



## 2010 Research Grant Program Winning Abstract

### Urothelial Cancer Stem Cells and mTOR Signaling

By **Edward Diaz**

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Bladder cancer represents the fifth most common cancer within the United States. Despite treatments, the 5-year relative survival rate for Stage 2, or muscle invasive disease, is 63%, and it is less than 43% when there is local invasion or metastasis. Rates have remained stable over the last decade, illuminating the need to improve current treatment paradigms and expand the current armamentarium.

Urothelial carcinoma, which represents 90% of bladder cancers, is composed of a heterogeneous population of cells, among which researchers have identified cancer stem cells (CSCs). These cells demonstrate the ability for self-renewal and multipotency. One specific urothelial CSC lineage (CD44<sup>+</sup>, CK5<sup>+</sup>, CK20<sup>-</sup>) has been identified through a mouse xenograft model. Detection of the gene signature associated with this CSC lineage in patient samples was shown to correlate with shorter time to tumor recurrence. This aggressive behavior of CSCs is corroborated by other studies that demonstrate CSCs to be particularly resistant to conventional therapies such as radiation and chemotherapy. These findings underscore the need to better understand the biology of this cellular subgroup.

The mammalian target of rapamycin (mTOR) signaling cascade is a key pathway in the progression of different cancers. In bladder cancer, our group showed that mTOR activation correlated with reduced disease specific survival and increased pathologic staging, and administration of the mTOR inhibitor rapamycin to a human bladder cancer xenograft model yielded a 55% reduction in tumor volume. Another study in which our group collaborated revealed expression of mTOR pathway mRNAs preferentially in a basal (stem-like) cell subset of urothelial carcinoma cells. This provides early evidence that mTOR signaling may be important to the progression of bladder cancer, possibly via its activity in stem cells.

Our hypothesis is that the mTOR signaling cascade is crucial to the tumorigenic potential of bladder cancer stem cells. Our specific aims are:

1. Isolate CSCs from primary bladder cancer specimens. CSCs will be isolated according to studied CSC molecular markers (CD44<sup>+</sup>, CD133<sup>+</sup>).
2. Optimize a protocol to propagate bladder CSCs in vitro.
3. Study the expression pattern of mTOR cascade proteins within urothelial CSC populations, and effects of inhibiting the mTOR pathway with rapamycin on CSC cellular function.

Tissue and cells will be harvested from radical cystectomy specimens. Tumors will be dissociated into a cell suspension using a papain-based protocol. Cells will then be filtered through a BD 70- $\mu$ m cellular filter, and plated on BD non-treated glassware. Non-treated glassware has been shown to diminish differentiation in embryonic and cancer stem cells. The urothelial origin of the cells will be confirmed by immunohistochemical



detection of urothelial carcinoma markers cytokeratin 7 and uroplakin III, and absence of CD31 (vascular marker) and CD34 (vascular and myofibroblastic marker), using BD Pharmingen™ antibodies for all markers except uroplakin 3. After brief propagation, cells will be sorted according to CD44 expression using a CD44-PE antibody (BD Pharmingen), and CD133 expression using a CD133-APC antibody (Miltenyi). Sorting will be performed using BD Falcon™ 2052 tubes and a BD FACSAria™ II flow cytometer. Sorted cells will then be propagated in vitro, or further expanded within a xenograft model with subsequent propagation in vitro. BD Matrigel™ matrix will be used as an injection vehicle for the xenograft model.

There are no reports documenting optimal media for propagation of urothelial stem cells in vitro. Media should facilitate cellular proliferation, limit differentiation, and preserve multi-potency. Extrapolation from other CSC protocols suggests the ideal formula would require supplemental growth factors and serum free conditions. Growth factors available from BD, including EGF, FGF, PDGF, and TGF-beta, have been identified as possible factors that may allow successful propagation of CSCs in vitro. We will systematically test combinations of growth factors in a base medium of serum free DMEM in order to identify a combination that best promotes cellular proliferation, while preserving multi-potency. BD chamber slides will be used to grow isolated CSC populations in the various media. This will allow monitoring of morphologic changes, such as development of tumor spheres or surface adhesion. Cell proliferation will also be assessed using a BrdU labeling kit (BD Pharmingen). After multiple passages, retention of CSC markers will be assessed through fluorescence activated cell sorting (BD FACS™). The ability of CSCs to recapitulate tumor histology will be confirmed in an immune deficient mouse model.

Characterization of mTOR signaling in CSCs will be done via immunofluorescence and Western blot analysis using BD Pharmingen antibodies against mTOR, S6, phosphorylated S6, Ki67, and an antibody against phosphorylated mTOR from Cell Signaling Technology. CSC populations will be incubated with rapamycin at multiple concentrations of the reported therapeutic range (in vitro: 0.01–1000 nmol/L) in order to identify dose-dependent effects of rapamycin on cell proliferation (BrdU labeling, BD Pharmingen) and apoptosis (Annexin V staining, BD Pharmingen). The extent of inhibition of mTOR by rapamycin will be determined by Western blot analysis of phospho-S6 (BD Pharmingen) and phospho-S6 kinase (Cell Signaling Technology).

Establishment of an in vitro model to isolate, propagate, and study urothelial cancer stem cells, and analysis of the role of mTOR activation in those cells, will provide strong evidence for clinical investigations targeting the mTOR pathway in urothelial carcinoma.

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