Extracellular vesicles for tissue repair and regeneration: evidence, challenges and opportunities

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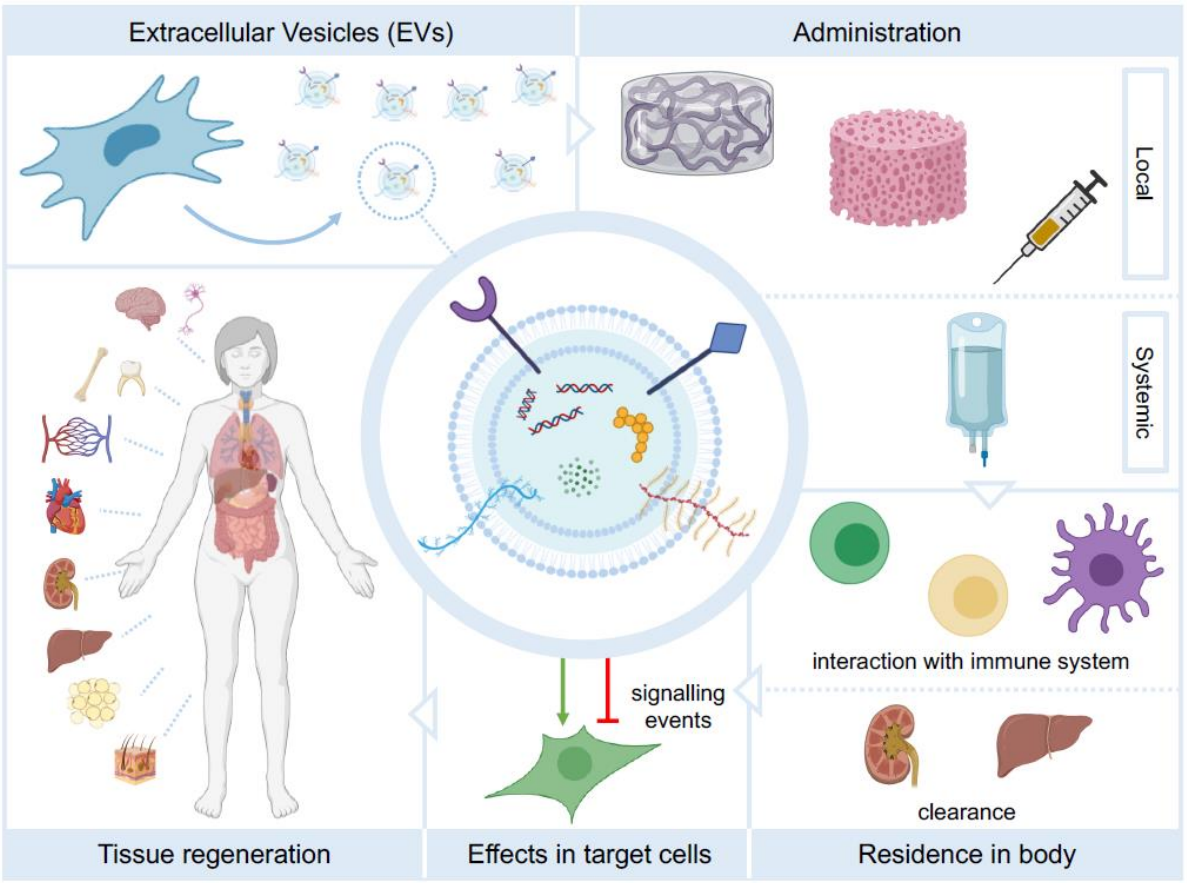
Graphical abstract



Abstract

Extracellular vesicles (EVs) are biological nanoparticles naturally secreted by cells, acting as delivery vehicles for molecular messages. During the last decade, EVs have been assigned multiple functions that have established their potential as therapeutic mediators for a variety of diseases and conditions. In this review paper, we report on the potential of EVs in tissue repair and regeneration. The regenerative properties that have been associated with EVs are explored, detailing the molecular cargo they carry that is capable of mediating such effects, the signaling cascades triggered in target cells and the functional outcome achieved. EV interactions and biodistribution *in vivo* that influence their regenerative effects are also described, particularly upon administration in combination with biomaterials. Finally, we review the progress that has been made for the successful implementation of EV regenerative therapies in a clinical setting.

Keywords

Regenerative medicine; Mesenchymal stem cells; Therapeutic cargo delivery; EV engineering; EV-functionalized biomaterials; Clinical trial.

Contents

1. Introduction

Regenerative medicine aims to restore the function of damaged organs or tissues. To accomplish this goal, different strategies are being explored. The majority of these approaches center around the use of various types of primary cells, such as stem cells, and their secretomes. These can be applied as a liquid infusion or in combination with biomaterial matrices. Non-hematopoietic mesenchymal stromal cells (MSCs) form a key class of cells in this respect. Since their discovery in the 1970s, MSCs have shown great promise for a broad range of regenerative medicine applications in various types of tissue defects and diseases [1]. This is mainly due to their reported immunosuppressive function and inherently low immunogenicity, their ability to home to sites of injury, and their ability to differentiate into numerous other cell types [2]. In addition to the well-established differentiation potential of MSCs (into osteoblasts and, chondro- and adipocytes), differentiation towards other cell types, such as cardiomyocytes and neuronal cells, has also been reported [3-5].

Despite stem cell transplantation being considered as one of the most promising approaches in regenerative medicine, the clinical application is facing a number of challenges. Firstly, the safety profile of stem cell treatments remains the biggest hurdle due to risks such as tumorigenicity, proinflammation, and rejection by the host [6]. Additional challenges for systemic administration include maintaining cell viability, increasing stem cell permanence, *in vivo* stem cell differentiation, specific delivery to the desired target site, and integration into the target tissue [6]. Also, the production of sufficient quantities of cells, quality control and heterogeneity of the stem cell population are examples of potential issues that need to be overcome [7]. Altogether, this has hampered the clinical success of stem cells in regenerative medicine applications [8]. Furthermore, human MSCs (hMSCs) were initially believed to integrate into damaged tissue, where they would proliferate and differentiate, thereby prompting regeneration [9, 10]. However, their structural contribution to regenerate tissues turns out to be more limited than originally thought, as only a small fraction of the administered cells is able to successfully engraft in the host and differentiate into the desired cell type [11]. Instead, as shown in the seminal work by Gnecchi *et al*., MSCs mediate their effects primarily in a paracrine manner [12]. They were shown to secrete cytoprotective factors which supported cardiomyocytes in an acute myocardial infarction model [12]. Identifying the factors responsible has been a major topic in the field, also as these could serve as cell-free strategies for regenerative applications [13]. Indeed, multiple candidates have been proposed, ranging from soluble growth factors, to cytokines, but also cargo delivered via EVs [14]. Early work by Timmers *et al.* showed that conditioned medium from MSCs could reduce myocardial infarction. The work suggested that the responsible component was a large complex as it could not pass through a 1000 kDa membrane [15], although we recently found in a similar experiment that ultrafiltration also depleted smaller, soluble factors in an off-target manner. It was subsequently shown that this component contained marker proteins for EVs [16]. Ever since, numerous publications have explored the regenerative potential of EVs in various settings.

EVs were originally described as an elimination apparatus for cells to dispose of undesirable material [17]. Extensive research into understanding their origin and biological function has highlighted the relevance of EVs in biological processes with opportunities for diagnostics and treatment of various human diseases. EVs are heterogeneous cell-secreted particles, enclosed by a phospholipid-bilayer membrane. The most studied subtypes are commonly

divided into two major sub-groups, based on their size and biogenesis. Microvesicles are generated by budding off from the plasma membrane and represent a subgroup of larger vesicles, typically varying in size from 0.1 to 1-2 µm in diameter. Exosomes on the other hand are formed by reverse budding of endosomal multivesicular bodies and are secreted from cells upon fusion of these bodies with the plasma membrane. Exosomes are smaller in size than microvesicles, with a reported diameter ranging from 30 to 150 nm [18]. Due to the overlap of microvesicles and exosomes in size (100–150 nm), density (1.08–1.19 g/ml) and protein markers, it is highly challenging to obtain pure vesicle populations. Differential centrifugation protocols likely result in an enrichment of the subtypes, not a pure population. In this review, in accordance to the MISEV guidelines, EVs will be used as the generic term for particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate [19, 20].

EVs contain a wide range of macromolecules, including proteins, lipids, and nucleic acids. It is thought that the cargo resembles the parent cells from which the vesicles originate to a certain extent and that the exact composition is what conveys the biological function of EVs. For example, significant research efforts in the field of EVs have uncovered a potential regenerative function, implicating EVs in processes such as angiogenesis, lineage specific differentiation, regulation of immune responses, and extracellular matrix organization [21].

In this review paper, we aim to evaluate the potential of EVs as regenerative entities and highlight the achievements that the EV-field has made in the realm of regenerative medicine. We will also review the recent progress in tackling the remaining challenges related to successful implementation of EV regenerative therapies in clinical applications.

2. Extracellular vesicles as regenerative entities

EVs have recently gained considerable attention as potential regenerative entities, as demonstrated by the continuously increasing number of studies evaluating their applicability for the repair and regeneration of various organs and tissue types [21]. An extensive overview of these studies, including the EV isolation method and basic characterization, possible EV modifications, EV source and recipient cell types and *in vivo* models, dose and mechanisms of actions, grouped based on the tissue/organ to be repaired, can be found in Supplementary table 1. A more concise summary is depicted in Table 1. A thorough and detailed comparison of these studies is challenging due to the highly heterogeneous experimental parameters. These include various source and recipient cell types, different doses used and diverse EV isolation and purification methods, which may result in reproducibility issues [22]. It seems that independent of the tissue type, studied EVs tend to have a predominantly decreasing effect on cell apoptosis and tissue inflammation while increasing cell proliferation, survival, angiogenesis and lineage-specific differentiation, both *in vitro* and *in vivo*, in a dose-dependent manner. Yet, the source and dose of the EVs, the precise culture conditions of the parent cells, as well as potential modifications to either parent cells or EVs, determine the potency of effects observed. Moreover, the exact molecular mechanisms responsible for these effects are still commonly underexplored and therefore remain somewhat elusive, as we will discuss later in this review paper.

[Insert Table 1 here]

2.1 Types of source cells for production of regenerative extracellular vesicles

MSCs are by far the most popular option as an EV cell source in regenerative applications. This is likely due to the long history of using these cells in regenerative medicine and the increasing evidence of their therapeutic actions via paracrine mechanisms. This has led to the assumption that MSC-secreted EVs are therapeutic entities themselves. When it comes

to MSCs, there is considerable variation stemming from the MSC tissue of origin as well as the characteristics of the MSC donor. This is also reflected in the released EVs and their therapeutic potential [23-28]. Additionally, it has been shown that the differentiation of MSCs towards certain lineages can also direct the regenerative potential of the produced EVs [29-39]. Despite the popularity of MSCs as EV-producers, a plethora of other cell sources, including embryonic, induced pluripotent and tissue-specific stem cells, as well as primary precursor and mature cell types - typically either from the tissue to be regenerated or from immune/endothelial origin - have been widely and successfully utilized as EV sources in regeneration (see Table 1) [40-42]. Therefore, further consideration is desirable when choosing the cell source for EV production. From a clinical perspective, MSC-EVs are often considered as the best option due to the well-recognized and beneficial immunoregulatory properties of these cells and the EVs produced by them. However, there is still limited evidence for the preferential immune-evasiveness of MSC-EVs over the EVs from other cell types, as discussed in detail in section 3.1. Moreover, when it comes to the therapeutic efficacy, extensive comparative data of different EV types is currently lacking, which further encourages the field to avoid focusing exclusively on specific cell types when evaluating the regenerative potential of EVs.

2.2 Regenerative potency of extracellular vesicles versus source cells

EVs have been shown to carry various macromolecules, including nucleic acids, proteins and lipids. Studies have shown that there is some selectivity in the components that are loaded into secreted vesicles. As such, certain components are enriched in EVs compared to their parent cells, whereas others will be present in a reduced quantity [43-48]. Notably, Larssen *et al.* showed that EVs can be traced to their parental cell type using a proximity assay and analysis of EV-associated markers only [49]. This makes for the contents of EVs to be linked to, though distinct from, the composition of the cell of origin.

The great promise of EVs as therapeutics for regenerative applications relies partly on the assumption that EVs outperform their parent cells or are at least equally potent when administered *in vivo*. Chen *et al.* showed that EVs from adipose tissue-derived MSCs are comparable to the parent cells in enhancing fat graft retention [50]. For bone and cartilage regeneration, endothelial progenitor cell- and bone marrow-derived mesenchymal stromal cell (BMSC)-EVs have been observed to stimulate bone regeneration and to protect cartilage and bone from degradation, respectively, in a comparable manner to administration of source cells [51, 52]. Similarly, in a hepatic ischemia-reperfusion mouse model, no difference was observed in the ability of human BMSCs (hBMSCs) and their EVs to stimulate liver regeneration [53]. Moreover, in the case of neuronal regeneration, human placenta-derived MSC-EVs and hBMSC-EVs supported myelin regeneration and functional recovery from cerebral apoplexy, respectively, at similar levels to parent cells [54, 55]. There is also evidence that EVs perform better *in vivo* compared to the parent cells. In a rat myocardial infarction model, the beneficial effects of MSC-EVs on myocardial repair were superior to MSCs [56]. In a similar model in mice, induced pluripotent stem cell (iPSC)-derived cardiomyocyte EVs outperformed cell injections, significantly improving cardiac function [41]. However, in contrast to these results, EV-treated BMSCs, but not BMSC-EVs alone, improved the healing in a mouse calvarial defect model [57]. Although the safety profile of EVs may favor their clinical utilization over cell therapies, particularly with non-autologous cells, the regenerative potency of EVs as stand-alone products still warrants further evaluation.

2.3 The contribution of extracellular vesicle subpopulations to regeneration

Independent of the cell type of origin, EVs are inherently a heterogeneous population of particles consisting of subpopulations with differing size and molecular profile. Therefore, it is likely that only a subset of the administered EVs is responsible for the observed effects.

Although the majority of studies evaluating EVs for regenerative applications use the bulk EV preparation and do not make a distinction between the subgroups, a few studies have specifically focused on MSC microvesicles. As mentioned earlier, the substantial overlap in biochemical and physical properties between microvesicles and exosomes, makes it difficult to obtain pure vesicle populations. Current protocols for vesicle isolation are likely to result in a subtype enrichment rather than a purified population. Nonetheless, several studies have indicated the microvesicle-enriched fraction to induce angiogenesis [32, 58], improve survival of allogeneic kidney transplantation [59], protect from acute kidney injury [60] and promote wound healing [61]. In addition, Lopez-Verrilli *et al.* demonstrated that MSC-exosomes increase neurite outgrowth in cortical neuron cultures, whereas the microvesicle-enriched fraction had an opposite effect, implying a different function of these EV subgroups [26]. MSC-exosomes were also shown to be superior to microvesicles in stimulating renal regeneration in an acute kidney injury model [62]. In contrast to these studies, Peng and co-workers observed that despite similar uptake levels, human embryonic stem cell-derived microvesicles affected the levels of Müller progenitor cell pluripotency and early retinal proteins, whereas the corresponding exosome fraction had no effect [63]. Furthermore, in another study evaluating the effect of MSC-exosomes and -microvesicles on the inflammatory status of osteoarthritic chondrocytes, no major differences between the EV subgroups were detected [64]. All in all, it seems that the regenerative contributions of the different EV subgroups are highly dependent on the subtype used and the application. Likely, this is due to differences in composition of the various vesicle subtypes. In this respect, Kowal *et al*. differentiated several vesicle subpopulations using an immuno-based isolation protocol [65]. Subsequent proteomic comparison revealed similarities in content, but also unique constituents. As such, immunoselection may represent a promising alternative for selection of specific vesicle subtypes with desired bioactivity [66].

Overall, evaluation of results from reported microvesicle and exosome comparison is still greatly hampered by unrestricted use of the EV nomenclature. Despite the guidelines from the International Society of Extracellular Vesicles on the Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018) [19], which recommend using the term “extracellular vesicle”, most EV studies still misleadingly refer to “exosomes”. Yet, it remains extremely challenging to separate this particular EV subpopulation from other vesicles secreted by cells. Apart from the differences stemming from the different biogenesis pathways, other EV subpopulations differing with respect to their molecular signature and mode of action in the regeneration, are likely to exist. Although regeneration is often referred to as if it were a single process, underlying molecular and cellular events are likely to differ between different tissues. Whether the apparently broad efficacy of MSC-EV preparations is due to common, “generic” signaling factors present in all MSC-EV preparations or due to the presence of multiple diverse MSC-EV subpopulations is not yet established. This is an area requiring more research and in-depth evaluation, preferably going towards the single-particle level, to facilitate the translation of the EVs as regenerative entities.

2.4 The regenerative cargo within native, unmodified extracellular vesicles

The regenerative potential of EVs is mainly attributed to the regulation of apoptosis, cell proliferation, differentiation, angiogenesis, and inflammation [21]. The exact mechanisms underlying the therapeutic effects of EVs remain to be fully elucidated. Yet, EVs affect various signaling cascades in the recipient cells either via release of cargo or by activation of specific cell surface receptors on the target cells. Several well-characterized signaling pathways have been related to EVs, including mitogen-activated protein kinase, Wnt/β-catenin, PI3K/Akt, Notch, TGFβ/Smad, STAT and Hedgehog signaling, as included in Table 1 and Supplementary table 1. However, due to the cell-type specificity and overall sensitivity of the signaling events to the experimental parameters, as well as the tendency of the studies to evaluate one or only a few pre-determined pathways or signaling factors, drawing a detailed picture of the mechanistic landscape responsible for the EV-induced regenerative

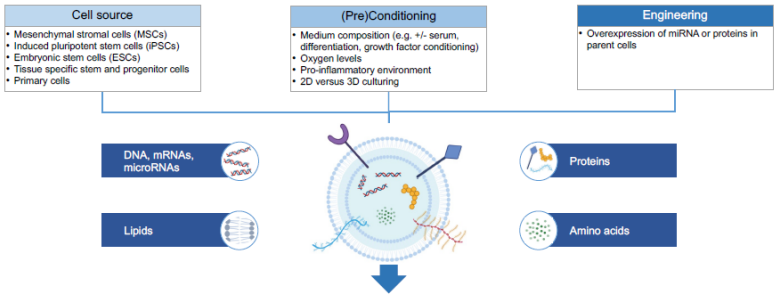
responses is challenging with the existing information. Nevertheless, several factors have emerged as promising contenders of conveying regenerative potential. Below, we discuss the three most important discoveries: the transfer of microRNA (miRNA), messenger RNA (mRNA), and proteins by EVs. It is, however, important to keep in mind that there is likely to be bias towards reporting positive results regarding functional transfer of EV cargo, which may give an overly optimistic picture.

2.4.1 MicroRNAs

An increasing number of EV studies evaluate the miRNA cargo of the vesicles. MiRNAs are a subtype of small (19 - 24 nucleotides), non-coding RNA molecules which target mainly mRNA molecules to regulate gene expression at the post-transcriptional level [67]. Predominantly, miRNAs suppress the expression of target mRNAs by inducing their degradation or translational repression. However, miRNAs have also been shown to stimulate gene expression under specific conditions [68]. Overall, it has been estimated that over 60% of all mRNAs are influenced by miRNAs [69]. Significant quantities of miRNAs have been detected in human body fluids, including blood plasma, serum, urine, saliva, and semen [70], with a fraction of them being contained within the lumen of EVs [71].

Since their discovery, key regenerative processes have been attributed to the mode of action of miRNAs, including cell proliferation, differentiation, migration and apoptosis. Several miRNAs have been proposed as potential contenders for inducing a regenerative and therapeutic effect in different tissues and organs. For example, miR-124 and miR-9/9* have been shown to induce direct conversion of fibroblasts into neuronal-like cells by the down-regulation of BAF53a, an evolutionarily conserved program of post-mitotic neuronal development in mice [72]. Another example of the regenerative capacity of miRNAs is miR-1. This miRNA has been reported to protect myocardium from apoptosis, oxidative stress, and fibrosis and to promote cardiac regeneration [73, 74]. However, one of the key issues in exploiting miRNAs as therapeutic contenders is reducing the nuclease-mediated degradation that can occur prior to achieving target modulation.

It is thought that EVs provide both general and specific protection for circulating miRNAs. EVs have been demonstrated to shield miRNAs by encapsulation, protecting these RNAs against adverse conditions, such as low pH and RNase-mediated degradation [75, 76]. As such, EVs are thought to function as vehicles for intercellular miRNA transfer and therefore as a mode of intercellular communication by which they exert biological effects and regulate target cell activity [77]. The miRNA cargo of EVs is highly heterogeneous and selectively sorted by the cells of origin [78]. Several studies have demonstrated the horizontal transfer of miRNA from isolated and purified EVs to the receiving cells. For example, Shojaati *et al*. concluded EVs from MSCs reduce corneal fibrosis and inflammation via the delivery of miRNAs [79]. In addition, a recent study showed that EVs from adipose-derived stem cells transfer miRNA-31 to promote angiogenesis by down-regulating the anti-angiogenic gene FIH1 (factor-inhibiting HIF-1, hypoxia-inducible factor-1) [32]. To further illustrate the diversity of EV-associated miRNAs that have been implicated in regenerative medicine, Figure 1 shows several examples of EV miRNAs that have been proposed to enhance tissue regeneration in animal models.



	Liver	Heart	Cardiovascular	Kidney	Brain & neuron	Bone and tooth	Skin	Adipose
Target tissue/organ								
Endogenous miRNA cargo	**miR-125b** (Hedgehog ↓); **miR-223** (NLRP3 and caspase-1 ↓); **miR-92a-3p** (αSMA, Collagen-Ia1, Fibronectin and TIMP-1 ↓); **miR-1246** (IL-6-gp130-STAT3 ↓)	**miR-210** (Efna3 ↓); **miR-21** (PTEN ↓ and pAkt ↑); **miR-210** (ephrin A3 and PTP1b ↓) **and -132** (RasGAP-p120 ↓)	**miR-31** (HIF-1↓); **miR-126** (Spred1 ↓ and Erk1/2 MAPK ↑); **miR-199b-5p** (Jagged1 ↓ and VEGFR2 ↑)	**miR-146a** (IL-12 ↓); **miR-100, -146a, -21, -221 and -143**	**miR-133b** (CTGF and RhoA ↓); **miR-17-92 cluster** (PTEN ↓ and PI3K/Akt/mTOR/GSK-3β ↑); **miR-184** (Numbl ↓) **and -210** (EFNA3 ↓); **miR-21 and -19b** (PTEN ↓)	**miR-3960**; **miR-126** (SPRED1 ↓ and Raf/ERK ↑); **miR-26a**; **miR-196a** (ALP, OCN, OPN, and Runx2 ↑); **miR-155** (Spi1, Mitf, and Socs1 ↓); **miR-27a-3p** (APC ↓ and Wnt/β-catenin ↑); **miR-22-3p** (FTO ↓)	**miR-21, -23a, -125b, and -145** (TGF-β2, TGF-βR2, and SMAD2 ↓); **miR-21 and -29c**	**miR-450a-5p** (WISP2 ↓)
Endogenous protein cargo	**Glutathione peroxidase 1**	**HSP-70** (pAkt/mTOR ↑)	**VEGF and MMP-2**	**SP1** (SK1 ↑)	**Neprilysin** (amyloid β ↓); **TGFβs**; **SNAP25**	**RUNX2, OSX, C/EBPα and PPARγ; Galectin**	**DMBT1**; **14-3-3 proteins** (MMP1 ↑)	**STAT3** (Arginase 1 ↑)
Engineered miRNA cargo	**miR-122** (IGFR, CCNG1 and P4HA1 ↓)	**miR-30d-5p** (Beclin-1,Atg5 and LC3 ↓ and P62 ↑); **miR-133** (Snail ↑); **miR-125b** (SIRT7 ↓); **miR-214** (CaMKII ↓)	**miR-675** (TGFβ1 and p21 ↓); **miR-126** (HES1 ↓); **miR-31** (HIF-1↓)	**miR-let7c** (Collagen IVα1, metalloproteinase-9, TGFβ1, and TGFβR1 ↓)	**miR-133b** (RhoA ↓, ERK1/2, STAT3 and CREB ↑); **miR-17-92 cluster** (PTEN ↓ and PI3K/ Akt/mTOR/ GSK-3β ↑); **miR-21** (PTEN/PDCD ↓); **miR-219** (PDGFRα and ELOVL7 ↓)	**miR-140-5p** (RalA ↓ and SOX9 ↑); **miR-375** (IGFBP3 ↓); **miR-92a-3p** (Wnt5a ↓); **miR-122-5p** (SPRY2 ↓); **miR-381-3p** (TAOK1 ↓); **miR-95-5p** (HDAC2/8 ↓); **miR-320** (SOX9 ↑ and MMP13 ↓)	**miR-181c** (TLR4 ↓)	
Engineered protein cargo		**CXCR4** (IGF-1α and pAkt ↑, caspase 3 ↓); **WNT3a** (GSK3β, TCF, LEF and WNT/β-catenin ↑) **and WNT5a** (p-ERK1/2, p-JNK and IL-6 ↑); **GATA-4**; **Lysyl oxidase homolog-2 and biglycan**	**C-kit and SCF**			**Wnt-3a; aptamer; Wnt5a and Wnt5b** (YAP ↑)	**Wnt3a**	
References (respectively)	[80]; [81]; [82]; [83]; [84]; [85]	[86]; [87]; [88]; [89]; [90]; [91]; [92]; [93]; [94]; [95]; [96]; [97]	[32]; [98]; [99]; [98]; [100]; [101]; [32]; [102]	[50]; [103]; [104]; [105]	[106]; [107]; [108]; [109]; [110]; [111]; [112]; [113]; [107]; [114]; [115]	[116]; [51]; [117]; [118]; [119]; [120]; [121]; [122]; [27]; [123]; [124]; [125]; [126]; [127]; [128]; [129]; [130]; [117]; [123]	[131]; [132]; [133]; [134]; [135]; [136]	[137]; [122]

The identification of miRNAs in EVs is based on results either from broad screens or by focusing on specific miRNAs. Some studies have attempted to relate EV-miRNA content to signaling pathways regulated in target cells by these miRNAs and, ultimately, to the EV-induced functional outcome. However, since EVs are complex entities carrying other cargo capable of acting as signal transducers and messengers (e.g. proteins, lipids, other RNA species), evaluation should not be limited to miRNAs.

2.4.2 Messenger RNAs

Horizontal transfer of mRNA by EVs has emerged as a mechanism of paracrine exchange of genetic information by MSCs and other cells [77]. Subsequent translation of the mRNAs in recipient cells is thought to result in new functionalities and increased regenerative potential. For example, Tomasoni *et al.* demonstrated that BMSC-derived EVs ameliorate renal dysfunction and repair tubular damage of acute kidney injury via transmission of insulin-like growth factor-1 receptor (IGF-1R) mRNA [138]. Target cells exposed to EVs acquired the human IGF-1R transcript and translated this into the corresponding protein. Another example of horizontal mRNA transfer by EVs was reported by Choi *et al.*, who employed kidney-derived MSC-EVs containing mRNA of three splicing variants of vascular endothelial growth factor (VEGF-A), basic fibroblast growth factor (bFGF) and IGF-1 [139]. When applied to an acute renal ischemia model in mice, enhanced renal function was observed by increasing proliferation of peritubular capillary endothelial cells. In addition, Ju *et al.* showed that MSC-EVs delivered human hepatocyte growth factor (HGF) mRNA to rat tubular cells which was translated into the HGF protein, stimulating cell dedifferentiation and growth by Erk1/2 signaling [140]. Furthermore, an *in vitro* model of acute kidney injury confirmed the regenerative potential of horizontal transfer of mRNA by EVs [140]. Hu *et al.* demonstrated that permeability of injured human lung microvascular endothelial cells for proteins was restored by the transfer of angiopoietin-1 mRNA by MSC-EVs [141]. In addition, restoration of lung protein permeability and reduction of inflammation in *E. coli* endotoxin-induced acute lung injury in C57BL/6 mice after administration of hBMSC-EVs was reported [142]. Zhu *et al.* attributed this therapeutic effect in part to the horizontal transfer of Keratinocyte Growth

Factor (KGF) mRNA by the EVs [142]. KGF has been shown to reduce lung edema and inflammation in several lung injury models. Figliolini *et al.* demonstrated that adipose stem cell-derived EVs prevent muscle damage and inflammation in a mouse model of hind limb ischemia, by the transfer of neuregulin 1 mRNA [70]. It should be noted that not all of these studies demonstrated that the observed regenerative effect was solely induced by horizontal transfer of mRNA by EVs, and not by concomitant transfer of the target protein by EVs. In addition, functional transfer of mRNA by EVs is not always observed nor does it seem to be confined to certain EV subpopulations. For example, Kanada *et al.* encapsulated reporter mRNA into EVs and demonstrated that mRNA was delivered to recipient cells by both studied EV subtypes, but was rapidly degraded without being translated [143]. Overall, the functional transfer of EV cargo is an area requiring more in-depth research.

2.4.3 Proteins

In addition to genetic transfer as a mechanism of conveying regenerative potential of EVs, delivery of proteins that could potentially modulate target cells has been proposed. EVs may shuttle proteins in their lumen that are capable of regulating several coalescent pathways. Studies of the proteome of MSC-EVs revealed that they contain proteins that mediate tissue regeneration through angiogenesis, coagulation, apoptosis, inflammation, and extracellular matrix remodeling [144]. Furthermore, the presence of proteins taking part in cell proliferation, adhesion, migration and morphogenesis was shown. In several studies, both *in vitro* and *in vivo*, the delivery of proteins to target cells by EVs has been demonstrated, thereby conveying their regenerative potential within different disease models. A list of examples of functional proteins that were previously shown to be transferred by EVs is given in Figure 1. An additional mechanism by which EVs can influence target cells is through proteins on their surface. These enable the EVs to dock to receptors on the surface of target cells [145]. As such, intracellular signaling in the target cells can be affected [146].

2.5 Tweaking extracellular vesicle cargo through culturing conditions and cell differentiation

Emerging evidence suggests that the regenerative effects of EVs could be potentiated by altering the conditions in which the donor cells are cultured, such as hypoxic and inflammatory conditions (Figure 1) [147, 148]. In addition, differentiation of parent cells can affect the regenerative potential of EVs [149]. Culture conditions and differentiation state can trigger intracellular alterations. As EV cargo resembles that of the parent cell, this can be influenced by controlling parent cell culture conditions. It has been well established that preconditioning human MSCs in hypoxic conditions results in enhanced biological activity of cells *in vitro* [150]. Interestingly, hypoxic conditions also positively influenced the regenerative potential of the secreted EVs. Bian *et al.* reported that the quantity of EVs released by MSCs was increased upon hypoxia stimulation [151]. In comparison to MSCs-EVs from normoxic conditions, EVs from hypoxic cells displayed higher cardiac regeneration potential in a rat myocardial infarction model. Work from Zhu *et al.* supports these findings [152]. They attributed the beneficial effect to enrichment of miR-125b, which mediated prevention of cell death and increased angiogenesis [152]. Furthermore, the same research group demonstrated that miR-210 was enriched in MSC-EVs upon hypoxia stimulation, and resulted in higher survival rates, smaller scar size, and better cardiac functions in a mouse model of myocardial infarction [153]. Gonzalez-King *et al.* reported that MSC-EVs overexpressing HIF-1 have an increased angiogenic capacity mediated by the jagged-1 protein [154].

In addition to potentiating EVs through the use of hypoxic culture conditions as summarized above, other strategies have been used to augment the potency of EVs. Shi *et al.* demonstrated that pre-conditioning of MSCs with 3,3-'diindolylmethane (DIM) upregulated Wnt11 expression in the secreted EVs [155]. Compared with the untreated MSC-EVs, pre-conditioning with DIM exerted an improved effect on wound healing in a rat deep second-

degree burn injury model. Simulating a pro-inflammatory environment for the donor MSCs promotes the release of EVs with enhanced anti-inflammatory effects. Harting *et al.* demonstrated that EVs derived from MSCs stimulated with TNF-α and IFN-γ resulted in an attenuated release of pro-inflammatory cytokines by splenocytes *in vitro* [156]. Among other potential mechanisms of action, these augmented EVs use PGE2 and COX2 to reduce inflammatory cytokines [156]. In addition, priming adipose-derived MSCs with TNF-α led to secretion of EVs, which enhanced proliferation and osteogenic differentiation in human primary osteoblastic cells [130]. Lastly, tweaking of EVs has also been achieved by culturing osteoblasts in the presence of mineralizing supplements. Davies and co-workers demonstrated that EVs derived from mineralizing osteoblasts induced mineralization in MSC-cultures, whereas EVs derived from non-mineralizing osteoblasts were not found to significantly enhance mineralization [157].

Another strategy to amplify the regenerative potential of EVs is by cultivating stem cells in a three-dimensional bioreactor. Jarmalavičiūtė *et al.* reported that culturing dental pulp stem cells (DPSC) on laminin-coated micro-carriers in a bioreactor resulted in an enhanced yield of EVs, which were able to suppress dopamine-induced apoptosis in dopaminergic neurons [158]. Interestingly, these effects were absent with EVs derived from stem cells grown under standard culture conditions. Furthermore, Yan *et al.* showed that compared to EVs derived from conventional two-dimensional cultures, EVs derived from umbilical cord MSCs cultured three-dimensionally in a hollow-fiber bioreactor were superior for cartilage repair [159]. This therapeutic effect was partly attributed to the activation of transforming growth factor β (TGFβ1) and Smad2/3 signaling.

Differentiation of MSCs towards certain lineages before collecting EVs can also dictate the regenerative potential of the produced EVs. EVs derived from DPSCs differentiated towards the odontogenic lineage were shown to be better able at inducing differentiation of DPSCs than EVs derived from naive DPSCs [29, 30]. Furthermore, EVs derived from hMSC that were differentiated towards the osteogenic, chondrogenic or adipogenic lineage, were subsequently shown to induce lineage specific differentiation in naive MSCs [31, 33, 34, 37, 39].

Overall, modifying the conditions in which the donor cells are cultivated, such as hypoxia or inflammatory conditions, or differentiation of the donor cells, could potentially enhance the favorable therapeutic effects of the secreted EVs. However, preconditioning the parent cells offers limited control in the specific cargo of EVs that is enhanced, as only naturally-occurring cargo can be enriched. Furthermore, the response of donor cells to their culture environment conditions may be a disadvantage, particularly when scaling-up cell cultures for production of EVs compatible with clinical applications. Large scale cell culture often comprises profound changes in the composition of the media used for cell culture, the substrates cells are seeded on, the shear forces they are subjected to during culture, the availability of nutrients and the increased levels of metabolism-related toxic products, among others (reviewed in [160]. These will most likely alter EV composition and, consequently function, and might even negatively affect their regenerative potential. Further studies comparing EV composition in small- and large-scale culture conditions are still needed to understand the real impact of cell culture scaling-up. All together this shows the importance of regulated and standardized culturing conditions as they may influence the content and properties of the secreted EVs. Uniformity will be key when designing a strategy for the production of EVs for therapeutic purposes.

2.6 Engineering extracellular vesicles to control cargo

In addition to cargo innately present within EVs, approaches to engineer the therapeutic content are also being explored. EVs can be designed to carry desired components, such as drugs, antibodies, proteins, and RNA. This could clear the way for more targeted delivery of

therapeutic cargo to injured tissue, as discussed in more detail in section 3.2. The use of these engineered EVs for regenerative medicine purposes has been reviewed previously [147]. Below, we will highlight approaches through which miRNAs, proteins and small molecules have been engineered into EVs.

2.6.1 MicroRNAs

As indicated above, the use of miRNAs shows great promise in regenerative medicine. Since miRNAs within EVs are selectively packaged by the secreting cell, enriching MSCs with specific miRNAs by transfection or transduction has been studied extensively. For example, Li *et al.* showed that transfecting MSCs with miR-133b resulted in an accumulation of approximately 2.5-fold higher levels of miR-133b in EVs, compared to EVs derived from non-transfected MSCs [113]. These miR-133b-enriched EVs improved functional recovery, reduced lesion volume, and preserved neurons after spinal cord injury in rats. Another example is the enrichment of miR-122 in EVs derived from lentivirus-mediated pre-miR-122 MSCs. Modification with miR-122 enhanced the therapeutic efficacy of EVs in the treatment of carbon tetrachloride-induced liver fibrosis [85]. Tao *et al.* demonstrated that EVs derived from miR-140-5p-overexpressing MSCs improved cartilage tissue regeneration and prevented osteoarthritis of the knee in a rat model [123]. Additional examples of EVs enriched in specific miRNAs used in regenerative medicine are given in Figure 1.

2.6.2 Proteins

Loading protein molecules into EVs after isolation from cell cultures is also an option to tailor EV content. Recently our group described the use of EVs for enzyme prodrug therapy. EVs were loaded with the enzyme β-glucuronidase to achieve local activation of an administered model anti-inflammatory drug [161]. Engineering the therapeutic protein content of EVs by modifying the donor cells has been studied to a lesser extent. Nevertheless, EVs are known to shuttle a variety of innately present proteins to target cells. To modify therapeutic protein cargo within EVs, the same strategy as for miRNAs can be used. For instance, Yu *et al.* transduced bone marrow MSCs with GATA-4, an important factor for the regulation of angiogenesis and cell survival [162]. EVs derived from these overexpressing GATA-4 MSCs reduced apoptosis, restored cardiac contractile functions and reduced infarct size in a regional myocardial ischemia/infarction rodent model [162]. Another example of engineered EV protein cargo was reported by Gee and colleagues, who encapsulated CRISPR-Cas9 protein into nanomembrane-derived EV-based ribonucleoprotein delivery system, named NanoMEDIC. NanoMEDIC induced genome editing by efficient transfer of CRISPR-Cas9 protein into various human cell types, including iPSCs, monocytes, T cells, iPSC-derived cortical neurons, and myoblasts [163]. As discussed above, it remains to be clarified if the functional effects observed are solely due to transfer of the target proteins by EVs or also promoted by concomitant transfer of its encoding mRNA.

2.6.3 Small molecules

Another potential strategy would be to load small molecular weight molecules, tailored to regenerative medicine applications, into EVs. By following this approach it is possible to define an optimized therapeutic window for the small molecules being delivered, with lower doses being typically still effective but with reduced cytotoxic side effects [164]. To the best of our knowledge, most studies in which small molecules were loaded into EVs were for applications in cancer treatment and reducing inflammation. As such, preconditioning MSCs with high concentrations of paclitaxel, an anticancer drug, resulted in a passive encapsulation within secreted EVs. These EVs were sufficient to inhibit tumor proliferation in mice [165]. Another example of loading small molecules within EVs was demonstrated by Sun *et al.* [166]. Mouse lymphoma cell (EL-4)-derived EVs were incubated with curcumin and applied in a lipopolysaccharide-induced septic shock murine model [166]. The curcumin

EL-4-derived EVs enhanced mouse survival significantly and reduced levels of IL-6 and TNF-α. Previous work from our group studied various passive and active methods to encapsulate porphyrins of different hydrophobicity into EVs. Hydrophobic compounds loaded efficiently into EVs and significantly increased their cellular uptake by >60%. Furthermore, active encapsulation techniques allowed up to 11-fold higher drug loading compared to passive methods [167]. Following these observations, EVs are good candidates for natural delivery vehicles of small molecules capable of mediating tissue repair. For instance, bisphosphonates, widely used for the treatment of bone-related diseases, are often formulated in liposomes [168], and are candidate drugs for future delivery via EVs.

2.6.4 Challenges in engineering extracellular vesicles

Re-engineering EVs to incorporate desired functionalities is an active field of research for which various strategies have been developed, including co-incubation, transfection, electroporation, sonication, and *in situ* synthesis [169]. As such, loading specific therapeutic cargo within EVs holds great promise for regenerative medicine applications. However, significant effort is required to increase our understanding of the molecular mechanisms underlying the packaging of cargo within EVs and the targeting towards specific disease sites [170]. Corso and colleagues reported significant differences in loading efficiencies and specificities for different proteins into EVs [171]. Currently, the EV-engineering field would greatly benefit from reporting on loading efficiency, since this will provide more insight into the potential of applied loading methods. In addition, overexpression of a specific therapeutic molecule in EV secreting cells may hold biological alterations in these cells themselves that have an impact in the loading of additional cargos into EVs beyond the molecule of interest. Consequently, the regenerative effects mediated by these EVs in target cells may be due to a wider range of molecules loaded into the vesicles. Once the molecular mechanisms are fully understood, regulated and standardized strategies for engineering the most effective regenerative EVs could be developed. Of note, several challenges and issues regarding engineering EV cargo, such as retention of small molecules within EVs, are extensively reviewed by others [172, 173].

2.7 Extracellular vesicle content and characterization – towards more efficient use in regeneration

Characterization of EV samples with bulk analysis methods, including various omics approaches, has identified a wealth of different biomolecules present in EVs. However, due to the small size of the vesicles compared to the cells from which they are derived, there is a physical limitation on the quantity of cargo contained within a single EV. Therefore, there is significant variation in content between EVs, being of a highly heterogeneous composition. In regenerative medicine, EVs will most likely contain a mixture of functional and non-functional content, of which the latter potentially reduces efficacy and necessitates the need to administer higher dosages. This factor complicates the use of EVs as regenerative agents, as the therapeutically active content will be difficult to standardize. In addition, current procedures for EV isolation are not optimized for the generation of the large EV quantities that are required for clinical applications.

Quantification of the biomolecule levels per vesicle may be more informative to judge the efficacy of EVs as therapeutics, rather than relying on bulk characterization methods. For example, miRNAs and mRNAs have been identified as likely candidates in the EV cargo that trigger regenerative effects. The first report demonstrating that EVs mediate miRNA and mRNA transfer between cells dates back to 2007 [77]. To date, more than a dozen different miRNAs have been implicated in various regenerative settings. However, recent reports indicate that the actual presence of individual miRNAs and mRNAs in EVs, and their transfer to the recipient cells, may be limited, up to the point where it is even questionable whether individual EV-associated miRNAs or mRNAs are capable of exerting biological effects [174-

176]. It is highly likely that the combined heterogeneous content of EVs triggers a complex interplay in the target tissue. However, this would require the delivery of distinct sets of EVs to the target cells. Recent advances in single-particle analysis techniques are anticipated to facilitate the identification and characterization of EV subpopulations and shed light on the biologically active EV subsets. Examples of such techniques include single-particle Raman trapping analysis [177], nano- and imaging flow cytometry optimized for single EVs [39], and single molecule microscopy [171].

Efforts to uncover potential solutions through which these issues can be circumvented are ongoing. The direction this research is taking is to pinpoint the exact cargo in EVs that triggers the observed regenerative effects. These effects can then be replicated either through pharmacological intervention, or by engineering synthetic EV equivalents with only the essential EV components.

2.8 Pathways implicated in regenerative effects are pervasive

The regulation of specific intracellular pathways is thought to be the basis for the regenerative potential of EVs. A multitude of published works have investigated the identity of these pathways in connection to the cargo present within the EVs. Combined, the number of pathways perturbed by EVs in different target tissues is immense (see Supplementary table 1). This may have implications for the value of EVs in the clinical setting. One may expect a storm of relatively poor controlled up- and downregulated pathways, not only in the target cells, but also in tissues not intended to receive treatment. As such, the use of EVs could lead to serious off-target effects. It is therefore of great importance to elucidate mechanisms involved and clarify to what degree they are modulated in target and non-target tissues. Since the utilization of EVs as therapeutics is hindered by the difficulties in ensuring delivery at the site of injury, an interesting strategy would be to engineer the EVs to target them towards their site of therapeutic action. Secondly, precise control of the intracellular pathways that are affected by regulating the therapeutic content of EVs would enhance clinical applicability.

2.9 The efficacy of extracellular vesicles in regeneration – dose matters

In preclinical studies, EVs have been applied extensively to several areas of regenerative medicine with, at first glance, tremendous success. However, as is typical in research, there is likely to be bias towards reporting positive results, which may provide an overly optimistic picture of the potential of EVs in regenerative applications. One critical factor that remains difficult to compare between these studies is how efficient EVs really are as regenerative entities. Therefore, it is important to discuss the regenerative effects triggered by EVs in the context of the quantity of vesicles added. Overall, the EV dosage that exerts relevant effects appears to be highly variable, typically ranging from 0.05 to 800 µg/mL or $1x10^5$ to $1x10^{15}$ particles/mL *in vitro*, and from 0.25 to 400 µg or $1x10^4$ to $1x10^{12}$ particles *in vivo* (Table 1). Most studies also report analysis of a single dose, rather than examining a dose response relationship between EVs added and effects observed. Moreover, it should be kept in mind that the reported EV doses may not be the true dose of EVs *per se* as the dosing is always based on either particle or protein quantities, both of which are only approximate measures of the true EV quantity and can be affected by contamination with non-EV material.

To illustrate this further, we compared studies that used the so-called tubule formation assay, in which human umbilical cord endothelial cells (HUVECs) are seeded on basement membrane mixtures. This assay has been a key method to assess potential regenerative effects of EVs *in vitro*. In Supplementary table 2, we summarize results from studies assessing the effects of EVs on tube formation. Results vary from no effect of MSC-EVs to a significant increase in tubule formation and stabilization. Culture conditions, such as medium used, configuration (2D vs. 3D) and exact MSC source appear to affect the potency of EVs

in this setting. Direct comparison of the EV doses and dose-dependent effects between different studies is challenging, since not all studies report on the number of cells that were used for production of EVs, the number of EVs that were applied in the tube formation assay or the number of cells to which these EVs were applied. Instead, in most studies, dosage is reported as a protein concentration. Since different EV isolation methods were used across studies, the quantity of free protein that co-precipitates with the EVs, and the number of particles within a given microgram of EV isolate, will vary. As such, reporting protein concentration is less indicative for the administered concentration than particle number [178]. It is also notable that the vast majority of studies employed differential ultracentrifugation or polymer precipitation as the sole isolation method, without further cleanup of the EV preparations - these methods are known to carry risk for co-isolating contaminating soluble protein [178, 179]. It is likely that the discrepancies observed in the effective EV dose are at least partially explained by the varying purity of the studied EV preparations. The problems related to artefacts stemming from the impurities of the EV preparations are demonstrated in our recent publication showing that the non-EV factors of the MSC conditioned medium, separated from the EVs by size-exclusion chromatography, are necessary and sufficient to stimulate angiogenesis and wound healing *in vitro*, suggesting that EVs might not always be responsible for the beneficial effects assigned to them [179]. Moreover, despite reports showing that EVs or EV-containing conditioned media are more potent than the corresponding EV-depleted conditioned medium [32, 36, 131, 180, 181], we showed that the process of EV depletion by ultrafiltration also depletes other soluble factors affecting the observed phenomenon and thus distorting the results [179]. In fact, there are several reports showing that the full conditioned medium is more potent or at least equally potent in the regenerative applications as the pure EV preparations [32, 64, 118, 182, 183], implying a synergistic effect of several secreted factors. In addition, the relative contributions of EVs and other soluble factors are likely to be highly dependent on the application, as evidenced in the study of Balbi and co-workers, showing that human amniotic fluid stem cell-derived EVs were able to recapitulate the cardiac regenerative effects exerted by full conditioned medium, but could not stimulate vessel formation [180]. Mitchell *et al.* indicated that conditioned medium (total secretome) and EV-associated factors from adipose-derived MSCs affected different aspects of tissue regeneration after muscle injury [182]. Specifically, only the total secretome but not the EV fraction was able to reduce the number of senescent cells *in vitro*, whereas the anti-inflammatory effects were mainly mediated by the EV fraction. *In vivo* in mice EV fractions had a stronger effect on skeletal muscle regeneration and decrease in macrophage infiltration after acute muscle injury, whereas the total secretome was responsible for the pro-angiogenic effect.

Supplementary table 2 also lists examples of studies where large doses of EVs were applied before effects could be observed: EVs secreted by 100 to 1000 times more MSCs than the number of HUVECs ultimately exposed. This begs the question of whether this signaling is physiologically relevant, and whether this EV dose is realistically achievable in the clinical setting. It is notable that the EV doses applied in animal models do not markedly differ from the EV quantity used in *in vitro* experiments, as shown in Table 1 and Supplementary table 1. However, in the *in vivo* situation, efficiency of delivery is unlikely to reach that of *in vitro*. Instead, substantial numbers of EVs become lost in the circulation, or are taken up by other tissues, which suggests that a higher EV dose compared to *in vitro* might be needed to achieve the desired regenerative outcome at similar potency. Therefore, the dose of EVs required to achieve the desired effects is one of the challenges that must be addressed. Is the amount of EVs that is needed to initiate regenerative effects achievable within the clinical setting, or will colossal quantities of EVs be required that are not readily within reach? Are the beneficial effects due to a minor subpopulation of active EVs, or to a concerted multi-modal effect? And to what extent have experimental issues affected previously reported data by co-isolation of bioactive soluble contaminants? Local application instead of systemic administration may be a vital delivery strategy to pursue in order to achieve the necessary

levels of EVs in target tissues. Targeted and biomaterials-based delivery strategies for EVs will be discussed below.

3. Extracellular vesicles for tissue repair and regeneration *in vivo*

3.1. Immune system evasion

One of the first aspects to take into consideration when utilizing EVs *in vivo* is their interaction with the host immune system. In describing the benefits of EVs as therapeutic vehicles, it is sometimes stated without further elaboration that EVs are “immunologically privileged” or able to “evade the immune system” as a result of their “biological membrane”. Indeed, they do not trigger anaphylaxis nor major toxicity upon *in vivo* administration, even when using xenogeneic EVs [184], and so are not inherently grossly immunogenic. However, the evidence so far collected on the immunological properties of EVs paints a more nuanced picture. The interactions of administered EVs with the host immune system are likely to depend on the presence or absence of alloantigens on the EVs, the presence or absence of immunomodulatory signals on the EVs, and the route of administration. What it means to successfully “evade the immune system” is time and context-dependent; all administered EVs will eventually be delivered or destroyed, and so the key question is whether they are able to reach their target over an appropriate timescale and in sufficient numbers to achieve the desired effect.

The interactions of a transplanted EV with the recipient immune system will ultimately depend on its population of membrane biomolecules, which in turn ultimately depends on the parent cell. Dendritic cells are antigen-presenting cells that typically present antigens to T-cells, invoking an adaptive immune response. EVs derived from activated dendritic cells contain peptide-loaded MHCII and are known to be capable of effective antigen presentation sufficient to activate an adaptive immune response *in vivo* [185], to the extent that they have been explored as a “cancer vaccine” in clinical trials [186]. Graft-derived dendritic cell EVs have been implicated in the rejection of allografts by the adaptive immune system [187]. Conversely, EVs from immature dendritic cells lack these molecules and therefore the capacity to strongly activate T cells [188]. Besides EVs from antigen-presenting cells, the interactions of vesicles with the host immune system will likely be determined by the presence of EV-associated alloantigens and immunomodulatory factors. For instance, EVs from bovine milk were previously described to be capable of triggering inflammatory responses *in vitro,* observed to a much lower extent when stimulating immune cells with MSC-EVs [189].

MSC-EVs are perhaps the most widely-explored for immune-evading drug delivery. MSCs are known to have immunomodulatory properties, and as such have been explored as an immunomodulatory therapeutic for graft-versus-host disease [189, 190]. Compared to other cell types such as fibroblasts, they persist for longer than expected in allogeneic tissue and do not cause rapid rejection or a dangerous immune response [191]. However, they should be considered immune-evasive rather than immunologically privileged, since they still cause a humoral and cellular immune response and are eventually cleared [191]. Given these properties, it has been suggested that their EVs might have similar immunoregulatory and immune-evasive behavior. It has been shown that human MSC-EVs can suppress the pro-inflammatory M2 macrophage phenotype *in vitro*, cause T-regulatory cell polarization [192] and can suppress pro-inflammatory Th17 cells [193]. Human MSC-EVs have been explored as a therapeutic for autoimmune diseases, with evidence of benefit [194-196], and their co-administration improves survival of allogenic skin grafts *in vivo* [197]. However, the capacity of human MSC-EVs to suppress pro-inflammatory immune cell phenotypes is not necessarily indicative of immunological evasiveness or protection for the EVs themselves. Although it is commonly stated that human MSC-EVs may have an immune-evasive

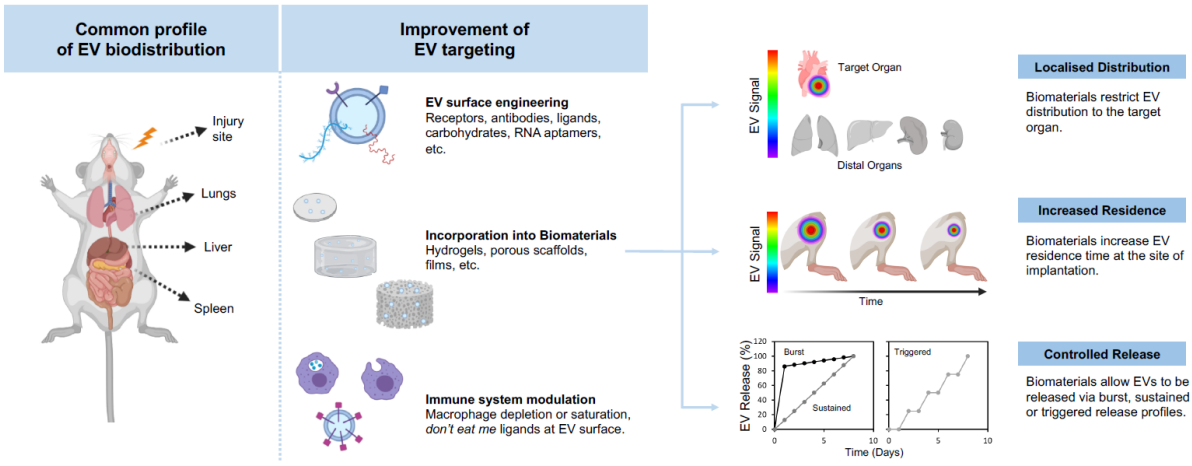
phenotype, it is not clear whether they persist in the circulation or in tissue for longer than EVs from other cell types. It appears that EVs from allogeneic cancer cells are rapidly cleared from the circulation by macrophages in the liver and spleen [198, 199], and uptake of EVs by macrophages *in vitro and in vivo* has been observed for EVs from other cell sources [200-202]. There is evidence that some EVs express surface proteins that prevent their uptake – CD47, a "don't eat me" signal that blocks SIRPα-dependent macrophage uptake, has been found on EVs from fibroblasts [203], T cells [204] and hMSCs [205]. In the case of fibroblast EVs, inhibiting CD47 expression was found to lead to a ~2-fold reduction in circulating exogenous EVs 3 hours after administration compared to EVs from wildtype cells. However, the extent to which human MSC-EVs evade clearance compared to other EV types remains unclear, as do the potential mechanisms that might underlie such an effect. Human MSC-EVs were found to accumulate in mouse organs 24 hours after administration at similar levels to EVs from other cell sources, with a particularly high proportion of human MSC-EVs ending up in the liver [206], indicating that they may not evade macrophage-dependent clearance. However, it is not clear to what extent these dynamics would be observed with EVs from autologous MSCs, which would have the advantage of antigenic compatibility.

A number of studies further support the concept of close interactions occurring between EVs and the immune system, reporting their accumulation in injured tissues in *in vivo* models of injury of different systems. For instance, dye-labeled EVs derived from MSCs or neural progenitor cells were found in the brain of murine models subjected to brain injury by stroke [108, 207-209]. In the work of Moon *et al.* EVs were found to accumulate in the affected brain area in a dose-dependent manner, and were rarely identified in other organs such as liver and lungs [108]. However, in the work of Zheng *et al.*, a quantitative analysis of the biodistribution of the DiI-labeled EVs from neural progenitor cells (NPC) after administration in sham and stroke-injured mice, showed that although DiI fluorescence could be detected in the brain, it was significantly higher in the liver and lungs [209]. Similar observations were reported in a mouse model of acute kidney injury, with DiD-labeled MSC-EV appearing to accumulate at higher levels in the liver and spleen compared to the injured kidney [210]. It is not yet clear if EVs preferentially accumulate at places of injury in general. EVs of different origin, labeled with lipophilic dyes, were reported in high levels in the kidneys of mouse models with kidney injury, compared to sham-operated or healthy animals [210, 211]. However, Zheng and colleagues did not find any difference in the redistribution of NPC-derived EVs to the brain of mice upon stroke compared with sham-operated controls [209].

Of note, it is important to recognize that the study of EV interaction with target tissues *in vivo* is impacted by the methods applied for vesicle labeling. The use of lipophilic dyes to stain EVs can lead to artefacts such as staining of co-precipitating lipoproteins in the EV isolate, desorption of the dye from the EV surface, and aggregation/micelle formation of excess dye (as reviewed in [212]). To address these issues, future studies should employ more rigorous isolation procedures to limit lipoprotein contamination and ensure that a minimum amount of dye is used. Alternatively, studies can produce EVs carrying genetically encoded fluorescent reporters or induce the parent cells to release fluorescently labeled EVs using a cytoplasmic label to avoid the issues associated with lipophilic dyes [212]. On the other hand, overexpression of reporter proteins fused to common EV marker proteins (e.g. tetraspanins CD63, CD9) can lead to changes in the molecular composition of the EV surface proteome, impacting the natural interaction of EV subpopulations with target tissues [213, 214]. Co-localized staining of the EV lipid membrane and RNA cargo has also been previously reported to ensure that only intact EVs are being visualized during fluorescent imaging and not lipoproteins or fragments of EV membranes [215].

Many studies link the biodistribution of EVs in injured tissues to their pro-inflammatory environment. Induction of neuroinflammation in a mouse model of lipopolysaccharide (LPS)-induced encephalitis increased the levels of radiolabeled EVs (derived from a macrophage

cell line) in the brain by 5.8-fold upon intravenous (i.v.) injection, compared to EV administration in healthy animals [216]. An increase in EV accumulation in the lungs, kidneys and heart was also observed, likely due to non-specific peripheral inflammation. However, the highest brain accumulation of EVs detected was only ~0.538% of the injected dose per gram of tissue, with most of the EVs being detected in the liver, spleen, lung and kidneys. Likewise, MSC-EVs might follow the pattern of their parental cells by accumulating in places of injury [210, 217], where pro-inflammatory events are taking place. Injection of a heterogeneous population of MSC- or lung fibroblast-derived EVs in mice, at 24 hours after whole body-irradiation, led to an increased accumulation of both EV types in bone marrow, liver and spleen for MSC-EV [217]. Accumulation of MSC-EVs in bone marrow significantly increased with increasing radiation dose, suggesting a link between EV tropism to an injured location and the extent of the injury. In this study, the percentage of CD11b+ cells and F4/80+ cells with internalized DiD-labeled EVs increased in the bone marrow and spleen after irradiation, further suggesting that increased EV accumulation at injury sites might be related at least in part to the activity of immune cells at that location, consistent with other evidence that macrophages mediate EV removal from the circulation. Recently, it was shown that lung-marginated Ly6C^{high} activated monocytes were able to take up higher levels of macrophage cell line-derived large EVs in a mouse model of LPS-induced sub-acute systemic inflammation, compared to control animals. In addition, Kupfer cells have been observed as being a major player in the uptake of EVs *in vivo*, in both physiological and injury conditions [211, 218]. The extent to which EVs found at the intended target organs are internalized by the tissue-specific cells that confer the organ its main physiological function, or by resident immune cells, remains to be demonstrated. This will ultimately impact the design of tissue repair/regeneration interventions, which may be more focused on immunoregulation or on repair and remodeling of the injured tissue, depending on the cell types that take up the administered EVs. Conversely, there is an opportunity to improve EV tropism to target organs, with a number of studies exploring systemic myeloid cell depletion [199], or Kupfer cell saturation by pre-dosing with placebo nanoparticles [219] to redirect the biodistribution of nanovesicles away from the liver (Figure 2).



3.2. Biodistribution and tissue targeting of extracellular vesicles

EVs are often described as having an intrinsic tissue targeting capability upon *in vivo* administration. One of the first works supporting this comes from the cancer field, where EVs originating from specific cancer cell lines were shown to accumulate in specific organs after *in vivo* injection, coincident to the preferential metastatic organs of their cells of origin, and depending on the integrins displayed at the surface of the vesicles [220]. Since then, additional studies have suggested a differential accumulation of EVs in certain organs in both healthy and disease *in vivo* models, often referred to in the literature as an intrinsic EV

homing capacity. Homing has been classically defined in the cell biology field as the capacity of a circulating cell to migrate into a target tissue in response to external environmental cues, most often chemotactic gradients [221]. This process assumes the engagement of surface receptors, the activation of signaling pathways and the effective signal transduction that ultimately translates into directed cell migration [221]. However, the occurrence of active sensing and signal transduction in response to external cues remains to be demonstrated for EVs. For this reason, the biodistribution of EVs into specific tissues is more accurately described as a targeting capacity, instead of a homing capacity.

It is currently believed that EV biodistribution *in vivo* is dependent on the EV cell of origin, reflected in the molecular composition imprinted in the surface of the secreted EV, and on the route and regimen of administration. In one of the first studies exploring biodistribution of EVs upon administration under physiological conditions *in vivo*, Wiklander and colleagues showed that EVs derived from HEK293T cells, labeled with DiR, accumulated preferentially in liver, spleen, gastrointestinal (GI)-tract and lungs upon i.v. injection in mice [206]. However, intraperitoneal (i.p.) or subcutaneous (s.c.) administration significantly decreased EV accumulation in liver and spleen, while increasing their levels in the pancreas and the GI-tract [206]. In the same study, i.v. administration of EVs at different doses, despite not changing their overall organ biodistribution, impacted the relative percentage of EVs accumulated, with higher doses leading to lower levels of EVs in the liver, but increasing levels in the lungs [206]. Most importantly, DiR-labeled EVs derived from C2C12 and B16F10 mouse cell lines and primary mouse dendritic cells accumulated at different percentages in liver, spleen, GI-tract and lungs after i.v. injection depending on their cell of origin [206]. In fact, dendritic cell-derived EVs were found at the highest percentage in the spleen, suggesting an accumulation dependent on interactions related to their immunological origin. Nonetheless, it should be emphasized that, regardless of their origin, EVs still accumulated primarily in the same organs in similar patterns, contrary to the idea of highly specific organ tropism and accumulation. The same observations were reported in other studies [119, 217]. For instance, Wen *et al.* showed that DiD-labeled small and large EVs derived from human lung fibroblasts accumulated at much higher levels in the lungs of mice upon i.v. injection, when compared to EVs from MSCs [217]. On the other hand, MSC-EVs were found to accumulate at higher levels in the liver and spleen. The fibroblast EVs accumulated at similar levels in the lungs, liver and spleen, again contesting the idea of highly specific EV tissue targeting.

The targeting capacity attributed to EVs has in some cases been linked to the presence of specific surface proteins. Integrins at the EV surface are suggested to be important mediators of their accumulation at specific tissues [220]. By a loss-of-function study, the presence of α4β7 integrin at the surface of EVs derived from a mouse T cell line was shown to condition their accumulation levels in gut mucosa in physiological conditions, a property of activated T cells expressing these proteins, without significantly affecting their accumulation in Peyer's patches, mesenteric lymph nodes, liver and spleen [222]. Tetraspanins, and their association with integrins, were also linked to *in vivo* EV targeting. Tspan8-containing EVs were enriched in the pancreas of mice after i.v. injection, whereas co-expression of β4 integrin led to their accumulation at similarly high levels in the lungs [223]. In addition, the glycosylation of EV surface proteins was shown to influence their biodistribution, with digestion of sialic acids improving the accumulation of ^{124}I-labeled EVs from mouse liver cells in the lungs, in comparison with non-digested EVs [224].

Altogether, these studies suggest that the degree of innate tissue-specific tropism of EVs is very limited, and usually not sufficient to determine their highest accumulation in organs other than the liver and spleen (Figure 2). EVs have also been suggested to accumulate in specific organs following injury (see above) [207-209, 211, 217]. In general, the reported increase in tissue-specific EV accumulation after injury is minor compared to the continuing non-specific accumulation in other organs and in many cases may be due to a nonspecific

increase in phagocytosis at the site of injury. Without appropriate controls, such as an EV population expected to be non-homing and non-therapeutic, a specific homing or signaling function for an EV population should not be inferred on the basis of their tissue accumulation alone.

Although highly specific tissue targeting of native EVs may be limited, their surface proteins do influence their biodistribution. Following this rationale, EV surfaces have been modified to contain different molecules that can promote their targeting to specific tissues (Figure 2). These include receptors, antibodies, ligands, peptides, RNA aptamers and sugar moieties, among others. The first study reporting EV engineering for improved targeting to an injured tissue was in 2011. In the seminal work by Alvarez-Erviti and colleagues, immature dendritic cell-derived EVs were engineered to express a fusion of the brain-tropic rabies viral glycoprotein peptide and the EV abundant protein LAMP2b for improved targeting of EVs to the brain, as indirectly assessed by an increased delivery of BACE1 siRNA [225]. Since then, several other works have attempted the engineering of regenerative EVs for improved biodistribution to injured organs, with only a few of them quantitatively verifying an actual increase in bioaccumulation of the modified EVs in the target organ. Wang *et al.* developed EVs tropic to the infarcted heart by fusing an ischemia-targeting peptide to LAMP2b, followed by labelling with DiR for *in vivo* tracking [226]. Compared to control EVs, increased levels of engineered EVs were found in the infarcted heart, supporting the effectiveness of the targeting. However, as before, very high fluorescence signals were also detected in liver, spleen and kidney [226]. In the work of Antes *et al.* the biodistribution of similarly engineered heart-tropic EVs was evaluated quantitatively [227]. EVs were engineered with an ischemia-tropic peptide attached to their surface via a streptavidin-polyethylene glycol (PEG) spacer anchored to the EV membrane by a diphosphine moiety. Targeted EVs were enriched in the hearts of myocardial infarction rat models, compared to control EVs. However, as seen before, it was found that the major organs of EV biodistribution remained the liver, lungs, and kidneys.

The core rationale for targeting of therapeutics to specific tissues is to reduce the necessary whole-body dosage, reducing side-effects and improving on-target efficacy. Although effective in improving EV delivery to a target organ, EV engineering for targeting does not seem to circumvent their high non-specific accumulation in the classical clearance organs, leading to loss of effective dose available upon *in vivo* administration. Whether the targeting that can currently be achieved will meaningfully impact the doses that can be used or reduce systemic side-effects is unclear. It is important to highlight that a reliable assessment of EV tropism *in vivo* requires a systemic evaluation of their biodistribution. These works clearly demonstrate that identification of EV accumulation at a target organ does not guarantee that they accumulate there preferentially compared to other organs.

However, reaching a target tissue is only the beginning of the intended functionality for EVs. They must productively interact with cells in the target tissue to alter their phenotype. This can be achieved by EV engagement with cell surface receptors, activating outside-in signaling pathways, or by delivery of their cargo to the cells by different mechanisms [228]. EVs have been reported to be able to deliver their cargo to target cells by directly coupling at their surface via gap junction proteins, such as connexin 43 [229], or by directly fusing to the cell membrane. Alternatively, they may be internalized by different mechanisms of endocytosis (e.g., receptor-mediated endocytosis, macropinocytosis, phagocytosis, among others), although the mechanisms that drive cargo unloading are still a matter of intensive study. In fact, it is still unclear what determines that a specific cargo carried by internalized EVs escapes the endocytosis pathways and is productively delivered to cells, instead of being degraded in lysosomes. The extent to which each one of the mechanisms of interaction between EVs and cells contribute to the alterations observed in the phenotype of the latter is still largely unknown. It is important to note, however, that receptor-mediated engagement of EVs with target cells may constitute a more specific mode of interaction,

being dependent on the presence of receptor-ligand partners at the surface of both EVs and target cells, being thus linked to the limited targeting capacity of EVs discussed above [228]. On the other hand, the less specific interaction mechanisms may contribute to EV loss for non-target organs. Immunofluorescence analysis of target tissues with detected EV accumulation has shown the presence of the lipophilic dyes used for EV labelling in locations compatible with a scenario of intracellular uptake of the vesicles once they reach the target tissue [207, 208, 210, 211]. However, fluorescence in extracellular locations has also been reported, compatible with an EV action dependent on engagement with cell surface receptors or adhesion to the extracellular matrix. Overall, studies exploring EV interaction with target cells *in vivo* are still lacking, and it is currently unknown if EV-cell interactions follow mechanisms similar to those reported *in vitro.*

In order to be an advantageous therapeutic delivery system, EVs must demonstrate characteristics comparable or superior to those of synthetic lipid nanoparticles (LNPs). Smyth *et al.* found that the biodistribution and rate of clearance of cancer cell-derived EVs was identical to that of synthetic liposomes and liposomes prepared from EV lipid extracts [230]. Interestingly, EVs loaded with siRNA, by parental cell engineering, were shown to be at least 10-fold more effective at delivering their cargo *in vivo* compared to other LNP vehicles [231]. Compared to EVs, synthetic LNPs are more cost-effective and more straightforward to manufacture on a large scale. Synthetic particles also have the advantage that they are fully chemically defined, more easily purified without potentially bioactive contaminants, and they can be loaded with therapeutic cargo during production rather than afterwards, simplifying the process and allowing high concentrations of drug to be readily loaded. The potential benefit of EVs over synthetic LNPs stems from their intrinsic targeting and immune-interacting proteins at the EV surface. However, any allogeneic protein is a potential antigen and immunological target. As such, synthetic LNPs lacking allogeneic proteins may be advantageous in this regard in terms of avoiding premature recognition and destruction, although LNPs may themselves be immunogenic [232]. Most studies in which organ specific EV targeting was achieved involved modification of base EVs to express targeting ligands. Incorporation of the same targeting ligands into base synthetic LNPs might be an interesting route for many therapeutic applications. A key advantage of EVs over synthetic lipid carriers are in applications where the EVs themselves have a therapeutic effect via their unique biological cargo.

3.3. Biomaterial systems for localized delivery of extracellular vesicles

Rather than relying on the innate targeting of EVs towards the intended tissues, alternative strategies employ the local administration of therapeutic EVs. This approach is of particular interest for tissues with external accessibility, such as the skin. Subcutaneous injection of EVs was previously reported to improve wound healing [61, 233] and the viability of skin grafts [234]. Intranasal administration of EVs has also been explored for local delivery to the brain. Although this is not a direct administration of vesicles to the intended target organ, it takes advantage of the easier retrograde transport via olfactory and trigeminal nerves directly to the brain, thereby avoiding the brain-blood barrier. This approach has been described both for the treatment of traumatic brain injuries and neurodegeneration [235-237]. Although more invasive from a procedural point-of-view, intracardiac injection of EVs has been reported for localized EV delivery to the heart, overcoming the challenge of short-term retention [180, 238].

Biomaterial-based delivery is another promising alternative method of administering EVs to a local anatomical site in need of regenerative treatment. Compared to local injection, incorporating EVs into a suitable biomaterial has the potential to control the biodistribution of EVs within the host, the dose of EVs administered, and the release profile of EVs from the biomaterial (Figure 2). Numerous studies have been performed that incorporate EVs into biomaterials such as hydrogels, films or porous scaffolds. Hydrogels have been most

extensively examined, possibly due to the ease of combining EVs with a hydrogel prior to gelation of the system. However, hydrogel-EV systems are often restricted to low-load bearing applications, such as treatment of ischemia, infarct or skin wounds. Load bearing applications, where the EV-material system is intended to remodel (e.g. vascular and bone regeneration), require more robust scaffolds such as ceramic, electrospun or decellularized tissue matrix scaffolds (see Supplementary table 3). Such systems often require more sophisticated methods of retaining EVs within the material structure compared to hydrogel-EV systems (see section 3.3.4).

The therapeutic benefit of EV-biomaterial systems is well documented, with all relevant studies reporting improved therapeutic effects of the combined systems compared to free EV injection or blank materials. This may point towards the publication bias of only positive results in the field, highlighted in a recent review detailing the role of MSC-derived EVs in regenerative medicine [239]. Rather than outlining the therapeutic effects reported by EV-biomaterial studies [240], this portion of the review focuses on highlighting key areas of interest to assist with the future development of more standardized and clinically translatable EV-biomaterial systems. We focus on the studies that tailor and/or characterize EV-biomaterial systems, and do not discuss the numerous studies that inject premixed EV and hydrogel systems without prior or subsequent characterization of the system (See Refs [30-32, 36, 86, 118, 127, 131, 241-255] for examples).

3.3.1 *Biodistribution of extracellular vesicles released from biomaterials*

Limiting the biodistribution of EVs to the required site of action within the host is one of the main benefits of utilizing EV-biomaterial systems. However, only a limited number of studies examine the biodistribution of EVs within the host, even at the site of implant. Han *et al.*, Liu *et al.*, and Zhang *et al.* examined the biodistribution of EVs at the EV-biomaterial implant site [100, 256-258]. Han *et al.* delivered PKH26-labeled EVs in PBS and in a hydrogel to murine infarcted hearts [256]. Twenty-one days following implantation, only the EVs delivered in the hydrogel were still detectable in the excised hearts. Similarly, this group delivered PKH26-labeled EVs in PBS and in a silk fibroin hydrogel to murine ischemic hindlimbs [100]. After 14 days only the EVs delivered in the hydrogel could still be detected in the excised muscle tissue. Liu *et al.* delivered PKH67-stained EVs to the myocardium of murine hearts in a hyaluronic acid (HA)-based hydrogel and demonstrated that fluorescent signal was present in explanted hearts after 24 hours [257]. However, no PBS control was included in the biodistribution analysis. Zhang *et al.* employed an *in vivo* imaging system (IVIS), a system capable of detecting fluorescent or bio-luminescent EVs *in vivo* (see review by Gangadaran *et al.* [259]), to visualize the biodistribution of luciferase-labeled EVs when delivered to murine hindlimbs via PBS or in a chitosan hydrogel scaffold [258]. Luciferase signal was visible in the hydrogel delivered group at 72 hours and absent in the PBS group. The studies that specifically examine the biodistribution of EVs at the site of implantation reveal that biomaterial-based delivery increases EV residence time compared to PBS delivery.

A number of studies have performed qualitative analysis of the gross distribution of EVs within a host animal using IVIS following implant of an EV-biomaterial system. Similar to the preceding studies, delivering EVs in a material increased residence time compared to injection in PBS. The following studies demonstrate that EVs largely remained localized to the site of implant, following either PBS or material-based delivery. Chen *et al.* used IVIS to examine the biodistribution of Vybrant DiO-stained EVs following subcutaneous delivery in mice via PBS or in a 3D-printed gelatin methacryloyl (GelMA) hydrogel scaffold [260]. The 3D-printed scaffold could better retain EVs at 7 days following implant compared to PBS injection. However, some distal fluorescence was observed in the extremities of mice in both groups at day 0, indicating that some EVs may have traveled distally from the site of implant. Gangadaran *et al.* administered DiR-labeled EVs to murine hindlimbs in PBS or in matrigel hydrogel [261]. IVIS revealed a significantly higher fluorescent signal in the gel group

between 12 and 48 hours compared to the PBS group, and that at 48 hours fluorescent signal was no longer observed for the PBS group. At all timepoints during the study, the fluorescent signal remained localized to the implant site in both groups. Henriques-Antunes *et al.* applied EVs labeled with Cy7-DPPE topically to a murine wound bed in either PBS or a light-triggerable HA-hydrogel [262]. PBS-delivered EVs were rapidly eliminated with 70% of their fluorescence lost after 2 days. In contrast, EVs delivered in the gel maintained the same concentration for 3 days prior to light application, which triggered the release of EVs in the wound bed. Fluorescence in both groups was largely localized to the wound area with more diffuse fluorescence observed at the PBS site of injection. Wei *et al.* implanted heparinized and non-heparinized electrospun poly ε-caprolactone (PCL) disks loaded with luciferase-lactadherin-labeled EVs subcutaneously in mice [263]. IVIS showed that the average radiance of luminescent EVs was reduced to zero in both groups after 6 days and that luminescence was restricted to the implant area. The evidence presented in the preceding studies suggests that biomaterials may not be required to prevent diffuse EV biodistribution, and that the main benefit of material-based delivery is increased residency time at the implant site.

Two studies have examined the effect of material-based delivery on the biodistribution of EVs in extracted organs distal to the implant site. Lv *et al.* administered DiR-labeled EVs via intramyocardial injection in mice via PBS or in an alginate hydrogel and analyzed EV retention using IVIS [264]. Over 14 days there was higher fluorescent signal at the implant site for the gel group compared to the PBS group, suggesting that hydrogel-based delivery enhanced EV retention in the murine heart. Assessing fluorescent signal in the explanted liver, spleen and lungs at day 3 revealed significantly less signal in the liver and spleen in the gel group compared with the PBS group, indicating that administration via PBS caused EVs to accumulate in distal organs and also that hydrogel administration restricted EVs to the implant site. Mardpour *et al.* administered PKH26-labeled EVs to the livers of mice in PBS or a PEG-based hydrogel and monitored the biodistribution of EVs using live imaging or by imaging the harvested organs [265]. After 1 day, PBS-delivered EVs were distributed throughout the abdominal cavity, while the hydrogel-delivered EVs remained localized at the injection site. Signal was still visible in the livers of the EV-gel group after 2 weeks, but was completely absent from the livers in the free injection group. The preceding studies highlight that when administering EVs to an internal organ, PBS delivery leads to diffuse transport of EVs away from the delivery site, and to accumulation of EVs in distal organs, while material-based delivery restricts EVs to the implant site. Future studies should therefore seek to analyze the biodistribution of biomaterial delivered EVs in the target organ and in explanted distal organs to confirm the restriction of EVs to the intended area, since gross examination of EV distribution using IVIS may not effectively characterize the true distribution of EVs within the host.

In summary, all of the studies that characterize the biodistribution of EVs following implantation of an EV-biomaterial system show increased residency time at the implant site and restriction of EVs to the implant site, with no transport of EVs to the distal organs. It should be noted however that reported retention times might be biased to some extent by the use of lipophilic dyes for EV labelling in most of these studies, with the disadvantage of these dyes often originating artifacts that confound EV detection, as discussed in section 3.1. Although an improved regenerative response at the implant site is not directly linked with increased residency time or restricted transport, all studies report improved regenerative metrics when implanting EV-biomaterial systems compared to free EV injections, thereby presenting compelling evidence for the utility of biomaterials in EV delivery.

3.3.2 Concentration and loading efficiency of extracellular vesicles in biomaterials

In most studies that develop EV-biomaterial systems, the concentration of EVs that are incorporated into the biomaterial is reported in micrograms of protein per microliter (see

Supplementary Table 3). As previously discussed in this article (see section 2.9), this is substantially influenced by sample purity and reporting EV concentration in such a manner gives no indication of the number of particles that are effectively incorporated into a given biomaterial. As a result, comparing the true concentration of EVs used across studies is challenging. Future studies should consider reporting EV concentrations as particles per scaffold, and report the particle-to-protein ratio as suggested in [19] so that both the concentration and purity of EVs within the biomaterial can be compared. However, the number of particles in solution can vary independently of EV concentration, owing to the presence of lipoproteins, and smaller EVs can go undetected with NTA. High-sensitivity flow cytometry may offer a solution to this issue by lowering the size detection limit to 40 nm and facilitating EV-specific detection via immunofluorescent staining [266].

Loading efficiency of EVs into the biomaterial system is a topic that requires attention, as it is often overlooked. Loading efficiency can be conveniently calculated by determining the quantity of EVs in the EV isolate solution before and after loading onto the scaffold. Cunnane *et al.* identified a reduction in protein content within the EV isolate after infusing scaffolds with EVs [267]. Results indicated that approximately 45% of the EV isolated protein content was retained within a tubular silk fibroin scaffold. Chew *et al.* determined EV loading efficiency by normalizing the levels of CTB-CD81 in the scaffold to the initial level of CTB-CD81 present in the EV isolate and expressing it as a percentage [268]. It was determined that the collagen sponges had been loaded with EVs at an efficiency of approximately 68%. Li *et al.* achieved a loading efficiency of 66% of EV isolated total protein content in a poly(lactic-co-glycolic acid) coated with polydopamine (PLGA-pDA) scaffold [33]. Future studies should determine loading efficiency in order to identify the optimal concentration of EVs required to load the biomaterial system that minimizes EV wastage. Basing loading efficiencies of EVs in biomaterials on particle number will help aid in the direct comparison between different studies.

3.3.3 Distribution of extracellular vesicles in biomaterials

Examining the distribution of EVs within the biomaterial system ensures that the desired dispersion of EVs is achieved and can be used to determine if sufficient EVs have been included in the biomaterial to achieve complete coverage. Studies have examined the changes in chemical composition of the biomaterial using spectroscopic techniques in order to confirm the presence of EVs within the biomaterial system [100, 256, 269]. Chemical analysis is useful for confirming that the EVs have been successfully incorporated into the system, however this analysis does not elucidate the distribution of EVs within the biomaterial. More detailed analysis methods are frequently employed by relevant studies, including fluorescent imaging and SEM. In the studies that perform fluorescent imaging of EV-biomaterial systems, the EVs are clearly visible and in most cases a good distribution and near complete coverage of EVs within the biomaterial is achieved [33, 215, 263, 265, 267, 270-272], although artifacts arising from shedding dye from the EVs labelled with lipophilic dyes cannot be excluded. SEM images of appropriate systems display nanoparticles on the material surface with the morphology typically displayed by EVs [33, 267, 271, 272]. However, SEM only shows a limited area of the system and should therefore only be used to support fluorescent imaging which can offer a broader image of EV distribution. More sophisticated methods of SEM-based imaging for EV detection can also be employed to examine EV distribution such as focused ion beam SEM [273] or density-dependent color SEM [161].

3.3.4 Release and retention of extracellular vesicles

The ability to achieve sustained release of EVs to the local target environment is one of the key advantages of EV-biomaterial systems. A large proportion of studies that develop EV-biomaterial systems characterize the release profile of EVs from the biomaterial (see

Supplementary table 3). A wide range of EV release rates have been reported; the fastest reported rate is 100% release after 14 days [124], while the slowest rate is 60% release after 36 days [100]. Similarly, a wide range of release profiles have been reported. The release profiles range from burst release of 60% in 2 days [274] to near perfect linear release of 10% over 14 days [275]. For EV-hydrogel systems, the release profile of EVs has been observed to follow the swelling profile of the gel [265]. This observation points towards diffusion-based release of EVs from hydrogel systems and presents the potential for tailoring EV release by altering the hydrogel swelling properties. Future studies should aim to characterize the entire release window of EVs from the biomaterial to observe the effect of material degradation. Furthermore, efforts should be made to increase the representativeness of *in vitro* assays such as including appropriate enzymes.

Sophisticated methods of incorporating EVs into biomaterial systems have been explored to better retain EVs within the system or to trigger EV release under specific conditions. It should be noted that around half of the studies that explore methods of EV retention are intended for vascular implants where physiological flow risks washing away the EVs [263, 267, 270]. Chen *et al.* crosslinked a vascular matrix scaffold with PEI/GNP via EDC to allow for binding of EVs [270]. Retention of EVs on the vascular graft was examined under physiological flow conditions using fluorescent imaging. The presence of PEI/GNP was shown to increase EV retention after 24 hours relative to the scaffold alone. Cunnane *et al.* incorporated EVs into a tubular silk fibroin scaffold using a rotational-vacuum device and examined the retention of EVs using fluorescent imaging following the application of physiological flow conditions for 1 hour [267]. EV coverage of the scaffold did not significantly decrease after 1 hour of flow, demonstrating the ability of the system to retain EVs. Wei *et al.* loaded EVs onto heparin-modified PCL graft surfaces [263]. The presence of heparin was shown to enhance the stability of EVs *in vivo* using an IVIS, as evidenced by increased retention of EVs to the graft surface over time. The stabilizing effect of heparin on EVs is attributed to heparin's high-affinity interactions with various lipid- or membrane-binding proteins. Li *et al.* coated PLGA scaffolds with pDA to adhere, carry, and retain EVs, with the aim of enabling a slow and local release profile of the vesicles [33]. The inclusion of a pDA film on the PLGA scaffold increased loading efficiency of EVs within the system from 29% to 66% of loaded EV protein content, and increased the release window of EVs from 3 days to beyond 8 days. Wang *et al.* retained EVs in a HA-based hydrogel by exploiting the electrostatic interactions that occur between negative potential EVs and the positive potential poly-ε-L-lysine used to crosslink the hydrogel [276]. The EVs were then released in a weak acidic environment due to breaking of the poly-ε-L-lysine linkages, resulting in pH-responsive long-term EV release. Henriques-Antunes *et al.* crosslinked a HA-based hydrogel with a photocleavable linker that had previously been attached to the thiol-groups of EVs, thereby forming an injectable EV-containing light disassembling hydrogel for the controlled release of EVs [262]. The release of EVs, determined using an IVIS, was shown to be dependent on gel irradiation time and the number of gel irradiations with UV light, with released EVs remaining bioactive. Future studies aiming to better retain and more controllably release EVs from biomaterial systems can build upon the techniques outlined in these studies. Particular attention should be afforded to characterizing the bioactivity of EVs following release as Nikravesh *et al.* [215] observed changes in EV size distribution following release from hydrogel systems, indicating that EVs may be coated with gel material upon release, which could obscure important surface molecules.

4. Clinical application of extracellular vesicles for tissue repair and regeneration

The knowledge accumulated over the last decades on EVs biogenesis, secretion and biological roles in physiologic and pathological conditions [146] have highlighted them as very promising biologic drugs (or biologics) for the treatment of a variety of human diseases. This has prompted the testing of EVs in the clinical setting in small studies. The seminal

work of Escudier *et al*. further motivated the clinical application of EVs, by demonstrating the safety of EV administration in humans and setting the standard for EV dosing in further studies [277]. Currently, dozens of studies involving EVs are registered in the clinical trial databases worldwide (clinicaltrials.gov; clinicaltrialsregister.eu; australianclinicaltrials.gov.au), but the majority is intended to monitor circulating EVs as biomarkers of disease, disease progression and effectiveness of applied treatments. Many of the studies dedicated to investigating the application of EVs as therapeutic agents are aimed at cancer treatment, with fewer studies applying EVs for tissue repair and regeneration (Table 2). Most clinical studies evaluating the regenerative potential of EVs are in Phase I or Phase II, aiming mainly at establishing the safety and efficacy of the EVs used in the treatment. Importantly, not all these studies are controlled, limiting the assessment of the true degree of EV treatment efficacy in human subjects. Autologous or allogeneic MSCs are the cell type preferentially used for EV production, and parameters indicative of inflammatory regulation are part of the primary outcomes for most of these trials. Interestingly, the urgency of the Covid-19 pandemic has prompted the initiation of several clinical trials using MSC-derived EVs aiming to ameliorate the severe inflammatory reactions many patients experience after infection with the SARS-CoV-2 virus [278]. Results of these trials will undoubtedly contribute to further understanding the real clinical value of EVs for immunomodulatory applications, including the efficacy they might have in the regulation of the inflammatory phase occurring after tissue injury. For instance, in one of these trials (Table 2) [279], EV inhalation significantly reduced the levels of C-reactive protein in circulation in Covid-19 patients, compared to placebo-treated patients. In addition, these studies will also provide evidence to evaluate the safety of EV administration in humans, and important information about dosing regimens (dose administered; interval of administration; route of administration) that can be more effective for future application in the regulation of the immune system in the context of a tissue injury. The immunomodulatory role of EVs was also demonstrated in another study where platelets and EVs were used to treat the inflammation associated with periodontitis (Table 2). The local administration of this biologic markedly reduced the inflammation area, compared with standard conservative treatments using antimicrobials and antiseptics.

[Insert Table 2 here]

4.1 The market for extracellular vesicles in tissue repair and regeneration

The excitement around the therapeutic potential of EVs is reflected by the increasing number of biotechnology and pharmaceutical companies introducing EVs in their portfolios or being founded to explore their clinical application. In Table 3 we summarize the main companies currently developing EV-based therapeutic products with indication for tissue repair/regeneration in the context of different diseases. Most of these products are still in the development or pre-clinical phase and comprise either naive vesicles from selected cell sources or vesicles engineered to carry therapeutic proteins and/or RNA cargo payloads. The principal strategy for the development of these engineered EVs is to rely on the genetic manipulation of producing cells to overexpress cargo to be enriched in the vesicles. On the other hand, Anjarium Biosciences is developing a novel hybrid nanoparticle where EVs are loaded by fusion with synthetic lipid vehicles carrying the cargo of interest [280]. Stem cells, particularly MSCs, seem to be one of the most selected cell types as the source of the EVs used in these therapeutic products, and following the observations in the *in vivo* studies described above, the rationale for this choice is mostly related to their broad immunomodulatory properties. In fact, neurologic diseases and injuries with associated exacerbated inflammatory processes, as well as autoimmune diseases are among the conditions for which more EV-based products are under development. Genetic diseases are also among the conditions more promising for the early use of EVs in therapeutics, particularly of engineered EVs, since these naturally-secreted vesicles are thought to be safer delivery vehicles for RNA or gene editing tools compared to other synthetic

nanoparticles [281]. Currently, Capricor Therapeutics, Aegle Therapeutics, United Therapeutics, and Exopharm are conducting clinical trials using EVs as the biological entity for tissue repair (Table 2) with most of the remaining companies predicting the first human studies in the next few years. On the other hand, Infusio, Exocel Bio, ExoCoBio have already available treatment programs or commercially-formulated EV-based products. Interestingly, the regenerative properties of the EVs that constitute these products is also drawing attention for their application in the cosmetics field (Table 3).

[Insert Table 3 here]

4.2. Manufacturing of extracellular vesicles for clinical applications

With the increasing application of EVs for therapeutic purposes, it has become clear that the workflow implemented in the laboratory setting for *in vitro* and *in vivo* studies is not compatible with the requirements for the production and formulation of clinical-grade EVs. For the production of cell conditioned media-derived EVs, large-scale cell cultures are required and, consequently, high-throughput isolation methods that guarantee the purity of the isolated EVs have to be used, all implemented under Good Manufacturing Practice (GMP) conditions. Furthermore, a consistent composition and potency of the EVs from each lot produced needs to be guaranteed. To fulfill this market need, companies specialized in the development of solutions for the production of clinical-grade EVs have recently surged, such as RoosterBio, Lonza/Exosomics S.p.A, VivaZome, EVerZom, Kimera Labs, CEVEC Pharmaceuticals, amongst others. Some of these companies are specialized in the isolation/generation, expansion and cryopreservation of cells at large scale, mainly of MSCs that secrete EVs at high yields and with consistent batch-to-batch composition. Others are focused on the development and implementation of methodologies for EV isolation or dedicated to perform a comprehensive analysis of the isolated EVs using omics approaches and testing their potency *in vitro*.

To produce the quantities of EVs generally required for a therapeutic application, large scale xeno-free cell cultures are required. Many studies exploring up-scaling of EV production in GMP conditions still use multilayered cell factories for cell culture, however this approach is not cost-effective. For adherent EV-secreting cells, seeding an initial high number of cells in hollow-fiber bioreactors and using culture media supplemented with human platelet-derived lysates is one of the most commonly used approaches for this purpose [282, 283]. Nonetheless, other systems being developed for large-scale cell culture will likely start being increasingly used for EV production as well, such as the stirred tanks with adherent cells seeded on microcarriers [284], and the bioreactors harboring 3D cell spheroids [58]. EVs are then commonly isolated by ultrafiltration, size-exclusion chromatography or to a less extent ultracentrifugation [282, 283, 285]. Conditions of cell culture and vesicle isolation can be further optimized to better fit each EV production model, however a landmark study in the field has previously shown that the yield of EVs obtained can vary by more than 50% over time, increasing production costs [282]. In addition, the isolation methods used for EV purification co-isolate other soluble contaminants, which may have therapeutic effects, but further compromise the definition of the composition of the EV product obtained. Historically, in the laboratory setting EVs have been preserved frozen in physiologically buffers. Likewise, at the large-scale level isolated EVs are also commonly formulated in sterile liquid suspensions in PBS, but formulations in alternative dispersants were also previously reported [286]. These suspensions are usually preserved frozen, typically at -80 ˚C, but lyophilization was also shown to be an effective strategy to preserve EVs (ASCE+ in Table 3 [286]). Nonetheless, more comprehensive studies comparing the effect of storage conditions in composition and, more importantly, function of EVs intended for clinical applications are still needed. This will help to define standard margins of EV loss and loss-of-function that still guarantee the application of an effective clinical product.

Due to the complexity and heterogeneity of EV preparations [287], a crucial point in the production of clinical-grade vesicles formulations is their quality control. Besides the assessment of standard parameters like vesicle size, morphology and enrichment of EV protein markers (e.g., CD63, CD81, CD9), total protein and RNA composition of the EVs isolated is often routinely assessed by proteomics and transcriptomics analysis, contrary to standard procedures in a laboratory setting. Alternatively, more limited EV analysis across batches has been reported based on the monitoring of the levels of signature molecules characteristic of the parental cells EVs are originated from, as a measure of EV preparation purity [282]. Moving forward, evaluation of batch-to-batch variation of EVs produced will likely rely more on the quantification of those molecules anticipated to mediate their functional role. In fact, one of the most important aspects that needs to be tested across different batches of EV preparations is their potency, in order to guarantee the production of an effective and homogeneous product over time. EV potency can be tested in *in vitro* functional assays, usually defined according to the final application and intended function for the EVs. For instance, MSC-derived EV products currently under development are commonly tested for their immunomodulatory properties *in vitro*, since many of them are intended for tissue repair/regeneration via control of inflammation. Protocols to determine and routinely test the mechanism of action underlying the functional effects observed should be further implemented for the validation of EV preparations isolated.

4.3 Regulation of therapeutic applications of extracellular vesicles

The development of any new pharmaceutical product involves the definition of the chemistry, manufacturing and control (CMC) of that product. This information is the basis of the documentation submitted to regulatory authorities for the approval of the novel product, and comprises every aspect of its development, from their active compounds composition, process of manufacturing at scale, mechanisms of action, biodistribution and toxicologic profile *in vivo*. Being biologics by nature, EVs are subjected to the specific CMC regulatory guidelines governing this class of medicines, including every step from the manufacture of such medicine to the analysis of their safety, efficacy and life cycle. Nonetheless, the complexity of EV composition prevents the definition of active compound(s) and their mechanism of action, and this most often prevents their categorization into a specific subtype of biologics [288], resulting in an ambiguous regulatory scenario. Furthermore, the approval of biologics for therapeutic use depends on the demonstration and definition of safety and efficacy parameters. However, compelling evidence of the efficacy of EVs to promote tissue repair and regeneration in human studies is still lacking. Learnings from other fields with more urgent need of a suitable treatment that justify the application of EVs in humans, such as the recent Covid-19 pandemic, and non-treatable cancers, will contribute to move EV therapies further into the tissue regeneration field. The EV market seems to be evolving ahead of regulatory authorities and objective regulations, with an increasing number of EV-based products coming closer to commercialization. This scenario has led in the past to the reports of EV-based product administration in humans with serious consequences for their health and well-being, with FDA raising the awareness to the risk of such practices [289].The development of EV-specific regulation guidelines or the adaptation to the EV field of existing biologics-regulating guidelines is needed and should be fast pursued. A closer engagement of biotechnology and pharmaceutical companies exploring EV-based regenerative therapies and the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, together with the scientific recommendation of experts and societies in the EV field could set the ground for the regulation of the EV market in clinical applications.

5. Conclusions

Research conducted to uncover the therapeutic potential of EVs has proven to be key for the consensual proposal of these cell-secreted products as regenerative mediators, particularly of those secreted by MSCs. However, how EVs promote tissue regeneration and what drives their regenerative effect is still far from elucidated. A range of selected molecules, particularly miRNAs and growth factor proteins or mRNAs, have been proposed as the natural mediators of EV regenerative capacity. These properties have been further enhanced by EV engineering with specific RNAs and proteins, which improved regenerative properties and increased therapeutic efficacy. Both *in vivo* and *in vitro,* EVs are thought to mediate tissue repair by promoting cell proliferation, differentiation and angiogenesis, and by inhibiting apoptosis and inflammation, although dissecting their exact effects, particularly in relation to the administered dose, is in most cases impossible. One of the factors that most likely contributes to the unknown mechanism of action of EVs in tissue regeneration is the lack of a wider application of large scale assays characterizing EVs content and functional activity, with most studies focusing on the characterization of selected cargo and biological functions. Another factor is the lack of standardized EV isolation methods that lead to the purification of defined EV subpopulations, with most works testing *in vitro* and *in vivo* a mix of different vesicle types. New subpopulations of vesicles [290, 291] and even non-membranous particles [292, 293] secreted by cells have been recently described and it is expected that further subpopulations of cell-secreted mediators are more finely characterized with increasing technological advances. A wider application of single vesicle analysis techniques in routine EV characterization assays dissecting the specific cargo of distinct EV subpopulations, and consequently their specific biological roles, would greatly help to better define the mechanism of action of EV preparations used for tissue repair and regeneration. The development of more specific methods of isolation that allow the purification of defined EV subpopulations should still be pursued in the field.

Interpretation of the regenerative effects mediated by EVs *in vivo* is even more difficult than in controlled functional assays *in vitro* due to their complex pharmacokinetics and -dynamics. As for conventional drugs, EVs can be eliminated by common systemic clearance mechanisms, but more importantly they establish many interactions with the immune system, which further influences not only their mechanism of action but also their biodistribution and the effective EV dose that reaches target tissues. It is still puzzling how the apparently low levels of EVs reaching tissues of interest promote a regenerative effect, when compared to doses most effective *in vitro*. This could be due to the pleiotropic effects these vesicles can have systemically in multiple cell types or the limitations of the *in vitro* assays themselves. To increase the efficiency of therapeutic dose delivery to a specific tissue, the natural tissue targeting feature of EVs has been explored and further improved by their surface functionalization with targeting moieties. Another promising strategy is the local administration of EVs integrated in different biomaterials, which allow further control of dosing parameters such as the controlled and time-sustained release of the vesicles at target sites. The potential of EVs is reflected by the increasing interest they generate in pharmaceutical and biotechnology companies worldwide. Further rigorous studies to determine the true therapeutic potential of EVs and their biological activity *in vivo* will drive evidence-based application of EVs in human patients.

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Declaration of interest

AMS and ND are employees of AstraZeneca R&D.

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