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Characterization and targeting of the human colon cancer-initiating cell compartment

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Colorectal cancer (CRC) is one of the leading causes of mortality in the western population. The majority of CRC associated deaths is due to metastasis in the late stages of the disease and thus it is imperative to find new targeted therapeutic strategies for its eradication. We and others have shown that the tumor initiating cell compartment in CRC is heterogeneous. A small sub-fraction of all colon cancer cells termed as long term tumor initiating cells (LT TICs) are responsible for tumor progression and metastasis in immunocompromised NOD/SCID-IL2RG^{null} (NSG) mice. To eliminate this most relevant cell population in human CRC, detailed knowledge about the mechanisms which regulate self-renewal, tumor initiation and metastasis formation is required. Therefore, this thesis addresses whether a fixed phenotype of long-term TICs is associated with their tumorigenicity and employs large scale molecular targeting to identify potential targets which drive proliferation and metastasis formation in TICs.

To study the biology of CRC TICs, TICs were enriched as three dimensional spheroid cultures from primary patient material under serum free conditions with the addition of cytokines. Upon xenotransplantation under the renal capsule of NSG mice, cultures derived from 3 individual patients reliably formed metastasis while spheroid cultures derived from 19 other patients never formed metastasis at all. These highly metastatic sphere cultures are characterized by down regulation of the epithelial molecules like E-Cadherin and keratins and an up regulation of the mesenchymal molecule Vimentin, indicating that these metastasizing spheroids are fixed in a mesenchymal-like state.

Upon addition of 10% FBS and withdrawal of cytokines, the phenotype of primary TIC enriched spheroid cultures changed dramatically. Cells did no longer grow as spheroids but formed an adherent cell layer and up regulated colon epithelial differentiation markers and down regulated stem cell markers in a patient specific manner. Despite this phenotypic differentiation, serum treated cultures maintained their tumorigenic potential upon serial transplantation, demonstrating that tumor initiation and self-renewal is not restricted to phenotypically immature spheroid cells. Moreover CD133 expression did not predict tumorigenicity and self-renewal of CRC TIC *in vivo*. This pronounced phenotypic plasticity further raises a challenge for the surface targeted elimination of human CRC TICs.

To identify genes regulating TIC proliferation and cell survival, large scale molecular targeting screens using pooled shRNA libraries were performed by using the Cellecta Decipher Library which targets 5043 cell signaling pathways associated genes with 5-6 barcoded shRNAs per gene transcript. To decipher which genes are involved in the proliferation of CRC spheroid cells, a negative shRNA screen was performed in a non-metastasizing spheroid culture in duplicates. The top 100 depleted shRNAs were enriched for

target genes related to apoptosis. From these a set of 25 candidate genes was selected using a selection criterion of 2 shRNAs showing the loss of viability phenotype. These 25 candidate genes were individually cloned and technically validated in the same sphere culture.

To identify genes which drive tumor-progression and metastasis formation, a long term *in vitro* to *in vivo* positive selection screen was performed in a non-metastasizing CRC spheroid culture. In the positive selection screen an enrichment of barcodes was observed for genes which have a selective advantage for proliferation upon knockdown. Interestingly from the *in vivo* screen, metastasis was obtained from originally non-metastasizing spheroid cells.

To further decipher which genes play a potential role in metastasis formation, originally non-metastasizing sphere cultures were transduced with a profound gene activating lentiviral vector which trans-splices into nearby genes. These originally non-metastasizing transduced spheroid cells formed metastasis upon xenotransplantation. Using high sensitive Linear Amplification Mediated (LAM) PCR, the exact integration locus of the vector was identified and the highest frequency of clones which contributed to tumor and metastasis formation was observed. The clonal pattern varied in all patients. Interestingly, hot spot integration regions for the same gene in two different patients apart from other dominant genes were observed, thereby predicting them as probable drivers of metastasis formation.

From the negative selection screen, Wnt genes conferred a loss of viability function which were also technically validated. As Wnt signaling is deregulated in colon cancer, it was observed that despite sphere cultures which have mutations in the APC and β-catenin gene, bind to GSK3β and Axin1. Furthermore upon inhibition of Wnt secretion, CRC spheroids failed to survive thereby asserting its role in their maintenance.

An *in vivo* transplantation model system which distinguished metastasizing versus non-metastasizing spheroid cells was established. Moreover an unstable phenotype of CRC TICs was observed. Potential targets which drive proliferation and metastasis formation were obtained from various molecular targeting screens and 25 genes from the negative high throughput shRNA screen were technically validated. Further, understanding the mechanism by which the identified candidate genes drive self-renewal, tumorigenicity and metastasis formation will serve as a basis for targeted manipulation and elimination of this most substantial cell population in CRC thereby create a new dimension of targeted therapies in future which would enhance long term patient survival.