Gene Signature of Cancer Stem Cells

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1. Glioblastoma Stem Cells

Increasing evidence indicates that tumors contain a rare fraction of cells; referred to as "cancer stem cells" (CSC) that acquired stem cell properties of self-renewal and maintain the propagation of the disease. Such cells are much more effective in the transfer of tumors to nude mice and are endowed with self-renewal properties similar to stem cells. They were first discovered in Leukemia and recently also in solid tumors of breast, colon, brain and liver cancer. Most of these types of CSC are characterized by cell surface markers which enable their isolation. In many cases the surface markers are similar to those of the stem cells or progenitors of the normal tissue that gave rise to the tumor. Our research aims at the analysis of differential gene regulation in the cancer stem cells compared to the rest of the cancer cells and its relevance and utilization for cancer development and treatment. We first studied the genetic signature of CSC, derived from Acute Myelogeneous Leukemia (AML) patients, by isolation of the $CD34^+CD38^-$ cell fraction, and compared their gene expression profiles to the $CD34^+CD38^+$ cell fraction in AML and the $CD34^+38^-$ in normal blood. We found 409 genes that were at least two-fold over- or under-expressed between the two cell populations ($CD34^+38^-$ and $CD34^+38^+$ of AML), including under-expression of DNA repair, signal transduction and cell cycle genes. This is correlated with the relative mitotic quiescence and drug resistance of stem cells. We also found elevated expression of ligands of Notch such as Jagged 1 and 2. Notch is known to be involved in cell differentiation of lymphocytes and several "stemness" factors, such as Notch or telomerase, show differential activation in normal hematopoietic versus leukemia stem cells. These differences could be exploited therapeutically even with drugs that are already in clinical use for the treatment of leukemia. The goal is to find a treatment that is effective on the cancer stem cells and not on the normal tissue stem cells.

Based on this we focused on the Notch pathway and showed that DAPT, an inhibitor of gamma-secretase, a protease that cleaves Notch, inhibits the AML stem cell growth in colony formation assays. Notch signaling pathway is important in controlling both developmental processes and tumorigenesis. It is known that Inhibition of the Notch signalling pathway results in suppression of cell proliferation and induction of apoptosis in the tumor cell lines that over expressed Notch. Furthermore, a clinical trial for a Notch inhibitor, MK0752 (Merck), has been launched for relapsed or refractory T-ALL patients and advanced breast cancers.

Inhibition of the Notch signalling pathway accelerated differentiation of HSC in vitro and markedly reduced long-term HSC and therefore may inhibit colony formation. Furthermore, Notch pathway is involved in differentiation in other tissues such as pancreas, liver and intestine.

We extended this study of CSC to Glioblastoma (GBM) tumors and developed a rapid assay for assessing the effect of various drugs on GBM stem cells. We examine GBM cell lines, in particular A172 as well as GBM derived from patients. We found that CD133 is a useful marker for GBM stem cells and we isolated GBM stem cells, obtained from surgical samples, by using magnetic beads-coated with antibody to this novel cell marker.

The $CD133^+$ GBM stem cells, formed neurospheres in culture, in contrast to the $CD133^-$ cell populations that do not form neurospheres (Figure 1). This property allows the analysis of the response of GBM stem cells by following the behavior of the neurospheres.

Various drugs, including Gleevec, the Secretase inhibitor DAPT (which also inhibited AML colonies) and peptide Amph1D disperse these neurospheres within 24-36 hrs leading to cell death, at relatively low drugs concentrations (Figure 2).

In order to further characterize the gene expression signature of the GBM stem cells we use microarrays to study the gene profiles that distinguish the GBM stem cells from non-stem cell populations. In addition, we have examined the microRNA (miR) profiles of GBM stem and non-stem
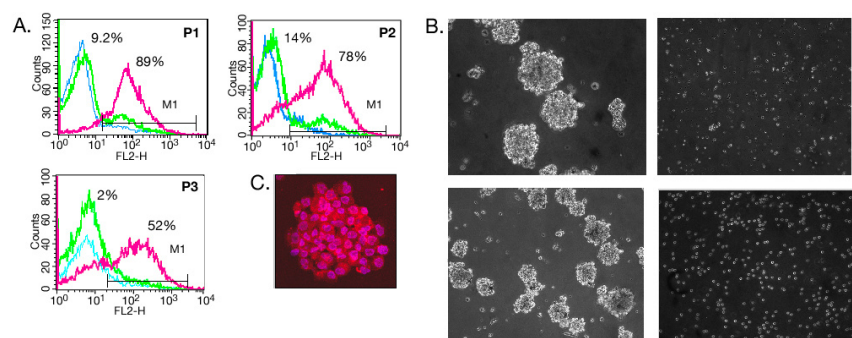

Fig. 1 Characterization of primary GBM tumor cells in culture.
A. Expression of the stem cell marker CD133 on primary tumor cells obtained from three GBM patients (marked p1-p3). Samples were dissociated, cultured, and stained with mAb for CD133 (pink, green) and IgG isotype control (blue). Indicated for each patient is the percentage of CD133 cells in the total population, prior (left hand number) and after magnetic sorting (right hand number). B. Neurophere like structures were found in the $CD133^+$ fractions of the primary GBM cells (left). $CD133^-$ fractions were unable to form neurospheres (right). C. Immunocytometry for CD133 expression on a single primary neurosphere, show high levels of CD133 (red), and purple counterstained with DAPI (x100).

Control | DMSO 1% | Gleevec 1μM | Gleevec 2.5μM
DAPT 5μM | DAPT 25μM | Amph1D 5μM | Amph1D 25μM
Amph1D 15μM | Gleevec 1μM+Amph1D 5μM | Gleevec 1μM+Amph1D 15μM
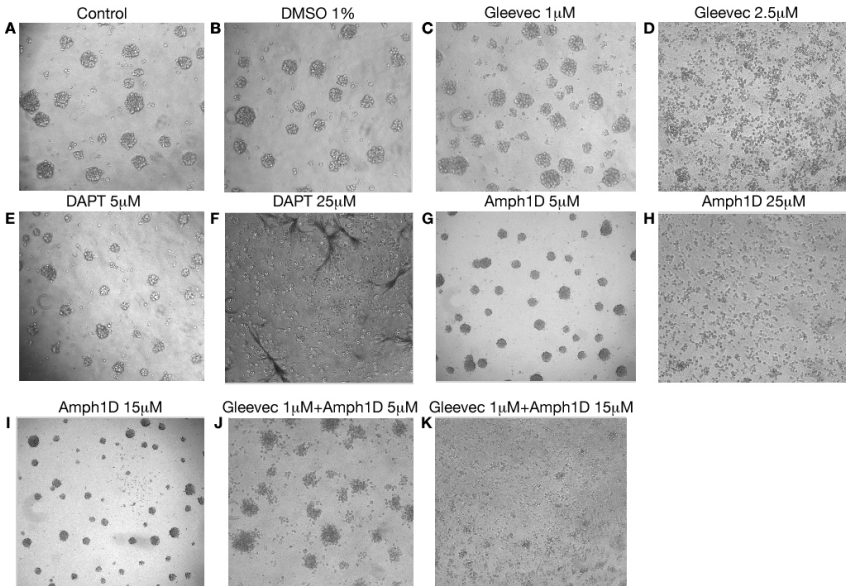
Fig. 2 *Drug assay on GBM stem cell neurospheres, derived from cell line A172. Cells were cultured in 96 well plates, 20,000 cells/well, with neurosphere medium. The drugs Gleevec, gamma-secretase inhibitor DAPT and amph1D peptide were added 4h after plating, at the concentrations indicated. Cells were visualized microscopically (objective x10). Cell mortality was determined using trypan blue. The experiment has been repeated several times with similar outcome.*
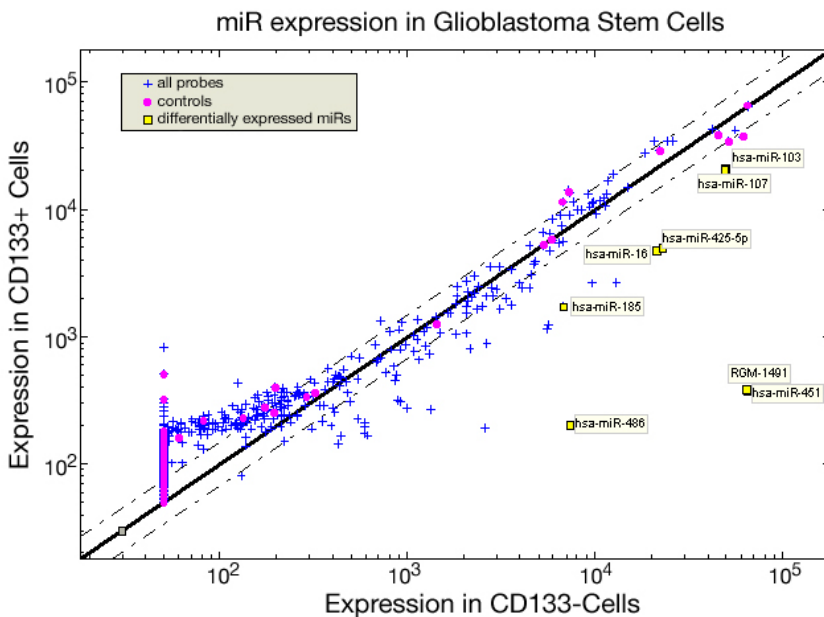

Fig. 3 *miR expression in GBM stem cells, CD133⁺ cells, compared to CD133⁻ cells in a primary GBM tumor. The assay used RNA, purified from the isolated GBM cell fractions CD133⁺ and CD133⁻. Down-regulation in expression of several miRs in GBM stem cells is indicated.*

cell populations, using MIR-arrays (Rosetta Genomics) .We found a marked down-regulation of several miRs in GBM stem cells implying that differentiation of the cancer stem cells to the tumor CD133- population is accompanied with up regulation of several miRs, some of which may be involved in regulation of normal brain stem cell differentiation (Figure 3).

In order to test the effect of such miRs on the GBM stem cells we are using plasmids that contain the miRs gene as well as the 22-long miR oligonucleotides in transfection experiments on the GBM neurospheres. We found that transfection of these miRs at 10-20 nM disperse the GBM neurospheres, leading to cell death.

We also identified a target site of SMAD on one of the miRs and we will try to decipher the pathway involved activation of miR by Smad in the GBM stem cells.

In addition, we found a synergistic effect of miR and other drugs (e.g. Gleevec) in dispersal of the neurospheres.

Our specific aims are: A) to characterize the effect of miRs and anti miRs on the viability of GBM, aiming at driving the stem cells to "differentiate" into a more drug- sensitive population and into a population deficient in self-renewal capability. B) To combine miR's effect on GBM with other agents that may enhance apoptosis like TRAIL or other miRs (e.g. the p53 target miR34), in order to increase specific apoptosis of GBM, using the neurosphere assay and additional assays. Although miR affects protein expression we know that RNA expression can also be modulated by the expression of miRs. We will perform microarray (Affymetrix) on GBM treated and untreated with miR 451, for example, to define changes in gene expression. We will develop methods, based on these changes to identify possible target sites of the relevant miRs in the RNA of defined proteins.
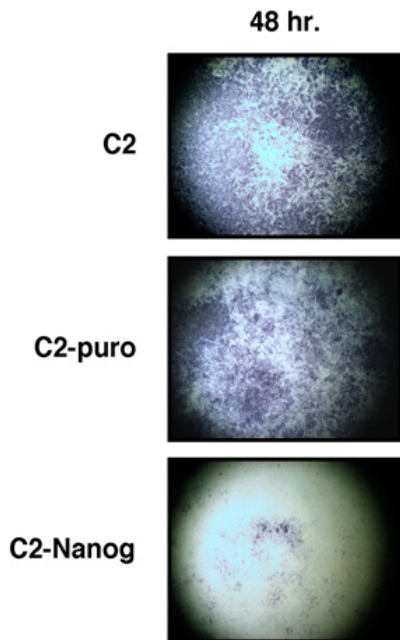
48 hr.

C2

C2-puro

C2-Nanog

Fig. 4 *Nanog expression inhibits osteoblast differentiation.*
C2 cells were infected with a retrovirus encoding Nanog (C2-Nanog), and a control virus (C2-puro) and selected for expression of Nanog and plated on gelatin. The next day, cells were transferred to osteoblast differentiation medium, containing BMP and cultured for 48 hours. After 48 hr. cells were washed, fixed and incubated in alkaline phosphatase (ALP) buffer and staining solution for 30 min for detection of ALP activity. The Figure shows that Nanog inhibits ALP staining in C2-Nanog cells.

2. Nanog inhibits the switch of myogenic cells towards the osteogenic lineage

To better understand the properties of pluripotency and inhibition of differentiation that are characteristic to stem cells, we study the effect of the stem cell specific transcription factor Nanog on differentiation of tissue stem-progenitor cells.

The homeodomain transcription factor Nanog has been implicated in inhibiting differentiation and controlling pluripotency of embryonic stem (ES) cells. We used ectopic expression of Nanog in the myogenic committed C2 cells to dissect these properties. Expression of Nanog in C2 cells does not alter terminal muscle differentiation but

has a profound effect on their switch to differentiate along the osteogenic lineage upon BMP treatment (Figure 4). Gene expression profiling revealed that ERK 1/2 phosphorylation, alkaline-phosphatase activity and osteocalcin expression were induced to much lower extent and remained suppressed even after 96 hr in Nanog expressing C2 cells, compared to control C2 cells. Hence, Nanog does not inhibit terminal differentiation of committed cells but it is an inhibitor of trans-differentiation that is dependent on *de-novo* activation of gene transcription.

Thus, similar to the role of Nanog as an inhibitor of ES cells differentiation, its overexpression in somatic cells precursors affects their capacity to switch their differentiation program (e.g. to bone) in response to specific factors, such as BMP, and as a result these cells can proceed mainly in their predetermined muscle differentiation pathway.

Selected publications

Kochupurakkal BS., Sarig, R., Fuchs, O., Piestun, D., Rechavi, R., Givol, D. (2008) Nanog inhibits the switch of myogenic cells towards the osteogenic lineage. Biochem Biophys Res Commun., 365, 846-850

Gal, H., Makovitzki A., Amariglio, N., Rechavi, G., Ram, Z., Givol, D. (2007) A rapid assay for drug sensitivity of glioblastoma stem cells. Biochem Biophys Res Commun., 358, 908-913

Weizer-Stern O., Adamsky, K., Margalit, O., Ashur-Fabian, O., Givol, D., Amariglio, N., Rechavi, G. (2007) Hepcidin, a key regulator of iron metabolism, is transcriptionally activated by p53.

Br J Haematol. 138, 253-262.

Gal, H., Amariglio, N., Trakhtenbrot, L., Jacob-Hirsh, J., Margalit, O., Avigdor, A., Nagler, A., Tavor, S., Ein-Dor, L., Lapidot, T., Domany, E., Rechavi, G., Givol, D. (2006) Gene expression profiles of AML derived stem cells; similarity to hematopoietic stem cells. Leukemia., 20, 2147-2154.

Piestun, D., Kochupurakkal BS., Jacob-Hirsch, J., Zeligson, S., Koudritsky, M., Domany, E., Amariglio, N., Rechavi, G., Givol, D. (2006) Nanog transforms NIH3T3 cells and targets cell-type restricted genes. Biochem Biophys Res Commun., 343, 279-285

Lapi, E., Iovino, A., Fontemaggi, G., Soliera, AR., Iacovelli, S., Sacchi, A., Rechavi, G., Givol, D., Blandino, G., Strano, S. (2006) S100A2 gene is a direct transcriptional target of p53 homologues during keratinocyte differentiation. Oncogene. 25 3628-3637.

Enk, CD., Jacob-Hirsch, J., Gal, H., Verbovetski, I., Amariglio, N., Mevorach, D., Ingber, A., Givol, D., Rechavi, G., Hochberg, M. (2006) The UVB-induced gene expression profile of human epidermis in vivo is different from that of cultured keratinocytes. Oncogene, 25, 2601-2614.