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Citation: Christodoulou, Ioannis, Goulielmaki, Maria, Devetzi, Marina, Panagiotidis, Mihalis, Koliakos, Georgios and Zoumpourlis, Vassilis (2018) Mesenchymal stem cells in preclinical cancer cytotherapy: a systematic review. *Stem Cell Research & Therapy*, 9 (1). p. 336. ISSN 1757-6512

Published by: BioMed Central

URL: <http://dx.doi.org/10.1186/s13287-018-1078-8> <<http://dx.doi.org/10.1186/s13287-018-1078-8>>

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REVIEW

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Mesenchymal stem cells in preclinical cancer cytotherapy: a systematic review

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Abstract

Mesenchymal stem cells (MSC) comprise a heterogeneous population of rapidly proliferating cells that can be isolated from adult (e.g., bone marrow, adipose tissue) as well as fetal (e.g., umbilical cord) tissues (termed bone marrow (BM)-, adipose tissue (AT)-, and umbilical cord (UC)-MSC, respectively) and are capable of differentiation into a wide range of non-hematopoietic cell types. An additional, unique attribute of MSC is their ability to home to tumor sites and to interact with the local supportive microenvironment which rapidly conceptualized into MSC-based experimental cancer cytotherapy at the turn of the century. Towards this purpose, both naïve (unmodified) and genetically modified MSC (GM-MSC; used as delivery vehicles for the controlled expression and release of antitumorigenic molecules) have been employed using well-established *in vitro* and *in vivo* cancer models, albeit with variable success. The first approach is hampered by contradictory findings regarding the effects of naïve MSC of different origins on tumor growth and metastasis, largely attributed to inherent biological heterogeneity of MSC as well as experimental discrepancies. In the second case, although the anti-cancer effect of GM-MSC is markedly improved over that of naïve cells, it is yet apparent that some protocols are more efficient against some types of cancer than others. Regardless, in order to maximize therapeutic consistency and efficacy, a deeper understanding of the complex interaction between MSC and the tumor microenvironment is required, as well as examination of the role of key experimental parameters in shaping the final cytotherapy outcome. This systematic review represents, to the best of our knowledge, the first thorough evaluation of the impact of experimental anti-cancer therapies based on MSC of human origin (with special focus on human BM-/AT-/UC-MSC). Importantly, we dissect the commonalities and differences as well as address the shortcomings of work accumulated over the last two decades and discuss how this information can serve as a guide map for optimal experimental design implementation ultimately aiding the effective transition into clinical trials.

Keywords: Adult mesenchymal stem cells, Umbilical cord matrix stem cells, Wharton's jelly, Tumor microenvironment, Experimental cancer cytotherapy, Gene delivery vehicles

Background

In the turn of the millennium after over four decades of intensive research in the genetics of cancer, an important paradigm shift has occurred, in which instrumental for the development of the disease is not only the link between cumulative instability of key genes and the rise of aberrant cell cycle control, but also the orchestration of a heterogeneous cell-based stromal network supportive of tumor invasion and metastasis. This switch in focus has led to the

elucidation of the composition of this microenvironmental niche and of the interaction dynamics of its multiple cellular and extracellular components. Concomitant have been the emergence of the epithelial-to-mesenchymal transition (EMT) process as a key cellular mechanism in tumorigenesis, as well as the important discovery that stem cells of mesenchymal origin (mesenchymal stem cells (MSC)) can home towards tumor sites. The latter has formed the basis of an exciting new anti-cancer strategy, namely cancer cytotherapy, in which allogeneic MSC can be used to specifically target and deregulate the local tumor microenvironment, as an answer to modern oncology's desperate need for a promising alternative to the current gold-standard

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therapy of combined chemotherapy (Additional file 1: Figure S1).

Towards this end, various MSC populations have been recruited in different experimental setups in dozens of in vitro and in vivo preclinical studies. Unfortunately, until recently, MSC-based cancer cytotherapy has to a large extent failed to deliver its initial promise of a high-end translational oncology option. In their vast majority, the 500 or more clinical trials employing the use of MSC take advantage of the immunomodulatory, anti-inflammatory, and tissue regenerative properties of these cells for treating, for example, autoimmune diseases, graft rejection and osteochondral degeneration, without, until very recently, any significant data on the anti-cancer efficacy of MSC in humans. The problem of translating experimental MSC-based cancer cytotherapy work into successful human clinical trials is multi-faceted. In the case where MSC are engineered to overexpress molecules with anti-cancer properties, tumor suppression is achieved with relatively high efficiency and reproducibility in the majority of experimental protocols applied. Nevertheless, genetic engineering of cells not only adds extra costly and time-consuming manipulation steps in vitro, but also raises safety concerns since it usually relies on the use of viral vectors that introduce instability and chances of mutation at the genome integration sites of the host. On the other hand, the use of naïve, unmodified stem cells, although more straightforward, is hampered by contradictory findings, largely driven by the immense biological variability inherent to cell-based products and therapies (compared to traditional pharmaceuticals), as well as by multi-parameter variability relating to the methods, processes and manipulation steps mediating transformation of lab findings to clinical-grade products.

With a mortality rate of nearly 60% for the over 14 million people diagnosed with cancer yearly worldwide, it is imperative that any novel therapeutic regime should be optimized to its full potential. Obviously, experimental cancer cytotherapy is at a nodal point, where the critical mass of information accumulated by the series of studies performed over the last two decades needs to be thoroughly revised, carefully analyzed, and properly evaluated in the context of immunobiology of the tumor microenvironment. With the first-in-human clinical trials assessing genetically modified MSC in gastrointestinal and lung cancer settings only very recently and more to follow, this task has become an essential one more than ever before [1]. More specifically, in order for the field to move forward, any new experimental attempt should be built upon (a) a clear understanding of the interactions between components of the local tumor environment during its progression from benign stromal tissue to a niche tuned to support growth, invasion, and metastasis of malignant cells and (b) careful

experimental design that minimizes unnecessary duplication of work as well as the emergence of technical heterogeneity.

With all the above in mind, we present here a systematic review of the advances in the MSC-based cancer cytotherapy field from its inception onwards, including the state-of-the art of the biology and relationship between the effectors (MSC populations) and targets (tumor niche components) implicated (discussed in the section “[MSC and the tumor microenvironment—attributes and inter-relationship in the context of cancer cytotherapy](#)”). Amalgamated in this review is a meta-analysis of the related bibliography, which was carefully designed and implemented in such a way to allow the extraction and decoding of the overwhelming number of experimental data presented therein and, hence enable the identification of commonalities and differences between studies, the dissection of emerging trends and patterns in reported findings, and possibly highlight any significant omissions or weaknesses in experimental design (presented in the “[Methodology of the meta-analysis](#)” and “[Overview and discussion of the meta-analysis’ main findings](#)” sections). Finally, in the concluding part (“[Conclusions](#)” section) of this review, an effort is made to both present the main findings as concise summary points in the context of state-of-the-art knowledge, which potentially can serve as a guide map, aiding the adoption of optimal conditions for the development of more efficient, translational cytotherapy protocols with extended clinical relevance.

MSC and the tumor microenvironment—attributes and inter-relationship in the context of cancer cytotherapy

The composition and dynamics of the tumor stromal microenvironment and the role of EMT in tumor development

Cells within the tumor parenchyma are not self-sustaining entities, but develop in a symbiotic manner by interacting via paracrine and juxtacrine signaling with the surrounding stroma. The stromal microenvironment is the non-neoplastic compartment of tumors and comprises a dynamic and highly heterogeneous network of tumor vasculature (including pericytes), infiltrating inflammatory and immune cells, extracellular matrix (ECM), (myo) fibroblastic cells (also known as tumor-associated fibroblasts (TAFs)), mesenchymal stromal/stem cells, and sometimes adipocytes [2], as well as reactive stroma [3]. Most if not all solid tumors have some degree of tumor stroma, and the presence of reactive stroma is often an indicator of poor prognosis [4]. Communication between cellular components of the tumor microenvironment is achieved through cytokines, chemokines, growth factors, and inflammatory and matrix

remodeling enzymes that contribute to the malignant properties of the cells.

Among the immune cells that infiltrate the tumor stroma and are present within the tumor microenvironment, selective T and B lymphocyte populations favor or suppress tumor growth and their detection has been associated with subsequent clinical outcomes [5–7]. Tumor-associated macrophages (TAM) and neutrophils (TAN) are usually pro-tumorigenic. TAMs participate actively in metastasis, being thus implicated in poor prognosis [8], while TANs enhance angiogenesis and immune suppression [9, 10].

Perivascular stromal cells, known as pericytes, are another essential component of the tumor stroma that secures vasculature maintenance by providing structural support to blood vessels [11]. Although pericytes are present within normal blood vessels, tumor vessels are characterized by excess pericyte coverage that contributes to their abnormal physiology.

Fibroblastic stromal cells within the tumor stroma, known as TAFs, have been linked to several processes that promote cancer metastasis and growth, including angiogenesis [12], EMT [13], and progressive genetic instability [14, 15]. TAFs exert their tumor-promoting actions through secretion of growth factors, cytokines, chemokines, structural protein components, and metabolites that act upon tumor cells [2]. Additionally, fibroblastic stromal cells can deregulate antitumor immune responses, as exemplified by experiments demonstrating that allogeneic murine tumor cells, when co-injected with fibroblastic stromal cells, can engraft across immunologic barriers [16]. Together, these studies suggest that tissue-specific fibroblasts are influential players in the progression of metastatic cancer and, at first glance, appear to benefit tumor growth and decrease overall patient survival. However, there is mounting experimental evidence that healthy tumor microenvironments suppress tumor growth, and it is only after acquisition of tumor-like genetic lesions that fibroblasts appear to promote tumor progression [14, 17, 18].

ECM is another key regulator of tumor development and progression by providing both a structural scaffold for the tumor stroma and also active soluble factors including growth and angiogenic factors, cytokines, and chemokines that regulate tumor behavior. During tumor development, the ECM is usually disorganized and collagen deposition as well as cross-linking with other matrix proteins such as elastins, laminins, or fibronectin has been associated with cancer invasion and metastasis [19]. Among the ECM-secreted growth factors, TGF- β is known to promote EMT in cancer cells, thus increasing local and distant invasive potential [20].

EMT is the process by which epithelial cells reduce the expression or function of proteins that promote

cell-cell and cell-basement-membrane adhesion and thus obtain a mesenchymal-like phenotype. Except for the regulation of metastatic properties, the EMT has been also associated with acquired drug resistance [21]. Furthermore, high expression of EMT-induced markers (vimentin, α -smooth muscle actin (α -SMA), N-cadherin, cadherin 11, SpArC, laminin and fascin) with simultaneous low expression of E-cadherin has been associated with poor prognosis in patients with breast cancer [22].

Principal common characteristics of isolated human MSC populations used in cytotherapy protocols

Stem cells possess a unique capacity of self-renewal, differentiation into multiple cell types and in vivo tissue repopulation [23]. These functions are triggered by signals that impel a stem cell to undergo either symmetric or asymmetric divisions [24]. Based on their ability to give rise to one or more different cell lineages, stem cells are characterized as totipotent (able to give rise to all cells constituting the developing embryo), pluripotent (e.g., isolated embryonic stem cells (ESC) that can differentiate into cells of all three germ layers), multipotent (capacity for differentiation towards most cell lineages), and unipotent (mono-specific differentiation). Mesenchymal stem cells (MSC) are multipotent stem cells that upon stimulation give rise to most body cell types including those of muscle, bone, fat, and cartilage lineages [25]. MSC were originally isolated from bone marrow (BM) aspirates; nevertheless, they can be isolated from many types of adult and fetal tissues using similar methodologies [26]. Adipose tissue (AT) provides a particularly abundant and accessible source, although many other adult tissue sites can also be utilized including kidney, skin, and the parathyroid gland [27]. In another study, it has also been suggested that a small population of MSC circulates within the peripheral blood and is highly mobilized during hypoxia [28]. MSC or MSC-like cells have been isolated from fetal tissues as well, including skin, cord blood (-CB), umbilical cord (-UC), and placenta [29]. Regardless of their origin, these MSC share similar defining characteristics including plastic adherence, cell surface marker expression (negative for hematopoietic markers CD34, CD35/positive for core markers CD29, CD44, CD73, CD90, CD105), and at least a tri-lineage differentiation potential (towards fat, bone, cartilage) under certain conditions [26]. The native functions are thought to include wound healing and support of hematopoiesis. More interestingly, when engrafted at sites of injury, MSC differentiate into connective tissue elements, support vasculogenesis, and secrete cytokines and growth factors that facilitate healing and tissue regeneration. In addition, due to their complex immunomodulatory properties, MSC can counteract inflammation, suppress host immune responses, and prevent fibrosis [30]. As a consequence of their diverse properties, MSC have been extensively utilized in the last decade in therapeutic

applications such as tissue repair and regeneration, as well as autoimmune disease [31]. For example, the immunosuppressive effects of MSC have been used for therapy of graft-vs.-host disease (first MSC biologic drug).

Another common feature of MSC is that they are able to localize to sites of hematopoiesis, inflammation, or injury as well as to solid tumors, by a mechanism characterized as homing. Tumor tropism distinguishes MSC from other mesenchymal cells, such as differentiated fibroblasts [32]. This homing ability of MSC to injured sites and their interaction with the local microenvironment has encouraged investigation into the possibility of using these cells, either unmodified or as gene or even drug delivery vehicles for targeted cytotherapy, most notably cancer treatment [33, 34]. Interestingly, it has also been shown that co-injection of MSC promotes growth of various tumors in vivo, possibly due to the immunosuppressive effects that help cancer cells escape immunosurveillance [16, 35].

Tumor tropism of MSC and the dynamic interaction between MSC and the tumor microenvironment, due to their complexity as well as their significance for cancer therapy, require a deeper understanding and merit further investigation and hence are discussed in more detail in the following sections of this review.

The tropism of MSC homing to tumors as the basis of cancer cytotherapy

Homing is the process by which cells migrate to, and engraft in, the tissue in which they can exert local functional effects. It is well established that MSC are recruited to sites of injury to support tissue repair, stem cell homeostasis, and immunomodulation. Tumors can be considered as chronic wounds and, thus, attract MSC in similar ways as injured tissues [36]. The homing ability of MSC towards tumors has been verified and studied in animal models in a variety of experimental settings, and it has been shown that MSC are active regulators of tumor progression [37, 38]. Homing and migration of MSC to tumor sites has been proved to be mediated by monocyte chemoattractant protein-1 (MCP-1 or CCL2) secreted by primary breast cancers [39], or stromal cell-derived factor 1 (SDF-1), a small chemotactic cytokine that activates leukocytes and is often induced by proinflammatory stimuli such as TNF- α or IL-1 [40] in response to prostate, colorectal, and breast cancer in vitro [41]. On the other hand, in cases of malignant gliomas, MSC recruitment is achieved through interaction with a large array of angiogenesis-related cytokines including IL-8, TGF- β , and VEGF secreted by cancer cells [42]. Moreover, a component of the extracellular matrix, matrix metalloproteinase 1 (MMP-1) stimulates MSC homing through cleavage and subsequent activation of the G-protein protease-activated receptor (PAR)-1 [43] (Fig. 1).

It is believed that the mechanisms orchestrating MSC homing to sites of injury, and consequently to tumors, resemble the migration of leukocytes towards sites of injury and inflammation, which is well studied. It has been demonstrated that bone marrow stroma-derived MSC (BM-MSC) express high levels of adhesion molecules, including integrins β 1 and α 4, which can mediate the engraftment of MSC to the bone marrow [44, 45]. The α 4/ β 1-VCAM-1 interaction has a central role in MSC communication with endothelial cells [46]. In the same context, the interaction between α 4/ β 1 on MSC and its binding site on fibronectin of the ECM has been reported to play a major role in transmigration of MSC into the extracellular matrix [47] (Fig. 1). Other mechanisms contributing to the homing ability of MSC include P-selectin, MMP-2 secretion, and a number of cytokines [46].

Once in the tumor, attracted MSC interact with the tumor microenvironment leading to its remodeling and promoting cancer progression. It has been reported that MSC that home to tumor sites are transformed into TAFs [2] and promote tumor angiogenesis and invasive properties [48]. MSC interact with tumor cells in myriads of ways and modulate their behavior. Interactions between tumor cells and tumor-associated MSC are highly plastic and bidirectional. The activity of MSC within the tumor stroma includes enhanced secretion of TGF- β that contributes to the EMT process and immune-suppressive activities. Moreover, they release VEGF, which contributes to neovascularization within the tumor microenvironment and they also produce SDF-1 to support tumor cell growth and survival [2]. MSC also secrete CC-chemokine ligand 5 (CCL5), also known as RANTES, which interacts with specific cytokine receptors such as CCR1, CCR3, or CCR5. CCL5 paracrine signaling was found to promote the migratory, invasive, and metastatic properties of breast cancer cells [49].

Another way of communication between MSC and cancer cells includes the exchange of microparticles, like exosomes. MSC-derived exosomes can modulate the function of tumor cells through induction of MMP-2 and ecto-5'-nucleotidase activity, thus enhancing the heterogeneity within the tumor microenvironment [50], while they also enclose tumor supportive micro RNAs, which enhance tumor growth [51]. Simultaneously, cancer cells secrete exosomes that stimulate BM-MSC to differentiate into pro-angiogenic myofibroblasts with tumor-promoting properties [52]. Nevertheless, in many cases, MSC have been shown to exhibit tumoricidal behavior within tumors, as it will be discussed below.

The tumor microenvironment is a highly dynamic, plastic, and heterogeneous welter of cells and cellular factors that provide support and protection to the developing tumor and thus its therapeutic targeting constitutes a popular anti-cancer approach [13, 53]. The ability of MSC

reports implicating umbilical cord matrix-derived MSC (UC-MS) in conflicting cytotherapy outcomes is, so far, scarce and limited to in vitro experimentation. For example, a study by Li et al. [68], reporting the promotion of proliferation and metastasis of MDA-MB-231 and MCF-7 breast cancer cells in vitro, is outweighed by a series of studies showcasing the opposite effect on the two cell lines in vitro and in vivo [69–73].

Besides conflicting results observed within specific MSC (effector) and tumor (target) combinations, contradictory findings have also been obtained with regard to the effect of different MSC populations on the same tumor target, strongly suggesting a close association between the antitumorigenic properties of the former and their developmental origin. With respect to breast cancer, for example, a great number of studies (mainly in vitro) have quite convincingly showed a robust tumor-promoting effect of both BM-MS [49, 56, 58, 74–78] and AT-MS [62, 65, 79, 80] on at least two different breast cancer cell lines. On the other hand, an equally large group of studies using UC-MS clearly demonstrate the opposite (onco-suppressive effect) on the same cell lines both in vitro and in vivo, as stated previously [69–73].

Finally, apart from the cases where the dubious efficacy of certain MSC against some tumors arises from contrasting reports in the literature, there are also cases where questions are raised over the therapeutic value of those naïve MSC that have been shown to possess strong onco-suppressive characteristics, albeit by yet unconfirmed reports. Such examples are the cases of AT-MS vs. pancreatic cancer [66], BM-MS vs. non-Hodgkin's lymphoma [81] and UC-MS vs. prostate [82] and bladder tumors [83].

Clearly, more focused work will lead to data accumulation, analysis of which will help in identifying trends and will fill in knowledge gaps regarding effectiveness of experimental cancer cytotherapy, at the same time shedding more light on the underlying biological mechanisms.

In the following sections, we discuss some of these mechanisms that may attribute to the complexity and adversity of experimental cancer cytotherapy outcomes and next present our meta-analysis strategy for decoding all available data from the field at its current state of maturity.

Possible mechanisms mediating the bimodal effects of exogenous MSC on tumors

Once MSC migrate to the sites of tumor development, they interact with components of the neoplastic parenchyma as well as with supportive stroma. The exact dynamics of this crosstalk is dependent on the developmental stage and origin of the MSC as well as the type and site of tumor targeted. Nevertheless, the final outcome, tumor support or

suppression, is most likely determined by the resulting tilt in balance between the respective mediating mechanisms (reviewed in [84]). In the first case, instrumental processes for MSC are the promotion of angiogenesis and contribution to the fibrovascular stromal network, mitigation of immune reactions, and stimulation of EMT and metastatic processes, while in the case where MSC exert a suppressive effect on the developing tumor, mechanisms such as cancer cell control, apoptosis induction, and regulation of Wnt and AKT signaling are involved and dominate.

Support of tumor vasculature and fibrovascular network

A significant amount of in vivo experimental data, including labeled MSC tracking, support the contribution of MSC to the tumor vascular and fibrovascular network, either directly by differentiating into pericytes, fibroblasts, and myofibroblasts that transform into TAFs [35, 85] or indirectly through secretion of specific growth factors [86]. A MSC-like population expressing the characteristic surface markers CD10, CD13, and CD90 has been identified within pericytes isolated from the stromal-vascular compartment [87, 88].

Once in the tumor microenvironment, MSC acquire expression of TAF antigens, such as α -smooth muscle actin (α -SMA), fibroblast-specific protein, vimentin, and SDF-1 in vivo and in vitro following co-culture with tumor cells or using tumor-conditioned media [35, 89]. In accordance with this data, human BM-MS have been found to promote angiogenesis and tumor blood vessel reorganization in a murine mammary adenocarcinoma model, with increased α -SMA expression, when hMS were injected in the tumor periphery or intravenously [90]. In another orthotopic mouse model of colon cancer, co-injected MSC were incorporated into the tumor stroma and expressed α -SMA, PDGFRb, desmin, FSP, and FAP as TAF markers [91]. However, when hAT-MS were co-injected with U87MG and H460 brain tumor cells in BALB/c nude mice, no vascular support was observed [63].

MSC also secrete various pro-angiogenic factors, such as VEGF, fibroblast-derived growth factor, PDGF, and stromal-derived factor-1 (SDF-1), that facilitate angiogenesis through promotion of endothelial and smooth muscle migration and proliferation towards the tumor site [92]. MSC-expressing VEGF caused increased microvessel density in pancreatic xenografts [93] and enhanced neovascularization in syngeneic mouse models of melanoma and lung tumors [94]. However, VEGF is not the basic factor that promotes angiogenesis and other pro-angiogenic cytokines must be involved in tumor vasculature expansion by MSC, as recombinant VEGF did not have the same effect on vessel growth as did the MSC-conditioned media [92]. VEGF, IL-8, angiogenin, and CCL2 were significantly enhanced by the

concomitant presence of MSC and lymphoma cells in C.B-17 severe combined immunodeficiency (SCID) mice, contributing to the migration of endothelial cells in transwell assays. However, when MSC were directly co-cultured with endothelial cells, a significant induction of endothelial cell apoptosis was recorded [81]. Other MSC-secreted growth factors implicated on tumor vasculature facilitation include hepatocyte growth factor, cyclooxygenase, IGF-1, PDGF- α , and transforming growth factor- α 1 [93]. Finally, it has been proposed that gastric cancer exosomes can trigger differentiation of UC-MS to carcinoma-associated fibroblasts and thus enhance the tumor fibrovascular network, an effect that can be eliminated through blockade of the TGF- β pathway [95].

On the other hand, a number of studies support the involvement of MSC in suppression of the tumor angiogenic network. MSC were found to migrate towards and inhibit growth of endothelial cell-derived capillaries in vitro through production of reactive oxygen species. The growth-suppressing effect of MSC was also observed in vivo, where established melanoma tumors injected with MSC exhibited lower vascular density [96].

Immunomodulatory effects on tumors

MSC are thought to promote tumorigenesis through their immunomodulatory behavior, which is characterized mainly by immunosuppressive effects that can be beneficial for cancer cells to escape the immune system surveillance [97]. MSC act directly on immune cells, including B and T lymphocytes, dendritic cells, and natural killer cells [97–99]. MSC can suppress T cell activity by either inhibiting their proliferation or, in case of activated T lymphocytes, by leading them to apoptosis [100, 101]. Inhibition of T cell proliferation has been found enhanced by different mechanisms, like interferon (IFN)- γ -mediated upregulation of an inhibitory cell surface marker, B7-H1 [102], or by Stro-1 expression [103]. In addition, Toll-like receptor (TLR) signaling has also been shown to contribute to the immunomodulatory properties of MSC, as expression of certain receptors can lead MSC to switch from a predominately immune-suppressive to a proinflammatory phenotype [104]. On the other hand, Puissant et al. showed that both AT-MS and BM-MS can inhibit lymphocytes only when they are in close contact [105], while in another study, UC-MS (isolated from Wharton's Jelly (WJ)-MS) were able to suppress proliferation of peripheral blood lymphocytes [106].

The in vivo immunomodulatory effects of MSC are still poorly studied within tumors. In a study by Djouad et al., the reported immunosuppressive action of MSC led to a higher incidence of melanoma formation in an allogeneic mouse model [16]. Based on the immunosuppressive properties of MSC, these cells have been

proposed to suppress the graft-vs.-leukemia effect and the graft-vs.-host response [107, 108].

Pro-metastatic effects and stimulation of EMT

The contribution of MSC to the establishment of distant metastases is rather controversial, ranging from promotion to suppression in a number of studies. For example, intravenous injection of MSCs derived from umbilical cord blood or adipose tissue reduced the formation of lung metastases in mice with established mammary tumors [109]. In contrast, mice that received subcutaneous co-injections of human breast cancer cells with human BM-MS displayed a marked increase in the numbers of micro- and macroscopic lung metastases. The pro-metastatic effect of MSC was found mediated through paracrine action, by the secretion of chemokine CCL5 from MSC in two out of four cell lines tested, as the metastatic potential was abolished when CCL5 expression was eliminated [49]. In a 3D model of hepatocellular carcinoma, co-culture with UC-MS led to increased secretion of MMP2, which in turn enhanced the invasive ability of cancer cells [110]. Additionally, hBM-MS secrete IL-17B, which may act through IL-17BR—a prognostic indicator of breast cancer progression and metastasis—to stimulate metastasis. This hypothesis was tested in a humanized model of breast cancer metastasis to bone, where co-injection of cancer cells with BM-MS increased the frequency of metastases with increased expression of IL-17BR [75].

MSC may also modulate EMT, a developmental process that is subverted by tumor cells, resulting in a more invasive phenotype. Co-culture of breast cancer cells with MSC resulted in upregulation of EMT-specific markers (N-cadherin, vimentin, Twist, and Snail) and a decrease in E-cadherin [111]. Another mechanism of MSC-mediated promotion of metastasis includes the formation of early metastasis through vasculogenesis or growth factor secretion. Accordingly, MSC facilitated the entry of breast cancer cells into the bone marrow through Tac-1 regulation of SDF- α 1 and C-X-C chemokine receptor type 4 (CXCR4) [112]. In support of this observation, Zhang et al. showed that MSC from the bone marrow can promote pulmonary metastasis of osteosarcoma tumors in mice, through a mechanism involving the CXCR4/VEGF axis [113].

Regulation of signaling pathways

In many cases, the tumoricidal action of MSC on tumors has been associated with suppressive effects on signaling pathways crucial for cancer progression, proliferation, and survival, mainly involving the PI3K/AKT and Wnt pathways. Inhibition of AKT was reported in a Kaposi's sarcoma model, where intravenously injected MSC migrated to tumors and effectively inhibited tumor proliferation [59]. MSC can also suppress the WNT/ β -catenin

pathway through induced expression and secretion of DKK-1 in human carcinoma cell lines. Interestingly, when DKK-1 was inhibited, tumor cell proliferation was restored [114, 115]. The ERK/MAPK signaling pathway has also been implicated in tumor promotion by MSC, since drug-mediated inhibition of ERK in breast cancer cells cultured in MSC-CM resulted in decreased proliferation of tumor cells [68].

Many studies have concentrated on the anti-cancer functions of neonatal MSC, highlighting the primitive characteristics that render them non-tumorigenic. Among these, WJ-MSCs show modest expression of pluripotency genes and high levels of several tumor suppressor genes, while they secrete a variety of growth factors and cytokines that inhibit tumor growth [70, 116, 117].

Regulation of cell cycle and apoptosis

Studies investigating the effects of MSC on tumors have shown that they either promote or inhibit apoptosis, leading to tumor attenuation or progression, respectively. Interactions between MSC and cancer cells in the bone marrow have been shown to promote survival of acute myelogenous leukemia through upregulation of anti-apoptotic bcl-2 with reduced rates of apoptosis in response to cytotoxic chemotherapy [118]. Furthermore, culture of colorectal cancer cells in the conditioned medium (CM) of MSC downregulated the expression of the apoptosis-related proteins Bax and p53 and upregulated the anti-apoptotic protein Bcl-2, leading to inhibition of apoptosis, while under the same conditions cancer cell cycle was promoted with an increased percentage of cells found in the S phase [60]. Except for promoting anti-apoptotic events, MSC have been reported to protect osteosarcoma cells from drug-induced apoptosis, through activation of the STAT3 pathway [119].

On the other hand, Lu et al. demonstrated that MSC had an inhibitory effect on mouse tumor cells and ascitogenic hepatoma cells in a cell-dependent manner involving Caspase 3, an apoptotic protein, and p21, a negative regulator of cell cycle, implying that MSC exert tumor inhibitory effects in the absence of host immunosuppression, by inducing G0/G1 phase arrest and apoptosis of cancer cells [120]. G1 phase arrest was also observed when leukemia cells were cultivated with MSC in vitro [34]. Similar results were obtained after intratumoral administration of MSC into melanoma-bearing mice, where interaction of MSC with the neocapillary network in the tumors caused cytotoxicity and apoptosis of the tumor-associated endothelial cells [96].

MSC-based cancer cytotherapy—current challenges and gaps of knowledge

Unquestionably over the last quarter of the century, the explosion of research in molecular cell biology and immunobiology of stem cells and cancer have shed light

on the composition and establishment of the tumor microenvironment, on mechanisms governing MSC homing to tumor sites and interaction with components therein, as well as on signaling cascades and molecular events mediating the pro- or antitumorigenic effects. In its majority, this bulk of knowledge has stemmed from applied work using MSC in variety of in vitro/in vivo tumor models. However, the complexity of the field and the heterogeneity and diversity of the outcomes have inevitably led to new elementary questions that remain to be resolved. Some of these are: Have research work efforts so far been correctly prioritized or have they been prone to partiality and duplication? What are the crucial areas that we may need to focus on both experimentally and conceptually in order to maximize clinical relevance/impact? To what extent is the discrepancy in results attributed to technical rather than biological heterogeneity? What are the mechanisms mediating anti-cancer efficacy in different MSC populations? Can we improve consistency and efficiency of primary outcome by optimization of experimental parameters? Is there an MSC type/source bearing “universally” consistent tumor suppressive action against a wider range of tumor targets and with fewer adverse effects in comparison to others? If so, what are the properties that give it an advantage, are they uniquely inherent, or can they be mimicked by specific experimental interventions/adaptations? Are there some MSC/tumor combinations which decisively give better results than others in experimental research work and should therefore be given priority in clinical trials? To what extent does genetic modification of MSC ameliorate the anti-cancer behavior of naïve MSC? Are specific genetic modification methodologies applied on MSC effector cells more efficient than others against tumor targets? Given the uncertainty of naïve MSC-based tumor cytotherapy and on the other hand the more robust performance of genetically modified MSC, should bench research and clinical trials focus primarily on the latter?

In the following sections of this review, we present a strategy for identifying the sources of heterogeneity, for evaluating their relative impact on cytotherapy outcome, and we summarize trends and patterns ultimately using our findings to address the aforementioned issues.

Methodology of the meta-analysis

Until this day, several reviews have focused on summarizing the aforementioned conflicting results involving the efficacy of MSC in cancer treatment and have, in some cases and to a limited extent, tried to dissect the causal relationship to those discrepancies [84, 121]. The novelty of this review lies in the specific strategy that we adopted for extracting, recording, and organizing experimental parameters, sourced from selected publications

in order to, first, investigate associations and patterns concerning reported discrepancies and, second, highlight the optimal conditions for the development of more efficient and reliable protocols for naïve MSC-based cancer cytotherapy.

As part of this effort to elucidate the conditions and factors that could possibly affect the behavior of MSC against tumors, we conducted a small-scale meta-analysis based on information extracted from the literature using a four-step strategy: (1) compilation of a relevant publication library, (2) deconstruction of literature methodology and reported findings, (3) classification and organization of extracted experimental data, and (4) data consolidation and statistical analysis (Additional file 2: Figure S2). In the first stage, the PubMed online bibliographic database was queried for the combinations of keywords “x AND y AND z” (where “x” was “adipose stem cell” or “bone marrow stem cell” or “umbilical cord stem cell” or “Wharton jelly stem cell”, “y” was “mesenchymal”, and “z” was “cancer therapy” or “cancer treatment”), using the following limiters: Humans, English, Journal article, and published from year 2000 onwards. The reference lists generated from the above searches were merged (replicates removed) into a single reference library comprising a total of 861 unique articles, using Endnote X2 citation manager software (Thomson Corp.). The list was then carefully examined manually for specific relevance to experimental cancer cytotherapy studies employing the use of human post-natal stem cells of non-hematopoietic origin, on human tumor cell lines or primary tumor cells. This resulted in a nearly 10-fold compacted library of 108 highly relevant publications whose reported findings formed the basis of our meta-analysis. In the second step, the articles were initially divided into two categories: (a) referring to studies using only unmodified (naïve) human MSC (n-MSC; $n = 55$) and (b) relevant to experimental cytotherapy models utilizing genetically modified human MSC (GM-MSC; $n = 53$). Subsequently, detailed information available in the methodology and results sections of the manuscripts were extracted and recorded in spreadsheets using Microsoft Excel. Strings of information were classified into five broad categories, namely type of stem cells used (effectors) and type of their tumor target (tissue/cell lines), characteristics of effector-target interface (e.g., dosing, route/timing/method of administration, culture format/animal model adopted), methodology applied for cell modification/labeling and endpoint analyses/assays, and key findings and primary outcome (i.e., pro-/antitumorigenic effect), thus populating a preliminary descriptive database. In the following step, this compartmentalized information was further fragmented and reassigned into over 20 categories (fields) designated “experimental parameters” (see field titles of Additional file 3: Figure S3) to construct a detailed database using Microsoft Access

2010. In this third step, data stratification (expansion of number of categories/fields with simultaneous simplification of field contents) allowed us to recognize individual effector-target combinations with matched primary outcomes, termed “experiments.” More than one such experiments per article were reported in some cases, leading to the identification of a total of 156 experiments, which were allocated in three distinct groups: (1) experiments describing the effect of unmodified (naïve) MSC ($n = 89$), belonging to the first category of articles above, (2) experiments of the second article category which refer to the use of naïve MSC as controls for evaluating GM-MSC anti-cancer efficacy ($n = 43$), (3) experiments (again included in the second article category) that describe the anti-cancer efficacy of GM-MSC without a direct comparison with naïve MSC ($n = 24$). The primary outcomes of these experiments were flagged as tumor promotion or suppression (or neutral, if no significant difference was observed between treatment and relevant controls) depending on the results of the in vitro/in vivo assays as described in the respective publications. In the final step, the experimental parameters were used to derive database queries and to perform statistical analyses, the outcome of which is discussed in the following section.

Overview and discussion of the meta-analysis’ main findings

Naïve MSC-based cancer cytotherapy: from trend identification to protocol optimization

Relevance of preclinical cancer cytotherapy targeting to global cancer incidence

Statistical analysis of the experimental data extracted from the literature has highlighted eight types of tumors as most frequently targeted in naïve MSC-based cancer cytotherapy experimental protocols (Fig. 2). In an effort to put these tumors into clinical context, they were compared to global incidence [122]. Interestingly, the list of the 10 most frequently targeted tumors has a corresponding total incidence of just over 60% globally; the remaining 40% includes important cancers (cervical, esophageal, bladder, N-H lymphoma) which conclude the top 10 global cancer incidence list, but nevertheless are not the main focus of experimental cytotherapy efforts. Moreover, a more thorough comparison of the data revealed a predisposition of the use of naïve MSC towards specific cancer types, as well as a notable inverse relationship between the frequency of cancer targets used in MSC-based cytotherapy experiments and the cancer types with the highest clinical incidence and mortality worldwide. Thus, while breast cancer with frequencies of 30% is the most popular target and is over-represented in comparison to its global incidence (19.6%), lung cancer, on the other hand, which is the most frequent and one of the most lethal types of cancer

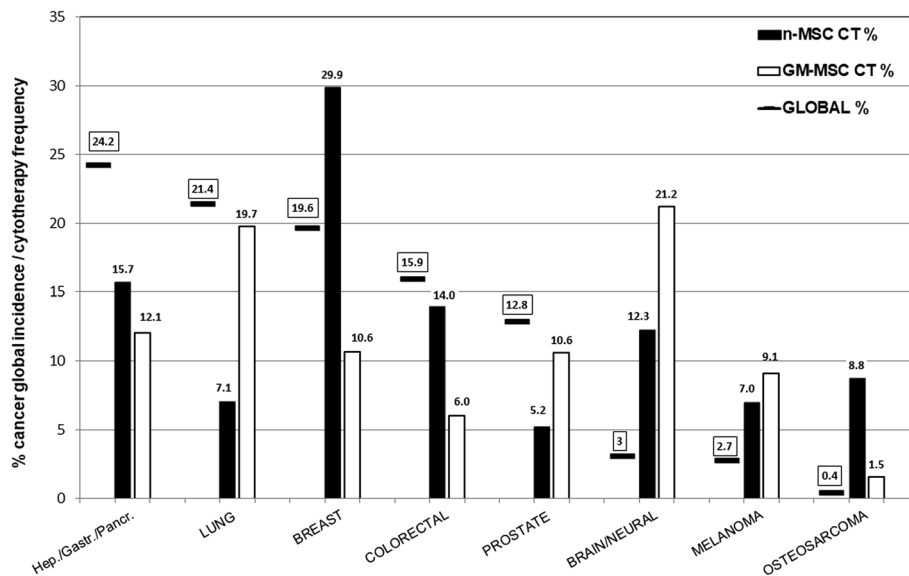


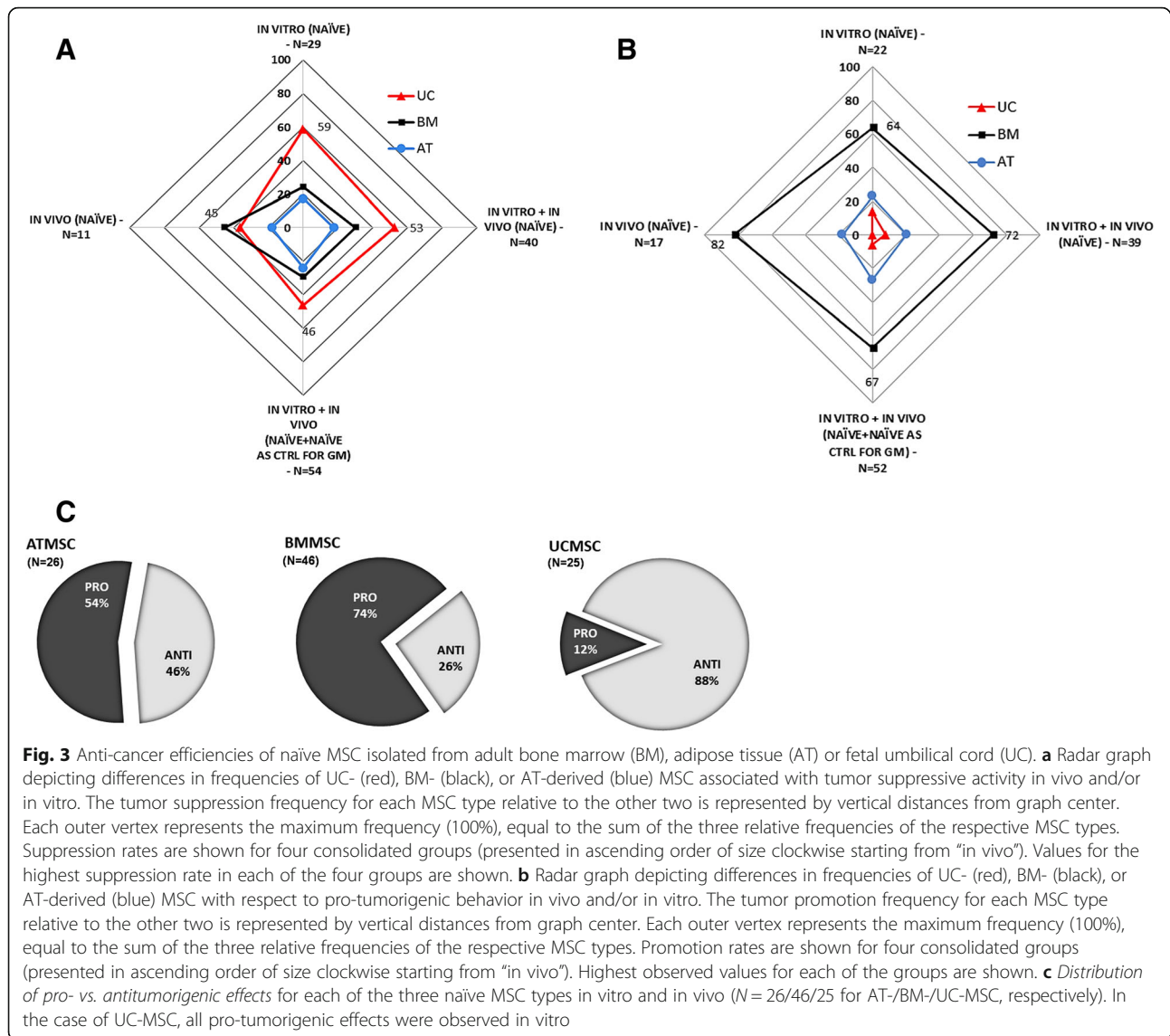
Fig. 2 Representation of common cancer types in MSC-based cytotherapy studies compiled in this review in comparison to global cancer incidences (clinical relevance). The most frequently targeted tumors in cancer cytotherapy studies have been ranked in descending order of worldwide cancer incidence (2012 data) [122] from left to right on the x axis. Global cancer incidence rates are depicted as solid line symbols (boxed values), while the frequencies of tumors targeted by unmodified/naïve MSC (n-MSC) or genetically modified MSC (GM-MSC; see also the “Genetically modified MSC as delivery vehicles for antitumorogenic molecules—overview and meta-analysis results” section) in experimental cancer cytotherapy (CT), as determined by our meta-analysis, are represented by black (n-MSC CT) or white (GM-MSC CT) columns, respectively. Sample sizes: $N = 79/N = 67$ for n-MSC- and GM-MSC-based work, respectively. For each cancer type, the difference in height between the solid line symbols and the column bars denotes the divergence in representation of global cancer incidence by CT work, with positive differences (global % > MSC CT %) signifying under-representation, and negative ones (MSC CT % > global %) over-representation of cancer incidence. For example, the two most under-represented tumors targeted by n-MSC CT are those of lung as well of liver/stomach/pancreas (HGP) (by 12.3% and 8.5%, respectively), while the most over-represented ones are those of breast and brain (difference of -11.3 and -8.3 , respectively), see the main text (the “Relevance of preclinical cancer cytotherapy targeting to global cancer incidence” section) for further discussion. Overall, the data suggest that in order for experimental CT work to become more clinically relevant, more focus should be put on the following, under-represented tumor targets: * Hepatic/gastric/pancreatic (HGP) tumors (using both n-MSC and GM-MSC). * n-MSC-based lung cancer cytotherapy. * Colorectal cancer targeted by both n-MSC (especially UC-MSC) and GM-MSC. * Prostate cancer targeted by GM-MSC (especially UC-MSC), as well as n-MSC. * GM-UC-MSC-based brain tumor cytotherapy

worldwide, is greatly under-represented in in vitro/in vivo experimentation (7.1% vs. an incidence of 21.4%). Actually, in cancer epidemiology, nearly one in two cancers are lung or hepato-gastro-pancreatic (HGP) cancers with a top-ranking, combined mortality rate of nearly 90%, yet they comprise less than 25% of cytotherapy targets. It is worth noting that HGP tumors are the only type featuring in the top three most frequently targeted by all three MSC types in cytotherapy studies, albeit with a frequency less than half than they affect patients globally (Fig. 2). Even more impressive is the under-representation of prostate cancer (the second most frequent cancer in adult male population), as well as the absence of studies regarding the use of UC-derived MSC on colorectal cancer. On the opposite side, we have the over-representation of brain tumors (glioma/glioblastoma), which is partly justified by the highly aggressive nature of these cancers (first year prevalence of 1.6%), as well as of melanoma (an extremely rare cancer). This misrepresentation is largely attributed to AT-MSC (Additional file 4: Figure S4).

The shortage of naïve MSC-based research on targeting highly frequent and lethal cancer types clearly necessitates more focused work to be carried out. Moreover, there is an excessive concentration of research efforts utilizing MSC against tumors (e.g., breast), which have failed to produce patient-beneficial results, as it is discussed below.

Differential effects of naïve BM-, AT-, and UC-MSC on tumor targets

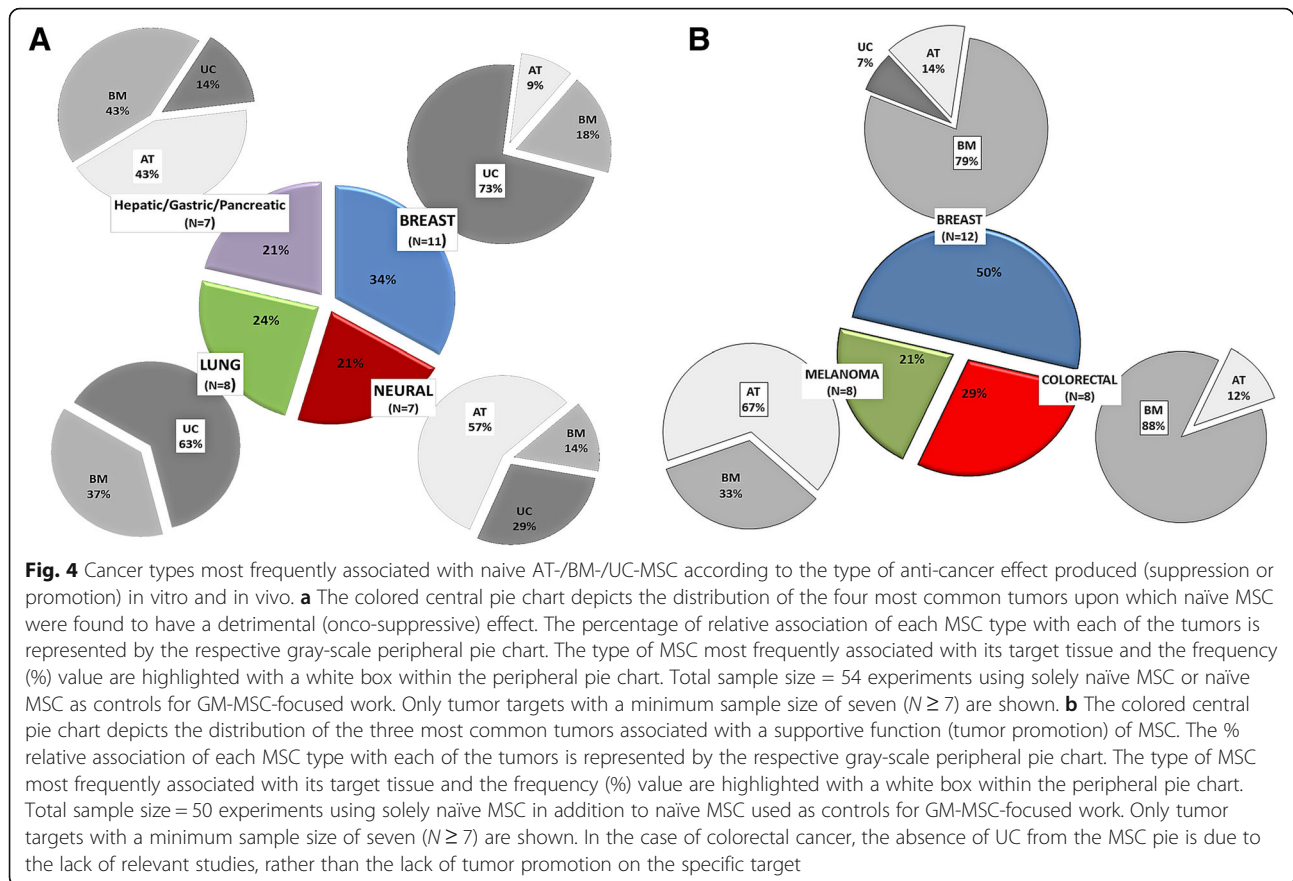
Following on from addressing the frequency, distribution and clinical relevance of cancers targeted in naïve MSC-based cancer cytotherapy, we set on to evaluate the anti-cancer behavior and efficiencies of the three MSC types relative to each other. Our meta-analysis showed a differential pattern of anti-carcinogenic behavior, with BM-MSC generally being pro-tumorogenic, and UC-MSC having a more clear onco-suppressive character (Fig. 3c). More specifically, overall, UC-MSC is the MSC type most commonly associated (about 50% frequency) with suspension of development of its tumor



targets (Fig. 3a), while concomitantly having the lowest association (<10%) with tumor promotion (Fig. 3b), which notably is observed only in relation to in vitro experimental work (Additional file 5: Figure S5). On the contrary, BM-MSC promote cancer progression in the majority (~70%) of the experiments conducted both in vitro and in vivo, at least twice as frequently as AT-/UC-MSC (Fig. 3b). On the other hand, AT-MSC's anti-cancer behavior is the least prominent relative to the other two MSC types (20.5% for AT-MSC, vs. 29.5%/49.5% for BM-/UC-MSC frequency of onco-suppressive action towards tumor targets in vitro and in vivo) (Fig. 3a).

Switching the focus to the specific types of tumors with markedly affected progression—either positively or negatively—by MSC administration, our meta-analysis revealed the following associations (Fig. 4). First, the

tumors most frequently suppressed by naïve MSC in the available cytotherapy in vivo and in vitro studies are those of the breast, lung, and brain, as well as the liver, stomach, and pancreas (Fig. 4a). UC-MSC are mainly responsible for suppressing breast and lung cancers (73% and 63% contribution, respectively), while AT-MSC are associated with glioma/glioblastoma inhibition in the majority of cases (57%), relative to the other MSCs. Second, with respect to tumor promotion, there is a strong association of BM-MSC with breast and colorectal cancer (79% and 88%, respectively) and of AT-MSC with melanoma. UC-MSCs, on the other hand, have minimal or no association with carcinogenesis induction for these three tumors (Fig. 4b). A parallel analysis based on consolidated MSC groups confirmed the pro-tumorigenic effect of BM-MSC on breast and colon, as well as the antitumorigenic effect of UC-MSC on breast and lung



cancers, and of AT-MSCs on neural tissue tumors (Table 1). Unfortunately, due to sample size constraints, the analysis could not resolve any possible differences between in vivo and in vitro effects. Nevertheless, it validated breast cancer, as not only the most common cytotoxicity target but also the only one being both suppressed and promoted by naive MSC (UC-MSCs and BM-MSCs, respectively).

Obviously, the origin of the MSC population used for administration in animal tumor models plays an instrumental role in determining the fate of the treatment. Our analysis has shown that, in terms of anti-cancer efficacy, naive BM-MSCs despite the plethora of studies referring to their use in cytotoxicity protocols, perform rather inefficiently. This is supported by the fact that they present the worst in vitro anti-cancer performance as discussed further on, they are frequently associated with tumor promotion both in vivo and in vitro (Fig. 3), and they are the only MSC type with validated tumor-promoting effects against at least three different tissues, including breast and colon (Fig. 4, Table 1). Most notable is the case of breast, in which tumor promotion was verified in vitro using three different cancer cell lines by five independent groups [33, 56, 58, 74, 76]. Furthermore, also worth noting is that, in contrast to

BM-MSCs, UC-derived stem cells show much greater potential as candidates for cancer cytotoxicity. This is supported by the following trends (Figs. 3, 4, and 5 and Table 1); UC-MSCs are (1) the only MSC type with no reports of tumor promotion in vivo (regardless of the xenograft model they are used in) and no confirmed reports in vitro in over 30 studies so far). This attribute alone makes the safest candidate for clinical trials for MSC-based cancer therapy. (2) UC-MSCs are the MSC type with the most robust antitumor activity (against breast and lung). The association with breast tumor suppression is particularly strong with many independent studies confirming it [69–73]. In comparison, the majority of studies targeting brain tumors using AT-MSCs attribute an antitumor behavior to the latter, while naive BM-MSCs, as stated before, have yet no proven therapeutic value. Moreover, with breast and lung accounting for 41% of tumors, the anti-cancer behavior of UC-MSCs is clinically relevant too. With respect to the last point made, one should take into account the limited number of studies available on other important tumors such as colorectal, prostate, and liver (see also Fig. 2) that do not allow any sound conclusions to be made regarding the effect of naive UC-MSCs on them. (3) UC-MSCs are the only MSC with essentially no

Table 1 Summary table of main experimental parameters and outcome as determined by the meta-analysis of cancer cytotherapy studies using naïve (unmodified) MSC

Consolidated groups	N	MSC	MSC N	Tumors most frequently targeted (%)	Tumor target frequency (%)	Mean antitumor effect*
In vivo (naïve)	30	AT	5	Brain/neural	60	ND
		BM	20	Breast	25	ND
		UC	5	Colorectal	25	ND
In vitro (naïve)	53	AT	10	Breast	40	ND
		BM	22	Breast	30	??
		UC	21	Breast	32	ANTI-
In vitro + in vivo (naïve)	83	AT	15	Brain/Neural	40	??
		BM	42	Breast	29	PRO+
				Colorectal	19	PRO+
		UC	26	Breast	31	ANTI-
In vitro + in vivo x(naïve+naïve as CTRL for GM)	126	AT	31	Brain/Neural	32	ANTI-/NEUTRAL
		BM	59	Breast	24	PRO+
				Colorectal	15	PRO+
				Liver	12	??
		UC	36	Breast	33	ANTI-
		Lung	17	ANTI-		

Findings based both on studies focusing solely on naïve MSC, as well as on selected studies where these cells were used as controls for experiments focusing on genetically modified MSC (GM-MSC) are shown. Results are listed in consolidated groups of increasing size (number of observations = N). MSC N column depicts MSC sample size. Primary outcomes include percentage of the most frequently targeted tumor types by the respective MSC, as well as the mean anti-cancer effect for each MSC (effector): tumor combination. The minimum accepted number of MSC (cut-off) most commonly associated with respective tumors was set to six *PRO+ denotes tumor-promoting effect exerted by >50% of the associated MSC ANTI denotes inhibition of tumor by >50% of the associated MSC ?? denotes no clear effect ND not determined due to insufficient data (N < cut-off)

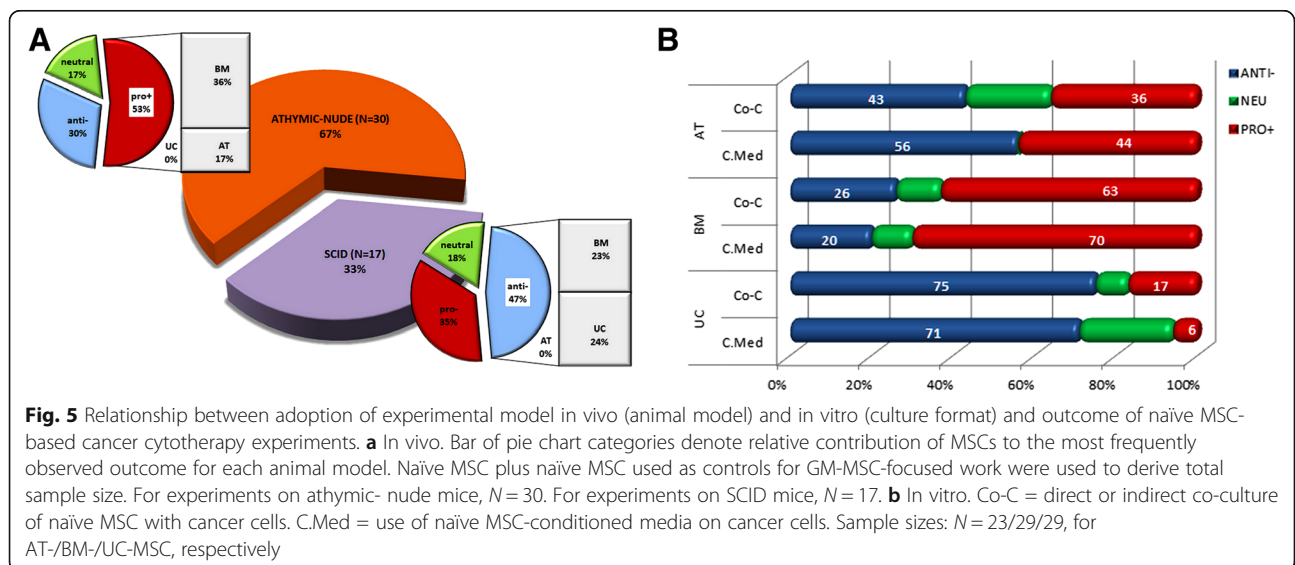


Fig. 5 Relationship between adoption of experimental model in vivo (animal model) and in vitro (culture format) and outcome of naïve MSC-based cancer cytotherapy experiments. **a** In vivo. Bar of pie chart categories denote relative contribution of MSCs to the most frequently observed outcome for each animal model. Naïve MSC plus naïve MSC used as controls for GM-MSC-focused work were used to derive total sample size. For experiments on athymic- nude mice, N = 30. For experiments on SCID mice, N = 17. **b** In vitro. Co-C = direct or indirect co-culture of naïve MSC with cancer cells. C.Med = use of naïve MSC-conditioned media on cancer cells. Sample sizes: N = 23/29/29, for AT-/BM-/UC-MSC, respectively

contradictory outcome reported (e.g., both pro- and antitumorigenic against the same tumor target). On the contrary, the latter is true for both BM-MSC and AT-MSC (BM-MSC vs. colon cancer *in vitro* [57, 60]; AT-MSC vs. brain tumors [62–64]). (4) UC-MSC are the only MSC type (as witnessed by *in vitro* experimentation, discussed further on) that can efficiently act upon its tumor target indirectly through its secretome, without any physical contact or crosstalk with components of the tumor microenvironment.

Exploring the role of experimental parameters on MSC-based cancer cytotherapy outcome

As discussed earlier, one of our main hypotheses was that the experimental conditions could be determining factors for the pro- or antitumorigenic behavior of MSC. Thus, technical variation can supplement biological heterogeneity as a co-founding factor for the discrepancies observed in tumor modulation by naïve MSC in cancer cytotherapy protocols. The propensity of MSC to either support or suppress tumor growth is the outcome of the combination of multiple parameters, such as donor-to-donor (epi-) genetic variability, heterogeneity in the isolation of the original MSC population, heterogeneity due to *in vitro* cell propagation, differences in the characteristics of the *in vivo* tumor model adopted, divergence in the choice of cell dosing (MSC: cancer cell infusion ratios), and timing of administration (co-infusion vs. MSC administration at early or advanced stage pre-established tumors).

Consequently, the classification of data extracted from studies in the literature as experimental parameters in our database was integral to our meta-analysis methodology approach. The parameters examined included choice of cell culture format/ animal model, cancer cell lines, *in vivo* cell administration scheme (route and timing), and MSC to cancer cell ratio. This was done in order to investigate the influence of each parameter alone or in combination in shaping cytotherapy outcomes and contributing to heterogeneity (Fig. 5, Tables 2 and 3).

Although we are anything but close to identifying the unequivocally ideal conditions under which MSC will be able to diminish tumor growth, findings that stemmed from analyses of core parameters in our study combined with the state-of-the-art knowledge could help build some generalizations regarding optimal conditions; these are discussed in the following lines and summarized in Table 3.

With regard to the experimental model adopted, our analysis showed that the animal model employed in *in vivo* cytotherapy protocols, and, in parallel, the cultivation conditions (culture format) of effector and target cells *in vitro*, could be decisive factors for the fate of MSC-based treatments (Fig. 5). At this point, it is worth highlighting that the *in vivo* work included in our analysis comprised

preclinical cancer cytotherapy models in which human primary MSC were implanted in immune deficient/immunocompromized rodent hosts (SCID or athymic nude mice variants) bearing human tumor xenografts. Syngeneic models or models bearing non-human MSC (which constituted 10–15% of the total) were not taken into consideration. Although this exclusion limited the amount of studies, hence, the number of data available for statistical analysis, it on the other hand constrained heterogeneity attributed to differences in native antitumor immune responses and, most importantly, in homology of expressed molecules between therapeutic cells and target cells [123]. Although both *xenograft models* included in our analyses provide excellent representation of human disease allowing the engraftment, growth, and interaction of human MSC and human cancer cells, the majority (two thirds) of the studies were conducted on athymic mice. The latter bear the advantage of being less immunodeficient than SCID variants, therefore more convincingly recapitulate immune system-affiliated antitumor therapeutic responses and disease progression [124]. As shown above, overall, the vast majority of tumor promotion effects are exerted by BM-MSC (Fig. 3b), while in the case of suppression there is no such clear contribution by a specific MSC type (Fig. 3a). With regard to the animal models used, one would expect tumor promotion to be more frequently observed in SCID mutants (severely deficient in functional B and T lymphocytes), rather than in athymic nude mice, which are less immunocompromized, bearing more active B cells and intact innate immunity through robust natural killer (NK-) cell responses. Yet, it is the majority of experiments (53%) in athymic mice in which tumorigenesis is promoted; largely responsible for this outcome are BM-MSC (Fig. 5a). This could be possibly explained by the immunomodulatory function of MSC which have proved to be able to inhibit T and B, as well as NK cells, suppressing thereby any possible adaptive immune responses [97–99]. Moreover, it has been proposed that active B cells, which in this case may escape MSC targeting, promote acute innate inflammation, which in turn impels malignant progression [125]. Furthermore, in the late 1990s, Barbera-Guillem and colleagues showed that immune complexes formed by antibodies and tumor-associated antigens (TAA) can promote tumor progression, through a mechanism that involves the activation of a crosstalk between polymorphonuclear (PMN) leukocytes and monocytes [126]. From this aspect, the potential interactions between injected MSC and the nude animal's activated B lymphocytes could trigger tumor progression through a mechanism that is T cell independent. In any case, all these mechanisms might be more prominently activated in the case of BM-MSC, since impressively, as aforementioned, UC-MSC do not promote tumorigenesis *in vivo*, and this is regardless of the immunological background of the xenograft host. This is most likely

Table 2 Contribution of experimental parameters/variables on cytotherapy outcome. The following parameters were examined

A. Tumor suppression vs. cancer cell lines (in vivo/in vitro)									
MSC	Breast cancer cell line								
		MCF-7		MDA-MB-231		All cell lines			
BM/AT/UC		17/ND/75 (75)		0/ND/60 (85.7)		36/20/56.3 (82)			
All MSC		36		46		34.4			
B. Tumor suppression vs. cell administration scheme (in vivo)									
MSC	In vivo cell administration scheme								
		Co-injection of MSC + cancer cells		Administration of MSC in established tumors					
BM/AT/UC		11.8 (50)/10 (0)/ND		64.7 (72.7)/50 (100)/64 (66.7)					
All MSC		14.3 (25)		61.5 (75)					
C. Tumor suppression vs. MSC: cancer cell ratio (in vivo)									
MSC	MSC: cancer cell ratio (in vivo)								
		MSC: cancer < 1		MSC: cancer ≥ 1					
BM/AT/UC		36.4/33.3/71.2		25/12.5/66.7					
All MSC		45.8		30					
D. Consensus of in vivo cytotherapy experiments									
Effect on tumor	MSC type	Mouse model	Admin. route	MSC dosage (millions)	Ratio (MSC to cancer cells)	Repeated dosing	Effector-target overlap period (days)	Total experiment duration (days)	
ANTI- (N = 19)	NA	NA	NA	1	1:2	NA	22 ± 19	30 ± 23	
PRO- (N = 27)	BM	Athymic/nude	s.c./s.c.	1	1:1.35	No repeat	39 ± 22	40 ± 24	

A. Type of tumor-specific cell line targeted. Tabulated values denote breast tumor suppression rates (%) caused by MSC after interaction with various breast cancer cell lines, including the two most common ones, MCF-7 and MDA-MB-231, in vitro and/or in vivo. Number of experiments $N = 11$ (MCF-7), 14 (MBA-MB-231), and 32 (all eight cell lines). ND not determined due to insufficient data ($N < 5$). Numbers in brackets (UC-MS) correspond to percentage of suppression if neutral effects are not taken into consideration. Data show a more robust tumor suppressive effect of UC-MS (in italics) which seems to be cancer cell line-independent. **B.** Timing of in vivo cell administration. Tabulated values denote percentages (%) of inhibitory effects caused by MSC on tumors in in vivo experiments, in which MSC were administered either simultaneously (co-infused) with cancer cells in animal models or sequentially in established tumors. The number of experiments recorded for the two modes of administration were $N = 27$ and 39, respectively. ND not determined due to insufficient data ($N < 5$). Percentages in brackets: sub-portion of samples in which a strong (over twofold) suppressive effect was observed. Data show that MSC have a more prominent suppressive effect when delivered to pre-established tumors. **C.** Cell dosing ratio. Tabulated values denote percentages (%) of tumor suppressive effects of MSC in in vivo experiments, depending on the relative number of administered MSC (effector cells) to cancer (target) cells (MSC to cancer ratio). The effects of two such ratios are compared: MSC: cancer < 1 (cancer cells in excess) vs. MSC: cancer ≥ 1 (MSC in excess). Percentages refer to the proportion of experiments with antitumorigenic results out of the total number of experiments in each ratio. The number of experiments recorded for the two ratios were $N = 24$ and 30, respectively. Data indicate a stronger, by over twofold, tumor suppressive effect of AT-MS when used at the lower MSC dosing ratio (values in italics). See also section 4.1.3 for further discussion. **D.** Combination of the typical, most frequently observed (> 50% frequency) parameters of in vivo experiments using naive MSC. Typical experimental parameters related to anti- or pro-tumorigenic effect of MSC are shown. NA data associated with the respective parameter gave no clear consensus. Highest suppression rates are highlighted in italics (where applicable).

attributed to the unique immunomodulatory properties of these cells, with their antigenicity not necessarily triggering tumorigenicity (further discussed in the following section).

In vitro cancer cytotherapy experimental models comprise two main types: (a) co-cultures of MSC and cancer cells, where cells communicate (two-way interaction) either directly through physical contact and the formation of gap junctions, or indirectly via exchange of soluble factors (crosstalk), and (b) monocultures of target (tumor) cells, which are grown in and react to culture media in which the effector cells (MSC) have been grown for 24–48 h (conditioned media (CM)). This second culture model basically allows only unilateral signal communication (from the effector to the target/responder cell). In both experimental settings, BM-MS had mainly pro-tumorigenic effects on cancer cells, while

on the contrary, UC-MS were mostly antitumorigenic (Fig. 5b). In vitro, the frequency of association of UC-MS with tumor-promoting events is very low (2–12 times lower than in case of adult MSC). More interestingly, the association of UC-MS with antitumorigenic activity is quite robust (> 70% frequency), irrespective of the culture model adopted. A moderate or strong tumor suppressive effect of UC-MS-CM has been observed against a group of cancer targets much more diverse than in the case of adult MSC; these include the bladder [83], breast [68, 71], larynx [127], lung [128], glioma [129], lymphoma [130], melanoma [131], osteosarcoma, and ovarian adenocarcinoma [70]. Consequently, UC-MS bear quite robust anti-malignant behaviors *in vitro*, with their secretome alone being strongly tumoricidal, suggesting that they possess superior inherent anti-cancer properties, being effective solely in a paracrine

Table 3 Possible causes of discrepancies in the antitumorigenic effects of MSC populations

Contributing parameters to outcome disparity of MSC-based cancer cytotherapy	Variability range of differentially expressed parameters	Proposed optimal experimental parameter
MSC isolation source	BM (human fetal or adult, mouse) AT (human), UC (human, rat)	Human UC
MSC in vitro/ex vivo expansion	MSC passage, MSC confluence, high serum or growth factor supplemented media, possible contamination with tumor cells	Determine maximum passage No. for MSC/check senescence status, minimize serum of animal origin
In vivo tumor model	Over 60 cell lines representing 15 tumor types (including sarcoma, hepatoma, adenocarcinoma, melanoma, glioblastoma, lymphoma) SCID, athymic nu/nu mice	At least two different tumor cell lines per cancer
MSC species origin	Syngeneic, xenogeneic	Athymic nu/nu mice (or nude variants) Human xenograft
MSC : Cancer cell ratio	BM-MS: 2:1-1:1-1:12 AT-MS: 1:1-1:10 UC-MS: 6:1-1:1-1:6 (ratios more frequently associated with tumor suppression)	Dependent on MSC type and in vivo or in vitro experiment. 1:1 and 1:2 should be used as starting ratios
Cell administration route	Orthotopic/intratumoral, subcutaneous (s.c.), intraperitoneal (i.p), intravenous (i.v)	Ideally, i.v. if homing also needs to be demonstrated. Otherwise, orthotopic, good for mimicking human carcinogenesis
Timing (latency) of MSC administration	Simultaneously with tumor cells, successive (variable time lag)	Successive (lag depends on type of tumor model; usually 7 days)
Repetition of administration	Single ("one-off") administration or repeated dosing	Repeated (once or twice); doses > 1 week apart

fashion with a non-essential need for physical cell contact. Taken together with their performance *in vivo*, UC-MSCs exhibit very low probability of eliciting tumor initiation or progression in experimental cancer cytotherapy, thus laying the foundation for their safe inclusion in clinical trials.

With respect to the diversity of tumors targeted, our analysis included over 15 tumor types represented by more than 60 well-characterized *human cancer cell lines*. The cancer types with the highest number of available cell lines were the breast and brain (eight and seven cell lines each, respectively). Nevertheless, it is interesting that only 12% of the studies used two or more representative cell lines to describe and confirm the observed effects on the tumor tested. In an effort to test whether MSC exhibit distinct behavioral imprints on different cell lines of the same cancer tissue, we focused on breast cancers, since not only they were the most popular tumor target, but also the one represented by a large number of cell lines, as stated above. We compared data from two of the most commonly used cell lines, MDA-MB-231 and MCF-7, vs. all eight available breast cancer cell lines (Table 2A). Interestingly, the tumor suppression rate overall did not differ significantly with respect to the cell line but as a function of the MSC used, with UC-MSCs having clearly a more profound effect than the other two MSC types. This is despite the differences in the characteristics of the cell lines examined, with MDA-MB-231, a triple negative, aggressive breast cancer cell line, being more prone to cytotoxicity, and MCF-7 more robust and resistant, as evidenced by chemotherapeutic treatments. Thus, although these results are only indicative, it would be logical to assume that for at least some cancers, the biological differences between MSC are a greater source of heterogeneity than the genotypic and phenotypic diversity of cancer cell lines. Nevertheless, it is proposed that the effects of MSC against a given cancer should be examined and verified by at least two cancer cell lines, ideally with distinct characteristics.

Our analysis has also highlighted the *cell administration scheme* adopted (i.e., the route and timing of MSC and tumor cell infusions) as one of the key factors influencing cancer cytotherapy outcome. Cross-examination of the data in Tables 2B and 2D reveals that simultaneous administration (co-infusion) of MSC and cancer cells in studies in rodents results in poor anti-cancer efficacy (14.3% suppression). In sharp contrast, delayed administration of MSC (usually infused orthotopically or intravenously, 7–10 days after initial tumor cell delivery) dramatically ameliorates both the occurrence and severity of tumor suppression (by over fourfold and threefold, respectively). This observation is consistent with the hypothesis that the presence of MSC during early tumor

establishment events may actually facilitate processes, such as angiogenesis, which are required for tumor initiation [93].

In terms of *cell dosing*, MSC-to-cancer cells ratios lower than one (< 1) (i.e., cancer cells in excess) seem overall to be better associated with tumor inhibition *in vivo* (45.8% vs. 30% for ratios ≥ 1) (Table 2C). Interestingly, the effect is more prominent for adult MSC, especially. AT-MSCs, while UC-MSCs seem to retain their high effectiveness towards cancer growth irrespective of the cell dosing ranges applied (Tables 2C and 3). In the case of BM-MSCs, tumor promotion occurring when relatively high numbers of MSC are used has been found to correspond to the immunosuppressive functions of these cells that are known to be more active at higher MSC numbers but lost at low doses [132, 133] (see also the “Linking MSC phenotypic differences to variation in anti-cancer efficacy” section).

As a final step in our analysis, we attempted to derive a *consensus of experimental parameters* affiliated to either tumor suppression or promotion by recording the frequently observed ($> 50\%$ frequency) value in each case. Although the data did not allow a consensus to be built in the first case, a set of parameters were identified that could be regarded, either alone or in combination, as “tumor promotion-statistically associated experimental parameters” in preclinical cytotherapy studies. Such factors are listed in Table 2D. Thus, according to our findings a single co-injection of tumor cells and human naïve BM-MSCs at a ratio of 1.5:1 to 1:1, subcutaneously (unnatural tumor growth site) in athymic mice would most likely maximize tumor initiation and growth.

MSC isolation and expansion in experimental cytotherapy—sources of variation and implications for clinical trials

The process of *in vitro/ex vivo* MSC expansion is necessary in order to obtain clinically significant cell numbers. This procedure is nevertheless time-consuming, costly, and poses cell contamination and loss risks, thus ideally the MSC source should allow the relatively easy, high-yield isolation of populations with propagation characteristics that minimize sub-cultivation time.

A large number of studies have unequivocally demonstrated that variations in *ex vivo* manipulation conditions, such as age and anatomical origin of donor tissue [134, 135] harvesting procedures [136], type of culture medium, including type of constituent growth serum [137, 138], initial plating and sub-culture seeding densities, and culture period/number of serial passages [139, 140], either separately or in combination [141], can have a major impact on proliferation and other vital processes (stemness/ differentiation, secretion) of all stem cell types, albeit to different extents.

The contribution of MSC expansion on the discrepancies observed regarding their antitumor behavior can only be speculated, since astonishingly, no preclinical cancer cytotherapy study so far has focused on evaluating the effect of variations in sub-cultivation characteristics of a given MSC type on specific cancer types. This would be particularly interesting as it would not only pinpoint the impact of this source of variation on cytotherapy efficacy but also on adequacy and would aid towards the establishment of some rudimentary good manufacturing practice (GMP) standards in light of the clinical trials [142].

With regard to the differences in *ex vivo* expansion between various MSC types, a significant amount of studies show that they are ontogeny-related, with many underlining the superiority of MSC isolated from fetal umbilical cord tissue, over those derived from adult tissues (bone marrow and adipose tissue) [143–145]. More specifically, the intervacular matrix of the umbilical cord (Wharton's jelly) contains the MSC population with arguably the best isolation and expansion characteristics among its other substructures (e.g., perivascular and subendothelial tissues) [146–149]. On the other hand, AT-MSC are regarded superior to BM-MSC, primarily due to the relatively easier, higher-yield harvest method, and secondarily due to their enhanced stability over prolonged sub-culture [150–153]. Indeed, BM-MSC have the least favorable characteristics in terms of tissue accessibility/abundance for harvesting purposes since their isolation relies on invasive, painful techniques that use aspiration needles to reach their anatomical source (usually the marrow cavity of the pelvic bones) and which are often associated with patient-to-patient variability and donor site morbidity [150, 152]. In sharp contrast, UC tissue is naturally collected during normal parturition, and unless it is stored for banking or research purposes, it is normally regarded as medical waste and discarded [149].

With respect to *ex vivo* growth kinetics, UC-MSC bear three distinct advantages over adult MSC. First, the isolation procedure results in larger pool of adherent, clonogenic cells (MSC), especially compared to BM-derived MSC. There are various studies reporting a yield of 10,000–15,000 MSC per cm of umbilical cord tissue in the starting culture [137, 146, 149, 154], while this can be further increased by protocol modification [155, 156]. With lengths of cord harvested ranging between 15 and 48 cm, a typical isolation yield would be about 400,000 highly proliferative UC-MSC [157–159]. On the contrary, adherent BM-MSC represent a quite rare population in the bone marrow, estimated at 0.0001–0.001% of total nucleated cells (1–10 colony-forming unit–fibroblast (CFU-F) per million of peripheral blood mononuclear cells) [160]. This actually equates to no more than a few hundred cells per ml of marrow aspirate, with a typical yield not

exceeding 5,000 cells per 5 ml of aspirate [139, 161]. Regarding adipose depots, the reported frequency of nucleated cells isolated is at least 0.4 million per milliliter of processed lipoaspirate. However, plating efficiency of these cells varies considerably (0.2–4 %) [161], so actual AT-MSC turnover can range from 0.24–10 million cells for liposuction volumes between 100–3000 mL (or a mean yield of 0.9–1.3 million stem cells from an average 300 ml (= 270 g) lipoaspirate) [152, 162]; this yield can be 500–2500-fold greater than that of BM-MSC [136, 163, 164]. Second, UC-MSC proliferate at a much faster rate, various studies report population doubling times (PDT) in the range of 26–42 h until the fifth passage (which incidentally is the most common sub-culture threshold used) while for adult MSC it is 50–65 h [136, 137, 139, 149, 165, 166], although there are studies reporting PDT exceeding 96 h especially in the case of AT-MSC [143, 167]. This equates to shorter culture periods required to obtain the desired cell yield and thus makes UC-MSC more suitable for clinical scale expansion (e.g., 30–40 days are required to complete five rounds of expansion, as opposed to 2 months in the case of adult MSC) [168]. A yield of about five billion UC-MSC from a single cord can be obtained within 5–6 passages when large five-cell stacks are used for propagation [138]. Third, UC-MSC can sustain their higher proliferation rates for extended time periods. They not only can be serially propagated up to eight or nine times while maintaining a short PDT (< 30 h), but can be additionally expanded up to 25–50 population doubling (PD) (over 20 passages) while maintaining a normal karyotype as well as their immunophenotypical and stemness characteristics [137, 158, 169, 170]. Differences of up to 22 PD have been observed between the proliferative lifespans of BM-MSC and UC-MSC [168]. Impressively, yields up to 10^{17} UC-MSC (40 PD) over a 2-month culture period have been reported (in sharp contrast to only 5×10^7 (8PD) for BM-MSC) [171]. Interestingly, growth in low O_2 environment (5%) [138, 172] or in xeno-free culture medium [173, 174] further enhances their proliferative lifespan, while extracts of these cells when coated onto cultures plates ameliorate late-passage proliferation of both UC-MSC and BM-MSC [168]. On the contrary, adult MSC show a limited potential for prolonged propagation, being able to maintain only up to 15–25 PD (9–12 passages) in culture [153, 165, 168]. A senescent phenotype, characterized by a plateau in the rate of PD accumulation, a sharp reduction in cloning efficiency, and an increase in positive β -galactosidase staining, has been observed in some cases as early as only six PD/passages [166, 175, 176]. In fact a cumulative number of 20 or more PD and a PDT > 60 h have been highlighted as thresholds for replicative senescence in BM-MSC, with recommendations to use cells at the lowest passage possible, preferably up to fifth to sixth [142, 177, 178].

With regard to the role of MSC expansion in cancer cytotherapy in specific, we could not draw any conclusions, since no detailed information/experimental data were available (besides number of passages, in some cases) regarding status of cells utilized (such as number of cumulative cell PD, PDT, CFU-F scores, senescence status). However, taking into consideration the growth kinetics and bearing in mind that the optimal MSC: cancer cell ratio should be 1:1 to 1:2 (Table 3), one could attempt to estimate the feasibility of scale up for clinical use for each type of MSC. Assuming that both a tumor (e.g., T1 palpable breast cancer) and a cancer cell (breast cancer cell lines) are spheroids with diameters of 2 cm [179] and 15 μm [180], respectively, one would need 12–24 billion MSC to treat a single tumor. According to our estimations, starting from isolation, AT-MSC would require seven serial passages over a month's period in order to generate this amount of cells. On the other hand, UC-MSC after 3.5 weeks and the same number of passages would accumulate 19 PD which would be sufficient for treating 8–16 tumors. Last, the ex vivo expansion characteristics of BM-MSC make these cells the least favorable for clinical use. The eight rounds of sub-culture required (equal to 22 PD) are not only the longest (at 56 days, essentially making very difficult to match any need for frequent, repeated dosing) but would also bring the cells close, if not beyond, their senescence threshold. Actually, presuming that this threshold is five to six passages (15–16 PD), then the maximum yield that BM-MSC could give is 100 times less than that required in our example, i.e., these cells can support protocols which require up to approximately 120 million cells.

Linking MSC phenotypic differences to variation in anti-cancer efficacy

In the introductory section, we gave a brief account of the characteristics that MSC share and form the basis of their adoption as valuable cytotherapy tools. Nevertheless, our literature meta-analysis has revealed that with respect to anti-cancer efficacy it is their differences in genotypic and phenotypic attributes that are more important. This notion is based on the observation that the most prominent disparity in the outcome of cancer cytotherapy is MSC (effector)-related. Some naïve MSC populations are more effective against specific tumor targets, where others underperform and may even prove deleterious for cancer treatment. A prime example of the latter case is the pro-tumorigenic effect of BM-MSC and AT-MSC on metastatic breast cancer. On the contrary, UC-MSC show an anti-carcinogenic response that is both strong and robust, i.e., less sensitive to experimental conditions such as choice of ex vivo model and cell dosing. According to our findings, based on the data available so far, fetal-derived

UC-MSC are safer to use and more frequently associated with suppression of tumor growth than MSC derived from human adult tissues. This is backed up by comparative studies evaluating the anti-cancer effects of MSC of different developmental origin [64, 129], although clearly more such studies should be carefully designed and carried out, in order to validate the notion that outcome of cancer cytotherapy is primarily MSC ontogeny-related.

There is sufficient evidence in the literature suggesting that the unique combination of the biological traits suited for giving UC-MSC their special role in human development actually allows them to serve a second purpose as naturally anti-cancer capable cytotherapy agents. UC-MSC are primitive cells falling in the developmental map between hESC and adult stem cells [181]. They share biologic characteristics with both stem cell types; however, they do not combine them with attributes that pose as safety risks for cytotherapy. Thus, similar to hESC and unlike adult MSC, they are consistently positive for *pluripotency and self-renewal markers* such as Oct-4, NANOG, LIF, SSEA-1, SSEA-4, Tra-1–60, and Tra-1–81 [116, 117, 182, 183]. In opposition to hESC, they do not form teratomas when injected in SCID mice [72, 154]. Moreover, as discussed earlier, they are capable of sustaining high proliferation rates for extended periods in culture (due to their longer telomeres and expression of telomerase), while simultaneously maintaining anchorage dependence, contact inhibition and serum dependence as growth requirements, i.e., showing no signs of *spontaneous transformation* [149, 154, 184, 185]. In contrast, human adult MSC have been linked to malignant transformation as well as karyotypic instabilities and epigenetic damage, raising safety concerns [176, 186, 187]. These can arise as a result of extensive ex vivo cultivation or prolonged exposure to tumor cell-generated stimuli. For example, spontaneous malignant transformation occurred in 45.8% of long-term (5–106 weeks) cultures of human BM-MSC, resulting in multiple fast-growing lung deposits when injected into immunodeficient mice [188]. Furthermore, in contrast to UC-MSC, a series of studies have provided evidence for transition of BM-MSC to a TAF-like myofibroblastic phenotype and hence contribution to tumor growth after long-term exposure to conditioned medium of breast and ovarian carcinoma cells [35, 89, 189]. Last, noteworthy is the implication of BM-MSC as originating cells for sarcomas, most notably the highly metastatic osteosarcoma [190, 191]. Nevertheless, there is also evidence in support of the view that adult MSC are safe for cytotherapy applications. With respect to BM-MSC, Bernardo and colleagues did not observe any sign of transformation or genomic instability after 25 passages/44 weeks in culture, respectively [192]. Apparently, this is attributed to their inability to escape replicative senescence due to their short telomeres [193]. Similarly, spontaneous transformation that was initially reported in human primary AT-MSC, following long-term (4–5 months) in vitro culture,

was later recognized as an artifact attributed to unnoticed minimal cross-contamination with a fibrosarcoma cell line cultured simultaneously in the same laboratory [194]. Last, a meta-analysis comprehensively summarizing the safety of 36 clinical trials using intravascular delivery of adult MSC for the treatment of non-neoplastic diseases did not find a significant association between MSC use and the appearance of malignancy [195]. Although the occurrence of malignancy as a long-term adverse event in response to systemic MSC infusion was as high as 24% (in non-randomized control studies), the difference compared to control patients was not significant, while none of the malignancies observed were formed de novo.

Our analysis has provided quantitative evidence for the poor anti-cancer performance of BM-MSC which are the MSC type most frequently (74%, Fig. 3c) associated with tumor promotion overall; moreover, their *secretome* scores very low in terms of tumor suppression (20%, Fig. 5b) which means that these cells lack intrinsic tumoricidal properties. On the contrary, UC-MSC bear strong tumoricidal abilities that are both intrinsic, i.e., naturally occurring without the need for cellular cross-talk, and extrinsic, i.e., arise after interaction with malignant tumor cells, dead tumor cells, or other benign cellular components of the tumor supportive stroma [196]. In both cases, the effects can solely occur in a paracrine manner, which nevertheless does not preclude direct physical cell contact, whilst they involve the secretion of multiple proteins (*secretome*). These include molecules that induce cell cycle arrest and cell death (e.g., tumor suppressors, caspases) on cancer cells [117], as well as variety of immunomodulatory effectors (e.g., pleiotropic cytokines and growth factors) [197–199] with mainly anti-inflammatory action. The antitumorogenic effect of UC-MSC *secretome* preparations (in the form of cell lysates or conditioned media) has been assessed on a variety of cancer cells in vitro (discussed in the previous section), most often through evaluation of their contribution to cell cycle arrest and apoptosis induction, e.g., by determining changes in expression/activation status of tumor suppressors (e.g., p53) [127, 130], initiator and executor caspases (caspase-9 and caspase-3) [8, 129, 130, 200], and negative/positive apoptosis regulators (Bcl-2, Survivin/Bax) [70, 127, 129, 130]. Upstream apoptosis-mediating mechanisms shown to be involved include JAK/STAT signaling [200] as well as regulation of Akt phosphorylation [83] and intracellular H₂O₂ production [130]. In fact it is possible that UC-MSC *secretome* affects the regulation of key antioxidant enzymes resulting in high levels of intracellular H₂O₂ in cancer cells, production of reactive oxygen species, and increased oxidative stress triggering apoptotic cell death, an anti-cancer mechanism similar to that used by many current chemotherapy drugs. Indeed, when UC-MSC

extracts are used in combination with doxorubicin, not only they do not increase resistance to the drug, but they also display an additive cytotoxic effect on target cancer cells [201, 202].

Lately, it has become evident that MSC *secretome*-mediated effects on cancer cells implicate the delivery of bio-molecular cargo (such as mRNA, miRNA, and secreted proteins) packaged into membrane structures called *extracellular vesicles (EV)*. MSC-derived EV (MSC-EV), which mainly comprise the nano-scale, endosome-derived exosomes, and the larger microvesicles, are considered as potent regulators of intercellular communication. The main biological potency of MSC-EV is maintenance of tissue homeostasis and mainly involves targeting housekeeping biochemical activities; however, they are also believed to play an important role in the dissemination of cancer phenotype. Data so far suggest that MSC-EV are an integral part of autocrine and paracrine signaling events modulating expansion of the tumor microenvironment, angiogenesis, and metastasis. Little is known about the exact mechanisms but data so far are indicative of complex regulation involving antagonistic effects [203, 204]. Thus, not only MSC-EV cargo composition seems to vary depending on the MSC source ultimately having a differential impact on tumor fate [205], but even more interestingly EV isolated from the same MSC source can have opposing effect depending on the type of tumor target [83, 206].

With respect to the immune system-affiliated antitumor effect of MSC, our meta-analysis did not allow us to make any clear deductions regarding the differences between fetal and adult MSC, since for the reasons explained earlier we chose to exclude data derived from syngeneic models which have proper antitumor responses (tumor infiltrating lymphocytes, macrophages, and myeloid-derived immuno-suppressor cells (MDSCs)) and better represent the contribution of the host microenvironment to the triadic interaction of MSC: cancer cells: stromal supportive cells. Nevertheless, the immunomodulatory properties of fetal and adult MSC have been quite well documented in comparison [105, 207, 208]; moreover, some excellent studies employing syngeneic models provide an insight into the immunomodulation-mediated tumor suppressive effect of UC-MSC [209–211].

Differences in *immunomodulation* are believed to be directly linked to the duality of results in experimental cytotherapy of cancer. For example, polarization of BM-MSC (through specific priming of Toll-like receptors (TLR)) into proinflammatory or immunosuppressive phenotype directly correlates to tumor suppressive or tumor-promoting effects, respectively [104, 212]. The immunomodulatory effect can be so powerful that can actually drive two opposing responses within the same MSC tumor model [213]. With respect to differences in immunomodulation properties of adult and perinatal MSC, three main assumptions can be

made by examining the available literature; (1) the immunosuppressive properties of human UC-MSc are significantly stronger than those of BM-MSc. (2) MSc respond to different thresholds of inflammation depending on the type and concentration of proinflammatory cytokines they are exposed to by regulating, in turn, the cytokine secretion and activation marker profiles on immune cells. (3) With respect to activation of immune cells, mitogen-induced and alloantigen-driven lympho-proliferation are modulated probably by different mechanisms, which are unique for BM-MSc and WJ-MSc. The evidence that points towards these conclusions originates mainly from *in vitro* work. Unstimulated UC-MSc exhibit a strong suppression of lympho-proliferation, even at a very low dose (1% UC-MSc, mononuclear cells (MNC)) compared to BM-MSc, independent of the inductive stimuli (presence of mitogen or alloantigen) [214]. Priming of UC-MSc (but not BM-MSc) with inflammatory stimuli (e.g., IFN- γ) further attenuates lympho-proliferation in a mixed lymphocyte reaction (MLR) containing allogeneic peripheral blood MNC. Nevertheless, mitogen-induced suppression of lympho-proliferative responses is further enhanced upon exposure of BM-MSc (and not UC-MSc) to inflammatory stimuli. UC-MSc (along with AT-MSc) suppress both mitogenically and allogeneically activated proliferation of purified T cells better than BM-MSc in a dose-dependent manner by secreting high levels of LIF; furthermore, they maintain and promote the expansion of T regulatory cells (T-regs) independently of the MSc/T cell ratio [183]. Last, with respect to B cell activation, UC-MSc when co-cultured with PHA-stimulated MNC do not influence acquisition of lymphoblast characteristics by B cells or their progression from non-activated to early activated stage, in contrast to BM-MSc and AT-MSc that exert an inhibitory effect [215].

The differences in immunomodulatory function between adult MSc and UC-MSc are not restricted to adaptive immune responses. Raicevic and colleagues have demonstrated a lower reactivity to bacterial or viral infections involving innate immunity mechanisms. In MLR containing CD3⁺ T cells, UC-MSc resist the neutralizing effect of inflammation or TLR ligation on their immunosuppressive properties by lacking TLR4 expression and by overproduction of HGF (almost 10-fold higher expression compared to BM-MSc and AT-MSc) [216].

Recently, the unique immunomodulatory properties of UC-MSc have been linked to secreted components of their ECM. In a study involving a mouse corneal transplantation model, Coulson-Thomas and colleagues showed that UC-MSc actively suppress inflammatory immune response of the host microenvironment by favoring M2 macrophage phenotype (anti-inflammatory) over M1 (proinflammatory) and by inducing maturation of T-regs and inflammatory cell death. They do this by secreting components of their unique glycocalyx which

constitutes the characteristic matrix in which the cells naturally reside and function [217]. One of the properties with high practical utility in terms of cytotherapy is their *low immunogenicity*, which is a key requirement for allogeneic transplantation. UC-MSc/WJ-MSc, like their adult counterparts, evade immune recognition by lacking co-stimulatory molecule expression (CD40L, CD80/CD86) which are normally implicated in activation of both T and B cell responses. However, crucially, UC-MSc distinguish themselves from adult MSc with respect to expression of major histocompatibility complex (MHC) molecules. UC-MSc express significantly lower levels of MHC class I (HLA-ABC) both at rest and following stimulation with IFN- γ , while MHC class II (HLA-DR) induction with IFN- γ treatment is substantially augmented in BM-MSc but is very negligible in WJ-MSc [169, 218]. The lower HLA class I and II expression profile of UC-MSc leads to dampened immune recognition and promotion of immune ignorance *in vivo*. It may also partially explain the lack of sensitivity of responder cell proliferation to UC-MSc doses, unlike BM-MSc where the balance between their immunosuppressive/-supportive properties is more emphatically determined by their direct environment (relative cell ratios and physical contact). Indeed, the fact that low MSc numbers may display a stimulatory instead of inhibitory activity on T cell proliferation has been shown using mixed lymphocyte cultures [207]. BM-MSc may express MHC class II antigens and act as antigen-presenting cells eliciting a strong T cell response in the presence of low levels of IFN- γ , i.e., in an inflammatory setting [219]. The expression of MHC class II molecules on the surface of MSc requires autocrine stimulation by endogenous, low levels of IFN- γ , but is decreased at high IFN- γ levels causing the loss of the antigen-presenting cell function of the MSc HLA-DR [220]. Interestingly, in a more recent study, it was shown that HLA-DR-negative BM-MSc can augment or hinder the proliferation of T lymphocytes depending on the cell ratio used. Optimal MSc-mediated inhibition required a high MSc: CD3⁺ ratio (1:4) and direct physical contact between the cells [183].

Human UC-MSc have been also found to differ from adult MSc with respect to the expression of HLA-G, a non-classical HLA class I molecule which plays a key role in maternal tolerance for the developing fetus [170]. UC-MSc, in contrast to BM-MSc, express high levels of HLA-G which upon IFN- γ stimulation increase even further. This high expression of soluble as well as membrane-bound HLA-G isoforms allow UC-MSc to suppress immune reactions by reducing allo-proliferation of T cells, by disturbing the cytolytic function of NK cells, and by preventing maturation of dendritic cells [221, 222]. HLA-G has been shown to mediate the suppressive function of UC-MSc on lympho-proliferation in MLR and

thus their escape from immune recognition by allogeneic peripheral blood lymphocytes [169]. The immunomodulatory activity of UC-MSc has been further demonstrated by their ability to suppress the expression of proinflammatory cytokines *in vitro* (such as TNF- α and IL-6) after co-culture with peripheral blood MNC, and *in vivo* by their survival as xenotransplants in immunocompetent mice not receiving any immunosuppression.

Despite their immunosuppressive properties and low immunogenicity, under certain circumstances, UC-MSc can elicit an immune response which should be taken into consideration when establishing a cytotherapy protocol (e.g., timing of MSc administration). In a clinically relevant miniature swine model, while a single injection of MHC-mismatched inactivated UC-MSc did not elicit a detectable immune response, repeated injections in the same region caused the opposite effect, as evidenced by the production of alloantibodies within 1 week [223]. Therefore, care must be taken in order to avoid sensitization, especially against orthotopic cancer cytotherapy.

Overall, ontogeny-related differences in the pattern of expression of HLA molecules (e.g., HLA-DR, HLA-G), as well as in the cytokine secretion profile (e.g., HGF, LIF, IL-6) between adult MSc and UC-MSc are largely responsible for the course of direct or indirect interaction with cellular components and bioactive factors of the tumor microenvironment driving the adoption of a tolerogenic/immunosuppressive or a more immunoreactive/proinflammatory phenotype, which ultimately determines the impact on tumor progression. With this in mind, the optimal dose, frequency, and timing of MSc administration need to be carefully considered for each cancer target.

Genetically modified MSc as delivery vehicles for antitumorigenic molecules—overview and meta-analysis results

Tumor specificity is the major obstacle hampering the effectiveness of current cancer treatment therapies, such as combination chemotherapy. Advanced drug targeting of tumor cells is particularly hard when treating highly invasive and/or metastatic tumors such as glioblastoma or pulmonary cancer. Uncontrolled drug distribution in the patient's body, i.e., insufficient concentration at the tumor site and toxic concentration in normal cells, which is attributed to inefficacy of anti-cancer therapy, is often the direct cause not only of severe side effects but also, ironically, of life-threatening complications. MSc tropism towards primary and metastatic tumor locations, which occurs independent of tumor type, MSc immunocompetence and route of delivery, combined with their relatively ease of *in vitro* manipulation to express molecules with anti-cancer action, naturally provides a solution

to drug specificity, bypassing the main issues of stability (short half-life), dosing (effective dose vs. maximally tolerated dose), and off-target toxicity (side effects) associated with their systemic administration. Historically, the therapeutic value of this unique “Trojan horse/smart bomb” approach for the controlled release of potent antitumorigenic agents *in situ* has been rather convincingly demonstrated in a large number of studies with genetically modified MSc (GM-MSc) targeting an extensive range of cancers in various experimental animal models [reviewed recently by [224, 225].

In this review, we have concentrated on studies referring to human tumor cells specifically targeted by human GM-MSc of adult bone marrow, adipose tissue, and fetal umbilical cord matrix tissue origin, herein termed GM-BM-MSc, GM-AT-MSc, and GM-UC-MSc, respectively (Additional file 6: Figure S6).

Our meta-analysis has revealed that the focus of GM-MSc-based cancer gene therapy experiments is on tumors difficult to treat, i.e., highly invasive and metastatic cancers (Fig. 2 and Additional file 6: Figure S6). Indeed, one of the most frequently used models is that of metastatic lung cancer (19.7%) which is in alignment with highest incidence and mortality of this type of cancer worldwide. Brain tumors, which are practically incurable, feature also quite often as targets in GM-MSc-based work (21.2% frequency) essentially serving as excellent proof-of-principle studies. Nevertheless, GM-UC-MSc are clearly under-represented in relation to brain cancer cell-mediated gene therapy. Indeed, according to our analysis there is a bias (also observed in the case of naïve MSc-based cytotherapy) in favor of BM-MSc (48% frequency of use) with only one in five (21%) of GM-MSc-based studies actually employing the use of UC-MSc over adult MSc. Interestingly, breast cancer's overall popularity as a target in *in vivo* anti-cancer work employing GM-MSc is more than threefold reduced (to 11%) relative to naïve-MSc-based experiments, probably justified by the strong performance of naïve UC-MSc against this type of tumor. Also in contrast to naïve MSc, the range of tumors targeted in GM-MSc-based cytotherapy is somewhat more limited. Sarcomas (Kaposi's, osteosarcoma), lymphomas (Burkitt's, non-Hodgkin's), and bladder and larynx tumors are notable omissions in GM-focused work.

In terms of anti-cancer efficacy, a number of studies have convincingly demonstrated the therapeutic potential of GM-BM-MSc against lung [33, 226–230] and brain [231–237] cancers and of AT-MSc and UC-MSc on brain [238–241] and breast [72, 73, 200, 242] tumors, respectively (Additional file 6: Figure S6). With regard to GM-AT-MSc and neural tissue tumors, the robust anti-cancer performance of the former counterbalances the erratic behavior of their unmodified precursors, while

in the case of breast cancer and UC-MSC the results obtained using GM protocols solidify the already encouraging results obtained using naïve UC-MSC.

Overview of GM strategies applied

High clinical relevance of tumor targeting in GM-MSC-based cancer gene therapy is especially important since the latter has showed consistently high anti-cancer efficacy in terms of inhibition of local tumor growth, suppression of metastasis, or prolongation of animal survival, irrespective of the experimental methodology. With regard to the latter, apart from the effector (MSC)-target (tumor) components, the choice of transgene (anti-cancer agent) that is incorporated into the MSC carrier, as well as the enabling genetic modification technology, complete the list of methodological components. The two main strategies adopted are (a) the rather straightforward overexpression of molecules with known antitumorigenic action or (b) gene-directed enzyme/prodrug treatment (GDEPT) (Table 4). In the first case, MSC are transduced with molecules which upon expression are usually secreted acting via different mechanisms on tumors and range from immune regulators such as interferons (e.g., IFN-alpha, IFN-beta, IFN-gamma) [33, 128, 200, 227, 229, 243–245] interleukins (e.g., IL-12, IL-21) [246–248] and chemokines (e.g., CXCL1) [228], to molecules with pro-apoptotic (e.g., sTRAIL) [226, 232, 234, 235, 249–253], anti-angiogenic (e.g., Alpha1-antitrypsin, NK4) [254, 255], or other properties (e.g., PEDF, TNF- α , HNF4- α) [54, 237, 256–258]. Among these, immune response modulators and pro-apoptotic agents are the most popular transgene classes used, with an adoption rate exceeding 60% (Table 4). The most frequently adopted representatives, IFN- β and TRAIL, which together account for more than 40% of all human transgenes used in GM-MSC-based experimental cancer cytotherapy, are incorporated with the aid of viral vectors (usually adenovirus and lentivirus, respectively) into BM-MSC and UC-MSC markedly enhancing their anti-cancer efficacy.

Tumor necrosis factor-related apoptosis-inducing ligand (*TRAIL*) is a type II transmembrane protein of the tumor necrosis factor (TNF) family of ligands that is expressed by a variety of immune cells and is capable of initiating apoptosis through engagement of its receptors which are differentially expressed in normal and cancer cells [259]. The attractiveness of TRAIL as a therapeutic agent for the treatment of experimental cancer lies in its ability to selectively induce apoptosis in a broad spectrum of tumor and transformed cells, but not most normal cells. TRAIL initiates a caspase 8-dependent apoptosis signaling cascade by forming homotrimers that bind cognate agonistic receptors DR4 (TRAIL-RI) and DR5 (TRAIL-RII) bearing a conserved death motif on

target neoplastic cells. Apart from the membrane-bound form, TRAIL can also exist and function as a soluble ligand. Its extracellular C-terminal domain can be proteolytically cleaved from the cell surface in a vesicle-associated or in a soluble form (sTRAIL) that is also able to oligomerise and trigger p53-independent apoptosis via binding to the cytoplasmic part of DR4 and DR5 on target cells [260]. And since most chemotherapeutic drugs and radiation act by inducing tumor cell apoptosis in a p53-mediated manner via activation of the intrinsic mitochondrial pathway, sTRAIL can be used complementarily to overcome resistance to conventional chemotherapy and radiotherapy. Nevertheless, clinical relevance of soluble TRAIL (sTRAIL) has been hampered by its short (< 1 h) pharmacokinetic half-life in plasma, suggestive of relatively low protein stability, as well as by its scavenging/partial elimination through binding to antagonistic decoy receptors (DcR1 and DcR2) expressed mainly by normal tissues which limit its availability at tumor sites [261]. Again, as in the case of IFN- β , local homing and delivery of TRAIL via GM-MSC can overcome these problems. Indeed, GM-MSC have been shown to successfully target and significantly reduce tumor growth in models of multiple myeloma [250], glioma/glioblastoma [232, 234, 235], lung [226, 249, 253], liver [229, 252], colorectal [251], and cervical [249] cancers. Among these are BM-MSC and UC-MSC carrying human recombinant sTRAIL against glioma/glioblastoma [232, 234] and hepatocarcinoma [252], respectively. The significance of TRAIL as an antitumor agent is highlighted by its inclusion in one of the very first clinical trials involving MSC-based cancer gene therapy (NCT03298763; [262]). Set to begin in 2018, this phase I/II clinical trial focuses on the evaluation of safety and antitumor activity of allogeneic BM-MSC-TRAIL on up to 46 metastatic non-small cell lung cancer (NSCLC) patients who are receiving GM-MSC cytotherapy in conjunction with conventional chemotherapy with pemetrexed/cisplatin. Interestingly, the trial addresses the issues of BM-MSC adequacy and variability by pooling cells from multiple donors before transduction [1].

In the second strategy, *GDEPT (also known as suicide gene therapy)*, GM-MSC act as carriers of genes encoding for specific enzymes that convert non-toxic prodrugs into active derivatives [263]. The prodrugs are administered systemically following in vivo infusion of GM-MSC and homing towards tumor sites. The cytotoxic effect of the activated drug is then exerted locally on cancer cells at the tumor site, thus minimizing off-target toxicity; of course the production of drug metabolites is also highly toxic for the MSC carriers themselves, which die in the process. The two enzyme/prodrug combinations most widely used are herpes

Table 4 Overview of the main MSC genetic modification (GM) strategies and their most commonly used constituents, as utilized in experimental cancer cytotherapy studies (N = 67)

GM strategy	Typical GM components				
	Antitumor agent(s)	Adoption rate (%)	Gene introduction vector *	Cellular vehicle *	Tumor target *
Antitumorigenic transgene overexpression	Anti-proliferative immune regulators (e.g., IFN-β)	41	AV	BM-MSC	Various
Gene-directed enzyme prodrug therapy (GDEPT)	Pro-apoptotic molecule (e.g., sTRAIL)	20.5	LV	Various	Various
	Cytosine deaminase (CD) + 5-fluorocytosine (5-FU)	18.6	RV	AT-MSC	Various
	Herpes simplex virus thymidine kinase (HSV-tk) + ganciclovir (GSV)	8.5	RV	AT-MSC	Brain

*Most frequently observed (> 50% frequency) parameters

simplex virus thymidine kinase gene (*HSV-tk*)/*ganciclovir* (*GCV*) and *Escherichia coli* cytosine deaminase (*CD*)/*5-fluorocytosine* (*5-FC*), with suicide genes introduced into the stem cell carriers via viral vectors (most often retroviruses) (Table 4). One obvious advantage of GDEPT over conventional cancer gene cytotherapy, apart from the conditional rather than constitutive type of action of the tumoricidal mechanism, is that it allows the elimination of disseminated cells, thus acting as a safety mechanism against any potential adverse effects (e.g., transformation events) related to the long-term persistence of homed and non-homed MSC in the patient's body. Most importantly, the main benefit of this anti-cancer approach is the amplification of the drug toxic effects by means of *bystander effect* which is described as the death of non-transfected cells (e.g., cancer cells) due to indirect effects caused by their neighboring transfected cells (MSC), causing more widespread cell death than if transfected cells alone were killed [264, 265]. A requirement for this phenomenon is the active or passive transference of the activated toxic metabolite to neighboring cells which involves the use of gap junctions [266, 267], endocytosis of apoptotic vesicles [265, 268], or paracrine effects that lead to immune system stimulation [269, 270].

The two main GDEPT systems differ to the mechanism by which they accomplish bystander effects and consequently diverge in terms of their tumor growth suppression efficiency. In the case of CD/5-FC system, activated (deaminated) 5-FU rapidly crosses the plasma membrane by passive diffusion and exerts its cytotoxic effects by interfering with both DNA and RNA synthesis. In contrast, the active phosphorylated form of GCV is a charged metabolite unable to readily diffuse out of the cell and thus relies on physical contact and gap junctional intercellular communication (GJIC) between therapeutic and tumor cell to exert its cytotoxic action by interfering with DNA polymerase function. In the case of CD/5-FC system, yeast or *E. coli*, the CD gene can be used for the activation of 5-FC, although the bifunctional fusion protein cytosine deaminase to uracil phosphoribosyltransferase (CD::UPRT) is preferred due to the higher bystander effect achieved in vitro and in vivo. CD::UPRT chimeric protein not only exhibits an enzymatic activity 10-fold greater than that of wild type CD and increases sensitivity to 5-FC by at least 100-fold, but also alleviates cytotoxicity resistance exhibited by some tumors [271, 272].

Various studies have attempted to compare the efficacy of CD::UPRT/5-FC and HSVtk-MSC/GCV systems (in the absence or presence of MSC carriers). Whereas in the first case, CD::UPRT/5-FC proves superior due to its greater bystander effect, the effect on tumors targeted by CD::UPRT-MSC/5-FC or HSVtk-MSC/GCV differs depend

ing on the expression status of enzymes of nucleotide metabolism and ATP-binding cassette (ABC) transporters, irrespective of GJIC status [239, 273, 274]. Thus, for example, while both systems are equally effective against 8-MG-BA gliomas, on the contrary, A375 melanoma and MDA-MB-231 breast adenocarcinoma cells are differentially sensitive to the two systems, with the former being more susceptible to CD::UPRT-MSC/5-FC and more refractory to HSVtk-MSC/GCV treatment, and vice versa.

TK/GCV and CD/5-FC GDEPT comprise the minority of GM-MSC-based strategies, almost exclusively using adult MSC as cellular vehicles (with AT-MSC used in three out of four cases). Cancer types targeted include melanomas [275], sarcomas [276], colon [277], prostate [67, 278, 279], and ovarian [274]. The TK/GCV system has been predominantly applied in combination with AT-MSC for treating neural tumors demonstrating very high efficacy [238, 239, 280]. Furthermore, in a phase I/II clinical trial started in 2015, the very first one worldwide to evaluate the safety and efficacy of GM-BM-MSC against advanced, recurrent, or metastatic gastrointestinal or hepatopancreatobiliary adenocarcinoma, the trial methodology was based on intravenous injection of HSV-tk-engineered MSC in patients, followed by repeated GCV injections [281].

It is worth highlighting that in some cases, in an effort to maximize tumor suppression efficiency, a *combination of strategies* is employed as evidenced for example in the case of UC-MSC-IFN- β plus 5-FU (cytokine-transduced MSC + chemotherapy drug) for the treatment of breast cancer [73], UC-MSC-sTRAIL plus 5-FU (apoptosis agent-transduced MSC + chemotherapy drug) [252], BM-MSC transduced with sTRAIL plus HSVtk/GCV (apoptosis agent-transduced MSC + GDEPT system) for glioblastoma treatment [231], and HSVtk/GCV in combination with CD::UPRT/5-FC (double GDEPT) used on AT-MSC against glioblastoma [239] or breast-to-lung metastatic cancer [273]. These approaches result in various degrees of synergy, ranging from mild, non-significant enhancement of tumor suppression, to additive or stronger synergistic effects depending on the tested cell line and experimental setup. For example, Matuskova and colleagues showed that sequential treatment (single treatment with CD/5-FC followed by HSVtk/GCV) leads to synergic cooperation of CD and HSVtk against MDA-MB-231 metastases to lung, an effect that is stronger at higher GCV doses [273].

Obviously, the multitude of combinations of antitumorigenic molecular cargo, cellular vehicle, tumor model, gene incorporation, and expression technology and method of administration enables the generation of a large number of individual GM-MSC-based anti-cancer strategies laying the basis for personalized cancer cytotherapy. Interestingly, a comparison of the different strategies applied in relation to a specific cancer model frequently used in experimental

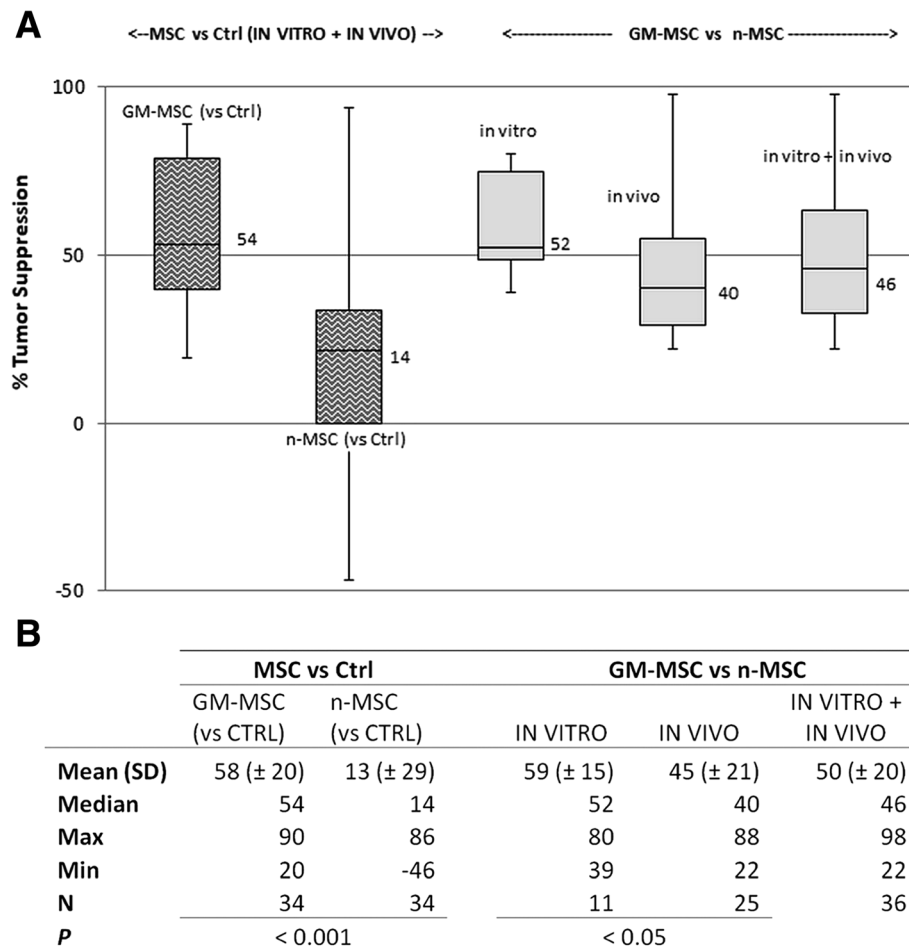


Fig. 6 Tumor suppression rates (%) for genetically modified MSC (GM-MSC) in comparison to naïve MSC (n-MSC) and non-MSC controls. Tumor suppression rates were determined using measured experimental outcomes (e.g., tumor size and weight, animal survival rate, proliferation assays) derived from 20 selected publications in which the anti-cancer efficacy of genetically modified human MSC was directly compared to that of unmodified, naïve MSC and non-MSC controls. **a** Whisker plots. Values next to whisker boxes depict median values. **b** Tabular summary of the data plotted in **a**. Sample size (*N*) relates to total number of experimental readouts (e.g., tumor size and weight, animal survival rate, proliferation assays) based on which % tumor suppression was calculated. *P* values correspond to *t* tests comparing the specific groups

cytotherapy studies reveals substantial accordance in the results obtained. For example, with respect to the popular xenograft lung metastasis model, administration of human GM-MSC in mice suppressed the metastases of human MBA-MB-231 breast cells to the lung (determined by measuring the increase in median survival of GM-MSC-treated mice) by 62%, 81%, and 73% ($72 \pm 9.5\%$) for BM-MSC-IFN- β [33], BM-MSC-TRAIL [226], or AT-MSC receiving double GDEPT (CD::UPRT/5-FC : HSVtk/GCV, 1:1) [273], respectively. Similarly, metastases of MDA-MB-231 breast cells were reduced by 53%, 42%, and 39% ($45 \pm 7.4\%$) in mice receiving BM-MSC-IFN- β [33], UC-MSC-IFN- β [72], or UC-MSC-IFN- β +5-FU combination treatment [73], respectively, as evidenced by the reduction of mouse lung masses one month after GM-MSC administration.

Regardless of the strategy followed, successfully marrying high efficacy with safety is the primary challenge in developing a *gene delivery system*. So far all GM efforts have relied on the heavy use of viral vectors (adenovirus—AV, retrovirus—RV, or lentivirus—LV). Generally, viral vectors are more efficient at transferring genes than non-viral vectors; however, clinical studies have revealed that the efficacy of virus-mediated suicide gene therapy was limited due to adverse effects, such as toxicity, oncogenicity, and the possibility of invoking an excessive antiviral host immune response [282]. For example, AV expression is transient and often produces a significant host immune response, whereas RV may cause incorporation errors or even oncogene activation [283, 284]. LV vectors are less likely to cause insertional mutagenesis, since the promoter can be

modified extensively. Furthermore, they have the ability to stably transduce both dividing and quiescent cells, which is a further significant advantage when using stem cells that are often quiescent or slow growing [285]. According to our analysis, LV is more frequently associated with MSC transduced with TRAIL (50% adoption rate compared to other antitumor agents). In this setting, LV has been shown to efficiently and stably transduce MSC without affecting their stemness, while at the same time, LV modification does not result in altering DNA copy number and arrangement in MSC [286]. Lately, advances in genetic engineering methods have led to the development of innovative non-viral nano-vectors that combine the low toxicity and immunogenicity of cationic liposomal carriers with the high transfection efficiency of viral systems [287, 288].

Naïve MSC vs. GM-MSC-based cytotherapy efficacy

As mentioned earlier, GM-MSC-based cancer cytotherapy has shown greater consistency in successfully suppressing tumorigenesis. Nevertheless, the difference in tumor suppression efficiency in comparison to n-MS-C has been largely discussed on a qualitative, rather than a quantitative basis. Our meta-analysis approach gives for the first time, to the best of our knowledge, a quantitative dimension to the advantage of GM-MS-C over n-MS-C-based cancer cytotherapy. With respect to the studies using GM-MS-C, they all report attenuation of tumor growth, irrespective of the type of MS-C effector, tumor target, GM strategy, or animal/culture model applied. On the contrary, as previously discussed, the frequency of n-MS-C studies showing a significant onco-suppressive effect varies greatly (26–88%, mean = 48%) depending on the type of MS-C employed (Fig. 3c). In other words, n-MS-C-based cancer cytotherapy is more “hit-or-miss” in terms of reliability with the final outcome largely dependent on the MS-C-tumor combination. In an effort to verify this observation and to provide a more accurate quantitative estimate of the impact of GM-MS-C over n-MS-C, we focused our meta-analysis on experimental data from selected studies referring to experimental cytotherapy work on human lung, breast and brain cancers, in which human GM-MS-C (of BM, AT, or UC origin) had been directly compared to n-MS-C as well as to matched non-MS-C controls within the same experimental setup [72, 73, 128, 200, 226, 227, 229, 232–237, 241, 242, 273, 289]. Such experimental readouts relating to anti-cancer efficacy included measurement of size and/or weight of the developing tumors, as well as changes in animal survival rates (in vivo), and determination of cell proliferation and/or viability (in vitro). The wider range of antitumor effects of n-MS-C is largely attributed to BM-MS-C. For example, intratumoral (intracranial) or systemic (intracarotid) administration of naïve BM-MS-C in athymic mice with established

gliomas resulted in reduction in median survival of the mice by 6 and 5 days (or by 20% and 16%), respectively, as compared to controls. On the contrary, infusion of BM-MS-C-IFN- β via the same routes extended animal survival by 12 and 8 days (or by 29% and 20%), respectively, over the U87-only control group [236]. Overall, according to our analysis the tumor suppression efficiency of GM-MS-C is higher by about 40% compared to that of n-MS-C, as depicted by the significant difference in mean and in median values of the two groups (Fig. 6). A point worth highlighting is also the fact that in vitro experimentation tends to generate tumor suppression rates (GM-MS-C/n-MS-C) that are generally higher than those obtained using rodent tumor models; this is particularly evident in the case of cancer cell apoptosis evaluation in vitro, where GM-MS-C show on average an eightfold higher pro-apoptotic effect, compared to their unmodified counterparts (data not shown). Nevertheless, the therapeutic advantage of GM-MS-C in vivo is strong, with the latter extending, for example regarding median survival time of tumor-bearing mice by an average of 16 days over n-MS-C, equivalent to a life extension by two to four years in humans (data not shown).

Taking all the above into consideration, it can be deduced that GM-MS-C exhibit greater antitumor efficiency when used as delivery vehicles in comparison with non-modified cells. For GM-BM-MS-C, there is enough preclinical evidence showing the efficacy of IFN- β on solid tumor suppression to warrant the move to clinical trials. Indeed, by mid-2019 a trial involving up to 21 patients with advanced ovarian cancer is expected to provide the first ever data on the maximum tolerability and safety of MS-C-IFN- β in humans (NCT02530047; [262]). With respect to GM-AT-MS-C, experimental cytotherapy data so far are suggestive of an increased antitumor efficacy in relation to gliomas, especially when combined with a form of GDEPT. Last but not least, in the case of UC-MS-C, genetic modification can only enhance their intrinsic suppressive action on tumor growth. Unfortunately, large gaps in GM-UC-MS-C work do not allow us to have a more complete picture regarding their anti-cancer performance against important cancers such as colorectal and prostate. Nevertheless, preclinical data so far suggest that breast and lung cancers comprise two potential targets for GM-UC-MS-C testing in humans. The hypo-immunogenicity and non-tumorigenicity of human UC-MS-C are additional features acting in support of the adoption of the specific cellular vehicle, in an allogeneic setting, for the transition from the bench to the bedside.

Conclusions

In our effort to decode the diversity of MS-C-based cancer cytotherapy outcomes, we methodically recorded and analyzed experimental data reported in the relevant

literature. Our approach, although carefully designed, is far from perfect as it is based on a relatively limited number of observations that restrict the statistical resolution to the descriptive statistics level. Nevertheless, the trends obtained are representative and agree with the literature. In summary, our findings allow the following conclusions to be drawn: (a) more studies focusing on frequently occurring tumors are needed. This holds truth for both naïve and GM-MS-C-based research work. For example, experimental data on naïve MSC targeting cancers such as hepatic, gastric and lung are rare, rendering any attempt to evaluate the MSC performance against the latter unsuccessful. Prioritizing these targets will provide more information towards a deeper understanding of the biology of MSC: tumor interactions, at the same time setting a solid basis for the development of more efficient MSC engineering protocols. Special emphasis should also be given on cancers for which there are preliminary studies with encouraging data that need confirmation, or there are no studies evaluating the anti-cancer performance of specific MSC types. These are pancreatic (naïve AT-/BM-MS-C), colon and prostate (naïve and GM-UC-MS-C), and brain cancers (GM-UC-MS-C). (b) More attention should be paid to experimental design and implementation to minimize the technical heterogeneity, which comprises a major contributor for inconsistencies and erratic outcomes observed in experimental tumor cytotherapy. Experimental parameters such as the use of multiple cell lines to represent each tumor target, the MSC to cancer cell ratio, timing and mode of cell administration used, and, last but not least, the type of animal model adopted are of particular importance. (c) Genetic modification of MSC offers a significant advantage in anti-cancer efficacy over naïve MSC, successfully alleviating the bimodal effects of some MSC populations against certain tumors shifting the balance robustly toward tumor suppression and can become the new standard once the enabling technologies become safer. More focused clinical trials are

needed towards this end. (d) Despite the omissions and inconsistencies, a critical mass of data from available studies so far suggest that the primary outcome of experimental cytotherapy is largely dictated by the biology of the interaction of the specific effector MSC with its target, with some MSC populations (e.g., UC-MS-C) generating a strong and robust tumoricidal response against specific tumors (e.g., breast), while others (BM-MS-C) are clearly unsuitable for use on the same target, since they display pro-tumorigenic behavior (Tables 5 and 6). Differences in antitumor efficacy of MSC are determined by the dynamics of their interactions with tumor niche components, which in turn are determined by their phenotypic and functional traits (e.g., secretome profile, senescence status, cell surface receptor expression levels), and ultimately depend on the developmental origin of these cells. (e) While a single stem cell type with a ubiquitous anti-cancer behavioral profile is unlikely to exist, our analysis indicates that MSC derived from the umbilical cord of the human fetus possess enhanced antitumorigenic characteristics compared to adult MSC, which are largely likely attributed to their unique immunomodulatory profile as primitive stem cells.

At this stage, any attempt to give a detailed account of the mediating mechanisms would be largely speculative, given the lack of studies focusing on the *in vivo* immunomodulatory effects of administered MSC within tumors, and the inadequacy of the present experimental models to recapitulate the multitude of highly plastic and bidirectional interactions that take place. Nevertheless, data patterns emerging from this meta-analysis (as discussed in the “Exploring the role of experimental parameters on MSC-based cancer cytotherapy outcome” and “Linking MSC phenotypic differences to variation in anti-cancer efficacy” sections) interpreted in the context of co-culture experiments examining MSC-immune effector cell interactions or focusing on the interplay between various tumor stroma cell classes during carcinogenesis can be summarized into a conceptual

Table 5 Summary of evidence-based cancer therapy by MSC as deduced by the meta-analysis of peer-reviewed experimental/preclinical research studies

MSC	MSC GM status #	Tumor target	Effect on tumor target	Evidence in support
AT	Modified (or naïve)	Neural	Suppression	**
UC	Naïve (or modified)	Breast	Suppression	***
UC	Modified (or naïve)	Lung	Suppression	**
BM	Modified	Neural	Suppression	**
BM	Modified	Lung	Suppression	**
BM	Modified (or naïve)	Pancreas	Suppression	*
BM	Naïve	Breast	Promotion	***
BM	Naïve	Osteosarcoma	Promotion	*

Primary or secondary/minor (in brackets) contribution of the genetic modification status of the MSC to the overall observed effect on tumor target
No. of studies * ≥ 3 , ** ≥ 6 , *** ≥ 10

Table 6 Summary of MSC suitability for experimental cancer cytotherapy applications

Cytotherapy evaluation areas [links to supportive evidence in the review]		MSC suitability for cytotherapy			
Adequacy	1	MSC donor tissue abundance/availability Ease of isolation/derivation Harvest yield [4.1.3, 4.1.4]	Most favorable attributes Best option ✓ (+ 1)	Some favorable attributes Alternative use (✓) (+ 0.5)	Detrimental effects questionable/ contraindicated ? (- 0.5)
Safety	2	Ease of sub-culture manipulation Expansion/upscaling potential [4.1.4]	UC	AT GM-BM	
	3	Genomic stability [4.2]	UC	AT	BM GM-MSC
Efficacy	4	Host integration/immunogenicity [4.2, 4.3.1, Table 4]	UC	GM-AT	
	5	Robust target-specific anti-cancer action [Table 5, Additional file 6: Figure S6]	UC (vs. breast) GM-BM (vs. lung) GM-BM (vs. neural)	GM-UC (vs. breast) GM-UC (vs. lung) GM-AT (vs. neural)	BM (vs. breast)
MSC total scores	6	% frequency anti- vs. pro-tumorigenic events [Table 1, Figs. 3, 4, 5b, and 6]	UC (breast, lung) GM-MSC	AT (neural)	BM (breast, colorectal)
			UC: 6 * (+1) = + 6 GM-BM: 2 * (+1) = + 2 AT: 1 * (+1) = + 1 GM-MSC: 1 * (+1) = + 1	AT: 3 * (+0.5) = + 1.5 BM: 1 * (+0.5) = + 0.5 GM-AT: 2 * (+0.5) = + 1 GM-UC: 2 * (+0.5) = + 1	BM: 3 * (-0.5) = - 1.5 GM-MSC: 1 * (-0.5) = - 0.5
Ranking:	Cord (+ 7) > fat (+ 3.5) > marrow (+ 1)				
Tissue-specific	Fetal (+ 7) > adult (+ 4.5)				
Fetal vs. adult	Naïve (+ 7.5) > GM (+ 4.5)				
Naïve vs. GM					

MSC, both naïve (UC/AT/BM), as well as genetically modified (GM-UC, GM-BM, GM-AT) were evaluated using an arbitrary scoring system based on the possession of favorable (positive scoring) or detrimental (negative scoring) characteristics with respect to six main cytotherapy evaluation areas pertaining to adequacy, safety and efficacy. The evaluation points/areas on which MSC are judged are listed on the left hand-side columns; the links in brackets provide the relative evidence presented throughout the review in support of the allocation of scores which are given to MSC according to the conformity of their traits to the specific cytotherapy requirements. This grading of the suitability of MSC across various aspects of cytotherapy results in the build of a matrix (shaded area of the table) in which each of the MSC entries in the first, second, and third columns are valued as + 1, + 0.5, and - 0.5, respectively. A score of + 1 is assigned to the MSC that possess the most favorable attributes (e.g., highest isolation yield, greatest expansion potential) and which are classified as an ideal cell choice. The MSC that gather fewer or to a lesser extent favorable characteristics, are regarded as a second, alternative MSC option and score + 0.5, while those associated with detrimental effects (e.g., mutation events, tumor promotion) get a negative score (- 0.5) and are considered of questionable value or contraindicated, for certain cytotherapy applications at least. Final ranking of the MSC types is done by calculating the total sum of points for each of them within the matrix and then listing them in descending order, i.e., from highest suitability score to lowest (bottom row of table, scores in brackets). Note that ranking is only indicative of the relative suitability of MSC for cancer treatment based on experimental work hitherto and does not take into account the relative misrepresentation of some MSC types (e.g., under-representation of UC-GM-MSC against colorectal, prostate, and neural tumors)

framework as follows: (i) Different types of exogenous MSC exert variable, often opposing, effects on the same types of tumor targets in vitro and in vivo. (ii) The tumor-promoting/-suppressive effect of MSC may involve direct contact with the components of the tumor microenvironment or may rely on paracrine/juxtacrine effects mediated by the secreted form of induced molecules. (iii) The type and extent of immune response regulation might be a function of the maturity and composition of the microenvironment as well as the differential sensitivity of various MSC types to these maturation levels. (iv) In any case, in an effort to fulfil their primary role of maintaining tissue homeostasis, MSC interfere with innate and immune response both in vitro and in vivo ultimately adopting a pro- or anti-inflammatory phenotype [290]. (v) The inflammatory response shares various molecular targets and signaling pathways with the carcinogenic process, such as increased cell proliferation rate, apoptosis, and angiogenesis. MSC are able to influence such cancer hallmarks by directly adopting a polarized inflammatory phenotype themselves or by directly polarizing infiltrating immune cells, most notably macrophages, into M1 (proinflammatory) or M2 (anti-inflammatory/immunosuppressive) types. (vi) Exogenous adult MSC exhibit a propensity towards adoption of the proinflammatory phenotype under the dictation of mutationally corrupted cancer cells emitting signals (secreted chemokines) deceptively associated with epithelial injury and infection. Once recruited into the tumor microenvironment BM-MS and AT-MS are induced to differentiate towards stromal TAF/CAF (activated myofibroblasts or adipocytes, respectively) that further propagate tumor development by providing mitogenic epithelial growth factors directly to cancer cells, by facilitating EMT or further recruitment of immune cells ([291], references within). Adult MSC may be more susceptible to transformation into TAF due to their genomic instability (accumulated oxidative damage) and elevated senescence status (shorter telomeres). (vii) In vivo, fetal UC-MS exhibit a more robust anti-cancer behavior by sustaining a healthy microenvironment by actively skewing immune responses to an anti-inflammatory profile (involving TLR3—rather than TLR4—ligation, emergence of T-reg and activation of immune complexes), resembling events occurring during tissue regeneration and resolution of inflammation. (viii) In vitro data showing the strong anti-proliferative effect of culture media containing secreted UC-MS metabolic products (secretome) against cancer cells suggest that UC-MS constitutively produce trophic factors that possess intrinsic antitumor properties. The secretome alone can sufficiently mount a strong dose- and time-dependent anti-proliferative response against certain tumor cells mediated by apoptosis signaling (Christodoulou and Goulielmaki, manuscript in preparation). (ix) The unique antitumorigenic

properties of UC-MS most likely are attributed to a key combination of secreted molecules normally associated with the structural and functional traits of the host organ tissue and its specialized role in the feto-maternal interface (e.g., LIF/IL-6: stemness; HLA-G: immune tolerance; glyocalyx matrix hyaluronan; structural integrity).

Overall, the marked anti-cancer efficacy of UC-MS, in addition to their “off-the-shelf” availability, which comprises the relatively greater abundance (ease of isolation, enhanced expansion potential) and safety (reduced risk of malignant transformation and hypo-immunogenic profile which allows them to be used in an allogeneic setup), compared to their adult counterparts highlights UC-MS as instrumental tools in cancer cytotherapy (Table 6). Clearly, the outcome of clinical trials will be crucial before these promising cells find their way into the clinic. Nevertheless, after almost two decades of experimentation, it seems that the cancer cytotherapy field is slowly coming off age.

Additional files

Additional file 1: Figure S1. Comparison of research activity (peer-reviewed journal articles) on selected cancer treatment options over the last two decades. The four graphs (solid lines) depict the evolution of publication rates of journal articles reporting research work in PubMed on four types of therapies relative to cancer treatment (radiotherapy, combination chemotherapy, immunotherapy, and MSC-based cytotherapy) over a period of 20 years. Graphs are based on reported rates over seven 3-year periods, with a forward projection of one period (dashed trend line). In all four cases, the rates increase exponentially ($y = a e^{b x}$); nevertheless, the growth for cancer cytotherapy publications (purple) occurs at a relatively higher rate as shown by the steeper trend line and higher value of b constant in the respective equation. This publication rate for MSC-based cancer cytotherapy studies has grown over sixfold faster, compared to that for combined chemotherapy, for example, rising from 1:100 to 1:15 over the last decade. This trend is suggestive of increased interest in this field that will most likely be sustained in the near future. PubMed search filters: English only, research articles only. (TIF 30030 kb)

Additional file 2: Figure S2. Overview of meta-analysis methodology (TIF 12282 kb)

Additional file 3: Figure S3. Example of a database form used to record experimental data used in the meta-analysis. Field titles correspond to the parameters comprising each of the in vitro and in vivo experiments as described in the methodology and results sections of the relevant articles. (TIF 9196 kb)

Additional file 4: Figure S4. Distribution of the three most frequently associated tumors in relation to MSC effectors. Sample sizes: adipose-derived MSC (AT-MS) = 32, bone marrow-derived MSC (BM-MS) = 56, umbilical cord-derived MSC (UC-MS) = 34. (TIF 4256 kb)

Additional file 5: Figure S5. Comparison of distribution of anti-cancer effects for naïve MSC vs. naïve MSC used as control cells for genetically modified MSC-based cancer cytotherapy studies (Naïve + GM). Each of the 100% stacked columns shows the relative distribution of anti-cancer effect observed (anti- vs. pro-tumorigenic vs. neutral) (TIF 103676 kb)

Additional file 6: Figure S6. List and frequency distribution of studies employing the use of genetically modified stem cells (GM-MS) of human adipose tissue (AT), bone marrow (BM), and fetal umbilical cord (UC) matrix origin. In each row of the table, the length of black-gradient filled horizontal bars is proportional to the total number of studies (value within bar) relevant to specific GM-MS/tumor combinations; the list of respective citations is shown under the bars. Cancer types are ranked in descending order of world incidence (see also Fig. 2). Only tumors whose

use is described by three or more independent studies are shown. Arrows at the beginning of each row of the table symbolize deviation of the frequency of tumor targeted in experimental cytotherapy work from their respective incidence/frequency of occurrence globally (yellow = difference within 5%; green, up = difference > 5% in favor of cytotherapy—tumor over-representation; red, down = difference of > 5% in favor of incidence—tumor under-representation). */**/# Studies referring to cervical cancer/ ovarian cancer/ use of UC-blood MSC, respectively. (TIF 9450 kb)

Abbreviations

5-FC: 5-Fluorocytosine; AT-MSC: Adipose tissue-derived mesenchymal stem cells; BM-MSC: Bone marrow stroma-derived mesenchymal stem cells; CD:UPRT: Cytosine deaminase to uracil phosphoribosyltransferase fusion protein; CFU-F: Colony-forming unit—fibroblast; CM: Conditioned media; ECM: Extracellular matrix; EMT: Epithelial-to-mesenchymal transition; GDEPT: Gene-directed enzyme/prodrug treatment; GM-MSC: Genetically modified mesenchymal stem cells; GSV: Ganciclovir; HLA: Human leukocyte antigen; HSV-tk: Herpes simplex virus thymidine kinase; i.v.: Intravenous; IFN: Interferon; MHC: Major histocompatibility complex; MLR: Mixed lymphocyte reaction; MNC: Mononuclear cells; MSC: Mesenchymal stem cells; n-MSC: Naïve (unmodified) mesenchymal stem cells; PD: Population doublings; PDT: Population doubling time; s.c.: Subcutaneous; SCID: Severe combined immunodeficiency; TAF: Tumor-associated fibroblasts; TLR: Toll-like receptor; TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand; UC-MSC: Umbilical cord matrix-derived mesenchymal stem cells; WJ: Wharton's jelly

Acknowledgements

We would like to thank Prof. Fragiskos N. Kolisis, former Director of the Institute of Biology, Medicinal Chemistry & Biotechnology of the National Hellenic Research Foundation (NHRF) for his initiative and major contribution in establishing the Program on Applied & Translational Stem Cell Research within the Institute that has enabled this work among others to be carried out.

Funding

The research work of MG was supported by the Hellenic Foundation for Research and Innovation (HFRI) and the General Secretariat for Research and Technology (GSRT), under the HFRI PhD Fellowship grant (GA. no. 2400). MD who was involved in this work received funding from IKY State Fellowships for Excellence on Postgraduate Studies in Greece.

Availability of data and materials

Datasets analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

IC conceived, drafted, and wrote the manuscript. MG and MD contributed to the literature search and bibliographic database compilation. MP revised the manuscript for important intellectual content and use of language. GK and VZ critically reviewed and edited the manuscript. All authors have read and approved the final submitted version of the manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

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Published online: 07 December 2018

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