Abstract 1245

Standardized 3D spheroid systems facilitate drug screening and biomarker identification with high relevance for cancer drug discovery

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ABSTRACT

Whilst patient-derived cancer organoid culture is not yet compatible with high throughput studies, cell-line-derived multicellular spheroids represent an attractive solution to recapitulate the structural and molecular complexity of cancer cell lesions with higher physiological relevance compared to 2D culture systems, thereby generating more predictable results and reducing the expenses attributed to current preclinical attrition rates.

As an illustration of the technological services developed by our team, we focused on colorectal cancer, which is one of the most commonly occurring cancer in humans. When stage I and II tumors are curable, metastatic stages of the disease still represent a clinical challenge. Along the last decades, the use of cytotoxic drugs in association with targeted therapies improved the overall survival of patients with metastatic colorectal cancer. However, a large subset of patients continues to be unresponsive, likely due to the high heterogeneity of colorectal cancer lesions. Sustained efforts are then needed to identify and target relevant biomarkers of crucial interest for the development of treatment strategies.

CDK8 is a non-mitotic Cyclin-Dependent-Kinase which functions as an oncoprotein in colorectal cancers. On the one hand, most published drug discovery projects report attractive activities for CDK8 inhibitors in enzyme assays and murine models, but these compounds generally display weak activity in conventional cell-based assays. On the other hand, the signaletic map involving CDK8 in colorectal cancer has mostly been investigated using RNAi mediated suppression studies and kinase dead mutants, emphasizing an important need to pursue the exploration of the oncogenic role of CDK8 using complementary pharmacological approaches implemented in the most relevant physio-cellular context. These two statements led us to investigate how modulating key culture parameters may impact the predictivity of colorectal cancer cell-based approaches used to select CDK8 inhibitors and/or identify biomarkers.

METHOD

The impact of three CDK8 inihibitors on the viability and proteome of HT-29 colorectal cancer cells was evaluated using cells cultured as 2D monolayers maintained under conventional oxygen pressure, or as 3D spheroids maintained under hypoxia (pO2 = 5%) in ULA plates. The effect of inflammation on the viability and proteome of cells was assessed by adding (or not) interferon-gamma to the medium prior (40ng/ml, 4h) and during (20 ng/ml) the pharmacological exposure.

All cells were grown during 4 days in 96-well plates using filtered Mc Coy medium completed with 10% BSA, afterward these were exposed to CDK8 inhibitors (Senexin A, Sorafenib, SEL120) at 10µM, 0,1% DMSO. DMSO wells were used as negative pharmacological controls.

Cell viability was measured following a 3 days long exposure with CDK8 inhibitors, using CellTiter Glo 3-D viability assay (Promega) according to manufacturer's instructions, and our PolarStar Omega luminofluorimeter (BMG LabTech). « No cell » wells were used as control to measure assay background. Staurosporin 2,14µM was used as positive control.

For proteome profiling, cell exposure to CDK8 inhibitors lasted 18 hours. Cell pellets were washed with PBS, lyzed in presence of protease inhibitors and processed using the human L2000 high density array from RayBiotech according to manufacturer's protocol. Array analysis was performed by RayBiotech's support team.

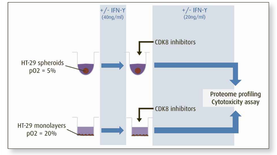

Figure 1
Method summary

The signal intensities of biomarkers in one sample were ranked by their quantiles. The average values were calculated for each rank across all the samples. The quantile normalization procedure replaced each data point with corresponding average at its rank. The data were log-transformed and limma (Linear Models for Microarray Data) was used to analyse the main effects of culture method (3D vs. 2D), interferon (Interferon-G vs. None) and medium (with DMSO as control).

We did not include interactions into this analysis. The biomarkers with adjusted p-values less than 0.05 were considered as significantly differently expressed. All the analyses were conducted using R programming language V 3.5.1 (R Core Team 2017).

RESULTS

HT-29 cells form highly cohesive spheroids in 4 days with high repetitivity and reproducibility. A number of 1000 cells per well enables us to reach a targeted spheroid diameter of 400 +/- 50µm.

The 3D spheroid format under hypoxia and interferon-gamma are two culture parameters that significantly increase the in vitro cytotoxic activity of SEL120-34A in an independent manner (p= 0.0286, Mann Whitney test, two-tailed). Combined together, these two parameters synergically redirect HT-29 cells to death at lower compound concentration ranges. An observed concentration-dependent increase of RLU in wells containing senexin-treated spheroids suggests the drug interferes with Celltiter Glo reagent, preventing the quantification of reliable viability values.

Amongst the 2000 biomarkers screened in this proteome study, nine are differentially expressed across culture methods (3D-hypoxia vs. 2D) and four across treatment with interferon-G (Interferon-G vs. None). Of particular interest, interferon-stimulated spheroids maintained under hypoxia overexpress IL-15 and underexpress cystein rich protein 1, two important proteins which negatively regulate colorectal carcinogenesis and colorectal cancer cell migration and invasion[1,2].

REFERENCES

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2. Bahri, R. *et al.* IL-15 suppresses colitis-associated colon carcinogenesis by inducing antitumor immunity. Oncoimmunology 4, (2015).
3. Rzymski, T. *et al.* SEL120-34A is a novel CDK8 inhibitor active in AML cells with high levels of serine phosphorylation of STAT1 and STAT5 transactivation domains. Oncotarget 8, 33779–33795 (2017).

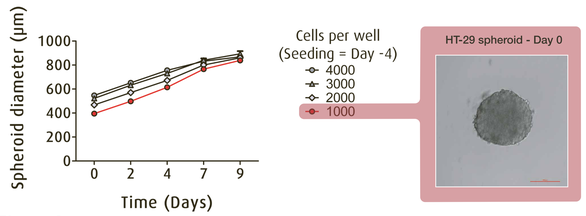


Figure 2
Calibrating spheroid size.
Spheroid's diameter according to the number of cells seeded in ULA 96-plates, and growth kinetic.

ID	logFC	AveExpr	t	P.Value	Adj.P.Val
IL-15	-7.0503397	3.531728	-23.085142	0.0000000	0.0000000
LAF4	0.4637917	9.177102	7.080113	0.0000066	0.0066110
Inhibin beta	0.5418209	9.209812	6.383819	0.0000199	0.0107088
Insulin R	-1.4768279	7.539016	-6.336693	0.0000214	0.0107088
Cysteine-rich Protein 1	0.4076597	9.130133	5.882021	0.0000457	0.0182497
Keratin 38	0.9977050	7.736214	5.424974	0.0001005	0.0334795
CNTF	-5.1561134	2.584614	-5.098680	0.0001797	0.0464610
GDF5	-1.2605397	7.827801	-5.014907	0.0002091	0.0464610
Hck	0.7234573	8.206876	5.014433	0.0002093	0.0464610

Table 2
Differential expression across culture methods
3D-hypoxia vs. 2D

ID	logFC	AveExpr	t	P.Value	Adj.P.Val
Neuropeptide B	-6.2360273	3.823299	-8.406918	1.00e-06	0.0019362
Cathepsin S	6.0029147	3.103584	6.965517	7.90e-06	0.0078861
Oxytocin-europhysin1/OXT	-5.1042340	3.810204	-5.513642	8.61e-05	0.0445895
ACAA1	-0.6537397	8.571239	-5.492784	8.93e-05	0.0445895

Table 3
Differential expression across treatment with interferon-G
Interferon-G vs. None

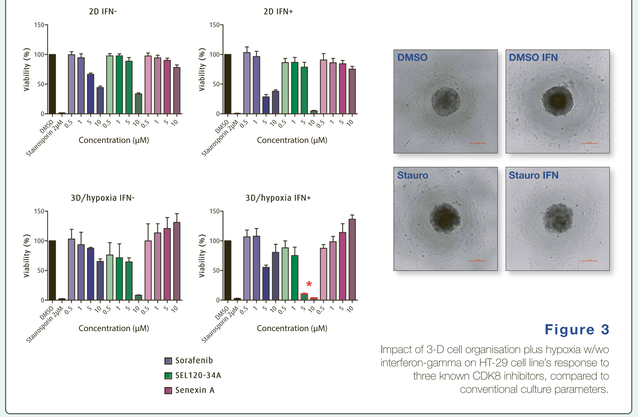
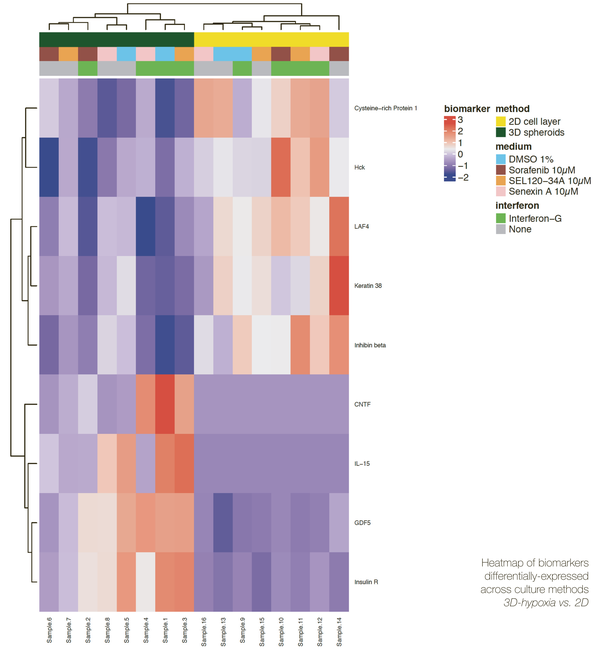


Figure 3
Impact of 3-D cell organisation plus hypoxia w/wo interferon-gamma on HT-29 cell line's response to three known CDK8 inhibitors, compared to conventional culture parameters.

Sample	Culture mode	Cytokine	Medium
Sample.1	3D spheroids-hypoxia	Interferon-G	DMSO 1%
Sample.2	3D spheroids-hypoxia	Interferon-G	Sorafenib 10µM
Sample.3	3D spheroids-hypoxia	Interferon-G	SEL120-34A 10µM
Sample.4	3D spheroids-hypoxia	Interferon-G	Senexin A 10µM
Sample.5	3D spheroids-hypoxia	None	DMSO 1%
Sample.6	3D spheroids-hypoxia	None	Sorafenib 10µM
Sample.7	3D spheroids-hypoxia	None	SEL120-34A 10µM
Sample.8	3D spheroids-hypoxia	None	Senexin A 10µM
Sample.9	2D cell layer	Interferon-G	DMSO 1%
Sample.10	2D cell layer	Interferon-G	Sorafenib 10µM
Sample.11	2D cell layer	Interferon-G	SEL120-34A 10µM
Sample.12	2D cell layer	Interferon-G	Senexin A 10µM
Sample.13	2D cell layer	None	DMSO 1%
Sample.14	2D cell layer	None	Sorafenib 10µM
Sample.15	2D cell layer	None	SEL120-34A 10µM
Sample.16	2D cell layer	None	Senexin A 10µM

Table 1
List of 16 samples used for proteomic profiling with the Human L2000 High Density Array



Heatmap of biomarkers differentially-expressed across culture methods
3D-hypoxia vs. 2D

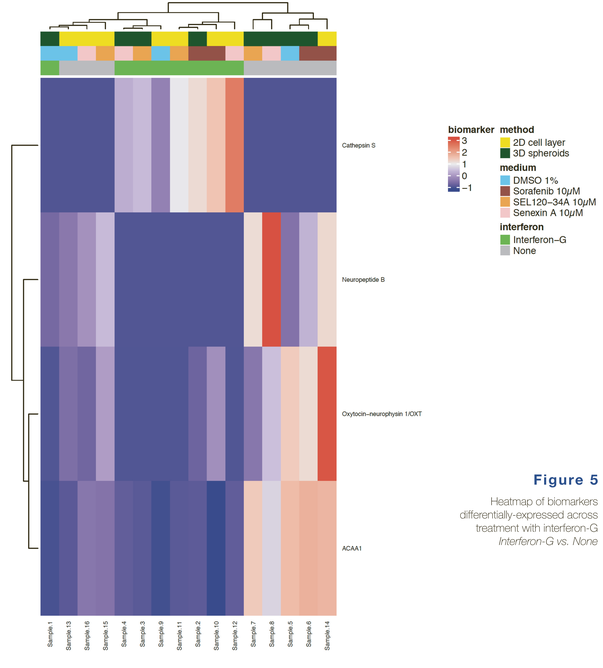


Figure 5
Heatmap of biomarkers differentially-expressed across treatment with interferon-G
Interferon-G vs. None

CONCLUSION

Integrating the 3D cell organisation, oxygen pressure level and inflammatory context in culture conditions markedly impacts HT-29 cell responses to SEL120-34A, resulting in increased drug cytotoxicity and differentially expressed biomarkers. Our results suggest a potential therapeutic interest of this drug in colorectal cancer in addition to AML[3].

We have developed a 3D multicellular spheroid platform which is compatible with standardized preclinical testing and high density proteomic profiling studies. We are currently developing a set of complementary 3D models capable of reliably recapitulating physiological responses that are complementary to animal studies, including microfluidics.