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REVIEW

SUBJECT COLLECTION: CELL BIOLOGY AND DISEASE

Made by cells for cells – extracellular vesicles as next-generation mainstream medicines

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ABSTRACT

Current medicine has only taken us so far in reducing disease and tissue damage. Extracellular vesicles (EVs), which are membranous nanostructures produced naturally by cells, have been hailed as a next-generation medicine. EVs deliver various biomolecules, including proteins, lipids and nucleic acids, which can influence the behaviour of specific target cells. Since EVs not only mirror composition of their parent cells but also modify the recipient cells, they can be used in three key areas of medicine: regenerative medicine, disease detection and drug delivery. In this Review, we discuss the transformational and translational progress witnessed in EV-based medicine to date, focusing on two key elements: the mechanisms by which EVs aid tissue repair (for example, skin and bone tissue regeneration) and the potential of EVs to detect diseases at an early stage with high sensitivity and specificity (for example, detection of glioblastoma). Furthermore, we describe the progress and results of clinical trials of EVs and demonstrate the benefits of EVs when compared with traditional medicine, including cell therapy in regenerative medicine and solid biopsy in disease detection. Finally, we present the challenges, opportunities and regulatory framework confronting the clinical application of EV-based products.

KEY WORDS: Extracellular vesicles, Next-generation medicine, Precise diagnostic biomarkers, Regenerative medicine

Introduction

Current medicine has only taken us so far in beating disease and tissue damage. Extracellular vesicles (EVs) have been lauded as the next generation of medicine (Bazzan et al., 2021). EVs are lipid membrane-surrounded structures that are secreted by all cells. They contain hundreds of biomolecules that act synergistically and activate multiple cell types – made by cells, for cells (Tang et al., 2020). Conventional pharmacological interventions use single molecules with limited mechanisms of action, typically administered to the body in an untargeted, shotgun fashion. In contrast, EVs shuttle a 'cocktail' of biomolecules and influence specific cells and tissues in numerous and coordinated ways (Tang et al., 2020). Harnessing this pluripotent attribute of EVs would revolutionise the way tissues damaged by injury or disease (such as acute and chronic lung injuries, chronic wounds, fistula, ischemia and myocardial infarction) are treated and repaired.

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To date, several subtypes of EVs have been identified, which include exosomes, microvesicles, microparticles, ectosomes, oncosomes, apoptotic bodies and exomeres (Gurunathan et al., 2021). These subtypes are determined by the origin, pathway of secretion and size (Fig. 1). One major subtype comprises relatively large vesicles, typically ranging between 100 nm and 1 µm in diameter, referred to as microvesicles. Microvesicles are generated at the plasma membrane and are also known as shedding vesicles or ectosomes, or as oncosomes when they are shown to contain transforming oncogenic cargo (Desrochers et al., 2016). The biogenesis of microvesicles is regulated by ARF6 and RHOA-dependent rearrangement of the actin cytoskeleton, which promote the outward budding and shedding of microvesicles from the plasma membrane (Li et al., 2012). Another subtype of EVs, referred to as exosomes, comprises smaller vesicles ranging from 30 nm to 150 nm in diameter. These smaller vesicles are formed as intraluminal vesicles (ILVs) within endosomal multivesicular bodies (MVBs) and are released from cells upon the fusion of MVBs with the plasma membrane (Harding et al., 1983). The trafficking of MVBs to the plasma membrane is regulated by Rab guanosine triphosphatases (GTPases), including Rab7A, Rab11A, Rab11B, Rab27A, Rab27B and Rab35 (Tai et al., 2018). Apoptotic bodies are another subtype of EVs; they range from 1000 nm to 5000 nm in diameter and are typically released from membrane blebs of apoptotic cells (Caruso and Poon, 2018). Recently, cells have been found to release non-membranous nanoparticles that are less than 50 nm in size, which have been termed exomeres; however, the exact mechanisms involved in the biogenesis of exomeres are currently unknown (Zhang et al., 2018a). Since a consensus on the specific markers for each EV subtype has not yet emerged, and because there is overlap between the size ranges of these vesicles, it has been suggested that the generic term 'EVs' be used for all lipid bilayer-enclosed particles that are naturally released from cells and unable to replicate (Thery et al., 2018) (Fig. 1).

EVs play essential roles in intracellular communication and tissue homeostasis (Nagelkerke et al., 2021). The potential for EVs to revolutionise medicine is evident in both the number of clinical trials that involve EVs and the exponential growth of government, industry and private investments, which exceeds investments in any other area of medicine (https://bioinformant.com/product/exosome-market-report/). We have analysed the US National Library of Medicine clinical trial database (www.clinicaltrials.gov; accessed on 19 July 2021) and have found that, currently, 83 applications are registered within the study object 'extracellular vesicles' and 223 within the study object 'exosomes'. Of these, 70 studies are examining extracellular vesicles as biomarkers and 13 studies are using extracellular vesicles as therapeutics. Regarding exosome-based products, 191 studies are evaluating exosome-based diagnostic tests, and 32 studies are testing exosome-based therapeutics. Currently, the EV field is one of the fastest growing

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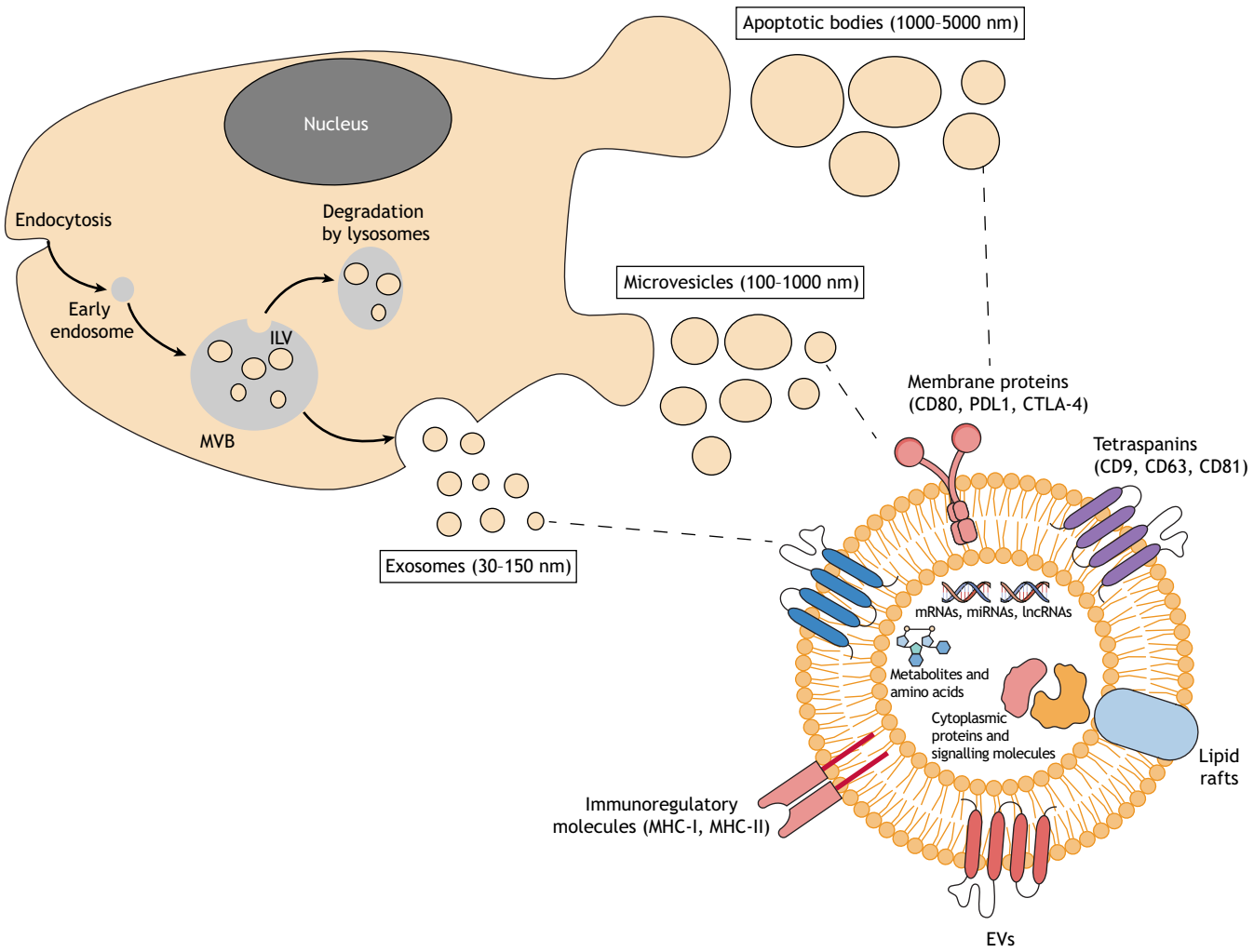

Fig. 1. Schematic of EV biogenesis and the molecular components of EVs. The biogenesis routes of three main types of EVs – exosomes, microvesicles and apoptotic bodies – are illustrated. Exosomes (30–150 nm) arise from late endosomes and form as ILVs within MVBs. The resulting MVBs are either degraded by lysosomes or secreted as exosomes. Microvesicles (100–1000 nm) arise by direct budding or exocytosis of plasma membrane, and apoptotic bodies (1000–5000 nm) are generated by blebbing of the plasma membrane during apoptosis. The key molecular components of EVs are lipids, proteins and nucleic acids, which depend on the biogenesis route, the source and the pathological stage of the parent cell. CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; MHC, major histocompatibility complex.

scientific areas, with more than 18,000 EV-focused articles found on PubMed (https://pubmed.ncbi.nlm.nih.gov/), of which 74% have been published in the last five years (https://bioinformant.com/product/exosome-market-report/). This number is growing exponentially and is likely to quadruple in the next five years.

Since the composition of EVs is finely tuned by the individual cells that secrete them in response to the local microenvironment and various stimuli that act on cells (Bazzan et al., 2021), there are two key areas emerging for the use of EVs: next-generation therapeutic agents and early disease diagnosis biomarkers with a high specificity. Here, we outline the advantages of EVs in these two areas, provide insights into their mechanism of action and present some examples of EV-based products in clinical trials. We also identify challenges that hinder progress towards clinical applications of EVs, and we describe emerging evidence of translational progress of EV-based products in preclinical and clinical applications.

EVs as next-generation therapeutics for tissue regeneration

The tissue-reparative function of EVs in processes such as angiogenesis, lineage-specific differentiation, regulation of immune responses and extracellular matrix organisation is attributed to the action of the multiple biomolecules that EVs carry and deliver to recipient cells to selectively stimulate multiple signalling pathways (Tang et al., 2020). For example, transfer of the microRNA (miRNA) miR-126 and proteins (VEGF and MMP-2) from EVs released from adipose-derived stem cells (ASCs) downregulates Spred1 and activates ERK1 and ERK2 (ERK1/2; also known as MAPK3 and MAPK1, respectively) mitogen-activated protein kinase (MAPK) pathways in endothelial cells, triggering angiogenic signalling (Togliatto et al., 2016). Another study has shown that EVs released from ASCs also contain miR-31, which can promote angiogenesis in endothelial cells by downregulating the anti-angiogenic gene *FIH1* (also known as *HIF1AN*; Kang et al., 2016). EVs influence various signalling pathways in recipient cells either through the release of cargo or the activation of specific cell-surface receptors on the recipient cells. EVs enter the recipient cells by endocytosis and release their cargo into the cytosol of the recipient cells through membrane fusion (Joshi et al., 2020).

EV composition is closely linked to the composition and physiological state of their parent cells. Thus, EVs produced by

mesenchymal stem/stromal cells (MSCs), which are used in regenerative medicine (stem cell-based therapy), inherit regenerative capabilities from the MSCs from which they are derived (Keshtkar et al., 2018). MSCs have been shown to successfully regenerate various tissues, including muscles, nerves, myocardium, liver, cornea, trachea and skin, in preclinical and clinical trials (Han et al., 2019). Indeed, MSCs can improve heart disease by promoting cardiomyogenic differentiation and preventing ischemic cell death (Siciliano et al., 2015). However, one study has shown that the occurrence of cardiomyogenic differentiation is not significant and that the effects of MSCs may be more limited than initially thought (Noiseux et al., 2006), suggesting that the regenerative effects of MSCs are mediated by the EVs they release (Hodgkinson et al., 2016). Indeed, bone marrow MSC (BM-MSC)-derived EVs have been shown to exhibit a similar cardioprotective potency as BM-MSCs themselves (Shao et al., 2017). For instance, transfer of miR-182 by EVs derived from BM-MSCs to macrophages can inhibit toll-like receptor 4 (TLR4) and mediate macrophage polarisation, which are key factors for cardiac repair (Zhao et al., 2019). Therefore, EV-based therapies are now considered a second-generation stem cell-based therapy, as they avoid problems associated with the use of stem cells, such as only low numbers of cells reaching target sites and risk associated with tumorigenesis after stem cell transplantation (Musial-Wysocka et al., 2019). Thus, in the context of regenerative medicine, EV-based therapies offer substantial advantages, including (1) rapid uptake and fast, transient action, (2) inability to self-replicate (reduced risk of tumorigenesis) (Racchetti and Meldolesi, 2021), (3) low immunogenicity and toxicity, and (4) resistance to hostile environments (such as the acidic pH in certain tissues; Ban et al., 2015) and maintenance of their activity during storage (Tang et al., 2020).

Given their numerous benefits, EVs released from MSCs have been widely used in various therapeutic applications, including the treatment of wounds, osteoarthritis, kidney injury, liver fibrosis, hepatic failure and lung injury (Tsiapalis and O'Driscoll, 2020), with particular interest in the improvement of skin regeneration during wound healing (Fig. 2). For instance, transfer of miRNA (specifically miR-125a) from EVs derived from ASCs to human umbilical vein endothelial cells (HUVECs) enhanced their angiogenesis by targeting the angiogenic inhibitor delta-like 4 (DLL4) signalling pathway (Liang et al., 2016). EVs derived from ASCs also activate the Akt signalling pathway, which promotes the migration and proliferation of dermal fibroblasts and keratinocytes (Ferreira et al., 2017). Furthermore, the long noncoding RNA (lncRNA) MALAT1 in these EVs promotes fibroblast migration and wound healing (Cooper et al., 2018). Moreover, EVs released from human umbilical cord MSCs promote differentiation of human macrophages (monocytic cell line THP-1) to the anti-inflammatory M2 phenotype and convert activated T lymphocytes into the T-regulatory phenotype, thereby exerting immunosuppressive effects (Ferreira and Gomes, 2018). In addition, human umbilical cord MSCs preconditioned with lipopolysaccharide (LPS) release EVs that can activate anti-inflammatory M2 macrophages in the inflammatory state, hence reducing inflammation while maintaining a homeostatic microenvironment for tissue repair (Ti et al., 2015). These 'specialised' EVs are characterised by an increased amount of miRNA let-7b, which is known to regulate macrophage polarisation by attenuating the TLR4–nuclear factor-κB (NF-κB) pathway and activating the Akt–STAT3 pathway (Teng et al., 2013) (see Table 1 for more detail).

In summary, EVs thus provide a new therapeutic option to regenerate tissues damaged by injury or disease. Advances in the fundamental understanding of EV biogenesis and its fate in biological systems, as well as new developments in technologies to produce therapeutic EVs, such as different approaches to scale up production of EVs (for example in bioreactors; Ng et al., 2019), have led to substantial progress in moving EVs to clinical trials. For example, companies including ExoCoBio (South Korea), ExoPharm (Australia) and Exogenus Therapeutics (Portugal) are currently conducting clinical trials of their proprietary EVs for skin regeneration purposes (Nagelkerke et al., 2021). ExoCoBio already have an available commercially formulated EV-based product consisting of exosomes derived from ASCs, called ASCE+, for the treatment of atopic dermatitis (AD) (Shin et al., 2020). ASCE+ has been shown to normalise altered epidermal gene expression that occurs in AD and to stimulate the production of epidermal ceramides, enhance keratinocyte differentiation and contribute to the formation of a proper epidermal barrier in an AD mouse model (Shin et al., 2020). ExoPharm is investigating the safety and efficacy of exosomes from platelets (PLEXOVAL) for wound healing in phase I clinical trials (Nagelkerke et al., 2021). Similarly, Exogenus Therapeutics has developed a biological consisting of small EVs (less than 200 nm diameter) derived from umbilical cord blood cells (Exo-Wound) for the treatment of chronic wounds (Nagelkerke et al., 2021; Cardoso et al., 2021). These EVs produce anti-inflammatory effects in a mouse model by downregulating inflammatory genes, including those encoding iNOS (also known as NOS2), IL-6, TNF and CXCL1, and upregulating proteins associated with anti-inflammatory M2 macrophages (CD163 and arginase-1) in wounds at day 3 of treatment (Cardoso et al., 2021). In addition, these EVs have a positive effect on skin tissue remodelling, which is associated with increased cell proliferation, cell adhesion and extracellular matrix proteins (such as collagens and fibronectin) at day 15 of treatment. Exogenus Therapeutics have also developed a scalable method for the isolation of these EVs (Cardoso et al., 2021). However, this product is still in the early stages of development, and further research is required before it enters clinical trials.

Use of EVs for osteogenesis and bone remodelling

Osteogenesis and bone remodelling is another area where EVs show a significant potential. A stimulation of osteogenesis and control of bone remodelling can be achieved by several miRNAs that are present and enriched in EVs, which act on multiple pathways to promote the production of bone morphogenetic proteins (BMPs), regulate RUNX2 (the key transcription factor in osteogenic differentiation) and modulate the Wnt–β-catenin signalling pathway. There are several mechanisms by which miRNAs that are found in EVs secreted by BM-MSCs promote osteoblast differentiation, specifically: (1) miR-15b targets and downregulates WWP1, attenuating KLF2 degradation and inhibiting NF-κB in an ovariectomised rat model, which results in an increase in osteogenic differentiation of BM-MSCs *in vitro* and the attenuation of bone loss *in vivo* (Li et al., 2020); (2) miR-20a directly binds to the 3′ untranslated region (3′UTR) of BAMBI, counteracting its inhibitory effects on osteogenic differentiation of BM-MSCs, and regulates the expression of osteogenic genes encoding BMPs and RUNX2 in an osteoporotic rat model, thereby promoting osteogenesis (Liu et al., 2021b); (3) miR-29b-3p blocks SOCS1-mediated inhibition of the NF-κB pathway by inhibiting KDM5A in an ovariectomised mouse model of osteoporosis (Zhang et al., 2021). The increased expression of KDM5A, which is found in

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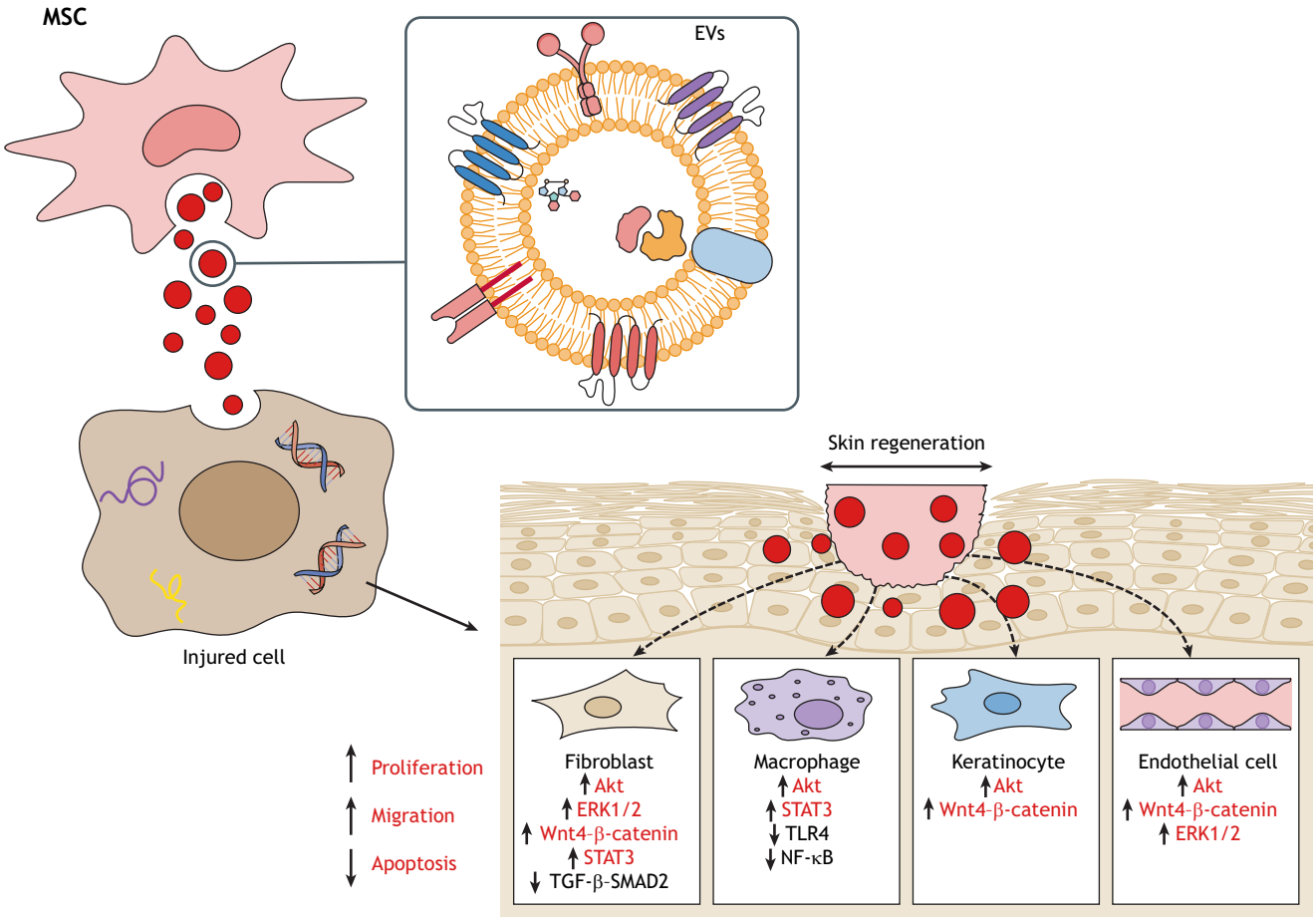

Fig. 2. Schematic illustration of the role of EVs derived from MSCs in the skin regeneration process. By transferring multiple therapeutic cargos to the chronic wound, EVs derived from MSCs can activate multiple pathways in the recipient cells (fibroblasts, macrophages, keratinocytes and endothelial cells). In recipient cells, the signalling pathways for proliferation and migration are stimulated (Akt, ERK1/2, Wnt4–β-catenin and STAT3), whereas those inducing apoptosis are inhibited (TGF-β–SMAD2, TLR4, NF-κB). The specific EV components that exert these effects are listed in detail in Table 1.

osteoporotic patients, inhibits the expression of SOC1 by reducing histone H3 lysine 4 trimethylation (H3K4me3) and lysine 27 acetylation (H3K27ac) in the SOC1 promoter region; this results in loss of the suppressive effect of SOCS1 on the NF-κB pathway and leads to osteoporosis. Therefore, a decrease of KDM5A expression by transfer of miR-29b-3p can inhibit the NF-κB pathway, leading to osteoporosis reversal and potentiated osteogenic differentiation in a mouse model (Zhang et al., 2021).

In addition to miRNAs, EVs secreted by MSCs contain other biomolecules, such as proteins and mRNAs, that can promote osteogenesis and cartilage repair (Gao et al., 2018). For example, transfer of Fas protein from BM-MSC-derived EVs reduces intracellular miR-29b levels in a mouse model of lupus (Fas-deficient MRL/*lpr* mice), which enhances epigenetic regulation of Notch signalling and thereby promotes osteogenic differentiation (Liu et al., 2015). Therefore, the transfer of Fas protein has been shown reduce osteopenia and facilitate bone formation in MRL/*lpr* mice. Another study has shown that the expression of CD73 (also known as NT5E) on EVs secreted by MSCs can induce Akt and ERK1/2 signalling, which results in an increase in cellular proliferation and infiltration, enhancement of matrix synthesis, and the modulation of immune reactivity (production of inflammatory cytokines) in chondrocytes from female rats (Zhang et al., 2018c). These processes lead to osteochondral regeneration and subsequent cartilage repair. In addition, mRNAs, miRNAs and several angiogenic factors – such as MCP-1 (CCL2), MCP-3 (CCL7) and SDF-1 (CXCL12) – that are found in BM-MSC-derived EVs collectively contribute to bone remodelling in a mouse model (Furuta et al., 2016). Further study has revealed that the activation of MAPK signalling pathways in recipient osteoblasts could be a key factor in the osteogenic activity of EVs secreted by BM-MSCs (Zhao et al., 2018).

In summary, a unique and large library of miRNAs, proteins and mRNAs that are present in and on EVs can control and regulate osteogenesis by targeting different molecular pathways. However, the ability to produce EVs that contain the desired cargos remains a key challenge and is currently a subject of several funded projects around the world that aim to bring EVs to mainstream clinical applications. Amongst different funding bodies, the European Commission Horizon 2020 programme has provided one of the largest grants to multinational teams (Table S1). In the context of osteogenesis, one of the flagship European Union-funded programmes, EVPRO (Extracellular Vesicles Promoted Regenerative Osseointegration), has used EVs encased in hydrogel and immobilised directly on a nanostructured prothesis surface to enhance the integration and longevity of hip prostheses (http://www.evpro-implant.eu/). This project extends the work of Pansani et al., which focuses on the use of EV-coated titanium implants to reduce the risk of inflammation and promote bone formation (Pansani et al., 2021).

Table 1. Summary of therapeutic effects of EVs derived from three common MSC sources

EV source	Recipient cells	Therapeutic cargo	Affected signalling pathways	Functions	References
Human umbilical cord blood	Fibroblasts and endothelial cells	miR-21-3p	Activation of PI3K–Akt and ERK1/2 signalling by targeting phosphatase and tensin homolog (PTEN) and sprouty homolog 1 (SPRY1)	Promotion of proliferation and migration in fibroblasts; enhanced angiogenic activities of endothelial cells	Hu et al., 2018
Human umbilical cord MSCs	HUVECs	angiopoietin-2 (Ang-2)	Targeting of the angiopoietin–TIE signalling pathway; downstream pathways were not investigated	Promotion of proliferation, migration and tube-formation of HUVECs; promotion of angiogenesis	Liu et al., 2021a
Human umbilical cord MSCs	Fibroblasts and keratinocytes	Wnt4	Activation of Wnt4–β-catenin and Akt pathways	Inhibition of heat stress-induced apoptosis; promotion of proliferation	Zhang et al., 2015
Human umbilical cord MSCs preconditioned with LPS	Human monocytic cell line THP-1	miRNA let-7b	Suppression of TLR4–NF-κB signalling and activation of Akt–STAT3 pathway	Regulation of macrophage polarisation	Ti et al., 2015
Human umbilical cord MSCs	LPS-stimulated macrophages	miR-181c	Suppression of the TLR4 pathway, subsequently reducing NF-κB/p65 activation	Alleviation of inflammation	Li et al., 2016
Human ASCs	Fibroblasts	lncRNA MALAT1	Targeting of the Akt pathway	Stimulation of cell proliferation and angiogenesis	Cooper et al., 2018
Human ASCs	Epidermal keratinocyte-like cells (HaCaT cells)	miR-21	Increase in MMP-9 protein expression via the PI3K–Akt pathway	Improved migration and proliferation; accelerated wound healing process	Yang et al., 2020
Human ASCs	Human skin fibroblasts	lncRNA H19	Inhibition of miR-19b and upregulation of SOX9 in recipient cells to activate the Wnt4–β-catenin pathway	Accelerated cell proliferation, migration and invasion; promotion of skin wound healing in mice	Qian et al., 2021
Human ASCs	HUVECs	miR-125a	Decrease in DLL4 expression	Promotion of angiogenesis	Liang et al., 2016
Human ASCs	Fibroblasts, peripheral blood mononuclear cells (PBMCs), and IFNγ- and TNF-treated fibroblasts	miR-34a-5p, miR-124-3p, miR-146a-5p, miR-132, miR-21, miR-29a	Suppression of the Notch1 pathway, targeting of TNF, IL-24, Mef2c	Induced macrophage polarisation; promotion of extracellular matrix protein synthesis	Heo et al., 2021
Human BM-MSCs	HaCaT cells and human dermal fibroblasts	Not identified	Inhibition of TGF-β–SMAD2 signalling pathway	Promotion of cutaneous wound healing	Jiang et al., 2020
Human BM-MSCs	PBMC-derived macrophages	miR-223	Targeting of PKNOX1	Reprogramming of proinflammatory macrophages (M1) to anti-inflammatory macrophages (M2); accelerated wound healing in mice	He et al., 2019
Human BM-MSCs	Human normal and diabetic wound fibroblasts	STAT3	Activation of Akt, ERK1/2 and STAT3 signalling pathways	Enhanced growth and migration of recipient cells; increased tube formation of endothelial cells	Shabbir et al., 2015

EVs as biomarkers with high specificity

Since the cargo of EVs mirrors the molecular composition of the parent cells, EVs provide information about the pathophysiological state of the cell of origin (Boukouris and Mathivanan, 2015). Additionally, the biomolecular cargo inside EVs is stable in biological fluids and protected against exogenous RNases and proteases (Boukouris and Mathivanan, 2015). Notably, EVs are able to cross the blood–brain barrier (BBB), spread in peripheral blood and reach different organs and tissues (Marostica et al., 2020). EVs can enter the endothelial cells of the BBB by three different endocytic processes: receptor-mediated transcytosis (as occurs in the transport of transferrin), lipid raft-mediated uptake and micropinocytosis (Chen et al., 2016). For example, the transferrin receptor (TfR) found on EVs can bind to the TfR at the surface of endothelial cells. Next, EVs are trapped inside the clathrin-coated pit, and these coated vesicles are transported by cytoskeletal actin filaments and directed towards either the lysosomal pathway (for degradation) or endosomal pathway (for transfer to the plasma membrane and fusion with the membrane to release the cargo) (Heidarzadeh et al., 2021). Given their ability to cross the BBB, EVs derived from brain cells have been detected in various biological fluids, including blood, and thus can be used as potential biomarkers for diseases affecting the brain (e.g. glioblastoma, Alzheimer's disease and Parkinson's disease) (Galazka et al., 2018).

Because of their complex composition, EVs offer unique opportunities not only for early and precise detection of a disease, but also to obtain insights into the disease stage and its pathogenesis (Bongiovanni et al., 2021). For instance, a recent study has shown that EV miRNAs in synovial fluid are different in the joints of rheumatoid arthritis patients with high- and low-grade inflammation (Foers et al., 2021). Moreover, there is an upregulation of disease-associated miRNAs in brain-derived EVs in Alzheimer's patients, which has been exploited to establish the first early diagnostic blood test for a neurodegenerative disease (Cheng et al., 2020). Furthermore, analysis of the proteomes of EVs from blood plasma of preoperative glioblastoma grade II–IV patients has demonstrated that such analysis can detect the stage of the disease and track its progression, which was difficult or impossible to achieve otherwise (Hallal et al., 2020). In particular, the expression levels of T-

complex protein 1 ring complex (TRiC) protein subunits and chaperonin-containing T-complex proteins, including CCT2, CCT3, CCT4, CCT5, CCT7 and TCP1, are higher in glioblastoma plasma EVs compared to levels in healthy brain plasma EVs. Expression of CCT2 and CCT7 is high in malignant glioblastomas (glioblastoma grade IV, GBM/GIV) but lower in less aggressive glioblastoma subtypes, which indicates that these proteins can be used to assess disease severity (Hallal et al., 2020). Indeed, the increase in TRiC expression has been shown to relate to tumorigenesis and impact molecular pathways that contribute to tumour progression such as p53 (also known as TP53) and STAT3 activity (Roh et al., 2015; Vallin and Grantham, 2019; Kasembeli et al., 2014; Trinidad et al., 2013). Besides proteins, previous studies have also shown that miRNAs and small noncoding RNA can reveal the stage of tumour progression in glioblastoma. For example, the expression levels of miR-320, miR-574-3p, miR-301a and the small noncoding RNA RNU6-1 are higher in high-grade glioblastoma plasma EVs (Manterola et al., 2014). Some miRNAs in EVs released from primary glioblastoma cells, such as miR-451 and miR-21, have been found to mediate the aggressive properties of glioblastomas. The transfer of miR-451 and miR-21 from EVs released from primary glioblastoma cells to microglia increases the production of cytokines, chemokines and matrix metallopeptidases, which contribute to the growth of glioblastoma cells while lessening the immune response (van der Vos et al., 2016) (Fig. 3).

Currently, conventional solid biopsy is the gold standard for pathological diagnosis; however, this method is invasive and sometimes difficult to perform. Liquid biopsies are emerging as a less invasive, highly individualised and precise method for diagnosis and treatment (Macias et al., 2018). Among the different sources of liquid biopsies, EVs are ideal as they are found in almost all body fluids, including blood, urine, cerebrospinal fluid, saliva, pleural effusion, ascites fluid, amniotic fluid and breast milk, as well as bronchoalveolar lavage fluid (Zhou et al., 2020). Moreover, as EVs are secreted from living cells and reflect the pathophysiological stage of their cell of origin, they are more representative of the physiological state than, for instance, cell-free DNA, which can be produced during necrosis or apoptosis (Cai et al., 2015).

EVs derived from tumours are especially useful for the early detection of cancer, the assessment of prognosis, prediction of response to cancer treatment, as well as monitoring therapy (Bongiovanni et al., 2021). Particularly in the case of tumours in the brain or central nervous system (CNS), solid biopsy is more invasive and dangerous compared to the accessible and potentially rich sources of cancer biomarkers in tumour-derived EVs (Lane et al., 2018; Wang et al., 2019). Furthermore, as EVs can cross the BBB, EVs derived from CNS tumours can be detected in the circulation and in cerebrospinal fluid, which considerably facilitates the detection of tumours in such a difficult to access part of the body (Morad et al., 2019). In comparison to other liquid biopsy tests for cancer, such as those measuring circulating tumour DNA (ctDNA) or circulating tumour cells (CTCs), EVs offer several advantages, including their superior stability, sensitivity and specificity (Yu et al., 2021). For example, exosomal nucleic acid from plasma does not require preservatives after collection and can be stored for 2 days at 25°C or processed through many freeze–thaw cycles without significant degradation of DNA or RNA (Enderle et al., 2015). While ctDNA testing relies on a single type of analyte, EVs carry multiple analytes that are informative of the cancer state, including DNA, RNA, proteins, lipids, oligosaccharides and metabolites, which improves the sensitivity and specificity of early cancer detection (Yu et al., 2021). Indeed, EVs have been used as biomarkers to monitor the effectiveness of the therapy for numerous cancers, including lung cancer, prostate cancer, breast cancer, melanoma, lymphoma and glioblastoma (reviewed in Zhou et al., 2021). For example, the expression of miR200c-3p, miR-21-5p and miR-28-5p in circulating EVs is different between responders and non-responders to anti-PD-1 (also known as PDCD1) or PDL1 (CD274) therapy in non-small cell lung cancer, demonstrating a correlation between the miRNA content of EVs and immunotherapy outcome (Shukuya et al., 2020).

In preclinical tests, EVs have been shown to be effective as diagnostic biomarkers for diseases where early detection is critical, including cancer (prostate, breast, lung or bladder), Alzheimer's disease, Parkinson's disease, dementia, preeclampsia and autoimmune diseases (Boukouris and Mathivanan, 2015). The potential of EVs as highly specific biomarkers is evident from the US Food and Drug Administration (FDA) approval of the ExoDx Prostate IntelliScore test (EPI; a urine exosome gene expression assay) for the early detection of prostate cancer in men 50 years or older (Bongiovanni et al., 2021). This non-invasive urine test analyses the expression signature of three exosomal RNA transcripts (*ERG*, *PCA3* and *SPDEF*); it has been used by more than 50,000 patients in their decision process and is included in National Comprehensive Cancer Network guidelines for early detection of prostate cancer (Yu et al., 2021). However, thus far, only a few EV-based biomarker applications have been approved for commercialisation, as there are still numerous limitations impeding the routine application of EV-based testing in the clinics (Bongiovanni et al., 2021). For example, the isolation, differentiation and molecular profiling of tumour-derived EVs circulating in the blood is difficult due to the presence of other biomolecules, such as proteins, protein aggregates, free nucleic acids, cytokines and platelets, as well as EVs from different cells (Caby et al., 2005). Since circulating EVs are released from various cell types, unless they carry a distinct cargo, it is still a challenge to confirm their exact origin (Li et al., 2019b).

Despite these limitations, the promise of EVs as biomarkers is undeniable since they offer substantial benefits compared with conventional biopsies. One example is the risk of false-positive results in prostate cancer when measuring the level of prostate-specific antigen (Jahn et al., 2015). Since EVs offer a unique repertoire of biomarkers that can assess the molecular status of a tumour in real time, they present new opportunities to develop diagnostic tests to detect diseases early on, track disease progress and help understand disease aetiology.

Challenges and progress in clinical application of EVs

Despite the undoubted potential of EVs to transform medicine, there are some limitations that hamper the progress of EVs into clinical practice. These include (1) a lack of reliable methods for mass production, isolation and purification of EVs; (2) a lack of consensus regarding the quality control of EVs; (3) inadequate understanding of pharmacokinetics and pharmacodynamics of EVs; (4) limited understanding of the fate of EVs in different biological systems including *in vitro* and *in vivo* models; and (5) insufficient data on the safety profile of EVs.

Regarding the first issue, there is currently no widely adopted protocol for the isolation of EVs in large quantities for clinical use. Indeed, different research laboratories employ different protocols for EV isolation, purification, quantification and characterisation (Saenz-Cuesta et al., 2015). Since different isolation methods result in different subpopulations of isolated EVs, it is essential to

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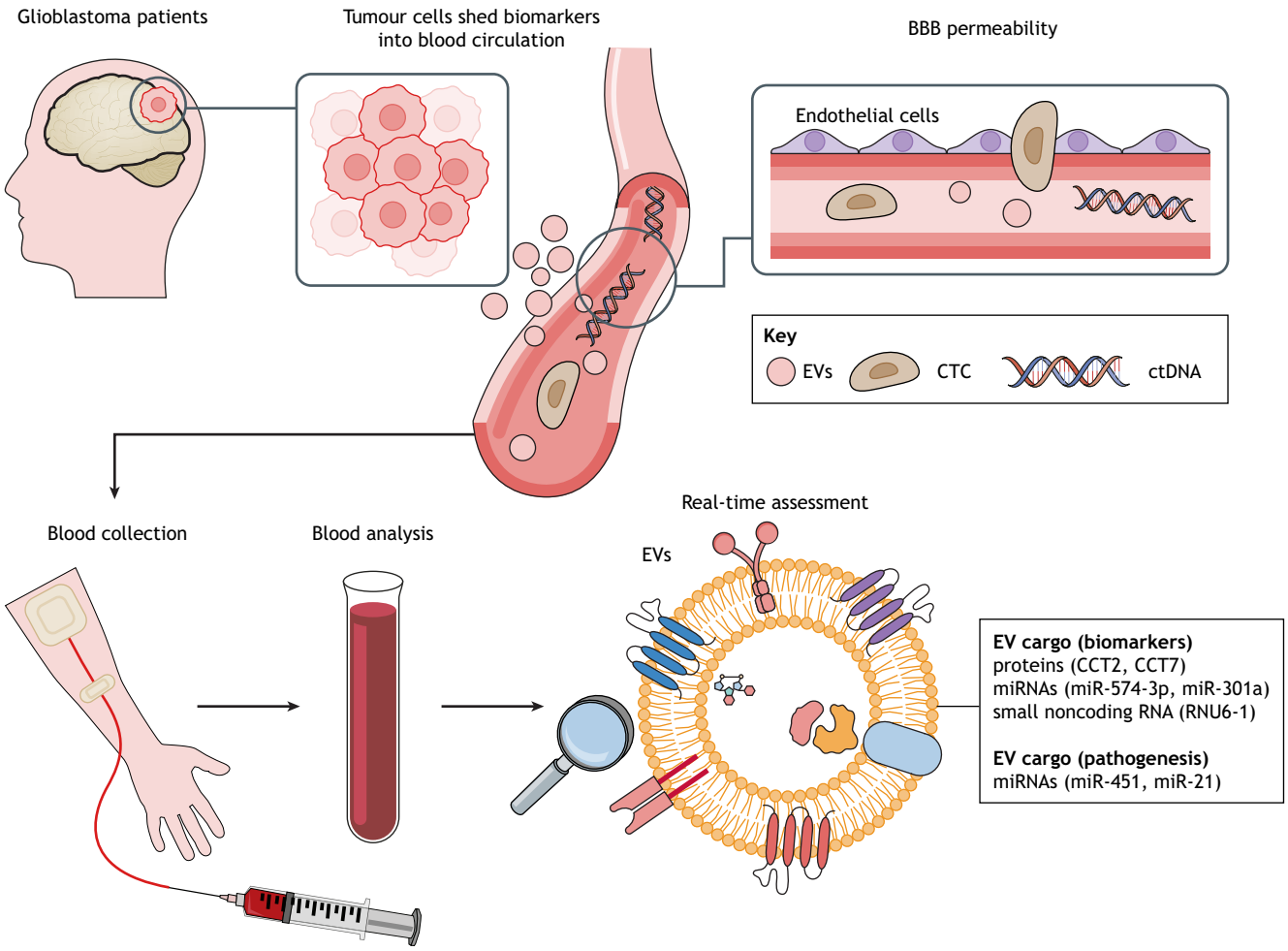

Fig. 3. Illustration of the potential use of EV cargos as biomarkers to diagnose glioblastoma patients. In glioblastoma patients, a disrupted BBB allows any biomarkers, such as CTCs, ctDNA and EVs, to enter the blood circulation system. Blood samples from patients can be easily collected and further analysed for disease diagnosis. Several components of EVs derived from the plasma of patients have been identified as potential biomarkers for disease progression (CCT2, CCT7, miR-320, miR-574-3p, miR-301a and RNU6-1). Furthermore, some miRNAs (miR-451 and miR-21) have been shown to be involved in the pathogenesis of glioblastoma and thus can provide information on the disease state.

standardise the protocols for EV isolation as well as their characterisation (Phan et al., 2021). Moreover, it should be noted that the final product of currently used isolation methods is only an EV-enriched preparation or formulation, which contains other components of the carrier medium, rather than pure EVs (Webber and Clayton, 2013). These components, such as plasma-derived high- and low-density lipoproteins, and uromodulin and albumin from urine, have a density similar to that of EVs, which makes them indistinguishable by current isolation and purification methods (Ramirez et al., 2018). In addition, the storage of EV preparations remains a concern, because EVs can degrade in the carrier medium, and their biological function might be compromised by long-term storage (Yuan et al., 2021). More research is needed to examine how different matrices, solvents and storage vials impact the biological properties of EVs and how to protect EVs from damage (Thery et al., 2018).

A further complication in the translation of EVs to clinics is that, regardless of origin and isolation method, there could be a layer of biomolecules adsorbed to the surface of EVs, referred to as the ‘corona’ (Monopoli et al., 2012). The corona cloaks the surface of EVs and potentially covers protein surface receptors, which subsequently impacts on their interactions with biological systems (Tóth et al., 2021). Kim et al. have shown that additional purification of EVs isolated via ultrafiltration using size exclusion chromatography columns substantially reduced the amount of protein and nucleic acid (DNA and RNAs) in the EV preparations (Kim et al., 2018). The results of this study suggest that proteins, DNA and RNA (and other biomolecules) adhere to EVs and form a coating, and that purification using chromatography columns is likely to sheer the coating off the EVs, which in turn suggests that the coating is a ‘soft corona’ (Kim et al., 2018). It is now accepted that the protein corona forms readily on the surface of EVs (Palviainen et al., 2020), and the enzymatic removal of EV surface proteins has been shown to impede the cellular uptake of EVs (Escrevente et al., 2011). Furthermore, as previously shown for other classes of nanoparticles, the corona remodels (changes its composition) during the transition of nanoparticles through different organs or biofluids (Khanal et al., 2020). Since the corona can affect the interaction between EVs and recipient cells, remodelling of the corona can have major impact on the downstream biological effects of EVs. Despite a substantial amount of work on corona formation on other classes of nanoparticles and evidence that the corona determines the fate of nanoparticles in biological systems (Walkey et al., 2014), this area has only recently been recognised in EV research and remains in its infancy.

A scalable amount of well-characterised EVs is another requirement for EV-based therapeutics. This is not a hurdle in cases where there is an abundant source of EVs. For instance, bovine milk and red blood cells are readily scalable, robust and near unlimited sources for EVs that can satisfy the initial expectation of low, affordable cost for a large number of patients (Dang et al., 2020). Specifically, EVs derived from bovine milk have been shown to mediate immunoregulatory effects in murine macrophages (Pieters et al., 2015), facilitate increased osteoblast differentiation in mice (Oliveira et al., 2016), and enhance the drug delivery and reduce off-target toxicity (Aqil et al., 2016). Recently, oral administration of EVs derived from bovine milk to mice has been shown to reduce primary tumours in colorectal cancer and breast cancer but accelerate metastasis in breast cancer and pancreatic cancer (Samuel et al., 2021). However, the exact mechanism and key molecules that drive these opposing effects in the cancer models are still elusive. In some cases, the source of EVs is limiting; for example, a reliable method for the expansion of MSCs, a commonly used source of EVs for applications in regenerative medicine, is still missing, and current methods are labour intensive and involve numerous procedures (Gowen et al., 2020). Furthermore, substrates used for cell expansion, such as microcarriers (to provide surface matrices that enable attachment and proliferation of adherent cells), have specific diameter (curvature), stiffness and surface chemistry, which collectively affect cell behaviour and thus the production and composition of EVs (de Almeida Fuzeta et al., 2020). These aspects, although rarely considered, are fundamental for the establishment of reliable protocols for EV production for specific applications. In addition, the use of conditioned medium from cell cultures for the isolation of EVs can give rise to issues with batch-to-batch reproducibility and consistency in the resulting EV preparations. For instance, cellular confluence, early versus later passage of cells, oxygen concentration, cytokines, heparin and serum content of the medium can all affect the quality and quantity of EVs released (Lener et al., 2015).

To exploit EVs as a therapeutic tool, it is essential to examine their biodistribution and their mechanisms of action both *in vitro* and *in vivo* (Gowen et al., 2020). Previous studies have shown that EVs released from different cell types exhibit different biodistribution patterns (Hoshino et al., 2015; Wen et al., 2016; Zhang et al., 2018b). However, the majority of existing studies have shown an uptake of EVs into the major organs of clearance (liver, lungs, kidneys and spleen) rather than into the organs to which EVs are specifically targeted normally (Kang et al., 2021). This issue could be due to some limitations in our currently used methods. Firstly, techniques used to detect EVs in tissues and organs require the extensive accumulation of fluorescent or radiolabelled EVs (tens to hundreds of µg of total EV protein), which is difficult to achieve and potentially non-physiological (Kang et al., 2021). Secondly, depending on the isolation methods, the biodistribution of EVs has been reported to be different; for instance, intravenously administrated EVs isolated using ultrafiltration with liquid chromatography accumulate to a lesser extent in lungs when compared with EVs isolated using ultracentrifugation only (Nordin et al., 2015).

With any therapeutic treatment, the safety profile must be thoroughly investigated (Gowen et al., 2020). Theoretically, every study that employs vesicles of natural origin as a drug delivery system should perform an assessment of immunogenicity. Although numerous studies suggest that EVs are unlikely to cause an immunogenic response, only a few studies have extensively investigated the potential risk of immunogenicity or any toxic effects of EV treatments in animal models (Zhu et al., 2017; Montaner-Tarbes et al., 2018). A mouse model has shown that EVs derived from engineered HEK293T cells (transfected with miR-199a-3p) had no toxic effects, and only insignificant changes in immune markers were measured. At the same time, no changes in immune markers were observed in mice treated with EVs derived from unmodified HEK293T cells (Zhu et al., 2017). Furthermore, immunisation of pigs with 2 mg of EVs derived from serum of pigs infected with porcine reproductive and respiratory syndrome virus (PRRSV) did not induce clinical symptoms or any adverse effects, and viral replication in PRRSV-negative pigs was not detected (Montaner-Tarbes et al., 2018). Future studies should thus focus on performing a thorough safety evaluation of EV-based therapies, which might help to accelerate the clinical translation of EV products.

Although the above limitations currently affect the clinical translation, recent financial reports highlight a substantial increase in investment in the EV field. A shift towards EV-based medicines is exemplified by the establishment of EV-centric programmes by major biopharmaceutical and biotechnology companies, such as Cytiva (formerly GE Healthcare), HansaBioMed and Roche. In addition, between 2000 and 2016, a total of 524 US patents and 948 National Institutes of Health grants cited ‘exosome(s)’, ‘extracellular vesicle’ and/or ‘microvesicle(s)’ (Roy et al., 2018). Notably, the FDA has approved Direct Biologics to conduct a phase II trial for the use of ExoFlo™, a BM-MSC-derived EV product for the treatment of COVID-19 patients. However, it remains unclear which specific therapeutic cargo in this product is responsible for the therapeutic effects. Nevertheless, some proteins that are found in ExoFlo™ have been shown to reduce inflammation and promote cellular repair and regeneration (see https://directbiologics.com/exoflo/ for more details). For example, VEGF, which is found in ExoFlo™, can regenerate lung epithelial and endothelial cells via activation of the phosphoinositide 3-kinase (PI3K)–Akt signalling pathway (Gerber et al., 1998; Roberts et al., 2007), and hence facilitate the repair in lung injury through epithelial regeneration, which will be beneficial for COVID-19 patients. However, the biological activity of the product, the actual concentration of the active compounds in the administered dose and the long-term effects after administration remain unclear (Lim et al., 2020). According to the US National Library of Medicine clinical trial database (https://www.clinicaltrials.gov/), 120 participants received a single intravenous dose of ExoFlo™, and they were evaluated for the safety and efficacy for 14 days post treatment (https://www.clinicaltrials.gov/ct2/show/NCT04493242). Overall, the average pressure of arterial oxygen to fraction of inspired oxygen ratio (P_aO_2/F_IO_2) was improved in patients receiving the treatment, and no adverse effects were observed 72 h after ExoFlo™ administration (Sengupta et al., 2020). ExoFlo™ is thus a promising candidate in treating patients with severe COVID-19 owing to its ability to restore oxygenation (increased P_aO_2/F_IO_2 ratio and reduced oxygen support requirement), downregulate the cytokine storm and improve overall immunity function, as well as being safe to use.

In summary, to move EVs towards mainstream medicine we need to address current limitations in isolation, purification, characterisation, standardisation and storage of EVs; scale-up EV production; ensure batch-to-batch reproducibility and consistency; and assess the biodistribution, pharmacokinetics and safety profile of EVs. The increase in investment for EV research gives us confidence that EVs will soon pave the way for better diagnosis and treatment of serious diseases.

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Conclusions

While the benefits of EVs in enabling early disease diagnosis and transforming therapies are clear, there are still some barriers to overcome before we can harness the full potential of EVs to enter mainstream medicine (Soekmadji et al., 2020). Given that biologicals are defined as 'a medicine that contains one or more active substances made by or derived from a biological cell', EV-based products can be classified as biologicals (Lener et al., 2015). However, it remains to be determined which classifications EV-based products would fall into and whether exemption guidelines targeting EV-based products could be used. If EV-based products are subjected to regulation as biologicals, they should be classified according to their risk profile (Box 1). Since various scenarios can be anticipated for EV-based products, including EVs derived from unmodified cells, from modified cells without a transgene or from modified cells containing a transgene, the decision regarding the regulation procedure has to be made on a case-by-case basis (Lener et al., 2015). Moreover, an exemption from regulation requirements can be applied for EV-based products if they can satisfy some certain conditions (Box 2). Nevertheless, several requirements for the manufacturing of EV-based products must be fulfilled before their evaluation in clinical trials; these include approval of technical requirements (isolation, purification and characterisation) and quality risk management, as well as of the safety and release profile (safety pharmacology, pharmacodynamics and toxicology) (Lener et al., 2015). Although there are no specific regulatory guidelines for EV-based therapies yet, the safety standards for cell- and tissue-based products could be used as valuable roadmaps for the development of this new class of biologicals (Soekmadji et al., 2020). It is anticipated that the choice and characterisation of the origin of EVs are highly relevant for the development of EV-based

Box 1. The legal regulation of EVs in Australia

In most jurisdictions around the world, a sponsor intent on marketing a new therapeutic product for clinical use must register that product with the relevant therapeutic goods regulator. To do so, the sponsor must provide evidence that shows manufacturing compliance, reliable collection and testing procedures, and the ability to achieve product consistency (Therapeutic Goods Administration, 2018; https://www.tga.gov.au/dossier-requirements-class-2-3-and-4-biologicals). In Australia, all therapeutic goods that are or comprise human cell and tissue products (HCTPs) are regulated as 'biologicals' under the Regulatory Framework for Biologicals (RBF; see part 32A of the Therapeutic Goods Act, 1989; https://www.legislation.gov.au/Details/C2019C00066). Since EVs are secreted from human cells, it is expected that the Therapeutic Goods Administration (TGA) will consider EVs to be biologicals. In the US, the FDA has similarly indicated that all EV-based products would be classified as drug or biological products, then evaluated for safety and efficacy before being subject to registration and approval for clinical uses (de Jong et al., 2020).

In Australia, biologicals used as medical treatments must be registered on the Australian Register of Therapeutic Goods (ARTG). They must be classified according to their risk profile in Classes 1, 2, 3 or 4, where Class 1 and 2 are the least risk-laden and Class 4 is the most risk-laden. Currently, there is uncertainty about into which class, if any, an EV-based product would fall. Class 1 and 4 biologicals must be expressly specified or 'mentioned' in the regulations to be approved for use, whereas Class 2 and 3 biologicals are defined by their method of preparation and are not required to be listed in the regulation. At present, no EV-based product is currently mentioned in any class, and a search of the ARTG shows that no EV-based product is currently approved for use in Australia. Current TGA guidance suggests that a biological derived from non-pluripotent stem cells (including a cell with multipotent potential) would likely be treated as a Class 3 biological, making it likely that many EV-based therapies would be treated as Class 3 biologicals (https://www.tga.gov.au/publication/australian-regulatory-guidelines-biologicals-argb). The implication of this is that EVs will likely be regarded as medium-risk products that will require the sponsor to provide a detailed evidence dossier demonstrating the proposed product's compliance with several strict standards before they are approved for clinical use.

In any event, EV-based products that require scalable production and distribution would almost certainly require evaluation, approval and registration on the ARTG before they are supplied, because the method of preparation for these products requires 'more than minimal manipulation' of biological materials (https://www.tga.gov.au/publication/australian-regulatory-guidelines-biologicals-argb). However, the legislation supplies three interesting alternatives to registration and approval for some biological therapies: (1) one-off personalised treatments, (2) exempted goods, and (3) goods treated as biological medicines and excluded from RBF. These unregulated uses are addressed in Box 2.

Box 2. Exemptions from regulation requirements – unregulated uses of EVs

Certain HCTPs are exempt from the registration requirements if they are used, in a one-off, personalised treatment. These biologicals must be (1) collected by a registered medical or dental practitioner from a patient, (2) 'minimally manipulated', (3) intended for 'homologous use' and (4) used on the same patient (autologously) by the same practitioner for a single indication in a single procedure. However, whether EVs would be subjected to any manipulation that is not permitted, or 'homologous use' will depend on the specific isolation, purification and characterisation procedure. While it is difficult to predict, it is unlikely that EVs will be considered as 'minimally manipulated' and will therefore not qualify for this exemption because, for instance, they may be derived from MSCs that may have themselves been minimally manipulated when being isolated from their source (for example, adipose tissue). The consequence of this is that EVs will not be able to be introduced into the clinic through this 'unregulated' pathway and instead will require some oversight.

Excluded goods are biologicals that are (1) collected from a hospital patient by a registered medical or dental practitioner, (2) wholly manufactured under the supervision of the same practitioner and (3) used for the same hospital patient. If any step in the manufacture occurs outside the hospital, it must be carried out by a person under contract with the hospital. Furthermore, these biologicals may not be advertised to consumers. If EV-based products were to meet the above conditions, they may be administered to patients in hospitals without needing approval through the ARTG process. The consequence of this would be that EV-based treatments would be available in hospitals without any additional regulatory approval. However, such an application is unlikely to accelerate EV-based products towards mainstream medicine because these one-off treatments could not be readily scaled up.

Finally, some biologicals and HCTPs are treated as therapeutic goods if they are: (1) haematopoietic progenitor cells used for haematopoietic reconstitution, (2) *in vitro* diagnostic devices, (3) samples of human cells or tissues used solely for diagnostic purposes in the same individual, (4) blood and blood components, or (5) biological medicines including vaccines, recombinant products and plasma-derived products (https://www.legislation.gov.au/Details/F2011L00894). Since there are similarities between EVs and some of these excluded products (for example, blood and blood components), EVs could be categorised as biological medicines – not biologicals. However, it remains unclear whether the TGA would treat EVs as biological medicines now or in the future.

Both regulatory uncertainty and the evident challenges associated with the registration process present considerable barriers to the translation of EVs to the clinic. Nevertheless, it is possible that existing standards for HCTPs could be used as valuable roadmaps for the development and eventual widespread application of this new class of biologicals.

therapeutics (Soekmadji et al., 2020). Therefore, the current assessment of safety, toxicity, biodistribution, pharmacokinetics and/or pharmacodynamics, immunogenicity and tumorigenicity used for cell-based therapies could also be used for EV-based therapies (Lener et al., 2015). It has been suggested that EVs used as next-generation biomarkers will soon allow the detection of many diseases at early stage and to precisely track their progression (Kosaka et al., 2019; Pang et al., 2020), including for difficult-to-detect pancreatic cancer, where early detection is key to reduce disease burden and mortality (Pang et al., 2020). EVs are particularly promising alternatives to the current standard biomarkers, which have a limited ability to detect autoimmune diseases (Xu et al., 2020), cardiovascular diseases (Dickhout and Koenen, 2018) and neurodegenerative diseases including Alzheimer's disease (Quiroz-Baez et al., 2020; Li et al., 2019a). In addition, EV-based therapeutics have the added advantage that additional bioengineering strategies could be used to tailor the composition of EVs for a specific disease and even for individual patients, or to modify the EV surface to modulate their targeting properties, thereby enabling their preferential uptake in specific target cell types or increasing circulation time (Murphy et al., 2019). Taken together, we believe that EVs hold great promise to transform the future of medicine and enable the development of truly personalised treatments.

Competing interests

The authors declare no competing or financial interests.

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Table S1. EC-funded projects focused on EVs

Funded projects	Description review
ExocyTher (https://cordis.europa.eu/project/id/852791)	Development of a bioreactor producing a turbulent flow, to attempt to mimic shear stress in blood vessels that triggers the release of EVs
evFOUNDRY (https://cordis.europa.eu/project/id/801367)	Development of a device/technology for the continuous production of high-grade EVs from milk and parasites
VES4US (https://cordis.europa.eu/project/id/801338)	Exploiting of sustainable sources, *e.g.,* microalgae strains and plants, for the production of EVs with a focus on drug delivery
greenEV (https://cordis.europa.eu/project/id/895579)	Development of a platform for the manufacturing of non-mammalian nanovesicles for the encapsulation, release, and enhanced absorption of selected nutraceuticals
EVPRO (https://cordis.europa.eu/project/id/814495)	Development a unique approach to enhance the integration and longevity of hip prostheses by incorporating EVs encased in hydrogel directly to nanostructured prosthesis surfaces
EVICARE (https://cordis.europa.eu/project/id/725229)	Focusing on using EVs derived from progenitor cells to promote the repair of the cardiac tissue; the project aim to provide new mechanistic insights into how the myocardial tissue is affected by EV injection into the failing heart
MARVEL (https://cordis.europa.eu/project/id/951768)	Establishment of an isolation technology, *i.e.,* DNA-directed reversible immunocapturing technology, to capture subpopulations of EVs at large scale
BOW (https://cordis.europa.eu/project/id/952183)	Creation of hybrid magnetic nanoparticles cloaked with EV membrane with the aim to modulate circulation time and enable more precise targeting in the body