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COMPLEXITY IN CANCER-HOST CROSSTALK

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Hepatocellular carcinoma (HCC), the major form of liver cancer, is currently the second leading cause of cancer deaths worldwide. The most common causes of HCC are hepatitis virus B and C (HBV, HCV) infections, nonalcoholic steatohepatitis (NASH), and alcoholic steatohepatitis (ASH). Due to the current obesity epidemic and the advent of HBV vaccines and effective HCV therapies, NASH will soon become the leading cause of HCC in the US and Western Europe. NASH is the most severe manifestation of nonalcoholic fatty liver disease (NAFLD), currently affecting 30-40% of American adults. All of the above hepatides (HBV and HCV infections, NASH, and ASH) are chronic inflammatory diseases of the liver, and inflammation driven by the cytokines TNF and IL-6 plays a key role in HCC pathogenesis [1,2].

To understand how obesity, NAFLD, and NASH increase HCC risk, we initially combined exposure to the hepatic carcinogen diethylnitrosamine (DEN) with high-fat diet (HFD) or genetic obesity and have shown that both dietary and genetic obesity strongly enhance HCC induction [3]. Subsequently, we found that autocrine IL-6 signaling is critical for the genesis of HCC progenitor cells (HcPC), which serve as the precursors for HCC[4]. Although HcPC exhibit a similar transcriptomic profile to bile-duct-derived bipotential hepatobiliary progenitors, they actually originate from differentiated zone 3 (pericentral) hepatocytes [4]. Furthermore, we have shown that both DEN-induced and NASH-derived (see below) HCC are initiated by differentiated zone 3 hepatocytes which express the enzymes needed for metabolic activation of DEN and de novo lipogenesis (DNL) [5]. In response to chronic stress and damage, these cells accumulate oncogenic mutations, undergo de-differentiation, acquire proliferative potential, and give rise to cancer.

Since HFD-fed or genetically obese mice exhibit so-called bland steatosis but do not manifest classical signs of NASH and require treatment with a carcinogen (i.e. DEN) to form HCC, we sought to develop a more accurate model for NASH and its progression to HCC. Studies of human NASH led to the so-called two-hit and multiple-hit hypotheses, according to which lipid droplet accumulation in hepatocytes is the first hit, leading to appearance of bland steatosis, whereas mitochondrial dysfunction or endoplasmic reticulum (ER) stress are likely to constitute secondary hits that lead to liver damage, inflammation, and NASH [6]. We therefore chose to use *MUP-uPA* transgenic mice, which display transient liver damage due to ER stress caused by high expression of urokinase plasminogen activator (uPA) in their hepatocytes [7]. Early liver damage in these mice, which peaks between 4-6 weeks of age, is transient because liver regeneration results in downregulation of uPA expression. Nonetheless, by placing the mice on HFD for several months we found that ER stress is re-ignited and that the mice display classical NASH signs, including ballooning degeneration of hepatocytes, inflammatory and immune infiltrates, and a "chicken-wire" pattern of peri-cellular fibrosis [8]. NASH development is totally dependent on ER stress and can be prevented by treating the mice with the chemical chaperons phenyl butyric acid (PBA) or tauroursodeoxycholic acid (TUDCA) or by overexpression of the chaperon GRP78/BiP. Importantly, after 8-9 months on HFD, 85-90% of the mice display aggressive, poorly differentiated HCC [8]. Exome sequencing of 16 such tumors had revealed 50-100 coding region mutations [9], a mutation frequency similar to that of human HCC [10]. The mutational signature was also nearly identical between the mouse and human tumors [9]. Further validating the usefulness of the *MUP-uPA* model for studying the pathogenic pathways involved in human HCC was the observation that many of the mutations were not recurrent, affecting a number of different pathways [9]. By contrast, exome sequencing of DEN-induced HCC revealed an overwhelming abundance of a particular *Braf* mutation, replacing valine with glutamate at position 600 [4]. Curiously, the same mutation has been identified in 22% of human cholangiocarcinomas but not in human HCC [11].

Given the authenticity of the *MUP-uPA* + HFD model we invested much effort in understanding the underlying pathogenic mechanisms. One of the earliest molecular and cellular events to take place in HFD-fed *MUP-uPA* mice is a large increase in TNF production, mainly by newly recruited liver macrophages [8]. TNF signaling via the type 1 TNF receptor (TNFR1) was found to be of importance in NASH pathogenesis, operating through the killing of cholesterol-loaded hepatocytes, which promotes subsequent compensatory proliferation. In addition, TNFR1 engagement is critical for early tumor growth acting through IKKβ-driven NF-κB activation [8]. Consistent with these results, constitutive IKKβ activation in hepatocytes promotes HCC development [12].

Another important player in NASH-induced HCC is the *Sqstm1* gene, which codes for the autophagy adaptor protein p62 [13]. Hepatocyte-specific *Sqstm1* ablation results in a marked reduction in HCC development in HFD-fed *MUP-uPA* mice [14]. Curiously, the oncogenic function of p62 is not related to its role as an autophagy adaptor. Instead, it is mediated through the activation of NRF2 [14]. Exactly how NRF2 activation promotes HCC development is a matter of intense investigation but it should be noted that mutations in the genes encoding NRF2 and its negative regulator KEAP1 have been detected in nearly 15% of human HCC [10]. However, in HFD-fed *MUP-uPA* mice, NRF2 activation is non-mutational. Of further note, p62 accumulation is a hallmark of many chronic liver diseases that increase HCC risk [13].

Most recently we found that the adaptive immune system plays a critical role in the control of NASH to HCC progression. We were first intrigued by the finding that human NASH and ASH patients exhibit elevated circulating amounts of immunoglobulin A (IgA) [15]. We confirmed that HFD-fed *MUP-uPA* mice and other mouse models of chronic liver inflammation and fibrosis also exhibit elevated amounts of circulating IgA and have shown it to be produced by liver-resident IgA^+ plasmablasts and plasma cells, collectively referred to as IgA^+ plasmocytes [9]. Importantly, liver IgA^+ plasmocytes express the immunosuppressive molecules IL-10 and PD-L1 and are also present in human NASH-afflicted liver but are barely detectable in healthy livers [9]. Immunosuppressive plasmocytes start accumulating in the chronically inflamed and damaged liver long before visible HCC nodules are detected and their generation depends on TGF-β-driven class switch recombination, as well as IL-21 and other cytokines. In the livers of HFD-fed *MUP-uPA* mice, IL-21 is mainly made by follicular helper cells (Tfh), whose accumulation also correlates with NASH development [9]. Importantly, previous work in our lab on drug-resistant prostate cancer had shown that IgA^+ IL-10- and PD-L1-expressing plasmocytes accumulate in castration-resistant tumors in response to myofibroblast activation and lead to exhaustion of tumor-directed cytotoxic T cells (CTL) [16]. Interestingly, NASH and ASH are also accompanied by activation of liver resident myofibroblasts, also known as hepatic stellate cells (HSC). Activated HSC and other myofibroblasts produce large amounts of CXCL13, an important B-cell chemoattractant [17]. Most importantly, IgA ablation and other genetic manipulations that prevent the development of IgA^+ immunosuppressive plasmocytes severely attenuate NASH to HCC progression in *MUP-uPA* mice without affecting NASH development [9]. The decrease in NASH to HCC progression is due to enhanced activation of anti-tumorigenic $CD8^+$ CTL, whose ablation results in dramatic acceleration (7- to 8-fold reduction in tumor latency) and enhancement of HCC development [9]. Furthermore, introduction of T cells without B cells into HCC-bearing *Rag1*$^{-/-}$ mice that are deficient in both B and T cells results in tumor regression. Conversely,

$CD8^+$ T-cell depletion from IgA-deficient mice enhances HCC development. Collectively, these results strongly suggest that chronic liver damage and inflammation also promote HCC development through indirect effects on the adaptive immune system, in addition to their effect on the proliferation of early HCC progenitors (Figure 1). IgA^+ immunosuppressive plasmocytes carry out their tumor-promoting function by inducing the exhaustion of HCC-directed CTL, which play an important protective role in

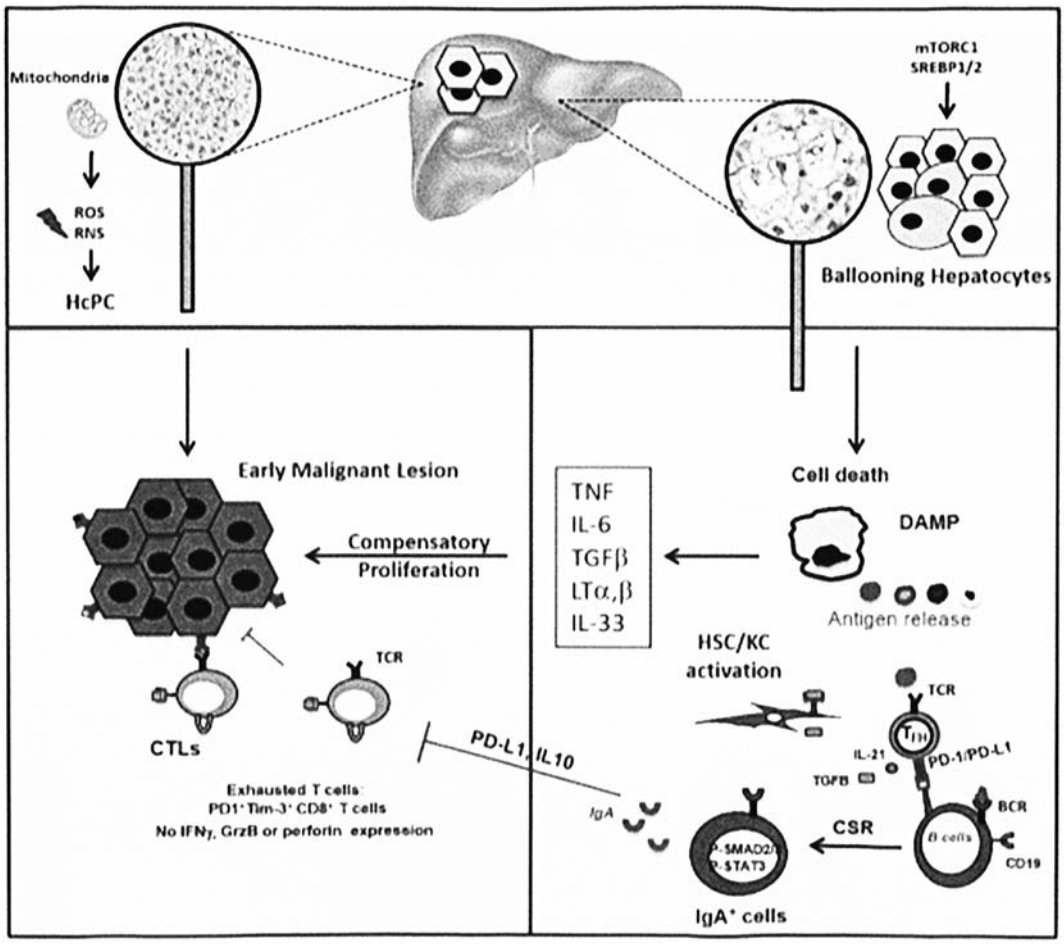

Figure 1 The pathogenesis of NASH-driven HCC.
NASH is initiated in response to metabolic alterations that promote the accumulation of lipids in hepatocytes and cause mitochondrial abnormailities that enhance the production of reactive oxygen species (ROS) that lead to genetic alterations that result in accumulation of oncogenic mutations as well as hepatocyte death. Release of DAMPs by dying hepatocytes triggers an inflammatory response that supports the growth of HCC progenitor cells (HcPC), leading to appearance of early malignant lesions. Such lesions are kept in check by HCC-directed cytotoxic T lymphocytes (CTL). However, the chronic inflammation that accompanies NASH also results in generation of immunosuppressive plasmocytes that interfere with CTL-mediated immunosurveillance, thereby promoting immune escape and tumor growth.

suppressing HCC development. Indeed, sequencing of T-cell receptor (TCR) α and β chains from HCC-associated $CD8^+$ T cells indicates that these cells have undergone clonal selection and expansion, suggesting that the majority of these cells recognize HCC-associated antigens. Importantly, our results provide the strongest support thus far for the immunosurveillance hypothesis, which was first proposed more than 50 years ago by Burnet and Thomas, according to which our immune system is responsible for rejection of nascent tumors. Our work also indicates that inflammation-driven immunosuppression is a key tumor-promoting mechanism.

Importantly, by treating HCC-bearing mice with a neutralizing PD-L1-specific antibody we were able to re-invigorate exhausted HCC-directed CTL and achieve rather impressive tumor regression. Rinvigoration of HCC-directed CTL was accompanied by a considerable reduction in the liver content of immunosuppressive IgA^+ plasmocytes whose development depends on their ability to engage PD-1 expressed by Tfh [9]. These results explain why antibodies that disrupt PD-1:PD-L1 interactions are quite effective in inducing the regression of aggressive stage 4 human HCC [18]. We all hope that the recent clinical approval of such drugs will revolutionize the treatment of non-resectable HCC, one of the most lethal cancers that is remarkably resistant to conventional chemotherapy.

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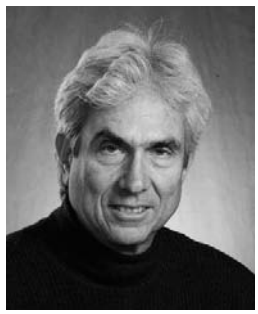

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CELL CONTEXT-DEPENDENT ROLES OF A CELL ADHESION MOLECULE, CADM1, IN HUMAN ONCOGENESIS

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Disruption of cell adhesion is a crucial step in invasion and metastasis of human cancer. The concept of this symposium is complexity in cancer – host crosstalk, because cancer is essentially a systemic disease. Once cancer cells develop solid tumors, they need to maintain the neoplastic tissues by cooperating with a variety of stromal components, including fibroblasts, lymphocytes, neutrophils, macrophages, endothelial cells and extracellular matrices (Figure 1). Here, we focus on the complexity in cancer from the viewpoint of complexity in cell to cell adhesion.

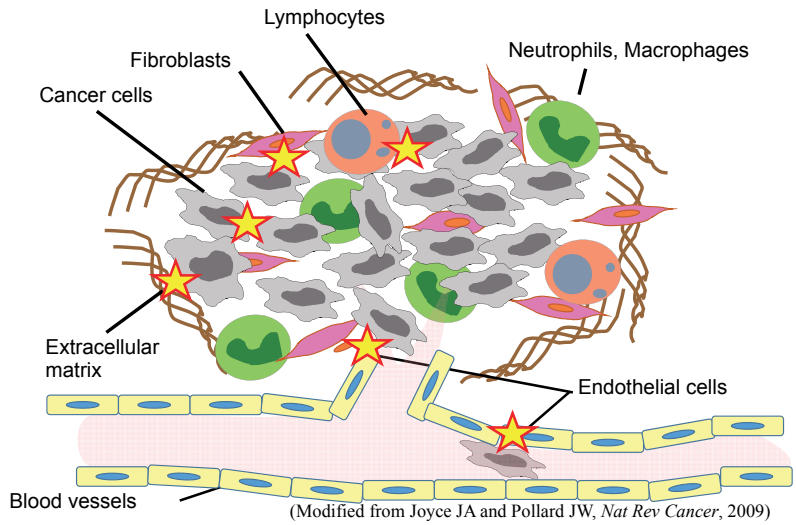

Figure 1 Complexity in cancer-host crosstalk from the view point of cell to cell adhesion

A variety of cell adhesion molecules, including cadherins, selectins, integrins, and immunoglobulin superfamily cell adhesion molecules (IgCAM), is shown to be involved in cell to cell interaction in epithelial tissue and solid tumors. An IgCAM, CADM1/Necl-2, is originally identified as a tumor suppressor by its suppression activity of tumorigenicity in nude mice [1]. CADM1 is involved in the formation and maintenance of an epithelial cell structure, whereas it is frequently inactivated in various cancers, including non-small cell lung cancer (NSCLC), in their advanced stages, suggesting that loss of CADM1 function promotes cancer invasion and/or metastasis [2]. This is supported by development of more invasive lung tumors when *Cadm1*$^{-/-}$ mice is crossed with mutant *K-ras* knock-in mice.

Here, we demonstrate that CADM1 inhibits HGF-Met-induced epithelial mesenchymal transition of MDCK cells by associating with Met on the cell membrane. CADM1 also suppresses Src-signaling by associating with Csk-binding protein (CBP) in the lipid raft on the membrane of colon cancer cells. Intervention of growth factor signaling by cell adhesion molecules, such as CADM1, on the cell membrane could provide a novel approach to suppress oncogenic pathways in cancer cells.

In contrast, CADM1 is overexpressed in adult T-cell leukemia (ATL) cells, conferring an invasive phenotype characteristic to ATL. CADM1 is now established as one of the specific cell surface markers to detect circulating malignant ATL cells in the patients by flow cytometry [3]. We have demonstrated that CADM1 directly associates with Tiam1 and constitutively activates Rac signaling in ATL cells, whereas invasive phenotype is drastically suppressed by introducing dominant-negative Rac mutants into ATL cells. CADM1 and Tiam1 are co-localized at the leading edge of ATL cells, playing the essential roles in formation of lamellipodia in ATL cell lines [4]. Moreover, we have found that CADM1 is overexpressed in small cell lung cancer (SCLC). Immunohistochemistry demonstrates that 33 of 45 (75%) primary SCLC tumors express CADM1 protein. Western blotting and RT-PCR analyses have revealed that CADM1 is significantly expressed in 11 of 14 SCLC cells growing in suspension cultures but in neither of 2 SCLC cells showing attached growth to plastic dishes, suggesting that CADM1 is involved in anchorage-independent growth in SCLC. Interestingly, CADM1 shows enhanced tumorigenicity in nude mice when transfected into SCLC cells lacking CADM1. Inversely, suppression of CADM1 expression by shRNA reduced spheroid-like cell aggregation of SCLC cells that were expressing a high amount of CADM1. These findings suggest that CADM1 enhances the malignant features of SCLC, as is observed in ATL [5].

These findings indicate that CADM1 has dual roles in oncogenesis; a tumor suppressor in epithelial cancer and an oncoprotein in ATL or SCLC. Our studies demonstrate that one of the possible molecular mechanisms underlying the distinct roles of CADM1 in epithelial cancers versus in ATL or SCLC is the distinct downstream cascades triggered from a

membrane protein, CADM1. In NSCLC and most other cancers of epithelial origin, CADM1 associates with a member of protein 4.1s and a member of MAGuKs and form a tripartite complex involved in cytoskeleton organization and epithelia-like cell structure and functions as a tumor suppressor [6]. By contrast, in ATL, CADM1 associates with Tiam1, one of the guanine nucleotide exchange factor (GEF) of Rac protein, which promotes cell mobility and invasive growth in ATL. Distinct downstream cascade we found here would provide a clear molecular basis to dual roles of CADM1 in oncogenesis.

In addition to the phenotypic regulation of epithelial cells, it has been reported that CADM1 is involved in the immunological responses between tumor cells and NK cells, as well as antigen-presenting cells and CD8+ T cells. Three independent groups have demonstrated that the CADM1 in tumor cells forms highly specific heterodimers with another IgCAM, class I-restricted T-cell-associated molecule (CRTAM), whose expression is only induced on the membrane of activated NK cells or CD8+ T cells [7]. This heterophilic trans-interaction between CADM1 and CRTAM enhances the cytotoxicity of NK cells and the secretion of γ-interferon from CD8+ T cells to attack the CADM1-expressing cells. Thus, it is possible to speculate that CADM1 could act as a tumor antigen, whereas loss of CADM1 expression might be advantageous for cancer cells to escape the immunological surveillance conducted by the host system.

CADM1 is a unique tumor suppressor that is involved in both adhesion-mediated epithelial like cell structure and immunological responses in most cancers of epithelial origin, whereas CADM1 inversely promotes invasive and metastatic growth in some specific types or context of cells, like ATL or SCLC. CADM1 provides an example to consider the importance of cancer complexity and cancer-host complexity from the view point of cell adhesion.

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CHEMOTHERAPY INDUCES ENRICHMENT OF IMMUNE-EVASIVE BREAST CANCER CELLS

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Breast cancer is a leading cause of cancer death in women, with an incidence of 250,000 new cases and > 40,000 deaths in the United States annually [1]. Chemotherapy is used in selected early stage and many advanced breast cancers [2]. Many patients initially benefit from chemotherapy but experience recurrence, metastasis, and ultimately death [3]. This is particularly true for triple-negative breast cancers, which lack expression of the estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2. Breast cancer stem cells, which are defined by their infinite proliferative potential and tumor initiating properties, contribute critically to tumor recurrence and metastasis [4, 5]. Breast cancer stem cells have increased chemoresistance through a variety of mechanisms, including increased drug inactivation, increased expression of drug transporter proteins, and enhanced DNA repair activity [6]. Chemotherapy may eliminate the bulk of cancer cells within a tumor, but leave behind breast cancer stem cells that are the source of recurrent and metastatic disease. Thus, novel treatment options that target Breast cancer stem cells are needed.

Hypoxia-inducible factors (HIFs) are associated with resistance to chemotherapy in breast cancer [7, 8]. HIFs are transcription factors consisting of a highly regulated HIF-1α or HIF-2α subunit and a constitutively expressed HIF-1β subunit [9]. In order to form a secondary (recurrent or metastatic) tumor, a breast cancer cell must have tumor-initiating or stem cell-like properties. HIFs play important roles in promoting specification and/or maintenance of the breast cancer stem cell phenotype in response to hypoxia [10-15]. Chemotherapy also induces enrichment of breast cancer stem cells in a HIF-dependent manner [8]. Chemotherapy induces HIF-dependent activation of the glutathione

biosynthesis pathway, leading to increased intracellular glutathione levels. Glutathione inhibits MEK-ERK signaling through copper chelation, leading to nuclear localization of FoxO3 and transcriptional activation of the *NANOG* gene, which encodes a pluripotency factor that specifies the breast cancer stem cells phenotype [16]. Chemotherapy also induces the HIF-mediated expression of glutathione *S*-transferase omega 1 (GSTO1) [17]. Knockdown of GSTO1 expression abrogates carboplatin-induced breast cancer stem cell enrichment, decreases tumor initiation and metastatic capacity, and delays tumor recurrence after chemotherapy. GSTO1 interacts with the ryanodine receptor RYR1 and promotes calcium release from the endoplasmic reticulum. Increased cytosolic calcium levels activate PYK2 → SRC → STAT3 signaling, leading to increased expression of pluripotency factors and enrichment of breast cancer stem cells [17].

In order to form a secondary (recurrent or metastatic) tumor, a breast cancer cell must evade the innate and adaptive immune systems. CD47 enables cancer cells to evade killing by macrophages [18], whereas CD73 and PDL1 mediate two independent mechanisms of evasion of cytotoxic T lymphocytes [19, 20]. Expression of CD47, CD73, and PDL1 is induced by hypoxia in certain cell types [21-25]. Treatment of human or murine triple-negative breast cancer cells with carboplatin, doxorubicin, gemcitabine or paclitaxel induces the coordinate transcriptional induction of CD47, CD73 and PDL1 mRNA and protein expression, leading to a marked increase in the percentage of $CD47^+CD73^+PDL1^+$ breast cancer cells [26]. Genetic or pharmacologic inhibition of HIFs blocked chemotherapy-induced enrichment of $CD47^+CD73^+PDL1^+$ TNBC cells, which were also enriched in the absence of chemotherapy by incubation under hypoxic conditions, leading to T cell anergy or death and increased dissemination of cancer cells to the lungs of immunocompetent mice [26]. Our results delineate a HIF-dependent transcriptional mechanism contributing to triple-negative breast cancer progression and suggest that combining chemotherapy with a HIF inhibitor may improve patient survival by preventing the counter-therapeutic induction of proteins that mediate immune evasion.

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TUMOR-DERIVED EXOSOMES PROMOTE ORGANOTROPIC METASTASIS AND SYSTEMIC DISEASE

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Metastasis to distant vital organs such as lung, liver, and brain is the most devastating feature of cancer progression, responsible for over 90% of cancer-associated deaths. In 1889, Stephen Paget first proposed that organ distribution of metastases is a non-random event, yet metastatic organotropism remains one of the greatest mysteries in cancer biology. Our recent studies uncovered that tumor-derived exosomes, alter the microenvironment at future sites of metastasis to form pre-metastatic niches, creating a favorable "soil" for incoming metastatic "seeds" [1,2]. However, by what mechanism this occurs, and the role of exosomes in tumor metastasis, remains unknown. To investigate the role of exosomes in organotropic metastasis, we have used two established organotropic human tumor models: the MDA-MB-231 breast cancer (BC) cell line, and its variants known to metastasize to the lung, brain and bone, respectively, as well as two liver metastatic pancreatic cancer (PC) cell lines, BxPC3 and HPAF-2 [3]. We first analyzed the biodistribution of fluorescently-labeled exosomes derived from lung metastatic, brain metastatic or bone metastatic MDA-MB-231 BC variants or PC cell lines, and found that BC exosomes follow the organ-specific distribution of the cells of origin, while PC exosomes home to the liver (Figure 1a). In each target organ exosomes are taken up by different cell types: fibroblasts/epithelial cells in the lung, Kupffer cells in the liver, and endothelial cells in the brain (Figure 1b). In the organotropic MDA-MB-231 model, prior education with the lung tropic exosomes redirected metastasis of the bone tropic cells to the lung, demonstrating the unique capacity of exosomes to determine the site of metastasis. Unbiased proteomic profiling of exosomes revealed distinctive integrin expression patterns, and analysis of plasma exosomes from BC and PC patients that later developed site-specific metastasis revealed that specific exosomal

integrins could predict metastatic spread (Figure 2). Recently, our group has identified unique exosome subpopulations and novel nanoparticles (exomeres) which packaged specific protein and genomic contents (Figure 3) [4]. The biodistribution patterns of these particles suggest distinct functional roles in the metastatic process (Figure 4).

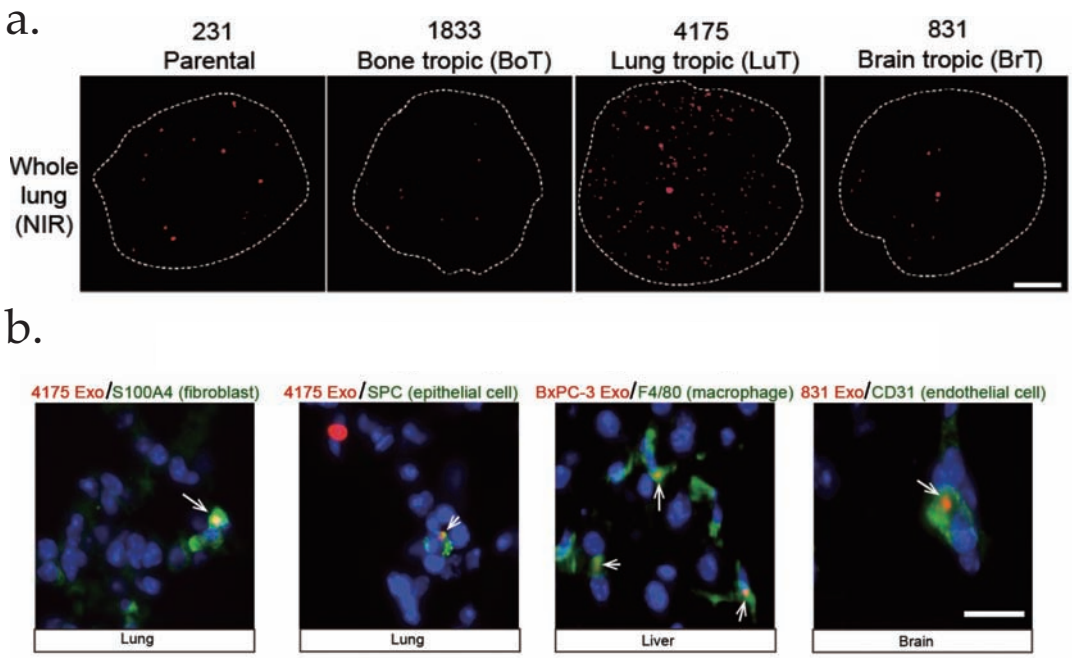

Figure 1 a. Exosomes from MDA-MB-231 sub-lines exhibit organ tropism
Exosomes injected(MDA-MB-231)
b. Distinct cell types uptake organotropic exosomes

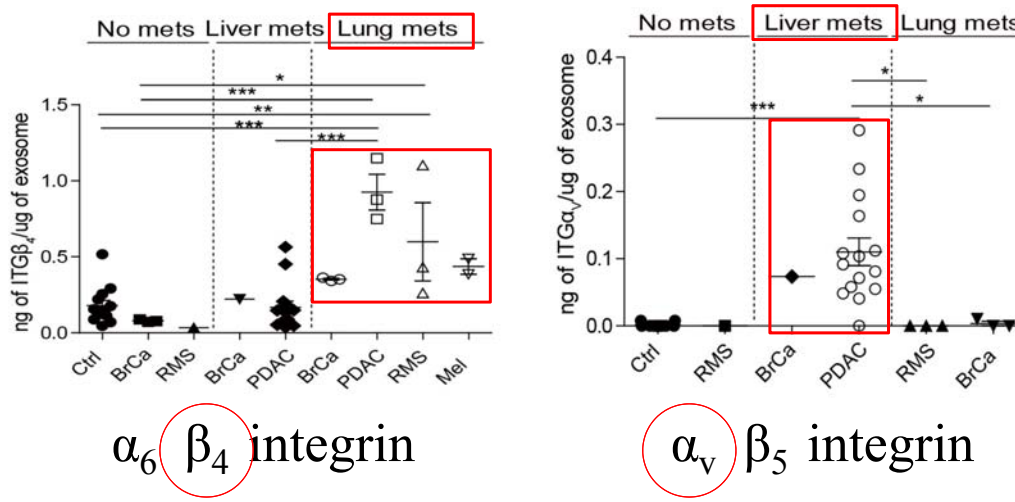

Figure 2 Blood sample analysis from Patients with lung mets or liver mets

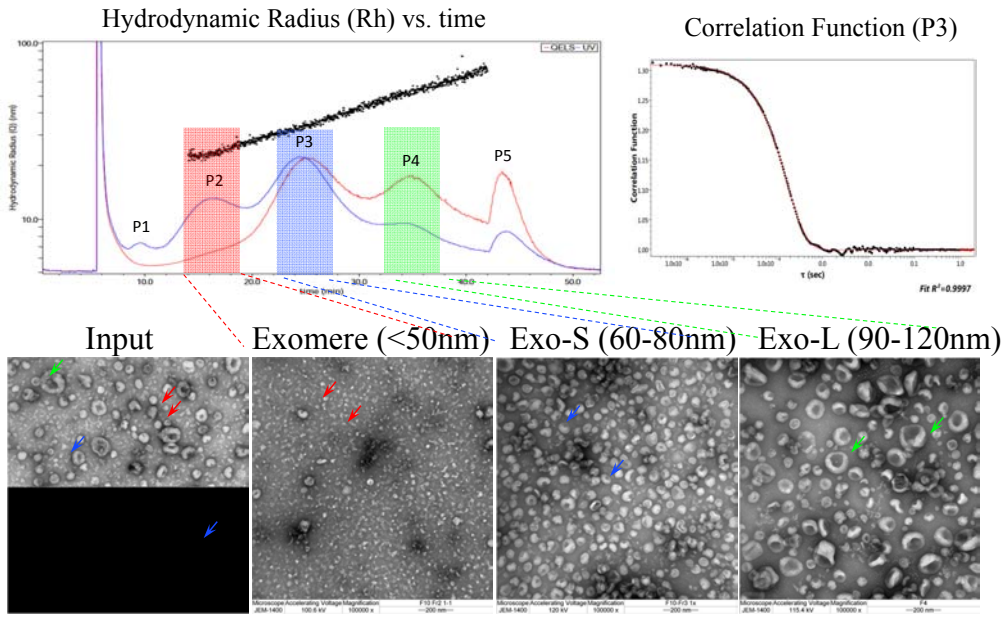

Figure 3 Identification of different subsets of exosomes by AF4 analysis

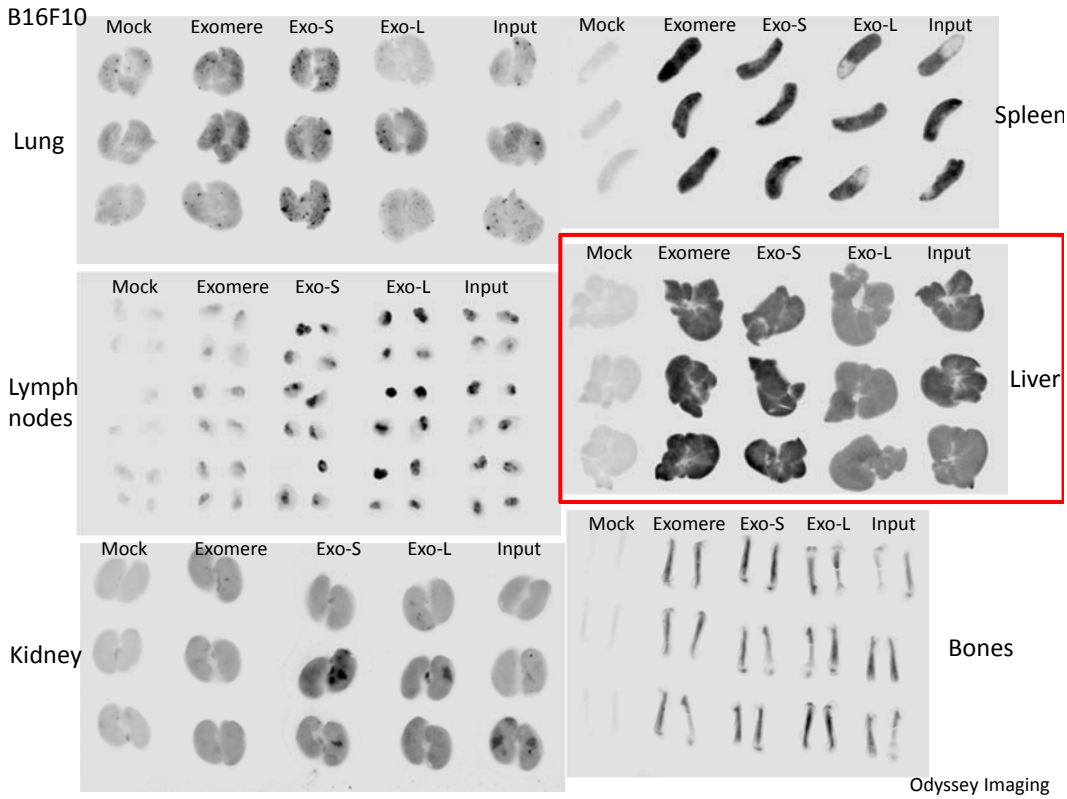

Figure 4

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EXTRACELLULAR VESICLES AS A NOVEL PLATFORM FOR CANCER DIAGNOSIS AND THERAPEUTICS

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Research has increased significantly on small vesicles secreted by healthy and diseased cells. Recent discoveries have revealed their functional and biomarker roles in cancer. Whether and how this knowledge of extracellular vesicles (EVs), known as exosomes and microvesicles, affects translational research and clinical practices have become pertinent questions.

EVs have been shown to be involved in such cell-cell communication between cancer cells and the surrounding microenvironment and to be important for the development of cancer metastasis [1, 2]. In addition, these cancer cell-derived EVs carry pathogenic components, such as proteins, messenger RNAs (mRNAs), microRNAs (miRNAs), DNAs, lipids, and transcriptional factors that can mediate paracrine signaling in the tumor microenvironment [3]. Understanding the metastatic mechanisms through EV transfer may open up a new avenue for cancer therapeutic strategies [4].

Our current understanding of cancer-EVs functions, cancer-EVs destroy our body's natural barriers. One example is EVs-mediated brain metastasis of breast cancer cells. A key event during brain metastasis is the migration of cancer cells through blood-brain barrier (BBB). However, the molecular mechanism behind the passage through this natural barrier remains unclear. We showed that cancer-derived EVs trigger the breakdown of blood-brain barrier (BBB) [5]. Importantly, EV-miR-181c promotes the destruction of BBB through the abnormal localization of actin via the downregulation of its target gene, PDPK1. PDPK1 degradation by downregulation of phosphorylated cofilin and the resultant activated cofilin-induced modulation of actin dynamics. Furthermore, we demonstrate that systemic injection of brain metastatic cancer cell-derived EVs promoted brain metastasis of breast cancer cell lines and are preferentially incorporated into the brain *in vivo*.

We also demonstrate that EVs derived from highly metastatic ovarian cancer cells promote peritoneal dissemination *in vivo* [6]. The EVs from highly metastatic cells strongly induce metastatic behaviour in moderately metastatic tumours. Notably, the cancer EVs efficiently induce apoptotic cell death in human mesothelial cells *in vitro* and *in vivo*, thus resulting in the destruction of the peritoneal mesothelium barrier. Whole transcriptome analysis shows that MMP1 is significantly elevated in mesothelial cells treated with highly metastatic cancer EVs and intact MMP1 mRNAs are selectively packaged in the EVs. Importantly, MMP1 expression in ovarian cancer is tightly correlated with a poor prognosis.

We currently found that prostate cancer-derived EVs are important for bone metastasis via differentiation and proliferation of osteocrast cells (unpublished). Prostate cancer-derived EVs play a significant role for bone metastasis by regulating bone remodeling process.

Furthermore, the circulating EVs have also been of interest as a source for liquid biopsies. EVs in body fluids provide a reliable source of microRNAs and proteins for cancer biomarkers. The tumor specific components in EVs effectively provide various messages on the physiological and pathological status of cancer patients. Our *ExoScreen* technology [7] allows us for developing a novel liquid biopsy for early detection of cancer.

EVs are secreted from cancer cells and delivered to recipient cells, modulating their phenotype. For example, EVs from cancer cells are delivered to endothelial cells, which enhances angiogenesis to obtain the oxygen and nutrition required for continued growth of the cancer. We propose the following therapeutic applications: inhibition of cancer cell EV production, disruption of EV uptake by recipient cells, and elimination of circulating cancer cell–derived EVs as shown in the Figure 1. These therapeutic strategies will prevent the delivery of EVs from cancer cells to microenvironmental cells, leading to the development of novel antiangiogenic and anticancer drugs. Thus, EVs will provide us the multiple strategies to fight against cancer, however, it is important to confirm safety issues and overcome technical problems to bring EVs in practical use.

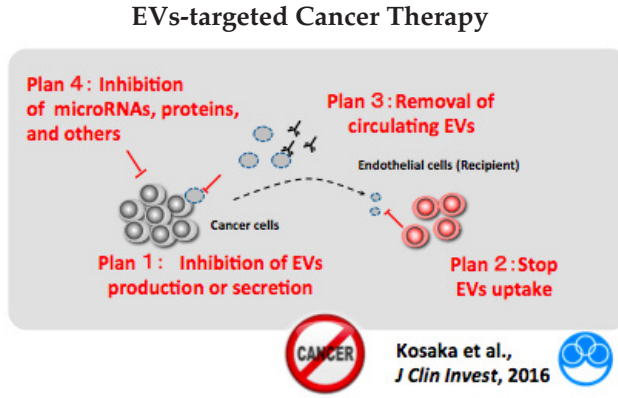

Figure 1 Therapeutic applications: inhibition of cancer cell EV production, disruption of EV uptake by recipient cells, and elimination of circulating cancer cell – derived EVs.

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THE FUNCTIONAL ROLE OF TUMOR STROMA AND EXOSOMES IN BIOLOGY AND TREATMENT OF PANCREATIC CANCER

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Trillions of exosomes and extracellular vesicles are naturally present in the blood and tissue [1]. Exosomes are nano-sized extracellular vesicles (40-150 nm), likely of endosomal origin, with a membrane lipid bilayer [2]. Although their precise physiological role remains largely unknown, is it suggested that exosomes mediate specific cell-cell communication and activate signaling pathways in cells with which they fuse or interact. Exosomes are detected in the tumor microenvironment, and emerging evidence suggests they play a role in facilitating tumorigenesis by regulating angiogenesis, immunity, and metastasis [1, 3]. While exosome production and cargo may vary depending on the cellular source, studies have identified that exosomes contain proteins, lipids, metabolites, mRNA, non-coding RNAs (ncRNAs), microRNAs (miRNAs) and genomic DNA [4-7]. Notably, circulating exosomes could be used as liquid biopsies and non-invasive biomarkers to potentially alert for early detection and diagnosis of cancer patients [1].

The process of vesicle shedding, by which exosomes are released into the extracellular space, appears to be amplified in proliferating cancer cells [1]. Tumor-secreted exosomes have been the focus of recent investigative efforts for their role as "vehicles", facilitating local intercellular communication (*e.g.,* autocrine and paracrine) as well as distal (*i.e.,* endocrine) signaling, through the transfer of their contents. Unlike liposomes and other synthetic drug nanoparticle carriers, exosomes contain many transmembrane and membrane-anchored proteins. These proteins likely enhance endocytosis and/or direct fusion with the plasma membrane of the recipient cells, thus enhancing potential content delivery [8-10]. Our studies have focused on harnessing the potential of exosomes as therapeutic vehicles, in particular for the treatment of pancreatic ductal adenocarcinoma

(PDAC) [11].

Despite current standard of care therapies, the prognosis for patients with pancreatic ductal adenocarcinoma (PDAC) is dismal [12-15]. The emergence of PDAC is on the rise and patients are in urgent need of effective therapies. Genetic analyses of PDAC show that mutations in a small GTPase, Kras, are encountered in the majority of patients [16-19] and are key drivers for initiation, progression, and metastasis [20-26]. While it is recognized that the Ras-mediated signaling pathway is a key mediator of pancreatic cancer progression, a direct and specific targeting of Ras has been elusive, dubbing Ras as an undruggable target. The generation of Ras pathway-inhibiting drugs and oncogenic Ras-specific RNAi molecules (siRNA and shRNA) have encountered targeting challenges and Ras remains an intractable pharmacological target for therapy and different approaches must be explored [27].

Genetic manipulation of oncogenic Kras in mice determined that dampening oncogenic Kras inhibits tumor progression despite the presence of other genetic defects [20, 22, 23, 28]. Effective delivery of RNAi molecules to non-liver parenchymal organs, especially the pancreas, remains a challenge. A major hurdle in employing specific RNAi constructs to inhibit Ras is the lack of an optimal delivery system [29, 30]. Using exosomes engineered to contain siRNA (iExosomes) that target the mutant form of the GTPase Kras found in pancreatic cancer, efficient and specific targeting could be achieved to control cancer progression in various mouse models of PDAC. The anti-cancer efficacy of iExosomes was associated with their enhanced retention of in circulation when compared to liposomes. iExosomes, prepared from fibroblast culture supernatant, were noted to present with the 'don't eat me' signal CD47, which enabled their protection from phagocytosis. Further, the enhanced macropinocytic activity of Kras mutant PDAC cells fascilitated the iExosomes uptake to the pancreas, contributing to the efficacy of iExosomes treatment in suppressing cancer and increasing the survival of mice with PDAC. These studies are prompting the engineering and use of exosomes in personalized therapy for cancer patients. This lecture will highlight some of the recent advances in the area of exosomes biology and tumor microenvironment, and its utility in the diagnosis and treatment of cancer.

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METABOLIC CROSSTALK BY AUTOPHAGY ADAPTORS IN THE TUMOR STROMA

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The analysis of the tumor genomic and transcriptomic landscape reveals the extreme difficulty in many occasions to distinguish between cancer-causing driver mutations from passenger mutations, which impairs our ability to design effective oncogene-selective therapies. Importantly, cancer cells create non-oncogenic addictions, irrespective of the mutational load of the tumor, which are required for the tumor to survive stressful conditions. This generates non-oncogenic vulnerabilities that can be identified as a possible way to target cancer cells in an oncogene agnostic manner. p62 is induced in many types of cancers, and specifically in prostate cancer and hepatocellular carcinoma (HCC), in which it has been shown to play a critical role as a non-oncogenic addiction [1]. We have recently discovered that the accumulation of p62 is necessary and sufficient for HCC induction in mice, through a mechanism that involves the activation of NRF2 and mTORC1 in hepatocytes, independently of autophagy [2]. We have also shown that p62 is required for prostate cancer through a mechanism that also implicates the ability of p62 to activate mTORC1 in response to nutrient sensing [3]. Interestingly, although p62 is upregulated in the liver parenchyma in patients of non-alcoholic steatohepatitis (NASH) and HCC, as well as in the prostate cancer epithelium, it is dramatically downregulated in the stroma of both types of cancer. This is important because the selective inactivation of stromal p62 creates a microenvironment that is conducive to tumorigenesis [4,5]. Results will be presented in which the detailed mechanisms whereby p62 acts as a tumor suppressor in the stroma will be discussed. Specifically, we will present the newly identified ability of p62 to regulate nuclear events in cancer associated fibroblasts, which has important implications in the design of novel therapies aimed at reprogramming the tumor stroma. In addition, we have

recently discovered that p62 also impacts tumor metabolism and progression through the regulation of metabolic and signaling cascades in the adipose tissue. These novel findings are central to our understanding of the metabolism in cancer patients.

Another important aspect of the stroma-epithelium crosstalk is the signaling and metabolic pathways that confer tumors the ability to withstand situations of nutrient stress. This is a common stage in many poorly vascularized and aggressive tumors that, surprisingly, correlates with poor patient prognosis. Importantly, it is becoming apparent that the stromal fibroblasts may exert a critical role in the control of nutrient utilization and metabolism in the tumor microenvironment. That is, cancer associated fibroblasts (CAFs) can provide nutrients to support the epithelial cells in the tumor and also modulate immune responses, which are critical for tumor regression upon immunotherapy. Interestingly, new results will be discussed that demonstrate that p62 deficiency in stromal fibroblasts promotes resistance to glutamine deprivation not only of stromal cells but also of the tumor epithelium. This is very important because glutamine is the most commonly depleted amino acid in solid tumors, and serves as the obligate nitrogen donor for numerous biosynthetic pathways. Our recent studies demonstrate that p62 directly controls the stability of the transcription factor ATF4. The precise mechanism involves the regulation of its stability through a process that involves the p62-mediated polyubiquitination of ATF4. In this way, the loss of p62 in stromal fibroblasts, a common event in cancer, results in the upregulation of ATF4. This is critical for the survival and proliferation of stromal and tumor epithelial cells, because p62 deficiency in the stroma activates glucose carbons flux through a pyruvate carboxylase-asparagine synthase cascade that is central to the generation of asparagine as a source of nitrogen for stroma and tumor epithelial proliferation. These most recent results, together with our data demonstrating the important role of p62 in the regulation of the vitamin D receptor in hepatic stellate cells demonstrate the existence of a new paradigm whereby p62 directly targets nuclear transcription factors to control metabolic reprogramming in the microenvironment and repress tumorigenesis.

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COMPREHENSIVE PHENOTYPIC CHARACTERIZATION OF INTESTINAL CANCER WITH MULTIPLE DRIVER MUTATIONS

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Colorectal cancer (CRC) is a leading cause of cancer-related death in the world. It has been shown by genome-wide analyses that *APC*, *KRAS*, *TGFBR2*, *TP53* are found in the most frequently mutated genes in CRC, which is consistent with a concept of classical multistep tumorigenesis. However, it has not yet been fully understood which combinations of driver mutations are responsible for induction of each process of malignant progression. To investigate the *in vivo* biological mechanism of the respective driver mutations in combination, we have generated compound mutant mice that carried driver gene mutations by crossing and comprehensively examined their phenotypes.

Apc simple mutant mice, $Apc^{\Delta 716}$ mice, developed benign intestinal adenomas caused by Wnt signaling activation [1]. On the other hand, $Apc^{\Delta 716}$ *Smad4*$^{-/-}$ and $Apc^{\Delta 716}$ *Tgfbr2*$^{-/-}$ compound mutant mice developed invasive adenocarcinomas, indicating that suppression of TGF-β signaling induces submucosal invasion of intestinal tumors [2, 3]. TGF-β signaling is important for differentiation of intestinal epithelial cells. Notably, organoid culture of intestinal epithelial cells derived from *Tgfbr2*-/- mice showed long crypt formation with increased number of CD44 positive undifferentiated cells, indicating that disruption of TGF-β signaling induces invasion of tumor glands through suppression of differentiation (Figure 1). Moreover, macrophages infiltrated to microenvironment express MT1-MMP,

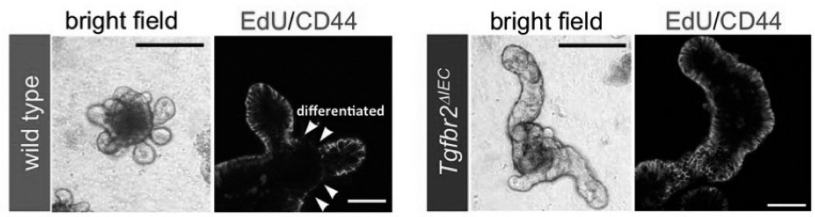

Figure 1 Intestinal epithelial cells from X-ray irradiated Tgfbr2-/- mice developed long crypt-like organoids in Matrigel with increased number of EdU labeled/CD44 positive cells (right), while wild-type mouse-derived cells formed typical mini crypt structure (left).

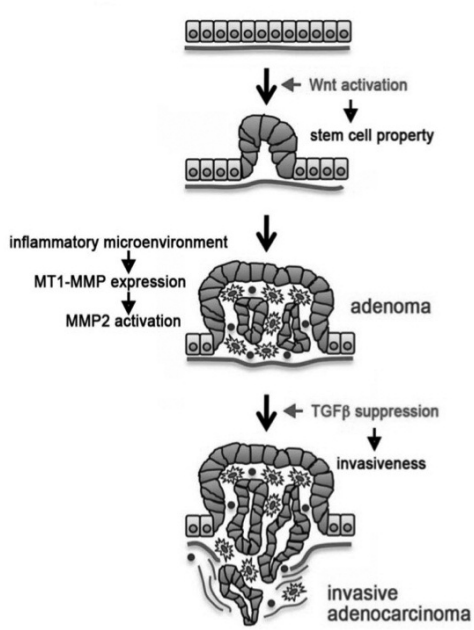

Figure 2 Inflammatory microenvironment activates MMP2 by macrophage-derived MT1-MMP, while TGB-β inhibition induces invasiveness by suppression of differentiation. These process may cooperate to induce submucosal invasion.

resulting in activation of MMP2. It is therefore possible that inflammatory microenvironment supports invasion of TGF-β-suppressed mucosa by degradation of basement membrane component (Figure 2).

About 75% of p53 mutations in cancers are missense type, suggesting a gain-of-function mechanism of mutant p53 for cancer development. Importantly, we found that $Apc^{\Delta 716}$ $Trp53^{R270H}$ compound mice developed invading adenocarcinomas in the intestine, which was not found in $Apc^{\Delta 716}$ $Trp53^{-/-}$ mice [4]. Moreover, mutant p53 expression caused drastic morphological changes of tumor organoids to complex glandular structure (Figure 3), which was associated with acquisition of invasiveness. Consistently, in the human CRC with p53 mutation at R273, we found increased irregular branching compared with p53 wild-type tumors. We also found that mutant p53 expression induces global transcriptome shift, and

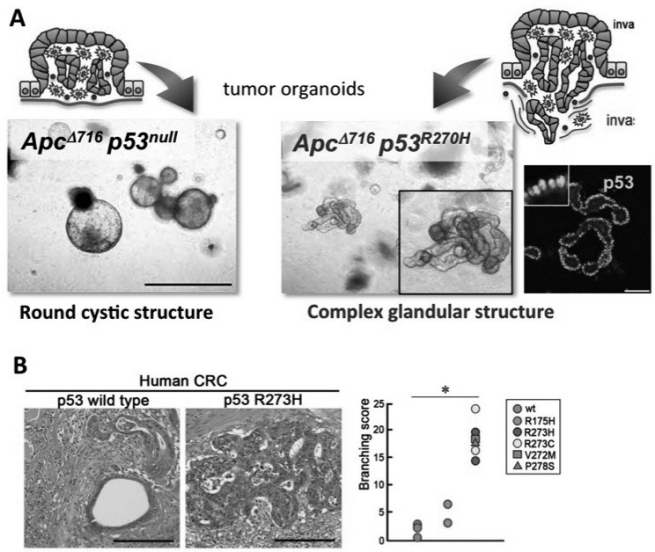

Figure 3 (A) Mutant p53 R270H induces complex glandular structure of tumor organoids by gain-of-function mechanism. (B) Increased irregular branching in human colon cancer with p53 mutation around codon 273 compared with that in p53 wild-type tumors.

ingenuity pathway analysis using expression data indicated that Wnt signaling pathway as well as inflammatory pathways are significantly activated by mutant p53 R270H expression (Figure 4). These results suggest that mutant p53 induces submucosal invasion of intestinal tumors through irregular gland formation possibly caused by Wnt acivation, and mutant p53-induced inflammatory microenvironment generation may also contribute to malignant phenotypes. However, *Tgfbr2* disruption and p53 R270H expression are not sufficient for more advanced stages of the colon cancer.

To further examine the malignant phenotypes induced by multiple driver mutations, we crossed mice carrying genetic alterations in *Apc* (A), *Kras* (K), *Tgfbr2* (T), Fbxw7 (F) and *Trp53* (P) in various combinations [5]. Consistent with the above studies, AT and AP mutations induced submucosal invasion of intestinal tumors. However, additional *Kras* mutation, namely AKT or AKP combination, is required for intravasation and EMT-like morphology in the primary tumors (Figure 5). These results indicate that activation of Wnt and Kras pathways together with TGF-β suppression or mutant p53 expression (AKT or AKP) is a minimum requirement of malignant progression of CRC. Therefore, activated pathways by these combinations will be an effective preventive strategy for CRC progression.

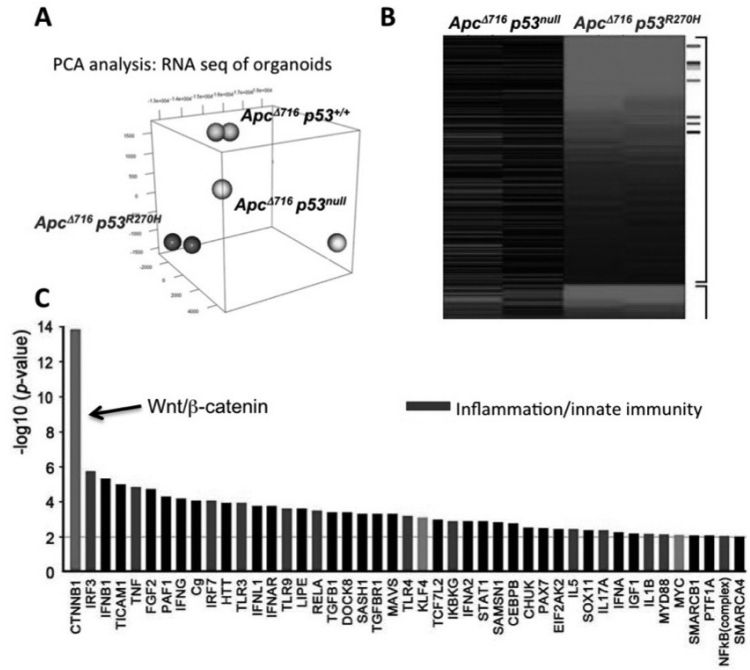

Figure 4 (A) PCA analysis of expression profiles of intestinal tumor organoids with different p53 status. (B) Differentially expressed genes in mutant p53 tumor organoids. (C) Significantly activated pathways by mutant p53 expression.

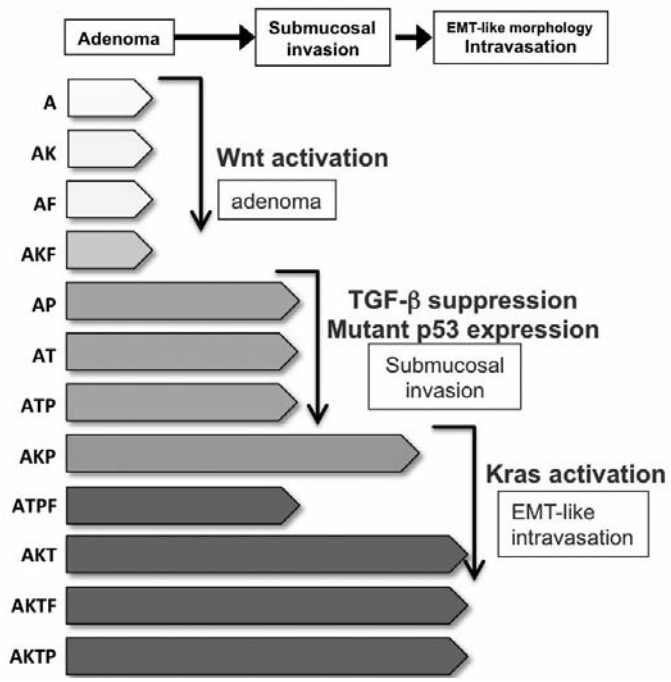

Figure 5 Schematic drawing of genotype-phenotype relationship in the mouse primary intestinal tumors.

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CELL PLASTICITY IN COLORECTAL CARCINOGENESIS

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The tumor microenvironment potentially impacts on different aspects of tumor stem cell biology. Different subpopulations of tumor stem cells might be able to interconvert in response to environmental triggers. Microenvironmental signals influence the de-differentiation process of transformed cells into cancer stem cells. Furthermore stromal-derived factors can lead to the activation of a transdifferentiation program resulting in epithelial-to-mesenchymal transition (EMT) and metastatic spread. We have recently developed a new mouse model of invasive colon cancer that closely recapitulates late stage human CRC [1]. Mice with an IEC-restricted deletion of p53 ($Tp53^{\Delta IEC}$ mice) that are repetitively challenged with the pro-carcinogen azoxymethane (AOM) develop invasive cancer in the distal colon within three months. In about 30 % of $Tp53^{\Delta IEC}$ mice lymph node metastases can be detected after 5-6 months. Interestingly, p53 controlled invasion does not depend on well-described downstream functions of p53 such as apoptosis, cell cycle control or genomic stability but rather on the development of an NF-κB dependent inflammatory microenvironment supporting the induction of EMT. Loss of IKKβ in IEC significantly blocks tumor invasion in this model. Interestingly, loss of IKKβ in myeloid cells does not decrease the incidence of p53-deficient invasive tumors, but instead it reduces the size of invasive lesions and decreases the proliferation rate of invading epithelia. This is paralleled by a diminished paracrine activation of STAT3 in invading tumor cells due to lower expression levels of STAT3 activating cytokines in recruited IKKβ-deficient myeloid cells. Strikingly, mice with myeloid specific IKKβ deletion are completely protected from lymph node metastases. The interleukin (IL)-6 family of cytokines is defined by the shared use of the gp130 receptor β-subunit. Engagement of the gp130 receptor by either IL-6 or IL-11

induces transient activation of Janus kinases (JAK) and the latent transcription factor STAT3. To determine the contribution of Stat3 signaling to the development of lymph node metastases in AOM-challenged *Tp53*$^{\Delta IEC}$ mice, we generated intestinal epithelial cell (IEC) specific knockout mice of the common IL-6 cytokine family receptor β subunit gp130 (*gp130*$^{\Delta IEC}$) and crossed these to *Tp53*$^{\Delta IEC}$ mice. Expectedly, the loss of gp130 in IEC substantially decreased the formation of lymph node metastases. However, this was not accompanied by a loss of Stat3 activation in p53 deficient IEC, suggesting alternative gp130 independent activation of Stat3 in these tumor cells.

Over the past 30-40 years basically every review on the role of reactive oxygen species (ROS) in cancer states that ROS can induce DNA damage and mutations. It had been suggested that during chronic inflammation increased ROS production may cause mutations. Myeloid cells are a major source of ROS in acute and chronic inflammation as well as in tumor induced inflammation. Surprisingly - and despite the firm statement in a huge number of reviews - nobody has ever shown and formally proven using a genetic model that myeloid derived ROS indeed can caute to enhanced tumor progression. TNFuse mutations in neighboring epithelial cells *in vivo*. Moreover, it has even been speculated that mutagenesis is not a direct consequence of myeloid derived ROS but rather an effect of ROS production in epithelial cells in response to pro-inflammatory cytokines such as TNFα. Using relevant genetic models we have now systematically addressed this question.

We used a conditional *Gpx4* knockout in myeloid cells which causes spontaneously increased ROS production in myeloid cells that is comparable to *wt* cells during an inflammatory condition (Figure 1). Importantly, loss of Gpx4 does not affect myeloid cell survival and function allowing us to use these mice in tumor models. We intended to use these mice as a genetic tool to increase ROS production in myeloid cells rather than to decipher GPx4-dependent functions in macrophages during tumor development. We were

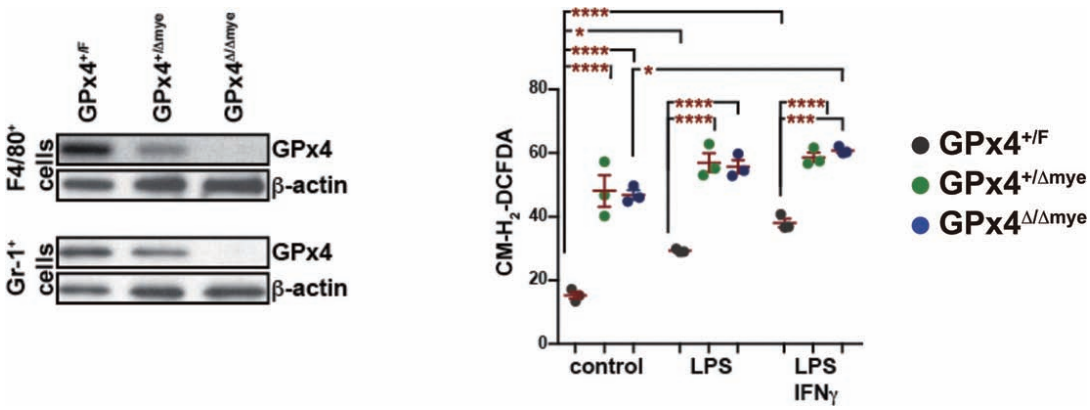

Figure 1 Myeloid specific deletion of Gpx4 (LysM-Cre)

then indeed able to demonstrate that these mice develop invasive cancer in a model of AOM-initiated intestinal tumorigenesis, which is not detected in *wt* animals (Figure 2). Invasive cancer can even be found when *Gpx4* is deleted from myeloid cells in established tumors using a newly developed tamoxifen-inducible LysM-CreERT2 mouse. Mechanistically we could show that increased mutational load in IEC is dependent on myeloid derived H_2O_2 rather than cytokine mediated effects in IEC (Figure 3, 4). Nevertheless, we provide evidence that also cytokines contribute to enhanced tumor progression. TNFα induces a feed-forward loop that culminates in enhanced macrophage recruitment and therefore increased ROS levels in tumors (Figure 5). Importantly, however,

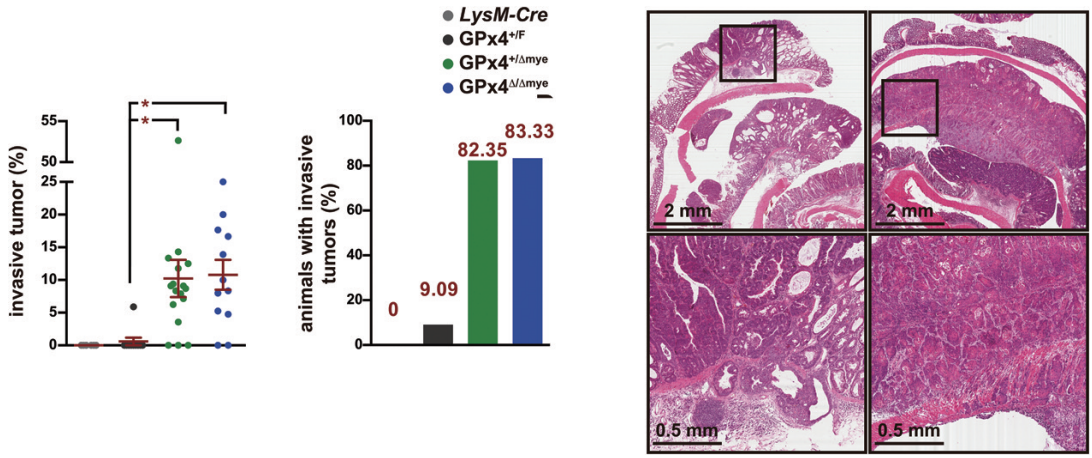

Figure 2 Loss of Gpx4 in myeloid cells promotes tumor invasion

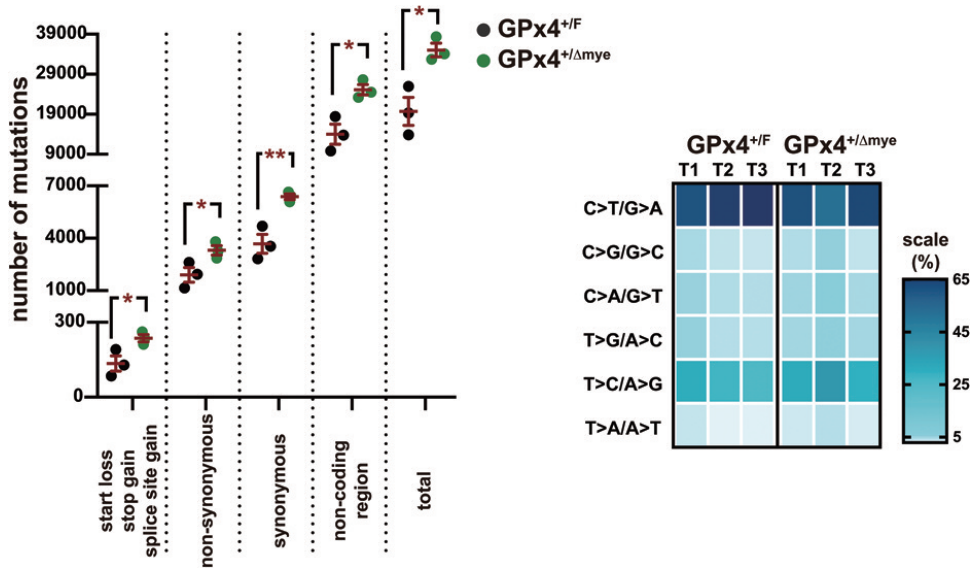

Figure 3 Increased mutational load in the absence of myeloid Gpx4

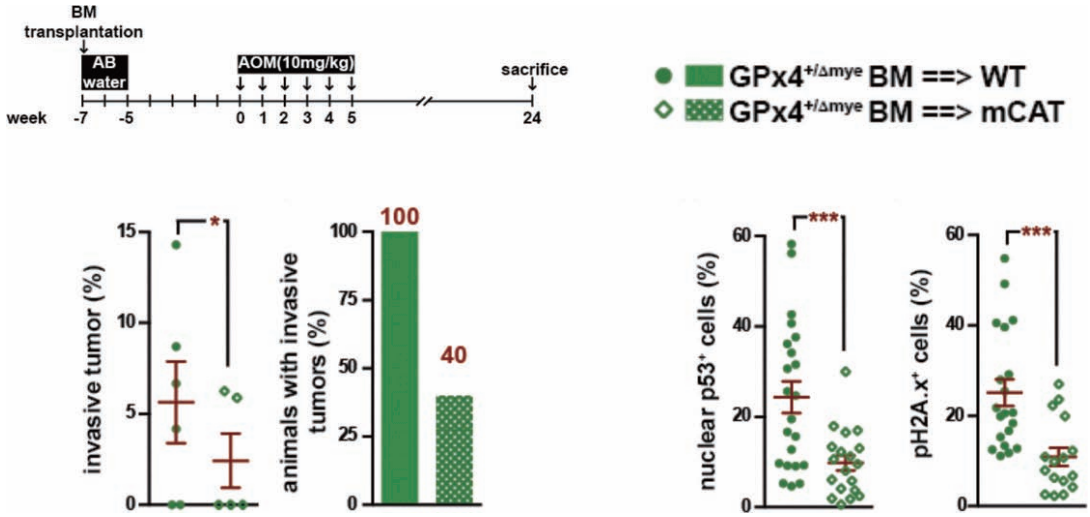

Figure 4 Catalase can block invasion

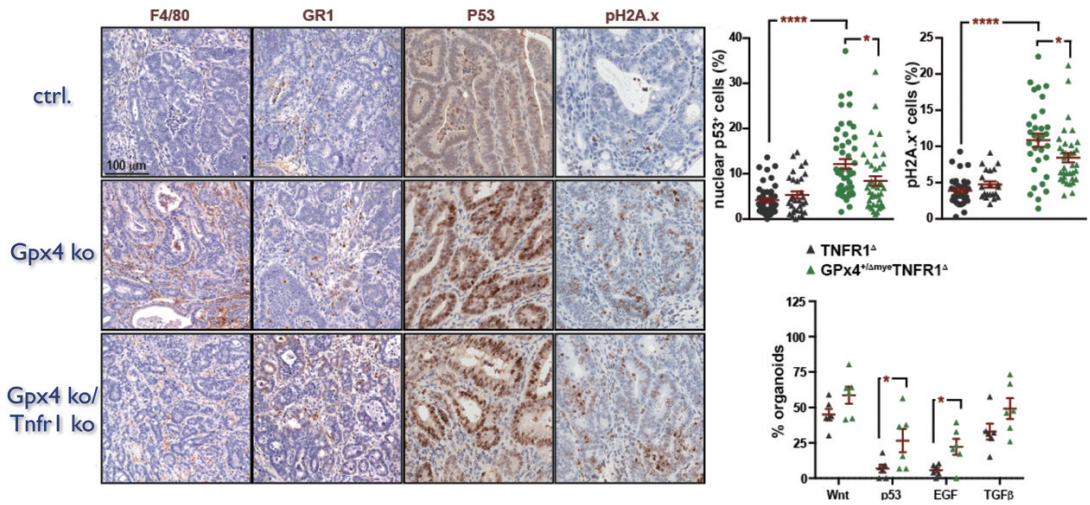

Figure 5 Loss of Tnfr1 does not affect mutational load

TNFα is not directly involved in DNA damage. We complemented our *in vivo* data with extensive *ex vivo* analysis of intestinal organoids and we can impressively demonstrate that repetitive treatment of *wt* organoids with H_2O_2 (but not TNFα) leads to the formation of a large number of mutations (verified by whole exome sequencing) closely mimicking the *in vivo* situation (Figure 6, 7). Importantly, we could further demonstrate that *Gpx4*-deleted mice develop tumors in a model of chronic inflammation whereas *wt* animals do not in the same time (Figure 8) and most importantly that *Gpx4* ko mice spontaneously develop tumors at various locations, lung being the most frequently affected organ (Figure 9).

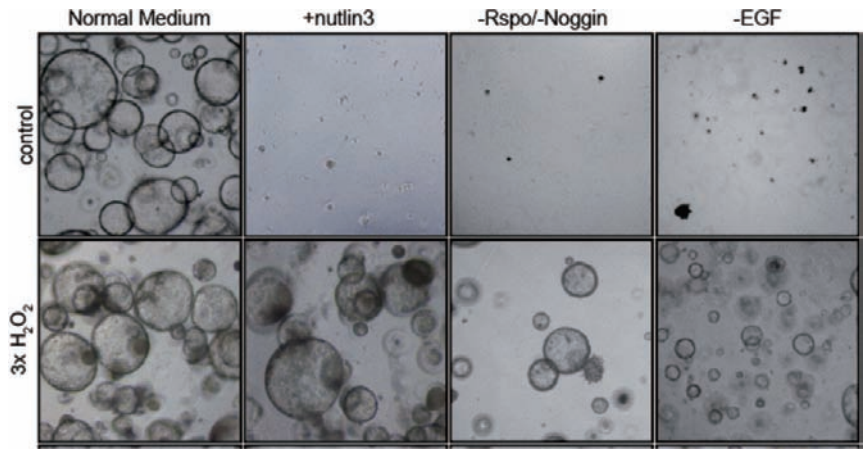

Figure 6 Repetitive H_2O_2 treatment induces mutations in organoids

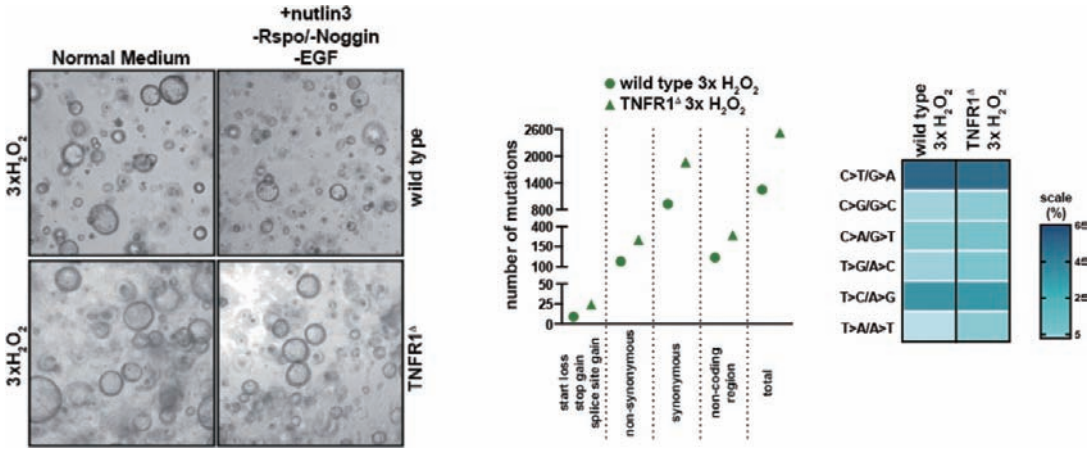

Figure 7 Loss of Tnfr1 does not prevent mutations upon repetitive H_2O_2 treatment

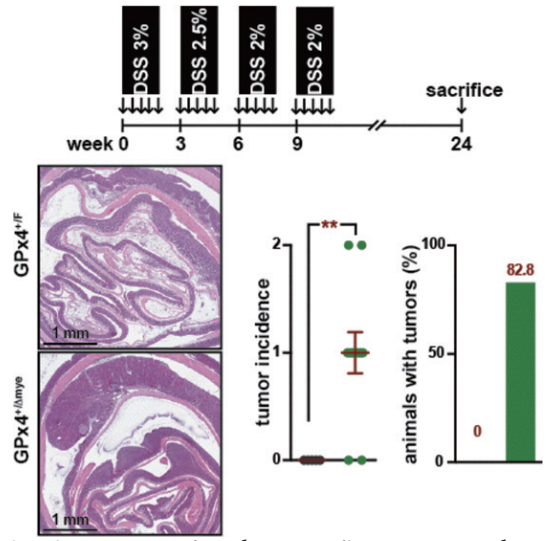

Figure 8 Gpx4^{Dmye} mice develop tumors after chronic inflammation without AOM

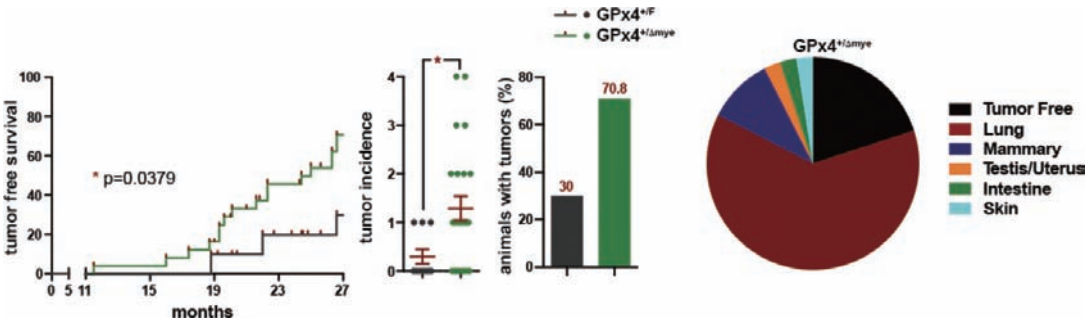

Figure 9 Spontaneous tumorigenesis in mice with myeloid Gpx4 loss

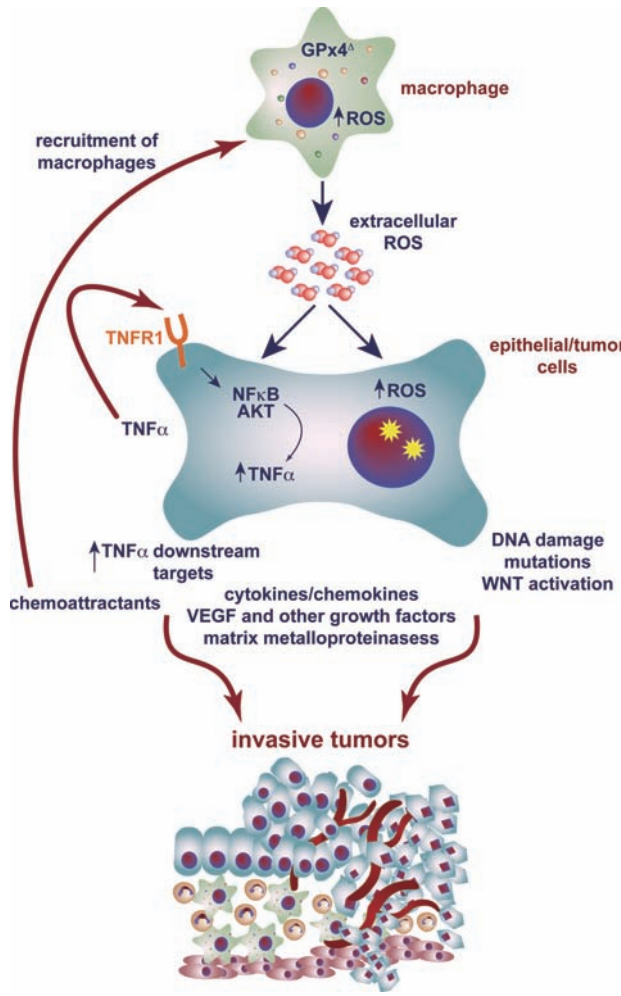

Figure 10 Summary

In summary, our study [2] actually proves for the very first time that chronic inflammation (=increased ROS production in myeloid cells) can initiate cancer and does not only contribute to tumor promotion and progression by shaping a pro-tumorigenic cytokine milieu in the tumor microenvironment. Moreover, we provided a detailed molecular mechanism and could distinguish ROS-dependent events from cytokine mediated effects: we could rule out a direct role of TNFR-dependent signaling, MyD88-dependent signaling as well as RAGE-dependent signaling for epithelial mutagenesis (Figure 10).

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TUMOUR-STROMA INTERACTIONS IN BREAST CANCER PROGRESSION

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The interaction of tumour cells with stromal cells and the extracellular matrix in the tumour microenvironment not only promotes tumour progression and metastasis (Figure 1), but also influences response to chemotherapy, endocrine therapy and targeted agents). As a consequence, there is an urgent need to identify strategies to efficiently target these interaction pathways for the prevention or suppression of metastatic disease and to overcome treatment-resistant progression in advanced breast cancer. We use a combination of *in vivo* RNAi screening, profiling of stromal cells in the tumour microenvironment, and *in vivo*, *ex vivo* and *in vitro* assays, particularly 3D *in vitro* co-culture assays, to identify key determinants promoting breast cancer metastasis.

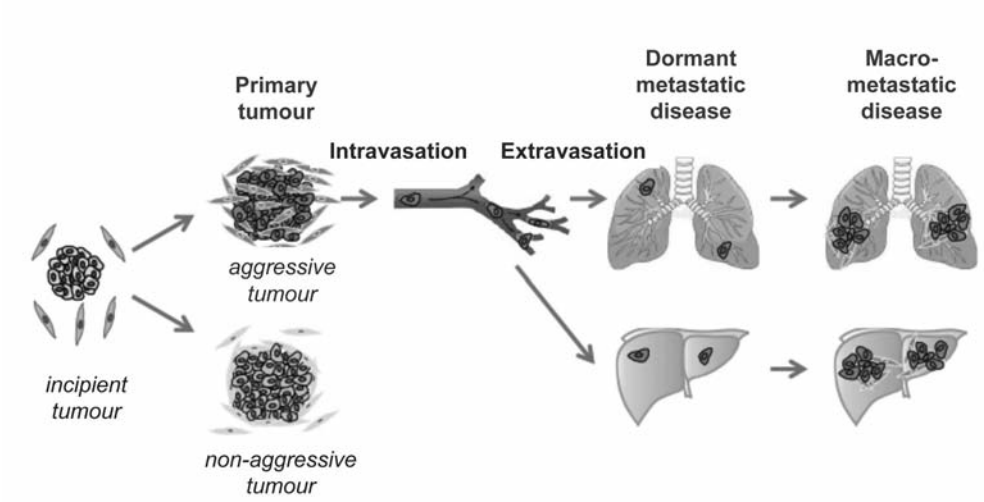

Figure 1

As examples of these approaches, we developed an in vivo shRNA screen to identify novel metastasis suppressor genes. A top hit in this screen was the sialyltransferase ST6GalNAc2 [1]. Mechanistically, ST6GalNAc2 silencing alters the profile of O-glycans on the tumour cell surface, facilitating binding of the soluble lectin galectin-3. This then enhances tumour cell retention and emboli formation at metastatic sites leading to increased metastatic burden, events that can be completely blocked by galectin-3 inhibition (Figure 2). Critically, elevated ST6GALNAC2 expression significantly correlates with reduced frequency of metastatic events and improved survival in human patients.

Subsequently, we have adapted this shRNA screening strategy to identify modulators of chemotherapy response. Chemotherapy remains the mainstay of treatment for advanced breast cancer; however, resistance is an inevitable event for the majority of patients with metastatic disease. Moreover, there is little information available to guide stratification of first line chemotherapy, crucial given the common development of multidrug resistance. From this approach, we identified JNK signalling as a key modulator of chemotherapy response. In agreement with the published literature, we demonstrated that JNK inhibition both promotes tumour cell cytostasis and blocks activation of the proapoptotic protein Bax, thereby antagonizing chemotherapy-mediated cytotoxicity [2]. To investigate the clinical relevance of this dual role of JNK signalling, we developed a proliferation-independent JNK activity transcriptional signature. Consistent with the dual role of JNK signalling, high-level JNK pathway activation is associated both with poor outcome in untreated patients and is predictive of enhanced chemotherapy response. These data highlight the potential of monitoring JNK activity as early biomarker of response to chemotherapy and emphasize the importance of rational treatment regimes, particularly when combining cytostatic and chemotherapeutic agents.

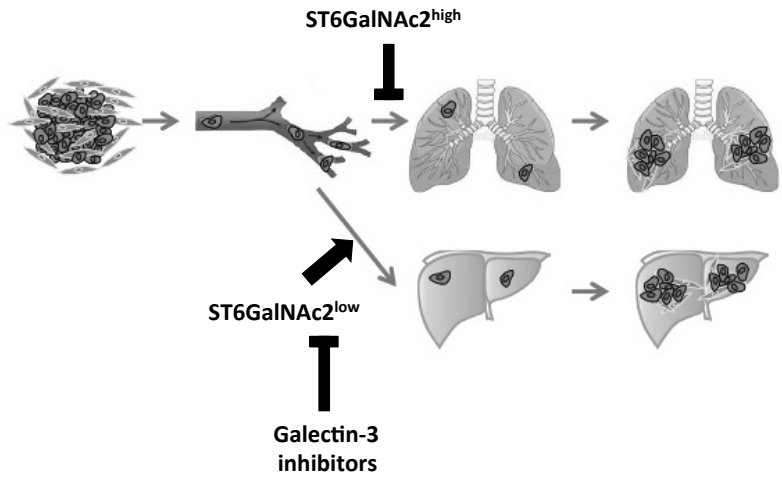

Figure 2

Although these studies provide valuable information as to how tumour cells modulate their response to the microenvironment and to therapeutics, they do directly address a key feature of breast cancers, namely 'how do the cancer cells drive the recruitment and activation of stromal cells?' and 'what are the molecular mechanisms driving stromal heterogeneity in breast cancers'. By profiling tumour cells and fibroblasts directly isolated from aggressive and non-aggressive mouse mammary tumours, we identified Wnt7a as a key factor secreted exclusively by aggressive breast tumour cells, which induces cancer-associated fibroblast (CAF) recruitment and activation [3] (Figure 3). Functionally, this results in extracellular matrix remodelling to create a permissive environment for tumour cell invasion and promotion of distant metastasis. Mechanistically, Wnt7a-mediated fibroblast activation is not dependent on classical Wnt signalling. Instead, we demonstrate that Wnt7a potentiates TGFb receptor signalling both in 3D in vitro and in vivo models, thus highlighting the interaction between t wo of the key signalling pathways in development and disease. Importantly, in clinical breast cancer cohorts, tumour cell Wnt7a expression correlates with a desmoplastic, poor-prognosis stroma and poor patient outcome.

Finally we have used genetically engineered mouse models to address how stromal cell activation promotes metastatic dissemination. Within the stroma of the primary tumour stroma, pericytes associated with the blood vessels and myofibroblasts share a number of traits, including the upregulated expression of the transmembrane receptor endosialin (CD248). By implanting mouse mammary cancer cells into wild-type and endosialin-deficient mice, in collaboration with Helmut Augustin's laboratory, we revealed that stromal endosialin does not affect primary tumour growth but strongly promotes spontaneous metastasis [4]. Mechanistically, endosialin-expressing pericytes in the primary tumour facilitate distant site metastasis by promoting tumour cell intravasation in a cell contact-dependent manner, resulting in elevated numbers of circulating tumour cells

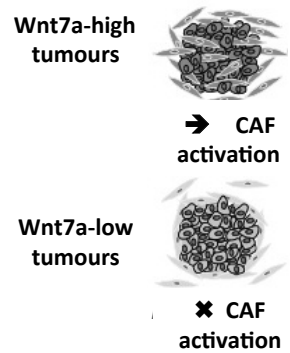

Figure 3

(Figure 4). Corresponding to these preclinical experiments, in independent cohorts of primary human breast cancers, upregulated endosialin expression significantly correlates with increased metastasis and poorer patient survival.

Our current studies are focused on (a) understanding the different functional roles played by activated and non-activated cancer-associated fibroblasts, and how these different fibroblasts interact with both tumour cells and other stromal cell populations, (b) the mechanisms by which a productive microenvironmental niche is initiated at metastatic sites, and (c) the mechanisms by which dormant breast cancer cells at secondary sites become reawakened leading to late metastatic relapse.

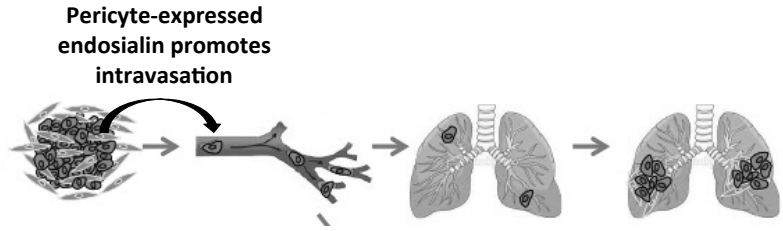

Figure 4

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THE ROLE OF SASP IN TUMOR MICROENVIRONMENT OF OBESITY-ASSOCIATED LIVER CANCER

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Obesity has become a worldwide health problem, and is known to increase a risk of several types of cancers. We previously identified that the enterohepatic circulation of the obesity-induced Gram positive gut microbiota metabolite, deoxycholic acid (DCA) induce the cellular senescence and senescence-associated secretory phenotype (SASP) of hepatic stellate cells, a phenotype that senescent cells secrete inflammatory cytokines, chemokines, proteases, growth factors and so on. Accumulating evidence has indicated that SASP is regulated by a combination of several transcription factors, epigenetic regulators and metabolic pathways, in response to DNA damage. DCA can create DNA damage by elevating the levels of reactive oxygen species (ROS), thereby inducing cellular senescence and the SASP to promote obesity-associated HCC. However, the trigger that initiates the SASP signals may vary depending on the physiological status, and thus, the precise mechanisms regulating the expression of SASP factors need to be elucidated in each biological and pathophysiological setting.

One of the mechanisms that may trigger the SASP could be Toll-like receptor (TLR) signaling. Previous *in vivo* studies have demonstrated that TLR4-mediated inflammatory signals induced by the Gram-negative bacterial component lipopolysaccharide (LPS), which is categorized as a pathogen-associated molecular pattern (PAMP) protein from the gut microbiota, are important for promoting liver fibrosis and fibrosis-associated liver tumorigenesis. However, in our obesity-associated HCC model, Gram-positive bacteria, but not Gram-negative bacteria, were found to be dramatically increased in obese mice fed an HFD, consistent with previous reports. Moreover, we did not observe the reduction of obesity-associated liver tumor formation in TLR4-deficient mice as compared with those in

wild-type mice when we performed the same protocol of neonatal DMBA treatment with HFD, suggesting that TLR4-mediated signals are unlikely to be involved in the acceleration of HCC development in our experimental setting.

Here, we show that the hepatic translocation of obesity-induced lipoteichoic acid (LTA), a Gram positive gut microbial component, promotes HCC development by enhancing SASP factor expression of hepatic stellate cells through Toll-like receptor 2 collaboratively with DCA. LTA also upregulated the expression of cyclooxygenase-2 (COX-2). Interestingly, COX-2-mediated prostaglandin E_2 (PGE_2) production suppresses the antitumor immunity in the tumor microenvironment, thereby contributing to HCC progression. Moreover, COX-2 overexpression and excess PGE_2 production were detected in HSCs in human HCCs with non-cirrhotic, non-alcoholic steatohepatitis (NASH), indicating that a similar mechanism could function in humans.

We showed the importance of gut-liver axis in obesity-associated HCC. Gut microbiota-driven COX-2 pathway produced lipid mediator, PGE_2 in the senescent HSCs in the tumor microenvironment which plays a pivotal role to suppress antitumor immunity, suggesting that PGE_2 and its receptor could be novel therapeutic targets for non-cirrhotic NASH-associated HCC.

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STROMAL CELLS: ESSENTIAL REGULATORS OF TUMOR PROGRESSION AND METASTASIS AND TARGETS FOR THERAPY

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Breast cancer is the leading cancer affecting women worldwide and metastatic breast cancer accounts for over 400,000 deaths per year. Metastatic burden is the main cause of mortality related to breast cancer. Myeloid cells are critical regulators of breast cancer tumor growth and metastatic progression and can have either a pro- or anti-tumoral phenotype [1-3].

Tumor-infiltrating inflammatory cells comprise a major part of the stromal microenvironment and support cancer progression by multiple mechanisms. High numbers of tumor myeloid cells correlate with poor prognosis in breast cancer and are coupled with the angiogenic switch and malignant progression. However, the specific roles and regulation of heterogeneous tumor myeloid populations are incompletely understood. CSF-1 is a major myeloid cell mitogen, and signaling through its receptor CSF-1R is also linked to poor outcomes. To characterize myeloid cell function in tumors, we combined confocal intravital microscopy with depletion of CSF-1R–dependent cells using a neutralizing CSF-1R antibody in the mouse mammary tumor virus long-terminal region-driven polyoma middle T antigen breast cancer model [2]. The depleted cells shared markers of tumor-associated macrophages and dendritic cells (M-DCs), matching the phenotype of tumor dendritic cells that take up antigens and interact with T cells. We defined functional subgroups within the M-DC population by imaging endocytic and matrix metalloproteinase activity. Anti–CSF-1R treatment altered stromal dynamics and impaired both survival of M-DCs and accumulation of new M-DCs, but did not deplete Gr-1+ neutrophils or block doxorubicin-induced myeloid cell recruitment, and had a minimal effect on lung myeloid cells. Nevertheless, prolonged treatment led to delayed tumor

growth, reduced vascularity, and decreased lung metastasis. Because the myeloid infiltrate in metastatic lungs differed significantly from that in mammary tumors, the reduction in metastasis may result from the impact on primary tumors. The combination of function alanalysis by intravital imaging with cellular characterization has refined our understanding of the effects of experimental targeted therapies on the tumor microenvironment.

There is growing evidence indicating that neutrophils have an important role in tumor development—from the inception of tumor formation and throughout the malignant progression [3]. Neutrophils both promote and prevent tumor progression. The contradictory role of neutrophils in tumor development may be explained be the fact that neutrophils can have both pro- or anti-inflammatory phenotypes. Depending on their phenotype, neutrophils are either antitumoral (N1) or protumoral (N2). The occurrence of N1/N2 neutrophils has only been shown in murine tumor models and will have to be confirmed more importantly, in humans. However, neutrophils with an activated phenotype, producing pro-inflammatory factors and stimulating T cell proliferation have been described in early human lung cancer, while immunosuppressive neutrophils has been reported in human colorectal cancer. Tumor-associated neutrophils (TANs) with a N1 phenotype have a pro-inflammatory cytokine profile, producing nitrate oxide and hydrogen peroxide (H2O2), and are cytotoxic towards tumor cells. N2 TANs, on the other hand, are characterized by high levels of arginase 1 (ARG1) and by their ability to inhibit effector T-cell functions. Our understanding of the polarization of neutrophils and how neutrophils contribute to tumor development has largely been elucidated from experimental mouse models.

Importantly, some breast cancer patients never develop metastatic breast cancer. The identification of a monocyte, macrophage and/or neutrophil subpopulation that prevents metastatic breast cancer could provide the backbone for the development of novel immunotherapeutic target molecules. We have shown that production of atypical T cell-suppressive neutrophils occurs during early tumor progression, at the onset of malignant conversion and that these cells preferentially accumulate in peripheral tissues, but not in the primary tumor, leading to metastasis [3]. Production of these cells results from activation of a myeloid differentiation program in bone marrow (BM) by a novel mechanism in which tumor-derived G-CSF directs expansion and differentiation of hematopoietic stem cells to skew hematopoiesis toward the myeloid lineage. With patient-derived xenograft (PDX) models, and large breast cancer tissue microarrays, we have revealed that tumor-derived cytokines may also inhibit metastatic progression through the recruitment of anti-tumoral activated monocytes.

Fibroblasts also regulate the microenvironment, producing the extracellular matrix as well as chemokines that recruit myeloid cells [4,5]. Increased collagen expression in tumors is associated with increased risk of metastasis, and triple negative breast cancer (TNBC) has the highest propensity to develop distant metastases when there is evidence of central fibrosis. Transforming growth factor-β (TGF-β) ligands regulated by cancer-associated fibroblasts (CAFs) promote accumulation of fibrosis and cancer progression. We have evaluated TNBC tumors with enhanced collagen to determine whether we can reduce metastasis by targeting the CAFs with Pirfenidone (PFD), an anti-fibrotic agent as well as a TGF-β antagonist [4]. In patient-derived xenograft models, TNBC tumors exhibited accumulated collagen and activated TGF-β signaling, and developed lung metastasis. We derived primary CAFs from 4T1 TNBC homograft tumors, TNBC xenograft tumors and tumor specimens of breast cancer patients. CAFs promoted primary tumor growth with more fibrosis and TGF-β activation and lung metastasis. We then examined the effects of PFD in vitro and in vivo. We found that PFD had inhibitory effects on cell viability and collagen production of CAFs in 2D culture. Furthermore, CAFs enhanced tumor growth and PFD inhibited the tumor growth induced by CAFs by causing apoptosis in the 3D co-culture assay of tumor cells and CAFs. In vivo, PFD alone inhibited tumor fibrosis and TGF-β signaling but did not inhibit tumor growth and lung metastasis. However, PFD inhibited tumor growth and lung metastasis synergistically in combination with doxorubicin. Thus, PFD has great potential for a novel clinically applicable TNBC therapy that targets tumor-stromal interaction.

Thus, the microenvironment contributes critically to drug response via regulation of vascular permeability and innate immune cell infiltration [6,7]. Clinically applicable therapy that targets tumor-stromal interaction may have important actions in reducing or preventing breast cancer progression.

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DEVELOPMENT AND MAINTENANCE OF THE STEMNESS OF COLON CANCER

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Cancer is composed of heterologous cells, and its heterogeneity is largely attributed to the cellular hierarchy generated by cancer stem cells (CSCs) or related cells, which are likely to be responsible for the refractory nature of cancer such as chemo-resistance or metastatic traits. Here we focused on mechanism of generation and maintenance of CSCs.

Lgr5 is one of the most representative markers of stem cells in various normal tissues, including the intestine. The importance of *Lgr5* as a stem cell marker can be extended to colon tumors. In fact, *Lgr5* likely serves as a hallmark of cancer stem cells (CSCs) not only in adenoma but also in advanced colon cancer. Therefore, it is likely that *Lgr5* stem cells or related progenitors, in normal tissues, after oncogenic mutations and exposure to tumorigenic insults, develop into tumorigenic stem cells, or CSCs.

To obtain a dynamic picture of how CSCs are generated from non-tumor epithelial cells during colon carcinogenesis, we performed gene expression analyses at the single cell level in a mouse model of inflammation-induced colon cancer. Apc(Min/+) mice were treated with dextran sulfate sodium to generate colon tumors, and the epithelial cells from tumors and non-tumors were used for multiple-gene qPCR and RNA-seq analyses at the single-cell level. Statistical analyses of the qPCR data indicated that *Lgr5*-positive stem cells were stratified into several groups, and comparison of the cells obtained from tumor and non-tumor tissues revealed the emergence of a novel *Lgr5*-positive stem cell group in tumor epithelia [1].

This tumor-specific group of *Lgr5*-positive cells showed enhanced tumorigenicity and gene expression profiles that are related to CSCs, including the activation of Wnt signaling. Functional analyses by CRISPR)/Cas9-mediated gene knockout revealed that, among the

Wnt targets that were specifically expressed in the tumor-specific stem cells, the long isoform of Tcf1 (Tcf1-LF) was required for the proliferation of colon tumor organoids. In fact, Tcf1-LF was required for a unique Wnt target gene expression profile. Furthermore, Tcf1-LF expression increased at an early stage of colon carcinogenesis and was associated with the nuclear accumulation of β-catenin, underscoring the importance of the induction of Tcf1-LF expression for the generation of colon cancer stem-like cells (Figure 1) [1].

We also identified several new genes that were specifically expressed in the tumor-specific stem cells, and their functional characterization is currently underway. To further investigate the roles of the identified CSC-related factors in full-blown human cancer, we examined their expression patterns in mouse xenograft models derived from an *in vitro* spheroid culture from surgical specimens of colorectal cancer, which was described below.

In addition to mouse models of inflammation-associated colon cancer, we studied tumor-derived human spheroids to study CSCs. Using the *in vitro* 3D culture (spheroid culture) from human colon cancer and serous ovarian cancer, we isolated and expanded CSCs (Figure 2). Through the transplantation of the in vitro cultivated CSCs into immuno-compromized NOG mice, we established *in vivo* models of colon and ovarian cancer (PDX models) [2,3]. By applying single-cell gene expression analyses, intra- and inter-heterogeneity of xenograft tumors were examined.

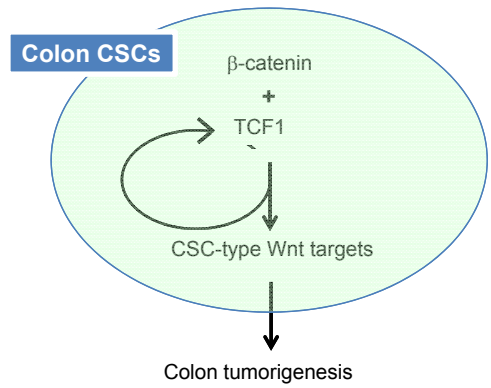

Figure 1 the generation of colon CSCs via the induction of TCF1-LF leads to colon tumorigenesis

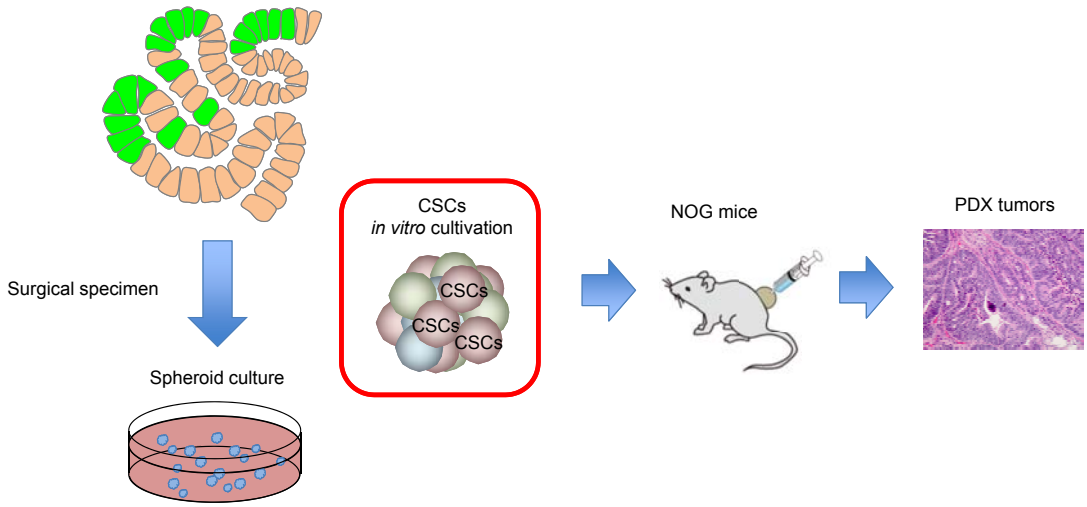

Figure 2 isolation and expansion of colon CSCs as spheroids and establishment of PDX models

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UNEARTHING MECHANISMS OF MALIGNANT PROGRESSION AND RESISTANCE OF CANCER STEM CELLS

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Adult tissue stem cells have the ability to self-renew long term and differentiate into one or more tissues. Many stem cells are used sparingly to replenish cells during normal homeostasis. However, even stem cells that are quiescent must be able to respond quickly to injury in order to fuel rapid tissue regeneration. How stem cells balance self-renewal and differentiation is of fundamental importance to our understanding of normal tissue maintenance and wound repair. Increasing evidence suggests that the regulatory circuitry governing this balancing act is at the root of some types of cancers.

The hair follicle is an excellent model system to understand how stem cells remain quiescent during times of minimal wear and tear, how these cells become mobilized during the cyclical bouts of hair growth and during the re-epithelialization of the epidermis that occurs in wound-repair, and how the normal process of stem cell activation goes awry in cancer. Hair follicles have the remarkable capacity to undergo cycles of growth (anagen), degeneration (catagen) and rest (telogen). In mice, the first several cycles are synchronized, making hair follicles an ideal system for understanding how stem cells interact with progeny and heterologous cell types in their niche to transition between quiescence and active tissue (hair) regeneration.

The quiescent hair follicle stem cells reside in a single layer of the bulge niche, located at the bottom of the non-cycling portion of the hair follicle. Within the bulge niche is a layer of BMP-expressing differentiated cells that anchor the hair and transmit inhibitory signals to maintain stem cell quiescence [1]. The stem cells adhere to a basement membrane flanked by a dermal sheath. Beneath the bulge is a mesenchymal stimulus called the dermal papilla. Stem cells at the base of the bulge, closest to this stimulus are the first to be activated at the

start of the hair cycle [2]. These cells communicate with the dermal papilla. As Valentina Greco, a former postdoc in my lab, now at Yale, showed, the 'primed' stem cells make increasing levels of WNTs; the dermal papilla makes increasing levels of BMP inhibitory signals, and together these two signals are needed to overcome the inhibitory BMP signal that otherwise keeps the stem cells in quiescence [3-6].

When the activating threshold is reached, the stem cells at the base of the bulge begin to proliferate and generate short-term progeny called matrix cells. The first group of these cells produces Sonic Hedgehog (SHH). As shown by Ya-Chieh Hsu, a former postdoc in my lab, now at Harvard, showed, SHH then acts in two ways: first it acts on the dermal papilla, which elevates BMP inhibitors and pro-activating FGFs; second, it acts on the quiescent upper bulge stem cells, which self-renew and produce a cellular shaft (the outer root sheath) which pushes the SHH-DP signaling center downward, resulting in a return of the bulge to quiescence until the next hair cycle [7]. Meanwhile the signaling center fuels the proliferation of the short lived progeny to produce the hair and its channel, the inner root sheath. After a period of hair growth, the lower two thirds of the hair follicle differentiates and degenerates, bringing the DP upward to make contact again with the bulge. In this way, the hair follicle becomes self-propelling to undergo these cyclical bouts of activity.

Over the years, we developed methods to purify the bulge stem cells and their progeny and we carried out RNA-seq on these populations straight from fluorescence activating cell sorting (FACS). The stem cells expressed high levels of SOX9, LHX2, NFATc1, FOXC1, IDs, TCF3 and TCF4 transcription factors, which distinguished them from their committed counterparts and from the epidermal stem cells residing in the basal layer of the epidermis [4,5,8-13]. We used conditional knockout technology to ablate each of these factors in the skin. Our findings showed that some factors, e.g. NFATc1, IDs and FOXC1, acted downstream from BMP signaling, and promoted stem cell quiescence. We found that TCF3/4 antagonize WNT-beta catenin to repress HF fate differentiation, while LHX2 suppresses sebaceous gland differentiation and SOX represses epidermal differentiation. In carrying out a combination of in vivo chromatin immunoprecipitation and high throughput DNA sequencing (ChIP-seq) with antibodies against not only the transcription factors but also epigenetic marks including H3K27ac, Med1, H3K27me3, H3K4me1, H3K4me3 and H3K36me2, we identified ~350 HFSC genes which are controlled by large open chromatin domains, or 'super-enhancers,' [10,14-18] a word coined by Rick Young at MIT. Within these super-enhancers are small regulatory elements, which we call 'epicenters,' that contain clustered binding sites for the cohort of HFSC transcription factors. In work carried out by two of my graduate students, Rene Adam and Hanseul Yang, we've cloned these regulatory elements and tested them in mice and found that they act to target reporter gene expression to the bulge stem cells [18].

In looking at the endogenous group of HFSC genes regulated by super-enhancers, we found that they encode the majority of the stemness genes, including the genes regulating SOX9, TCF3/4 and LHX2 as well as Integrins alpha 6 and beta4, LGR5, BMP6 and the receptors for BMPs, WNTs and FGFs. There are many other human disease genes that are found on this short-list comprising ~5% of the total number of genes expressed by HFSCs.

Intriguingly, when HFSCs were cultured, their chromatin landscape changed dramatically as a new cohort of super-enhancers were gained while others were lost. In seeking mechanisms, we discovered that many HFSC transcription factor genes were silenced in vitro while new wound-induced transcription factors appeared. Moreover, the new super-enhancers were regulated by these wound-induced factors, corresponding to the new proliferative migratory state of the cultured hair follicle stem cells. Interestingly, when HFSCs become activated to re-epithelialize the epidermis, they undergo a fate switch in which they turn off the expression of SOX9, LHX2, NFATc1 and other HFSC transcription factors and switch on the expression of KLF5, ELK3 and other epidermal transcription factors. In transit, however, they enter a state where they express certain features of both HFSCs and epidermal SCs (EpdSCs). We refer to this state as "lineage infidelity" [19]. In the work of Yejing Ge, a postdoctoral fellow in my laboratory, we show that lineage infidelity is a transient but essential transitionary state that both EpdSCs and HFSCs undergo during wound repair. Intriguingly, lineage infidelity is also seen during malignant progression, and in SCCs, it becomes permanent, a feature which we trace to very high levels of phosphorylated ETS2.

Published in *Cell* earlier this year, this work gave us new insights into the sensitivity of stem cells to their microenvironment and the importance of niche-stem cell interactions in dictating stem cell behavior. Hanseul Yang along with Daniel Schramek, a former postdoc in my lab now at the Lunefeld Cancer Center in Toronto, then asked the question of how the chromatin landscape changes as hair follicle stem cells acquires the mutations that lead to malignant progression. When they carried out ChIP seq analyses on the tumor-initiating (stem) cells of squamous cell carcinoma chromatin, they unearthed new super-enhancers regulating oncogenes such as Src and Myc. These super-enhancers were regulated by a new cohort of transcription factors whose genes were also regulated by super-enhancers, thereby generating a feed forward loop. Notably were ETS2 and ELK3, because these super-enhancer regulating transcription factors are known to be phosphorylated and activated by RAS-MAPK, the pathway upregulated in the SCCs. Indeed, mice engineered to express a phosphomimic ETS2, the epidermis became hyperproliferative, invasive and generated a chromatin landscape that was similar to the SCC state [20]. Again, these findings underscored the importance of a now new microenvironment characteristic of the SCC state. Moreover, in our recent study on lineage infidelity, Yejing cloned and tested

these strongly ETS2 sensitive regulatory elements and identify one that becomes specifically activated upon malignant transformation [19]. This new driver now provides a new tool with which to probe deeper into the nature of SCCs in the future.

In addition to exploring oncogenic regulation at the chromatin level, we've used *in vivo* ribosomal profiling to look into translational controls on the oncogenic state. In work performed by Ataman Sendoel, a current postdoctoral fellow in my laboratory, we've learned that global protein synthesis is downregulated in EpdSCs when they activate a single oncogene, in this case, ectopically expressed *Sox2* [21]. We were intrigued by our discovery that a small cohort of mRNAs are still efficiently translated in this condition, and their go-terms suggest that they encode cancer promoting proteins. We performed an RNAi screen to assess the relative importance of various translational initiation factors and ribosomal proteins on WT versus oncogenic stem cells. Surprisingly, the canonical initiation complex is more important for WT than for oncogenic stem cell growth. The reason turns out to be that the canonical initiator Eif2α is phosphorylated and repressed in SOX2-expressing EpdSCs. Under these conditions, the non-canonical EIF2A can compete for translation. It turns out to be important, as without EIF2A, SCCs fail to be sustained. We published this work earlier this year in *Nature.*

Finally, we looked at how the microenvironment changes during malignant progression. We discovered that wherever blood vessels invade the stroma and approach the tumor, the tumor-initiating cancer stem cells at the tumor-stroma interface respond to TGF-beta brought in by the perivasculature, rich in immune cells such as monocytes. The outcome is striking—these stem cells become slow-cycling and break down the basement membrane and invade the stroma. By contrast, stem cells more distant from blood vessels proliferate rapidly, generating the bulk of the tumor [22]. Does this matter? To test this possibility, we treated mice with cisplatin, the drug of choice for many human squamous cell carcinomas. Most of the tumor cells died, except for those cancer stem cells responding to TGF-beta. Moreover, these stem cells evaded the chemotherapy and regrew the cancer when cisplatin was washed out. This was the work of Naoki Oshimori, who has now moved on to start his own lab at Oregon Health Science Cancer Center [23]. The studies beautifully underscore the importance of heterogeneity in the tumor microenvironment.

In another recent twist on the impact of the microenvironment, we turned to exploring the role of stem cells in the memory that skin exhibits when it receives an inflammatory assault e.g. from a microbial infection, irritant or wound. We learned that epidermal stem cells, which experience an inflammatory assault and are present long after the pathology has returned to normal, are able to retain a memory of inflammation. Upon a secondary assault, e.g. wounding, the epidermis repairs the wound quicker than naïve skin. We show that this memory is not dependent upon B or T cells or macrophages, but rather is exhibited

at the chromatin level. The EpdSCs retain ~2000 open chromatin domains that act as inflammation sensors. Many of the genes associated with these domains are activated quickly upon receiving a secondary assault. This provocative work is that of Shruti Naik, a postdoctoral fellow in my laboratory, and Samantha Larsen, a graduate student, and was published in *Nature* in 2017 [24].

I don't want to leave you with the notion that genetic variation in tumor progression doesn't matter. Clearly, it does, as there are hundreds of mutations in these solid tumors. To this end, we've used our powerful in utero lentiviral technology [25] to carry out in some cases, genome-wide screens for oncogenic drivers [26], tumor suppressors [27] and oncogenic microRNAs involved in SCC progression [28]. Together, these multipronged approaches should be useful in our ever growing endeavors to understand the underlying basis for squamous cell carcinomas, which are not only one of the most common but also one of the most life-threatening cancers world-wide.

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ROLE OF ACTIN DYNAMICS IN REPROGRAMMING AND CELL FATE DETERMINATION OF CANCER AND MICROENVIRONMENTAL CELLS

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Cancer-associated fibroblasts (CAFs) are cellular components in the cancer stroma and considered to contribute to progression and survival of cancer cells due to its plasticity. CAF is characterized as an α-smooth muscle actin (α-SMA) positive cell and speculated to originate from not only resident fibroblast but also various cells such as adipocytes, epithelial cells, endothelial cells, bone marrow derived mesenchymal stem cells and hematopoietic stem cells. Among them, we have special interest in adipocytes because, in a subset of tumors, growth and metastasis occur as a result of the adipocyte-rich microenvironments. Cancer cells are known to stimulate lipolysis of adipocytes, leading to delipidation and acquisition of a fibroblast-like phenotype in adipocytes. This transition of adipocytes to CAFs are accompanied by loss of expression of adipocyte terminal differentiation markers and are associated with functional changes in the cells, which support aggressive phenotypes of tumors. However, the molecular mechanism of such adipocyte-CAF transition remains to be elucidated.

We previously reported that inactivation of MKL1, a monomeric G-actin binding transcriptional coactivator, via actin cytoskeleton dynamics drives adipocyte differentiation of multipotent fibroblastic preadipocytes (Nobusue et al., 2014). In addition, we found that the expression of ACTA2 which encodes α-smooth muscle actin is induced by MKL1 activation and is required for the maintenance of the fibroblastic phenotype of preadipocytes. We here investigate the role of MKL1 in the adipocyte-CAF transition. By using Tet-On system, we induced expression of MKL1 in matured adipocytes and found that they lost the expression of adipocyte differentiation markers and became expandable multipotent fibroblastic progenitor cells both in vitro and in vivo. In addition, withdrawal

of doxycycline to suppress the expression of MKL1 did not cause a reversal of the fibroblastic phenotypes, suggesting that MKL1 is a factor for reprogramming adipocytes into a fibroblastic progenitor state. We also demonstrated that MKL1 induction reprograms matured chondrocytes into fibroblastic progenitors.

Given that inactivation of MKL1 potentially suppresses the adipocyte-CAF transition in tumor tissue, we investigated the effect of Rho-kinase inhibitors which prevents nuclear translocation of MKL1 on the tumor formation in mouse osteosarcoma inoculation model (Shimizu et al., 2010). Blocking the adipocyte-CAF transition or promoting adipocyte differentiation in CAFs may provide a novel therapeutic approach for modulating tumor microenvironment.

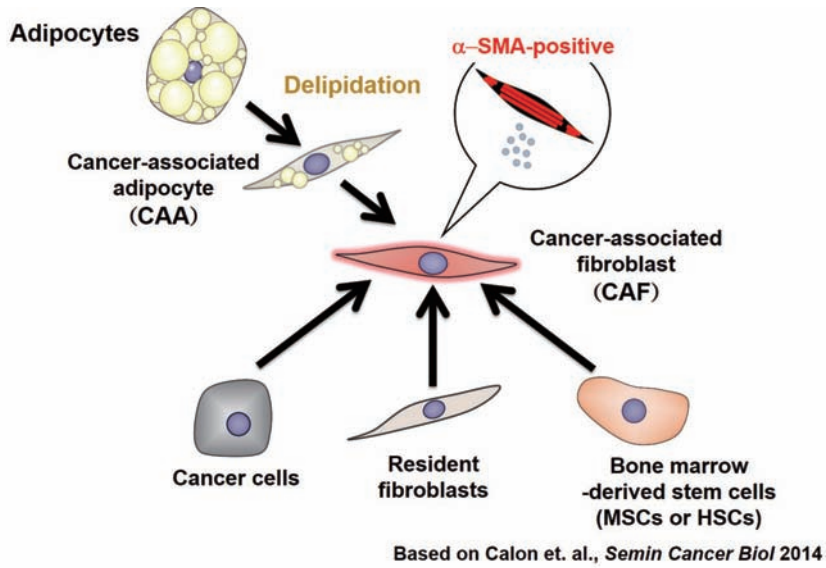

Figure 1 Cancer-associated fibroblasts (CAFs) are converted from various cells. CAFs are plastic and activated fibroblastic progenitor cells characterized by α-SMA expression. Adipocytes and the cancer-associated adipocytes (CAAs) are considered to be major sources of CAFs.

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ROLE OF THE MICROENVIRONMENT IN CANCER INVASION AND THERAPY RESPONSE

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The two most lethal aspects of cancer are its ability to invade into the surrounding tissue and its resistance to chemo- and radio-therapies. We are interested in understanding how communication between cancer cells and stromal cells contributes to these phenotypes. In particular, we will present data on the mechanism of cooperation between cancer cells and stromal fibroblasts (also known as Cancer-Associated Fibroblasts - CAFs). Many studies have identified roles for soluble factors, such as HGF and SDF-1, and exosomes, in cancer cell – CAF crosstalk, but we have focused on the role of the extracellular matrix (ECM) and direct contact in tumour – stroma communication.

Imaging of collectively invading co-cultures of carcinoma cells and patient derived stromal fibroblasts reveals that the leading cell is always a fibroblast, and that carcinoma cells move within tracks in the extracellular matrix behind the fibroblast. By using spheroid and organotypic invasion assays, we show that the generation of these tracks by fibroblasts is sufficient to enable the collective invasion of the squamous cell carcinoma (SCC) cells and requires both protease- and force-mediated matrix remodeling [1]. This study emphasizes the importance of matrix remodeling by stromal fibroblasts for the promotion of local invasion.

Many tumors show an initial response to targeted therapies before genetic resistance emerges, however little is known about how tumor cells tolerate therapy before genetic resistance dominates. Building on our knowledge of how fibroblasts can remodel the ECM to promote invasion, we show how the ECM can also generate a 'safe haven' in which melanoma cells can tolerate targeted therapy [2]. The Ras-MAPK pathway is mutated in the majority of melanoma, with BRAF mutations being most common. Tumors with BRAF

mutations show an initial response to BRAF inhibitors before genetic resistance emerges. However little is known about the spatio-temporal response to BRAF inhibitors *in vivo* and how this relates to the failure of targeted therapy. We have used both intravial ratiometic FRET and FLIM of an ERK/MAP kinase biosensor to investigate heterogeneity in signaling in both murine syngeneic and human melanoma models. We have longitudinally monitored responses to targeted therapy and identified areas that become refractory to drug action. BRAF mutant melanoma cells can rapidly become tolerant to PLX4720 in areas of high stroma. The rapid kinetics of this process indicate that it is not caused by genetic events. We demonstrate that PLX4720 has an unexpected effect on the melanoma-associated fibroblasts, leading to enhanced matrix remodeling. The remodeled matrix then provides signals that enable melanoma cells to tolerate PLX4720. Blocking these signals using a FAK inhibitor greatly enhances the efficacy of BRAF inhibition in several pre-clinical models. We propose that this safe haven enhances the population of cancer cells from which genetically resistance emerges by supporting a population of cancer cells from which genetically resistance emerges.

We have recently uncovered a novel mechanism of tumor – stroma cross-talk involving a pathological heterotypic cell-cell contact. Our early imaging studies had revealed a close physical association between fibroblasts and cancer cells [1]. This was intriguing because in normal skin, epithelial cells and fibroblasts do not contact because they are separated by a basement membrane. However, a signature feature of tissue damage and invasive squamous cell carcinoma is breakdown of the basement membrane; in this context, epithelial cells and fibroblasts can contact each other. In collaboration with Xavi Trepat in Barcelona, we now show that CAFs exert a physical force on cancer cells that enables their collective invasion. Force transmission is mediated by a heterophilic adhesion involving N-cadherin at the CAF membrane and E-cadherin at the cancer cell membrane. This adhesion is mechanically active; when subjected to force it triggers β-catenin recruitment and adhesion reinforcement dependent on α-catenin/vinculin interaction. Impairment of E-cadherin/N-cadherin adhesion abrogates the ability of CAFs to guide collective cell migration and blocks cancer cell invasion. N-cadherin also mediates repolarization of the CAFs away from the cancer cells. In parallel, nectins and afadin are recruited to the cancer cell/CAF interface and CAF repolarization is afadin dependent. Furthermore, heterotypic junctions between CAFs and cancer cells are observed in squamous cell carcinoma, but not adjacent normal mucosa. Taken together, these findings show that a mechanically active heterophilic adhesion between CAFs and cancer cells enables cooperative tumor invasion [3].

We have now extended our analysis of heterotypic cancer cell – CAF junctions to investigate downstream changes in gene expression. This has revealed that heterotypic

contact triggers dramatic changes in chemokine, cytokine, and other inflammatory modulators triggering anti-microbial and anti-viral responses. We propose that this represents a tissue level damage sensing mechanism analogous to molecular DAMPs. We speculate that heterotypic epithelial – fibroblast contact originally evolved to promote wound healing and prophylactically trigger host defense to infection, but that these become co-opted by cancer enabling invasion and modulating therapy responses.

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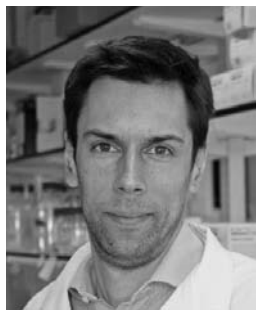

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CHALLENGES FOR COMPLEXITY OF CANCER BY SUPERCOMPUTER – FROM GENOME TO NETWORK

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The drastic cost down of sequencing has been accelerating the world-wide production of cancer omics data, e.g. TCGA (The Cancer Genome Atlas), ICGC (International Cancer Genome Consortium). It is now usual to sequence and analyze several hundreds of samples for one study. This situation made us face with cancer big data whose analysis should require large-scale computational resources (high performance computing, high-speed massive storage, data transfer, etc.) and new big data analysis methodologies. For example, US NIH "The Big Data to Knowledge Program" (B2K) and NCI "High Performance Computing Program" have been building the infrastructure and services for biomedical big data. This is a common public policy in leading countries.

In Japan, we have been using two supercomputer systems for cancer research. One is the supercomputer SHIROKANE at Human Genome Center of University of Tokyo (http://hgc.jp/english/). It is designed for life science applications, especially, sequence data analysis. The total computation power at peak is 550TFLOPS generated by two types of computation nodes (thin node: 5GB memory/core, fat not: 2TB memory/node). The 30PB Lustre File System enables fast data analysis for big data. It processes 4 million jobs/month. The other is "K computer" at RIKEN Advanced Institute of Computational Science (http://www.riken.jp/en/research/labs/aics/). Its computing power 10PFLOPS is generated by 705,024 CPU cores on 88,128 nodes that are interconnected as a torus fusion structure (similar to 3D Möbius band). The combined use of these two supercomputers creates an extraordinarily super computational platform for cancer omics data analysis.For these supercomputers, we developed and implemented various computational methods for Cancer Systems Biology from genome to network.

The first is genome data analysis. There are several well-established variant calling pipelines, e.g. GATK (https://software.broadinstitute.org/gatk/), the EMBL workflow for structural variation, the DKFZ workflow for SNVs, indels, and copy number (https://github.com/ICGC-TCGA-PanCancer/DEWrapperWorkflow), etc. Independently of these pipelines, our open source pipeline Genomon (https://github.com/Genomon-Project/) has been developed in collaboration with Dr. Seishi Ogawa (currently, Kyoto University School of Medicine) for blood cancer. It is realized as a suite of bioinformatics tools for analyzing cancer genome data (WGS, WES, RNA-seq). It enables us to perform very sensitive and accurate detection of most types of genomic variants (single nucleotide variants, short indels, mid-size indels and large scale structural variations), and transcriptomic changes (gene fusions, aberrant splicing patterns). It adopts an efficient job scheduling framework that realizes easy analysis of several hundreds of genome and transcriptome sequencing data simultaneously. The first contribution of Genomon was the discovery of aberrations of splicing machinery in myelodysplasia [1]. Then several tens of contributions came out by Genomon pipeline using supercomputers, including clonal hematopoiesis in aplastic anemia [2]. Detection of ITD (internal tandem duplication) is also in the scope of Genomon [3]. One of recent advancements of Genomon is the ability of accurate detection of mid-size (10bp-1000bp) indels which is considered as a "blind spot" of Illumina short read sequencing. It played an essential role in the study of adult T cell leukemia/lymphoma (ATL) that was directed by Dr. Seishi Ogawa [4]. 426 ATL patient samples were analyzed (48 WGS, 81 WES, 57 RNA-seq, 109 methylation analyses, 343 samples for validation) and the study revealed comprehensive understanding how/where HTLV-1 virus damages human genomes and leads to ATL. K computer helped WGS data analysis and SHIROKANE did other data analysis. Major driver alterations are found in TCR/NFκ-B pathway, T cell trafficking and other T cell-related pathways as well as immunosurveillance. Within them, Genomon found 2,857 structural variations from 48 WGS sequence data and recurrent intragenic deletions causing exon skipping in *IKZF2*, *CARD11*, *TP73*. Notably, Genomon identified structural variations (SVs) in the 3'UTR region of *PD-L1* for 27% WGS samples that is located at the immune checkpoint. Gene expressions of the samples with these SVs were found high. Then, 10,210 samples (mostly RNA-seq data) of 33 tumor types from TCGA were analyzed with Genomon on SHIROKANE. The data analysis and human investigation revealed SVs in the 3'UTR region of *PD-L1* for 12 tumor types. Through biological validations including genome-editing, a unique genetic mechanism of immune escape caused by SVs commonly disrupting the 3'URT region of *PD-L1* was discovered [5].

The second is computational strategy for unraveling gene networks and their diversity lying over genetic variations, mutations, environments and diseases from gene expression profiles of cancer cells. We developed methods for exhibiting how gene networks vary

from patient to patient according to a modulator, which is any score representing characteristics of cells, e.g. survival, drug resistance [5,6]. SiGN-L1 (http://sign.hgc.jp/signl1/) is network estimation software using sparse learning. It uses L_1-regularization for simultaneous parameter estimation and model selection of statistical graphical models such as graphical Gaussian models and vector autoregressive models. SiGN-L1 also implements the NetworkProfiler [5] that realizes the analysis of individual differences of gene networks with respect to the extra individual index called modulator. Network analysis with NetworkProfiler revealed system changes in epithelial-mesenchymal transition and *KLF5* (Krueppel-like factor 5) is identified as a new regulator of EMT (validated by Dr. Takashi Takahashi, Nagoya University). A further advancement was done by employing adaptive penalized logistic regression with which drug-sensitivity prediction was drastically improved [6]. We also developed a microRNA/mRNA gene network analysis with Bayesian network method that revealed subnetworks with hub genes (miR-30d, miR-195, TTF-1/NKX2-1, etc.) that switch cancer survival from 126 non-small cell lung cancer samples. We implemented the method as a software SiGN-BN that is gene network estimation software using the Bayesian network model and nonparametric regression. It is capable of estimating regulatory dependencies between genes as gene networks from expression data such as individual cell samples, gene knocked-down cell samples, drug-stimulated time series (time course) samples, and so on. SiGN series are implemented both on SHIROKANE and K computer. On-going gene network research is also presented, including a discovery of the first lncRNA, named MYMLR, modulating MYC gene for selective target regulation using K computer (validated by Dr. Takashi Takahashi, Nagoya University School of Medicine). This study analyzed 7988 gene networks for each "modulator" non-coding RNA lung cancer samples from 40 studies. The computational methodology named GIMLET for this study will be published soon [8]. Before K computer, we could not even imagine such large-scale network analysis.

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PATHOLOGY FROM THE MOLECULAR SCALE ON UP

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High parameter single cell analysis has driven deep understanding of immune processes. Using a next-generation single-cell "mass cytometry" platform we quantify surface and cytokine or drug responsive indices of kinase target with 45 or more parameter analysis (e.g. 45 antibodies, viability, nucleic acid content, and relative cell size). Similarly, we have developed two advanced technologies that enable deep phenotyping of solid tissue in both fresh frozen and FFPE formats (50 – 100 markers). We have recently extended this parameterization to mRNA with the capability to measure down to 5 molecules per cell in combination with any other set of previously created markers.

I will present evidence of deep internal order in immune functionality demonstrating that differentiation and immune activities have evolved with a definable "shape". This shape is altered during immune surveillance and "imprinted" during, and after, pathogen attack, traumatic injury, or auto-immune disease. Hierarchies of functionally defined trans-cellular modules are observed that can be used for mechanistic and clinical insights. I will focus upon pathogen attack, traumatic surgical intervention in human, and auto-immune processes for the presentation.

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MICROBIOME, METABOLISM AND MUCOSAL IMMUNITY

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Complex and dynamic interactions exist between the gut microbiome, metabolism, and mucosal immunity to maintain intestinal health. Sufficient perturbations to these interactions contribute to inflammatory and autoimmune diseases. Environmental conditions and diets, for instance, that accompany western lifestyles profoundly impact the composition and function of the gut microbiome, which may lead to breaks in immune tolerance and a "broken circuit" of inflammation in genetically susceptible individuals. The consequences of changes in the gut microbiome are dictated by the mechanisms that sense and respond to dietary and microbial signals. We are utilizing several novel approaches to examine interactions between host and microbes with the goal of understanding the mechanisms by which microbes and their metabolites contribute to mucosal homeostasis and intestinal immunity. Here, we focus on examples of how chemical, human genetic, multi'omic, and computational approaches have contributed to this goal.

We and others characterized short-chain fatty acid (SCFA) sensing and signaling in the intestine [1-2]. Produced by microbial metabolism of dietary fiber, SCFAs are sensed by the G-protein coupled receptor GPR43 and signal to regulate immune responses and protect against inflammation. Butyrate, but not other SCFAs, was shown to inhibit intestinal stem cell proliferation and inflammation. Butyrate is further metabolized by host cells, raising the possibility that a butyrate byproduct was the signaling molecule. Using stem cell proliferation and cytokine production as function assays, we developed a chemical screening approach to test the activity of structurally similar molecules [3]. Our results suggested that butyrate, and not predicted byproducts of butyrate metabolism, is directly involved in the mechanism inhibiting stem cell proliferation and inflammation.

Employing a longitudinal study of human cohorts, we recently tested the hygiene hypothesis, which posits that changes in early microbial exposure lead to altered immune maturation and increased incidence of autoimmune diseases [4]. The incidences of type 1 diabetes and allergy are much higher in Finland and Estonia than in neighboring Russia. Taking advantage of this "living laboratory", the DIABIMMUNE project collected clinical metadata, serum, and monthly stool samples from birth to age 3 of 1,000 children born in these three countries. While the children in the cohort were genetically similar, they were exposed to vastly different living environments. We analyzed microbiome samples to identify early-life environmental triggers of autoimmune and allergic diseases. Our study found that in the first year of life, Bacteroidetes were more common in microbiomes of Finnish and Estonian children, while Actinobacteria were highly abundant in those of Russian children. We determined that the largest functional differences between microbiomes of Finnish and Russian children were in lipopolysaccharide (LPS) biosynthesis pathways. We further demonstrated that *E. coli* was the major contributor to lipid A biosynthesis in all three countries. In Finland and Estonia, Bacteroidetes were also large contributors. Next, we compared the immunogenicity of LPS from these species, finding that LPS from Bacteroidetes is less immunostimulatory compared to LPS from *E. coli*. Structural changes in LPS were shown to influence immunogenicity, suggesting that the more potent *E. coli* LPS contributes to immune education and decreased risk of autoimmune disease in Russian children.

Because IBD is influenced by the composition of and interactions between microbes, the intestinal epithelium, and the immune system, we use multi'omics approaches to integrate multiple factors that contribute to disease [5]. We are part of the group leading the second phase of the Human Microbiome Project (HMP2), an IBD data resource that collects a variety of host and microbial multi'omics data on stool, biopsy, and serum samples from IBD and non-IBD control subjects. By comparing metagenomes and metatranscriptomes, we showed that on average, the functional potential of a microbial species is proportional to its functional activity (that is, DNA levels generally correlate to RNA levels) and that more abundant microbes show a higher DNA to RNA correlation. For example, *P. merdae* was the species with the highest correlation and was detected across Crohn's disease (CD), ulcerative colitis (UC), and non-IBD samples. This indicated that relative abundance of *P. merdae* coincided with increased transcriptional contributions from this species. *D. invisus*, an oral bacterium with similar relative abundance as *P. merdae*, showed the lowest DNA to RNA correlation, suggesting *D. invisus* is not actively transcribing in the gastrointestinal tract. HMP2 data also allows us to contrast metabolic function potential and activity. We identified specialized pathways that are contributed by specific microbes in the contexts of IBD and non-IBD. *F. prausnitzii* almost entirely contributed to the super-pathway of hexuronide and hexuronate degradation across samples. Importantly, metatranscriptomics

allows for the identification of disease-specific changes in microbial transcriptomes. For instance, the *B. vulgatus* L-lysine biosynthesis VI pathway was more abundant on the transcriptional level in IBD samples than non-IBD samples. Our data highlight the importance of analyzing both the abundances and activities of gut microbes through metatranscriptomics. Similar analyses of metabolomics and metagenomics reveal microbial metabolites associated with disease. We identified classes of microbial metabolites that are differentially abundant in IBD and correlated this with species relative abundances.

Finally, we use computational approaches to predict microbial function in maintaining intestinal homeostasis. For example, we hypothesized that identifying gut microbes genetically capable of utilizing mucins for energy might provide insight into which microbes are critical to maintain intestinal homeostasis [6]. Computational analyses identified mucin-utilization genes in metagenomics samples from HMP subjects. These species were shown to robustly utilize mucin as an energy source in functional assays. We demonstrated that one of these species, *Peptostreptococcus russellii*, protects against DSS-induced colitis *in vivo*. Several Peptostreptococcus species contain a gene cluster that allows the microbes to metabolize tryptophan into indoleacrylic acid (IA). We showed that IA promotes intestinal epithelial barrier function and anti-inflammatory responses. A diminished ability of gut microbes to produce IA was found in stool samples from IBD subjects.

We are using these novel approaches to ultimately address challenges in IBD treatment. Currently, anti-integrin therapies produce suboptimal patient responses. In a prospective study with CD and UC patients receiving anti-integrin therapies, we associated specific microbes and their functions with clinical remission [7]. Seven bacteria, including *R. inulinivorans* and a Burkholderiales species, and clinical factors such as smoking status were predictive of outcome. This study demonstrated that microbiome changes occurring early in disease course predict response to treatment.

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IMMUNOGENOMICS IN THE CANCER PRECISION MEDICINE ERA; CHARACTERIZATION OF T CELL REPERTOIRES IN TUMOR IMMUNE MICROENVIRONMENT BY TCR ANALYSIS

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The recent development of high-throughput DNA sequencing technologies has driven the rapid progress in genomics research and enabled us to address a multitude of biological and pathological conditions, such as cancer, autoimmune diseases, and drug-induced toxicities. In addition, the development of cancer immunotherapies, particularly drugs modulating the immune checkpoint molecules, clearly demonstrated the importance of host immune cells in the fight against cancer. However, the molecular mechanisms by which these new therapies kill tumor cells still remain unclear. Hence, we have been proposing the significance of the new filed "immunopharmacogenomiccs" to characterizing the systemic and tumor immune environment including T cell and B cell repertoires by next-generation sequencers. This field has been enormously helpful for better understanding of the changes/alterations of our immune responses during the course of various disease conditions and treatments. I here report the deep sequencing of T-cell receptors that enabled us to capture the immune microenvironment in tumors.

We have analyzed T cell changes in melanoma tumors before and after anti-PD-1 antibody treatment and found that tumors in responders exhibited a substantial increase of *CD8*, *GZMA* and perforin 1 (*PRF1*) expression levels as well as oligoclonal expansion of tumor-infiltrating T lymphocytes (TILs) in the tumor tissues of the responders. In one patient who showed myocarditis, myositis, and myasthenic crisis after one-shot of anti-PD-1 antibody treatment, we identified infiltration of clonally expanded T cell populations in the skeletal muscle, implying the very strong T cell immune response against muscular cells.

Secondly we analyzed tumor microenvironment in 22 kidney cancer tissues before and at 3 months after cryoablation. We found that proportions of abundant TCRB clonotypes (defined as clonotypes with ≥ 1% frequency among total TCRB reads) were significantly increased in the post-cryoablation tissue samples than those of pre-cryoablation tumor samples. Expression analysis of immune-related genes showed significantly elevated transcriptional levels of *CD8*, *CD4*, Granzyme A (*GZMA*), and *CD11c* along with high *CD8*/*FOXP3* ratio in the post-cryoablation tissue samples, suggesting that cryoablation could induce strong immune reactions in tumors with oligoclonal expansion of anti-tumor T cells, which probably circulate systemically.

Furthermore, we collected surgically-resected tumor tissues from five breast cancer and six mesothelioma patients, and characterized 3 different portions of individual tumors through somatic mutation analysis by whole exome sequencing, T cell receptor beta (TCRB) repertoire analysis of tumor-infiltrating lymphocytes (TILs), and found that tumors revealed significant correlation between the number of predicted neoantigens and the diversity of TILs ($P = 0.0009$), suggesting that certain TILs might recognize the cancer-specific antigens including neoantigens derived from non-synonymous somatic mutations of cancer cells.

In addition, to investigate the similarity/difference in TILs in original tumor tissues and those of *in vitro* expanded populations in squamous cell carcinoma of head and neck (SCCHN), we performed whole exome analysis, expression profile analysis of immune-related genes, and T cell receptor (TCR) analysis for TILs in original tumors as well as *in vitro* expended TILs in surgically-resected HPV-negative fresh tumors with SCCHN. Three of eight SCCHN tumors we examined were found to have an unusually high number of non-synonymous somatic mutations (900-4,000 mutations) probably due to mutations in mismatch repair genes, *MSH2* or *MSH4*, or a DNA polymerase gene, *POLE*. Highly frequent TCR clonotypes in expended $CD8^+$ TILs from these three tumors revealed high similarity to those in original tumors. However, for remaining tumors with the lower mutational load, T cell clonotypes between TILs in original tumor tissues and those expanded *in vitro* were almost entirely different. Our findings might provide clinically useful information for identification of tumor-antigen-specific T cell clones.

This kind of immunogenomics analysis is extremely important to uncover the changes in immune microenvironment during the cancer treatment and may improve the clinical outcome of immunotherapy including development of TCR-engineered T cell therapy.

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AT THE CROSS-ROAD BETWEEN THE GUT MICROBIOME AND CANCER IMMUNOSURVEILLANCE

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The tumor microenvironment is influenced by anticancer therapies, and even more so by those affecting the gut homeostasis. We reported that a deviated repertoire of the intestinal microbiome called « dysbiosis », caused by broad spectrum antibiotics, compromised the efficacy of cyclophosphamide (CTX), an immunomodulatory alkylating agent exerting cytotoxic effects against cancer [1].

Cancer immunotherapy is successful against different hematologic and solid tumor entities.

Administration of immune checkpoint inhibitors (ICI) unleash T lymphocyte-mediated immune responses by suppressing the interaction of T cell inhibitory receptors with their cognate ligands on tumor or stromal cells. The most widely used ICI are monoclonal

antibodies (mAb) targeting programmed cell death protein 1 (PD-1) and its ligand PD-L1. PD-1 blockade is highly efficacious against advanced melanoma, non-small-cell-lung cancer (NSCLC), head neck cancer and renal cell carcinoma (RCC).

Recent work in mice has highlighted the key role of the gut microbiota in mediating tumor responses to chemotherapeutic agents and immunotherapies targeting PD-L1 or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) [2].

Therefore, we explored the possibility that dysbiosis in the context of cancer patients could influence primary resistance to PD-1 blockade in tumor bearers.

Antibiotics altered the gut microbiome composition, severely compromising survival of patients diagnosed with stage IV lung and kidney cancer patients in second line therapy [3]. Metagenomic analyses of fecal samples performed by shot gun DNA sequencing of patients stools at diagnosis predicted best clinical response and progression free survival at 3 months to anti-PD-1 mAb in lung cancer patients and best outcome in both cohorts [3]. The commensal bacterium that was most significantly enriched at diagnosis in patients predisposed to respond was *Akkermansia muciniphila*, a mucine consuming metagenomic species associated with lean healthy volunteers and epithelial homeostasis. The fecal presence of *A. muciniphila* was detectable in 69% (11/16), versus 34% (15/44) of patients showing a partial response or primary progression respectively (p=0,007).

Accordingly, preclinical models of dysbiosis induced by broad spectrum ATB in tumor bearing mice or enforced gavage with non-responding patients' stools in germ-free mice led to the loss of animal responsiveness to anti-PD1 Ab therapy while administration of responders feces induced anticancer efficacy of the mAb. Similarly, compensating dysbiotic feces with A. muciniphila restored the capacity of recipient mice to respond to PD1 blockade [3]. Our data suggest that central memory CD4+ T-cells expressing CCR9 and CXCR3 accumulate 48h after PD-1 blockade in mesenteric lymph nodes and later on in tumor beds, accompanied by a loss of Treg infiltration. These antituùor effects of PD-1 blockade in eubiotic animals or in dysbiotic recipients compensated by A. muciniphila were dependent on IL-12p70, a Th1 cytokine [3].

Taken together our data suggest the importance of a healthy gut in the therapeutic efficacy of various anticancer therapies including immune checkpoint blockers, cyclophosphamide or platinum compounds. Restoration of a healthy gut architecture relies on microbial homeostasis. Modulation of the gut microbiome composition in cancer patients holds great promise in resetting the cancer-immune set point and circumventing primary resistance in advanced malignancies. We show compelling preclinical and clinical evidence to propose fecal microbial transplantation or the use of live biotherapeutics for prospective clinical investigations in oncology [4].

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BREAST TUMOR EVOLUTION: HETEROGENEITY AND SELECTION

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The immune system and the tumor microenvironment play key roles in shaping tumor evolution. They can promote the selection for subpopulations of cancer cells and eliminate less fit clones. Immunotherapy is one of the most promising cancer therapies and almost the only one that can effectively cure even distant metastatic disease in a relatively non-toxic manner. However, its success has been limited to a subset of patients. In breast cancer, only ~20% of patients with triple negative breast cancer (TNBC) benefit from immune checkpoint inhibitor (e.g., anti-PD-L1) therapy potentially due to heterogeneity among tumors for mechanisms by which they escape immune surveillance. Escape from immune surveillance is a prerequisite for invasive and subsequently metastatic progression. Thus, we hypothesized that the in situ to invasive carcinoma transition is a critical step for immune escape in breast cancer and understanding mechanisms of this immune escape would facilitate the design of more effective immunotherapies for both early and advanced stage disease. In DCIS (ductal carcinoma in situ) cancer cells are physically separated from the stroma by the basement membrane and the myoepithelial cell layer that form the milk ducts and intra-epithelial leukocytes are rarely detected [1]. In contrast, in invasive and metastatic tumors cancer cells and leukocytes are intermingled, thus, only cancer cells that can evade the immune system and survive in this environment will play a role in disease progression hence shaping subsequent tumor evolution.

To begin dissecting mechanisms of immune escape during early stages of breast tumor progression, we have analyzed the composition of leukocytes in normal breast tissues, DCIS, and invasive ductal breast carcinomas (IDC) of all major breast tumor subtypes (e.g., luminal, $HER2^+$, and TNBC) by polychromatic FACS. We found significant differences in multiple cell types including decreased $CD8^+$ to $CD4^+$ T cell ratio and increased neutrophils

in tumors, and higher fraction of macrophages in TNBC [2]. We have also analyzed the gene expression profiles of T cells from each of these tissue types and identified progression stage-specific differences. We detected an overall decrease in the frequencies of activated granzyme B positive ($GZMB^+$) $CD8^+$ T cells and an increase in suppressor Tregs as tumors progress. Multicolor immunofluorescence of DCIS and IDC, including matched DCIS and locally recurrent IDC cases in the same patient, confirmed a decline in activated $GZMB^+CD8^+$ T cells with invasive progression. We also found that TCR clonotype diversity was higher in DCIS than in IDCs. Interestingly, we detected a few relatively frequent TCR clones that were shared among different DCIS patients, one of which was predicted to recognize a protein from the Epstein-Bar virus.

In order to investigate potential mechanisms underlying the more immune suppressive environment in IDC, we analyzed the expression and copy number of immune checkpoint genes and proteins by immunofluorescence and FISH, respectively. We found high epithelial expression of PD-L1 in triple negative IDC with amplification of *CD274* (encoding PD-L1) observed in 3/10 cases, whereas tumor epithelial cells in DCIS had low expression of PD-L1 and no amplification of *CD274*. These results suggest a selection for breast cancer cells with *CD274* copy number gain during invasive progression. To identify other genomic regions encoding for immune regulatory proteins that may also show differential amplification between DCIS and IDC, we analyzed the copy number of 17q12 chemokine cluster located in close proximity of *ERBB2* (encoding HER2). By analyzing $HER2^+$ tumors in the TCGA, we found that co-amplification of the 17q12 chemokine cluster (CC) with *ERBB2* was enriched in $HER2^+ER^+$ luminal tumors, whereas there was either no

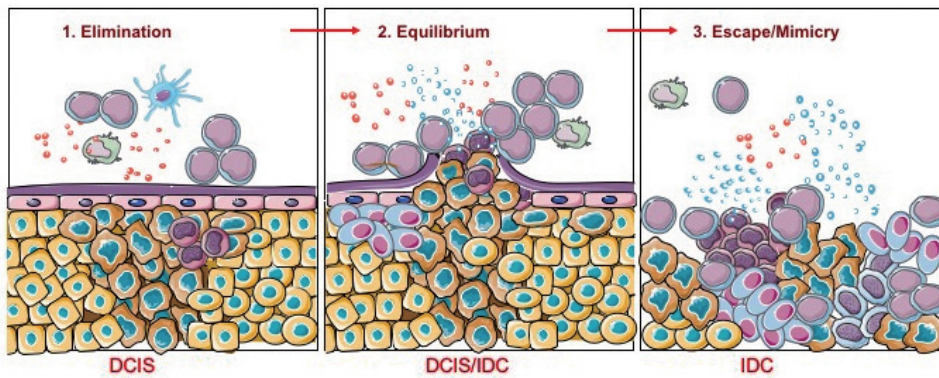

Figure 1 Hypothetical model of immune escape during in situ to invasive carcinoma transition. In DCIS with intact myoepithelial cell layer and basement membrane occasional invasive cells are eliminated by the immune cells keeping tumors in the DCIS stage. In microinvasive lesions there may be an equilibrium between cancer cells and anti-tumor immune response. Tumor cells invading the surrounding tissue have successfully escaped with immune cells either by upregulating immune suppressive mechanisms or loosing neoantigens that trigger a response.

gain but even a loss of the CC cluster region in $HER2^{+}ER^{-}$ breast tumors. Interestingly, we found higher expression of T cell activation and exhaustion-related genes in tumors that lack CC gain. Moreover, when assessing a cohort of $HER2^{+}$ samples by multicolor FISH and immunofluorescence, we found an inverse correlation between CC amplification and frequency of $GZMB^{+}CD8^{+}$ T cells.

Overall our results show co-evolution of cancer cells and the immune microenvironment during tumor progression. Our results demonstrate that analyzing immune-related changes during DCIS to IDC transition will improve our understanding of immune editing in breast cancer, which will aid the design of more effective immunotherapies and selection of patients who are more likely to respond to treatment.

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GENETIC SCREENS TO IDENTIFY IMMUNOTHERAPY TARGETS

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Despite the dramatic clinical success of cancer immunotherapy with PD-1 checkpoint blockade, most patients don't experience sustained clinical benefit, suggesting that additional therapeutic strategies are needed. To discover new immunotherapy targets, we developed a pooled, loss-of-function *in vivo* genetic screening approach using CRISPR/Cas9 genome editing in mouse transplantable tumors treated with vaccination and PD-1 checkpoint blockade. We tested 2,400 genes expressed by melanoma cells for those that synergize with or cause resistance to checkpoint blockade, and recovered the known immune evasion molecules, PD-L1 and CD47. Loss of function of multiple genes required to sense interferon-γ caused resistance to immunotherapy. Deletion of Ptpn2, a pleotropic protein tyrosine phosphatase, improved response to immunotherapy. Cellular, biochemical, transcriptional and genetic epistasis experiments demonstrated that loss of function of Ptpn2 sensitizes tumors to immunotherapy by enhancing interferon-γ-mediated effects on antigen-presentation and growth suppression. Thus, strategies that increase interferon-γ sensing by tumor cells could improve the efficacy of immunotherapy. More generally, *in vivo* genetic screens in tumor models can identify new immunotherapy targets, model resistance mechanisms, and accelerate the rational selection of combination immunotherapy.

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LATENCY, IMMUNE EVASION AND OUTBREAK OF METASTATIC STEM CELLS

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Metastasis frequently occurs long after an apparently successful treatment of a primary tumor. Cancer patients with no clinical evidence of active disease may relapse with distant metastasis months, years or even decades later. Metastasis grows from disseminated cancer cells (DTCs) that remain latent until unknown conditions allow their outgrowth [1]. It is thought that tumors release large numbers of cancer cells into the circulation since early stages of tumor growth, long before diagnosis and treatment. Although a majority of the dispersed cells are eliminated in the circulation or soon after infiltrating distant tissues, a minority may survive as latent seeds in target tissues. People who are clinically considered free of disease after treatment of a primary tumor may still carry thousands of DTCs in the bone marrow and other organs. DTCs survive predominantly in a growth-arrested state that is resistant to cytotoxic and targeted anti-tumor therapies that are effective against proliferating cancer cells. The presence of these pathogenic cells does not represent an existence of metastasis. However, DTCs constitute latent metastasis, which is a major concern in the clinic. A better understanding of the biology of latent metastasis should yield therapeutic strategies for its elimination and the prevention of cancer relapse.

Approaching Metastasis through Mechanistic Biology

We approached the problem metastasis through the analysis of mechanisms that regulate cell behavior and are altered in cancer. Our attention has been focused for many years on delineating the TGF-β pathway and the basis for its contextual action in normal and cancer cells [2]. TGF-β signaling regulates differentiation in embryonic stem cells, lineage identity and cell cycle progression in progenitor cells, death in pre-malignant cells, and metastasis in carcinoma cells. Previously we showed that the TGF-β receptors activate

SMAD transcription factors that directly control lineage identity genes [3]. We recently defined a regulatory network involving the Wnt pathway under the control of p53 tumor suppressor family, and the TGF-β/Nodal pathway [4]. This network drives mesendoderm differentiation of mouse and human embryonic stem cells. *Wnt3* and its receptor *Fzd1* are critical p53 family target genes in this context. WNT3-activated TCF3 and nodal-activated Smad2/3 depend on each other for co-occupancy of target enhancers in mesendoderm differentiation genes. The p53 family coordinates essential WNT-TCF and Nodal-SMAD inputs for mesendoderm differentiation in the early embryo [4].

The role of TGF-β as a regulator of lineage identity and differentiation is also manifest in adult stem and progenitor cells. In adult pancreatic progenitors TGF-β-activated SMAD signaling enforces epithelial identity through induction of SOX4 expression and regulates the cell cycle by inducing CDK inhibitors [5] (Figure 2). However, in mutant KRAS pre-malignant cells, TGF-β triggers an identity crisis by simultaneously inducing SOX4 and Snail, a master effector of the epithelial-to-mesenchymal transition (EMT). This contradictory combination triggers apoptosis and acts as a tumor suppressor mechanism [5] (Figure 2). Tumor progression requires the elimination of the tumor suppressive effects of TGF-β, which pancreatic cells can achieve by mutational inactivation of SMAD4 or TGF-β receptors, or alternatively, by downstream alterations in TGF-β/SMAD signaling that

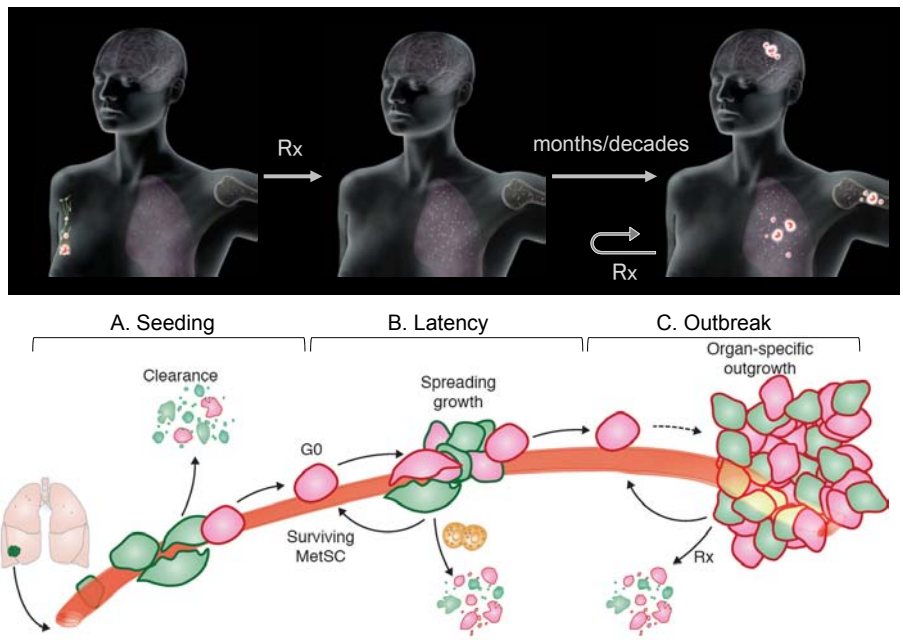

Figure 1 Metastatic colonization steps.
In our working model, cancer cells suffer extensive clearance after extravasation. MetSCs can survive by entering quiescence (G0) or initiate outgrowth by spreading along capillaries. However, growth is inhibited by NK cells and by other defenses. Emergence of immune suppressive clones with organ-specific colonization traits eventually triggers aggressive outbreak. Targeted therapy leaves resistant clones that resort to metastasis re-initiation mechanisms. Our Aims target key mediators of lung adenoCa metastatic colonization.

remove the tumor suppressive effects. Carcinoma cells can then repurpose the TGF-β-SMAD pathway for metastatic dissemination, latency, or outbreak (Figure 2). For example, TGF-β-SMAD signaling stimulates breast cancer cells to express angiopoietin-like 4 for extravasation in lung [6] and interleukin 11 and osteopontin for osteoclast activation in bone [7]. Tumor-derived TGF-β can also act on the stroma to impart immune suppression or to induce the expression of tenascin C and other extracellular matrix proteins that build a metastatic niche [8]. These insights shed light on the role of TGF-β as a key mediator in physiology and disease, and as a potential target for therapy.

Latent Metastasis

Although metastatic progression through the latent stage is a major concern in the clinic, little is known about the identity of latent DTCs and the mechanisms that allow them to remain quiescent, evade immunity, retain tumor-initiating capacity, and evolve into aggressive metastasis [1]. The lack of suitable experimental models has inhibited progress in this area. We recently developed mouse models of latent metastasis using cancer cells from early-stage lung and breast tumors [9]. These models are allowing us to define mechanisms that suppress the outgrowth, support the long-term survival, and enable the eventual outbreak of latent metastatic cells.

Latency-competent lung and breast cancer cells have stem cell-like properties. We refer to these cells as metastatic stem cells (MetSCs) [10] (Figure 1). SOX2 and SOX9 lineage-

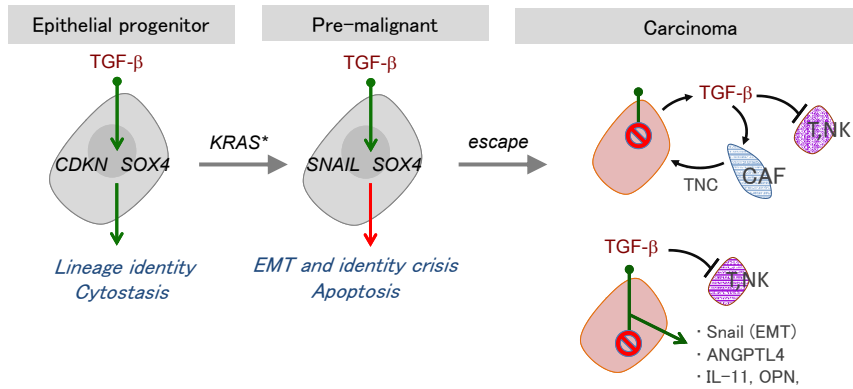

Figure 2 The TGF-β – SMAD pathway in tumor suppression and metastasis.
A basis for the dual role of TGF-β in certain types of cancer was recently elucidated. In pancreatic epithelial progenitors the TGF-β–SMAD signaling pathway regulates lineage identity (e.g. though induction of SOX4 epithelial identity transcription factor) and growth homeostasis (through induction of CDK inhibitors). However, TGF-β is a tumor suppressor for pre-malignant pancreatic progenitors that harbor the obligate oncogene KRAS. KRAS primes these cells for a strong induction of the EMT master transcriptional regulator SNAIL. An EMT occurring side-by-side SOX4 expression in these cells creates an identity crisis of simultaneous pro-mesenchymal and pro-epithelial signals, leading to apoptosis. Oncogenic progression requires the inactivation of this tumor suppressive response by loss of SMAD4 or by downstream loss of apoptotic responses. Once free from TGF-β tumor suppressive responses, carcinoma cells can use the altered TGF-β responsive to their benefit during different stages of metastatic progression.

determining transcription factors impart a progenitor state to MetSCs. These cells reside in perivascular niches and can self-impose a slow-cycling state by avoiding WNT growth stimuli and responding to TGF-β in this context [10] (Figure 3). This state includes the downregulation of including MICA and ULBP family members, membrane proteins that serve as ligands for the receptor NKG2D on NK cells and cytotoxic T cells. Through these and other mechanisms, quiescent metastatic cells cam evade NK cell-mediated clearance.

Latent MetSCs frequently enter the cell cycle, but are cleared by natural killer (NK) cells unless they gain additional immune evasive capacities. We propose that metastatic latency is a highly dynamic stage, in which frequent attempts by disseminated MetSCs to reinitiate outgrowth are thwarted by immune surveillance. Our working hypothesis is that the latent metastatic state does not necessarily involve a long-term permanence of MetSCs in replicative quiescence, but rather involves MetSCs that frequently enter the proliferative state but fail to form a growing colony because NK cells detect and eliminate the incipient growth. Proliferation entails the exposure of MetSCs to recognition and elimination by immune cells, whereas cancer cells that re-enter quiescence are spared (Figure 3). As a

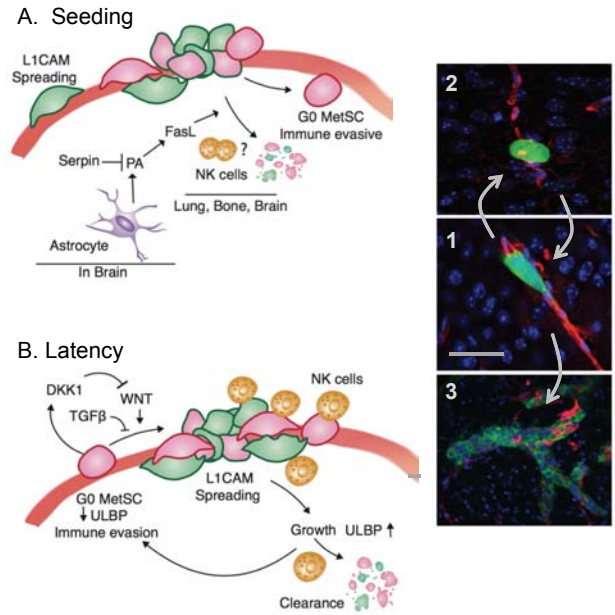

Figure 3 Metastatic seeding and latency.
(A) NK cells and astrocytes clear a majority of infiltrated cancer cells. MetSCs survive by entering immune evasive G0 and, in the brain, by producing serpin inhibitors of plasminogen activator. Inset: MetSCs spread on capillaries after extravasation (1), and enter G0 and round up near capillaries (2), or spread and proliferate along the capillary surface (3); L1CAM mediates spreading and is required for outgrowth. (B) In this model, MetSCs that enter G0 constitute a highly dynamic latent state, with ongoing cycles of growth re-initiation and NK-mediated clearance. Entry into G0 leads to downregulation of ULBPs, ligands for NK and CTL receptors. This cycling would allow selection of organ-specific colonization traits.

corollary, antibody-mediated depletion of functional NK cells in mice immediately allows the accumulation of over metastasis, providing us with a functional assay for the study of regulators of metastatic outbreak. In sum, latency is not created by MetSCs that remain quiescent for long periods but rather by a equilibrium between transiently proliferative disseminated MetSCs and immune effectors that cull MetSCs that reinitiate proliferation.

Organ-specific Metastatic Colonization

Metastatic outbreaks and formation of aggressive metastatic colonies have other requirements besides the evasion of immune surveillance. A requirement that may be relevant for the initiation of metastatic colonization in different organ sites is the spreading of MetSCs on the surface of capillaries. MetSCs that remain in contact with capillaries after extravasation are observed to spread on the abluminal surface of the vessels as a condition for proliferation [11]. The localization of MetSCs in the perivascular niche ensures their access to oxygen, nutrients and paracrine factors. However, spreading on capillaries licences these cells for the initiation of growth in the rich microenvironment (Figure 4). Spreading is mediated by the L1 cell adhesion molecule (L1CAM, also known as CD171). L1CAM-mediated spreading on capillaries provides cancer cells with stimuli that are critical for outgrowth immediately upon infiltration of target organs as well as after exiting from a period of quiescence under conditions of latency. These results identify an obligatory step in the initiation of metastatic colonization, and provide an explanation for the widespread association of L1CAM with metastatic relapse in the clinic.

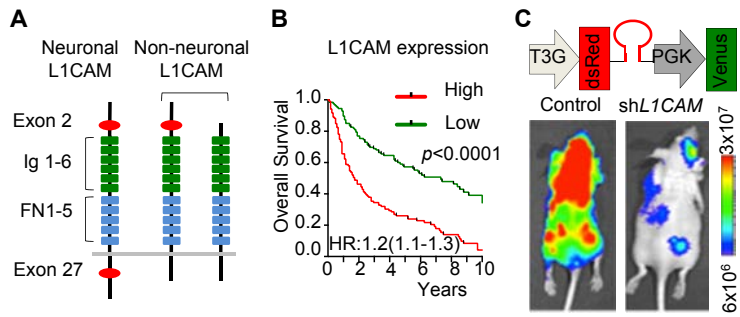

Figure 4 L1CAM and vascular cooption in metastasis.
(A) Schematic representation of neuronal and non-neuronal L1CAM isoforms. Red ovals represent exons 2 and 27, which are spliced out from the non-neuronal isoforms isoform. (B) Overall survival plot for patients with high and low L1CAM expression bifurcated at median L1CAM expression level. High L1CAM expression is associated with poor patient survival. (C) L1CAM is necessary for metastatic outgrowth post extravasation. L1CAM knockdown using a doxycycline-inducible construct (top) 9 days after intracardiac injection of lung adenoCa cells reduces metastatic outgrowth (bottom).

Overt metastasis in different organ involves different mediators of organ-specific colonization. Many such mediators have been identified in recent years that are expressed by the cancer cells or the affected host stroma [1]. For example, osteolytic bone metastasis by breast cancer cells depends on osteoclast activating factors, several of which are induced by stromal TGF-β [7]. It also depends on SRC amplification of CXCR4 signaling to activate the PI3K pathway form cancer cell survival [12,13]. In contrast, colonization of the lungs is enhanced by the expression of monocyte-binding receptors [14] and extracellular matrix proteins that support stem cell survival [8].

We recently focused on metastasis to the central nervous system. Brain metastases occur in 20-40% of advanced stage cancers and represent the most prevalent intracranial malignancy in adults. Current clinical management of brain metastases provides limited disease control and most patients succumb to tumor progression within one year of diagnosis. Recent work has elucidated cellular and molecular interactions responsible for brain metastasis. Circulating cancer cells infiltrate the brain parenchyma by expressing specific mediators of cell passage across the blood-brain barrier (BBB) [15]. The vast majority of cancer cells that infiltrate the brain perish, rejected by the most abundant cell type in the brain, the astrocyte. The astrocyte network, coupled together via gap junctions, serves a protective role in the CNS. In brain metastasis, reactive astrocytes generate the protease plasmin and cytotoxic cytokines. Brain metastatic cells counter this defense with serpin inhibitors of plasminogen activator [11] (Figure 3).

Astrocyte-cancer cell interactions are not uniformly antagonistic. Brain metastases contain abundant reactive astrocytes, and astrocytes can exert a beneficial effect on cancer cell co-cultures. We recently identified a role of astrocytes in promoting brain metastasis [16]. Breast and lung cancer cells express protocadherin 7 (PCDH7) to favor the assembly of carcinoma-astrocyte gap junctions composed of connexin 43 (Cx43). Once engaged with the astrocyte gap-junctional network, brain metastatic cancer cells employ these channels to transfer the second messenger cGAMP to astrocytes for activation of STING, an innate immune response pathway to cytosolic double-stranded DNA (dsDNA) (Figure 5A). STING activation stimulates the production of inflammatory cytokines IFNα and TNF in the astrocytes. As paracrine signals, these factors activate the STAT1 and NF-κB pathways in brain metastatic cells, which in this context support tumor growth and chemoresistance. We tested the orally bioavailable modulators of gap junctions meclofenamate and tonabersat as inhibitors of this paracrine loop, providing proof-of-principle for the applicability of this therapeutic strategy to treat established brain metastasis [16]. We are conducting clinical trials of meclofenamate in patients with relapsing brain metastasis of breast and lung cancers.

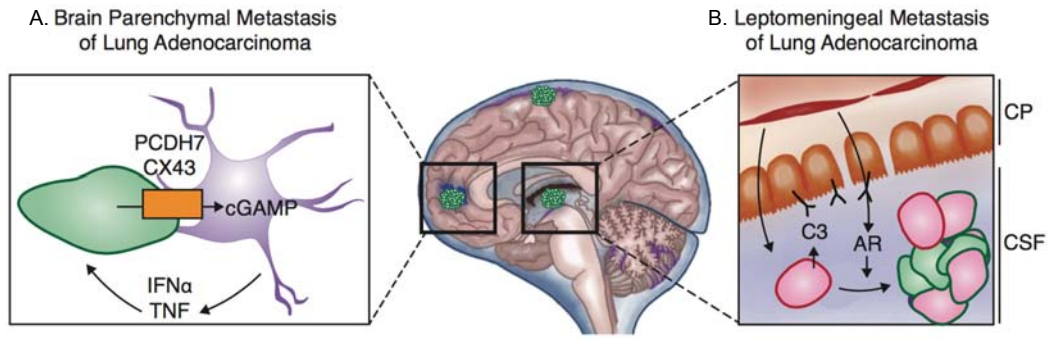

Figure 5 Metastatic colonization of the CNS.
(A) Brain metastatic cells coopt astrocytes via Cx3/PCDH7 gap junctions and cGAMP transfer. cGAMP triggers production of inflammatory cytokines in astrocytes that support BrM growth and chemoresistance. (B) Leptomeningeal metastatic cells that reach the CSF through the choroid plexus (CP) and produce complement C3, which inhibits the blood-CP barrier for entry of plasma growth factors (e.g. amphiregulin, AR).

The leptomeninges surround the brain and spinal cord and contain the cerebrospinal fluid (CSF). Cancer spread into the CSF compartment, called leptomeningeal metastasis, presents a formidable clinical challenge. Metastases to this fluid-filled space disseminate rapidly over the entirety of the central nervous system, growing on and invading into the brain, spinal cord, cranial and spinal nerves, resulting in rapid neurologic disability and death. The majority of leptomeningeal metastases arise from primary breast and lung cancers. Approximately 5-10% of patients with solid tumors harbor leptomeningeal metastasis, and this number is expected to rise. The molecular basis of this morbid, increasingly prevalent complication of cancer remains unknown. To address this problem, we selected lung and breast cancer cell lines for the ability to infiltrate and grow in the CSF [17]. Complement component 3 (C3) was upregulated in four leptomeningeal metastatic models and proved necessary for cancer growth within the leptomeningeal space. In human disease, cancer cells within the CSF produced C3 in correlation with clinical course. C3 expression in primary tumors was predictive of leptomeningeal relapse. Mechanistically, we found that cancer cell-derived C3 activates the C3a receptor in the choroid plexus epithelium to disrupt the blood-CSF barrier (Figure 5B). This effect allows plasma components including amphiregulin and other mitogens to enter the CSF and promote cancer cell growth. Pharmacologic interference with C3 signaling proved therapeutically beneficial to suppress leptomeningeal metastasis in these preclinical models [17].

Perspectives

By exploiting mouse models of metastasis and patient-derived samples in combination with a mechanistic dissection of cancer-relevant cell regulation pathways like the TGF-β pathway and others, we were able to identify genes and mechanisms whose expression mediates metastasis in mouse models and associates with organ-specific relapse in patients. Some of these pro-metastatic pathways are mutationally altered in cancer, whereas others are altered at the epigenetic level [18]. This work additionally illuminated the close mechanistic links between drug resistance and metastasis [19,20]. The ability to biologically dissect and molecularly deconstruct the basic determinants of metastasis is allowing the design and clinical testing of innovative therapeutic strategies for the prevention and treatment of metastasis.

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ABERRANT REGULATION OF MAPK SIGNALING PATHWAYS IN CANCER

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Mitogen-activated protein kinase (MAPK) cascades are the major signaling systems that dictate cell fate decisions such as proliferation, survival, and apoptosis. There are at least three subfamilies of MAPKs, named ERK, p38, and JNK in human cells (Figure 1). While the classical ERK MAPK is preferentially activated in response to mitogenic stimuli and plays a central role in cell growth and survival, two relatively newly identified types of

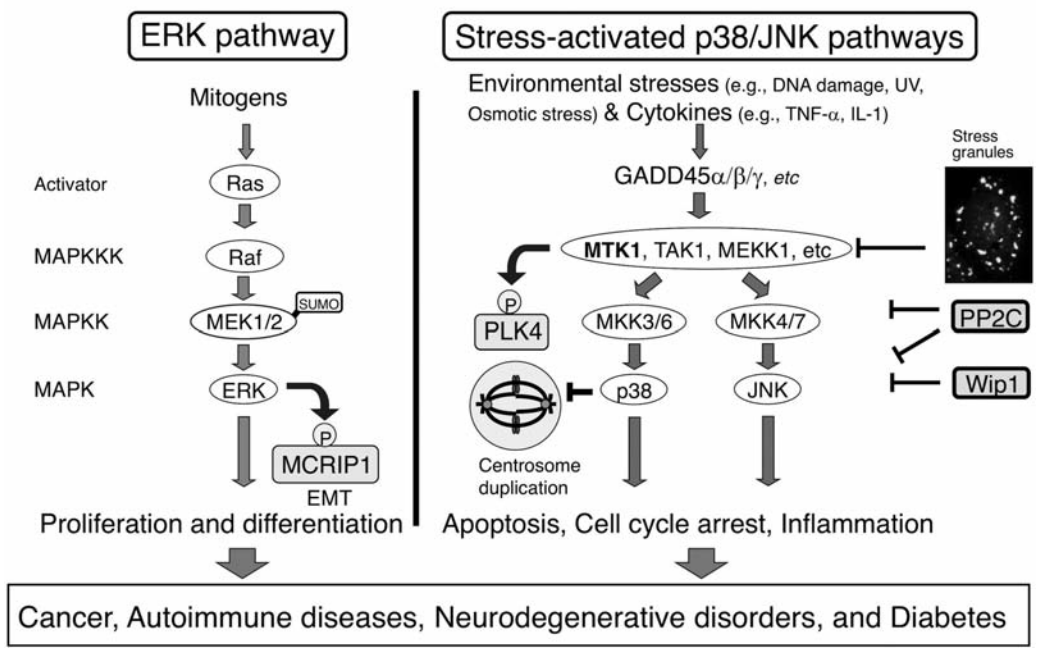

Figure 1 Human MAP kinase signaling cascades

MAPKs, p38 and JNK, are more potently activated by a variety of environmental stresses (*e.g.*, UV- and γ–irradiation, DNA-damaging reagents, oxidative stress, osmotic stress, heat shock and *etc*.) and are thus collectively called stress-activated protein kinase (SAPK). Besides cellular stresses, the SAPK pathways are also activated by cytokines such as IL-1, TNFα, and TGF-β. Perturbation of these critical cellular signaling systems is involved in a variety of life-threatening diseases, including cancer. Therefore, these signaling systems are of clinical importance.

The ERK cascade is a key transducer of mitogenic signals, and is frequently hyper-activated by various oncogenes in human cancer [1]. This pathway not only upregulates growth-related genes, but also down-regulates several anti-proliferative and tumor suppressive genes (*e.g.*, E-cadherin) in various cellular processes including the epithelial-to-mesenchymal transition (EMT). EMT is a cellular trans-differentiation program whereby epithelial cells lose their epithelial characteristics and acquire a migratory, mesenchymal phenotype. This phenomenon is involved in a wide range of biological process, including embryonic development, tissue repair, and tissue fibrosis. Furthermore, inappropriate reactivation of the EMT program in malignant epithelial cells is considered to be a major mechanism for the induction of tumor invasion and metastasis. Therefore, comprehensive understanding of the molecular basis of EMT is crucial for the development of novel therapeutic interventions for cancer. Although TGF-β signaling is a prominent mediator of EMT, accumulating evidence has revealed that various other signaling pathways also play a role in this process. In particular, it has been reported that hyper-activation of the ERK pathway is sufficient to induce EMT in many, if not all, types of cells. Besides ERK signaling, C-terminal binding protein (CtBP), which is a core component of the transcriptional co-repressor complex that contains histone modifying enzymes (*e.g.*, histone deacetylases and methyltransferases), is also involved in epigenetic gene silencing of E-cadherin during EMT. However, the functional relationship, if any, between ERK signaling and CtBP remains obscure.

By developing a novel technique to isolate ERK substrate proteins, we identified a previously uncharacterized protein of unknown function, designated MAPK-regulated corepressor interacting protein 1 (MCRIP1), as a novel and specific substrate of ERK. MCRIP1 is conserved in all vertebrates from fish to human, and is expressed in all tissues examined. Interestingly, we found that MCRIP1 bridged ERK signaling and CtBP-mediated gene silencing [2]. CtBP is recruited to promoter elements of target genes by interacting with the DNA-binding transcriptional repressor ZEB1. We found that MCRIP1 bound to CtBP, thereby competitively inhibiting CtBP-ZEB1 interaction. When phosphorylated by ERK, MCRIP1 dissociates from CtBP, allowing CtBP to interact with ZEB1. In this manner, the CtBP co-repressor complex is recruited to, and silences the E-cadherin promoter by

inducing histone modifications. Our findings demonstrate MCRIP1-mediated functional interaction between ERK signaling and CtBP during EMT, and delineate a molecular mechanism as to how oncogenic ERK signaling induces epigenetic silencing of tumor suppressor genes such as E-cadherin (Figure 2).

The p38 and JNK MAPKs play pivotal roles in cellular stress responses such as apoptosis [3]. Cell fate decisions that are influenced by the p38 and JNK pathways might be important for minimizing the chance of tumorigenesis. There is increasing evidence that these pathways are involved in tumor suppression, and are indeed aberrantly regulated in cancer. In this talk, we also report an unexplored role of the p38 and JNK pathways in the regulation of centrosome number [4].

Centrosomes, which consist of a pair of centrioles surrounded by an amorphous pericentriolar material, serve as the microtubule-organizing centers that are essential for the formation of mitotic spindles in animal cells. In order for cells to undergo normal bipolar cell division, the single interphase centrosome must duplicate precisely once per cell cycle. The strict control of centrosome numbers is indispensable for accurate chromosome segregation at cell division and for maintenance of the stability of genomes. The presence of more than two centrosomes (*i.e.*, centrosome amplification) results in the formation of multipolar mitotic spindles and consequentially in chromosome segregation errors. Centrosome amplification significantly increases the frequency of lagging chromosomes during anaphase, thereby promoting chromosome missegregation. Since

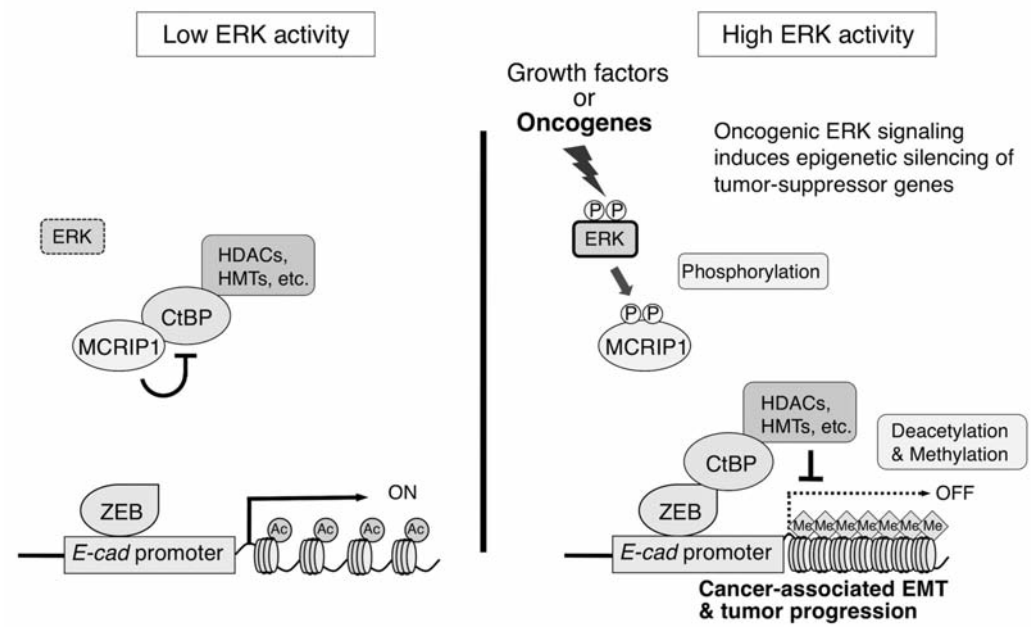

Figure 2 A novel ERK substrate, MCRIP1, plays an important role in epigenetic gene silencing of E-cadherin during EMT by regulating CtBP

chromosome missegregation results in both numerical and structural abnormalities of chromosomes, ablation of the numeral integrity of centrosomes induces chromosomal instability, and thus is considered to be a major cause of cancer development and progression.

We have recently demonstrated a direct functional link between the SAPK pathways and PLK4. We found that, upon stress stimuli, stress-responsive MAPKKKs such as MTK1 and TAK1 directly phosphorylated and activated polo-like kinase 4 (PLK4), a master regulator of centrosome duplication. Stress-induced, MAPKKK-mediated, PLK4 activation provides survival signaling and promoted centrosome duplication. At the same time, however, the p38 and JNK SAPKs and the tumor suppressor p53 protein, which are also activated by various stress stimuli, cooperated to counteract PLK4 activity, thereby preventing centrosome amplification. Importantly, we demonstrated that simultaneous inactivation of SAPKs and p53 in cells exposed to stress allowed unchecked activation of PLK4, leading to centrosome overduplication and chromosomal instability, both of which are hallmarks of cancer cells. This co-operation between SAPKs and p53 explains why both p53 and the MKK4 MAPKK (a SAPK activator) are frequently mutated simultaneously in human cancer cells, in which centrosome number is often increased after stress (Figure 3).

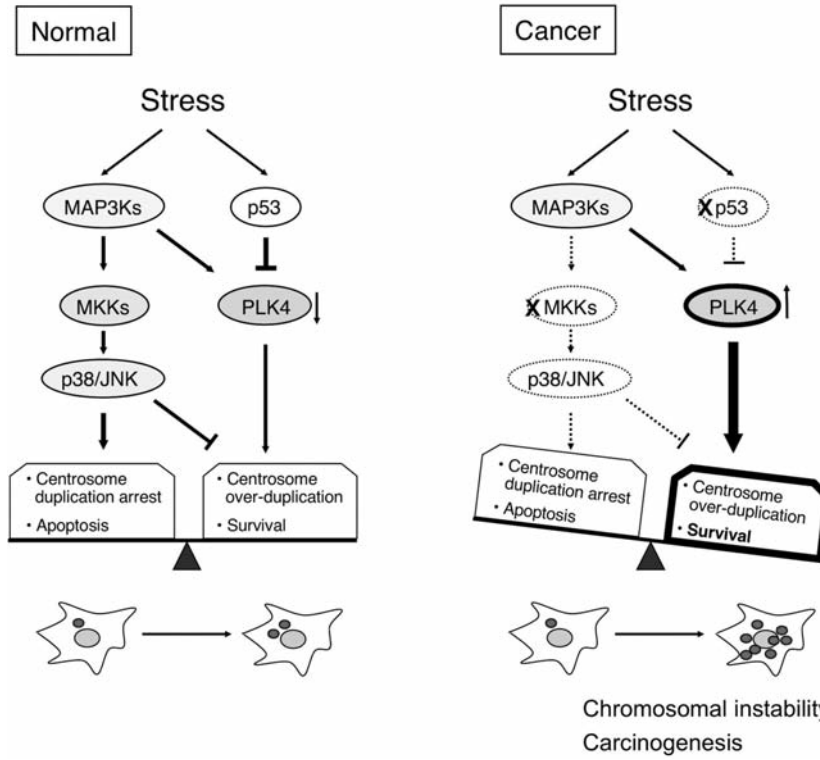

Figure 3 Combined inactivation of p53 and p38/ JNK induces centrosome over-duplication

Mutational inactivation of the MKK4 MAPKK has been shown to drive carcinogenesis (known as "driver" mutations) and is indeed frequently observed in a variety of human cancers. However, the mechanism by which MKK4 prevents carcinogenesis remains to be elucidated. Our results provide the first evidence that centrosome integrity is synergistically regulated by the two important tumor suppressors, MKK4 and p53, and reveal a molecular mechanism that underlies centrosome amplification in cancer cells. Based on these findings, we proposed that MKK4 is a novel type of tumor suppressor whose function is manifested particularly when p53 is also inactivated.

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PI 3-KINASE LINKS OBESITY, INSULIN RESISTANCE AND CANCER

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Retrospective studies have shown that cancers in certain tissues occur at higher rates in individuals who are obese and/or insulin resistant. Many possibilities for explaining this correlation have been proposed. Of particular interest, some of the cancers that correlate with obesity and insulin resistance occur in tissues that express the insulin receptor at relatively high levels, including the liver, pancreas, breast and endometrium. This observation raises the possibility that the ambient high levels of serum insulin that are characteristic of patients with insulin resistance might be contributing to the growth of tumors in these organs. Consistent with this idea, agents that lower serum insulin, including the anti-diabetic drug metformin have been shown to reduce cancer incidents or deaths in patients with insulin resistance/type 2 diabetes [1-4]. Phosphoinositide 3-Kinase (PI3K) is activated by insulin and other growth factors to mediate cell growth [5]. The PI3K enzyme encoded by the PIK3CA gene is one of the most frequently mutated oncogenes in human cancer. This same enzyme mediates insulin responses in liver, muscle, fat and other tissues. The most common mutations in this gene enhance the ability of PI3K to bind to the insulin receptor substrates, IRS1 and IRS2 and thereby enhance the ability of PI3K to be activated by insulin and IGF1. This observation raises the possibility that elevated levels of serum insulin could enhance the growth of tumors that express the insulin receptor, especially when the tumor expresses a mutant form of PIK3CA. Consistent with this idea, retrospective studies have shown that cancers that correlate with obesity and insulin resistance (conditions where serum insulin levels are high), such as endometrial, breast and colorectal cancers, frequently have activating mutations in PIK3CA. These observations suggest a model in which tumors with mutations in PIK3CA are highly sensitized to

insulin-dependent growth and that the elevated serum insulin levels in individuals with insulin resistance in liver, muscle and fat promotes anabolic metabolism in the tumor [6]. This condition would allow the tumor to take up glucose more readily than muscle or fat in insulin resistant individuals.

We have generated mouse models to interrogate the role of PI3Ks in metabolic control and in the generation of cancers. These studies indicate that inhibitors of PI3Ks could be effective in treating cancers in specific mutational backgrounds. More than 20 PI3K inhibitors that target the gene product of PIK3CA have entered clinical trials for treating solid cancers. Although these inhibitors look promising and two have been approved, a variety of toxicities have created challenges [7]. Some of the toxicities, such as hyperglycemia, are on target since inhibition of the PIK3CA gene product is expected to cause insulin resistance. Importantly, the acute insulin resistance that occurs in response to PI3K inhibitors not only raises serum glucose levels, but also induces insulin release from the pancreas, which raises insulin levels. As discussed above, the increased serum insulin has the potential to activate PI3K in the tumor, and it is possible that very high insulin levels could reactivate PI3K in the tumor despite the presence of a PI3K inhibitor. We are exploring therapeutic interventions that might prevent this rebound of insulin-dependent PI3K reactivation in tumors.

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TGF-β SIGNALING IN CANCER

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Transforming growth factor-β (TGF-β) elicits both tumor promoting and suppressive functions during progression of cancer. We here demonstrate our recent findings on TGF-β signaling in progression of cancer, focusing on the induction of epithelial-mesenchymal transition (EMT) by TGF-β. TGF-β induces EMT through activation of Smad as well as non-Smad signaling pathways. Inhibition of TGF-β signaling by inhibitory Smad, Smad7, or small-molecular-weight TGF-β receptor inhibitors results in suppression of cancer metastasis in mouse models [1]. Multiple transcription factors are involved in TGF-β-induced EMT. In addition, cross-talk with other signaling pathways plays important roles in the induction of EMT. Mesenchymal-epithelial transition (MET) is the inverse process of EMT; TGF-β-mediated EMT is a crucial event in the invasion of cancer, while MET occurs when cancer cells form metastatic foci at distant organs.

Cooperation of Ras and TGF-β Signaling in the Progression of Cancer

Snail, a key regulator of EMT, is induced by TGF-β in cancer cells. We showed that induction of Snail by TGF-β was dependent on functional cooperation with active Ras signals, and knockdown of Ras strongly attenuated the Snail induction by TGF-β in pancreatic cancer Panc-1 cells [2]. Overexpression of active Ras into HeLa cells resulted in strong induction of Snail expression by TGF-β, while most other direct targets of TGF-β, including *SMAD7* and *SERPINE1*, were not increased by Ras signaling. These observations indicate that Ras and TGF-β signaling cooperate in the induction of Snail and progression of the EMT process.

We then performed global mapping of the accessible chromatin regions using formaldehyde-assisted isolation of regulatory element (FAIRE)-sequencing [3]. To study

the functional cooperation between TGF-β and Ras, we used the mouse mammary epithelial cell line EpH4 and its Ras-transformed derivative, EpRas. TGF-β and Ras modulated chromatin accessibility either cooperatively or independently; chromatin accessibility to the epithelial marker gene regions, including *Cdh1* (encoding E-cadherin) and *Esrp2*, was reduced upon TGF-β and Ras stimulation, while that to some mesenchymal marker gene regions, including *Cdh2* (encoding N-cadherin) and *Mmp13* (encoding matrix metalloproteinase 13), was increased (Figure 1). We have also found that Etv4 and Etv5, ETS family oncogenic transcription factors, bound to more than one-third of the accessible chromatin regions in EpRas cells upon TGF-β treatment. Expression of *Mmp13* and cell invasiveness induced by TGF-β appeared to be regulated by Etv4 and Etv5. Thus, transcriptional regulation during EMT induced by Ras and TGF-β involves alterations of accessible chromatin regions, which is, in part, regulated by Etv4 and Etv5.

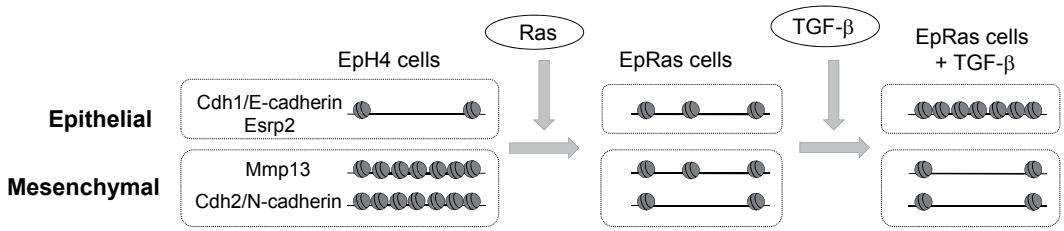

Figure 1 Regulation of EMT-related gene expression and alteration of chromatin accessibility by Ras and TGF-β. Chromatins are closed by Ras and TGF-β at the *Cdh1*/E-cadherin and *Esrp2* gene loci. In contrast, *Cdh2*/N-cadherin and *Mmp13* gene loci become accessible upon Ras and/or TGF-β stimulation. Modified from Arase et al. [3]

Roles of TTF-1/Nkx2-1 in Progression of Lung Adenocarcinoma

Thyroid transcription factor-1 (TTF-1/Nkx2-1) is a tissue-specific transcription factor in lung epithelial cells, and expressed in lung cancer. TTF-1 is used as a diagnostic marker of lung cancer; TTF-1-positive lung cancer patients show better prognosis than TTF-1-negative patients. We have found that TTF-1 inhibited the TGF-β-induced EMT, induced the expression of E-cadherin, and restored epithelial phenotype in lung adenocarcinoma cells. This effect of TTF-1 was accompanied by down-regulation of TGF-β target genes, including *SNAI1* and *SNAI2* (encoding Snail and Slug, respectively) [4]. Knockdown of TTF-1 expression enhanced TGF-β-mediated EMT. Thus, TTF-1 exhibits a tumor-suppressive effect through inhibition of response to TGF-β. In addition, we have shown that TTF-1 repressed the production of TGF-β2 in A549 cells, while TGF-β in turn decreased the expression of TTF-1, suggesting that modulation of TTF-1 expression is a potentially interesting therapeutic strategy for treatment of lung adenocarcinoma (Figure 2A).

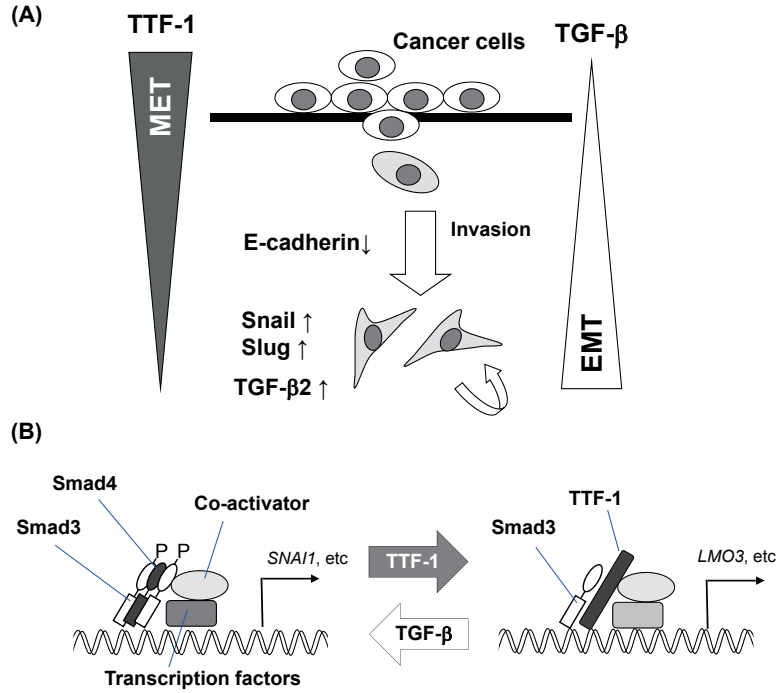

Figure 2 TGF-β signaling and TTF-1 in the induction of EMT and MET.
(A) TGF-β signaling induces EMT, whereas TTF-1 induces MET in lung adenocarcinoma cells. (B) Smad4-independent functions of Smad3 regulated by TTF-1. The Smad3/Smad4 complex interacts with other transcription factors and transcriptional co-activators (or co-repressors), and regulates transcription of certain target genes, including *SNAI1*. In the presence of TTF-1, Smad3 interacts with TTF-1 and other transcription factors, and regulates certain other genes, including *LMO3* [5].

We have further investigated the mechanism through which TTF-1 inhibits the functions of TGF-β. TTF-1 disrupted the nuclear Smad3/Smad4 complex without affecting the nuclear localization of phospho-Smad3. Genome-wide analyses by ChIP-seq revealed that TTF-1 co-localized with Smad3 on the chromatin and modulated the binding patterns of Smad3 throughout the genome. Intriguingly, Smad3 bound to the chromatin with TTF-1, but not with Smad4, at some Smad3-binding regions, which could be observed independently of TGF-β stimulation (Figure 2B). Thus, TTF-1 competes with Smad4 for interaction with Smad3, and the Smad3/TTF-1 complex regulates the transcription of certain genes independently of Smad4. These findings propose a new model of Smad signaling with TTF-1 [5].

Our ChIP-seq analyses in lung adenocarcinoma cells using next generation sequencers led to identification of new target genes of TGF-β and TTF-1, which appeared to be involved in cancer cell metabolism and cell growth and survival through cross-talk with other signaling pathways. RNA-binding motif protein 47 (RBM47) is a target of TGF-β in

cells that have undergone EMT. Expression of RBM47 was inhibited by TGF-β in lung cancer cell lines. RBM47 suppressed the expression of cell metabolism-related genes, which are the direct targets of Nrf2, suggesting tumor-suppressive roles for RBM47 through suppression of the Nrf2 activity [6].

Another novel target of TGF-β and TTF-1, identified by the ChIP-seq analyses in lung adenocarcinoma cells, was tuftelin 1 (TUFT1), which turned out to be a key regulator of the mTOR complex 1 (mTORC1) signaling pathway [7]. Expression of TUFT1 was induced by TGF-β stimulation, and TUFT1 induced cell proliferation and motility, leading to tumor growth and cancer metastasis in mouse models *in vivo*. Silencing of TUFT1 caused dispersion of the lysosomes in the cytoplasm, and inhibition of mTORC1 signaling. TUFT1 promoted perinuclear lysosomal localization by interaction with RABGAP1, a GAP for certain Rab GTPases, suggesting that TUFT1 may also be a potential therapeutic target for lung and some other carcinomas.

Whole-body Profiling of Cancer Metastasis by CUBIC-based Cancer Analysis

We have recently reported a new imaging technology, termed CUBIC-based cancer (CUBIC-Cancer) analysis, which allowed transparantization of animal tissues and whole-body/organ imaging of cancer (collaboration with Prof. Hiroki R. Ueda). By CUBIC-Cancer analyses, spatiotemporal quantification of metastatic cancer progression at single-cell resolution is possible. Although importance of the EMT in the invasion and intravasation of cancer cells has been demonstrated, involvement of the EMT in the extravasation of cancer cells has not been clearly determined. Immunodeficient mice were intravenously injected with A549 lung adenocarcinoma cells pre-treated with or without TGF-β. CUBIC-Cancer analyses clearly showed that colonization of the metastatic cells gradually progressed over 14 days after elimination of the cells at the early time points. Statistical analysis revealed that TGF-β stimulation significantly increased the number of metastatic foci from 1 to 14 days, demonstrating that EMT promotes not only intravasation but also cell survival and extravasation of cells at metastatic sites (Figure 3) [8].

Acknowledgments

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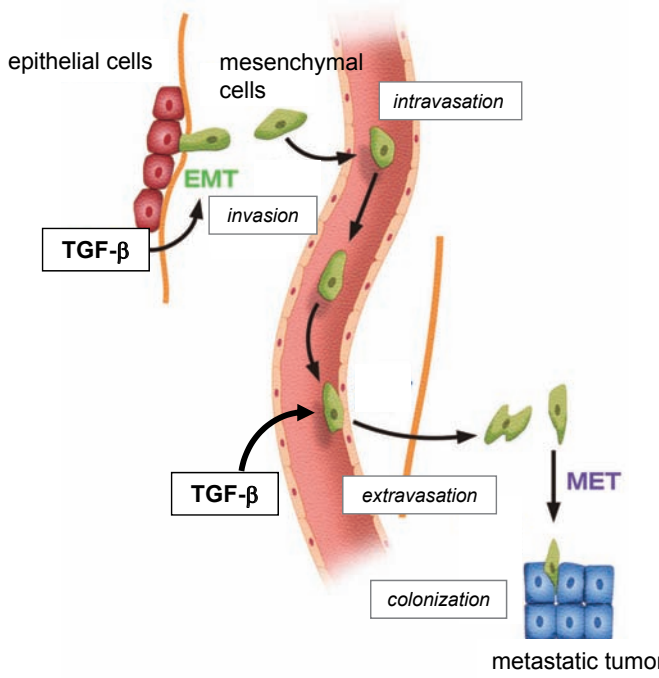

Figure 3 EMT in cancer progression. TGF-β signaling induces invasion and intravasation. In addition, CUBIC-Cancer analyses suggested that TGF-β signaling supports the cell survival and extravasation [8]. E-cadherin expression can be observed in metastatic cells, suggesting that MET occurs at the metastatic sites.

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THE DEPENDENCE RECEPTOR PARADIGM: FROM AN ORIGINAL MECHANISM OF CELL DEATH TO CLINICAL TRIAL

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A few years ago, an original concept of cell biology was proposed: whereas the classic dogma postulates that transmembrane receptors are inactive unless bound by their specific ligand, they proposed that some receptors may be active not only in the presence of their ligand, but also in their absence. In this latter case, the signaling downstream of these unbound receptors leads to apoptosis. These receptors were consequently named "dependence receptors", as their cell expression renders the cell's survival dependent on the presence in the cell environment of its respective ligand [1]. To date, we have identified or have been involved in the identification of most known dependence receptors.

Beyond the basic interest of studying a receptor capable of transducing two antagonizing signals –i.e., one "positive" in the presence of ligand leading to cell differentiation, proliferation or migration and one "negative" in the absence of ligand leading to cell suicide-, we have proposed that this dual function could lead these receptors to have key roles both during embryonic development and in the regulation of tumorigenesis.

In the context of their involvement in embryonic development, we hypothesized that the pro-apoptotic activity of these dependence receptors is crucial for the development of the nervous system as a mechanism to "authorize" neuronal guidance, migration or localization in settings of ligand presence. Along this line, we found that the Sonic Hedgehog (Shh) receptor Patched is a dependence receptor and its ability to induce apoptosis in the absence of Shh is crucial for the adequate development of the neural tube [2,3]. We also showed that the netrin-1 receptors DCC and UNC5H regulate death/survival of specific neurons during the development of the nervous system [4,5]. This implication is

not limited to the developing nervous system as we showed the importance of the apoptosis induced by UNC5H in the formation of blood vessels - angiogenesis- [6,7].

In the context of cancer, we have formulated the hypothesis that these receptors are tumor suppressors that would limit tumor progression by inducing apoptosis of tumor cells outside of settings of ligand accessibility/availability [8,9]. We are particularly interested in the receptors that bind netrin-1 –i.e., DCC and UNC5H-. We showed that both DCC and UNC5H are dependence receptors in cancer cells: whereas in the presence of their ligand netrin-1, they transduce classic "positive" signals, in the absence of netrin-1, they actively trigger apoptosis [1,7,10-14]. Interestingly, DCC and UNC5H are considered as tumor suppressors because their expression is lost in many cancers [8,15,16], suggesting that the presence of these receptors is a constraint for tumor progression. This was actually formally proven by showing that in mice the invalidation of UNC5H3, the overexpression of netrin-1 in the digestive tract or the specific inactivation of the pro-apoptotic activity of DCC resulted, in agreement with the dependence receptors paradigm, in a similar tumorigenesis [17-19]. Thus, aggressive cancers that develop are cancers for which the dependence receptor pathways are blocked through mechanisms including the genetic loss of these receptors (Figure 1).

However, a loss of dependence receptors is not always the selective advantage used by tumor cells to escape this survival dependence on the presence of the ligand. Indeed, we showed that in many cancers such as metastatic breast cancer, lung cancer, neuroblastoma, lymphoma or melanoma [20-24] tumor cells acquire the preferred autocrine expression of netrin-1. This selective advantage for the tumor is much more appealing in terms of putative therapeutic strategy. Indeed, the titration of the ligand by a molecule that interferes with the interaction between a dependence receptor and its ligand should lead to

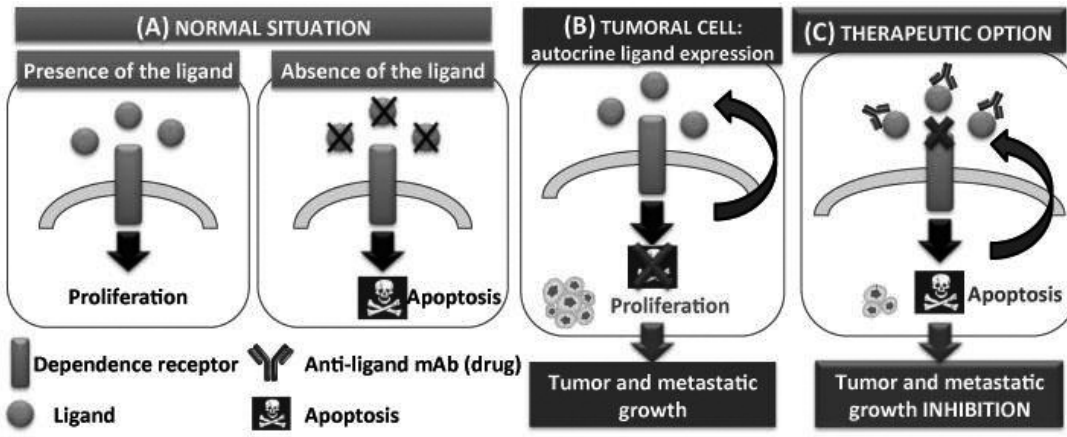

Figure 1 The dependence receptor paradigm and the associated therapeutic strategy.

tumor cell death (Figure 1). Along this line, we showed that titration of netrin-1 by a drug candidates allows tumor cell death in vitro and triggers regression of tumors and metastases in mice [20-24]. Of interest, this gain of ligand is probably not limited to netrin-1 but may possibly be extended to the other ligands of other dependence receptors. Indeed, we recently showed that an autocrine expression of NT-3, a ligand of the TrkC dependence receptor is detected in various cancer types and can be targeted to trigger tumor growth and metastasis inhibition [25]. A similar observation was made for the pair Sema3E/PlexinD1 where Sema3E, the ligand of the dependence receptor PlexinD1 is up-regulated in breast cancer [26]. Of interest, this gain of ligand is not only promoting survival of the cancer cells but also in some case of the endothelial cells as recently demonstrated with Jagged 1 being up-regulated in lung cancer as a mechanism to block the pro-death activity of Notch3 behaving as a dependence receptor in this settings [27]. We thus have been trying to develop drugs based on the interference on the interaction between dependence receptors and their ligands as anti-cancer strategies. A humanized anti-netrin-1 antibody has been preclinically developed [28] and the first human trial (Phase I) using an agent interfering between netrin-1 and its receptors has started in 2017 in all solid tumors (https://clinicaltrials.gov/ct2/show/NCT02977195). Thus, from a basic cell biology concept, we hope to be able to provide evidence that targeting ligand/dependence receptor interaction is beneficial to treat cancer.

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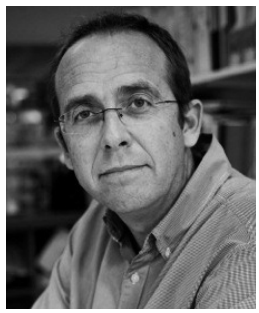

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ASK FAMILY KINASES IN STRESS SIGNALING AND CANCER

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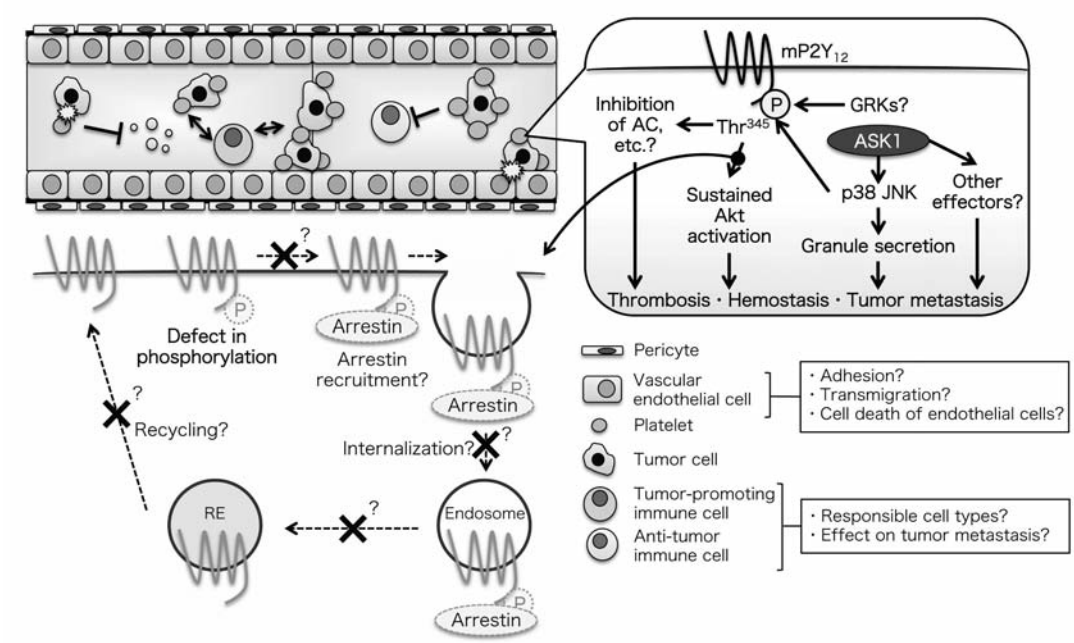
Stress-responsive signaling cascades function as a rheostat for appropriate cellular responses; therefore, their dysfunction culminates in tumor initiation and progression [1]. The most important stress-responsive signaling cascades are mitogen-activated protein kinase (MAPK) pathways, composed of extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase (JNK). Apoptosis signal-regulating kinase 1 (ASK1) is a Ser/Thr kinase that is activated in response to various stressors, such as cytokines and oxidative stress and is responsible for activation of p38 and JNK [2]. Although ASK1 reportedly exerts both tumor-promoting and tumor-suppressive effects on tumor progression [3], it remains unclear whether ASK1 modulates tumor metastasis and/or serves as an upstream regulator of p38 and JNK. In our presentation, we reported drastic attenuation of lung tumor metastasis in ASK1 knockout mice [4]. Intriguingly, tumor metastasis was also attenuated in wild-type mice that received bone marrow from ASK1 knockout mice but not myeloblast-specific (i.e., granulocytes, monocytes, and macrophages) ASK1 knockout mice. We, therefore, focused on platelets among the non-myeloblastic bone marrow-derived cell types. Importantly, platelet-specific ASK1 knockout mice exhibited attenuated tumor metastasis, as well as unstable hemostasis and impaired thrombosis, indicating the involvement of platelet-intrinsic ASK1. Additional analysis of *in vitro* platelet aggregation suggested that ASK1 regulates adenosine diphosphate (ADP) signaling in platelets. ADP signaling is mediated by two types of G protein-coupled receptors (GPCRs), $P2Y_1$ and $P2Y_{12}$, in platelets [5]. We newly identified the phosphorylation site (Thr345 in mice) of $P2Y_{12}$ as a target of ASK1-JNK/p38 axis. This site was found to be located at the C-terminal putative postsynaptic density 95/discs

large/zonula occludens-1 (PDZ)-binding motif and is required for sustained Akt activation. Hence, the results in our study [4] offer insight into the positive regulation of ADP signaling through $P2Y_{12}$ phosphorylation and ASK1-mediated MAPK signaling in platelets.

Further study will be needed to investigate the precise molecular mechanism by which the newly identified $P2Y_{12}$ phosphorylation site affects $P2Y_{12}$ function, as well as other downstream pathways, apart from Akt (e.g., inhibition of adenylate cyclase). In general, GPCR phosphorylation by GPCR kinases (GRKs) leads to desensitization, arrestin recruitment, and internalization followed by either receptor recycling or degradation [6]. $P2Y_{12}$ was shown to be phosphorylated by GRK2 and GRK6, resulting in desensitization; however, the phosphorylation sites targeted by these kinases have not been identified [5]. Thus, GRKs may also phosphorylate Thr345 (in mice) or other sites of $P2Y_{12}$. It would be interesting to examine the relationship between ASK1-JNK/p38 axis and GRKs in the regulation of $P2Y_{12}$ phosphorylation and function. Moreover, it remains unknown whether $P2Y_{12}$ Thr345-phosphorylation is relevant to arrestin recruitment, receptor internalization, and/or resensitization, similar to the bleeding-patient mutation (P341A) at the corresponding PDZ motif of human $P2Y_{12}$ [5].

Of note, ASK1 knockout mice that received bone marrow from wild-type mice also showed attenuated tumor metastasis, suggesting that ASK1 may regulate tumor metastasis in X-ray-resistant recipient cells. We reported in this symposium that ASK1 in endothelial cells (ECs), which are one of the X-ray-resistant cells, also facilitates tumor lung metastasis. Hence ASK1 may mediate adhesion of tumor cells to ECs followed by tumor cell



transmigration and/or EC death induced by direct interaction with tumor cells. Furthermore, we also observed promising results suggesting that metastasis-suppressive immunity is enhanced in ASK1 knockout mice compared with wild-type mice. The responsible cell types and the precise regulatory mechanism of tumor metastasis therein are now under investigation.

In sum, our findings reveal a previously unidentified function of MAPK pathway in GPCR fine-tuning in platelets [4]. We hope this knowledge will greatly contribute to the development of novel molecular mechanism-based strategies against tumor metastasis and thrombosis.

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CONCLUDING REMARKS

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The 48th International Symposium of the Princess Takamatsu Cancer Research Fund has provided cancer researchers here with a great opportunity to discuss and decipher the biological features of cancer from the aspect of cancer-host crosstalk and its complexity in nature.

Cancer arises by having genetic and/or epigenetic alterations caused by exposure to various environmental insults of either endogenous or exogenous origins. As a consequence of the evolutional process, cancer cells further acquire an invasive and metastatic nature after the stepwise accumulation of multiple genetic and epigenetic alterations. Heterogeneity (complexity) and plasticity are other characteristic features of cancer cells, and acquisition of these phenotypic characters in cancer cells is considered to be achieved, in part, by complex interactions between cancer cells and other cellular surrounding components, including fibroblasts, endothelial cells of blood and lymphatic vessels, immune cells and macrophages. Direct or indirect interaction with metabolites of intestinal microbiota could also be involved in initiation and promotion of cancer cells. Drastic therapeutic outcomes of immune checkpoint inhibitors, for example, have proved the substantial contribution of a variety of immune cells for development of human cancers in an *in vivo* setting. These facts surpass our previous comprehension regarding the biological nature of cancer, and nowadays it is conceivable that complex cellular interactions within cancer tissues *in vivo* are indispensable for survival and progression of cancer cells.

Cancer is now defined as a systemic disease that occurs and develops under complex cellular networks *in vivo*, and understanding these complex cellular networks from various

aspects by means of comprehensive and integrative approaches would enable us to decipher the complex nature of cancer. In light of the importance of various types of cancer networks, we created a program for the 48th International Symposium of the Princess Takamatsu Cancer Research Fund under the title of "*Complexity in Cancer-host Crosstalk*". Throughout the symposium, a considerable number of important subjects on cancer-host crosstalk were presented, and fruitful and constructive discussions were exchanged on this occasion. Some of the representative issues that have been extensively discussed during the symposium can be summarized as follows.

1. Heterogeneity in cancer cells and cancer stem cells.

Heterogeneity in cancer cells is widely known to be evolutionally achieved through the process of tumorigenesis, and pathogenesis of heterogeneity was also revealed to be a complex and diverse process. Single cell transcriptome analysis demonstrated the presence of heterogeneity in colon cancer stem cells as well. Cell-cell interaction in tumors was shown to play a pivotal role in stomach carcinogenesis using genetically-engineered compound animal models in mice. Diverse biological functions of HIF-1 and metabolic reprogramming by autophagy adaptor p62 was also presented. TGF-β-induced quiescent stem cells at the perivascular region were reported to be present in squamous cell carcinoma (SCC), and these heterologous cells could be responsible for acquisition of resistance to chemotherapy.

2. Signal diversity in cancer cells.

Functional crosstalk among various intracellular signals could also confer cells with differential responses to extra-cellular stimuli. ASK1/ASK2 signals regulate tumorigenesis via apoptosis and innate immunity. High insulin levels could reactivate PI3K in tumors despite the presence of a PI3K inhibitor. Therefore, a ketogenic diet along with a PI3K inhibitor could be more potent in suppressing tumor growth. TGF-β and TTF-1 reciprocally cooperate in epithelial mesenchymal transition (EMT), and TTF-1 disrupts the Smad3/Smad4 complex. Dependence receptors (DRs) are regulators of tumor growth and metastasis, and Netrin-1, a DR ligand, was shown to be up-regulated in cancer-associated fibroblast (CAF).

3. Stromal – epithelial tumor cell interactions

Interaction between epithelial tumor cells and mesenchymal stroma cells is considered to play key roles in tumor development, as described earlier. Activation of hepatic stellate cells in the liver plays a key role in development of non-alcoholic steatohepatitis (NASH). Intriguingly, inactivation (senescence) of hepatic stellate cells was also reported to be

involved in a subset of liver carcinogenesis related to fatty liver. Crucial roles of CADM1 in tumor epithelial – stromal cell communications, tumor-stromal interaction by MLC phosphorylation and metabolic reprogramming by p62 downregulation in cancer development were also reported in this symposium. In addition, MKL1, which modulates actin dynamics, was demonstrated to be involved in adipocyte-CAF transition. Presence of heterogeneity in CAFs, which was revealed by single cell transcriptome analysis, was also beautifully demonstrated. More detailed mechanistic analyses are warranted in the future to dissect molecular mechanisms involved in epithelial tumor cell-mesenchymal fibroblast interaction.

4. Immune microenvironment:

As evidently proved by beneficial clinical outcomes of immunocheckpoint inhibitors, such as anti-PD-1 and anti-PDL-1 antibodies, the immune microenvironment plays a key role in regulating tumor cell survival. Many novel mechanisms involved in the immunologic microenvironment of cancers were presented during the symposium. IgA+ plasmocytes that express PDL-1 and IL-10, NK activation by CADM1, chemotherapy-induced immune evasion through HIF-1, and immune-modulation by CAFs were also presented. Metastatic stem cells (MetSCs) were reported to stay in a slow-cycling state and thereby evade immune surveillance.

Furthermore, immune-pharmacogenomic approaches to characterize T-/B-cell repertoires in tumor tissues, comprehensive genetic screens for identification of immunotherapy targets, dynamic changes in neo-antigen/TCR profiles between ductal carcinoma *in situ* (DCIS) and invasive ductal breast carcinoma (IDC), and switching to a more suppressive immune environment in IDC were demonstrated in an attractive and enthusiastic manner. As for other cellular components related to immune reaction of cancer cells, T-cell suppressive neutrophils, activation of a myeloid differentiation in bone marrows, G-CSF-directed expansion and differentiation of myeloid lineage hematopoietic stem cells (HSCs), and dynamic changes in leukocyte and macrophage populations were also presented in this symposium. Future explosive expansion of this field of cancer research is further warranted to dissect the complexity of tumor cell-immune cell networks and to provide us with novel therapeutic modalities.

5. Gut microbiota and their metabolites

Metabolites from microbiota could work as novel factors to modulate or to intermediate epithelial cell transformation in several organs, such as the gastrointestinal tract organs and the liver as partly detailed in the previous section. Moreover, understanding host-microbe chemical interaction, a crossroads between gut microbiome and cancer immune

surveillance, dysbiosis, T-cell response against gut microbiome could be responsible for acquisition of novel characteristic phenotypes in cancer cells, such as resistance to anti-PD-1/PDL-1 antibody treatment.

6. EMT-MET transition, invasion, metastasis and metastatic stem cells

Both epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) are essential phenotypic changes in cancer cells for their invasion and metastasis. Transcriptional regulation of MMPs and L1CAM by HIF-1 and also the MAPK pathway were reported to be involved in this process. Modulation of metastasis and cell plasticity by gp130-dependent- or –independent activation of Stat3 and biological roles of exosomes in various aspects of metastatic events (namely; dormancy, pre-metastatic niche formation, extravasation, and dissemination) were presented. Mouse models of latent metastasis by metastatic stem cells (MetSCs) was quite a novel and exciting finding. ASK1 in platelets and vascular endothelial cells were demonstrated to coordinately promote lung metastasis.

7. Novel approaches to clarification of cancer network

Since cancer-host crosstalk was revealed to be highly complex, novel analytical methodologies need to be invented. A computational approach to dissect complex networks, molecular imaging using a next-generation single-cell mass cytometry with high resolution, and an *in vivo* RNAi screening have been introduced. *In vivo* imaging of p21/p16 and MetSCs, and also a new *in vivo* imaging technology with transparentization seem to be two of the most promising and attractive technologies for resolution of cancer-host crosstalk *in vivo*. Further technical innovation in this field is eagerly awaited.

Throughout the symposium, we were extremely delighted and stimulated to have been exposed to many more innovative researches, approaches and technologies to decipher the *"Complexity in Cancer-host Crosstalk"*, which is one the most complex natures of cancer *in vivo.* Comprehensive understanding of the complex and heterogeneous nature of cancer pathogenesis could lead us to conquering this enemy and future implementation of personalized precision medicine, especially in cancer detection at early stages, effective treatment and prevention of metastasis.

Lastly, on behalf of the organizers, I would like to express my sincere thanks to the Princess Takamatsu Cancer Research Fund for hosting this invaluable occasion, and also thank our distinguished speakers and guests for bringing us great success and new ideas. Finally, I as a chairman of the organizing committee would like to see all the speakers and participants again in the not-too-distant future to discuss and tackle these difficult subjects.

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