 in neuroblastoma cell lines (Fig. 1).[53] CD81 is a four-pass transmembrane protein that belongs to the family of tetraspanin proteins.[54] It interacts with integrins and other tetraspanin proteins and plays multiple functions in cell adhesion.[54] The purified extracellular domain of GPR56 has also been reported to inhibit cell adhesion, but the mechanism by which this occurs has not been investigated.[55] Our finding that GPR56 interacts with TG2 provides further support for potential roles in cell adhesion (see below). As suggested by motifs in their extracellular segments, other LNB-7TM proteins have also been implicated in cell adhesion. CD97 contains an RGD peptide sequence that mediates association with the integrins α5β1 and αvβ3 to stimulate endothelial cell invasion and angiogenesis.[56]

Does GPR56 signal through G proteins? GPR56 and CD81 form a complex with Gαq (Fig. 1).[53] Whether GPR56 signals directly through Gαq has not been investigated. The signaling mediated by other LNB-7TM proteins is also poorly understood, mainly due to a lack of known ligands. Before our report, another endogenous ligand for LNB-7TM proteins was CD55 (decay-accelerating factor).[57] CD55 binds to CD97, but the physiological implication of this interaction is not clear. Latrophilin is also an LNB-7TM receptor with a known exogenous ligand, although its endogenous ligand has not been found.[41] This receptor binds to black widow spider toxin (latrotoxin) and stimulates exocytosis in neuronal cells. Latrophilin was shown to form a complex with Gαq and, upon activation by latrotoxin, induces a series of downstream events that may be mediated by Gαq: these include activation of PLC, IP_3 production and mobilization of intracellular Ca^{2+} stores.[58] This suggests that LNB-7TM proteins might signal through G proteins in the same way as classical GPCRs. Further investigations will be needed to understand the signaling pathways through other LNB-7TM proteins such as GPR56.

Tissue transglutaminase (TG2). Through biochemical purification, we identified TG2 as a ligand of GPR56 (Fig. 1).[33] TG2 was the first transglutaminase identified based on its ability to incorporate primary amines into proteins.[59,60] It is localized in both the cytosol and the extracellular space. In the cytosol, it has been claimed to function as a G protein (Gh) for α-adrenergic receptor and to activate phospholipase C (PLC).[61] Extracellularly, it is activated by Ca^{2+} and acts as a crosslinking enzyme in the extracellular matrix: it catalyzes the formation of covalent bonds between glutamines of one protein and the lysines of another.[60]

TG2 has also been implicated in cancer progression. Its expression and activity levels have often been reported to be downregulated during tumor progression,[62-65] although there are some reports of up-regulation.[66] The function of TG2 in tumor progression is not understood. Some reports showed that TG2 could inhibit tumor growth. Application of recombinant TG2 to rat mammary adenocarcinomas significantly delayed the tumor growth[67] while ectopic expression of TG2 in a highly malignant hamster fibrosarcoma cell line significantly reduced tumor incidence.[68] Recently, the role of TG2 in tumor progression was directly tested using $TG2^{-/-}$ mice. The results showed that tumor growth was enhanced in the knockout mice relative to wild-type controls,[35] suggesting that TG2, like GPR56, might function as a suppressor of tumor growth.

How might TG2 suppress tumor growth? TG2 is a major crosslinking enzyme in the ECM. Its crosslinking activity stabilizes the matrix and prevents ECM proteins from being processed by proteases, such as MMPs.[35,69] TG2 is implicated in multiple steps during wound healing: it might help to stabilize the fibrin clots and to activate TGFβ by incorporating latent TGFβ binding protein 1

(LTBP-1) into matrix.[70] Inappropriate upregulation of TG2 was found to correlate with abnormal ECM accumulation and fibrosis.[71,72] In tumors, addition of exogenous TG2 results in increased crosslinking of collagen I in the ECM, which may somehow inhibit tumor growth.[35] In addition, exogenous TG2 has also been shown to inhibit angiogenesis, possibly due to the increased crosslinking in the tumor ECM and reduced matrix turnover around blood vessels.[35] The crosslinking activity of TG2 could also lead to changes in properties of ECM: it directly binds to fibronectin,[73,74] a major component of ECM, through its N-terminus and enhances cell adhesion mediated by fibronectin.[75] TG2 also associates with integrins β1 and β3 and, therefore, might serve as an intermediate and enhance interactions between fibronectin and integrins.[76] Whether or not this function of TG2 also occurs in vivo and, if it does, what the implications for this are in tumor progression have not been investigated.

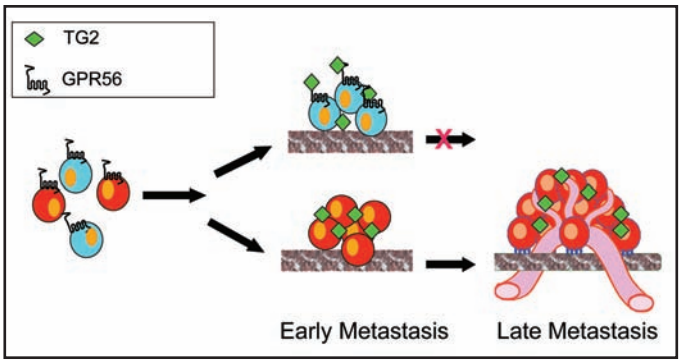

Figure 2. Working hypothesis for the roles of GPR56-TG2 interactions in metastasis. A pool of cancer cells with high (red) and low (blue) metastatic potentials disseminate into a distant organ. The expression levels of GPR56 in highly metastatic cells are down-regulated in the metastases, whereas those in poorly metastatic cells remain high. The TG2 expressed in the tissue suppresses the growth of those cells with high levels of GPR56 but not those cells with low levels of GPR56.

WORKING HYPOTHESES: METASTATIC CELLS SURVIVE AND GROW AS METASTASES BY EVADING GPR56-TG2 INTERACTION.

As mentioned earlier, we hypothesize that highly metastatic cells might somehow evade potentially growth suppressive effects from a foreign microenvironment in the invaded tissue and grow as metastases. Downregulation of GPR56 might be one of the mechanisms. Cells with high levels of GPR56 might interact with TG2 in the invaded tissues (such as the lung) and their growth would be inhibited. Cells with low levels of GPR56, however, could avoid this inhibition and grow as metastases (Fig. 2).

Many questions remain unanswered. First, are additional (not necessarily later) genetic or epigenetic alterations needed for completing the final stage of metastasis? Some experimental data have suggested that they are. For example, significant gene expression differences have been found between populations of cancer cells with different metastatic abilities. Many of those differences, such as GPR56, were reported to play causal roles in metastasis.[30-33] This view has recently been challenged on the basis of gene expression profiling studies using human tumor materials. Those studies found that gene signatures present in primary tumors are sufficient to predict their propensity to develop metastases,[77,78] suggesting that the metastatic abilities are predetermined in the primary tumors and no additional gene expression changes are needed. A model to explain the evidence from both sides was proposed and suggested that both the initial and additional changes may be required for metastasis to succeed.[79] The initial changes may be required for tumor cells to disseminate and spread to distant organs and the additional changes are for the survival and growth of metastases in different environments. Therefore, whereas all metastases share similar initial changes, they might have different additional changes to survive and grow in different microenvironments.

The possible occurrence of initial and additional changes were further investigated by Klein and colleagues.[80-82] They collected single cytokeratin-positive (CK^+) cells from the bone marrow of cancer patients. These cells were known as an important risk factor for reduced survival in cancer patients.[83] They also display characteristics of cancer cells, suggesting that they might be disseminated from the primary tumors.[80] The CK^+ cells from patients with no detectable metastases (M0 patients) are therefore probably from early dissemination, whereas those from patients with metastases (M1 patients) might be disseminated from the later stage of cancer progression. To delineate the genetic changes in tumor progression, the authors compared the CGH (Comparative Genome Hybridization) profiles of the CK^+ cells from M0 and M1 patients and different areas of matched primary tumors.[81,82] They discovered that some CK^+ cells from M0 patients had the CGH profile of normal cells, suggesting that these cells disseminate very early during tumorigenesis. Furthermore, the CK^+ cells from M0 patients that do contain CGH abnormalities have very different CGH profiles than the cells from matched primary tumors, suggesting that these cells might have collected additional genetic changes after dissemination. A subpopulation of these cells might gain the ability to survive and grow as metastases, which could be why the CK^+ cells in M1 patients have more homogeneous CGH abnormalities than those from M0 patients. Collectively, the authors proposed a model for the systemic progression of cancer: cancer cells disseminate early during tumorigenesis, these disseminated cells go through a dormant period and acquire diverse genetic changes, and finally a subpopulation of those cells succeed in forming metastases. This model is consistent with the notion that additional genetic or epigenetic changes after dissemination are required for metastases to form.

It is not clear when the downregulation of GPR56 occurs during cancer progression. Our unpublished data suggested that the metastatic derivatives express similar levels of GPR56 as the poorly metastatic parental line when cultured in vitro. Only when they were injected in vivo either subcutaneously or intravenously to form tumors, their expression levels of GPR56 were significantly reduced compared with the parental line (Fig. 2).[33] Therefore, the expression level of GPR56 appears to be regulated by signals from the tumor microenvironment, and the parental line may respond differently to these signals than the metastatic derivatives. What these signals are will need further investigation.

Another question concerns the functions of GPR56 and TG2 in normal tissues. GPR56 and TG2 are both expressed in multiple tissues, therefore, if TG2-GPR56 interaction suppresses tumor cell growth, it might keep normal cells in a quiescent state as well. GPR56 mRNA has been found to be up-regulated in both neuronal and hematopoietic stem cells.[46,47] Stem cells are known to be slow-cycling cells, therefore TG2-GPR56 might be responsible for maintaining this relatively quiescent state of stem cells. This possible interpretation of TG2-GPR56 functions could also be applied to metastasis, since TG2-GPR56 interaction might inhibit the replication of cancer cells during metastasis.

Finally, the above hypothesis assumes that TG2 cooperates in the suppression of tumor progression by GPR56. Although this possibility needs to be further investigated, the fact that both proteins have been implicated in suppression of tumor progression suggests that they could be functionally linked during metastasis. GPR56 might localize TG2 at the tumor cell surface and cause local ECM remodeling and inhibition of tumor growth. In particular, the localized TG2 might also alter the effect of TG2 on cell adhesion and invasion, which have multiple implications for tumor progression and metastasis. GPR56 might also change the activity of TG2 and lead to changes in ECM composition or structure. Finally, TG2 might directly signal through GPR56 and induce suppression of cell growth. All these possibilities will need to be explored in the next few years.

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