From whole genome siRNA screens to systems biology:
The glycosaminoglycan degradation pathway in cancer metastasis

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The fatality of cancer predominantly results from the dissemination of primary tumor cells, followed by blood circulation and the formation of metastases in distant organs. The formation of metastasis requires that cancer cells complete several sequential steps, starting with their detachment from the primary tumor. We aimed to identify proteins involved in the very first steps of cancer metastasis and implemented a cell based assay for cell detachment (Figure 1) in our functional genomics pipeline (Wiemann et al., 2004; Arlt et al., 2005; Sauermann et al., 2007).

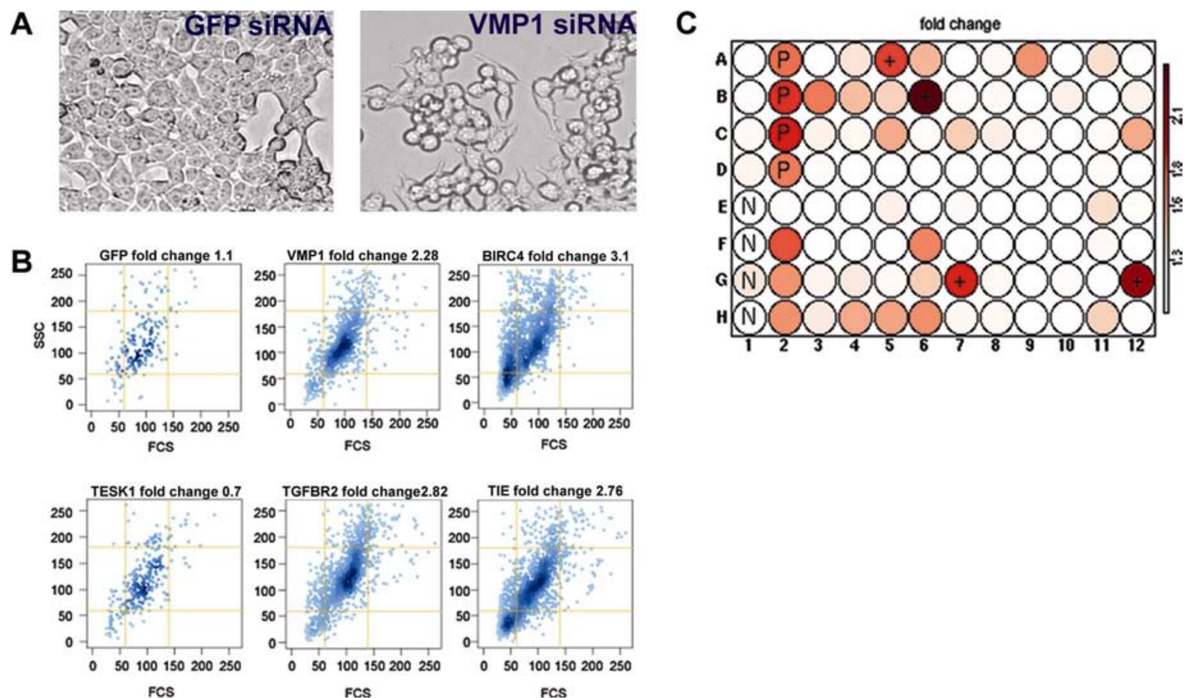

Figure 1: A genome-wide siRNA screen to identify proteins involved in cell adhesion. A: Downregulation of Vmp1, a protein involved in the formation of initial cell-cell contacts, results in loss of adherence B: Measured effects on cell detachment by FACS counting. In the first panel the effects of control siRNAs are shown. Each dot within these plots represents one cell. GFP was taken as neutral control and VMP1 as positive control. Cell detachment due to apoptosis was monitored through the shift of the dots, as shown for the apoptosis suppressor BIRC4. The second panel represents two of the identified candidates (TGFBR2 and TIE). TESK1 is a testis specific protein, as expected the siRNA transfection has no effect on cell detachment. C: Data analysis.

After counting the cells of the main population (in the centre of the dot plots in B), the fold change of each well was calculated and the results were visualized through plate plots with color coded fold changes.

A genome wide siRNA screen for cell detachment

For the assay, 21.167 siRNA pools (SMART pools, Dharmacon) were used to transfect cells cultured in 24 well plates. Three days later, the detached cells were transferred into 96 well plate and counted by flow cytometry. As a positive control, we used Vmp1, a protein which is involved in the assembly of cell junctions (Sauermann et al., 2007). Only living cells were considered for data analysis to rule out false positives caused due to apoptosis. To identify proteins which induce cell detachment after down regulation, we calculated the fold change after data processing. Proteins which showed the same or a higher fold change than our positive control were regarded as potential candidates. This way we came up with 997 candidates that significantly induced cell detachment upon down regulation and used different bioinformatic tools to evaluate our results.

Data integration

First, we applied a category analysis for significantly enriched GO-terms. We could associate the GO-terms for some of the candidates with cell detachment, for example Collagen, Extracellular Matrix Structure Constituent, Phosphatase Activity and Positive Regulation of NFkB Import into Nucleus. Then, we continued with Ingenuity analysis. Ingenuity is an application software for large data sets which generates networks based on a PubMed created database. A set of 430 candidate proteins were excluded by the program because of missing PubMed entries. For the remaining candidates we got two networks with 100% overlap with the assay candidates, and these results currently help us to work out the relevant cellular processes for cell detachment.

As we are highly interested in the integration of the non-characterized proteins into these networks, we developed a method which allows a classification in KEGG pathways (Kanehisa et al., 2004) based on InterPro domains (Mulder et al., 2007). However, for many proteins we did not have KEGG annotations. In order to get a signature for the KEGG pathways we used InterPro domains. First we determined the number of proteins with InterPro IDs in the siRNA library as well as in the candidate list, and then figured out the matching InterPro IDs in the library and the list for each KEGG pathway. We performed a Fisher test to calculate the ODDs ratio and the p-value. Using this method, we found the KEGG pathway for Glycosaminoglycan Degradation to be overrepresented in our candidate list. In total, we found 31 proteins which belong to the this KEGG pathway. It is well known that misregulation of Glycosaminoglycans (GAG) can aid in

promoting the malignant phenotype in cancer. Therefore, we validate the entire pathway in a 3D cell culture system for the impact on metastasis relevant processes (Figure 2).

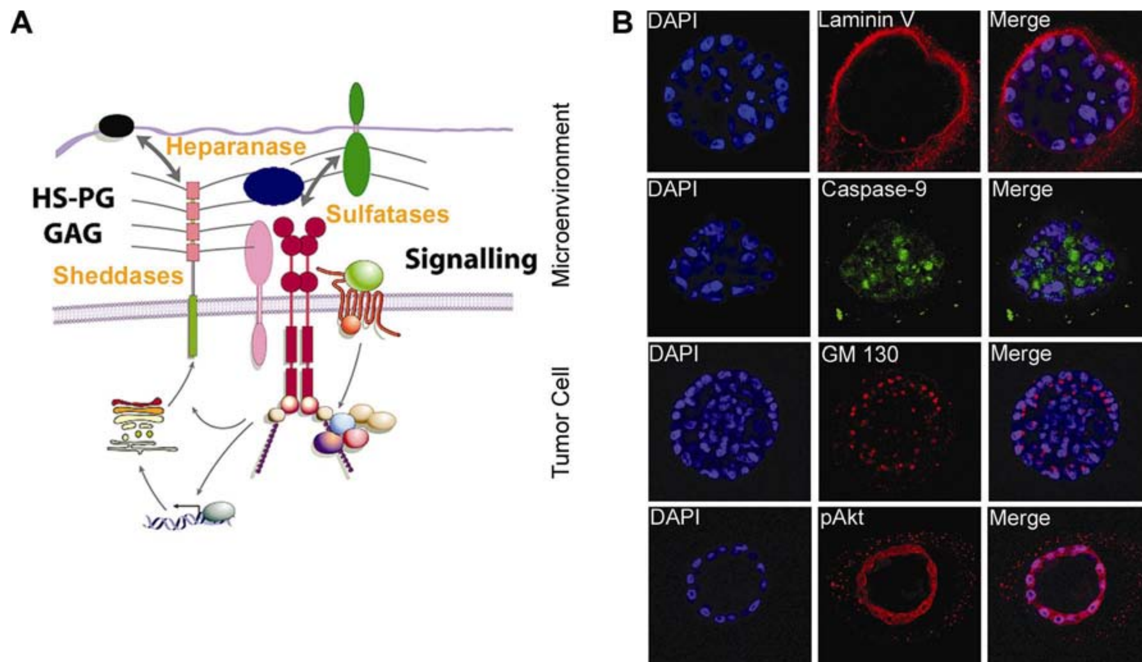

Figure 2: Model system to study GAG degradation and cell signalling. A: Schematic view of the system. The early processes of cell dissemination are strongly influenced by the microenvironment of tumour cells, permitting them to leave the primary tumor. Modulating proteins (indicated in yellow) of HS-PGs and GAGs are major factors affecting the balance of cells within their immediate environment and impact metastasis-relevant signalling pathways. B: The 3D cell culture system descibed by Debnath and Brugge is used to study the system. Here is shown the formation of acini using markers for the extracellular matrix (Laminin V), apoptosis (Caspase-9), cell polarization (GM130) and signalling (pAkt).

References

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