2013 Final Progress Report for Tal Danino
Title: Engineered minicells and probiotics for cancer therapeutics and diagnostics

In 2013 under Misrock funding, several directions in our project goals progressed and have been iterated on based on what has been learned to date. The candidate published a first author paper in the area of synthetic biology, and has a manuscript in review at *Science*. Our experimental progress is summarized below. In short, minicells have been determined to act as *static* genetically engineered delivery vehicles, which are still being pursued in different directions from the original proposal, but the major shift of our work has been towards probiotics which can be engineered as active sensors.

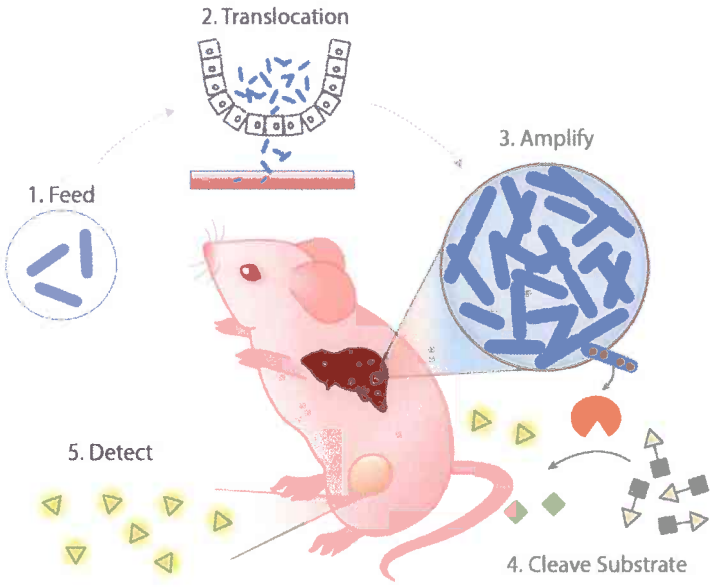

Figure 1: Programmable Probiotics (PROP) for noninvasive cancer detection. a) The PROP-laZ diagnostic platform is made up of probiotic E. coli Nissle 1917 (EcN) bacteria transformed with a dual-stabilized, high expression lacZ vector. (1) PROP-Z is delivered orally. (2) Bacteria rapidly (24 hours) translocate across the GI tract and (3) specifically amplify within metastatic tumors in the liver. (4) PROP-Z express high levels of the lacZ enzymatic marker, enabling urinary detection via injected cleavable substrates.

Previous work on engineered minicells and transition to safe probiotic delivery. Our ultimate goal is to engineer a genetically programmable vehicle to deliver cancer therapeutics to tumor environments. While minicells were proposed to be a promising balance between nanoparticles and liver bacterial vectors, our work showed that we could not independently induce minicells in microfluidic devices. We continue to pursue the use of minicells both as static, targetable delivery vehicles and as freshly created drug delivery vehicles that are produced in situ within the tumor. As an alternative to minicells, we proposed using probiotic bacteria that can be orally administered as our safe delivery vehicles. We chose the probiotic E.coli Nissle, a probiotic currently prescribed in humans and developed a platform called Programmable Probiotics (PROP), which we used to produce large quantities of an enzyme called lacZ which can enable detection of the tumor via urinalysis (PROP-Z, Fig. 1). This manuscript is currently in revision at *Science*.

We sought to apply PROP-Z probiotics in a pre-clinical assay to measure its capacity to overcome the clinical challenge of detecting cancer metastases, which are ultimately responsible for 90% of all cancer-related deaths but remain difficult to detect because of their small size and multiplicity. The portal venous system flows from the GI tract to the liver, thus, we hypothesized that after oral administration, probiotics would follow blood flow patterns and directly colonize liver metastases. To test this prediction, we chose a syngeneic model of colorectal cancer liver metastases (Fig. 2A). To directly visualize probiotics within the liver metastases, we orally administered PROP-Z bacteria expressing a chromosomally-integrated luminescence cassette. These small doses of bacterial were sufficient to detect luminescent signals in metastases as small as 1 mm, and we observed bacterial infiltration in virtually all tumor cores (Fig. 2B,C). To quantify the specificity of colonization, we developed a quantitative PCR-based assay to measure PROP-Z bacteria in various organs following oral administration. Over a period of 7 days, we measured the number of bacteria in liver metastases and found a striking colonization level of 10^6 bacteria (Fig. 2D). In stark contrast, PROP-Z bacteria infiltration in control organs (spleen and kidneys) was below the limits of detection for our q-PCR assay (< 500 bacteria/g). As confirmation of this negative result, we assayed for off-target colonization by performing colony counts of entire organs, and observed zero PROP-Z bacteria in any control organ tested. As a result of zero off-target colonization, PROP-Z treated mice survived without any noticeable adverse effects for at least 9 months. Collectively, these findings constitute the first demonstration of GI translocation and specific colonization of liver metastases by oral delivery of the probiotic EcN, setting the stage for chaperoned delivery of gene circuits to systemic tumors via oral delivery.

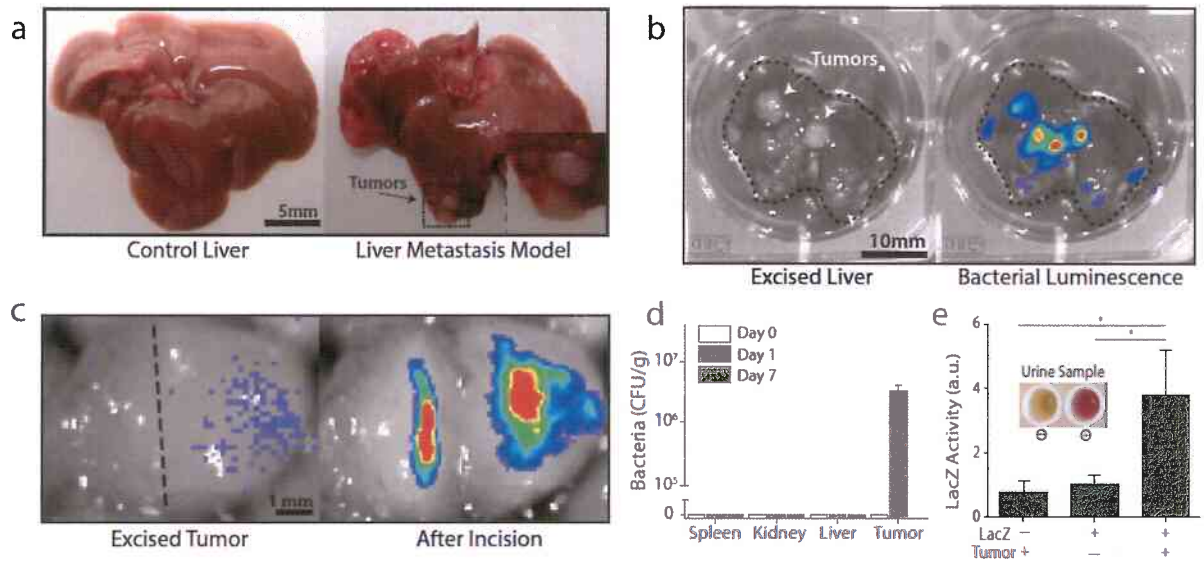

Figure 2. Rapid, specific, and minimally invasive detection of millimeter-scale metastatic tumors. We generated liver models of metastasis to test the performance of our diagnostic on a relevant clinical problem—the early detection of small tumors difficult to detect using traditional methods. **a)** Healthy (top) and metastatic livers (bottom), showing small tumor nodules. **b)** 24 hours following oral administration, PROP-Z bacteria were detected in tumor nodules via IVIS measurement. **c)** PROP-Z bacteria colonize the inner core of metastatic nodules are often not

visible from the exterior (top) but are revealed by cutting to the interior (bottom). Dashed line (top) indicates cut site. **d)** To determine safety and specificity, we colony counted PROP-Z in organs following oral administration. We observed 10^6 bacteria colonize tumors after 24 hours with zero colonization (0 CFU/g) in off-target organs and no growth over time (*means +- s.e.m, n=4 each*).**e)** PROP-Z bacteria detect the presence of metastatic tumors via tumor-specific growth leading to high-level expression of lacZ. We quantified enzymatic activity by injecting Lu-gal, a lacZ substrate that when cleaved produces luciferin, and assayed the urine for luminescence (*means +- s.e.m, n=6 for each condition, 2 tail students t-test, $p<0.05$*).

In order to precisely quantify the capacity of our diagnostic to detect small metastatic tumors noninvasively from the urine, we performed luciferase assays using the systemically administered substrate LuGal. LuGal is a safe, commercially available luciferin-galactoside conjugate that, when cleaved by lacZ, acts as a substrate for luciferase to produce luminescence. Using only a small sample (1µl) of the collected urine, we detected millimeter-scale metastatic tumors as early as 24 hours following oral administration of PROP-Z bacteria. In quantifying diagnostic performance on multiple replicates including healthy mice, mock surgeries, and unmodified EcN bacteria, we measured a total in vivo signal-to-noise ratio (SNR) of approximately 4 (Fig. 2E). This SNR was sufficient for the CPRG substrate to achieve readily visible urinary color changes, specifically in the presence of small tumors (Fig. 2E, inset). Since clinically relevant point-of-care diagnosis is dependent on a strong SNR to accurately classify a disease state based on patient samples, the most critical aspects of any diagnostic technology are its sensitivity and specificity. Our PROP-Z platform derives sensitivity from its multiple sources of amplification: exponential bacterial growth, aggregation of signal from multiple metastases, enzymatic cleavage by lacZ, and renal concentration in the urine. While the PROP-Z platform can also achieve notable sensitivity, our specific implementation of the platform is primarily bounded by the pharmacokinetics of the injected substrate(s), as well as its background cleavage by the tumor microenvironment. Thus, the high specificity (10^6-fold enrichment) observed with our diagnostic is achieved in part due to its interaction with the immune system, in that growth of the probiotic chaperone is permitted only within the tumor microenvironment, and prevented in all other sites.

In translating any new medical technology, the central questions involve the degree to which the platform will function safely and effectively in human patients. The PROP-Z system features a probiotic chaperone that is currently prescribed in humans and can be cleared readily with antibiotics. In addition, safety has also been previously demonstrated using attenuated S. typhimurium bacteria in human clinical trials. A remaining open question regarding the efficacy of our PROP-Z platform relates to the trafficking of microbes in the human gut. It has yet to be determined to what extent the specific microbiome of a patient may influence the rate of EcN translocation, nor can it be predicted how an individual's immune response might affect the specificity of colonization. As a modular, evolving platform, subsequent iterations of the process may extend to support paper test modalities, incorporate substrates for colorimetric and/or MRI-based diagnosis, and be programmed to integrate with other synthetic biomarkers for cancer. In the present proof-of-concept study, we have already established a diagnostic strategy compatible with the existing clinical paradigm of urinalysis. Ultimately, we envision that complex synthetic gene circuits may reach beyond the broad diagnostic domains discussed here, and be

extended to applications in therapeutic strategies that utilize self-triggered gene circuits, such as quorum sensing, to deliver clinical payloads to the densest tumor regions.

Publications.

Danino T, Prindle A, Hasty J, Bhatia S "Measuring growth and gene expression dynamics of tumor-targeted S. typhimurium bacteria" *Journal of Visualized Experiments*, 2013 Jul 6;(77):e50540. doi: 10.3791/50540.

In submission.

Programmable Probiotics: Genetic Circuit Chaperones for Noninvasive Cancer Detection