In Vitro Modeling of Tissue-Specific 3D Microenvironments and Possibile Application to Pediatric Cancer Research

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Abstract: A large body of evidence indicates that three dimensional (3D) cancer models are superior to two-dimensional (2D) ones in better representing the *in vivo* phenomena. Indeed, 3D models allow recapitulating *in vitro* the *in vivo* features observed in solid tumors (e.g. cell polarity, cell-cell/cell-matrix interactions, biochemical/metabolic gradients, anchorage-independent growth and hypoxia). Moreover, it is well established that the microenvironment plays a fundamental role in regulating tumor development and behavior, including drug resistance. Thus, innovative models able to mimic this complexity represent attractive tools in cancer research. In this review article, we provide a comprehensive review of the application of 3D culture systems in pediatrics' cancer research. In particular, 3D *in vitro/ex vivo* models of the most common pediatric tumors, such as leukemias, lymphomas and malignancies of the nervous system, will be considered.

Keywords: Three dimensions (3D), microenvironment, pediatric cancer, pediatrics' oncology.

1. INTRODUCTION

For almost all cancers, if death rates continue to decline, statistics indicate that the number of new cases/year and the number of mortality deaths are still too high. In 2014, in effect, the estimate of cancer incidence in the USA will be about 1.66 million new cases, while deaths will reach the number of 585.720 patients [1]. In Europe (2012), the predicted number of new cases of cancer was 3.45 million, and 1.75 million deaths [2], while, for 2013, the predicted number of cancer deaths decreased to 1.31 million [3]. For children and adolescents (birth to 19 years old) in USA, for 2014, the predicted number of newly diagnosed cases of cancer is 15,780 and about 1,960 deaths from cancer will occur. The annual incidence rate of cancer for this young population is 186.6 per 1 million [4]. Altogether, these data demonstrate that we are far away from assuring the cure of cancer patients.

Tumorigenesis is a multistep process, and it was initially considered as a succession of mutations occurring in oncogenes, in tumor suppressors and, ultimately, in microRNAs, all events which lead to a successive uncontrolled proliferation of tumor cells, induction of angiogenesis and lymphangiogenesis, and further metastatic processes [5]. The high heterogeneity of cancers between individuals is thought to be multifactorial due to genetic diversity, the presence of cancer stem cells**,** and the impact of the tumor microenvironment [6]. It is well-known that genotypic alterations alone are not sufficient to explain tumor progression and metastatic processes, and that the tumor microenvironment can be profoundly involved in regulating tumor growth, local invasion of the stroma, angiogenesis/lymphangiogenesis, intra/extravasation, metastasis, drug resistance, as well as tumor reversion [7-11].

In the present review, we have considered the concept of the tumor microenvironment in adult solid neoplasms and in the *in vitro* models available to study the main hallmarks of adults' cancer, and childhood malignancies. There are some differences between adults' and childhood's tumors; for example, the incidence of tumor types is different. In adults, carcinomas/solid tumors are the most frequent types of neoplasms (about 80%), whereas in childrens/ adolescents, leukemia accounts for about the 30% of the total tumors, followed by malignancies of the Central Nervous System (CNS) (20-25%), by lymphomas (16%) and neuroblastomas (7.6%) [12]. In adults, parenchymal cells are mature and terminally differentiated, and then only a limited number of cells highly proliferate, whereas in young people (and mostly in children), a certain number of organs/tissues present an incomplete terminal maturation, and their cells, instead of differentiating, continue to proliferate and die. This increases the difficulty of chemotherapeutic

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treatment of the tumor's proliferating cells without side effects on healthy tissues. Finally, in children, tumors may require quite a few events to progress, as compared to the adult ones [13]. In the last section of the review we have illustrated and discussed specific 3D cell-based systems suitable for modeling the most common pediatric tumors, such as leukemias, lymphomas, CNS's cancers and neuroblastoma.

2. THE IMPORTANCE OF THE TUMOR MICROENVIRONMENT

The normal tissue's microenvironment is characterized by specific architecture, extracellular matrix (ECM) and parenchymal cell types, as well as by the presence of vasculature, immune cells, and specific physical and biochemical cues (e.g. interstitial pressure, biomechanical factors, cell metabolism's

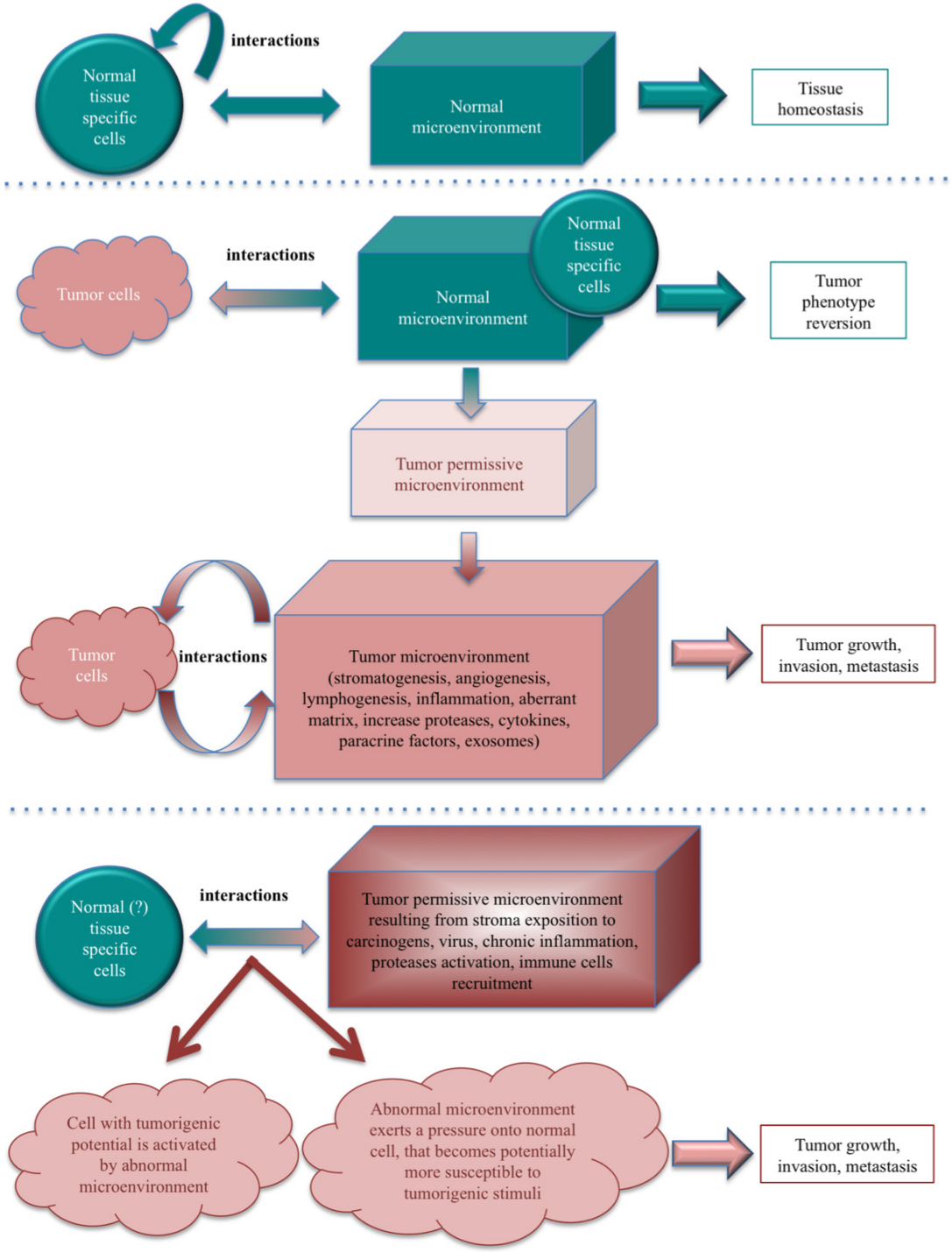

Figure 1: The stroma microenvironment is an active contributor to tissue homeostasis and pathology. Microenvironment exerts a selective pressure onto tumor cells, as well as tumor cells that interact with the microenvironment may selectively constraint it to progress toward tumorigenic process or to regress to "normal" state.

products, anti-oxidative agents, gas components, pH). Tissue homeostasis results from the balance between cell-cell/cell-matrix interactions and from the composition and organization of the stroma. Alterations of cell (normal versus malignant) or/and microenvironment stability/features can result either in the malignant phenotype reversion or in tumor growth (Figure **1**). In such reversible configurations, the cell microenvironment is often proposed as a "tumor suppressor" element, since it is thought to limit cancer progression. Different cellular and macromolecular entities are implicated in tumor suppression (e.g. immune, epithelial, or myoepithelial cells, proteases, junctions such as desmosomes) [9, 14-16]. Drugs such as tamoxifen can also suppress the tumor phenotype by interfering with the mammary ECM [17]. On the other hand, when the microenvironment is modified, its composition/architecture varies, in such a way that it becomes more permissive to tumor growth, and begins to play a crucial role in regulating tumor evolution and propagation. In the pre-tumor/tumor microenvironment, it is really possible to observe a loss of tissue architecture, aberrant cells and ECM (in quality and quantity, *desmoplasia*), neoangiogenesis/lymphangiogenesis, inflammatory and fibrotic processes, recruitments of tumor-associated fibroblasts and macrophages, infiltration of leukocytes, high presence of cytokines, growth factors, hormones, morphogens and proteolytic enzymes. Tumor cells and inflammation are often intimately co-involved in the further tumor development. Indeed it is well-known that the local inflammatory tumor microenvironment has an impact on cancer development; its role is so fundamental to tumor progression that it now represents a hallmark of cancer [6]. Moreover, recent findings have demonstrated that tumors can affect distant tissues, by inducing a chronic inflammatory response *in vivo*, leading to increased systemic levels of oxidative DNA damage [18]. The stroma itself may possess an oncogenic impact, independently of the presence of tumor cells [19]. Moreover, the tumor microenvironment is also involved in the drug resistance process that it is an important cause of cancers'relapse and fatal onset.

The components of the tumor microenvironment vary according to the tumor type, but usually two main compartments can be identified: i) the cellular compartment consisting of tissue specific cells (fibroblasts, pericytes, smooth muscle cells, endothelial cells, mesenchymal cells, immune and/or inflammatory cells) and ii) the matrix-related compartment comprising a solid phase (extracellular matrix) and a more "fluid-phase" (soluble factors: cytokines, chemokines, trophic factors). There is a dynamic interplay between tumor cells, normal cells and their surrounding matrix that involves biomechanical and biochemical signals, integrated into complex molecular interactions and intra- and extracellular signaling networks [20], outlined in Figure **2**.

2.1. The Main Cellular Actors in the Host Stroma

2.1.1. The Non Malignant Cell Compartment

The reciprocal interactions between malignant cells and their neighboring host stromal cells actively regulate tumor features, growth, invasion, angiogenesis and metastasis. These interactions can also be mediated by paracrine factors. Moreover, tumor cells recruit/activate non-malignant cells (bone marrow-derived cells, inflammatory and immune cells, cancer associated fibroblasts, pericytes, endothelial cells), which interfere with the normal, physiologic microenvironment. The (abnormal) tumor microenvironment itself favors tumor progression by altering cell function and ECM cues.

Bone marrow derived Mesenchymal stem cells (MSCs) present an innate tropism for tumor [21] as a result of tumor cells' chemotaxis, mediated, for example, by growth factors (VEGF, EGF, HGF, bFGF and PDGF). The MSCs homing can also be regulated by chemokines and proteases (urokinase, metalloproteases). MSCs can be recruited by tumor cells, and when they reach the tumor microenvironment, they differentiate in tumor-associated fibroblasts, pericytes and myofibroblasts [22-23]. Reciprocally, these mesenchymal cells can attract tumor cells in the bone marrow district. Their exact role as tumor suppressor or promoter is still in discussion because of the complexity of their intercellular and molecular interactions with the tumor microenvironment [24].

Inflammatory cells. Chronic inflammation are recognized as an important step in cancer progression [25]. The inflammatory infiltration in tumors results from the recruitments of leukocytes mediated by cytokines and chemokines synthesized by malignant cells. This process is important for the further angiogenic process. Inflammatory and immune response is intimately linked in determining tumor behavior.

Immune cells. Among all functions of the immune system, the immune-surveillance should, as a rule, also eliminate cancer cells. Cells dedicated to such targets

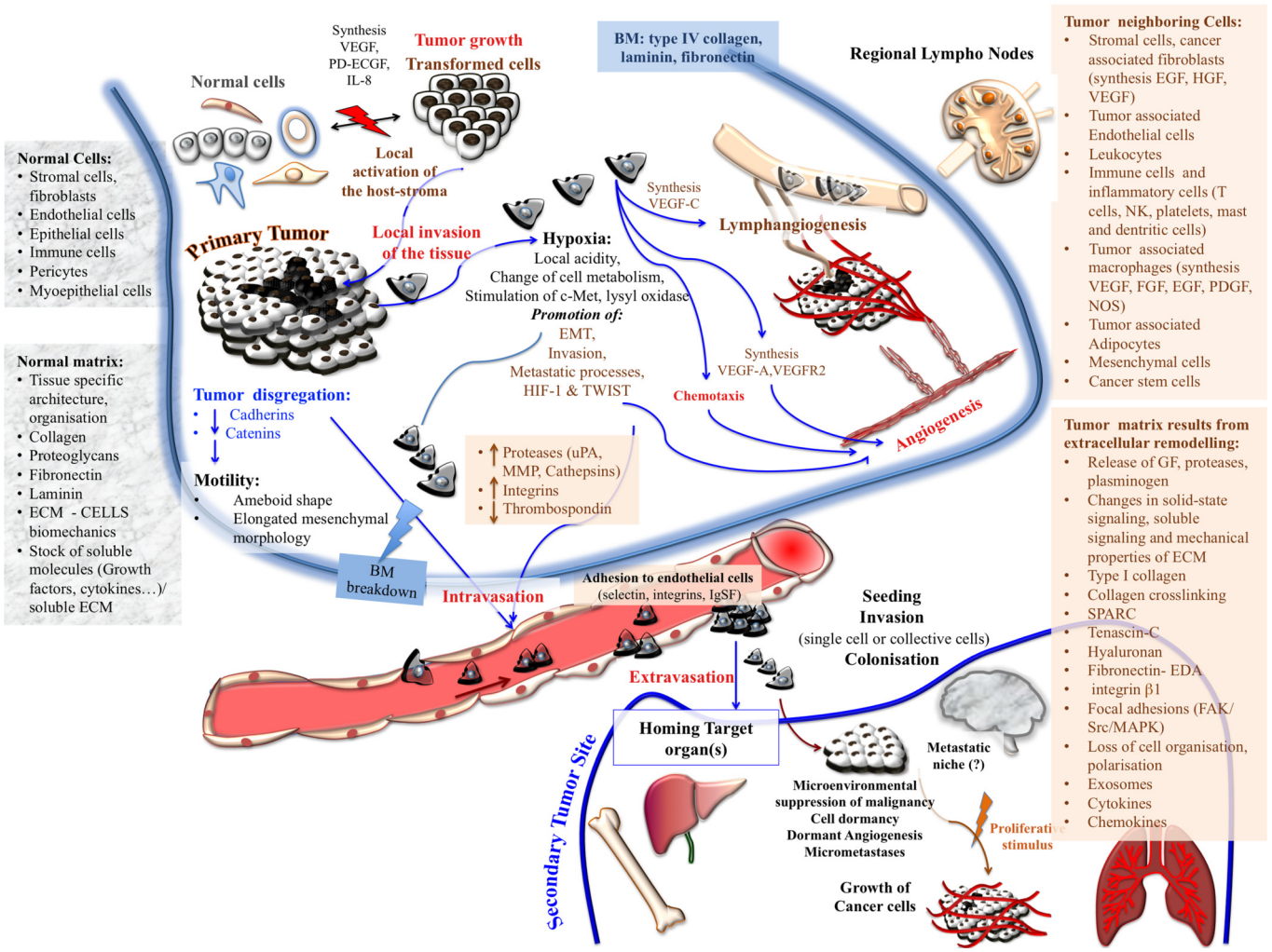

Figure 2: The main cancer hallmarks. The heterogeneous tumor microenvironment embraces a cohort of cells (stromal cells, immune and inflammatory, stem cells, etc.), gradients of bioactive molecules, macromolecular, biophysical and biochemical cues (ECM, growth factors, cytokines, metabolites), which allow interactions and resulting signaling networks to contribute to tumor progression. Whereas normal cells and ECM may be conducive to tissue homeostasis and tumor phenotype reversion, the tumor microenvironment leads to a cascade of events that usually finishes with the dissemination of tumor derived cells into other tissue districts of the body. These changes regard either cells behaviors and features (for example switching from normal fibroblasts or myoepithelial and epithelial cells to myofibroblasts, cancer associated fibroblasts, mesenchymal cells etc) or activation of an adaptive response by altering cell metabolism and syntheses (from oxidative to glycolytic pathways) as well as gene expression (hypoxia inducible factor 1 (HIF-1), proteases, integrins, cadherins, growth factor, cytokines, chemokines, pro-angiogenic morphogens), due to hypoxic and acidic environment and resulting in ECM remodeling, basal membrane collapse, activation of angiogenesis/lymphangiogenesis and subsequent migratory/metastatic processes to target organs. Tumor and tumor microenvironment characteristics vary between cancer types, patients and in the course of the pathology. For leukemia, for example, osteoblastic/endosteal and hematopoietic stem cells niches, in addition to the cohort of cells already described, play a crucial role.

are mainly lymphocytes T, monocytes/macrophages, mast cells and natural killers (NK). However, in addition to preventing tumor growth, the immune system is involved in the opposite induction of cell transformation (tumorigenesis), the so called process of cancer immune-editing [26]. A way by which immune cells can promote tumorigenesis is the increase in MMP synthesis, involved in ECM remodeling and, therefore, in the remolding of the stromal microenvironment [27].

Tumor Associated Macrophages (TAMs). TAMs are present in many tumors as a significant cell population; moreover their high number also corresponds to a poor prognosis' marker in many

cancers. Monocytes are recruited into the tumor by chemotaxis (mainly by chemokines and factors such as CCL2, CCL-5, CCL8, CXCL-12, VEGF, MIP1a, and MIF). Macrophages can produce VEGF-C and VEGF-D, MMP-2 and IL-8, playing, by this way, an important role in lymphangiogenesis and ulterior metastatic processes mediated by the lymphatic system. They can also produce a number of angiogenic growth factors, cytokines and proteases, which are implicated in tumor progression. Macrophages also interact with tumor cells, reciprocally exchanging/sensing biochemical cues (mediated by CXCL-12 and EGF or NO and CXCL-12) [28, 29].

Dendritic cells. These cells are physiologically involved in the innate and adaptive responses of the immune system. Moreover, they are recruited in the tumor environment regulate the angiogenic process by secreting TNFα, TGFβ, GM-CSF and IL-12 [30]. Whereas they can play a crucial role in inhibiting tumor growth, their normal activities and maturation can be hindered by tumor associated macrophages, reducing in such a way the immune surveillance necessary to counteract tumor growth [31-32]. The capability of this cell type to regulate angiogenesis might also be linked to their predisposition to differentiate into endothelial-like cells in response to tumor signals [33]. Moreover, because they also secrete metalloproteases (MMPs) and their inhibitors (TIMP), they can play a role in tumor progression and metastasis [34]. Mast cells are immune cells involved in immunological and inflammatory responses. They can either down regulate the tumorigenic process by secreting interleukins, or promote tumor growth by interfering with stroma-epithelium interaction and ECM degradation, encouraging angiogenesis (e.g. by secreting factors such as VEGF, FGF-2 and IL8) and lymphangiogenesis, and inhibiting antitumor activity [10, 35].

Cancer-associated fibroblasts. Within a tumor mass, different types of sub-populations of fibroblasts are usually found (i.e. dormant fibroblasts, myofibroblasts, cancer-associated fibroblasts), that are molecularly different from their normal fibroblastic counterparts [36]. They synthesize great amounts of ECM components (type I collagen and fibronectin) and are largely responsible for *desmoplasia*. These abnormal fibroblasts secrete matrix proteases and relative inhibitors (whose crucial role is either linked to their proteases' activity, or to their non-proteolytic activity), which are paracrine factors that regulate tumor growth (EGF, TGFβ, HGF, PDGF, IL1, IL6, IL8), as well as angiogenesis (VEGFA and FGF) [37]. Cancer-associated fibroblasts produce CXCL12 that allow the recruitment of endothelial progenitors from the bone marrow [38]. These fibroblastic cells originate mainly from the trans-differentiation of resident fibroblasts, but also from bone marrow mesenchymal cells [36]. One part of these cells probably also derived from the epithelial-mesenchymal transition (EMT) that takes place during tumor invasion. Cancer-associated fibroblasts represent a noticeable part of the tumor mass and influence tumorigenesis (initiation, progression, invasive and metastatic processes) [9, 39]. They sustain cancer cells' survival by removing their metabolic wastes [40], and their presence also represents an unfavorable parameter for cancer patients [41].

Adipocytes - cancer associated adipocytes. These cells are responsible for energy storing, and can directly provide lipids to tumor cells; adipocytes also play an important role in regulating the tissue microenvironment, because of their capacity to synthesize a number of biologically active molecules, referred to as adipokines, and which include cytokines, growth factors, hormone-like factors and chemokines [42]. These tumor-associated adipocytes were shown to promote the growth of malignant tumors in breast, colon and prostate tissues) [43-45]. Moreover, adipose stromal cells might also be recruited in the tumor microenvironment and contribute to the differentiation of progenitors into pericytes and adipocytes [46].

Tumor associated endothelial cells. These cells are involved in the angiogenic process that promotes cancer progression. Moreover, the hypoxic environment within the tumor's tissue, together with neoplastic, stromal and inflammatory cells secreting VEGF, stimulate the angiogenic switch of quiescent endothelial cells to proliferating cells. This leads to the building of a new, abnormal architecture and poorly functional blood vessels [47, 48], that fail to become quiescent because they lost the adequate control of pro- and anti-angiogenic factors. Besides, these neo-endothelial cells synthesize growth factors favoring tumor (IGF, PDGF, GM-CSF).

Perycites. The recruitment of pericytes into the tumor's microenvironment is mediated by PDGF-B receptor expression [49]. These cells participate in the maintenance of functional vessels, while their reduced number in tumor's vessels seem to sustain intravasation processes.

2.1.2. The Malignant Cell Compartment

The hypothesis of Cancer stem cells (CSCs) existence is based on tumor's heterogeneity; more recent evidence seems to indicate that these tumor cells' subsets might be liable to tumor progression [50, 51]. Their presence was shown in leukemia as wells as in solid tumors, even if at a lesser level [52, 53]. The hypotheses regarding their origin are still under investigation [50]. The heterogeneity in CSCs' (and in their progeny's) phenotype and function between patients and tumor's type, as well as inside the same individual tumor, increases the difficulty in better characterizing them and targeting them for therapy. Moreover, these cells might be present in a pre-metastatic niche, either as Epithelial to Mesenchymal Transition (EMT) derived cells, or as a trigger for the recruitment of metastatic cells in a hospitable niche [52].

All the host cells are, at the beginning, without apparent malignant properties, but, probably due to the wrong microenvironment pressure, to their dynamic intercellular interactions, and to their active communication with tumor cells, they express an atypical phenotype [54].

2.2. ECM Compartment

The ECM, in addition to representing a support structure for cells, is also responsible for regulating vital biological processes (such as differentiation, proliferation, viability, migration, cell interactions/communication), and serves as reservoir for a number of factors. Matrix properties that are directly involved in the regulation of such events are: i) its own biochemical composition (e.g. collagen, laminin, fibronectin, tenascin C, hyaluronic acid (HA), proteoglycans, cadherins, integrins, osteopontin, galectins, fibulin-1), ii) the presence of matrix proteases involved in ECM remodeling/breakdown (MMPS, urokinase plasminogen activators, cathepsins), iii) its organization (at macro-, micro- and nanoscale), or iv) its capacity to interact with biologically active molecules (growth factors, cytokines, chemokines), usually stored for interacting with ECM macromolecules, and also v) its biomechanical properties, that are directly linked to the regulation of gene expression. MMPs are enzymes involved in ECM turnover, and, in such a way, within the tumor context, they play a noticeable role as regulators of tumor progression [34, 55]. ECM also presents mechanical properties (stiffness, compliance) that regulate cell behavior, owing to mechanotransduction pathways and mechanosensing cell capacities. These ECM characteristics influence (prevent or favor) tumor invasion, as a consequence of alterations in tensegrity, namely in the equilibrium in continuous tension and discontinuous compression forces existing at the matrix, cell, and intracellular levels and that stabilizes cell functions [56-58]. Alterations in ECM stiffness/ compliance were shown, for example, in murine breast where: in normal murine breast elastic modulus is about 170 Pa, in the tumor surrounding stroma it increases up to 920 Pa whereas it reaches about 4000 Pa in tumor [59].

Integrins function as mechano-transducers and influence gene expression and cell behavior. Integrins show a crucial, and often contradictory, role on regulation cell migration, invasion, proliferation and survival; they orchestrate host cells' behavior and thus tumor progression [59-61]. The integrin subtypes that are more frequently overexpressed in tumors are, for example, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha 5\beta 1$, $\alpha 4\beta 1$, $\alpha v\beta 6$ and $\alpha 6\beta 4$. The expression and clustering of integrins in turn can be modulated by the ECM stiffness, resulting in a more tumorigenic phenotype [59]. In addition to the tissue architecture stability, the matrix integrity is fundamental too and regulates the release of pro and antiangiogenic cues, by mean of its type IV collagen, fibronectin and thrombospondin-1 components [62, 63]. In effect, when ECM proteins are hydrolyzed by matrix proteolytic enzymes, pro- and anti-angiogenic factors, and other ECM-sequestered factors, turn out to be bioavailable [62, 64].

Tumor microenvironment was shown to be also involved in drug resistance. Resistance to chemotherapeutics may be determined by tissue organization, cell adhesions/interactions, ECM viscosity (and relative drug diffusion in the interstitial space), hypoxic and acidic intratumoral context, or by soluble mediators produced by tumor, host cells and their cross-talk [8, 9].

Another factor that points to the importance of the microenvironment in tumor progression is evinced in the appearance of second cancers after radiotherapy. Radiotherapies may have opposite effects on the tumor: whereas they locally present the expected anti-tumor effect, they can also alter the tumor microenvironment (increase in growth factors', cytokines' or tumor promoting factors' secretion), and might contribute, in such a way, to local invasion and metastatic processes in pre-irradiated zones [65]. This was confirmed by several observations, which suggests that the effect of radiation depends on a

number of parameters (the characteristics of the irradiated tissue, the age of the patient, the dose and dose rate, the volume of targeted tissue, and hereditary factors) [66]. The effects of radiation might be mediated by genomic instability of cells that survive to the therapy, by the selection of hypoxic resistant cells, by the activation of the tumor microenvironment's production of growth factors and proteases, as well as by the possible radio-resistance of a subset of cancer stem cells [67].

3. DEVELOPING MORE BIOMIMETIC CANCER MODELS

In order to overcome the high incidence of tumors, to increase the efficacy of treatments (in addition to traditional surgery, chemotherapy and radiotherapy), and to reduce therapy-related side-effects and resistance, it is necessary that oncology research points to the development of new therapeutic strategies, (such as, for example, specific immuno-therapy and molecular-targeted therapy), in order to better focus onto specific molecular/microenviron-mental objectives [68]. This will, further, provide a more specific patient-targeted therapy, as tumorigenesis reflects a complex and heterogeneous situation, closely related also to individual characteristics. Unfortunately, whereas in preclinical studies alternative strategies were shown to be efficient, when translated to patient's treatment, they often showed an unsuccessful outcome. This may be due to the "gap" still existing between *in vivo* (animal-based) models (immuno-compromised or transgenic/humanised mice), *in vitro/ ex vivo* culture models, and in humans (i.e. clinical application); thus, the lack of physiologically relevant models for the study of human tissues' functions and behavior is now well recognized, at the different levels of basic and applied research and risk assessment [69]. Moreover, in addition to ethical reasons, the application of the 3R's [70] recently included into most of the new international legislative bodies (see, for example, EU REACH Regulation, 2006 - at http: //ec. europa.eu/enterprise/sectors/chemicals/reach/index_en .htm - and European Directive 2010/63 EU - available at http: //eur-lex.europa.eu/LexUriServ/LexUriServ.do? uri=OJ: L: 2010: 276: 0033: 0079: en: PDF), encourage/ compel the reduction / elimination in the use of animal models in basic and applied research studies. In order to overcome such limitations, new and relevant *in vitro* models need to be developed. Because of the complexity, variability and heterogeneity of tumors (intra-tumor's physical, biochemical cues and molecular gradients -zonation of tumor- and differences between patients and tumor types), it clearly emerges that a battery of human-derived *in vitro/ex vivo* models must be developed/optimized to better respond to clinical needs.

In conclusion, it clearly appears that it is important (and urgent) to increase our knowledge of the tumor microenvironment, in order to better control and target it. This is the reason why *in vitro* models, able to reproduce all the fundamental cues and features of the tissue-specific native microenvironment, the complex network of cell-cell's/cell-matrix's, and cell-bioactive molecules' interactions, need to be developed. *In vitro* models should, then, take into consideration (and resume) the complexity of the tumor microenvironment. To this aim, it should be better to consider a tumor as a complex organ, in which a wide variety of cells coexist, that secrete a high quantity of soluble factors (growth factors, cytokines, chemokines, proteolytic enzymes, etc.), that interfere with cells' behavior but, also, with the physiological tissue remodeling process or with tensegrity; these last, in turn, control/may affect cells' behavior and, consequently, ECM features.

3.1. 2D *versus* 3D Cancer Models

Cell culture systems were initially designed in order to simplify the complex *in vivo* context, and they allow a specific control on environmental conditions and have the advantage of being more reproducible and cheaper than *in vivo* models. The majority of *in vitro* models are characterized by the maintenance of cells in 2 dimensions (2D) (artificial, rigid, plastic surface of traditional Petri dishes). Several mechanistic *in vitro* studies have been performed in such 2D culture systems, which are very distant from the physiologic *in vivo* conditions where cells develop and function; even if they represent a fundamental source of information into key biologic phenomena linked to neoplastic transformation (elucidation of specific signaling pathways' perturbation, genetics of oncogenes and tumor suppressor genes, mechanisms of action of tumor promoters and mutagens, etc.), these systems are often too limited to correctly mimic the complexity and heterogeneity of the tumor/tumor's microenviron-ment interaction, and fail in simulating real cell behavior in living organisms. Whereas, *in vivo*, cells naturally grow in a three dimensional (3D) context that, besides allowing the correct polarization and orientation of cells, provide them with the surrounding matrix, that represents a dynamic environment that furnish cells with an architectural support, temporally-regulated

biochemical and biomechanical cues, and specific interactions with neighboring cells and matrix; whereas, in the classical 2D *in vitro* configuration, culture conditions impose an unnatural cell polarization, a loss of cell organization - with disrupted interactions between cells and cell/ECM, caused by the disruption of normal tissue architecture - and the loss of native ECM, with the consequence of affecting, in such a way, biomechanical cues and critical biological processes, such as cell proliferation, viability, migration, differentiation, and specific cross-talks, as well [69, 71-74]. Moreover, the "flat" 2D conditions, lacking native ECM, does not mimic the natural tumor's microenvironment that normally interfere with drug bioavailability, rendering, in such a way, a hardly effective extrapolation of drug activity from *in vitro* studies to clinical applications. As for normal tissues, culture models may be more or less complex, and reflect, more or less accurately, the *in vivo* specificities of the native tissue. Briefly, from the simpler to the more complex *in vitro* models, biological studies can be performed on isolated subcellular fractions, microsomes, single isolated cells, cells in suspensions, 2D cultured cells (homotypic or heterotypic cultures), and 3D cultures (multilayered confluent cells, multicellular spheroids, with or without scaffolds, in mono- or heterotypic configurations, tissue slices, organotypic systems) [69, 75, 76]. Often, the more the system model is simplified, the less it is able to maintain, at long-term, cells' viability and functions. As in the case of untransformed, normal cells, cancer cells also, when maintained in 3D culture, exhibit behavior and gene expression patterns closer to the *in vivo* conditions [77-79]. Already in the '70s, Bissel's group and others demonstrated how tissue architecture, 3D context, composition of ECM, correct cell polarization, and epithelial adhesion are fundamental for reproducing relevant *in vitro* models able to mirror, as much as possible, the physiological or pathological features of breast tissue [80, 81]. They showed that the 3D culture better recapitulates different tumors' behavior than did 2D configurations. For example, it was demonstrated that the 3D context was able to reverse tumor phenotype to normal (with specific pathways' inhibitors), and to sustain the cross-talk between integrin and EGF receptors; moreover, tumor cells cultured in 3D showed, with respect to 2D-cultured controls, an increased pro-angiogenic capacity and a higher resistance to IFN, chemotherapeutic agents and irradiation [82, 83]. Other parameters that are highly modified by the switch from 2D to 3D configuration are the cell migration, cell morphology and cell signaling [84, 85]. Some other advantages of the 3D *vs*. 2D culturing techniques for investigating tumor's behavior are also illustrated and discussed by Ferrarini *et al.* [86]. Moreover, some biological events that can be preferentially studied in 3D models are presented in Table **1**.

Table 1: Examples of *in vitro* Models Applied to the Study of the Main Cancer Hallmarks

Biological events	Models	Outcomes	References
Cell invasion, migration, motility and metastasis	• Matrigel™ *based assay* • *Type I collagen assay* • *Type I collagen overlaying 3D assay* • *Multicellular spheroids* • *Acellular matrices* • *Co-cultures of bone sections with tumor cells in roller tube system.* • *Co-cultures of Non-Small-Cell Lung Carcinoma Cells with bronchial mucosa* • *Microfluidic models* • *Nanoimprinted scaffolds*	• The outgrowth of cells from multicellular spheroids (consents to isolate the migration process from the complex multicellular context). • Hydrogel based assays allow investigation of factors and pathways involved in migration as well as dynamics regulating this process. • ECMs produced by fibroblasts at different stages of tumor progression allow to reproduce the matrix/tumor cells interactions and their involvement in tumorigenesis/invasiveness. • Co-culture systems seem efficient in reproducing ➢ bone tropisms of tumor cells originating from breast or prostate. ➢ bronchial mucosa invasion by lung carcinoma cells. • Microfluidic models allow interesting studies of metastatic process by culturing in 3D different cell types (tumor cells, cells of target organ etc). A limit of these models (for metastasis investigation) is that it is necessary to know in advance the main step of the different processes (intra-, extravasation, target organs). Concerning the easier tumor growth, cell migration approaches, they allow a good survival of 3D cocultures. • Micro- and nano-engineering represent the future of tissue engineering for conducting cell behavior, up today few of these models are applied to tumor biology.	[242, 319-329]

Table 1 Continue …..

Biological events	Models	Outcomes	References
Angiogenesis	• Matrigel™ *or collagen based assays with cells or aortic explants.* • *3D sprouting angiogenesis assay.* • *Organotypic co-culture assays*	• Outgrowth or migration of cells is evaluated after different period of time. • Co-culture assays, where fibroblasts can serve as ECM producer; the three dimensionality is mainly provided by the fibroblast layer. • After some days, sprouting/branchings or neo-capillaries (tube like structures) may be observed in response to biological stimuli (growth factors). • It is important to choose the good (anatomical) source of endothelial cells. • None of the actual *in vitro* model accurately reproduce the whole *in vivo* process of angiogenesis, mainly because of the complexity of the neovascularisaztion events that, for example, also includes the regulation of the neoangiogenesis by the fluid flows.	[11, 99, 116, 330-334]
Drug responsiveness/ resistance / testing	• *Multicellular aggregates in static or dynamic conditions* • *Multilayered postconfluent culture* • *Hydrogel based cultures* • *Tissue slices*	• Cells express more differentiated functions and resemble more closely to *in vivo* situation. • Cell maintained in 3D often exhibit an increase resistance to chemotherapeutic drugs as compared to cells maintained in 2D configuration. According to the tumor cell type and the configuration of the 3D models, cells can gain or lose drug sensitization reproducing the native features of tumor. • Synthetic scaffold/hydrogels leading to better monitor ECM mechanics may allow to take into account the influence of the physicochemical properties of ECM onto drug bioavailability and diffusion.	[71, 87, 116-117, 335-339]
Tumor growth	• *3D free floating multicellular spheroids (mono and heterotypic), gel embedded spheroids, in static or dynamic culture conditions*	• Several tumor features (cell proliferation, intercellular contacts and interactions, cell-matrix interactions, hypoxia, HIF-1a expression and relative consequences) can be reproduced. • Coculture in dynamic conditions also consent to recreate, at least in part, the complex bone marrow microenvironment and are applicable in studying hematopoietic niche in health or illness.	[90, 115, 120 122, 337]
Microenvironment influence	• 3D culture in/onto natural and/or synthetic scaffolds	• ECM mechanics (compliance, stiffness) can be better controlled by using synthetic scaffolds.	[77, 78, 99, 115-121]
Tumor reversion	• Culture of tumor cell in/on normal basal membrane or in low stiffness	• ECM leads to the recovery of “normal” cell behavior (slower growth, epithelial like polarization ecc).	[59, 340-341]

3.2. 3D Tumor Models

A first step in increasing the complexity of the microenvironment is the culture of multilayered post-confluent cells. In these systems, cells are maintained on V-bottomed microplates and grow as multilayers (up to 15 layers can be assembled), harboring properties that look like those of native tumor, thus suggesting that these models could be suitable for drug screening [75]. The 3D culture of multilayered cells was further developed by Simon and collaborators [87], by using breast cancer-derived cells, but a deeper characterization of cell model’s properties is required in order to ascertain the capability of correctly reproducing the tumor environment.

The 3D culture models of tumor cells that are at present most extensively used are represented by the culture of multicellular spheroids (MSs), that consists in the self-aggregation of (neoplastic) cells in multicellular, rounded aggregates (spheroids). These spheroids can be obtained by different methods (e.g. coated-plates, hanging drops, liquid-overlay techniques, hydrogel templates, micro-patterned wells). Spheroids can also spontaneously form in dynamic bioreactors (such as spinner flasks or Rotary Cell Culture System’s - RCCS™ - devices), or by microfluidics-based technologies, with or without scaffolds [88-90]. Spheroids show a particular cellular microenvironment, since they are able to reproduce, at least in part, key 3D tissue-like features known to influence cell behavior and gene expression (i.e. organization, zonation, ECM deposition, cell-cell and cell-matrix interactions). Due to a low mass transfer condition, these peculiar tissue-like 3D constructs display various similarities depending on tumor mass, and, in particular, gradients of nutrients, metabolites, catabolites’s wastes and growth factors, associated with low O_2, and high CO_2 concentrations

[91]. If spheroids grow up to 500 μm diameter, a central necrotic core appears, reproducing in such a way the hypoxic and necrotic processes usually observed in solid tumors [92, 93]. Cells grown in MSs usually exhibit a lower sensitivity to therapeutic drugs and a more specific response to particular therapeutic agents as a result of new signaling pathways activation [94, 95]. In living organisms, in addition to the involvement of particular proteins (such as the drug/ABC transporters, enzymes and HMGB1), or to the increased efficiency in DNA repairing mechanisms and resistance to apoptosis, multi-drug resistance may also be due to ECM's organization and composition (*desmoplasia*), cell-cell adhesion and lower growth rate, which are parameters that mirror the *in vivo* context, in which tissue architecture and cell adhesion, as well as the absence of blood vessel and the low hydrostatic and osmotic pressure, limit the diffusion of therapeutic drugs [83, 96, 97] .

In addition, when cells grow in spheroids, they form an outer layer of proliferative cells and a more central quiescent zone where cells do not proliferate. Since chemotherapeutic drugs often target the dividing cells, this quiescence may justify the "resistance" to cyto-static drugs observed in MSs [89, 98]. Furthermore, *in vivo,* drug bioavailability and delivery are also regulated by the ECM's attributes (configuration, composition, structure), that can alter/disrupt cell signaling [99, 100]. Heterotypic MSs more closely mimic the heterogeneity of tumors and the heterotypic inter-relations between cells, and, in addition to monotypic culture, they allow the investigation of important tumor endpoints such as angiogenesis, tumor cell migration and invasion, and drug responsiveness [101]. Examples of heterotypic spheroids may encompass tumor cells with fibroblasts, macrophages, and endothelial cells [102, 103]. For example, heterotypic MSs of melanoma cell lines and endothelial cells allowed to demonstrate that–invasion and angiogenesis processes may be potentiated by cadherins [104]. Co-cultures of fibroblasts and cheratinocytes can be also used to form 3D models of reconstructed skin, in which melanoma or other skin cancer cells can be embedded. Within these 3D skin reconstructed models, melanoma's cells from different stages of tumor progression have the same properties they show in the patients' skin. For instance, melanoma cells at the initial radial growth phase are unable to invade the dermis from the epidermis, whereas, in advanced stages such as the vertical growth phase and the metastatic phase, cells readily invade the dermis [105]. A number of recent studies have assessed the effects of novel anti-cancer drugs in 3D models of reconstructed skin [105, 106]. Alternatively, multicellular aggregates can be formed by a unique cell type for generating tissue-like structures and, then, they can be further co-cultured with tumor' cells, in order to observe the reciprocal interaction of both cell types [107]. Important limits of these MS-based models are: i) some cells are not able to aggregate in order to form multicellular spheroids; ii) the spatial arrangement of cells could be casual and could not match the *in vivo* co-localization of specific cells; iii) cell ratio inside multicellular spheroids does not always reflect the real proportion of cells in the tumor's environment; iv) co-cultures should involve different tumor cells (in addition to non-tumor cells), in order to better mimic tumor's heterogeneity; v) 3D MS's culture require very efficient dynamic bioreactors, which can allow a long-term culture of the spheroids, so that the maturation of the neo-synthesized ECM may take place and achieve characteristics close to the situation *in vivo,* vi) cell amplification and aggregation can be time consuming experimental procedures, vii) while heterotypic cultures can mimic the reciprocal cross-talk of tumor's with neighboring cells (thus mirroring the *in vivo* tumor context better than in other *in vitro* model systems), these co-culture conditions make it more difficult to distinguish among all cell types, which of them is responsible of the biological effects observed in response to external stimuli.

3.3. Mimicking the ECM

One of the most important challenges for finding the 3D culture model more suitable for each specific experimental purpose, is to also engineer ECM, so that neo-constituted matrices are obtained with structural and biochemical features similar to those of the specific native matrix of the tissue under study. This means that it is important to reproduce both the mechanical (architectural, topographic, physical features and tissue mechanics: ECM dimensionality, collagen fiber size and orientation, ECM stiffness/compliance), and the biochemically- and biologically-active ECM's characteristics (ligand-binding sites) [108, 109].

One of the most used "biological" ECM is that extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma's cells (most diffused trade names: Matrigel™, Cultrex BME™). This tumor-derived matrix has the characteristics of a basement membrane-like matrix, since it is mainly composed of type IV collagen, laminin, perlecan, nidogen/entactin and trophic factors [110, 111]. It was shown to sustain 3D cultures of

breast-, colon-, prostate-, and lung-derived tumor cells, allow expression of some of their original tissue-specific functions and to allow co-culturing, thus confirming, for example, that, in an *in vitro* prostate tumor model, stromal cells might activate tumor growth and invasion's process [74, 79, 112-114]. However, this extractive matrix, besides being variable in quality from batch to batch, fails, at least in part, in recreating the native mechanics of normal basement membrane, and does not correspond at all to the other matrices found within connective tissues and parenchyma. This mouse-derived matrix does not represent the ECM of all tumors, since, as reported before, each tumor possesses its own characteristics, which also vary in accordance with its evolution. Therefore, it is of interest to be able to isolate/customize/bioengineer the tissue-/tumor- specific 3D matrices. Several strategies were developed to respond to this need, and a number of natural (hyaluronan, chitosan, collagen, alginate, fibrin, laminin-rich ECM proteins) or synthetic matrix/gel/ hydrogels/scaffolds were proposed (e.g. Poly(lactide-coglycolide), poly(lactic acid), poly(vinyl alcohol), poly(ε-caprolactone) or polyethylene glycol) (e.g. Poly(lactide-coglycolide), poly(lactic acid), poly(vinyl alcohol), poly(ε-caprolactone) or polyethylene glycol) [77, 78, 99, 115-121]. One advantage of scaffold-based strategies is that it is possible to better manage matrix stiffness/compliance, porosity, and biochemical composition.

Among the various available ECM substitutes, collagen gels seem to represent an interesting option, especially if cross-linked with other matrix components [120]. Unfortunately, as in the case of Matrigel™, analogous, collagen hydrogels also have a noteworthy difference between the different preparations. In collagen gels, monotypic or heterotypic cultures can be developed, thereby increasing the possibility of better mirroring the *in vivo* conditions by integrating tumor's neighboring cell types [122]. Culture of fibroblasts in collagen gels have allowed a better understanding of the reciprocal interactions between cells and ECM, and, namely, the regulation of cell behavior mediated by the mechanical properties of ECM [123].

Type I collagen can be electrospun and, therefore, it can support cell growth. By acting on ECM biomechanics, it also provides oriented fiber alignment-signals - or topological cues - known to regulate cell behavior. Hydrogels with a mixture of Matrigel™ and type I collagen were shown to present stiffness properties ranging from normal to breast tumor tissue [59], and to sustain multicellular cultures and tissue-like morphogenesis [124]. Scaffolds formed of chitosan and alginate's mixture were shown to better favor the tumor progression of human-derived glioma cells than does each component alone [125]. Moreover, scaffolds/ hydrogels can be functionalized to favor more native cell behavior and more tissue-like function [99] . Hence, the choice of natural matrix-derivatives and configurations is also of importance, since, as in *in vivo* tumors, it undoubtedly influences cell behavior, from cancer reversion to promotion steps.

Alternatively, some *in vitro* models have been developed, by using decellularized matrices and, even if their use for cancer investigations is still limited, they could represent an important source of natural ECM. The major advantages of these decellularized matrices are that they can be prepared from different tissues, maintaining the 3D architecture of the native tissue and its original composition. These matrices were used for investigating the behavior of breast tumor-derived cells (proliferation, morphology, migration) and their drug sensitivity, that were shown to be quite similar to what can be obtained with the *in vivo* xenograft models [126]. In particular ECM, prepared by using different explants obtained from human intestinal mucosae (ranging from healthy colon to colorectal carcinoma), can also be used to investigate tumor cell's behavior in response to a more or less permissive microenviron-ment [127]. The main limit of this ECM is that they are natively complex, their exact composition is not fully known, and this may render difficult the interpretation of cell's behavior in response to a particular stimulus.

Synthetic matrices could also be regarded as the future of engineering tumor microenvironments, but the high number of biomaterials proposed in the literature need to be further optimized before being effectively applied in the tumor biology fields [128]. In effect, in the design of these matrices, several key factors should be taken into account, and, among them, landscape/ topography, fiber orientation, porosity (macro-, micro-, nanoscale conditioning), matrix viscoelasticity, matrix remodeling, and growth factors' and cytokines' binding sites. It is now easier than before to monitor / engineer matrix compositions (collagen, proteoglycans, other macromolecules), to include adhesion peptides (the integrin-binding Arg-Gly-Asp motif *RGD*, for example), to functionalize the surface of the scaffolds/hydrogels, to control biomaterials' mechanical properties [129], to model cell behavior in space and time, and to develop patterning strategies providing a biomaterial substrate conducive to support a particular geometric configura-tion.

3.4. Which Cells for 3D Cultures?

Another important point that it is necessary to keep in mind is the choice of the cells to be used in the 3D *in vitro* models. There is a high number of available cell lines that express some specific tumor features, and a number of them have already been well characterized [130]. Nevertheless, the native heterogeneity of tumors renders difficult the choice of one suitable cell line, since cell lines could represent only one clone among the whole mosaics of tumor cells. Moreover, the use of freshly isolated cells from patients' tumors allows to work on primary cells, by avoiding the phenotypic drift related to the culture conditions. However, the risk exists that the experimental procedures employed for cell isolation and culture may exert a selective pressure, giving rise to clones able to survive and to eliminate more labile cells, with the consequent loss of the native cell heterogeneity typical of neoplastic tissue.

Interesting approaches based on the use of the native tissues were developed for different tumor-derived tissues, overcoming, in such a way, the limitation related to cell isolation. These models are mainly represented by the culture of tissue slices or explants. Both these approaches allow the maintenance of the 3D tissue architecture, cell organization, matrix arrangement and composition, i.e. the complexity and heterogeneity of the tissue microenvironment. Moreover, both models allow working with human tissues (healthy- or tumor-derived tissues), solving the problem of interspecies differences that occur when animal models are used. This allows a more accurate prediction of the human *in vivo* cell behavior. Tissue slices were applied to cancer research in order to analyze therapeutic strategies and drug responses, as well as to gain new insight into the cell invasion process [131-133].

The more complex culture of tissue explants is appealing, since it offers the possibility of maintaining a more consistent section of tissue (thickness of more than 1 mm *vs.* 250 μm, for explants and tissue slices, respectively), thus increasing the possibility of preserving multilayer cell-cell and cell-matrix interactions [134]. Once correctly established and optimised, the model based on the use of tissue explants can allow long-term experimental procedures, thus representing a potent tool for preclinical investigation of therapeutic drugs targeted on individual patients. Today, the limit of these tissue slices- and explants-based models is the difficulty of optimizing the protocols of *ex vivo* culture, since 3D hydrodynamic conditions and high mass transfer are essential to sustain cell viability and native tissue functions.

Even if 3D cultures lack some aspects of the whole *in vivo* environment (e.g. systemic interrelations, immune response, hormonal inputs), they differ from 2D conditions, that offer more cell signaling information and less tissue-like features. With respect to 2D cultures, 3D models also better resemble animal *in vivo* models, bridging the gap between these two models, routinely used in cancer research. However, 3D *in vitro /ex vivo* models still need to be optimized in order to overcome a number of limitations (Table **2**). Among these limits, time consuming procedures and lack of reproducibility are the most urgent to solve, especially if these models will be used for drug screening purposes in pharmaco-toxicology field.

4. LEUKEMIA

Leukemia is the most common neoplasms in children and accounts for almost a third (30%) of the tumors at diagnosis. Pediatric leukemias occur predominantly as acute leukemias, with acute lymphatic leukemias (ALL) representing the largest proportion (about 80% of all childhood leukemias) followed by the acute myeloid leukemias (AML, accounting for about 10% of all childhood leukemias) [12, 135]. Leukemic cells inhibit hematopoiesis and infiltrate organs, eliciting the main clinical manifestations, such as anemia, bleeding and infections. Leukemic infiltration of lymph nodes, the spleen and the liver may alter their function and increase their size. Moreover, testes and CNS are often infiltrated by leukemia cells [136].

4.1. Normal and Leukemic BM Microenvironment

Several lines of evidence suggest that the bone marrow (BM) microenvironment plays a central role in the control of leukemia initiation, progression, and drug-resistance. The BM microenvironment consists a complex 3D highly vascularized architecture of multiple cellular components, soluble factors, all embedded in an ECM. The cellular component of BM can be divided into hematopoietic cells and the BM stromal cells (BMSCs). The hematopoietic stem cells (HSCs) are localized within the BM in two main niches: the osteoblastic niche (at the bone-BM interface) and the vascular niche (around the blood vessels). This

Table 2: Main Shortcomings of 3D Culture Models and Proposals to Overcome them

Shortcomings of 3D culture models	
Weakness	***Counteractions to circumvent limitations***
Models are often limited to short term studies	• Develop effective bioreactors
Whole tumor complexity is lost	• Increase multicellular cultures approaches • Increase microengineering approaches (microfluidics, microfabrications, micropatterning) • Recover the native tissue mechanical cues • These necessary developments, are difficult to reach because of the high variability between tumor types, and inside tumor itself; a promising strategy may be to develop patient-biopsies cultures, but they will be hard to handle in the high throughput context and they are poorly available
Differences/variability between 3D models in pre-clinical assays	• Standardization of procedures and methods • Standardization for preparation of good/constant quality scaffold/hydrogels • The high number of available models and their relative strength and weakness force an aware choice of the model for defined application (because of tumor heterogeneity and the actual lack of perfect *in vitro* model, it is necessary to keep in mind that each *in vitro* system could model only partly the conditions in living organisms)
Application of Tissue engineering to tumor biology	• Microfluidic approaches offers good prospective applications, but need further investigation to render them easy to use, less expensive when thinking to high-throughput applications
Lack of suitable methods for drug screening in high throughput contexts	• Both cell models and analytic protocols should be optimized and standardized. • Models should consent to cover as much as possible the whole tumor /tumor microenvironment characteristics (these models are still lacking) • Scaffold/hydrogel/ medium etc (all components of the *in vitro* models should be tested for its interactions with drugs to eliminate experimental bias)
Loss of systemic regulations	• This gap could be bridge with the development of multi-compartmental bioreactors or bioreactor organized as parallel or series networks (but use of animals will remain necessary even if reduced)
Cost-effectiveness	• Standardization of procedures • Optimization of techniques for biological investigations • Research of more suitable models to reach equilibrium between reliability and cost

localization is required to maintain their self-renewal capacity [137, 138]. BMSCs include several cell types, such as endothelial cells, perivascular reticular cells, osteoclasts, osteoblasts, mesenchymal stem or stromal cells (MSCs), all of them important for HSC maintenance. Moreover, other stromal cells (glial cells, neuronal cells, adipocytes) can regulate hematopoiesis [139].

MSCs play an important role in BM, since they give rise to several cell types, including endothelial cells, neurons, astrocytes, myocytes, chondroblasts, osteoblast and adipocytes [140, 141]. This heterogeneous population is responsible for the production and deposition of ECM, formed mainly by type I and IV collagens, proteoglycans, glycosaminoglycans such as HA, and glycoproteins such as fibronectin, osteopontin, laminins and thrombospondins [142]. http: //www.ncbi.nlm.nih.gov/pmc/articles/PMC3566109/-pone.0054778-Hines1. Moreover, ECM not only provides the structural scaffold for cellular element of BM, but also represents a reservoir of cytokines, chemokines, and growth factors, produced by hematopoietic cells and BMSCs. The interaction between tumor and stroma occurs through the expression of receptors on the surface of leukemic cells binding the stromal cells or through adhesive ligands present both on stromal cell or ECM. Collectively, the BM architecture allows myelopoiesis, lymphopoiesis and immunoregulation processes in close physical proximity [143]. Accumulating evidence suggests that BM niches promote proliferation of a subpopulation of leukemic stem cells (LSCs), enhance their survival, and protect them from chemotherapy. Thus, the BM microenvironment has been proposed as a sanctuary for minimal residual disease, responsible for the occurrence of relapses, in several leukemias, including AML and ALL [144-146]. These events are mediated by the interaction between leukemic tumor cells/LSCs with specific components of ECM, with soluble factors present in the BM microenvironment or BMSCs. The processes of homing, mediated by soluble factors such as SDF-1, and the adhesion to the

stromal niche, thanks to adhesion molecules like CD44 or VLA4 integrins, are crucial events. The sequestered leukemic cells can proliferate thanks to several growth factors, cytokines, pro-angiogenic factors present in the BM stroma [145]. Interestingly, the adhesion of leukemic cells to the stromal niche elicit a better survival rate. Several mechanisms have been proposed to explain this feature, such as the induction of the cell adhesion-mediated drug resistance phenomenon (CAM-DR) [147, 148], the induction of a reversible quiescent state, that favors cell survival because most standard chemotherapies act on proliferating cells [145] and the increase of hypoxic areas which contributes to chemoresistance of leukemic cells http: //www.ncbi.nlm.nih.gov/pmc/ articles/PMC3414410/ - R10 [149]. The discovery of this complex network, in which the leukemic cells are only one of the participating players, suggests that more effective molecular therapies should target not only leukemic cells but also their microenvironment [150, 151]. Thus, useful leukemia models should reproduce not only a generic 3D environment, but also the specific leukemia microenvironment, represented by BMSCs, specific components of ECM or soluble factors embedded.

4.2. 3D Leukemia Models

Relatively few 3D models have been specifically set up for AML and ALL, thus multiple myeloma (MM) and chronic myelogenous myeloid leukemia (CML) and 3D models have also been considered in this review.

4.2.1. Leukemia Cell Lines on Scaffolds

The widely used 3D model of cellular spheroids, formed by spontaneous aggregation of cells, is well suitable for adherent cells but not for leukemia cells, that grow in suspension. Thus, leukemia cells necessarily need a support, such as a scaffold, eventually coated with an ECM component. Blanco and collaborators [152] evaluated the adhesion and the proliferation of several AML cell lines on different polymeric scaffolds, coated with ECM proteins. They showed that the polyurethane (PU) scaffold coated with collagen type I had the best performance, allowing the cell growth for over 6 weeks in the absence of exogenous growth factors.Vu and collaborators [153] produced an alginate hydrogel able to mimic 3D soft marrow tissue. The addition of the RGD peptide, the integrin-binding motif, to the hydrogel promoted further K562 leukemic cell growth and enhanced cell differentiation.

4.2.2. Co-Culturing Leukemia Cell Lines and BM Stroma Cells

Increasing complexity can be achieved by co-culturing a leukemia cell line with BM stroma cells. Dainiak and collaborators [154] used, as a scaffold, a polyacrylamide-based macroporous hydrogel (MH), functionalized with the type I collagen and with a structural analogue of the cell adhesive peptide RGD. MHs scaffolds with differently functionalized surfaces have been seeded with the human acute myeloid KG-1 leukemia cell line cultured with human embryonic fibroblast on MHs. The authors found out that leukemia cancer cells formed multicellular aggregates on collagen or RGD-like functionalized MHs but not on plain MHs and that KG-1 aggregates were more resistant to the treatment with the chemotherapeutic drug Arabinofuranosyl Cytidine *(*Ara-C).

The increased resistance to chemotherapeutic agents in a 3D environment, with respect to the corresponding 2D model, is a common feature not only in leukemia cell but also in other tumor types [78]. Moreover, the co-culture system results in a further increase of the chemoresistance. Indeed, Alitawi and collaborators [155] developed and characterized an *in vitro* 3D *co*-culture system where leukemia cell lines were seeded with human BM MSCs on a polyglycolic acid/poly-l-lactic acid (PGA/PLLA) copolymer scaffold. The effects of cytotoxic agents, such as doxorubicin or cytarabine, were evaluated and results were compared to those obtained by culturing leukemic cells alone in suspension (2D monoculture), or growing over a monolayer of expanded hu-BM-MSCs (2D co-culture). The authors demonstrated that 2D and 3D co-culture conditions showed less cytotoxicity of chemotherapy when compared to the 2D monoculture condition and that the 3D co-cultures achieved the highest resistance to chemotherapy. Moreover, these authors tested the capacity of free diffusion of molecules with different weights inside the PGA/PLLA scaffold, showing that it allows free diffusion of molecules up to 1000 Da [155]. This is one of the major issues in designing a 3D model for chemotherapy, since the 3D model need to allow media components and cytotoxic agents to diffuse freely to target cells.

An innovative 3D model was set up by Usuludin and coworkers [156]. They developed a hematopoietic co-culture system using the hollow fiber bioreactor (HFBR, purchase from FiberCell Systems Inc., Federick, MD, USA). An immortalized human BM stromal cell line, HS-5, was first established and maintained for up to 28

days in the HFBR. Subsequently, K562 erythro-leukemia cells were added to the bioreactor. The HFBR co-cultures enhanced proliferation of leukemic cells, compared to a standard 2D condition. Moreover, in 2D cultures, cells differentiated along the erythrocyte and monocyte pathways, but retained a subpopulation of myeloid progenitors. On the contrary, HFBR co-cultures showed multilineage differentiation pathways, such as the megakaryocytic, and the monocytic lineages, while erythroid differentiation was inhibited. Importantly, the cells expanded in the HFBR culture were mostly adherents, emphasizing the importance of cell-cell contact for leukemic cell proliferation.

4.2.3. 3D Leukemia Models with Primary Cultures

A very interesting application for leukemia 3D models is the *ex vivo* expansion of the leukemic cells isolated by patients. It has been demonstrated that primary leukemia cultures better survive and proliferate in a 3D mimicry with respect to classical 2D techniques [157, 158]. One of the most important advantages consists of having a sufficient number of neoplastic cells to identify the most effective drugs. Patient specimens that are derived from BM biopsies or peripheral blood often contain too few neoplastic cells in order to test drug effectiveness. Accordingly, 3D *ex vivo* expansion of primary tumor cells would allow the identification of personalized drugs.

Kirchner and collaborators [157] reconstructed a 3D BM ambient, by coating BMCs in fibronectin/Matrigel^{TM} mixture on a collagen I/fibronectin scaffold. In this 3D issue culture model, they succeeded in expanding multiple myeloma (MM) clones isolated by BM mononuclear cells (BMCs) of aspirates, so that it was possible to test the anti-tumoral efficacy of well-known drugs, such as Melphalan and Bortezomib. Intriguingly, Hou and coworkers [158] have succeeded in reproducing a 'biomimetic osteoblast niche' with bio-derived bone as a scaffold, coated with osteoblasts obtained by differentiating MSCs of CML patients. The reconstructed osteoblast niche maintained stem/ progenitor cells from BM of patients CML for a long period of culture, more efficiently than the 2D culture system.

4.2.4. Ex vivo Maintenance of BM Biopsies in Bioreactors

Up to date, Ferrarini and collaborators set up the first 3D dynamic culture system for BM biopsies derived from patients with hematological tumors. The authors used the Rotary Cell Culture System (RCCS^{TM}) Bioreactor, a device specifically designed to culture cells in a 3D milieu in microgravity. The RCCS^{TM} was applied to *ex vivo* cultures of MM specimens [134], obtaining long-term cultures of myeloma tissue explants. Stained histological slices showed a preserved architecture in which viable MM cells were embedded in their native microenvironment, comprehensive of the lamellar bone and vessels. The *ex vivo* cultures of MM specimens in the RCCS^{TM} bioreactor were also used to test the antitumoral efficacy of the proteasome inhibitor Bortezomib, a well-known drug used in MM therapy. Remarkably, the beta2-microglobulin levels, a proliferative marker commonly used for staging of MM, in patients' sera before and after the drug therapy paralleled those observed in supernatants from the *ex vivo* cultures in presence/ absence of Bortezomib. Thus, these results showed an interesting concordance between the effects exerted by Bortezomib *in vivo* and *ex vivo.* Collectively, the results obtained on *ex vivo* cultures, showed that it was possible to maintain the original tissue architecture and the specific microenvironment for each patient, potentially allowing the maximum degree of predictability in drug response.

4.2.5. 3D Microfluidic Tissue Models

At the intersection of tissue engineering and microfluidics recent advances show that it is possible to grow *in vitro* 3D tissues by reproducing the nutrient and waste transport function of the microcirculation and the emulation of the shear stress effects. One of the most important advantages is also the use of very small amounts of reagents and cells and the real-time monitoring of cellular parameters. Several microfluidic devices have also been set up to emulate tumor growth in the BM microenvironment.

Zhang and collaborators [159] have seeded the microfluidic culture chambers with a human osteo-blastic cell line. This cell line easily adhered to the support and produced a measurable extracellular matrix (ECM), thus mimicking a tumor osteoblastic niche, which then facilitated the seeding of mononuclear cells from BM biopsy of MM patients. Real-time monitoring microscopy and flow cytometry post-analysis showed that this *in vitro* MM tissue model permitted the preservation and proliferation of primary human MM cells. Moreover, it recapitulated the essential interactions between malignant cells and the recreated endosteal niche, all within a perfused micro-environment intended to mimic the fluidic features of the BM. The relevant clinical advantage, proposed by

the authors, is the requiring of very small amounts of patient-derived BM cells (< 1.10^6 cells, for an eight-chamber microfluidic device), allowing an accelerated evaluation of new therapeutics for the personalized treatment of multiple myeloma.

Opposite to this dynamic microfluidic system, Khin and collaborators [160] have very recently set up a static 3D microfluidic device, allowing a co-culture of MM cell lines with the adherent BM-derived stromal cell line HS-5 or a co-culture of MM primary cells with patient mesenchymal cells. Static microfluidic devices do not allow the mimicking of the fluidic flow, however they avoid the possible generation of several artifacts, caused by the continuous flow (e.g. removal of soluble signals important for cell-cell communication, unvaried concentration of media components) typical of dynamic systems [161]. MM and stromal cells were mixed into the collagen/media mix and seeded in commercially available 3D cell-culture slides (m-slide Chemotaxis 3D *Ibitreat* from Ibidi, LLC). 3D co-cultured cells were exposed to Melphalan and Bortezomib, in order to set up a preclinical assay for chemosensitivity. Digital images obtained with live microscopy were analyzed to detect cell death after drug exposure. Using this platform, the authors were able to predict the chemosensitivity to Melphalan and Bortezomib in several MM patients [160].

4.2.6. 3D Leukemia Models for Testing Motility/ Invasion

Infiltration of leukemia cells in several organs is a common feature of the disease. Specific assays for testing motility and invasion in the 3D milieu can be of interest. In cancer research, the most popular cellular invasion assay is the Transwell (or Boyden) chamber assay. Cancer cells are seeded on top of a thin gel containing ECM molecules, in turn positioned above a filter. The invasion capability is evaluated by counting the number of cells able to pass to the other side of the filter [162]. This assay is very appealing because of its speed and the ease in the quantitative analysis. However, the biological process is conducted in a limiting 2D milieu and only the end point is monitored. To overcome these problems the 2D well-known transwell assay has been adapted in order to study migration in 3D matrix. In 3D motility assay, cells are embedded within a thick matrix of seeded on top (collagen or Matrigel™). Results are obtained by counting the number of migrating cells in the ECM gel [163]. Several authors have used the transwell chamber assay in 3D matrix for testing invasion ability of leukemia cells [164, 165]. This method was later refined, by observing leukemia cell motility in 3D Transwell with time-lapse microscopy. The image analysis allowed not only quantifying cell motility in the samples, but also to describe the type of motility, i.e. amoeboid or rolling type [166].

A similar and simplified method with respect to the 3D Transwell assay consists of applying collagen or Matrigel™, containing specific chemoattractants to be studied, directly into the cell culture plate and adding cell leukemia suspension on top. Cellular invasion was scored by evaluating the number of cells at different positions in the 3D matrices, by using an inverted microscope and a digital depth meter [167, 168]. A more complex 3D model for testing cell motility was developed by de Silva and collaborators [169], where they have fabricated an Inverted Colloidal Crystals (ICC) scaffold, with a 3D honeycomb-like structure from colloidal crystal templates. The internal dimensions of hydrogel-made pores, as well as the gel stiffness, were tuneable, thus rendering these ICC scaffolds suitable for cell invasion studies. The authors tested the migration ability of the acute promyelocytic leukemia NB4 cell line, which can differentiate with all-trans retinoic acid (ATRA) into mature, highly mobile neutrophils [170]. Fluorescent marked NB4 cells were seeded on the ICC scaffolds and the invasiveness was calculated by analyzing confocal images at different depths. The authors succeeded in clearly demonstrating the ability of differentiated NB4 to enter the scaffold, while non-differentiated cells were only very occasionally found below the scaffold surface [169].

4.2.7. 3D Models for Expanding Normal HSC, a Tool for Clinical Therapy

Several 3D models have been developed in order to mimic normal BM niches [171-173]. Artificial analogues of BM can accelerate the understanding of hematopoiesis in humans or can be suitable for *ex vivo* simulation of some aspects of human immune response. Moreover, in the oncological field, these 3D models can be useful for *in vitro* production of human hematopoietic cells. Indeed, the HSCs transplantation, a widely used therapy for several hematological malignancies, presents two important limitations: an insufficient number of matching donors and the difficulty of obtaining enough cells [174]. Thus, expanding HSCs, obtained from donors, can be a suitable strategy to overcome these obstacles.

5. LYMPHOMA

Lymphomas represent the third most common childhood malignancies. In under 15-year-olds they occur at a median age of 10 years and 8 months; non-Hodgkin lymphoma-NHL (including Burkitt lymphoma) (6.6%) and Hodgkin lymphoma-HL (4.8%) are the most frequent diagnoses in this age group. The same pattern is seen in adolescents and young adults. Lymphomas, especially HL, are practically non-existent among young children [12]. More than 90% of pediatric NHLs are high grade lymphomas divided into four major histologic subtypes: diffuse large B-cell lymphoma, Burkitt lymphoma, lymphoblastic lymphoma and anaplastic large cell lymphoma.

The two most common HL in children are the nodular sclerosis HL and the mixed cellularity HL. Lymphomas start generally in lymph nodes and diffuse through the lymphatic vessels to other lymph nodes or to the spleen, or to extranodal area, such as bone, BM, liver and lungs. Other lymphomas, in particular NHL, arise outside the lymph system, especially in the digestive tract (stomach, intestines, liver), thyroid gland or skin.

5.1. Lymphoma Microenvironments

With respect to solid tumor, dealing with lymphoma cancers is more complicate, since they can proliferate both in suspension and in several tissues [175]. As demonstrated in other tumors, the progression of lymphomas involves a complex interactions between neoplastic cells and their microenvironment, including the tumor's vascular system and the stromal cells. Due to the complexity of lymphoma malignancies, residing in different tissues, the microenvironment is characterized by different populations. In BM, lymphoma cells are interacting with the BMSCs, already discussed in the leukemia paragraph. In secondary lymphoid organs, these non-malignant stromal cells consist of a heterogeneous group of cells, such as macrophages, follicular dendritic cells, follicular reticular cells, fibroblasts, B small lymphocytes, plasma cells, Th1 and Th2 cells, T regulatory cells (T-regs), eosinophils, mast cells and granulocytes. They are recruited and/or induced to proliferate by tumor cells and produce soluble or membrane-bound molecules involved in tumor cell growth and survival. The tumor-host communication is a complex network, involving adhesion molecules, chemokines, and chemokine receptors. The resulting tumor growth depends on the balance between the inhibitory and growth-promoting signals [176, 177]. Thus, in each tissue, lymphoma cells presents an unique network of signals, and therefore is hard to get targeted therapies [175, 178].

5.2. 3D Lymphoma Models

The literature on 3D lymphoma models is very poor. This gap can be partially filled by considering that most of the 3D leukemia models are also suitable for lymphomas. However, to the best of our knowledge, the 3D reconstruction of the lymphoma microenvironment, at least in lymph nodes, is completely missing. Much work remains to be done in this area.

5.2.1. Scaffold-Free Lymphoma 3D Models

As leukemic cells, lymphoma cells can not form cellular spheroids, typical of adherent cells. However, a method was recently proposed of forming 3D multicellular aggregates (MALC) of lymphoma cells [179] *in vitro*, by modifying the well-known hanging drop technique [180]. The t(14,18)+ follicular lymphoma RL cell line was used for this purpose and the growing cells formed compact oval-shaped aggregates, with ~1 mm diameter, a dimension never reached with the standard 2D culture [179]. The gene expression profiles either from RL 2D cell suspension or from RL cells grown as MALC were analysed. Cells from MALC presented more anti-apoptotic gene expression profiles, and they were more resistant to *NK*-mediated lysis than cells in 2D culture, suggesting an innate predisposition to immune escape [179]. Later, MALC of RL cell lines were used, by the same authors, to test the anti-tumoral activity of obinutuzumab and rituximab, two antibodies recently proposed in lymphoma therapy [181]. As already demonstrated in several tumor types, the cells grown in a *3D* model showed different drug responses than those grown in *2D,* highlighting the importance of developing 3D models for the testing of chemotherapeutic drugs, not only in NHL, but also in all type of cancers.

5.2.2. Lymphoma Cell Lines on 3D Scaffolds

Birgersdotter and collaborators [182] developed a 3D model of HL by using a cell culture matrix based on an oligopeptide, which has been extensively tested for 3D mammalian cell culture [183, 184]. This oligopeptide consists of regular repeats of alternating ionic hydrophilic and hydrophobic amino acids (arginine, aspartic acid and alanine), and associates to form stable beta-sheet structures in water. The presence of monovalent cations results in the spontaneous assembly of the oligopeptide into a stable, macroscopic membranous matrix, composed of ordered filaments

that form porous enclosures. The authors cultured the HL cell line L1236 in the 3D oligopeptide and its gene expression profile was analyzed, in parallel with 2D culture condition and with fresh lymph node biopsies from HL patients. The authors found that the 3D culture modulated gene expression of the L1236 cell line, better mimicking the *in vivo* expression profile [182].

5.2.3. Co-Cultures of Lymphoma and Stromal Cell Lines on 3D Scaffold

Caicedo-Carvajal and coworkers [175] have developed a 3D cell co-culture system in order to optimize the growth of mantle cell lymphoma (MCL) cell lines. The scaffold was made of four layers of polystyrene (PS), a geometry generating a characteristic 3D porous structure. 3D PS Scaffold was seeded with a MCL cell line: HBL2, of lymph node origin. HBL2 cells were co-cultured with human dermal fibroblasts (hDFb) in the 3D milieu, showing and enhanced cell proliferation, with respect to the 2D culture. The authors suggest that this 3D system allows the expansion of primary tumor cells of patients, from blood or other tissues in which hematological cancer cells are present, and that these expanded cells can be used for customized drug screening assays.

6. CNS PEDIATRIC TUMORS

The heterogeneous group of pediatric CNS tumors are the most common solid cancer in the pediatric age and the second most common tumor after leukemias, representing 20 to 25% of all childhood cancers [12]. Pediatric CNS cancers comprise a diverse group of tumors with different histology, arising at various sites within the central nervous system. The largest subgroups are astrocytomas (43% of all brain and CNS tumors in children), which are diagnosed throughout childhood. Most of the astrocytomas are diagnosed as low-grade (grade I: juvenile pilocytic astrocytomas, grade II: diffuse astrocytomas), while a small percentage as high-grade (grade III: anaplastic astrocytomas and grade IV: glioblastoma multiforme). The second most common group is represented by the embryonal tumors (19% of all childhood brain and CNS tumors). Most of these are medulloblastomas and cerebral primitive neuroectodermal tumors (PNET). Finally, about 10% of childhood brain and CNS tumors are ependymomas and choroid plexus tumors [12, 185].

6.1. CNS Pediatric Tumor Microenvironment

High-grade brain tumors display striking cellular heterogeneity. Some of the cells show increased tumorigenicity and stem-cell-like capacity, and they have been proposed as the cells of origin for tumor recurrence. Thus, the existence of cancer stem cells (CSCs) has been demonstrated in several high-grade CNS tumors, such as glioblastomas, ependymomas and medulloblastomas, even if the CSCs presence in lower grade tumor is controversial [186, 187]. However, also in CSN cancer, the tumor-associated parenchymal cells, such as endothelial cells, microglia, immune cells and neural precursor cells, also play an important role in the evolution of the disease.

Resident activated microglia or new recruited macrophages from blood, are the most abundant nontransformed cells present in brain tumors. It has been shown that the M1 phenotype, with anti-tumoral properties, is the most prevalent in low-grade CNS tumors, while the M2 phenotype, also designed as Tumor Associated Macrophages (TAMs) positively correlates with the histological malignancy of the tumor [188, 189]. It has been demonstrated that TAMs promote glioma cell proliferation, as well as angiogenesis and invasion [190, 191].

CD8 and CD4 T cells are also present in brain tumors. Some authors identified the CD8/CD4 T cell ratio as prognostic factors, as demonstrated in non CNS tumor. Indeed, in colon cancer it has been demonstrated that the CD8/CD4 ratio is higher in patients with better clinical outcomes [192]. Consistent with these findings, it has been shown that pilocytic astrocytomas showed the highest CD8/CD4 ratio and that the average ratio of CD8/CD4 in ependymomas, multiforme glioblastomas, medulloblastomas was progressively lower [188]. However, the correlation between lymphocyte infiltration and clinical outcome is still controversial.

More recently, the focus is shifting on the regulatory T cells (Tregs), identified by the antigen FoxP3, with pro-tumoral activities. For instance, it was shown that FoxP3 expression correlates with the tumoral progression in several types of astrocytomas [193], and that temozolomide, the standard chemotherapeutic agent for glioblastoma, induced a reduction of Tregs, both in the blood and in the tumor tissues [194]. Therefore, Tregs have been identified as a novel targets in glioma treatment.

Recently it has been demonstrated that glioblastomas can interact with normal neural precursor cells (NPCs). Interestingly, NPCs are recruited from brain tumor tissue, thanks to chemoattractant

molecules, such as CXCR4, and their number inversely correlates with tumor-size and survival. Thus, an antitumorigenic action for NCPs has been postulated. *In vitro* experiments seem to sustain this hypothesis, since cultured NPCs release soluble factors that can inhibit glioblastoma proliferation, induce differentiation and cell death [189]. Brain tumors are highly vascularised and this rich vascular network is not only a matter of tissue oxygenation, but, more importantly, of endothelial cells, pericyte, and astrocyte (the neurovascular system) presence, that support tumor progression. This microanatomical structure, designed as perivascular niche (PVN), contain stem cells in both normal and brain tumoral tissues. This complex network of cells allows multiple interactions, promoting CSCs proliferation, enhancing their survival, and protecting them from chemotherapy [187, 189].

Together with the perivascular niche, it has also been postulated the presence of the hypoxic niche [186], as already observed in leukemia and other tumors. Hypoxia promotes the acquisition of stem-like properties, can facilitate the CSCs maintenance, and promotes neoangiogenesis [186, 187]. Of note, we must not forget the role of ECM in the pathogenesis of brain tumoral disease. For instance, it has been demonstrated that proteoglycan expression is altered in human glioblastoma and that this modification promotes an abnormal activation of the receptor tyrosine kinase (RTK), involved in the progression of the disease [195].

6.2. 3D Models For CNS Pediatric Tumors

6.2.1. Tumor Brain Spheroids or Neurospheres

Spheroids of brain tumor cells (neurospheres) have been largely employed by scientists for at least three decades. Both stabilized brain cancer cell lines and primary cells, obtained from biopsy of CSN tumors, can be used to form spheroids [196]. As frequently happens in the 3D world, neurospheres are more representative of the tumor with respect to the corresponding monolayer cultures. For instance, it has been demonstrated that genomic profiles of primary cells of glioblastomas, cultured in 2D stardard condition, very often deviate from the parental cancer profiles, whereas genomic profiles are generally preserved in sheroids [197]. As with other types of solid tumors, neurospheres are also widely used for tumor biology or drug screening studies, being suitable for tests on proliferation, cell death, invasion and motility [198]. After the discovery of neural stem cells [199], research on neurospheres has undergone a major impetus. Indeed, neurosphere cultures have shown to be a suitable method to isolate and propagate neural stem cells. The neurosphere assay**,** developed with the aim of confirming that neurospheres contain neural stem cells, was initially proposed by Reynolds and Weiss in 1992 [200]. They were able to culture primary cells isolated from the normal striatum tissue of the adult mouse brain, in the presence of epidermal growth factor. At the beginning of the culture, most of the cells had died, but few cells survived, proliferated and formed a free-floating 3D spherical cluster of detached cells, the neurospheres. The proliferating cells initially expressed characteristic markers of stem cells, such as nestin. Moreover, the neurospheres continued to proliferate in secondary cultures, after their mechanical dissociation and plating as single cells. Interestingly, after several days of culture, the secondary neurospheres were able to differentiate into astrocytes, neurons and oligodendrocytes with phenotypes characteristic of the adult striatum *in vivo* [200, 201]. In the following years, scientists have demonstrated the presence of neural stem cells in several regions of embryonic and adult brains both in human and mouse [201]. The neurosphere assay is now a widely used technique suitable to isolate neural stem cells; it permits to examine the three fundamental characteristics of neural stem cells: proliferation, self-renewal, and multipotency [201]. Later, neurospheres containing stem-like neural precursors were also obtained from human glioblastoma multiforme specimens by Galli and collaborators [202]. The authors demonstrated the ability to proliferate, to undergo self-renewal and to generate neurons, astrocytes, and oligodendrocytes, as the normal neuronal stem cells do. Moreover, these cells injected orthotopically in immunocompromised mice can form cancers with a histological architecture similar to that of the human tumor [202]. In subsequent years, scientists have succeeded in obtaining neurospheres containing cancer stem cells from ependymomas, medulloblastomas, and primitive neuroectodermal tumors and from high grade gliomas (grade III anaplastic astrocytomas and grade IV glioblastomas) but not from lower grade gliomas [203-206]. Detailed protocols for obtaining them are now available in the literature [200, 204, 207-209]. Thus, the neurosphere cultures have represented a suitable 3D model for deepening understanding of the role of brain tumor stem cells in radio- and chemoresistance mechanisms, as well as in the brain tumor response to growth factors, hypoxia, and pharmacological agents [210-214]. The possibility of performing gene/protein

expression analysis as well as to section neurospheres for histology or immunocytochemistry allows detailed molecular studies. Moreover, the isolation and propagation of neuronal stem cells from a specific specimen allows studying patient-specific pharmacologic approaches.

6.2.2. Co-Culturing Neurospheres and Immune Cells

Co-culturing approaches with neurospheres and immune cells, normal constituents of the tumor microenvironment, are also of particular interest. Iwasaki and collaborators studied the infiltrative capacity and the cytolytic process of lymphokine-activated killer (LAK) cells against a human glioma spheroid model. Multicellular tumor spheroids were co-cultured with LAK cells on a rotary shaker. Histological analysis revealed that LAK cells are able to infiltrate directly in the core of the spheroids, causing tumor cytolysis [215].

Kees and collaborators have studied the role of microglia, isolated from patients with glioma, in the control of proliferation and invasion of spheroids derived from glioblastoma cell lines. They have used a collagen co-culture model, seeding both glioma spheroids and microglia cells. They demonstrated that human microglia has tumor-promoting activities that are overridden by pharmacological treatments [216].

More interestingly, the pro-tumoral effects of the altered microglia from brain tumor patients on brain tumor initiating cells (BTICs), a subpopulation expressing stem cell markers, can be reversed after the treatment with Amphotericin B (AmpB). Indeed, cultures of monocytes and microglia from glioma patients were not able in inhibiting the spheres formation of autologous BTICs, but this was modified by AmpB treatment [217].

In a recent paper, Etminan and co-worker have co-cultured glioma spheroids with dendritic cells (DCs), with the aim of testing the antitumoral efficacy of the photodynamic therapy. Indeed, after the treatment, immature DCs were recruited into the glioma spheroids. They then matured, likely activating specific T cells with anti-tumoral activity [218].

6.2.3. 3D Co-Cultures of Tumoral CNS Cells and Normal Brain Cells

3D co-cultures of normal brain cells and glioblastoma-derived cells have been set up by Biggs and coworkers [219]. Normal brain cells were isolated from cortices of Wistar rats and allowed to re-aggregate and to mature for 20 days, by placing them on a semi-porous membrane at an air-liquid interface, and subsequently generating characteristic brain Hi-Spots [220, 221]. Histological analysis showed that both neurons and astrocytes were present in these 3D cultures, with a preserved physiological architecture. On this reconstructed 3D neural tissue, fluorescent marked glioblastoma cells were seeded. The authors could observe their spread throughout the Hi-Spots, forming new cell aggregates, and able to last for many days (25 d) in culture. This platform was then tested for evaluating anticancer compounds, thus representing a simple system to verify drug effectiveness on brain tumors in an orthotopic environment [219].

Orthotopic 3D co-cultures of tumor brain spheroids in normal brain tissues have been also set up to test invasiveness. In this case neurospheres have been implanted on the center of human [133] or rat brain slices [222]. The histological evaluation of the confrontation revealed either an invasive pattern or a non-invasive pattern (see the relevant paragraph for a better description).

6.2.4. 3D Tumoral CNS Cultures on Scaffolds

One of the earlier research studies on 3D culture of brain tumor cells was presented in 1977 by Carllson [223]. He studied the mitotic activity of glioma cells embedded in an agarose gel, in relation to the position of the cells in the colony, finding that the fraction of cells that incorporated tritiated thymidine decreased nearly exponentially with the depth in the colonies [223]. Over time, it has become increasingly clear that matrices in which cells are embedded is not simply a scaffold to hold them on, but a it is communicating structure important in determining cell function and behaviour [224].

Thus, scientists have tried to mimic specific components of the brain ECM, in order to better reproduce the microenvironment. Many compositions of the matrix have been studied. One of the widely used constituents is the collagen [225-227], eventually mixed with other molecules, such as agarose [228], the ECM proteins tenascin [229] or chondroitin sulfate [230]. Among the ECM components, an important role has been demonstrated for HA, able to regulate the glioma cell phenotype [231] or to increase stem-like properties of glioblastoma cells [232]. For this reason, hydrogels containing HA have been proposed as a scaffold for neuronal tumors [233].

In the recent years, scientists have set up HA-based scaffolds in combination with other molecules, such as a different type of collagens [230, 234], the kappa-elastin (HA-κE), present in the basement membrane of blood vessels [235], gelatine, PEG [231], and chitosan [232]. Besides these natural extracellular matrix materials, which are expensive and potentially could transmit pathogens [236], several natural polymers have been also studied, such as chitosan-alginate scaffold [125], or alginate hydrogel functionalized with D- or L-aminoacids [237]. Thus, thanks to these experimental models it was possible to study the impact of the individual components of the ECM on tumor biology [230-232] and to assess pre-clinical drug and radiation sensitivity screening [238, 239].

6.2.5. 3D Models for Testing Motility/Invasion of CNS Tumors

Gliomas are highly infiltrative tumors, thus several 3D culture methods have been set up in order to study this parameter. The widely used 3D transwell or spheroid invasion/motility assays in soft matrices, like collagen, Matrigel™, fibronectin or agar are extensively applied in brain tumor research [225, 226, 240-244]. Moreover, several other matrix compositions have been tested with the aim of better reproducing the *in vivo* microenvironment. Thus, invasion assays have been performed in HA-based hydrogel [230, 233-235].

An intense interstitial fluid flow is present in gliomas, during the processes of angiogenesis, and invasion. Therefore, the impact of the interstitial fluid flow on glioma cell invasion can be of interest. With this purpose, modifications of the classical 3D transwell invasion assay have been presented recently [245]. Glioma cells, with different invasive properties (noninvasive and invasive) were seeded in a gel containing HA or collagen I and placed on porous membrane, like a classical transwell assay. The above setup was modified, by establishing, in the casted gel containing the seeded glioma cells, a fluidic flow with an average speed of 0.7 μm/s through the cell/gel compartment, mimicking the interstitial fluidic flow. Interestingly, the authors showed that the interstitial flow promotes cell invasion in several glioma cell lines and that the flow effect was dependent on the CXCR4 receptor, involved in brain cancer invasion and progression [246].

Invasion assays with spheroid co-cultures (confrontation assays) has also been developed. In this case, neurospheres are placed in contact with normal tissues, which are used as the target tissues that may be invaded and destroyed by invading neoplastic cells [247]. Either embryonic chick tissue, foetal brain aggregate (heterologous), or normal connective tissue from the tumor-bearing patient (autologous) [240, 242, 248-251] have been used as the target tissues.

After adhesion of the tumor cells to the normal tissue, histological evaluation of the confrontation will reveal either an invasive pattern or a non-invasive pattern. In the invasive pattern, the tumor-derived cells invade into the host tissue and replace it, as it is often seen with high grade gliomas [252, 253]. In the non-invasive pattern the tumor-derived cells live together with, and generally surround the host tissue without replacing or destroying it, as seen with non-invasive benign meningioma [253, 254]. Several upgraded protocols have been proposed, which consist in implanting fluorescent stained spheroids, obtained from human brain tumors, on the center of human [133], or rat brain slices [222]. The invasion process can be evaluated over the time by using a confocal microscopy. The advantages consist in a better representation of the invasion process, due to the fluorescent tag applied to the tumor cells, as well as in a better representation of extracellular matrix molecules normally encountered by invading glioma cells [133, 222]. Oellers and collaborators described a similar refined protocol [255], presenting a co-culture of glioma cells with myelinated axons *in vitro*, with the aim to study the interactions between migrating glioma cells and nerve fibers. Glioma cells were positioned close to the long retinal axons obtained derived from explants of embryonic chickens. Migration of cancer cells has been monitored by confocal microscopy and high-resolution video microscopy [255]. Overall, the invasiveness results obtained by confrontation assay are correlated with malignancy *in vivo* [256]. Moreover, this assay is also suitable for study of the involvement of specific genes, for testing various anti-tumoral molecules/drugs or the effect of radiotherapy on brain tumor invasion ability [250, 257, 258].

Besides these classical assays, scientists have also proposed particular protocols to measure motility and migration in brain tumors. For instance, Agudelo-Gracia and collaborators have studied motility of glioma cells cultured on nanofiber scaffolds, that mimic the neural topography [259]. Neurospheres obtained form glioblastoma cell lines or tumor explants were manually placed within nanofiber-coated wells to analyze the migration, by analyzing the total area and perimeter

covered by the migratory cells. The authors found that cell migration of glioblastoma neurospheres was reduced by STAT3 inhibitors. Notably, these inhibitors at the same concentrations failed to inhibit migration in a standard 2D system [259].

6.2.6. Bioreactors

One of the first experiments on brain tumor cells 3D growing in a bioreactor was presented by Ingram and collaborators [260]. Several malignant glioma cell lines were introduced in the NASA rotary cell culture system, forming spheroids with an increasing expression of the adhesion molecule CD44 over the time in culture [260]. An exhaustive study on brain tumor aggregates generated using the 3D RCCS^{TM} has been presented recently by Smith and collaborators [261]. Several types of brain cancer cell lines (pediatric glioblastomas and high grade gliomas, pediatric central nervous system primitive neuroectodermal tumor, pediatric medulloblastomas) were grown in conventional 2D cultures and the RCCS^{TM}. Gene expression and microRNA profiles were analyzed, as well as drug sensitivity to Vorinostat, a histone deacetylase inhibitor. The principal finding was that 3D culture in RCCS^{TM} better recapitulates the histological architecture and the molecular profiles of primary brain tumors with respect to 2D cell culture. Similar findings were also obtained with primary explant culture in the RCCS^{TM}, which retains features of the primary brain tumor. Moreover, the 3D glioblastoma aggregated of the RCCS^{TM} cultures demonstrated a reduced sensitivity to Vorinostat with respect to the 2D system, and likely better recapitulate the drug response observed *in vivo* [261].

Panchalingam and coworkers [262] developed bioreactor protocols for expanding cancer stem cells from human glioblastoma specimens. Human glioblastoma-derived cells were introduced in a bioreactor with paddle impellers (NDS Technologies, Palo Alto, CA). The expanded cells were characterized using both flow cytometry and a differentiation assay. The obtained results showed that a high percentage of the expanded tumor cells possessed stem cell features and that the expression profile was preserved with respect to the brain cancer tissue of origin.

Of note, glioma rat cells have been expanded in hollow-fiber bioreactors. However this model system was not set up for brain cancer studies but for monitoring the cellular effect during ischemia.

6.2.7. Microfluidic System

In the literature, there are very few examples of studies carried out in microfluidic on 3D brain tumors. One of the few studies was presented by Lee and collaborators [264], which studied glioma cell migration in soft matrices. As in the static cell growth conditions, the choice of the matrix is also a crucial point for getting the best biomimetic microenvironment in this device. These authors have used a HA hydrogel to seed the glioma cell line A-172. The HA hydrogel can be remodelled by MMPs secreted by glioma cells during migration, and the authors have studied the concentration gradient effect of VEGF in this parameter. The authors observed an intense spreading of glioma cells in the microchannel containing VEGF; moreover these cells were more able to degrade MMP-sensitive hydrogel with respect to control cells [264].

A second type of study in microfluidics, which preserves the 3D cell structure, are those dealing with brain slices. The microfluidic technology seems to better preserve the viability of the explanted tissues for some days, by improving the oxygen/nutrient penetration into slices. Therefore, this device can be of interest for developing personalized cancer therapies [265, 266].

An interesting microfluidic experimental model was proposed by Ma and collaborators [267]. They tested the cytotoxicity of two anticancer drugs, temozolomide (TMZ) and ifosfamide (IFO), on glioblastoma multiforme brain cancer cells in conjunction with liver metabolism. The microfluidic devices contained two separate chambers connected in tandem, one for seeding liver cells with different cytochrome P450 (CYP) subtypes and the second for seeding tumor brain cells. Both chambers contained polylactic acid (PLA) as a scaffold, to allow the 3D culture. TMZ, which is inactivated by liver metabolism, was more ineffective in the presence of 3D liver cultures. Conversely, ifosfamide (IFO), which requires CYP-dependent activation for its antitumor activity, showed the maximum cytotoxicity in the presence of CYP overexpressing liver cells. Thus, this model could better predict drug effects, by considering the contribution of liver metabolism on drug activities [267].

The limited production on microfluidic devices applied to 3D cellular models might suggest, on one hand, that there are technical difficulties occurring, and, on the other hand, that scientists may prefer different

3D models. Indeed, 3D models permit the culture of a higher number of cells, and they are not subject to potential biological artifacts, due to mechanical stress for the high hydrodynamic shear forces [268].

6.2.8. Brain Tumor Explants of Surgical Specimens

The cultivation of tumor explants allows the maximum preservation of the microenvironment and in personalizing the culture. Of course, they have limited survival time and scientists are committed to finding new protocols to lengthen this time. Some tissues are more suitable, such as lung and colon [269], unfortunately, *ex vivo* cultures of brain tumor tissues are more difficult to obtain, because of their friability. Tumor blocks transferred in culture medium can be maintained up to 48 hours with preserved histoarchitecture of glioblastoma. In contrast, starting from 72 hours, tumor tissues present cells in apoptosis [270]. Few attempts have been made to lengthen the time of vitality in culture of brain tumor explants, such as those performed in microfluidic devices or in bioreactors (see the relevant paragraphs).

7. NEUROBLASTOMA

Neuroblastoma (NBL) is the most frequent extracranial solid tumor of childhood, occurring in very young children, as the median age at diagnosis is 17 months [271]. NBL present a large number of clinical manifestations that range from spontaneous regression to very aggressive malignant growth, so that NBL disease is classified into low-, intermediate-, or high-risk categories, based upon clinical and biological features. The accurate stage of patients at diagnosis and the classification on risk groups is important to tailor the therapy, in order to reduce toxicity and improve outcomes. NBL accounts for about 7% all childhood neoplasms, but it is responsible for 10% for the deaths from pediatric malignant diseases [272].

One of the most important prognostic factors is age, with an inverse relationship between age and outcome. Other prognostic factors are the presence of metastasis and some genetic factors of the tumor as MYCN amplification, 11q deletion and structural aberrations [271, 272]. Tyrosine kinases -TRK family of neurotrophin receptors greatly correlates with clinical outcomes: TrkA expression is associated with a favorable clinical behavior, whereas TrkB with unfavourable outcome [273]. For babies aged younger than 18 months without the amplification of MYCN, even those with metastases, prognosis is better than for older children [271].

Neuroblastomas originate from primordial neural crest cells, thus tumors occur mostly in the adrenal medulla and the rest arise anywhere along the chain of the sympathetic nervous system. They can present as mass lesions in the chest, neck, pelvis or abdomen. Almost 50% of the patients have metastatic disease at diagnosis, especially in liver, skin, lung, BM and bone [271].

7.1. Neuroblastoma Microenvironment

The tumor microenvironment strongly contributes to the evolution of NBL disease. One of the most explored components of the NBL microenvironment is the immune system. It is well-known that high risk NBL can escape the immune system [274]. Several mechanisms have been proposed, such as the downregulation of the human leukocyte antigen (HLA) or adhesion molecule expression on the surface of NBL cells, and the expression of chemokines by tumor cells or infiltrating stromal cells that can down-modulate immune responses or recruit macrophages to disable these lymphocytes [275].

Targeting immune cells, modulating surface molecules involved in the immuno-response, interfering with the cytokine activities, all are promising therapies in fighting this disease [275-277]. Other studies have pointed out the importance of hypoxia level in the progression of the disease. Indeed, it has been demonstrated that the adaptation to low tension of oxygen induces the expression of several genes (the 'hypoxia gene signature'), which allows the cells to survive. Moreover, during hypoxia, NBL cells are more resistant to apoptosis, leading to pleiotropic drug-resistance [278, 279].

Profiling several tumor specimens of neuroblastoma patients, Fardin and coworkers [280] have found that the hypoxia gene signature is associated with most aggressive subtypes, without regard to the *MYCN* amplification. These authors have proposed that the hypoxia gene signature can be a new potential prognostic factor for neuroblastoma.

The angiogenic switch, intimately correlated with the hypoxic condition [281], is also involved in NBL progression. Indeed, it has been demonstrated that several angiogenic factors, such as VEGF, are expressed in NBL tissues *in vivo*; moreover, a positive correlation was observed between VEGF expression and poor outcome [282].

Relapse and metastases constitute the major challenges in clinical management of NBL. Tumor cells disseminating from primary sites to various organ sites have several possible fates: death, progression toward metastasis or formation of dormant micrometastasis. Dormant micrometastasis could progress to an actively growing macrometastatic lesion and cause a late metastatic relapse in the high risk group of NBL patients [283]. Thus, specific microenvironment factors involved in the progression of micrometastasis or in the attraction of human neuroblastoma cells in the site of metastasis have been studied in several organs, such as lung [284], bone [285, 286] or BM [287].

7.2. 3D Models for Neuroblastoma

7.2.1. Neuroblastoma Spheroids

The NBL spheroids have been used for more than two decades. As demonstrated in other types of tumors, NBL spheroids better recapitulate the salient aspects of *in vivo* neoplasm growth [288, 289]. For instance, Kumar and coworkers [288] have studied the different protein expression profile when NBL cells are cultured in 2D with respect to 3D. The proteomic analysis was performed by *2D* gel electrophoresis and followed by mass spectrometry identification. Several polypeptides involved in metabolism, cell structure, cell stress response, signal transduction and transport were over-expressed in NBL spheroids. The differential proteins identified suggest that NBL spheroids better recapitulate in vivo tumor physiology.

The main fields of application of NBL spheroids are studies on drug discovery [290], radiosensitivity/ radioresistance [291], targeted radiotherapy [292], new therapeutic strategies [293] or mechanisms of tumor initiation and progression [294].

Neuroblastoma cell lines are also used as a model for neurodegenerative diseases, due their ability to differentiate into neuronal like cells. Indeed, it is well established that the 2D cultured human NBL cell line SH-SY5Y [295] can be differentiated into several neural cell phenotypes using agents such as retinoic acid (RA) [296] or brain-derived neurotrophic factor (BDNF) [297].

The use of neuroblastoma SH-SY5Y spheroid cultures further improved the model of neuronal differentiated tissues. Jung and coworkers [289] have evaluated the differentiating action of RA on SH-SY5Y spheroids. They showed that the expression of neuronal markers (synaptophysin, neuron-specific enolase- NSE), the contents of cell adhesion molecules and ECM proteins, and the ability to extend neurites were much higher in RA-treated spheroids than in RA-treated monolayer cells [289]. Interestingly, Seidel and collaborators [298] succeeded in differentiating SH-SY5Y spheroids without the need of differentiation promoting agents, by cultivating them in a gyratory shaker. This neuronal 3D-culture model was then used to study the contribution of the protein TAU in the neurodegenerative processes.

7.2.2. 3D Invasion Assays

Invasiveness is an important feature of all metastatic tumors, so that invasion assays are widely used tools in neuroblastoma research. In 3D conditions, Matrigel™ invasion chamber assay is one of the most popular method [299-305], together with the invasion assays through 3D collagen gels [306]. Thanks to these studies, the effects of ionizing radiation [307], differentiating agents [299, 301], antitumoral drugs/molecules [303-305] or the contribution of specific oncogenes [300, 302] on invasiveness have been explored.

7.2.3. NBL Cells on Scaffolds

Neuroblastoma 3D culture embedded in soft matrices, like agarose, Matrigel™ or collagen, is widely used by scientists [299-305, 308]. Valero and coworkers [309], have studied the impact of different soft matrices on neuronal differentiation of the Neuro2a neuroblastoma cell line (N2a), evaluated with a novel impedimetric biosensor. The authors showed that neither agarose nor bare collagen was able to support the differentiation of N2a cells. On the contrary, collagen-laminin mixture was able to do it by promoting the appearance of long neurites, touching the adjacent neurons.

Besides the common matrices, new biomolecules have been explored, such as the bacterial nano-cellulose, excreted by Gluconacetobacter xylinus [310], or synthesized materials such as poly (L-lactic acid) PLL [311], photocurable polylactide (PLA) resin [312], poly(DL-lactide-co-glycolide) acid [313], carbon structures [314] or polymeric microspheres [315]. However, the main applicative field, by using these new materials, is not the NBL cancer research, but the neural tissue engineering, thanks to the properties of NBL cells to form neuron-like tissues.

7.2.4. Bioreactors

Literature is poor regarding papers presenting data obtained with bioreactors in the NBL cancer research

field. However, the application potential of this experimental model is interesting, as suggested by Redder and coworkers [316, 317]. These authors have demonstrated that the study in the microgravity rotary bioreactors of the cellular *in vitro* aggregation kinetics and organoid morphology correlate to the neuroblastoma malignant potential. Amplified- MYCN or TrkB expressing neuroblastoma cell lines, with unfavorable outcomes, aggregated much more rapidly than unamplified MYCN or TrkA expressing neuroblastoma cell lines. Moreover, the authors observed differences in organoid morphology between the two groups. The authors then proposed the microgravity assay as a rapid and reproducible *in vitro* assessment of neuroblastoma malignant potential.

By using a rocking bioreactor system, an interesting application in NBL medical treatment was proposed by Rujkijyanont and coworkers [318]. CD56+ natural killer (NK) immune cells isolated from peripheral blood cells of normal donors and NBL patients were cultured and expanded in the rocking bioreactor. After the culturing, expanded CD56+ cells showed a marked increase of cytolytic activity on all the NBL cells studied. This procedure can represent a new therapeutical strategy in order to generate autologous or allogeneic CD56+ cells able to kill NBL cells but with a low risk of *Graft versus Host Dise*ase- GVHD.

CLOSING REMARKS

Since the 1980s, 3D culture has known a great evolution, that completely refined the scientific approach to cancer biology. The “third” dimension provided to *in vitro* cultured cells a new context, favoring normal and cancer cells/matrix interactions and cross-talk, thus mimicking, in a more natural way, the tumor microenvironment. This cancer hallmark plays a crucial function in regulating tumor behavior and survival, but it still needs to be better understood in order to be used in targeted cancer therapy. The 3D cell culture is a potent tool to model normal and pathological tissues, in order to increase our basic knowledge in tumor evolution (initiation, progression and invasion), as well as to better target oncological therapy.

However, even if great scientific and technical efforts have been made, there is not yet, at present, the perfect *in vitro* model able to exactly mimic tumor cells and its microenvironment, as it exists in the patient. As indicated by the literature, several 3D culture models are able optimally to represent many cancer hallmarks /outcomes. Nevertheless, none of these models can reproduce with accuracy the whole and complex *in vivo* tumor context, even if each one provides valid scientific information to assess tumor biology and anti-tumoral drugs efficacy/resistance. We still lack the ideal model that can be translated from lab benches to preclinical trials and to personalised cancer treatment. But the questions are: can such a model exist? Can/must a singular *in vitro* model mirror the complexity of a wide range of cancers? Can a so complex condition be translated into a simplified *in vitro/ex vivo* system? In effect, because our knowledge on cancer(s) is still partial, a number of queries are not answered.

Thus, for the next steps, it is imperative that oncology research includes multidisciplinary competences (engineers, physicists, biologists, healthcare doctors, oncologists, pharmacologists, toxicologists, etc.), in order to better concentrate efforts and define the basic, pre-clinical and clinical endpoints, as well as the experimental strategies to reach such objectives.

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