CHAPTER 1

Therapeutic Potential of Mesenchymal Stromal Cell-derived Small Extracellular Vesicles

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1.1 Concepts in Regenerative Medicine

Degenerative diseases are classically associated with irreversible tissue loss. In this context, it is widely assumed that the potential of endogenous stem and progenitor cells, which normally control tissue homeostasis, is insufficient to promote tissue regeneration. Consequently, stem and/or progenitor cells with developmental potentials comparable to endogenous stem cells have been applied to different preclinical models as well as to a variety of different patient cohorts suffering from degenerative diseases.

In the early 2000s, as interest in stem cell biology grew exponentially, a number of observations implicated plasticity in somatic stem cell compartments, *i.e.* the capability of somatic stem cells to generate differentiated cells far beyond their normal developmental routes. Observations implied, for example, that immature brain cells, under the right extrinsic conditions, could create blood cells and *vice versa*.[1,2] Mesenchymal stem/stromal cells

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(MSCs) especially became an attractive research object. MSC-like fibroblasts were originally described by Friedenstein and colleagues in the late 1960s.[3,4] These fibroblastoid cells, initially grown from adult bone marrow, can be easily expanded as plastic adherent cells. Within differentiation assays they can develop towards the osteogenic, adipogenic and chondrogenic lineage, all being mesodermal derivatives,[5] thus fulfilling the potential of mesenchymal stem cells, whose existence had been predicted before.[6] Quickly it became evident that MSCs can be raised from various tissues types including fat, umbilical cord, umbilical cord blood and placental tissue. In addition, some MSC types, including the mesodermal adult progenitor cells (MAPC) and the unrestricted somatic stem cells (USSCs) appeared as somatic stem cells with germ layer spanning developmental potentials comparable to the pluripotent embryonic stem (ES) cells, which also became popular at that time.[7] In contrast to the ES cells, however, MSC-like cells showed no teratogenic potentials and quickly were considered as therapeutic agents for degenerative diseases.[8,9] It was proposed that the administration of MSCs, systematically applied to the body and guided by environmental factors, home to affected tissues and differentiate to replace lost cell types. Since MSCs also generated cells mimicking features of neurons or myocardial cells, respectively,[10,11] they were quickly discussed as allogeneic off-the-shelf products for acute diseases like ischaemic stroke and myocardial infarction. Consequently, groups have started to study their interaction with allogenic immune cells. While it was initially assumed that MSCs would be rejected in principle, they were shown to modulate the activity of different types of immune cells. Specifically, without the need for direct intercellular contacts, they were found to reversibly inhibit proliferation of stimulated CD4 and CD8 T cells[12–15] and to promote the generation, recruitment and regulation of regulatory T cells.[16,17] MSCs also effectively inhibit the proliferation of B cells and the differentiation of monocyte-derived dendritic cells.[18,19] Furthermore, MSCs inhibit the proliferation of IL-2 induced NK cells and prevent NK cell activation, downregulating NKp30 and NKG2D receptors.[20,21] Thus, MSCs suppress various types of immune effector responses and promote regulatory immune cell functions. Coupled to these functions, they secrete a number of different soluble factors including cytokines known to modulate immune responses, such as transforming growth factor β1 (TGF-β1), hepatocyte growth factor (HGF), tryptophan degrading enzyme indoleamine 2, 3-dioxygenase (IDO), prostaglandin E2 (PGE_2) and human leukocyte antigen (HLA) class I molecule G (HLA-G).[12,14,22–26] Consequently, in addition to their assumed cell replacement potential for the treatment of degenerative diseases, MSCs became increasingly discussed as therapeutic agents for inflammatory diseases, especially for acute Graft-*versus*-Host Disease (aGvHD).

Acute GvHD is triggered by allogeneic immune cells being transplanted as part of allogeneic haematopoietic stem cell (alloSC) transplants to myeloablative patients. The co-transplantation of immune cells including T cells is required to eliminate residual tumour (leukaemic) cells that have not been eradicated by the myeloablative treatment regimen before alloSC transplantation. Thus, alloSC transplantation provides a combined regenerative and

immunotherapeutic approach. Despite the fact that the therapy can be curative, up to 50% of patients receiving alloSC transplants develop mild to severe forms of aGvHD; severe forms are associated with high mortality rates.[27] First-line aGvHD patients are treated with steroids which are lympholytic and can successfully suppress disease inducing T cell functions in approximately half of the aGvHD patients. For the remaining, the steroid-refractory aGvHD patients until now, no second-line treatment has been approved. MSCs have been and are still considered as potential second-line therapeutic agents for steroid refractory aGvHD patients. Indeed, in 2004, Katarina Le Blanc and her team successfully treated a 9 year-old steroid-refractory aGvHD patient with allogenic MSCs.[28]

Since then, more than 1200 clinical MSC trials have been registered at the American National Institute of Health (NIH), most of them either intending to apply MSCs as regenerative or immunotherapeutic agents to a diversity of different patient cohorts (clinicaltrials.gov). From 2006 to 2009, the first phase 3 clinical trial (NCT00366145; [Remestemcel-L]) was conducted to assess the efficacy of human MSCs as second-line therapy for steroid-refractory aGvHD. Despite the reported safety of Remestemcel-L, the overall response rate beyond day 28 was comparable within the Remestemcel-L group and the placebo group.[29] However, clinical studies on paediatric aGvHD patients or on patients with high aGvHD risks revealed better outcomes in MSC-treated than in placebo-treated groups.[30] As a consequence, a phase 3 clinical study had been performed (NCT02336230), single-armed and prospective, to treat steroid-refractory paediatric patients with Remestemcel-L. The MSC product was dosed 2×10^6 cells kg^{-1} bodyweight and applied over a 4 week period twice per week. Within the MSC treated group, 74% of the patients showed improvements beyond day 28. In contrast, in the control group the aGvHD symptoms of only 45% of the patients were improved beyond day 28. The overall response improvement was sustained in a huge proportion of the patients with confirmed improvement beyond day 180.[31,32] Still, with the exception of Japan, which has licenced a commercial MSC product (TEMCELL) for aGVHD treatment, and a conditional clinical approval for paediatric aGvHD treatment with Prochymal, another commercial MSC product, in Canada and New Zealand,[33,34] we are not aware of any other licensed MSC product at the moment.

1.2 MSCs Act in a Paracrine Rather than Cellular Manner

Starting with the observation that MSCs can mediate inhibition of T cell proliferation in the absence of physical intercellular contacts[12] and coupled with the fact that they secrete various cytokines, it became evident, over the years, that MSCs exert proportions of their therapeutic functions *via* their secretome, both in pro-regenerative and in immunotherapeutic approaches.[35]

Initially, studies that investigated whether cells need to engraft into affected tissues to achieve their therapeutic effects showed that, in most cases, systemically administered MSCs end up in the lungs of treated animals and

were rarely recovered in high numbers in their assumed target tissues.[36–38] The group of Darwin Prockop, for example, demonstrated that intravenously applied human MSCs improved the heart function following myocardial infarction in mice. Specifically, applied MSCs decreased inflammatory responses, reduced infarct sizes and improved cardiac functions. Upon exploring the bio-distribution of the administered human MSCs by screening the animals, human DNA was almost exclusively detected in the animals' lungs and only marginally in other tissues. Furthermore, MSCs trapped in the lung were shown to secrete TNF-α-induced protein 6 (TNAIP6 or TSG-6), an anti-inflammatory cytokine. Subsequent experiments in which recombinant TSG-6 was administered to infarcted mice confirmed that TSG-6 administration decreases myocardial infarct-induced inflammatory responses and reduced infarction sizes. Supporting the important role of TSG-6, human MSCs following siRNA mediated knockdown of the TSG-6 expression failed to improve myocardial infarction sizes.[36] Comparably, MSC administration was found to improve induced acute kidney injuries without engrafting in high amounts into affected organs. The regenerative effect of the applied MSCs was associated with the secretion of insulin-like growth factor-1 (IGF-1). MSCs genetically engineered to silence IGF-1 expression limited the MSCs pro-regenerative effects.[39] Thus, it became obvious that against the initial assumption, MSCs hardly home to affected organs and tissues to replace lost cell types, but rather improve clinical symptoms by their secretome.

As a consequence of these and related observations, the originally proposed stem cell-related characteristics of MSCs have been questioned. Consequently, MSCs are currently preferably defined as "mesenchymal stromal cells" instead of "mesenchymal stem cells". Recently, Arnold Caplan, one of the MSC pioneers, recommended to define them according to their paracrine mechanism of action more accurately as "medical signalling cells".[40] Regardless of what the preferred definition for the abbreviation "MSC" will finally be, it has become a major goal to identify the active components that exert the pro-regenerative/immunomodulatory activities of MSCs.

1.3 MSC-EVs Mediate Their Therapeutic Effects *via* Extracellular Vesicles

Considering that paracrine activities mediate the MSCs' therapeutic effects, a number of studies explored the therapeutic effects of MSC-derived conditioned medium (CM). Indeed, MSC-CMs were shown to mediate cardio-protective effects in different murine myocardial infarct models.[41–43] Gnecchi and colleagues demonstrate that CMs of MSCs engineered to express high Akt levels promote myocardial tissue repair and functional improvement.[42,43] The investigation conducted by Timmers and colleagues, who fractioned the MSC-CMs by filtration and revealed that only the unfractionated MSC-CM and a fraction not passing 1000 kDa filters contained cardio-protective activities, specifically activities that reduced infarct sizes and improved the systolic and diastolic cardiac performance.[41] Subsequently, the authors

demonstrated by transmission electron microscopy the presence of vesicular particles with sizes of 55–65 nm in such fractions. Simultaneously, the presence of exosomal marker proteins was confirmed, *i.e.* CD9, CD81 and Alix. Accordingly, the authors termed the vesicular particles exosomes.[44]

Similar to the effects in myocardial infarction models, Bruno and colleagues observed that upon fractioning human bone marrow-derived MSC-CMs by centrifugation, the activity was recovered in ultracentrifugation pellets. The transmission electron microscopic characterization of this fraction revealed the presence of vesicular particles with sizes ranging from 80 nm to 1 μm (mean of 135 nm), which the authors deciphered as microvesicles.[45] Comparable to the MSCs themselves, the vesicle-containing fraction was able to reduce apoptosis rates and increase the proliferation of tubular epithelial cells *in vitro*. *In vivo*, the administration of MSCs and the microvesicle-enriched fraction prepared from MSC-CMs showed comparable efficacies on functional and morphological recovery of glycerol-induced acute kidney injury (AKI) in severe combined immunodeficiency (SCID) mice. Supplementary to each other, these pioneering studies of the groups of Sai Kiang Lim and Dominique de Kleijn and that of Giovanni Camussi implied for the first time, that small extracellular vesicles (sEVs) exert the MSCs' therapeutic effects (Figure 1.1).[44,45]

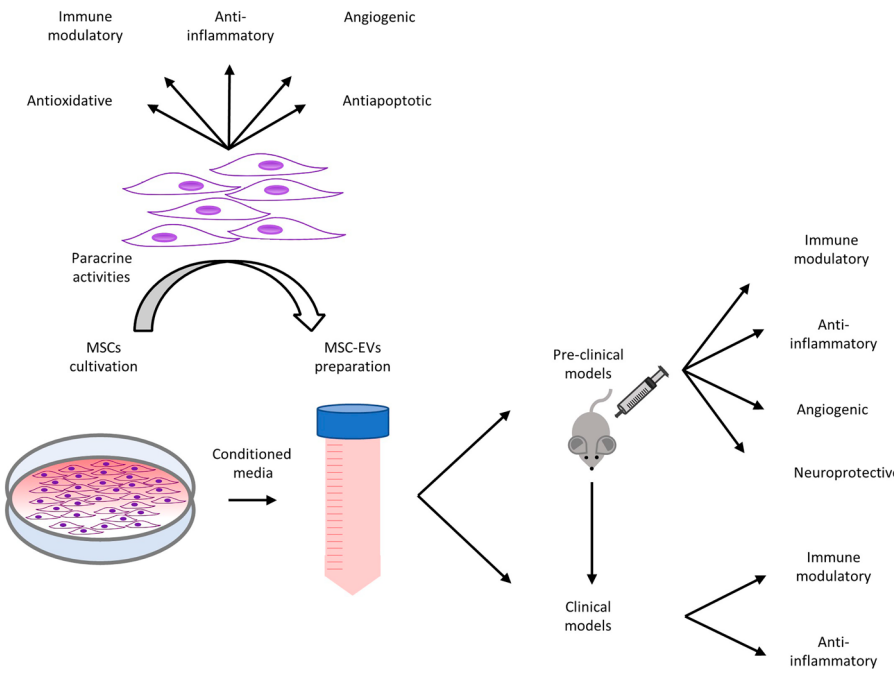

Figure 1.1 MSCs act *via* their EVs that can be harvested from MSC-conditioned media. Like MSCs, prepared MSC-EVs suppress disease associated processes and promote regeneration in a multimodal manner.

1.4 MSC-sEVs Improve Disease Symptoms in Many Different Disease Models

The finding that MSC-sEVs prepared from MSC-CMs are as therapeutically potent as their parental MSCs provided new perspectives for novel cell-free therapies. Compared to cellular therapies sEV-based therapies provide several hypothetical advantages, *e.g.* the lack of self-replication potentials of EVs, which in cellular therapies could result in tumour formation.[46] Importantly, due to their small sizes, which in sEV preparations are below 200 nm, sEV products can be sterilized by filtration. Furthermore, the overall handling and storage is less complicated than for cells.[47] Eventually, sEV products can also be produced from immortalized cell-line cells which cannot be used for the clinical applications. Consequently, sEV production might become scalable to industrial levels, which is not possible for cellular therapeutics. Still, there are some issues which need to be addressed; amongst others, sEV therapies need to be as efficient as cellular therapies.

To this end, using a mouse model for ischaemic stroke, we confirmed that also in this model systemically administered MSC-sEV preparations improve post-stroke symptoms to a comparable extent than their parental cells. Both induced neurological recovery by promoting neurogenesis and angiogenesis. Furthermore, the impact of the administration of MSC-sEV preparations was analysed on the composition of the peripheral blood. Notably, as in humans, ischaemic stroke induced leukopenia in ischaemic stroke mice with the residual T cells showing an activated phenotype.[48–50] Pointing towards contributing immunomodulatory effects, in ischaemic stroke mice treated with MSC-sEV preparations, the extent of leukopenia was reduced and the activation phenotype of the T cells suppressed.[48] In an ongoing study it was shown that administration of MSC-sEV preparations also reduced the invasion of neutrophils and other leukocytes into the ischaemic lesion sites. Notably, neutrophil depletion resulted in a comparable improvement as the administration of MSC-sEV preparations and also suppressed the invasion of other leukocytes into the infarcted area.[51] The fact that no additional benefit was observed when MSC-sEV preparations were administered in neutrophil depleted ischaemic stroke mice implies that MSC-sEVs exert at least a huge proportion of their therapeutic effects by the modulation of neutrophils in post-stroke animals.[51] For now, it remains an open question whether these effects are exerted directly or indirectly.

Site-by-site comparisons of MSCs and their EVs were also performed in a rat model for contusion spinal cord injury. Both were shown to be equally effective in reducing the level of neuro-inflammation for up to 2 weeks post-injury. Moreover, administration of MSC-sEV preparations immediately after spinal cord injury was shown to decrease the expression of pro-inflammatory cytokines in the spinal cord parenchyma in the very early phase of secondary damage.[52]

In addition to these site-by-site comparisons, the therapeutic potential of MSC-sEVs has been approved in a quickly increasing number of different preclinical models. For example, in the nervous system, positive effects of administered MSC-sEV preparations have been also observed in a rat ischaemic stroke model, in a mouse model for sciatic nerve regeneration and in spinal cord injury rats and a rhesus monkey model for cortical injury.[53–61] We and others observed therapeutic effects of administered MSC-sEV preparations on neonatal brain damage.[62–67] Also, in the neonatal setting, the beneficial effects of MSC-EVs were described on neonatal lung damage.[68–71] The therapeutic effects of MSC-sEV preparations on acute and chronic kidney injury models are studied by many groups; positive effects have already been reported in the early phase of MSC-EV research.[72–77] Other organ injuries can be improved by MSC-sEV administration including liver, adult lung and muscle injury models.[36,78–84] In addition, MSC-sEV preparations were found to promote blood flow in a rat model of critical limb ischaemia, skin burn healing and survival of allogeneic skin grafts.[85–88] Recently, locally applied MSC-sEV preparations were shown to alleviate noise-induced hair cell loss, partially restoring hearing loss.[89] There are dozens of other applications and we apologize to mention only a small selection and cite only a small proportion especially of the studies that were published more recently.

1.5 MSC-sEVs Have Successfully Been Applied to Patients

Coupled with the promising preclinical data, MSC-sEVs are also increasingly considered for therapeutic application in humans. At first, MSC-sEVs were applied in an individual treatment attempt to a patient suffering from treatment refractory aGvHD. During a period of 2 weeks, MSC-sEVs, confirmed to modulate pro-inflammatory responses of the patient's immune cells *in vitro*, were infused intravenously at 2- or 3-day intervals. The MSC-sEV treatment improved the GvHD symptoms considerably, resulting in the patient's stability for more than 4 months.[90]

In a randomized placebo controlled clinical trial performed in Egypt, 40 enrolled patients with chronic kidney disease (CKD) stage III and IV were divided in two groups. Two doses of MSC-sEV preparations were administered to the patients of the first group, while the placebo group received saline intravenously. Kidney functions were assessed by estimated glomerular filtration rates (eGFR), urinary albumin creatinine ratio, blood urea and serum creatinine levels. Inflammatory immune activities were evaluated by determining blood levels of TNF-α, TGF-β1 and IL-10. Compared to the placebo group, the MSC-sEV treated group exhibited improved CKD symptoms as reflected by creatinine clearance. The improvement was accompanied by significant increases of TGF-β1

and IL-10 concentrations as well as a significant decline of the TNF-α level in the plasma of MSC-sEV treated patients.[91]

In both cases, promising therapeutic effects were observed with no reported or observed side effects. Recently, bone marrow derived MSC-sEV preparations were applied to 24 patients suffering from COVID-19 without showing any side effects.[92] Unfortunately, the authors did not report details about their MSC-sEV product and too many questions remained open for the results to be informative for the scientific community.[93–95]

1.6 Immunomodulatory Activities of MSC-EV Products

Apparently, MSC-sEVs can act *via* different mechanisms to improve the symptoms of the respective diseases. Their exact mechanisms of action (MoA) are not yet clear. However, it appears that their ability to modulate immune responses and shift the immune system from the acute inflammatory state to its regulatory state, *i.e.* from defence to tolerance, is one of their key functions.[96,97] Among others, the suppressive effects of MSC-EVs on DCs have been reported *in vitro*. MSC-EV addition to human monocyte-derived DCs reduced expression of the DCs maturation and activation markers CD83, CD38 and CD80, decreased secretion of pro-inflammatory cytokines IL-6 and IL-12p70, and increased production of anti-inflammatory cytokine TGF-β.[98] Also *in vitro*, the addition of MSC-sEVs to classically activated (M1) bone marrow derived macrophages significantly reduced the mRNA levels of pro-inflammatory M1 markers, such as CCL5, IL-6 and TNF-α. Notably, MSC-sEVs can also modulate already M2 polarized macrophages; for example, they significantly suppressed Resistin-like alpha and CD206 induction and enhanced M2-derived Arginase 1 (Arg1) expression levels.[71] The influence of MSC-sEVs on macrophage polarization towards M2 immunomodulatory phenotype has been also shown in animal models of spinal cord injury and renal injury.[99,100] In acute kidney injury models, the MSC-EV treatment showed immunomodulatory effects by increasing anti-inflammatory cytokines and reducing the local invasion of macrophages and lymphocytes.[75,77,100,101] Indeed, immunomodulatory features of MSC-sEVs have now been described in several disease models, implying that immunomodulation is an important part of the proposed EV-mediated MoA contributing to the pro-regenerative effects of MSC-sEVs.[96]

Coupled to the observation that MSC-sEV preparations modulated immune responses in many disease models, including ARDS and sepsis,[96,102–104] we consider MSC-sEV therapies indeed could be an effective treatment option for COVID-19 patients.[105] However, coupled to the tolerance induction which appears beneficial in GvHD and other sterile, non-virus-induced diseases, serious adverse effects in the presence of replicating pathogens need to be considered. Hypothetically, viruses and bacteria could spread in an uncontrollable manner in tolerogenic environments. Although anti-bacterial

activities for MSCs and their secretome[106–109] as well as MSC-EV anti-influenza activities have been reported,[110] we are still cautious and consider this issue as one of several that should be addressed carefully before applying MSC-sEV products to patients with disease promoting, replicating pathogens.[105]

1.7 Other Activities Mediated by MSC-EV Products

In addition to their immunomodulatory properties, MSC-EVs also induce other pro-regenerative processes eventually required to promote successful tissue regeneration. For example, MSC-EVs have been mechanistically shown to increase ATP levels in damaged cells, reduce oxidative stress and severity of cell injury, and restore cellular metabolic activities.[111] Furthermore, MSC-sEV preparations exert pro-angiogenic activities.[48,112] In our aforementioned ischaemic stroke model, the applied MSCs and MSC-sEVs both promoted neurogenesis by increasing the survival of neural progenitor cells resulting in a higher neuronal density 4 weeks after ischaemia. Similarly, angiogenic processes resulting in the production of new endothelial cells were promoted. Thus, MSC-EV administration increased endogenous neurogenesis and angiogenesis.[48,113] In line with our study, functional recovery following administration of an MSC-sEV preparation was correlated in a rat traumatic brain injury model with angiogenesis promoting activities.[114] Pro-angiogenic potentials were also recovered in EV preparations of MSCs engineered to overexpress CXCR4. In a myocardial infarction model, MSC-EV administration resulted in the upregulation of VEGF, IGF-1α and pAkt and downregulation of caspase-3 in cardiomyocytes. Furthermore, the vessel formation was enhanced.[115] Also in kidney injury models, MSC-sEV treatment has been found to promote angiogenesis in addition to the expansion of endogenous renal cells.[45,77,116–118]

In summary, MSC-EVs apparently act in a multimodular manner. So far, mainly their immunomodulatory and pro-angiogenic processes have been investigated. However, they might also exert anti-apoptotic effects, restore energy and fulfil several other functions we might not be aware of yet. Due to the heterogeneity observed in the MSC field, it is less likely that all mechanisms are equally important in all of the different target diseases. It appears more reasonable that depending on the target disease different functional modalities are required. Also, MSCs are not uniform cell types. They share some common features,[119] but also can differ from each other phenotypically and functionally. MSC and MSC-EV heterogeneity will be discussed in the next paragraph in more detail.

1.8 Heterogeneity of MSC-sEV Preparations

An important consideration in translating MSC-sEVs into the clinic is that therapeutic potentials of individual MSC-sEV preparations may vary among independent preparations. MSCs have already been recognized as a

heterogeneous cellular entity. Regardless of their origin, MSCs from specific sources can differ in size and the expression level of *bona fide* MSC marker proteins.[119–122] According to our own experience, we observed tissue-specific as well as individual differences in the stromal MSC functions, namely their support of primitive haematopoietic cells.[123] Although we assume that there are different MSC subtypes that most likely differ in their therapeutic potentials, we are not aware of any generally accepted criteria for distinguishing different MSC subtypes. However, upon producing sEVs from bone marrow derived MSCs of different donors or even of independently expanded MSCs of the same donor, we reproducibly observe molecular variations as well as functional differences in the *in vivo* and *in vitro* immunomodulatory capabilities of the obtained MSC-sEV products. Specifically, upon comparing the cytokine content of independent MSC-sEV preparations we monitored differences in the concentrations of pro- and anti-inflammatory cytokines.[90] At the functional level, we have recently shown that independent MSC-sEV preparations, which share many properties regarding their sEV content, still differently and reproducibly modulate T cell activities *in vitro* and *in vivo*.[124,125] Recently, we investigated their functional properties to modulate immune responses in a multi-donor mixed lymphocyte reaction (mdMLR) assay as well as in their capability to suppress aGvHD symptoms in an optimized aGvHD mouse model. Remarkably, MSC-sEV preparations revealing *in vitro* immunomodulatory capabilities also were able to modulate aGvHD symptoms *in vivo*; however, a proportion of samples failed to show efficacy in both assays.[124] Comparably, without correlating the *in vivo* results to the results of the mdMLR assay, we also observed that independent MSC-sEV preparations provide different capabilities to modulate symptoms in an ischaemic stroke model.[51] Thus, in our hands, a relevant proportion of independent MSC-sEV preparations failed to mediate therapeutic effects in various animal models. Until reliable surrogate markers have been discovered and qualified, we see it as mandatory that therapeutic MSC-EV preparations are functionally tested, preferably in an assay that has been qualified as a potency assay. Of note, potency assays have to fulfil several requirements accepted by regulatory authorities and are not just functional assays providing information about potencies of therapeutic agents. Recently, we have discussed this topic in detail and for more information we would like to refer the reader to ref. 131.

Apart from the variability in the potency of our MSC-sEV products, potencies of other MSC-sEV products may depend on the origin of the parental MSCs. We do not know yet whether MSCs of any other source than bone marrow may provide accelerated therapeutic activities or even whether other cell types than MSCs release more potent EVs. Certainly, there are cell inherent features which define the sEV products' therapeutic potency, but also the production process starting with the cell culture conditions *via* CM harvesting procedures and intervals over the downstream processing methods will certainly affect the potency of the resulting sEV products. Since this discussion is beyond the scope of this chapter we would like to refer to white

papers addressing these issues.[47,94,126,127] Apart of the recommendations met in these white papers, we would like to recommend to anyone intending to approach the therapeutic EV field experimentally to also study and consider more general recommendations of the International Society of Extracellular Vesicles (ISEV).[128–130] Although the guidelines are very well written, they are condensed and might overwhelm EV novelists. Therefore, we strongly recommend that anyone intending to approach the EV field experimentally gets in contact with experts in the field. To promote EV research and support EV novelists, many national EV societies have been established in addition to ISEV. For example, the German Society for Extracellular Vesicles (GSEV) was founded in 2017.

1.9 Take-home Message

EVs are currently a hot topic and offer many promising aspects for the life science field. sEVs of various cell types, particularly of MSCs, appear to mediate pro-regenerative effects by modulating the immune system in various diseases. Preclinical data, supplemented by a few applications in humans, predict a huge therapeutic potential. However, the EV field is still very young, and we have to deal with several challenges to translate EVs accurately into routine clinics. Many of these challenges, including heterogeneity issues, have been recently discussed in a position paper of ISCT and ISEV in the context of MSCs-EV therapies for COVID-19 patients, but are also relevant for all other clinical sMSC-EV applications.[105]

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