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Predictive bioinformatics identifies novel regulators of proliferation in a cancer stem cell model

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ABSTRACT

The cancer stem cell model postulates that tumors are hierarchically organized with a minor population, the cancer stem cells, exhibiting unlimited proliferative potential. These cells give rise to the bulk of tumor cells, which retain a limited ability to divide. Without successful targeting of cancer stem cells, tumor reemergence after therapy is likely. However, identifying target pathways essential for cancer stem cell proliferation has been challenging. Here, using a transcriptional network analysis termed GAMMA, we identified 50 genes whose correlation patterns suggested involvement in cancer stem cell division. Using RNAi depletion, we found that 21 of these target genes showed preferential growth inhibition in a breast cancer stem cell model. More detailed initial analysis of 6 of these genes revealed 4 with clear roles in the fidelity of chromosome segregation. This study reveals the strong predictive potential of transcriptional network analysis in increasing the efficiency of successful identification of novel proliferation dependencies for cancer stem cells.

1. Introduction

The cancer stem cell (CSC) theory posits that, in at least some cancers, tumor cells are arranged in a hierarchical lineage with a minor population, the CSCs, capable of unlimited proliferation while the bulk of the tumor is comprised of partially differentiated cells with limited ability to divide (Shackleton et al., 2009). A consequence is that only a subset of tumor cells, the CSCs, have the ability to generate tumors when transplanted (Bonnet and Dick, 1997; Al-Hajj et al., 2003; O'Brien et al., 2007). A corollary of the CSC theory is that eradicating tumors and preventing recurrence requires elimination of CSCs. However, identifying specific pathways to target CSC's has been a difficult challenge. Therefore, we used a transcriptional network algorithm called GAMMA to identify novel candidate targets, then tested the effects of depleting their expression in an established CSC model system.

Cell division is essential for tumor growth. The core pathways that mediate division are highly conserved from lower eukaryotes to mammals. However, mammals have evolved supplemental pathways. Proteins that participate in these supplemental pathways may be generally dispensable for the division in normal cells but may promote the fidelity of chromosome segregation. However, through mutation and epigenetic changes that accompany tumorigenesis, these pathways may become essential for cancer cell proliferation. This idea is supported by the fact that at least some cancers are highly vulnerable to inhibition of certain mitotic regulators (Mita et al., 2008; Perez de Castro et al., 2007). In brief, CSCs may become "addicted" to certain supplementary cell division pathways. Our goal was to test if our bioinformatic analysis could identify components of these pathways whose depletion would inhibit CSC growth.

2. Materials and methods

2.1. Cell culture

BPLE, BPLER, HMLE and HMLER cells (generously provided by Drs. Fabio Petrocca and Robert A. Weinberg, Massachusetts Institute of Technology) were maintained in WIT-T culture medium (Cellaria). All cell lines were maintained in 75 cm^2 filter flasks in a humidified incubator at 37 °C with 5% CO^2. Cell lines were screened for mycoplasma by fixing cells on coverslips with 3:1 methanol:acetic acid and labeling with 1 μg/ml DAPI. Observation by fluorescence microscopy confirmed that all lines were free of mycoplasma contamination.

2.2. siRNA screen

2.2.1. Cell culture

Cells were passaged by trypsinization (0.05% trypsin, 0.53 mM EDTA, 0.085% PBS). Optimal initial cell density was empirically determined as one that would be near confluency after a 7-day incubation, without

Abbreviation: CSC, cancer stem cell.

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overgrowth. Cells were plated with 100 μl of media in quadruplicate at 800, 600, 500, 400 and 300 cells/well for BPLE and BPLER and 600, 500, 400, 300 and 200 cells/well for HMLE and HMLER. 100 μl of media were added after 2 days to mimic experimental treatments. Cells were fixed, permeabilized, stained and read 7 days after initial plating. Optimal initial concentrations were 600 cells/well for BPLE and BPLER and 500 cells/well for HMLE and HMLER (Fig. S1).

2.2.2. Transfections

Transfections were carried out using Lipofectamine RNAiMax (Invitrogen) 2 days after plating. siRNAs (Bioneer) were suspended in RNAse-free H_2O at a concentration of 4 μM. 1–3 siRNAs were combined for each gene target (Table S1). Transfections were carried out in quadruplicate with 0.5 μl (10 nM) siRNA mix used for each well and standard Lipofectamine RNAiMax protocol was followed. Transfection mix was made up in 100 μl of WIT-T media and added to each well bringing the total volume of the well to 200 μl of media.

To identify the optimal starting siRNA concentration BPLER cells were transfected in quadruplicate with 50 nM, 40 nM, 30 nM, 20 nM and 10 nM concentrations of siRNA targeting luciferase (negative control) and PLK1 (positive control). 10 nM luciferase siRNA transfection showed minimal growth inhibition and 10 nM PLK1 siRNA transfection had approximately the same level of inhibition as higher concentrations (Fig. S2A). This was repeated in all 4 cell lines with similar results (Fig. S2B).

2.2.3. Growth assays

Five days after transfection, the media was removed from each well, and cells were treated with 100 μl of 1% paraformaldehyde in PHEM buffer (60 mM PIPES, pH 6.9, 25 mM HEPES, 10 mM EGTA, 4 mM MgCl2) containing 0.05% Triton x-100 and 1:1000 dilution of commercial SYBRGold stock solution to fix and permeabilize the cells and label DNA. Plates were then incubated for 30 min and read on a Genios plate reader (Tecan) with the following settings: gain @ Optimal, 10 flashes/well, (FITC filter set), read from bottom, Lag 0, Integration 40. Growth was normalized by dividing the cell count of each well to the average of the control wells. 3 replicate experiments were performed for each cell line. After expression normalization, the Bioconductor package limma (http://www.bioconductor.org/packages/release/bioc/html/limma.html) was used to determine genes that showed significant differential expression under experimental conditions and cell types. A linear model was fitted to the expression data for each probe. Moderated t-statistics were computed by empirical Bayes shrinkage of the standard errors toward a common value. The p-values corresponding to the moderated t-statistics were adjusted for multiple testing by computing false discovery rates (fdr) using the method of Benjamini, Hochberg, and Yekutieli (Benjamini and Yekutieli, 2001). We used both fdr and fold change to select differentially expressed genes by requiring at least a twofold change (log2[fold] ≥ 1) and fdr ≤ 0.05.

Cell counts were determined using cell line specific equations generated by standard curves. Known cell counts ranging from 1250 to 35,000 cells/well were plated in quadruplicate and incubated at 37 °C for 10 h to allow cells to settle. The average fluorescent intensity was plotted against cell count and the resulting equation was used to extrapolate the cell count from the measured fluorescent intensity in the experimental groups (Fig. S3).

2.3. Cell cycle analysis

2.3.1. Immunolabeling

Cells were seeded on 25 mm coverslips in 6-well plates at approximately 12% confluency with 2 ml of media and transfected with 30 nM siRNA 24 h later. After another 48 h, cells were fixed in 1.5% % paraformaldehyde in PHEM buffer containing 0.05% Triton-X 100 solution for 15 min. Coverslips were blocked with 20% Boiled normal goat serum (BNGS) in MBST (10 mM MOPS, 150 mM NaCl, 0.05% Tween 20) for 20 min. Mouse anti-γTubulin antibody (Sigma: T5326) in MBST with 5% BNGS was applied overnight at 4 °C. Coverslips were washed 3 times with PBST for 5 min and labeled with FITC-conjugated Goat anti-mouse secondary antibody (Jackson Immunoresearch: 115-096-062) at 5 μg/ml in MBST with 5% BNGS for 60 min at room temperature. Coverslips were then washed 3 times in PBST and labeled with 0.05 μg/ml DAPI in water for 3 min. Coverslips were washed with water and then mounted on slides with 9 μl vectashield (Vector laboratories) containing 1 mM $MgSO_4$ and the edges of the coverslip sealed with nail polish.

2.3.2. Cell cycle profiling

100 fields on each coverslip were imaged with a 15-plane z-series at 0.25 μm steps spanning the chromatin. Each z-series was summed, and individual nuclei were counted and analyzed with Metamorph software (Molecular Devices) using the cell cycle plugin. Intensity gates for scoring cells in G1, S, G2/M were established in the control and applied to each of the experimental sets. Mitotic indices were determined by manually counting the proportion of cells containing condensed chromosomes.

3. Results and discussion

3.1. A bioinformatics approach to candidate identification

Stem-cell mitotic regulators remain poorly characterized, and their identification is complicated by several factors. First, stem cells are a minor population of the dividing cells within a tissue or tumor. Second, accessory mitotic regulators may be difficult to discern because their depletion only decreases the very high fidelity of chromosome segregation but are not indispensable for the division process itself. Third, proteins may have evolved functions in multiple areas of cell cycle control or cell physiology, making the characterization of their roles more difficult. To address these challenges, we used a predictive algorithm called Global Microarray Meta-Analysis (GAMMA) to identify candidate stem cell mitotic regulators. GAMMA is a bioinformatics approach that uses public microarray and RNAseq datasets from NCBI's Gene Expression Omnibus (GEO) repository to identify transcripts that are correlated across many experimental conditions (Wren, 2009; Dozmorov et al., 2011). Using a "guilt by association" principle, groups of transcripts that are highly correlated with each other are likely to share similar biological associations, such as playing a role in the same disease or phenotype, and being involved in the same pathway. Using a k-Nearest Neighbors approach, GAMMA identifies the 40 most correlated transcripts for each gene, then uses literature mining to identify what the correlated genes have in common in MEDLINE, in terms of their being co-mentioned with diseases, phenotypes, chemicals, and other genes (Wren et al., 2004). GAMMA has been successfully validated in several studies (Towner et al., 2013a; Towner et al., 2013b; Clemmensen et al., 2012; Lupu et al., 2011; Daum et al., 2009). Selecting for high GAMMA scores in genes related to cancer, stem cells, and mitosis, we evaluated 50 candidate genes with minimal previous characterization (Table 1).

3.2. siRNA screen of BPLER and BPLE

A significant challenge in identifying gene dependence in CSCs is the lack of reliable experimental comparisons in growth assays. When tumor cells are placed in culture, growth rates of various subpopulations of cancer cells may vary wildly as cultures are often heterogeneous. Furthermore, although CSC populations have been successfully isolated by fluorescence-activated cell sorting (FACS), these populations quickly lose their stem cell characteristics and differentiate in culture (Fillmore and Kuperwasser, 2008). To address these problems, we focused on BPLER cells, a tumorigenic cell line derived from normal breast epithelium sequentially transformed with SV40LT, hTERT and hRAS (V12) (Ince et al., 2007). These were compared with non-tumorigenic