Building quantitative systems pharmacology models from scratch: Filling in the gaps

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Abstract

While working for a commercial pioneer in the quantitative systems pharmacology space, a common refrain was that biologist don't take the right data when it comes to building mechanistic mathematical models of disease. Yet, the company was reluctant to publish primary research articles showing how existing data are used and what kind of data are needed. Over time, this blanket critique of biologists, to me, seemed a bit hypocritical. On the flip-side, a common critique levied by biologists about mathematical models is that they just reproduce that which we already know. So in re-entering academia with a single ticket to play at West Virginia University, I wanted to change that narrative or simply try not to be a hypocrite. Here, I will discuss our approach for using mechanistic mathematical models to better understand complex, dynamical systems that one encounters in biology and for filling in some of the gaps in our understanding of these systems in the context of disease biology.

Returning to academia from "the dark side"

Quantitative systems pharmacology (QSP) is an emerging field where mechanistic mathematical models are used by the pharmaceutical industry to develop new drug therapies and optimize their efficacy using dose-scheduling simulations [1]. My first real PhD job was with one of the commercial pioneers in the QSP space: Entelos, Inc. At Entelos, interdisciplinary teams of PhD engineers and life scientists created QSP models from scratch based on the available literature, which required reading a lot of papers. In the 5 years that I was at Entelos, I had read close to 3000 papers. Reading these papers, building mathematical models in collaboration with my life science colleagues, and consulting outside scientific experts [2, 3, 4] shaped the scientific perspective that I took with me when I returned to academia as an independent investigator.

Initially, I focused on how cells integrated signals internally by analyzing data generated by others (e.g., [5, 6, 7]) and, increasingly, my own group using mechanistic mathematical models (e.g., [8]). While there was a robust community that modeled signaling cascades within cells, two events encouraged me to take a different direction. The first event was feedback on a proposal where the reviewer mentioned that I should find a scientific space that doesn't compete with larger groups at more prestigious institutions. In other words, it is more difficult to compete for federal funding against investigators that reside at institutions that have hidden labor advantages over my current institution, as described recently in [9]. These hidden labor advantages may be greater start-up funds, larger internal pilot grants, endowed chair positions, independently funded research assistantships, and donations from private foundations. The second event was emerging literature illustrating the extensive and sustained rounds of mutations that occur during oncogenesis. Mutations in

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malignant cells can change the abundance of proteins that transmit information within cells or can alter the structure of these same proteins to alter with whom they interact. In contrast to a common conceptual view of intracellular signaling as an electrical circuit with a fixed topology, these alterations can collectively rewire signal transduction within a cell [10, 11]. Moreover, these events collectively suggested to me that developing predictive mechanistic models of how malignant cells integrate extracellular signals internally to elicit a cellular response would be a difficult path to take.

Shifting focus to tumor-immune cross-talk

Within tissues, signaling among different cell types is important for organizing the right response. While malignant cells may have rewired intracellular signaling pathways, all of the other normal cells within the tissue microenvironment should respond canonically to extracellular signals, assuming that we know what signals are transmitted between cells. One cell type that I had previously modeled was the dendritic cell [6, 7]. As scouts of the immune system, this specialized cell senses threats by recognizing molecular patterns associated with pathogens or traumatic cell death through genetically encoded sensors and secretes signals that activate other cells that are adapted to get rid of the threat, like Natural Killer (NK) cells and cytotoxic CD8+ T lymphocytes (CTL). One of these secreted signals is the cytokine Interleukin-12 p70 (IL12), which was originally called Natural Killer cell stimulating factor and also a subject of my prior modeling work [5]. A low-level of endogenous IL12 signaling, that is tonic IL12 signaling, within the tumor microenvironment underpins the enhanced anti-tumor immune response enhanced by immune checkpoint blockade [12].

Given that oncogenesis is an evolutionary process associated with mutation and selection, malignant cells that arise within a tissue may potentially interrupt this communication between dendritic cells and NK/CTL cells. Towards this aim, we set up a phenotypic screening assay - an assay where you model a cellular behavior and see if changing the experimental conditions changes the cellular behavior [13]. If it does, this is followed by identifying the factor responsible for the change in phenotype. The phenotype that we were modeling was a T cell response to IL12 in the production and release of IFNγ, a cytokine that enables CTLs to recognize their target cells. We had also recently published a quantitative study of a T helper cell line that responded to IL12 [14]. Experimentally, we tested whether the T cell response to IL12 changed in the presence of a mouse cancer cell line: B16F0. The B16 model and its' variants are interesting as they have been considered the gold standard for testing immunotherapies in pre-clinical mouse models [15], as essentially most interventions dont work in it.

To extract as much information as we could from this experiment involving putting two cell lines in a dish, a time-course and multiple read-outs were included in the experimental design. Interestingly, the time-course data suggested that the tumor cell was secreting something that was interfering with the T cell response to IL12. Having a mechanistic model of the T cell response to IL12 helped rule out some competing hypotheses, like a reduction in IFNγ production was due to a reduction in T cell viability or a reduction in the concentration of IL12. Turns out the B16 cells over-express one component of the IL12 receptor, IL12RB2, that sequesters extracellular IL12 and redirects the extracellular IL12 signal to not activate STAT4 within the cell but enhance cell viability by activating Akt [11].

To identify this secreted factor using methods that weren't observationally biased like antibody arrays, we incorporated a mass spectrometry-based proteomic component into the study. The analysis of the proteins secreted by the B16F0 cells suggested a short list of candidates that included a number of secreted proteins and extracellular vesicles. Given a head-to-head comparison of likely potential targets summarized in [16], we decided to focus on the secreted matricellular protein Cell Communication Network factor 4 (CCN4; previously known as Wnt-inducible Signaling Protein - 1 (WISP1)). Antibody neutralization and supplementing culture media with the recombinant protein were used to validate that CCN4 inhibited IFNγ production [13]. Extracellular vesicles also contained a complicated payload of proteins and coding and non-coding RNA that functionally altered T cell responses [17, 18]. While elaborating on the role of extracellular vesicles in establishing an immunosuppressive tumor microenvironment is intriguing, the fastest path to translating our findings to the clinic would be to target a secreted immunosuppressive protein, like CCN4, using an antibody.

Clarifying CCN4 biology

Thinking about the blanket critique that mathematical models just reproduce what is already known, to me the value of mathematical modeling is clarifying what we know and don't know about biology rather than just fitting data [2]. A recent study highlights that 90% of publications focus on only 20% of the genome [19]. So there are lots of genes and their products that we don't know what they do. For instance, CCN4 is not one of the 20% of genes that are well-characterized. CCN4 biology is still a bit murky, as there are only about 500 papers related to CCN4/WISP1 that have been published since 1993. Databases - like CellPhoneDB [20], CellTalkDB [21] or others reviewed in [22] - used to predict cell-cell communication from single-cell RNA seq data have no information about CCN4. Moreover natural language processing models, like ChatGPT, are unlikely to provide help as they draw from existing knowledge. Clarifying CCN4 biology requires additional experimental investigation.

In the context of biomedicine, a lack of information related to a gene is a weak motivator for further study. A stronger motivator is a clinical correlation. In the study of cancer, one of the first questions that people ask is whether expression of the gene of interest is changed in a tumor relative to normal tissue. In contrast to an earlier time where one would have to gather and analyze tissue samples themselves, recent public repositories of data obtained from tumor and normal tissue facilitate such comparisons. Leveraging these public databases, I found that CCN4 is upregulated in essentially every tumor sample from patients with invasive breast cancer but not in normal tissue [23]. In the context of melanoma, we also found that increased CCN4 expression correlates with a worse outcome (see Figure 1 in [24]) and that malignant melanocytes are a source of CCN4 within the tumor (see Figure 1 in [25]).

Observing that CCN4 was increased in invasive breast cancer and unregulated at the invasive front of melanoma nests motivated a follow-on study to clarify the association between CCN4 expression and invasion. Using a combination of wet experiments and mechanistic mathematical modeling, we found that disrupting adherens junctions, which occurs during invasion, increases CCN4 expression with an interlocked positive and negative feedback network motif [26] and summarized in Figure 1. Interestingly, genetic alterations that activate this pathway were significantly enriched in melanoma compared to random chance [24], as summarized in Figure 1C. For instance, copy number amplifications of *CCN4*, which would increase the amount of mRNA produced in response to a transcriptional cue, is enriched in 4% of samples. The majority of missense or truncating mutations in the *CDH1* gene occur in the extracellular cadherin domains, which has the potential to disrupt homotypic binding and promote constitutive pathway activity [27]. Mutations in *CTNNB1* preferentially occur in Exon 3, which is in the N-terminus and helps target the protein to the proteosome for degradation. Similar mutations have been found to increase nuclear accumulation of beta-catenin [28]. Truncating mutations in *APC* that lead to impaired function were enriched. As *APC* negatively regulates beta-catenin activity, these mutations would lead to enhanced pathway activity [29]. Given that mutations in these four genes mentioned all have the same functional response, it is interesting to note that these mutations are non-overlapping.

Ok, so increased CCN4 is associated with a worse outcome and CCN4 is upregulated in invasive cancers, but what does it do? Well, secreted proteins can have two different effects termed autocrine and paracrine effects. First, autocrine effects can influence the behavior of the cell that makes it. One can think of this as the out-loud self-talk that we sometimes use to encourage ourselves to complete a task - "You can do this!". Second, paracrine effects can influence the behavior of other cells in the local vicinity that are able to respond to the secreted signal. One can think of this as the cross-talk between different players of the same soccer team as they play an opponent - they yell "Man on, man on" to warn that an opposing player is approaching outside of their field of vision.

In cases where the particular secreted protein is linked to the 20% of genes that are well-studied, clarifying these two different effects is a bit easier. In such cases, the experimental tools have been refined and you have a strong guess as to what you're looking for. Unfortunately, CCN4 is not one of the well-studied genes. Extrapolating from other modes of intercellular communication like growth factor signaling, one might assume that secreted CCN4 elicits a cellular response by binding to a defined cell surface receptor. However that would be misleading, as the unsuccessful focus of the scientific community on identifying the "CCN4 cell surface receptor" was a barrier for progress within the field [30]. Instead, CCN proteins should be thought of as organizers of a bidirectional interaction network between a cell and the extracellular matrix by docking extracellular proteins with cell surface proteins like integrins. While this new perspective is helpful,

how CCN4 acts on specific cells within the tumor microenvironment remains unclear. Moreover, inconsistent experimental design in some related work further clouds the field (e.g., convoluted mouse-human experimental design and gamed concentrations [31, 32], transient overexpression using a strong CMV promoter [33], or use of xenograft models [34]).

To clarify CCN4 biology, we decided to address the self-talk or autocrine signaling first. In the context of melanoma, we published two papers to help clarify how CCN4 altered the behavior of melanoma cells [24, 35]. While all cells have the capability to express every gene, access to the DNA within a cell is modified during development to shape what genes are expressed by the cell and, ultimately, the cell's biological function. As malignant melanocytes and malignant breast cancer cells have very different developmental origins, we would expect that the action of CCN4 on breast cancer cells is likely different than on melanocytes. That said, we used an unsupervised machine learning approach to identify the expression of genes associated with epithelial-like and mesenchymal-like states [36]. Interestingly, CCN4 was one of only three secreted proteins that were associated with a mesenchymal-like state in both melanoma and breast cancer. We are working on clarifying the effects of CCN4 in these different cellular contexts.

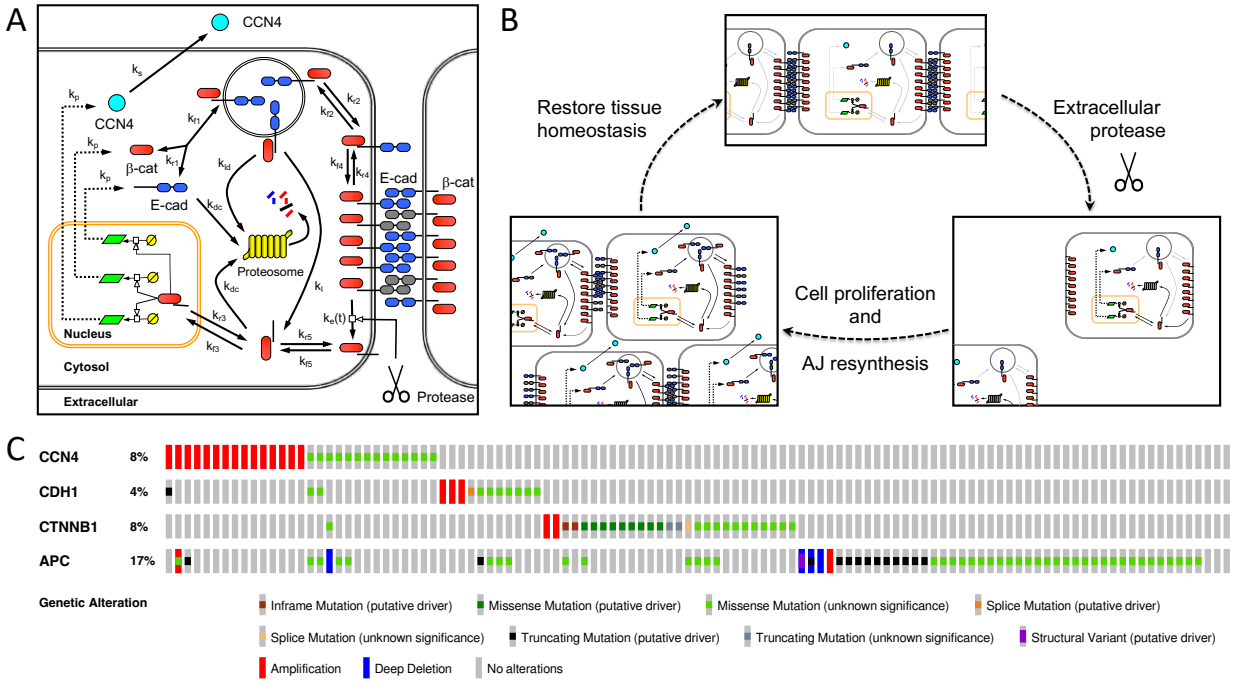

Figure 1: **Schematic diagram of the signaling network associated with non-canonical activation of beta-catenin upon disrupting adherens junctions and associated mutational landscape observed in melanoma.** (A) Schematic diagram of the network topology associated with the adherens pathway activity of beta-catenin. Adherens junctions are formed by homotypic interactions between the extracellular cadherin domains of a single pass transmembrane protein E-cadherin (E-cad: blue double oval). The cytoplasmic tail of E-cadherin provides a scaffold for a multi-protein complex that includes beta-catenin (β-cat: red oval). (B) Upon the proteolytic cleavage of adherens junctions, a cytoplasmic fragment of E-cadherin and associated catenins (tBE) are transported to the cytoplasm. In the cytoplasm, the cytoplasmic fragment of E-cadherin and associated catenins can either enter the nucleus or undergo proteasomal degradation. In the nucleus, this multi-protein complex promotes the transcription and translation of CCN4, beta-catenin, and E-cadherin, among other factors. mRNA is represented by light green parallelogram. Once synthesized, CCN4 (light blue circle) is secreted. Newly synthesized beta-catenin and E-cadherin reform the multi-protein complex and are transported to the cell membrane to re-establish adherens junctions. If no E-cadherin binding sites are present, the multi-protein complex is internalized and degraded. (C) Graphical summary of mutations that include both changes in single nucleotides and in copy numbers for skin cutaneous melanoma (TCGA, PanCancer Atlas) samples. Of the 363 samples contained in the dataset obtained through cBioPortal (retrieved 03/02/2023), the queried genes are altered in 110 (30%) of the samples.

In contrast to the different anatomical origins of these two malignancies, the immune system is distributed throughout the body and regulated by similar mechanisms irrespective of the anatomical location. Finding the same mechanism to suppress host immunity at work in different cancers may suggest a convergent evolutionary path to oncogenesis and an interesting therapeutic target. However, identifying cross-talk between different cell types for a relatively understudied gene is difficult. To do this, we took a two-pronged approach, one traditional and one data-driven.

First, we used a traditional approach in cancer immunology: remove the gene of interest in a transplantable syngeneic tumor cell line, implant the wild-type and knock-out variants of the tumor cell line in immunocompetent mice, and look for changes in tumor growth. If there are differences in tumor growth, then isolate the tumors, digest into single-cell suspensions, and characterize the difference in prevalence of immune cell types using flow cytometry. In a recent paper [25], we report findings using two different syngeneic mouse melanoma models, where knocking out CCN4 reduced tumor size and increased the prevalence of CTLs and NK cells in the tumor. We are currently working towards clarifying the molecular mechanisms that underpin these responses using a different approach.

One limitation of the traditional cancer immunology approach is that maybe you're missing something as you have to decide what to measure when you design the experiment. Sure single-cell RNA sequencing can address this point, but (A) it's really expensive (it's almost 10x the cost of sequencing each biological replicate by bulk RNA-seq), (B) you're only focusing on the cellular transcriptome of the cells that you can isolate following a particular enzymatic digestion protocol, and (C) it is a relative new technique so that it's difficult to know if one is observationally biased. A second limitation is based on using mouse models of cancer, where translating the findings in mice to humans can be unclear and a common source of critique. Sure there are some "humanized" mouse models but, to me, these are not a good choice here. If you observe something in these "humanized" mouse models, the results should be interpreted with caution due to potential artifacts of putting two different species together.

How about taking actual human data and predicting how increased expression of a gene influences the prevalence and functional orientation of different cell types within a particular tissue, like the skin in the case of melanoma or mammary gland in the case of breast cancer? Given public databases that contain gene expression information obtained from samples of melanoma and breast cancer tissue, we combined two different computational approaches to predict how a gene, like CCN4, alters the network of cells within a tissue during oncogenesis [37]. First, we used tissue deconvolution methods to infer the prevalence and functional orientation of stromal, immune, and malignant cell subsets within a collection of bulk tissue samples. Next, we used Bayesian network inference to predict how expression of a gene by malignant cells causally alters the prevalence and functional orientation of these different cell types within the tumor microenvironment. Specifically, Bayesian network inference predicted that increased CCN4 expression promotes a mesenchymal phenotype in breast cancer, inhibits the cytotoxic activity of NK cells, and increases the prevalence

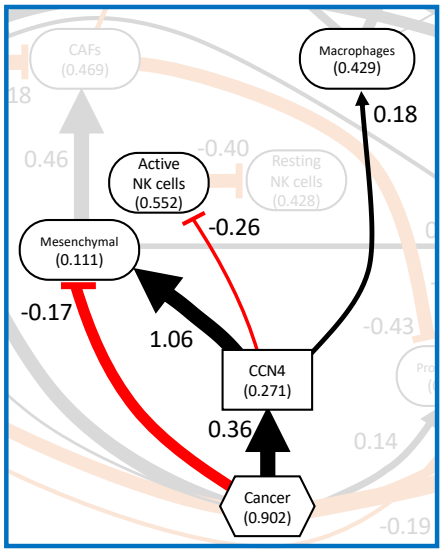

Figure 2: **Bayesian network inference predicts that CCN4 expression promotes a mesenchymal phenotype in breast cancer, inhibits the cytotoxic activity of NK cells, and increases the prevalence of macrophages in the tumor microenvironment.** A directed acyclic graph (DAG) representing the conditional probability distribution inferred using the digital cytometry features extracted from cross-sectional tissue samples included in the breast cancer arm of the TCGA, as described in [37]. The black arcs represent a positive causal relation while red arcs represent a negative or inhibitory causal relation. The width of the arc is proportional to the confidence in the existence of an arc and the number beside the arc annotates the extent of influence of the parental node on the target node. The number included within the node symbol represents the average normalized value of the digital cytometry feature within the dataset with values of all of the parental nodes set to zero.

of macrophages in the tumor microenvironment (see Figure 2). We validated the predictions using results obtained from syngeneic mouse models. While exactly how these causal effects are mediated are not clear at this point, these predictions enable narrowing the focus to specific cell types and simplifying the experimental design to in vitro study of isolated cell types. Overall, we think that this data-driven approach helps motivate focused experiments to clarify CCN4's role in suppressing anti-tumor immunity. Moving forward, we would also like to elaborate on this approach to infer cellular cross-talk within tissues, as summarized in [38].

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