Examining Breast Cancer Response to Metformin Using Tissue-Engineered *in vitro* Testbeds

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Statement of Purpose: In the United States, 1 in 8 women will develop breast cancer in their lifetime [1]. Sadly, the American Cancer Society estimates 280,000 new cases of invasive breast cancer will be diagnosed in 2021 alone. Recent studies show that breast tumor innervation, in which neurites invade adjacent breast tumors, plays a critical role in accelerating cancer progression and invasion and contributing to poor patient prognosis [2,3]. Metformin has recently been identified as a breast cancer therapy; however, this treatment contributes to vitamin B12 deficiency in patients [4]. In response, clinicians prescribe supplementation, but over-supplementation of vitamin B12 is linked to special cases of increased neural cell survival and neurite outgrowth [5, 6]. There lacks understanding if vitamin B12-induced neurite outgrowth occurs in breast cancer; however, we hypothesize that over-supplementation of vitamin B12 promotes breast tumor innervation and cancer metastasis [7]. *Here, we establish a tissue-engineered platform for modeling breast cancer in vitro, evaluate the effect of Metformin on cell viability and phenotype, and determine the concentration of Metformin most effective in metabolism and cytokine secretion reduction.* These discoveries will aid in future studies investigating vitamin B12-induced neurite outgrowth and metastatic potential in breast cancer and the potential identification of viable targets for breast cancer therapies.

Methods: Subcutaneous adipose tissue from male Sprague Dawley rats was harvested, decellularized based on a previously described protocol [8], and digested into a hydrogel. This gel was combined with a rat tail collagen-I hydrogel 1:1 to mimic the stiff breast tumor ECM. Gelation kinetics and immunohistochemistry were performed to establish collagen fibril polymerization. Normal (HC11) and cancerous (4T1) mammary epithelial cells were embedded in the composite hydrogel with and without 3T3-L1 pre-adipocytes to replicate the environment of early- and late-stage breast cancer, respectively. The hydrogels were cultured for 1 week in complete DMEM supplemented with 0, 1, 2.5, 5, or 10mM of Metformin. Cell metabolism following Metformin treatment was evaluated using alamarBlue, and VEGF secretion was determined via Luminex and normalized by concentration per ng double-stranded DNA. Immunofluorescence was performed against N-cadherin and αSMA. Preliminary co-cultures with male BALB/c mouse dorsal root ganglia (DRG) were conducted to establish for neurite outgrowth with 0mM vitamin B12. Immunofluorescence was performed against β-III tubulin.

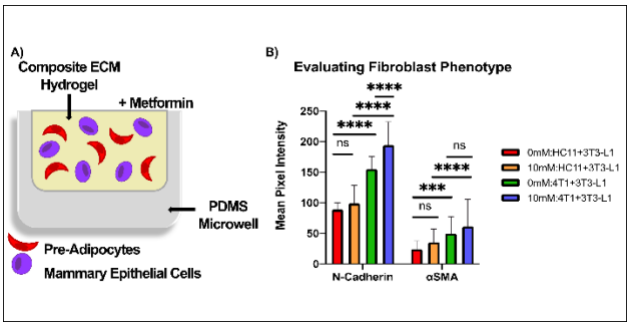

*Figure 1: Three-dimensional composite ECM hydrogel is used to study breast cancer in vitro (A), and pre-adipocytes exhibit change phenotype when cultured with cancer cells and treated with Metformin (B). ns not significant, ***p<0.005, ****p<0.0001.*

Results*:* The composite hydrogel underwent complete thermal polymerization, indicating a native tumor microenvironment culture model. Cultures of 4T1+3T3-L1 cells (Figure 1A) showed stable cell metabolism and significantly increased VEGF levels ($p<0.0001$) with 5mM and 10mM Metformin. Moreover, 3T3-L1 cells exhibited a significant increase in N-cadherin ($p<0.0001$) and αSMA ($p<0.005$) when cultured with 4T1 cells and significantly different N-cadherin levels ($p<0.0001$) with increased Metformin treatment (Figure 1B). This suggests that 3T3-L1 cells differentiate into myofibroblasts and act as cancer-associated fibroblasts in the presence of cancer cells and high Metformin dosage. Early neurite outgrowth was established in DRG cultures, predicting future successful neurite outgrowth studies. These results show that the composite ECM hydrogel allows for the physiologically relevant *in vitro* study of breast cancer. In future studies, 10mM Metformin will be utilized. Future DRG co-cultures will be used to understand vitamin B12-associated neurite outgrowth in breast cancer and the resulting effect on brain metastatic potential.

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