A 3D perineural invasion model on chip for investigating the interaction between Schwann cells and macrophages

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Background of research

1) Research Objective

Immunotherapy is a promising area of research in the treatment of metastatic cancer. However, prostate cancer generally does not show a significant response to immunotherapy compared with other types of advanced cancer. This may be due to the immunosuppressive microenvironment created by the high degree of innervations. Schwann cell (SCs) is the main component of nerve fibers and has the functions of nerve regeneration and immune regulation. In prostate cancer, 75% of patients have perineural invasion (PNI), and SCs is the main executor of PNI and promote tumor metastasis. However, the immunomodulatory effect of SCs within tumors is less clear. The objective of the proposed research is to develop a 3D perineural invasion model that closely mimics the *in vivo* microenvironment of nerve-cancer crosstalk to study the process of perineural invasion and investigating the interaction between schwann cells and macrophages. Furthermore, we hope this chip can be used to develop new strategies for combination drug therapy to decrease the resistance of immunotherapy.

Objective 1: Develop a 3D perineural invasion model on DMF chip

Objective 2: Discover whether Schwann cells can promote macrophages infiltrate to tumors.

Objective 3: Discover the underlying mechanisms of how Schwann cells polarize macrophages to the M2 phenotype and contribute to an intertumoral immunosuppressive environment.

Objective 4: Discover some blockers of perineural invasion or macrophage polarization to develop new strategies for combination drug therapy to reduce resistance to immunotherapy.

2) Background

Prostate cancer: a highly innerved cancer.

Prostate cancer (PC) is the most common malignancy in men, and it was the fifth leading cause of cancer death among males worldwide in 2020[1]. Approximately 80-90% of prostate cancer patients are diagnosed at localized or locally advanced stages. At present, there are few efficient clinical treatments for PC. At early stage, PC is prevalently treated with radical prostatectomy,

brachytherapy, cryotherapy, and focal therapies[2]. This cancer, however, frequently evolves towards a locally advanced disease. At this stage, the androgen deprivation therapy (ADT), associated or not with external beam radiotherapy, represents the backbone patient's treatment[3]. Nevertheless, oncologists still experience many frustrations because of the ineffectiveness of these approaches, mainly related to the therapy escape and disease progression. PC often becomes castration resistant (CRPC), which can be metastatic or not. Few approaches are actionable in these patients and the death toll remains paradoxically high, albeit the substantial improvements in early diagnosis and treatments.

Recent studies have shown that peripheral nerves (sympathetic, parasympathetic, and sensory nerves) interact with tumors and stromal cells, promoting the occurrence and progression of various solid and hematological malignancies[4]. In addition, as cancer progresses from precancerous lesions to obvious cancer, the nerve density has almost doubled compared to age-matched non-tumor tissue controls, such as prostate cancer, colon and rectal cancer, head and neck cancer, breast cancer, pancreatic cancer, gastric cancer, and lung cancer[4]. The progression of prostate cancer has been shown to depend on the development of autonomic nerves to the tumor microenvironment[5] (Fig.1). For example, denser autonomic nerve innervation is associated with a poor prognosis in PC. Adrenergic fibers are found to increase in normal prostate tissue around human tumors, while cholinergic fibers infiltrate tumor tissue. Sympathetic nerves activate adrenergic neural signals, which are necessary in the early stages of tumor progression and the initiation of angiogenesis, while parasympathetic nerves activate cholinergic neural signals, leading to tumor dissemination and metastasis[6]. In addition, multiple studies have shown that the sympathetic neurotransmitter norepinephrine (NE) promotes PC migration and metastasis by activating adrenergic β receptors (Adrβs)[7]. Surgical or chemical destruction (dopamine analog 6-hydroxydopamine (6OHDA)) of sympathetic nerves within the tumor can inhibit early growth[4].

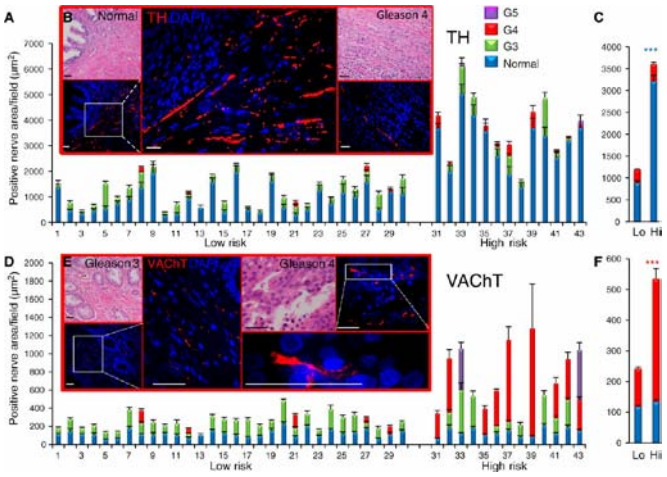

Fig 1. Human high-risk prostate adenocarcinomas are rich in adrenergic and cholinergic nerve fibers. A-C) the density of adrenergic fibers in patients with low-risk or high-risk prostate cancer; D-F) the density of cholinergic fibers in patients with low--risk or high-risk prostate cancer.

Cancer patients often suffer from neuropathic pain due to the neural innervation of cancer. Treatments such as radiation and chemotherapy may cause nerve damage, leading to pain[8]. Therefore, cancer treatment usually includes painkillers, some of which are very effective and addictive. Tumor immunotherapy is rapidly being combined with other therapies to improve patient survival rates. For example, immunotherapies targeting Programmed Cell Death Protein 1 (PD-1) and its ligand PD-L1 have shown good clinical effects in various types of cancer. However, patients with late-stage prostate cancer participating in clinical trials show overwhelming resistance to anti-PD-1 antibody treatment[9].

Potential reasons: why prostate patients are insensitive to immune checkpoint inhibitors such as anti-PD1 treatments.

There are two main reasons for this resistance. One is due to the high expression of PD-L1 in the tumor microenvironment. Although PD-L1 is rarely expressed on prostate tumor cells, it has been

reported that the prostate tumor stroma shows strong PD-L1 expression[10]. In prostate cancer, areas rich in autonomous tumor-infiltrating nerves express high levels of the PD-L1. This inhibitory immune checkpoint ligand binds to cytotoxic CD8+ T cells expressing the homologous receptor PD-1 and promotes their unresponsiveness. The area of high PD-L1 nerve expression is negatively correlated with the area of CD8+ T cell expression, and high PD-L1 nerve density is associated with recurrence[11].

On the other hand, the immunosuppressive tumor microenvironment is considered to be the basis for prostate cancer patients to develop resistance to anti-PD-L1/PD-1 monotherapy. Tumor-associated macrophages (TAMs) are major innate immune cells that constitute up to 50% of the cell mass of human tumors[12], therefore, they represent an interesting target for immunotherapy. Moreover, in vivo studies have demonstrated that macrophages mediate both chemo- and immunotherapy resistance through the secretion of soluble factors and the mediation of matrix deposition and remodeling that induce pro-survival and/or anti-apoptotic programs in the malignant cells and the TME[12]. Specifically, in PC, several studies have reported that TAMs infiltration into the TME supports PC cell proliferation and migration and is associated with disease progression and metastasis after therapy with ARSIs[13]. Wu et al. found that high infiltration of M1 macrophages and neutrophils were associated with poor prognosis[3]. Erlandsson et al. showed that men with high numbers of M2 macrophages in the prostate tumor environment had increased odds of dying of PC[14]. It is possible that the infiltrated M1 macrophages are polarized to M2 macrophages, together with other suppressor cells such as Tregs, promote an immunosuppressive environment.

Nerves modulate immune cells recruitment to create an immunosuppressive tumor microenvironment.

In most of the research, macrophages are recruited by cancer cells through cancer cell secreted colony stimulating factor to promote cancer migration and nerve invasion. Consistently, nerve innervation recruit macrophages into the tumor microenvironment by releasing numerous neurotransmitters and neuropeptides (Fig.2). In breast cancer, CD11b+F4/80+ macrophages have been shown to infiltrate the tumor parenchyma upon pharmacologic sympathetic activation of adrenergic receptors and to contribute to a 30-fold increase in metastasis to the lymph nodes and lung[15]. It has also been shown that noradrenaline stimulates IL6 production and activates macrophages and other stromal cells in the tumor microenvironment[16]. Furthermore, β3-adrenergic signaling induces ovarian cancer cells to secrete brain-derived neurotrophic factor, which serves to convert macrophages and neutrophils into immunocompetent M1 and N1 types to recruit and maintain hematopoietic stem cells and Mesenchymal stem cells[3]. However, little is known about the regulatory role of PNS glial cells, Schwann cells (SCs), in the immunosuppressive environment formation.

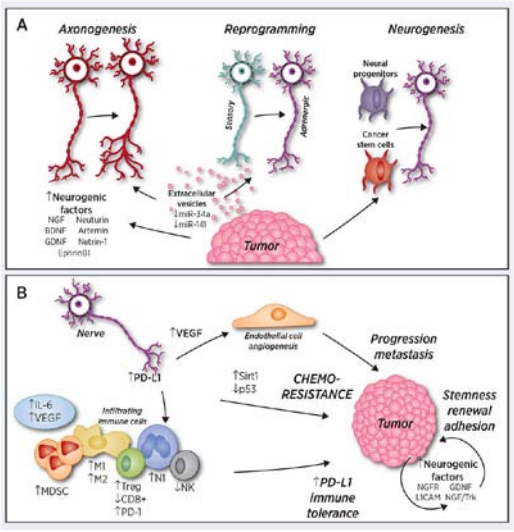

Fig 2. Reciprocal interaction between cancer and nerves. A) Cancer cells drive nerve alteration; B) Sympathetic innervation promotes the tumor microenvironment and tumor growth.

Schwann cells (SCs): a critical role in nerve regeneration (neuronal regeneration and immunomodulation function).

The Schwann cells (SCs), principal neuroglia of the PNS, is a critical feature in the context of peripheral nerve regeneration following traumatic injuries and peripheral neuropathies. After a nerve damage, SCs are rapidly activated by injury-induced signals and respond by entering the repair program (Fig.3). During the repair program, SCs undergo dynamic cell reprogramming and convert into a repair phenotype aimed at promoting nerve regeneration and functional recovery[17]. The repair-SCs promote the disintegration of distal cut axons and their clearance, together with invading macrophages. The macrophages are recruited to the injured site by repair-SCs. Macrophages play a critical role in peripheral nerve injury. Besides contributing to myelin clearance, they participate in the inflammatory response, foster axon debris removal, and regulate the injured site microenvironment, which allows for efficient regeneration. During the peak phase of myelin clearance after injury, repair-SCs express high levels of several growth factors and chemoattractant cytokines including MCP-1, GDNF, interleukin-6 (IL-6) and LIF. These factors act by promoting the recruitment and the induction of both pro- and anti-inflammatory macrophages (M1- and M2-macrophages)[18].

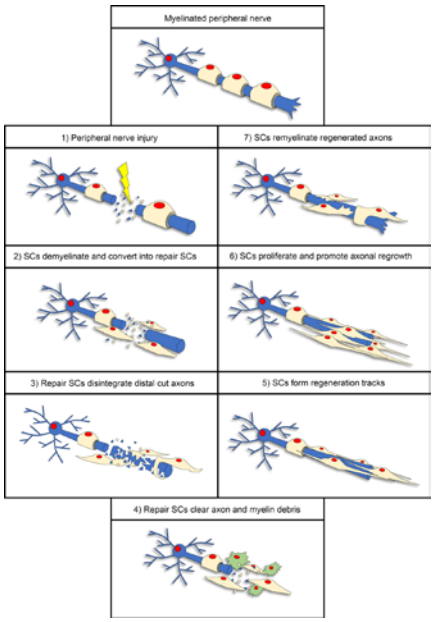

Fig 3. Repair program in the peripheral nervous system.

Schwann cells (SCs): the main executor of inducing tumor perineural invasion.

In tumor microenvironment, SCs also promote tumor growth and cancer cell invasion through various mechanisms. The repair-like SCs has been demonstrated in pancreatic and lung cancer. Numerous studies have shown that repair-like SCs can promote cancer invasion in a contact-dependent manner, a process termed perineural invasion (PNI)[19]. Deborde et al. found that nonmyelinating SCs in pancreatic ductal adenocarcinoma are activated by c-Jun to converted into repair-like SCs, similar to their reprogramming during nerve repair. Repair-like SCs activated by cancer cells form tracks that serve as cancer pathways to promote cancer cell migration and invasion[20]. PNI has become a key pathological feature of many malignancies, including those of the head and neck, pancreas, colon and rectum, prostate, biliary tract and stomach[21]. For many of these malignancies, PNI is associated with high morbidity, is a marker of poor outcome and reduced survival, and is accompanied by resistance to immunotherapy. PNI is a common phenomenon in prostate cancer and is thought to be responsible for most extracapsular spread of prostate cancer. The study found that approximately 75% of prostate cancer patients have PIN, and the five-year progression-free survival rate drops to 70% for patients with PNI, compared with 90% for patients without PNI[21].

As an immunomodulator, SCs exhibit immune functions in the context of neurorepair, including recognition and presentation of antigens and recruitment of immune cells, especially

macrophages[22]. However, in the cancer environment, after activated by cancer cells, the immunomodulatory function of repair like SCs are less clear, including chemoattraction and polarization of immune cells, which may also support cancer by enhancing immune tolerance and immunosuppression. Therefor, the proposed project attempts to solve these two questions:

Question 1: Are SCs-recruited macrophages the major source of tumor-associated macrophages?

Question 2: How SCs regulate macrophages to form an immunosuppressive environment to promote tumor development after being activated by cancer cells?

Digital microfluidic (DMF) platform is an ideal platform to construct in vitro PNI models.

In vitro cultured cells and animals are widely used to model PNI and test potential therapeutics[23]. For instance, although in vivo studies have the advantage of emulating physiological complexity and maintaining the whole organism intact, it is difficult to accurately decouple the specific cell–cell interactions of interest from interference of other cells and tissues. For the in vitro models, The Transwell assay is the simplest method to study PNI and is mainly used to examine the chemotaxis of neural-secreted cytokines or chemokines on cancer cells. However, it cannot fully replicate cancer cell activities in the extracellular microenvironment, especially the direct cell-cell interactions occurring in the PNI process.

The tissue engineering 3D culture system consists of natural and/or synthetic polymers that mimic physicochemical cues present in tissue extracellular matrix (ECM) and provides an optimal platform for studying cell-cell and cell-ECM interactions. However, they lack advanced fluid/droplet manipulation platforms to achieve in vivo-like 3D structures. The 3D models they constructed are too simple to represent the complex nature of in vivo physiology[19][20].

The microfluidic techniques are promising platforms for PNI model construction, which has been widely used for neurodegeneration disease modelling[24] (Fig.4). But conventional microfluidic techniques are usually necessary to design an intricate microchannel network. And it usually includes complex components, like valves, some external modules such as syringe pumps, and detectors, which complicate the fabrication and assembly process of the chip system. On the other hand, we all know that all the tissues in the body are highly organized and dynamic and compose of various types of cells. Although currently utilized microfluidic-based approaches enable cell-cell interactions in 3D, they lack the ability to control the organization of multiple cell types and cannot build complex 3D architectures, which typically requires manual manipulations of cells and biomaterials.

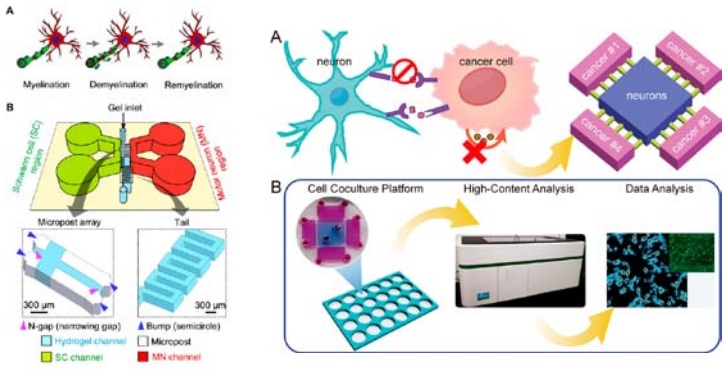

Fig 4. Neurodegeneration disease model in traditional microfluidics (left). Neuron and cancer cell interaction platform based on traditional microfluidics (right).

Compared to the traditional microfluidics, the digital microfluidic (DMF) is an innovated and powerful platform for droplet operation. The DMF platform is a microfluidic technique that performs various operations on droplets and particles and can directly rive and reorganize distinct hydrogels containing different cells to establish complex 3D microstructures[25]. The system is simple to make, does not require complicated microchannel networks and external modules, and can track the movement and behavior of single cells in real time. Furthermore, the detachable substrate allows in-depth biological analysis. Li et al.[26] reported cell invasion in a digital

microfluidic microgel system (CIMMS) with the ability to isolate subpopulations of invading cells for RNA-seq analysis. In this system, microgels were first formed with collagen I core surrounded by a shell of basement membrane extract (BME). When droplets of cell suspension were actuated touching the edge of microgels, the device was rotated 90° to settle the cells on the microgel. As soon as the cells were attached, the device was returned to its original orientation, generating an invasion model for further analysis by confocal immunofluorescence microscopy and transcriptome sequencing (Fig.5). Prof. Shih-Kang Fan's group has engineered in vitro liver tissues on DMF, containing liver cell, endothelial cells, and macrophages in GelMA. This liver tissue can be co-cultivated for more than 2 weeks, and its structure and function are very similar to those in vivo. This liver tissue was used for toxicity testing of acetaminophen (APAP) at different concentrations (Fig.6).

Therefore, the DMF platform would be an ideal platform to construct PNI models to study Schwann cell and macrophage interactions due to its ability to control the organization of multiple cell types and build complex 3D architectures.

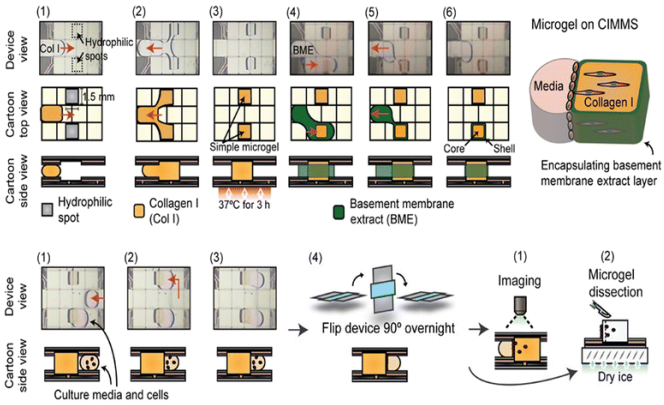

Fig. 5 DMF-based cell invasion analysis by introducing microgels, which are formed with a collagen I core surrounded by a shell of basement membrane extract.

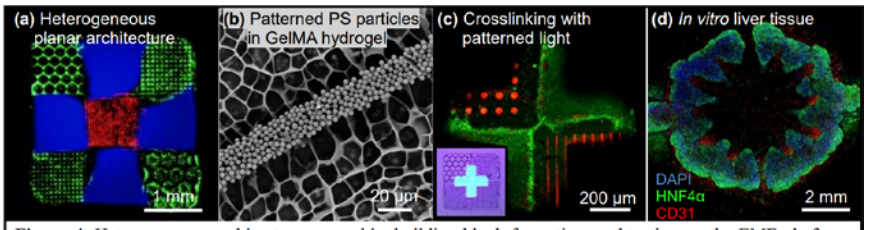

Figure 4. Heterogeneous architectures assembly, building block formation, and testing on the EMF platform. **(a)** Contacting 9 PEGDA droplets containing dye (blue) and particles (green and red) for architecture (3 mm × 3 mm × 100 µm) assembly and crosslinking by UV light. **(b)** SEM of 3 µm PS particles patterned by DEP in 5% GelMA hydrogel. **(c)** Building blocks with arbitrary geometries (shown cross-shaped) prepared by partial crosslinking of assembled droplets with adjustable UV light patterns (shown cross in inset). **(d)** Engineered liver tissue containing liver cells (Huh-7, green), blood vessel cells (HUVEC, red), and macrophages (THP-1) is constructed in GelMA (3-10% for stiffness) hydrogels for acetaminophen (APAP) toxicity testing.

Fig. 6 d) Engineered in vitro liver tissues for organs-on-a-chip, containing liver cells (Huh-7, HNF4α-stained, green), endothelial cells (HUVEC, CD31-stained, red), and macrophages (differentiated THP-1) in GelMA, are prepared, co-cultivated for more than 2 weeks.

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