Cell culture medium formulation and its implications in cancer metabolism.

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Abstract: Historic cell culture media were designed to ensure continuous cancer cell proliferation *in vitro.* However, their composition does not recapitulate the tumor's nutritional environment. Recent studies show that novel media formulations alleviate the non-physiological constraints imposed by historic media, and lead to cell culture results more relevant to tumor metabolism.

A tumor is a complex, dynamic and disordered structure within an organism, composed of mixed populations of normal and cancer cells. To understand the role of the biological units of this system, researchers embraced a reductionist approach over than 60 years ago when they started to culture cells in isolation. Since then, most of the experiments in cancer research have been performed with cell lines cultured as monolayers, often referred to as *in vitro.* Such experiments substantially contributed to the advancement of our knowledge in cancer cell biology, and it is undeniable that culturing cancer cells is informative, and has advantages that overall exceed its obvious limitations. Tumors consist of different niches depending on vascularization, genetic clonality, and infiltration of immune and stromal cells. Since a cell culture dish overtly differs from the growth conditions of cells in tumors, researchers continuously attempted to refine culture conditions by modulating oxygen concentration, by allowing cells to form self-contained three dimensional structures, i.e. spheroids, or by supplying extracellular matrixes with different chemical-physical properties. Recently, more complex co-culture systems have also allowed to study the interaction between different cell types *in vitro*.

However, most of the experiments currently ongoing are still performed in historic cell culture media, some of which were formulated at least half a century ago, and whose composition clearly differs from the nutritional environment that cells withstand in tumors. For example, Eagle's Minimal Essential Medium, MEM, and its Dulbecco's modified version, DMEM, were designed to supply cancer cells with only those nutrients essential for their continuous proliferation. These media are widely used and in 2016 more than half of the published cell culture-based studies employed one or other of these media [1]. Another frequently used cell culture medium, F12, was optimized for the clonal growth of Chinese Hamster Ovary (CHO) cells under reduced serum supplementation [2]. In general, currently available commercial cell culture media were formulated to allow continuous and accelerated growth of specific cell types, and not to recapitulate the metabolic environment of the tissue of origin, or the proliferation rates of tumors [2]. As a result, media often lack metabolites normally present in human fluids, while others, such as glucose, glutamine or pyruvate, are often found at supra-physiological concentrations (Supplemental Table 1). On the contrary, compounds irrelevant for human pathophysiology, such as L-alanyl-L-glutamine dipeptide (e.g. GlutaMAX™), are commonly supplemented at millimolar concentrations, with uncharacterized, yet inevitable consequences on cell metabolism.

Only in recent years, has it been shown that excessive concentrations of nutrients affect the metabolism of cultured cells and lead to discrepancies in metabolic phenotypes between cultured cells and tumors. For example, the proliferation of cancer cells has been shown to depend less on mitochondrial respiration when cultured with excessive concentrations of pyruvate, as indicated by their decreased sensitivity to metformin [3] (Figure 1). Additionally, high concentrations of cystine found in historic media enhance glutamine consumption and dependency by cancer cells in culture [4]. Undoubtedly, the effects

of culture media with non-physiological levels of nutrients are not limited to cancer cells. BrainPhys™ is an example of a medium developed to recapitulate specific functional phenotypes observed in the brain. In 2015, Bardy et al. formulated BrainPhys™ with a reduced concentration of neuroactive ions and amino acids in comparison to DMEM/F-12 and Neurobasal™ media. This specialized medium enabled researchers to study the electrical activity of neurons derived either from primary tissue, or from induced pluripotent stem cells, as well as in brain explants cultured *ex vivo* [5]. The logic applied in the designing of BrainPhys™ raises questions on what is currently known about the availability of nutrients and metabolites in specialized tissues, and in the tumor microenvironment. Are cells in tumors exposed to nutrient concentrations comparable to those of plasma? Do adjacent cells exchange nutrients directly through gap junctions or microtubes [6] or *via* extracellular interstitial fluid? Answers to these broad questions remains largely speculative, however recent evidences suggest that in poorly vascularized pancreatic adenocarcinomas, the concentrations of specific nutrients in the interstitial fluid significantly deviates from the circulating levels [7].

Under a reasonable assumption that the circulating levels of metabolites constitute a relevant source of nutrients for most normal and neoplastic tissues, researchers have cultured cancer cells in serum or lymph fluid [4], [8]. However, the availability, complexity, and undefined nature of these bio-fluids limit their applicability in cancer research. In 2015 we formulated a medium with glucose, pyruvate and amino acids concentrations similar to human blood (Serum-like Modified Eagle's Medium, SMEM, Supplemental Table 1 [9]). SMEM has lower concentrations of the amino acids found in DMEM, it has additional proteinogenic (e.g. alanine, glutamate) and non-proteinogenic amino acids (e.g. ornithine, citrulline), but still lacks many polar metabolites normally found in human plasma. In 2017, Cantor et al. described the effects on cancer cells of a more complex medium formulation with amino acid derivatives, ketone bodies, end products of organismal catabolism (e.g. urate) and other components at concentrations found in human plasma (HPLM, Supplemental Table 1 [10]). Urate is the end product of purine catabolism, and Cantor et al. reported that it can regulate the biosynthesis of the pyrimidine nucleotides by inhibiting uracil monophosphate synthetase (Figure1). This enzyme is also responsible for the activation of the drug 5-flurouracil, therefore cancer cells cultured in HPLM have been shown to be less sensitive to this anticancer drug. These observations suggest that the formulation of the cell culture medium might have profound implications in the target identification and drug development processes, in particular when these focus on cell metabolism [11].

Plasmax™ is a more complex iteration of the afore mentioned SMEM and, similarly to HPLM, it aims to recapitulate more closely the nutrient composition of human plasma [12]. Plasmax™ formulation contains 66 organic components. Amongst these, arginine and pyruvate are ~10 fold less abundant in this medium than in historic ones, such as DMEM. In triple negative breast cancer (TNBC) cells, pyruvate stabilizes the hypoxia-inducible factor 1α (HIF1α) in a dose dependent manner, and at the concentrations supplemented in historic media (0.5-1mM) it induces a pseudo-hypoxic response even under atmospheric oxygen availability. Concomitantly, in cells cultured in media such as DMEM or RPMI, the high concentrations of arginine reverse the direction of the reaction catalyzed by the urea cycle enzyme, argininosuccinate lyase. This metabolic feature was not observed in cancer cells grown in Plasmax™, nor in mammary orthotopic xenografts. Furthermore, in only four days of culture in Plasmax™ the metabolic profile of TNBC spheroids resembled the metabolic landscape of orthotopic xenografts more closely than that obtained with historic media. This indicates that the metabolism of established cancer cell lines, isolated and cultured for many passages under the non-physiological selective pressure of historic media, can be rectified towards a more tumor-like state. In addition, these observations suggest that *in vitro* models could be further refined by culturing cells freshly isolated from patient-derived material directly in a more physiological medium.

In commonly employed media, essential components (e.g growth factors, proteins, non-polar nutrients, and trace elements) are largely provided by the serum. Hence, their concentration varies between different batches, thereby impairing the reproducibility of results between laboratories. To achieve a chemically defined medium that allows cells to be cultured without serum supplementation, essential components must be included in the formulation. Plasmax™, as well as some advanced commercial media, contain trace elements such as Fe, Se, Zn, Cu in the form of salts. Human metabolism largely depends on circulating levels of these elements bound to organic small molecules and proteins such

as transferrin, selenoproteins, ceruloplasmin, and albumin. Therefore, the physiological availability of these important metabolic catalysts can be achieved by supplementing relevant concentrations of trace elements, coupled with appropriate carrier molecules. Essential components are not limited to trace elements. Vitamins, hormones, lipids, proteins, and growth factors, normally contributed by serum, should also be considered in the attempt to achieve tumor-relevant media formulations.

Finally, it is common cell culture practice to incubate the cells with a fixed amount of medium for extended periods. This can lead to the exhaustion of heavily consumed nutrients (e.g. glucose) and an accumulation of metabolic products (e.g. lactate) far beyond the physiological ranges reported in humans (Supplemental Table 1). This consideration applies in particular to physiological media where the concentration of nutrients has not been artificially increased to overcome this problem. An option to prevent both nutrient exhaustion in spent medium and excessive concentrations of nutrients in fresh one, is offered by a bioreactor, called nutrostat [13], which provides a constant flow of fresh and exhausted media. While this approach is widely employed in microbiology and biotechnology, it is far less practical for multiplex experiments used in cancer research. A gross approximation of a steady state level of nutrients and metabolic end-products in the cell supernatant can be achieved by adjusting the ratio between cell number, volume of medium, and time between medium renewals. This can be applied with only minor modifications of current cell culture practice, for example by increasing the frequency of medium renewal, or by adjusting the volume of medium proportionally to the number of cells.

In summary, for decades cell biologists have used media disconnected from physiology. In the last decade, the growing focus on cancer cell metabolism has contributed to the exacerbation of some of the artifacts observed by culturing cells in historic media. But cell metabolism does not exist in a vacuum and the nutrient composition of media alters the gene expression [12], [14] and the epigenome [15] thereby affecting the relevance of *in vitro* models to cancer biology. The implications of using nutritionally skewed media in cancer research will become more evident with the use of refined and more physiologically relevant culture media by a broader research community. In parallel, more efforts to understand the nutritional environment of specialized tissues and tumors will provide us with more defined templates for designing better cellular models of cancer.

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Figure Legends

Figure 1: Metabolic reactions of cancer cells cultured in historic and physiological media. Arrows and text highlighted in red indicate reactions or metabolite levels enhanced in historic media, such as DMEM. Text with dashed outline and clear filling indicates nutrients and metabolites not present in DMEM. 5-FU: 5-fluorouracil, AcCoA: acetyl-coenzyme A, ASS: argininosuccinate, ATP: adenosine triphosphate, Citn: citrulline, Fum: fumarate, FUMP: 5-fluorouracil monophosphate, HIF1α: hypoxia-inducible factor 1α, αKG: α-ketoglutarate, MetF: metformin, NAD: nicotinamide adenine dinucleotide, Oaa: oxaloacetate, Orn: Ornithine, Pyr: pyruvate, UMPS: uridine monophosphate synthetase.

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