Summary: PROX1 - IDENTIFYING INHIBITORS OF PROX1 IN COLORECTAL CANCER

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At least 50% of the Western population develops a colorectal tumor by the age of 70, and in about 1 in 10 of these individuals, progression to malignancy ensues (1). The lifetime risk of developing colorectal cancer (CRC) is thus estimated at approximately 5-6%. As a result, CRC is the second leading cause of cancer related morbidity and mortality in Europe (2). **Based on the high incidence rate and malignancy of CRC, there is a high need to identify molecules involved in its pathogenesis that can serve as potential novel therapeutic targets.**

Activation of the APC/-catenin/Tcf pathway is an initiating event in the majority of human CRCs and the ß-catenin protein is one of the key determinants in the pathogenesis of colon cancer (1). Levels of free ß-catenin are usually strictly controlled in the cell through a multiprotein complex that comprises for example the adenomatous polyposis coli (APC) and the kinases GSK-3 and CKIα. These enzymes are capable of phosphorylating ß-catenin, thus inducing its degradation. Not surprisingly, in about 80% of individuals suffering from the sporadic version of CRC, both *APC* alleles are mutated and thus inactivated (1). Unfortunately, although activation of the APC/-catenin/Tcf pathway is crucially involved in the vast majority of human CRCs, this signal transduction system is also responsible for the normal proliferation of intestinal crypt cells and thus, its inhibition leads to serious consequences. **Alternative targets for therapy must therefore be sought for new treatments of colorectal cancer.**

The homeobox transcription factor **PROX1** is critical for the establishment of the lymphatic endothelial cell phenotype, retinal progenitor cell differentiation, and pancreas and liver development (3). Importantly, **our laboratory previously discovered that PROX1 is overexpressed in approximately 70% of human CRCs as well as in mouse models of intestinal cancer with abnormal ß-catenin/Tcf signaling** (4). PROX1 expression is associated with the transition from benign to malignant tumor phenotype, and it is only present in cells with high nuclear ß-catenin content, while in normal colonic and small intestinal epithelium PROX1 is expressed in a rare subpopulation of enteroendocrine cells. We have also found that PROX1 expression is associated with a more malignant phenotype among various human colon adenocarcinoma cell lines, and have shown that suppression of PROX1 inhibits the growth of tumor xenografts, whereas PROX1 overexpression strongly enhances the growth and malignant potential of tumors in a transgenic mouse model of colon cancer (4). Importantly, deleting PROX1 specifically from the gut has no detectable phenotypic consequences, thus **showing that this transcription factor serves an ideal target for new therapies.**

In our project we aimed at **finding compounds that specifically inhibit PROX1 activity in CRC cells.** As PROX1 seems to activate or suppress genes in a tissue-dependent manner, first we determined the PROX1 binding sites in a CRC cell line. Importantly, PROX1 showed the expected inhibitory activity on these target sites, but surprisingly, its activity largely disappeared when the target sequences were stably integrated into the genome. As an alternative, we set up a cellular system where PROX1 can be silenced under the influence of Doxycycline. This model provides a useful tool to directly compare the behaviour of CRC cells with or without PROX1 activity. As a synthetic lethality screen, we screened a chemical library. We were interested in the potential drugs that had a higher killing activity towards PROX1-positive as compared to PROX1-negative CRC cells. Although in our first screen some promising hits emerged, in the secondary confirmatory experiments they showed only a very modest preference of damaging PROX1-positive cells. Thus, as the third alternative we carried out a **CellArray screen** with our Doxycyclin-inducible cells. In this approach the cells attach to siRNA-containing Matrigel spots on a glass slide where they get transfected and specific genes are thus silenced. The pre-requisite of this method is the good

adherence of the test cells to the spots on the glass slides. As one of the main effects of PROX1 is the reduction of cell adherence, our CRC cells are poorly adherent and we will need to optimize the Matrigel spots.

PROX1 seems to have tissue-specific targets and although its importance in lymphangiogenesis is well known (3), its mechanism of action has not yet been solved. To get an insight into the effects of PROX1 in CRC at the molecular level, we set out to analyze the CRC-specific target genes based on our previous microarray results. In our experiments **we confirmed more than 20 different PROX1 regulated genes both at the RNA and at the protein level. Furthermore, we also analyzed the effects of upregulation and downregulation of some of these genes by using cDNA overexpression or sh/siRNA silencing constructs.** Although the microarray results turned out to be highly reproducible, none of the genes alone seemed to be responsible for the effects of PROX1 (e.g. stimulation of cell proliferation indirectly, inhibiting cell adherence). Currently we are testing the combinatory effects of these genes.

Our previous studies showed that PROX1 plays a critical role in the adenoma to carcinoma transition of CRC. To define the role of PROX1 in this process, **we set up an adenoma model system where PROX1 expression can be manipulated** and the consequences can be easily monitored. According to our preliminary results, neither high Wnt-activity nor the PROX1 expression is enough to induce cell invasion, but instead, another triggering stimulus is required. We are now analyzing the possible factors that may be involved in this process. Importantly, **in a collaborative work with Dr. Ben-Neriah we could also show that in CKIα$^{-/-;}$ p53$^{-/-}$ mice, p53 acts as a critical suppressor of invasion. When p53 is deleted, PROX1 is turned on and PROX1 then belongs to the invasion gene set that appears to be responsible for the invasion (5).**

Taken together, the project has produced the necessary tools to carry out a large-scale high-throughput synthetic lethality screen. These experiments could lead to the discovery of new drugs or critical genes that can be used for further therapeutic developments to inhibit PROX1 and thus improve the therapy of colorectal cancer patients.

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