Engineering a Three-Dimensional *In Vitro* Model of Bone Sarcoma for Drug-Testing

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Statement of Purpose: The current anticancer drug testing paradigm is costly and inefficient, as potential drug candidates take years to advance through standard preclinical and clinical testing, but only few ultimately gain regulatory approval. Ideally, ineffective therapies should be eliminated early, but current *in vitro* drug testing based on widely-used monolayer cultures remain a poor predictor of drug efficacy as cells are separated from their three-dimensional (3D) *in vivo* microenvironment. We hypothesize that an electrospun poly(ε-caprolactone) (PCL) scaffold will provide critical 3D structural cues for the culture of bone sarcoma cells that support their native phenotype *ex vivo*, and therefore elicit a clinically-relevant drug response. In this study, we aimed to investigate (1) the 3D morphology and growth characteristics of an Ewing's sarcoma (EWS) cell line (TC-71) cultured within an electrospun PCL fiber mesh, (2) the ability of these 3D-cultured cells to exhibit an *in vivo*-like protein expression profile, and (3) the 3D model-derived drug response to doxorubicin, a cytotoxic chemotherapy integral to bone sarcoma treatment.

Methods: Scaffolds were electrospun to produce uniform discs (1 mm thick; 8 mm diameter) composed of 11.6±1.7 μm nonwoven PCL fibers. 250,000 TC-71 cells were seeded per scaffold and resulting constructs were cultured in 12-well plates. For 2D monolayer culture, 50,000 cells were seeded in 12-well plates. Male non-obese diabetic SCID$^{-/-}$ and CB17/SCID$^{-/-}$ mice were used to generate subcutaneous xenografts (10^6 cells injected/animal). Scanning electron microscopy (SEM) was used to characterize the morphology of the 3D-cultured cells. Growth profile was obtained by measuring DNA content or tumor volume as an index of proliferation for cells in 2D and 3D or *in vivo*, respectively. To assess the extent to which the 3D-cultured cells mimic the *in vivo* phenotype with respect to the clinically-investigated IGF-1R/mTOR signaling pathway, and differ from those cultured as monolayer, isolated proteins were analyzed by western blotting. Sensitivity of cells to doxorubicin in the three EWS models was evaluated by measuring cell viability (normalized to untreated controls) after exposure to increasing doses of the drug.

Results: At Day 2 in culture, TC-71 cells adhered to the scaffold in sparse clusters and eventually formed large, sheet-like aggregates by Day 20 (Fig. 1). Review by an experienced sarcoma pathologist identified the small, round-cell morphology characteristic of human EWS tumor. In contrast to 2D monolayer culture, TC-71 cells proliferated at a substantially slower rate within the 3D PCL scaffold and were more closely aligned with cells grown *in vivo* (Fig. 2). Beyond bearing close morphological resemblance and a similar growth profile

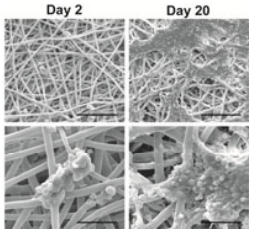

Fig.1 TC-71 cells in 3D PCL scaffold at low (upper panel; scale bar 200 μm) and high (lower panel; scale bar 50 μm) magnification.

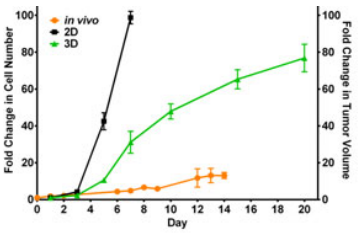

Fig.2 Growth profile of TC-71 cells in 2D monolayer and 3D PCL scaffold, as well as tumor volume in xenografts.

to that *in vivo*, striking differences were observed in the expression of proteins associated with the IGF-1R/mTOR signaling pathway (Fig. 3). Expression of phosphorylated IGF-1R was observed to be higher in the 3D EWS model than in 2D culture, suggesting constitutive activation of IGF-1R signaling (similar to that *in vivo*). Additionally, proteins associated with putative mechanisms of resistance to IGF-1R-targeted therapy (c-kit and HER2/neu) were more highly expressed in the 3D EWS model and xenografts, as compared to cells in 2D monolayer culture. The potential utility of this 3D EWS model as a drug-testing platform was investigated by comparing the response of TC-71 cells to doxorubicin where cells in 3D (IC_{50}: 2.738 μM) and *in vivo* (IC_{50}: 0.184 μM) demonstrated greater drug resistance than did cells in 2D (IC_{50}: 0.0122 μM) (Fig. 4).

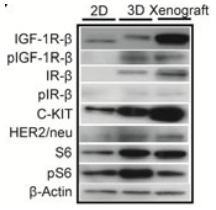

Fig.3 Western blot of IGF-1R/mTOR pathway-related proteins in TC-71 cells in 2D monolayer culture, 3D PCL scaffold, and *in vivo*.

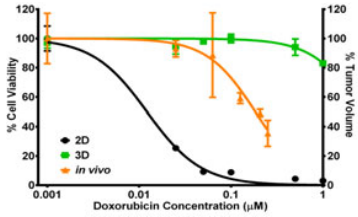

Fig.4 Response of TC-71 to doxorubicin in in 2D monolayer culture, 3D PCL scaffold, and *in vivo*.

Conclusions: We established an *ex vivo* 3D EWS model that closely mimics the *in vivo* tumor morphology, growth kinetics, and protein expression profile. EWS cells cultured in 3D PCL scaffolds were not only more resistant to traditional cytotoxic drugs than were cells in 2D monolayer culture but also exhibited remarkable differences in the expression pattern of the IGF-1R/mTOR pathway. This 3D model may have broad applicability for mechanistic studies of bone sarcomas and exhibits the potential to augment preclinical evaluation of antineoplastic drug candidates for these malignancies.

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