

REVIEW ARTICLE

The paradigm of stem cell secretome in tissue repair and regeneration: Present and future perspectives

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Abstract

As the number of patients requiring organ transplants continues to rise exponentially, there is a dire need for therapeutics, with repair and regenerative properties, to assist in alleviating this medical crisis. Over the past decade, there has been a shift from conventional stem cell treatments towards the use of the secretome, the protein and factor secretions from cells. These components may possess novel druggable targets and hold the key to profoundly altering the field of regenerative medicine. Despite the progress in this field, clinical translation of secretome-containing products is

Abbreviations: α -SMA, alpha-smooth muscle actin; γ GT, Gamma-Glutamyl Transferase; 3D, three dimensional; A549, adenocarcinomic human alveolar basal epithelial cells; ADMSC, adipose tissue-derived mesenchymal stem cell; ADSC, adipose-derived stem cell; AGF, angiogenic growth factors; ALI, acute lung injury; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMI, acute myocardial infarction; ANG, angiotensin; AQP5, aquaporin-5; ArCr, cartilaginous area; ArOs, osseous area; ASC, adipose stromal cell; ASC-ST, adipose MSC-derived secretome; ASMC, amniotic stromal mesenchymal stem cells; AST, aspartate transaminase; ATG5, autophagy-related protein 5; ATMPs, advanced therapy medicinal products; AuBK, aurora B kinase; BBB, Basso, Beattie, Bresnahan ratings; Bcl2, B-cell lymphoma 2; BDNF, brain-derived neurotrophic factor; Beclin 1, coiled-coil myosin-like BCL2-interacting protein; bFGF, Basic fibroblast growth factor; Bleo, bleomycin; BLM, bleomycin; BMP, bone morphogenetic proteins; BMSCs, bone marrow mesenchymal stromal cells; BrdU, bromodeoxyuridine; B-ST, UCMSCs exposed to healthy brain extract; CAT, catalase; CD, control diet; CD31, cluster of differentiation 31; CD44+/CD24-, breast cancer cell phenotype associated with stem/progenitor cell properties; CD68, cluster of differentiation 68; Cfl-2, Cofilin-2; CH, human articular chondrocytes (CH); CHS, cordycepin-induced HaCaT secretome; CLL2, chemokine (CC-motif) ligand 2; CM, conditioned medium; CNS, Central Nervous System; Col1A2, human collagen alpha-2 type I; Col3A1, collagen alpha-1(III) chain; Cox-2, cyclooxygenase-2; CXN43, connexin 43; DCF, dichlorofluorescein; DCX, doublecortin; dECM, decellularised extracellular matrix; DMEM, Dulbecco's Modified Eagle Medium; DNA, deoxyribonucleic acid; DPSCs, dental pulp stem cells; ECM, extracellular matrix; ECS, extracellular space; ECV-304, immortal endothelial cell line; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial-mesenchymal transition; EPCs, endothelial progenitor cells; ER, endoplasmic reticulum; ESC-MSC, human embryonic stem cell-induced mesenchymal stem cell; EV, extracellular vesicles; F4/80, marker for macrophages and dendritic cells; FBS, foetal bovine serum; FGF, fibroblast growth factor; FR, Dermal Fibroblasts; G1, growth phase of cell cycle; GDNF, glial cell line-derived neurotrophic factor; GMP, Good Manufacturing Practices; GPX1, glutathione peroxidase 1; GSH-PX, glutathione peroxidase; HA, hyaluronic acid; HaCaT, immortalized, human keratinocyte line; hAFs, human amniotic fluid-derived stem cells; hBM-MSC, human bone marrow mesenchymal stem cell; hBMSCs, human bone marrow stromal cells; HCEC, human cardiac microvascular endothelial cells; HDF, Human Dermal Fibroblasts; HDMECs, human dermal microvascular endothelial cells; hDPSC, human dental pulp-derived stem cell; Hepatic I/R, hepatic ischemia/reperfusion; hESC-MSC, human embryonic mesenchymal stem cells; hESC-MSC-IMRC, human embryonic stem cell-derived MSC-like immune and matrix regulatory cells; HFD, high-fat diet; HFFs, foreskin fibroblasts; hFSSC, human foetal skin-derived stem cells; hGFs, human gingival fibroblasts; Hif-1 α , hypoxia-inducible factor-1 α ; HiPSCs, human induced pluripotent stem cells; HIRI, hepatic ischaemia-reperfusion injury; hMSC, undifferentiated human mesenchymal stem cells; hMSC-EC, human endothelial cell-differentiated mesenchymal stem cells; HNF4 α , hepatocyte nuclear factor 4 α ; HO-1, heme oxygenase-1; HSCs, hepatic stellate cells; HSP, high-scale production; HUCMC, human umbilical cord matrix cells; hUCMSCs, umbilical cord mesenchymal stem cells; HUVEC, human umbilical vein endothelial cells; IB-ST, UCMSCs exposed to injured brain extract; IL, interleukin; IMRCs, immune and matrix regulatory cells; IPA, Ingenuity Pathway Analysis; IPF, idiopathic pulmonary fibrosis; IRI, ischaemia-reperfusion injury; IV r-TPA, recombinant-tissue plasminogen activator; IV, Intravenous; Ki67, Antigen Kiel 67; LC3I, cytosolic form of LC3; LC3II, LC3-phosphatidylethanolamine conjugate; LSC, lung spheroid cell; LSP, low-scale production; LV, left ventricle; LVEF, left ventricular ejection fraction; LX2, human hepatic stellate cell line; M Phase, mitotic phase of cell cycle; MCP-1, monocyte chemoattractant protein-1; MDA, malondialdehyde; MDR1+, human P-glycoprotein encoded by the multidrug resistance 1 (MDR1) gene; MI, myocardial infarction; MKI67, antigen Kiel 67; MMP, matrix metalloproteinase; MMSC, placental multipotent mesenchymal stromal cell; mNVCM, neonatal mouse ventricular cardiomyocytes; mRNA, messenger RNA; MSC-CM, secretome from human bone marrow-derived mesenchymal stem cells; MSCs, mesenchymal stem cells; NAC, N-acetyl cysteine; NaCl, sodium chloride; NGF, nerve growth factor; NOX4, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4; NRF2, nuclear factor erythroid 2-related factor 2; NT-3, neurotrophin-3; OA, osteoarthritis; OC, osteocalcin; OPN, osteopontin; P53, tumor protein P53; p62, sequestosome-1 (SQSTM1); PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; PCNA, proliferating cell nuclear antigen; PD, pharmacodynamic; PDGF, platelet-derived growth factor; PD-L1+, presence of programmed death-1 ligand 1 (PD-L1) protein in a tumor; PIGF, placental growth factor; PK, pharmacokinetic; p-NF-Kb, phosphorylated form of the nuclear factor-kB (NF-kB) transcription factor; PPD, phenotypic drug discovery; ProSPC+, positive AT2 cells, proliferation of surfactant protein C-positive AT2 cells; p-STAT3, phosphorylated signal transducer and activator of transcription 3; PTX-3, Pentraxin 3; rMSCs, rat MSCs; RNA, ribonucleic acid; ROS, reactive oxygen species; SCI, spinal cord injury; SD, Sprague Dawley; SD-MSCs, skin-derived MSCs; SERCA2, sarco-/endoplasmic reticulum calcium ATPase 2; SGZ, subgranular zone; SMAD3, mothers against decapentaplegic homolog 3; SOCS3, suppressor of cytokine signaling 3; SOD, superoxide dismutase; SOD1, Superoxide dismutase 1; SPC, surfactant protein C; Sr-90, Strontium-90; ST, USMSCs secretome without brain extract; TAA, thioacetamide; TBI, traumatic brain injury; Tbil, total bilirubin; TGF- β , transforming growth factor β ; TIMPs, tissue inhibitors of metalloproteinases; TLRs, toll-like receptors; TNBC, triple negative breast cancer; TNF- α , tumour necrosis factor α ; TSP-1, thrombospondin-1; UCMSCs, umbilical cord mesenchymal stem cells; uPAR, urokinase-type plasminogen activator receptor; UPS, unconventional protein secretion; VEGF, vascular endothelial cell growth factor; VEGF-A, vascular endothelial cell growth factor A; VEGF-C, vascular endothelial cell growth factor C; vWF+, von Willebrand factor; WHI, wound healing index; XFS, xeno-free medium; Ym-1, rodent-specific chitinase-like protein (CLP) lacking catalytic activity.

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limited by several challenges including but not limited to ensuring batch-to-batch consistency, the prevention of further heterogeneity, production of sufficient secretome quantities, product registration, good manufacturing practice protocols and the pharmacokinetic/pharmacodynamic profiles of all the components. Despite this, the secretome may hold the key to unlocking the regenerative blockage scientists have encountered for years. This review critically analyses the secretome derived from different cell sources and used in several tissues for tissue regeneration. Furthermore, it provides an overview of the current delivery strategies and the future perspectives for the secretome as a potential therapeutic. The success and possible shortcomings of the secretome are evaluated.

KEYWORDS

drug discovery, extracellular matrix, regenerative medicine, secretome, target discovery, therapeutic potential

1 | INTRODUCTION

As life expectancy continues to increase due to the improvement in medical science, the margin between the supply of available tissues and organs and the resulting demand for tissues and organs continues to widen.^{1,2} This disparity causes extended patient waiting times for patients requiring organ transplants,³ and unfortunately in some cases, patient death occurs. The supply-demand disparity has resulted in the increased demand for tissue engineering and regenerative solutions, to regenerate and repair damaged tissues and organs as to restore their native functionality.

Regenerative attempts have largely focused on material sciences as well as improving bio-fabrication tools and technology to modulate cellular functions in attempts to regulate the body's native regenerative potential.^{4–7} Within this regenerative domain, the importance of stem cells and their cellular secretions has become increasingly notable. Therefore, the exploration of human proteomics and secretomics is growing rapidly as it has begun to profoundly alter the field of regenerative medicine.

Interest in the study of secreted proteins and factors began gaining traction in the early 2000s and over the past 10 years, secretome studies have grown exponentially owing to the advancements in proteomic platforms and instrumentation. The driving force behind such advancements is the fact that the secretome plays a crucial role in health and disease progression.^{8–13} The term secretome originated from Tjalsma et al., 2000, who described the secretome as 'both the components of machineries for protein secretion and the native secreted proteins'.¹⁴ The definition has widened and for this review, the secretome will be defined as the totality of soluble and insoluble organic and inorganic molecules, lipids and extracellular vesicles (EVs)^{15–17} released from cells, tissues or an organism under a defined time and under controlled conditions.^{15–17} As an important distinction, soluble factors of the secretome are defined as molecules that are able to freely diffuse through the extracellular fluids found in the extracellular space (ECS) to mediate cell signalling across various

distances^{18,19} and comprise factors including but not limited to chemokines,^{19,20} cytokines,^{2,19–26} hormones,^{2,22–25} angiogenic factors,^{2,22–25} enzymes^{2,22–26} and growth factors.^{2,19–26} In contrast, the structural components of the extracellular matrix (ECM)^{2,22–25,27} and vesicular fractions such as exosomes and microvesicles are categorised as the insoluble factors of the secretome.^{20,28,29} Exosomes, a critical component of the secretome, have gained a lot of attention in recent years due to their ability to mediate unique intercellular communication and influence biological processes.^{30,31} After fusing with the plasma membrane, these cell-derived vesicles are released into the ECS to elicit their effect, through their cargo.^{30,31} Since the cargo contained in these vesicular fractions are tissue-specific, they can contain various cargo such as soluble organic factors such as nucleic acids (both DNA and RNA)^{2,15,17,22–26} and proteins,^{2,15,17,22–26} organic insoluble factors such as lipids^{2,15,17,22–26} and inorganic factors such as phosphate,³² calcium³² and various metabolites with differing solubility.³³ Altogether, the secreted factors influence several biological processes, including but not limited to signalling, homeostasis, immunomodulation, development, tissue repair and regeneration, ECM organisation, inflammation, apoptosis, adhesion, angiogenesis, adhesion, proliferation and proteolysis. Fundamentally, it is the collection of these secreted factors and proteins that determines the phenotype of the cell.^{30,31,34}

Cell-based therapies rely heavily on the delivery of either allogenic or autologous cells to the injury site to promote tissue restoration due to their cellular plasticity and ability to differentiate into multiple cell lineages.^{35–37} Despite the success, cell-based therapies are limited by the reduced proliferative functionality of the cells, especially in pathological microenvironments.²⁰ Stem cells, on the other hand, possess multipotent differentiation capacity as well as immunomodulatory and anti-inflammatory properties.^{38–40} The benefits of stem cell therapy are controlled by homing to the injury site, cellular differentiation for injury tissue grafting and the release of cellular factors.^{17,21} Despite the plethora of benefits associated with stem cell therapy, several limitations remain such as immunocompatibility,

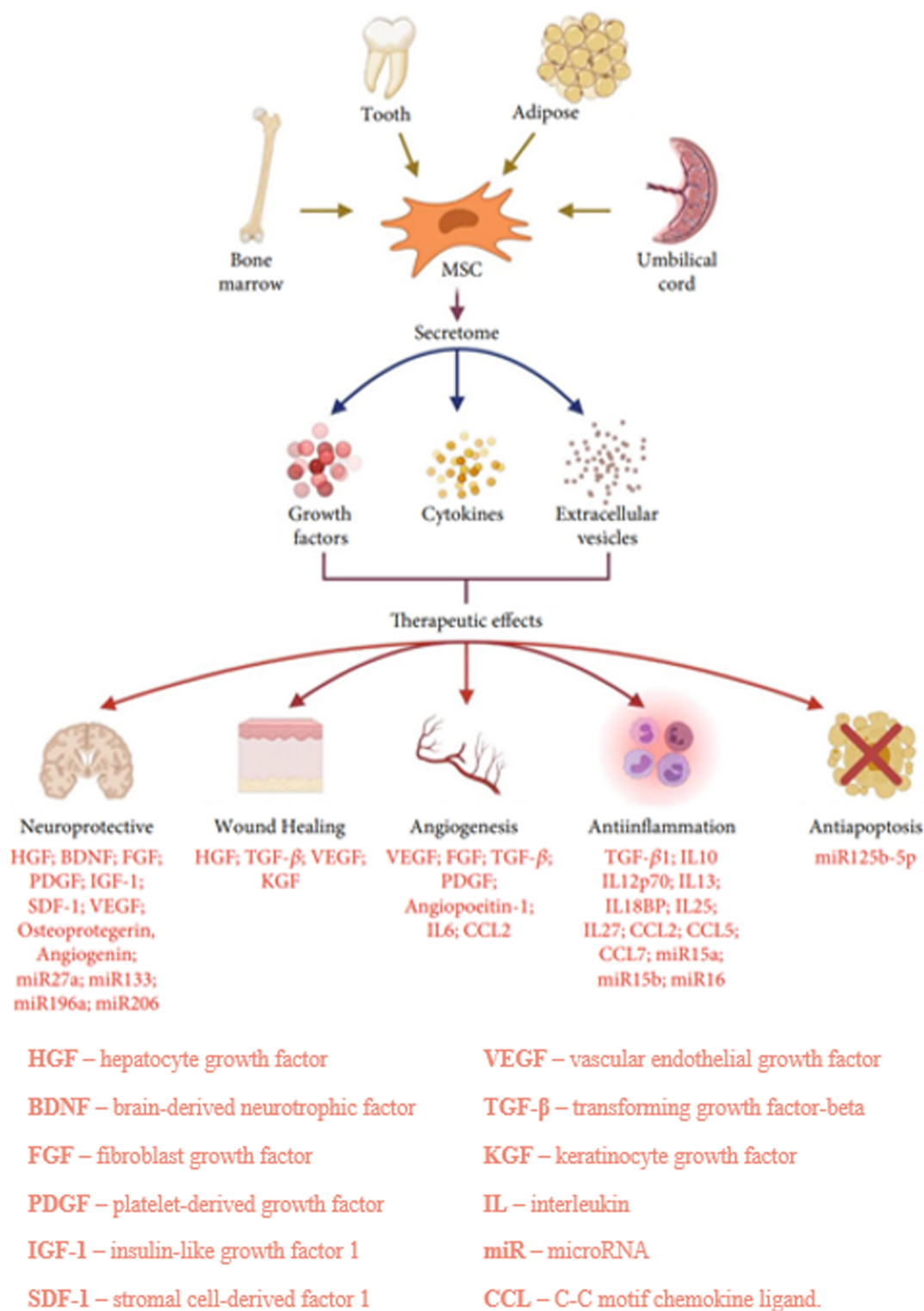


FIGURE 1 A schematic diagram showcasing a selection of mesenchymal stem cell (MSC) sources, their secreted molecules and factors and various potential therapeutic effects. The intercellular communication of stem cells, including MSCs, heavily relies on the components of the secretome to achieve their potential therapeutic effects. This image has been adapted from Foo et al., 2021⁶⁶ under the Creative Commons Attribution 4.0 International Public License.

infection transmission and tumourigenicity.^{41,42} Additionally, large quantities of cells are required which results in constant growing and passing of cells in vitro, which has the potential to result in

abnormal cellular behaviour and properties. Despite stem cell studies, particularly those utilising mesenchymal stem cells (MSCs), producing favourable results in clinical trials, stem cell-based therapies have

been further restricted due to a lack of clarity surrounding the cell isolation and *in vivo* expansion protocols, the source of stem cells, culture conditions, mode of infusion, dosage, administration frequency and delivery route, amongst others.^{43,44} Emerging research has suggested that the secreted factors from stem cells are the cause of their therapeutic effect, rather than the cells themselves due to the poor integration of the cells into the damaged tissue.^{6,17,21,37,45–55} Therefore, the secretome can be collected and harvested to elicit a therapeutic response.

Considering the above, secretome studies have transitioned into a form of cell-free-based therapies owing to the key study performed by Gnechi et al., 2005. In a cardiac model, the research team demonstrated that the paracrine secretion of cytoprotective factors from the MSCs provided evidence of the regenerative potential of the secretome.⁵¹ Another study performed by Ankersmit et al., 2009 indicated that tissue damage was inhibited in an acute myocardial infarction (AMI) rodent model after the infusion of stressed peripheral blood mononuclear cell (PBMC) suspensions.⁵⁶ Furthermore, this research team also indicated that stem cells and PBMC secretions showed comparable regenerative abilities⁵⁷ whilst improving cardiac outcomes and reducing the infarct area in rodent and porcine AMI models.⁵⁸ Additionally, the regenerative and versatile therapeutic abilities of PBMCs were shown in acute spinal cord injuries, cerebral ischaemia and skin wound rodent models after the animals were treated with the secretome of stressed PBMCs.^{59–61} Such studies indicated that the secreted factors were able to elicit their therapeutic abilities rather than relying on the cells to differentiate into the required cell type. These studies together with exosome analysis have enriched our understanding of cellular communication and coordinated cellular behaviour which has begun to open many doors for potential novel therapeutic strategies such as disease diagnosis and monitoring, biomarker identification and targeted disease therapy.⁶²

Stem cell studies, particularly those concerned with analysing the secreted factors, have uncovered factors that have several biological functions including but not limited to angiogenic, mitogenic, anti-scarring, anti-apoptotic and chemoattractant characteristics.^{63,64} Therefore, it is no surprise that the secretome, from multiple cell sources, has generated a myriad of interest owing to its potential benefits in pharmaceutical and clinical sciences (Figure 1). The benefits of secretome factors include but are not limited to immunity,⁶⁵ antimicrobial properties,⁶⁵ regeneration and wound healing,^{65,66} angiogenesis,^{65,66} anti-oxidative stress,⁶⁵ anti-apoptosis,^{65,66} reducing safety concerns associated with cell implantation, circumvention of surgical stem cell removal,²⁰ reduction in tumourigenic potential due to the absence of self-replicating features, immunocompatibility^{17,21,37,52,65,67} as well as the ability to be stored for extended periods without the need for toxic cryoprotectants.^{17,21,37,68}

This review will consider how the secretome can provide clinically relevant pharmaceutical targets and act as clinical novel therapies. In addition, this review will consider the advancements and achievements made in this field, focussing on the implication of the secretome in health and disease as well as the secretome as a biotherapeutic agent.

2 | THE SECRETOME: IMPLICATIONS IN HEALTH AND DISEASE

It is important to acknowledge that the composition of the secretome is largely governed by the cell type, the differentiation state of the cell, the age and metabolic state of the individual^{37,69} as well as the physiological environment surrounding the cells, the residing niche of the cell and the microenvironment stimuli.^{2,17,19,34,43,45,70–81} This environmental state ultimately reflects the cell's functionality depending on the signalling messages it receives, which can result in different proteins or different quantities of proteins being secreted.⁸² It is these secretory pathways that can result in the healthy or diseased state of the tissue or organ as well as enhancing or reducing disease progression.⁸

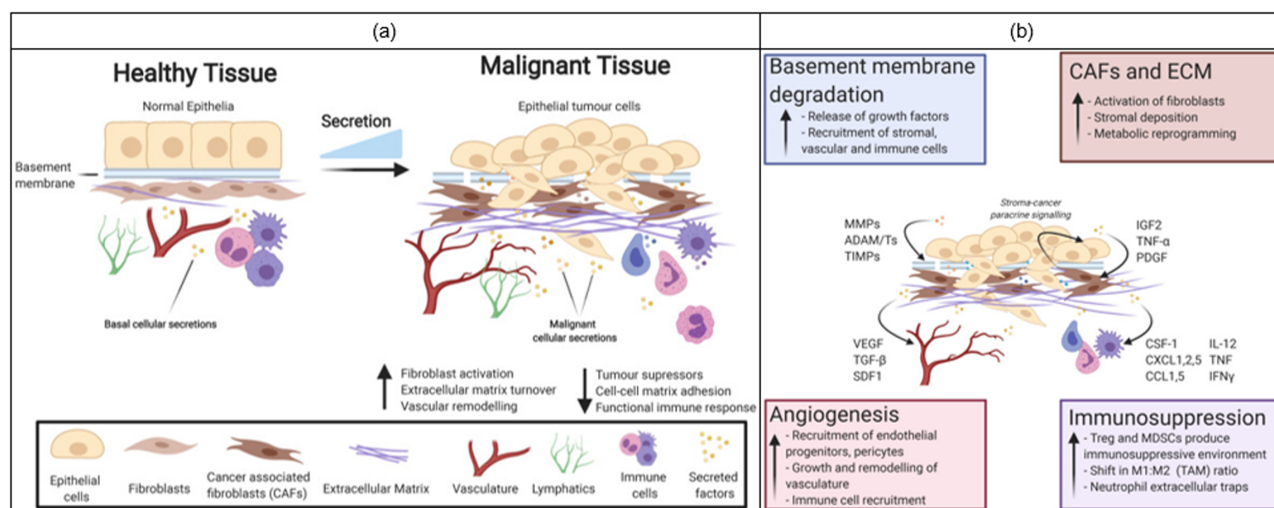
2.1 | The secretome in healthy tissue regulation

The bulk of eukaryotic secretory proteins pass through conventional and well-reported secretory pathways from the endoplasmic reticulum (ER) to the Golgi complex onto the trans-Golgi network and finally to the plasma membrane.^{83,84} A signal at the protein's N-terminal or transmembrane domain is required to initiate this process.^{84,85} In healthy tissues, the secretome is regulated by several regulatory proteins to maintain homeostasis and cellular communication, essential for the health of the organism.^{84,86,87} Despite the number of proteins that are secreted through the process discussed above, several proteins are secreted through non-conventional pathways not involving the ER-Golgi transport route^{84,88–90} or pathways where proteins do not bear the signal peptide.^{84,91} These pathways are described as unconventional protein secretion (UPS) pathways. This indicates that the secretome has various functions and the secreted factors can act in several manners including endocrine, paracrine or autocrine, both systemically and locally.⁸⁷

2.2 | The secretome in disease progression

Apart from a small number of anomalies, most UPS pathways are promoted by several cellular stresses including but not limited to mechanical stress,⁹² nutrient starvation,⁹³ ER stress^{94,95} and inflammation.⁹⁶ It is these UPS pathways that cause the initiation and progression of disease and, therefore, UPS-related diseases continue to increase.

The induction of unconventional protein secretion can be triggered by sterile inflammation connected to diabetes, allergic and autoimmune diseases and Alzheimer's disease.^{96–100} Another example is the pro-tumourigenic environment of cancer cells. A genomics analysis performed by Robinson et al., 2019 indicated that the largest differential expression in several cancer lines was the loss of genes encoding for tumour suppressors and cell-cell/matrix adhesions and immune responses as well in comparison to their healthy tissue counterparts. In addition, overexpressed genes were largely associated with ECM composition, modification and structure. Therefore, this



ADAM/T – a disintegrin and metalloproteinase/with thrombospondin motif, CCL – C-C motif chemokine ligand, CSF-1 – colony-stimulating factor 1, CXCL – C-X-C motif chemokine ligand, IFN – interferon, IGF2 – insulin-like growth factor 2, IL – interleukin, MDSC – myeloid-derived suppressor cell, MMP – matrix metalloproteinase, PDGF – platelet-derived growth factor, SDF1 – stromal cell-derived factor 1, TAM – tumour-associated macrophage, TGF – transforming growth factor, TIMP – tissue inhibitor of metalloproteinase, TNF – tumour necrosis factor, Treg – regulatory T cell, VEGF – vascular endothelial growth factor.

FIGURE 2 Cellular graphics illustrate the secretome's intricate and dynamic roles in the progression of the cancer state. (A) Visualisation of secretome alterations driving cancer development, including vascular remodelling, extracellular matrix (ECM) deposition and reduced cell-matrix adhesion due to the increased secretion of altered secretome components. (B) Illustration of pro-tumourigenic processes resulting from secretome dysregulation, including enhanced basement membrane degradation, angiogenesis at the tumour site, ECM deposition, activation of cancer-associated fibroblasts (CAFs) and immunosuppression. This image has been adapted from Ritchie et al., 2021⁸⁷ under the Creative Commons Attribution License.

study highlighted the involvement of the cancer cell secretome in the progression and pathophysiology of the tumour (Figure 2).¹⁰¹ Figure 2 highlights the shift in the secretome to promote a pro-tumourigenic environment. Pathways affected include increased fibroblast activation, ECM deposition and accumulation and vascular remodelling as well as the reduction of tumour suppressors, immunosuppression and loss of cell-cell matrix adhesion.^{87,101} Comparably, in Duchenne muscular dystrophy, a genetic mutation results in dystrophin protein not being expressed. This results in progressive muscle inflammation, weakness and degeneration due to alterations to the extracellular environment.⁸ Therefore, it can be noted that UPS pathways hold great potential in providing druggable targets for the development of therapeutics for human disease treatment.

2.3 | The role of the secretome in the various phases of repair and regeneration

Whilst the complex molecular mechanisms underlying the repair and regeneration are not universally described or understood across all tissue types,¹⁰² there is a consensus that a tissue injury triggers a coordinated cascade of signalling molecules to facilitate the repair process.¹⁰³ Although the stages of tissue repair are tissue-specific and are largely governed by the extent of the injury, there are four main stages controlling tissue repair and regeneration. Despite being distinct phases, the phases overlap and are described as hemostasis, inflammation, proliferation and remodelling. Each phase is characterised by various cellular interactions that are controlled by the local

release of cellular secretions such as chemokines, growth factors, cytokines and various inhibitors.^{104–106} Immediately after the primary insult has occurred, constriction of the blood vessels in combination with the activation of the coagulation cascade by platelets and clotting factors signals the hemostasis phase. This phase is critical in the formation of the fibrin clot to prevent excessive blood loss and establish a temporary matrix to facilitate cellular migration and proliferation. During this phase, cell secretions can include thrombospondin, von Willebrand factor, fibronectin and sphingosine 1 phosphate whilst transforming growth factor β (TGF- β) and platelet-derived growth factor (PDGF) are important mediators in recruiting immune cells to enter the wound and initiate the initial repair to the ECM.¹⁰⁶ The inflammation phase then commences within 24 h post-injury. This phase is characterised by the recruitment of further immune cells as important mediators against infection and the clearing of the damaged ECM components.^{105,107} During this phase, cell secretions can include various cytokines, chemokines, growth factors and enzymes such as interleukin (IL)-8, (IL)-1, (IL)-6, tumour necrosis factor (TNF)- α , PDGF, fibroblast growth factor (FGF), insulin-like growth factor 1, elastase and matrix metalloproteinase (MMP)-8.¹⁰⁶ The proliferative phase then begins and is responsible for the formation of granulation tissue and an established ECM, composed of various proteoglycans, collagen and fibronectins, to replace the temporary ECM secreted during the hemostasis phase. This phase ensures that cell proliferation occurs to begin closing the wound site. During this phase, cell secretions can include various MMPs, PDGF, TGF- β , TGF- β 1, TGF- β 2, TGF- β 3, TNF- α , basic FGF (bFGF), vascular endothelial cell growth factor (VEGF) as well as anti-angiogenic factors, such as endostatin, angiostatin and

thrombospondin and pigment epithelium-derived growth factor.¹⁰⁶ The last phase, remodelling, is responsible for the strengthening of the granulation tissue over time and the maturity of this new tissue into a scar. There is also a notable decrease in cell density and metabolic activity. During this phase, cell secretions can include the deposition of reticular collagen, type I collagen, lysyl oxidase, zinc-dependent endopeptidases, tissue inhibitors of metalloproteinases (TIMPs), MMP-1, MMP-2, MMP-9, TGF- β and TGF- β 1.¹⁰⁶

Whilst the phases presented apply to all tissues, variations such as the inherent capacity of the tissue to regenerate, the microenvironment and the secretion of regenerative factors contribute to the variation in repair and regeneration currently observed in patients.

3 | THE SECRETOME AS A BIOTHERAPEUTIC AGENT

MSCs are an effective tool for tissue repair as they migrate to the injury site and suppress the inflammatory response, thereby promoting repair and regeneration.¹⁰⁸ Furthermore, repair and regeneration are attributed to the biofactors that are released from MSCs and act in a paracrine manner¹⁰⁹ that can activate cell-cell signalling¹⁹ to mediate a biological response.

3.1 | Central nervous system regeneration

Low regenerative capabilities plague the adult central nervous system (CNS), which results in an increasing prevalence of people enduring a variety of CNS pathologies, including but not limited to spinal cord injury (SCI), ischaemic stroke and traumatic brain injury (TBI). Since CNS disorders and injuries have a multitude of causes and symptoms as well as differences in pathophysiology, repairing damaged and diseased tissues and organs remains problematic.

Of the difficulties, activation of biological cascades often intensifies the primary injury.^{110,111} Currently, most clinical approaches focus on palliative solutions, rather than promoting tissue repair and regeneration.¹⁵ Novel therapies have begun to be developed for regenerative applications, including biomaterials,¹¹² molecular therapies^{113,114} and cell-based therapies.^{115–117} Despite CNS cell-based therapies producing favourable results, several challenges such as transplantation safety, remain.¹⁵ As such, attention has shifted towards the cell-secretome.

3.1.1 | Spinal cord injury

Following the primary insult to the spinal cord, secondary molecular insults cause lifetime functional deficits due to the formation of a glial scar, which inhibits native regeneration.^{118–123} Despite the improvement in the understanding of SCI pathophysiology, surgical intervention still presents with its own issues such as reperfusion of the injured tissue.^{123–125} Considering this, novel neuroprotective

strategies need to be designed to complement the existing surgical interventions whilst mitigating the effects of the secondary molecular insults. As such, strategies involving the cell-secretome for functional recovery are being explored.

In a recent study, Vawda and co-workers used secretome factors from bone marrow mesenchymal stromal cells (BMSCs) or human umbilical cord matrix cells (HUCMCs) to differentiate between their therapeutic efficacy post-SCI. In adult Wistar female rats, a C7-T1 compression/contusion injury was induced, which was followed by the prompt administration of intravenous (IV) concentrated secretome factors. In this study, HUCMCs were shown to be more effective at reducing the secondary molecular insults post-SCI and lesion volume whilst improving vascular functionality. However, there were only marginal effects in parenchymal haemorrhage and vascular permeability. Furthermore, these effects did not result in prolonged functional recovery. This highlights that HUCMC factors may not be successful when utilised as a standalone therapy.¹²⁶

In a different study performed by Tsai et al., 2018, the use of BMSCs secretome factors as a potential treatment for SCI was investigated to assess if the BMSCs secretome factors would result in significant functional improvements in an SCI rat model. It was found that female Sprague Dawley (SD) rats that underwent a T8-10 laminectomy exhibited improved locomotion from one-week post-injury and beyond ($p < 0.05$). SD rats dosed with the BMSCs secretome factors continued to exhibit improved functional recovery, indicated by higher Basso, Beattie, Bresnahan (BBB) ratings, for the 6-week duration of the study (weeks 2–6; $p < 0.01$). This finding differed from the results presented by Vawda et al., 2019, despite only one cell type being utilised in this study.¹²³ Furthermore, at 6 weeks post-injury, the secretome-treated rats displayed higher axonal densities as well as more nerve fibres and preserved tubulin in comparison to the SCI rats. The findings indicated that the factors improved neuroprotective effects whilst providing a microenvironment for axonal regrowth after SCI.¹²⁷

From the work presented, it is evident that the secretome shows potential as a SCI therapeutic, however, not all research presented resulted in long-term functional recovery. Due to this, it is imperative that further research is conducted before clinical translation can occur.

3.1.2 | Traumatic brain injury

TBI is a non-degenerative, non-congenital acquired form of brain damage from an external force, which can result in temporary or permanent impairment of psychosocial, physical and cognitive functions.^{128–130} As with SCI, the varied prognosis is governed largely by the secondary molecular insults rather than the primary injury.^{131–133} The secondary molecular insults, which are triggered by neuroinflammation, are a major contributor to apoptosis and necrosis of neural cells which results in extensive injuries¹³⁴ and prevents native regeneration. Unfortunately, there are no current therapeutics for TBI treatment as treatments are unable to adequately modulate



the process of neuroinflammation.^{135,136} The current care for individuals who have sustained a TBI is palliative. As with SCI, novel neuroprotective strategies need to be designed to complement the existing palliative care options whilst mitigating the effects of the secondary molecular insults. A potential avenue is the exploration of the cell-secretome for functional recovery.¹³⁷

In research performed by Xu et al., 2020 adipose MSC-derived secretome (ASC-ST), were collected and investigated to establish if MSCs (ASC-ST) could regulate neuroinflammation and improve functional outcomes in an SD rat TBI model. After a TBI had been induced, a modified neurologic severity score test was performed on all groups. In comparison to the vehicle control, the secretome group exhibited superior performance and achieved lower scores from Day 7 to 28 after the induction of TBI. A noteworthy finding was that the scores for the MSCs (ASC-ST)-treated group were approximately less than half of the scores that were recorded the day after TBI induction. In addition, using a Morris water maze test, the MSCs (ASC-ST)-treated group were able to locate the platform quicker. Once the platform was removed, they exhibited superior memory than the TBI-vehicle group over a 60 s period. Furthermore, MSCs (ASC-ST) were able to diminish nerve fibre damage 14 days after TBI induction when assessed using fractional anisotropy. Additionally, MSCs (ASC-ST) treatment was shown to assist in mediating the ongoing inflammatory responses due to an increase in brain permeability. Although further investigations are necessary, MSCs (ASC-ST) treatment was shown to have a potentially protective effect on neural cells by arresting apoptosis which may improve cytotoxic oedema. These findings show that injected MSCs (ASC-ST) can be used to treat early-stage secondary molecular insults that cause neuroinflammation post-TBI. It was shown that MSC (ASC-ST) treatment could improve functional outcomes post-TBI, improving neural cell apoptosis, improving nerve fibre damage as well as reducing neural oedema.¹²⁹

In a separate study performed by Liu et al., 2020, it was hypothesised that there would be a beneficial therapeutic effect in response to the secretome released in response to brain tissue damage of umbilical cord mesenchymal stem cells (UCMSCs). In this light, three secretome extracts were prepared for testing: (1) USMSCs secretome without brain extract (ST), (2) UCMSCs exposed to healthy brain extract (B-ST) and (3) UCMSCs exposed to injured brain extract (IB-ST). The results indicated that the IB-ST group exhibited improved cognitive function compared to the ST group ($p < 0.01$) and demonstrated an improvement in memory as well as an increase in the excitatory postsynaptic slope potential and population spike amplitude. Furthermore, on Day 7 and Week 4 post-TBI, there was a 35% and 55% increase in the number and density of Bromodeoxyuridine (BrdU) positive cells in the dentate gyrus for the IB-ST group over the ST group, respectively. Additionally, an increase in Sox2 and Hes1 was noted at 7 days post-TBI for the injured brain extract group in comparison to the ST group, indicating an increase in cellular proliferation. In terms of the neural differentiation 3 days post TBI in the dentate gyrus, the differentiation of neuroblasts was significantly higher for the IB-ST group over the ST group ($4100.5 \pm 563.2/\text{mm}^3$ vs. $1350.2 \pm 225.3/\text{mm}^3$, $p < 0.01$) and after 7 days post TBI, an

increase in the mature neuron density of the subgranular zone (SGZ) of the ipsilateral hippocampus was noted for the IB-ST group over the ST group ($3265 \pm 446.8/\text{mm}^3$ vs. $1201.0 \pm 143.6/\text{mm}^3$, $p < 0.01$). Considering the migration of newborn neurons post TBI, an increase in the ratio of neuron migration from the SGZ to the granule cell layer after 4 weeks was 72.3% higher for the IB-ST group over the ST group and after 8 weeks, there was a greater number of neurons in the granule cell layer for the IB-ST group over the ST group ($3600 \pm 445.2/\text{mm}^3$ vs. $650.0 \pm 69.9/\text{mm}^3$, $p < 0.01$). Lastly, the IB-ST group displayed a reduction in apoptosis in the microenvironment of the dentate gyrus as well as an upregulation of hippocampus neurogenesis proteins and neurogenesis-associated factors.¹³⁸

From the research above, the secretome of MSCs may elicit a beneficial effect post-TBI and may assist with improving TBI outcomes. In addition, although more research is required, the secretome may provide a new therapeutic avenue for treating TBI considering the promising research that has been presented.

3.1.3 | Cerebral infarction

A cerebral infarction, more commonly known as ischaemic stroke, can be described as a sudden decrease in blood flow to the brain resulting in tissue necrosis and death. It is usually caused by an embolic or thrombotic occlusion in the main arteries supplying blood and nutrients to the brain. This causes loss of tissue function, which is followed by the loss of supporting structures and neurons. The reduced blood flow initiates a cascade that commences with the depletion of electrical function and disruptions to the membrane function from calcium-dependent excitotoxicity followed by the production of oxidative free radicals which leads to membrane destruction and cell lysis.¹³⁹ Despite recombinant-tissue plasminogen activator (IV r-TPA) being the benchmark treatment for ischaemic stroke, high-risk patients—many of which fall within the incidence group—are unable to receive treatment due to contraindications based on comorbidities. In addition, the benefit of IV r-TPA is time dependent with little to no effect being observed after 3 h.¹⁴⁰ Therefore, the cell-secretome may be an innovative direction for potential functional recovery.

In an in vitro model, Loiola et al., 2021 set out to assess if the secretome derived from endothelial progenitor cells (EPCs) from stroke patients would improve angiogenesis and ischaemia models. The researchers considered the effects of Good Manufacturing Practices (GMP) on the yield of the EPC stroke patient secretome produced. It was found that high-scale production (HSP) produced a higher yield of proteins in comparison to low-scale production (LSP) (HSP: 34.9 ± 1.6 mg vs. LSP: 207 ± 13.6 μg). However, in comparison to VEGF-A, the EPC stroke patient secretome did not produce a significant difference in cellular migration (264.3% [203.4–349.6]), proliferation (139.6% [134.8–183.4]) and tubulogenesis (120.7% [103.3–142.4]) when compared to the control.¹⁴¹

In a separate study, Tai et al., 2021 aimed to assess whether the secretome from human embryonic mesenchymal stem cells (hESC-MSC) would influence angiogenesis, neurogenesis and neurological

deficit in an experimental stroke model. Results indicated that the secretome group exhibited better forelimb usage and an improvement in motor function recovery when compared to the saline control. In addition, the secretome group had a notable reduction in both the cortical, striatal and total infarction volumes. Furthermore, the secretome group exhibited an increased expression of both angiogenesis (CD31) and neurogenesis markers (Ki67, Nestin, Reelin and DCX) in the striatum and cortex regions in comparison to the saline group.¹⁴² These findings, particularly the upregulation of neurogenesis markers, are in accord with what Liu et al., 2020 found in their TBI study, indicating that the secretome may be the missing link in brain tissue recovery regardless of the application. In a different study by the same research team, they set out to establish the effects of the hESC-MSC secretome on inflammation, neurogenesis and apoptosis in the hippocampus in a stroke model. As above, although the secretome group exhibited an increased expression of neurogenesis markers (Ki67, Nestin, Reelin and DCX) in the hippocampus in comparison to the sham, the results were not statistically significant in the one-way ANOVA analysis. Results from the inflammatory response in the hippocampus, contrastingly indicated that there was a significant upregulation of IL-1 β and IL-6 transcripts ($p < 0.001$) after stroke in comparison to the sham, however, IL-10 expression remained unchanged ($p = 0.20$). This indicates that the secretome may be able to modulate the inflammatory response in the hippocampus post-stroke. Considering the impact of the secretome on apoptotic markers in the hippocampus indicated that although there was an increase of significance in Bax ($p < 0.01$), Bim and Bcl2 transcripts did not indicate significant changes in comparison to the sham ($p = 0.16$ and $p = 0.86$, respectively). There was no significant change in the expression of NT-3, NGF, GDNF and BDNF in comparison to the sham in the hippocampus. Lastly, the one-way ANOVA analysis results indicated that the secretome group had an increase in significance in surviving neuron percentage (CA1, CA3 and DG) in comparison to the sham control ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively) after stroke.¹⁴³

Surprisingly, the use of the secretome produced contradictory results for applications in cerebral infarction. Considering the research conducted by Taei et al., despite the hESC-MSC secretome showing favourable results in the striatum and cortex regions, the results in the hippocampus were largely indifferent. This could indicate that the benefit of the hESC-MSC secretome may be brain region dependent, which could potentially limit its future usage as a successful cell-free therapeutic.

3.2 | Connective tissue regeneration

For this review, connective tissue will include both bone and cartilage. This distinction has been made as the tissue comprising bone can be described as mineralised connective tissue.¹⁴⁴ Unlike with the CNS where the tissues exhibit limited native regeneration, some connective tissues do have the ability to regenerate. Although bone tissue displays spontaneous regeneration in response to fractures, not all

fractures can be adequately repaired.¹⁴⁵ On the other hand, cartilage does not have the ability to self-regenerate due to the inherent lack of a blood supply.¹⁴⁶ In attempts to assist with the regeneration of connective tissues, potential therapies have been developed, however, they are plagued by their own limitations. It is therefore evident that a new avenue needs to be considered to improve the clinical outcomes that are currently being observed in connective tissue regeneration.

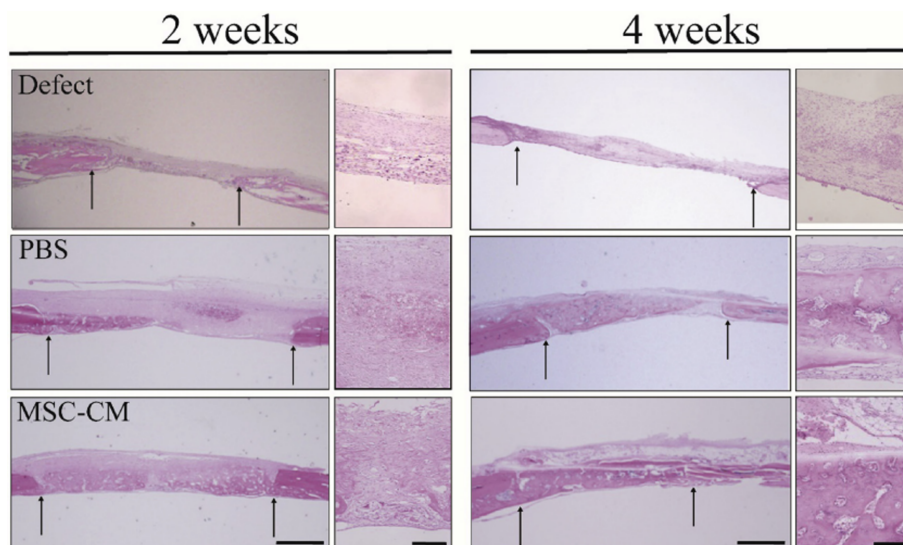
3.2.1 | Bone regeneration

Bone regeneration is a spontaneous, yet complex physiological process that is activated in response to the native healing of bone fractures.¹⁴⁵ Bone regeneration is a multifarious process, requiring the coordination of several cellular role players for optimal tissue regeneration and connective tissue mineralisation.¹⁴⁷ The appearance of blood, inflammation and blood coagulation at the fracture site,¹⁴⁸ commences the regenerative process and is ultimately responsible for the primary strength of the new tissue. Whilst small fractures are readily repaired without scarring and strength restoration, clinical conditions (native fractures or orthopaedic/oral and maxillofacial surgery) where large-scale repair is required often result in regenerative process compromise, delayed or insufficient repair.¹⁴⁵ Although most of the current bone regeneration strategies such as bone grafts (gold standard) and distraction osteogenesis produce adequate results, autogenous grafts are plagued by blood loss, donor site pain, potential donor site infection,^{149,150} lack of cellularity,^{149,150} rejection, disease transmission and high cost.^{151–154} On the other hand, tissue engineered scaffolds have emerged and have successfully reduced some of these limitations, however, they are limited themselves by host site reaction and immune response, as well as a limitation in translational material testing, which has reduced their clinical applicability.^{145,151} As such, the use of the secretome may assist in overcoming these inherent issues.

In a study conducted by Dilogio and co-workers, they attempted to identify the effect of using human bone marrow-mesenchymal stem cell (hBM-MSC) secretome to treat critical-sized femur defects in SD rats. The findings indicated that the dosing of the secretome alone was sufficient in enhancing bone healing as there was no statistical difference between the secretome alone and the secretome in combination with bone morphogenetic proteins (BMP) and MSC. This result was confirmed by evaluating the osseous area (ArOs) where the secretome-only group exhibited the highest significant ArOs formation when compared to the other groups. In terms of the cartilaginous area (ArCr), the secretome alone had the greatest decrease in ArCr in comparison to the other groups but showed an increased rate of ossification at 4 weeks. Lastly, the secretome alone had the lowest total callus area when compared to the other treatment groups.¹⁵⁵ These results indicate that although the secretome alone performs well in certain instances, BMP and MSC could still convey a beneficial advantage in fracture healing.



FIGURE 3 Histological analysis comparing bone regeneration in the secretome (MSC-CM) group to the phosphate buffered saline solution (PBS) control group. After 2 weeks, bone regeneration in the secretome group had almost completely covered the defect area, while only connective tissue was observed in the control PBS group. By 4 weeks, lamellar bone was evident at the defect site for the MSC-CM group, whereas the PBS control group showed only initial replacement of immature bone. Host bone edges are marked with arrows. Bars: 1000 μ m (left), 200 μ m (right). This figure is reproduced from Ogata et al.,¹⁵⁶ with permission from Elsevier.



In research conducted by Ogata et al., 2018, the research team hypothesised that the secretome from human bone marrow-derived mesenchymal stem cells (MSC-CM) delivered in an Atelocollagen (Terudermis®) implant would enhance early endogenous stem cell migration at the lesion site to induce bone regeneration. The results indicated that 2- and 4 weeks post calvarial bone defect surgery, the percentage of newly regenerated bone was 53% and 62.6%, respectively for the secretome group, which was significantly greater in comparison to the other experimental groups. Interestingly the new bone after weeks 2 and 4 in the secretome group was shown to be made up of lamellar bone similar to native structures. Furthermore, as shown in Figure 3 from the data presented by the research team, the defect, stained using haematoxylin and eosin, was completely closed after week 4 in the secretome group, whereas only partial closure was noted in the other groups. This image illustrates the potential therapeutic benefit of the secretome in accelerating bone closure and regeneration after defect induction at the lesion site. The secretome group also induced cellular migration of labelled rat MSCs (rMSCs), from the caudal vein to the implant site, 1 week after injection and displayed the greatest fluorescent intensity in all the experimental groups.¹⁵⁶ This study highlighted the fact that the secretome may act through paracrine signalling to elicit its therapeutic effects.

The utilisation of the secretome as a bone tissue regenerative strategy seems to be a relatively new direction and as such, not much research is available. However, although the secretome does seem to be beneficial in accelerating bone tissue regeneration, more research is required to confirm its clinical applicability.

3.2.2 | Cartilage regeneration

Despite the important role of cartilage, cartilage displays a limited native regenerative capacity due to the lack of a blood supply, often requiring surgical intervention as a solution.¹⁴⁶ Current therapies include intra-articular injection of various preparations but are

associated with chondrocyte toxicity,^{157,158} cartilage damage^{157,158} and limited therapeutic benefit.^{157,159–161} Surgery is another option but is associated with possible immunologic response and varied tissue availability,^{157,162} whereas cell-based therapies are plagued by the inability to balance proliferation and differentiation in chondrocytes¹⁵⁷ as well as ethical concerns (stem cell therapies).^{157,163} Tissue-engineered constructs have also been considered but are limited by inadequate mechanical strengths, leaching of organic solvents, uneven pore densities, biocompatibility and incomplete regeneration and repair.¹⁵⁷ Therefore, an innovative approach to facilitate regeneration is required.

An in vitro study conducted by Palamà et al., 2020 aimed to analyse the ameliorative effects of the secretome from human bone marrow stromal cells (hBMSCs) grown in either foetal bovine serum (FBS) or a xeno-free medium (XFS) in an osteoarthritis (OA) model. To mimic the inflammatory OA environment, human chondrocytes were dosed with IL-1 α and then with the XFS or FBS-derived secretome. The results showed that the XFS-derived secretome was able to reduce the inflammatory environment by inhibiting the IL-1 α -induced expression of Cox-2, IL-6 and IL-8, whereas the FBS-derived secretome maintained the inflammatory environment. This result was attributed to the upregulation of several cellular proteins involved in angiogenesis (VEGF, ANG, TSP-1), immunomodulation (ANG, PTX-3, TSP-1), wound healing (TSP-1, uPAR, OPN) and remodelling processes (OPN, VEGF, ANG) for the XFS-derived secretome. In addition, XFS-derived secretome was shown to produce more EVs which further prevented the inflammatory effects of IL-1 α and conveyed a chondroprotective effect in vitro in comparison to the FBS-derived secretome. Furthermore, XFS-derived secretome was shown to reduce NF- κ B inflammatory activity after 40 h.¹⁶⁴ This study highlighted that XFS-derived secretome could have beneficial effects in degenerative diseases such as OA as well as general bone repair. A further benefit of the XFS-derived secretome is that it is free from contaminating serum due to its chemical origins hence making it a ready-to-use, clinical-grade product.¹⁶⁴

In a separate *in vitro* study, Niada and co-workers set out to investigate the possible anti-catabolic and anti-hypertrophic action of the secretome derived from adipose stromal cell (ASC) on TNF α -stimulated primary human articular chondrocytes (CH). The secretome reduced osteocalcin (OC) and collagen X expression by 18% on day 1 and 37% on day 3 as well as lowered MMP-13 activity by 61%. Furthermore, MMP-3 activity was halved. Additionally, the secretome did not influence chondrocyte proliferation and viability. This study indicated that the secretome was able to limit the destructive effects of TNF- α (inflammatory cytokine) and MMPs.¹⁶⁵ This study also suggests that the secretome can be prepared and stored in advance to create a ready-to-use product, which agrees with the views of Palamà et al., 2020.

Despite the benefits of the secretome presented in this section, *in vivo* studies focussing solely on the regeneration of cartilage using the secretome seem to be lacking. As such, these results must be confirmed in animal models before their clinical applicability can be decided. Furthermore, more research directed towards the regeneration of cartilage is needed before we see the production of a potential therapeutic.

3.3 | Skin, wound and oral regeneration

Similar to the liver, the skin is an organ that also has full regenerative abilities.¹⁶⁶ Skin regeneration is a sequential, overlapping process involving many role players that activate gene expression through endocrine signalling. Homeostasis, the first phase, begins immediately after the injury and involves vascular constriction and the formation of the fibrin clot. The wound tissue and the clot then coordinate growth factor and pro-inflammatory cytokine release. Inflammatory cells (lymphocytes, macrophages and neutrophils), utilising chemotaxis, then sequentially infiltrate the wound after bleeding has been controlled to initiate the inflammatory phase.^{167–170} With the assistance of the macrophages, the proliferative phase then begins and overlaps with the inflammatory phase. This stage is characterised by angiogenesis as well as the movement and expansion of the epithelial cells across the surface of the wound. In addition, deposition and synthesis of the ECM occurs in the dermis. The remodelling phase then occurs which is distinguished by the maturation and regression of capillaries as well as the remodelling of the ECM back to the native tissue state.^{167–170} However, in skin lacerations, slower regeneration occurs which is coupled with the dysregulation and remodelling of the ECM and excessive deposition of altered collagens and other ECM proteins.^{27,171} In terms of oral wounds, the process is similar to that of skin with notable differences including the presence of different cell phenotypes and saliva creating a moist environment¹⁷² as well as the presence of microorganisms.¹⁷³ There are several factors that contribute to poor oral wound healing, but the most common factor is the oral cavity's inability to achieve homeostasis, at any of the healing phases, after an injury has occurred.¹⁷⁴ Currently, therapeutic attempts to reduce scarring have been largely unsuccessful. It is evident that the ECM is a good target for therapeutic intervention and

opens the door for the secretome to potentially improve the regenerative attempts in these tissues.

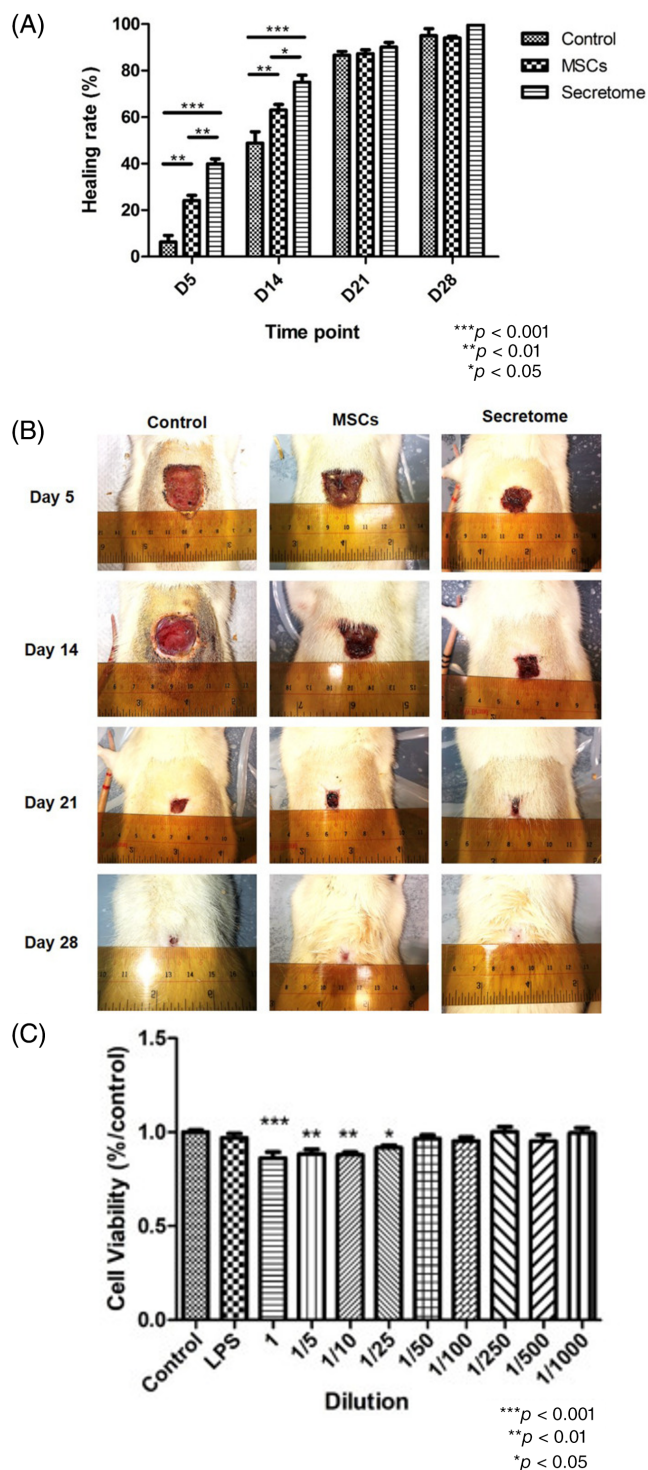
In research performed by Ajit et al., 2023, the research team assessed the efficacy of adipose tissue-derived mesenchymal stem cell (ADMSC) secretome enriched with pro-angiogenic growth factors (AGF), VEGF and Hif-1 α , on the growth of blood vessels and wound healing in a rabbit model of large, full thickness, acute wounds. The results indicated that surface wound healing was superior in rabbits dosed with the secretome enriched with AGF in comparison to the sham. Specifically, after 10 days, the wounds treated with the VEGF-enriched secretome exhibited 47% closure as compared to the sham control which exhibited 19% closure. In addition, the healing rate was also higher in rabbits treated with the AGF-enriched secretome. Furthermore, after 28 days, notable angiogenesis and epithelialisation with mature tissue granulation and low fibroblast and inflammatory cell populations were noted in rabbits treated with the AGF-enriched secretome. Additionally, secretome treatment groups displayed a significant increase in the wound size covered by mature collagen than the control groups. Likewise, secretome groups displayed an extensive capillary network with broader vessels and increased vessel numbers over the control groups.¹⁷⁵ The results of this study are particularly significant due to the histopathological similarities between rabbit models and human skin. Nevertheless, more research is required to assess the long-term safety, efficacy and scar formation of secretome preparations.

In a study published by Vriend and co-workers, the researchers proposed that a hydrogel prepared from decellularised porcine skin extracellular matrix (dECM) and impregnated with the secretome derived from adipose tissue-derived stromal cells (ASCs) would improve the vascularisation, wound repair and skin flap viability in a Wistar rat pedicled skin flap model. Findings suggested that there was no significant difference between the treatments in wound surface area as all wounds reduced over the course of the study. In addition, results for the wound healing index (WHI) indicated that the loaded hydrogel exhibited higher index values over 14 days in the distal and medial areas which indicated poorer wound healing in comparison to the control, whereas the proximal area showed no difference between treatment groups. Additionally, there was a lack of blood perfusion in the distal area across all groups after 7 days, however, lower perfusion was present in the loaded hydrogel group in the medial after 7 days and blood perfusion remained constant from 7 to 28 days. It was likely that the larger wound area impeded the perfusion of blood in the hydrogel treatment group ($p < 0.01$). Interestingly, the ASC secretome (not loaded in the hydrogel) exhibited an increase in vessel density ($p < 0.0001$) in the distal area after 7 days when compared to the hydrogel-loaded group. In terms of cell proliferation, measured using Ki67 staining, the number of proliferating cells remained the same across all groups in the areas tested (distal, medial and proximal). Correlating results were noted for the width, length and angle of collagen fibres.¹⁷⁶ Although this research provided unexpected evidence that the loaded hydrogel did not have a significant impact on the wound healing of the skin flap, the ASC secretome was shown to have a significant impact on angiogenesis. It is therefore essential that further

research be undertaken to verify these results and determine the efficacy of this secretome preparation for its pharmaceutical application.

In a study conducted by Ma et al., 2021, the researchers set out to compare the efficacy of ADMSCs and the secretome derived from ADMSCs to enhance wound healing in a full-thickness skin excision SD rat model. Results indicated that although both the ADMSCs and the ADMSC secretome significantly increased the rate of wound

FIGURE 4 Images illustrating the accelerated wound healing, enhanced cell viability and macrophage metabolism in response to the adipose tissue-derived mesenchymal stem cells (ADMSCs) secretome. (A) A graph showing the percentage of wound healing over 28 days, with the secretome significantly enhancing wound healing on Days 5 and 14, compared to the MSC treatment. (B) Photographs depict complete wound closure and accelerated wound reduction on Days 5 and 14, consistent with the wound healing rate analysis. (C) Cell viability results demonstrate suppression of RAW 264.7 cell metabolism at the highest concentration of the secretome tested. This image is adapted from Ma et al., 2021¹⁷⁷ under the Creative Commons Attribution-NonCommercial 3.0 Unported License.



healing ($p < 0.05$) as shown in Figure 4A, the ADMSC secretome was shown to further enhance the effect after Days 5 and 14 in comparison to the ADMSCs. Additionally, from Figure 4B, it is evident that full wound closure was achieved on Day 28 in the secretome group. Furthermore, whilst VEGF deposition increased in both groups, VEGF deposition was the highest in the ADMSC secretome group. A similar trend was observed in the cell proliferation data as the number of PCNA-presenting cells was higher for the ADMSC secretome group when compared to the ADMSC group ($p < 0.05$). Additionally, the ADMSC secretome group was shown to reduce the quantity of TNF- α and IL-6 to a greater extent than the ADMSCs alone ($p < 0.05$). In an in vitro study, an increase in cell viability ($p < 0.05$) was noted for the ADMSC secretome between the dilutions of 1/10–1/1000 in dermal fibroblast (FR) cells, however a concentrated preparation of the ADMSC secretome displayed toxicity in FR cell metabolism. Interestingly, the concentrated ADMSC secretome had no effect on FR cell proliferation but a broad dilution of the ADMSC secretome significantly increased the cellular proliferation ($p < 0.05$). In terms of the FR cellular migration, the more concentrated dilutions of the ADMSC secretome resulted in a significantly faster FR migration ($p < 0.05$). Interestingly, the more concentrated dilutions of the ADMSC secretome resulted in significant suppression of RAW 264.7 cell metabolism ($p < 0.05$) but resolved at the more dilute concentrations as shown in Figure 4C. Inflammation studies indicated that there was a significant decrease in the quantity of MCP-1 in response to a variety of ADMSC concentrations (1/50–1/1000) ($p < 0.05$) when compared to the control. Such results could indicate that the secretome can modulate inflammation and reduce the immune response whilst maintaining cell viability. In addition, TNF- α expression was significantly reduced after exposure to specific ADMSC concentrations (1/50, 1/100, 1/250, 1/500) when compared with the control ($p < 0.05$).¹⁷⁷ Whilst this study indicated that the ADMSC secretome had an improved therapeutic outcome, it also highlighted that the concentration of the secretome cannot be assumed to be safe and therefore additional research is required to establish the therapeutic window of secretome preparations before their clinical applicability can be determined.

In a study performed by Rong et al., 2019 the research team aimed to investigate if the secretome derived from human foetal skin-derived stem cells (hFSSC) would improve skin injuries induced by

radiation in a rat model. In vitro indicated that human umbilical vein endothelial cells (HUVEC) displayed the greatest percentage of Ki-67-positive cells (88.4 %) in response to the hFSSC secretome treatment when compared to the other two controls ($p < 0.01$). Furthermore, in response to the hFSSC secretome, a significant promotion of tube formation with an increase in branching point ($p < 0.001$) and tube length ($p < 0.001$) in the HUVECs was noted in comparison to the controls. From the in vivo Strontium-90 (Sr-90) radiation skin injury model, the results indicated that the rats treated with the hFSSC secretome experienced reduced desquamation, erythema, crusting and scarring with an increased wound healing rate (week 7: 96.3% wound closure) in comparison to the controls. It was noted that rats treated with the hFSSC secretome had more sweat glands, hair follicles and sebaceous glands (cutaneous appendages) in comparison to the controls (43.1 ± 5.6 , $p < 0.001$). Lastly, the hFSSC secretome treatment group displayed the highest expression of TGF- β 3 and Col3A1 ($p < 0.01$), Ang-1 ($p < 0.01$), Ang-2 ($p < 0.05$), VEGF ($p < 0.01$) and PIGF ($p < 0.001$) with the lowest expression of TGF- β 1 and Col1A2 ($p < 0.05$) when compared to the controls.¹⁷⁸ These results indicate that the hFSSC secretome may promote in vitro cellular proliferation and tube formation, may accelerate angiogenesis and wound healing in rats and could regulate the protein and gene expression in wound healing.

In research published by Robert et al., 2019 the aim was to utilise the secretome from skin-derived MSCs (SD-MSCs) alone and in a hydrogel system to assess if accelerated cutaneous wound healing would occur in a mouse model. Results from the in vitro experiments indicated that in response to the SD-MSCs derived secretome, organised tube formation was significantly enhanced in human umbilical vein endothelial cells (HUVECs) and the number of meshes ($p < 0.0001$) and nodes ($p < 0.0001$) for the secretome-treated group was similar to the positive control after 12 h and remained after 24 h. Although the researchers wanted to confirm these results in vivo, no significant improvement in wound closure, epidermal thickness and thickness of granulation tissue was observed in an in vivo model.¹⁷⁹ The results indicated that the secretome derived from the SD-MSCs could aid in the initial stages of wound repair owing to its in vitro pro-angiogenic properties, however, further work is required to assess its in vivo effectiveness.

In research published by Ormazabal and co-workers in 2022, it was investigated whether the secretome derived from human endothelial cell-differentiated mesenchymal stem cells (hMSC-EC) and undifferentiated human mesenchymal stem cells (hMSC) would improve wound healing in a high-fat diet (HFD) type-2 diabetes model. The findings suggested that although increased wound healing was observed for the hMSC-EC secretome group in both the control diet (CD) and HFD groups, delayed healing was noted in the HFD group. Although both groups exhibited wound closure by Day 9, the CD group exhibited 80%–90% closure by Day 2, whereas the HFD group exhibited the sample closure by Day 4. In addition, the histological analysis indicated that the hMSC-EC secretome group exhibited greater wound healing in comparison to the controls. Interestingly, secretome protein profiling indicated that the hMSC-EC secretome

exhibited an upregulation of 11 hallmark mediators of angiogenesis/vasculogenesis. Of these proteins, VEGF-C, FGF-7, ANG-1 and ANG-2 and MMP-9 were mixed and assessed for their wound repair properties. In vitro results indicated that the protein mixed increased proliferation in ECV-304 endothelial cells but not HaCaT cells, whereas in vivo findings showed a significant increase in the wound closure rate in the HFD mice in comparison to the CD mice after 9 days.¹⁸⁰ Although some of the results presented in this paper were favourable, some results were inconclusive and require further research to establish the feasibility of the hMSC-EC secretome and protein mixture as potential therapeutic candidates.

Recent in vitro research published by Kunhorm and colleagues intended to investigate whether cordycepin-induced HaCaT secretome (CHS) would improve the biological and wound healing properties of Human Dermal Fibroblasts (HDF). The results indicated that the proliferation of HDF cells increased in a dose-dependent manner MKI67 expression increased after CHS treatment. The CHS treatment was also shown to exhibit reactive oxygen species (ROS)-scavenging properties in HDF cells through its ability to significantly reduce the fluorescent intensity of dichlorofluorescein (DCF) comparably to that of N-acetyl cysteine (NAC). In addition, significant genes involved in ROS-scavenging for example glutathione peroxidase (GPX1), catalase (CAT) and superoxide dismutase 1 (SOD1) were upregulated in a dose-dependent manner. From a wound-healing assay, HDF cells treated with the CHS treatment exhibited increased cell migration, suggesting its potential wound-healing properties. Furthermore, HDF cells treated with CHS were shown to have increased the mRNA expression level of ECM components whilst decreasing the genes responsible for ECM degradation. Moreover, CHS treatment activated autophagy in the HDF cells indicating its ability to promote cellular homeostasis.¹⁸¹ Whilst the data presented here is convincing, more research is required to confirm these findings in in vivo models to determine their clinical applicability in wound regeneration.

In research performed by Ahangar et al., 2020 the researchers aimed at delivering the secretome derived from human gingival fibroblasts (hGFs) to assess if improved oral wound healing would occur in an excisional wound repair murine model. The results indicated that excisional wounds treated with the secretome from the hGFs displayed significant improvements to the wound area (Day 3/7: $p > 0.001$ and Day 14: $p > 0.01$), a decrease in wound width (Day 3: $p > 0.001$ and Day 7: $p > 0.05$) as well as a higher percentage of re-epithelialisation (Day 3/7: $p > 0.05$) when compared to the controls. Results from an in vitro study on foreskin fibroblasts (HFFs) and human keratinocytes (HaCaTs) indicated that in response to the hGFs secretome, a significant increase in cell proliferation was noted for the HFFs by 16%, $p < 0.0001$ and the HaCaTs by 30%, $p < 0.0001$ after 24 h when compared to the controls. After 48 h, scratch results indicated that migration in the HFFs in response to the hGFs secretome was 81.7% in comparison to the 53.6% closure in the control medium ($p < 0.0001$). In addition, a decrease in neutrophil quantity was noted on Day 3 and 7 by 49.2% ($p < 0.0001$) and 61.1% ($p < 0.0001$), respectively, for the hGFs secretome group when compared to the control wounds. Macrophage infiltration, monitored using F4/80, was



also reduced in the hGFs secretome group ($p < 0.05$) and Ym-1 expressing macrophages increased at Day 3 ($p < 0.0001$) and Day 7 ($p < 0.05$) when compared to the controls. A highly significant reduction in the levels of TNF- α was also noted at Day 3 ($p < 0.0001$) for the hGFs secretome group in comparison to the control groups. An improvement in the density of capillaries and structures was noted on Day 7 ($p < 0.0001$) for the hGFs secretome group in comparison to the controls and there was an increase of significance in the migration, proliferation and vessel-like formation in human dermal microvascular endothelial cells (HDMECs) when exposed to the hGFs secretome. In terms of collagen production, the production of both collagens I and III were increased on Day 7 ($p > 0.05$) in the hGFs secretome group in comparison to the controls. In the *in vitro* assessment, the HFFs cells exhibited a decrease in production of collagen I ($p < 0.0001$) and III ($p < 0.0001$) when exposed to hGFs secretome when compared to the negative control.¹⁸² These findings indicated that the hGFs secretome was able to enhance wound healing and re-epithelialisation, decrease inflammation, increase angiogenesis and reduced collagen I and III expression.

In a bioinformatics study by Salkin et al., 2022 the team compared the secretome derived from human dental pulp-derived stem cell (hDPSC) with the secretome from hDPSC transfected hDPSC cells (TGF- β 1 Secretome) to promote osteogenic-adipogenic differentiation and gingival wound healing. Results from Ingenuity Pathway Analysis (IPA) indicated that for the TGF- β 1 Secretome there was an induction of osteogenesis pathways, improved hDPSC immunophenotypes, downregulated adipogenesis pathways and was shown to be associated with *in vitro* wound healing pathways.¹⁸³ Despite this study indicating the potential therapeutic benefit of the transfected TGF- β 1 secretome in regenerative dentistry and orthopaedics, *in vivo* studies are required to determine the therapeutic clinical translation for patients.

Whilst the results presented in this section are somewhat inconsistent, more research—especially directed towards oral wound regeneration—is required to confirm the presented favourable outcomes for therapeutic efficacy. Moreover, there is a definite need for wet lab-based research, directed towards oral wound regeneration as a large number of the available research is bioinformatics-based which makes it difficult to ascertain patient safety and applicability.

3.4 | Well-perfused organ regeneration

For this review, the liver and lungs will be considered well-perfused organs. Whilst the liver is the only visceral that has the ability to self-regenerate, the other visceral organs, including the lungs, are able to adapt to tissue loss but will never return to their original state.¹⁸⁴ Whilst the liver has this remarkable ability, in cases of severe and acute injury causing the loss of hepatocytes,^{185,186} collagen deposition results in the formation of fibrosis, which impedes regeneration.^{186–188} On the other hand, the lungs commonly respond to injury through a non-regenerative process of scar tissue development at the lesion site, which causes stiffening of the lung tissue and

contributes to loss of function and the increase in morbidity numbers.¹⁸⁹ Whilst different strategies and therapies for well-perfused organ regeneration have been developed, no real success has been made in this field. It is critical that novel approaches be considered to improve clinical outcomes.

3.4.1 | Hepatic regeneration

Of all the solid organs (e.g., the kidney, lungs and pancreas), the liver is the only solid organ that can regenerate the tissue to maintain the 100% liver-to-bodyweight ratio after tissue loss.¹⁸⁴ Briefly, hepatic regeneration begins with the resident hepatocytes entering the mitotic cycle as well as the production of a plethora of growth factors and cytokines that trigger DNA synthesis.^{190–192} In the proliferation stage, the hepatocytes enter G1 and further activation of growth factors occurs resulting in the restoration of the liver parenchyma.^{190,192,193} In the last step, although not widely understood, proliferation signals are terminated and the formation of the hepatostat (adjustment of liver size to 100%)^{190,192,194} and controlled ECM deposition occurs.^{190,192} However, in times when the regenerative abilities are overwhelmed, altered ECM deposition may occur which can lead to fibrosis and loss of native organ functioning and regeneration.^{195–197} Currently, there is no clinical treatment available that can reverse liver fibrosis at any stage and a liver transplant is the standard treatment for chronic liver disease.¹⁹⁸ Therefore, a new avenue is required that can aid the regenerative process and assist in repairing liver fibrosis.

In a study performed by Jiao et al., 2021, the research team investigated the function of the secretome derived from adipose-derived mesenchymal stem cells (ASC) in promoting hepatic regeneration after a liver ischaemia-reperfusion injury (IRI) in pigs with partial resection. The results indicated that the ASC-secretome-treated group had lower hepatocyte swelling, inflammation and vacuolisation when compared to the ASC non-secretome control ($p < 0.01$). This result indicated that the secretome from ASC cells significantly improved the histopathology of the liver after injury. The secretome group had lower serum TNF- α and IL-1 β levels when compared to the saline control after Days 1 ($p < 0.01$) and 3 ($p < 0.05$) post-surgery. In addition, IL-6 serum levels were lower on Day 1 and 3 ($p < 0.05$) post-surgery in comparison to the control. Considering serum levels of IL-10, both groups had increased serum levels on Day 1 and 3 post-surgery ($p < 0.01$). This result showed that the secretome from ASC cells significantly reduces inflammation after hepatic injury. The levels of alkaline phosphatase (ALP) were significantly reduced in the treatment group on Days 1 and 3 post-surgery ($p < 0.05$) in comparison to the control groups (Day 1: $p < 0.05$ and Day 3: $p < 0.01$). Alanine aminotransferase (ALT) serum levels were lower after surgery (Day 1: $p < 0.01$, Day 3: $p < 0.05$) in comparison to the other groups ($p < 0.01$). The serum levels of ANG-1, ANG-2 and VEGF increased significantly after surgery when compared to the controls. Results after the liver IRI with partial liver resection indicated that when compared to the control, the group treated with the secretome displayed

a significant increase in the mRNA expression levels of HGF and Cyclin D1 in the hepatic tissue Day 1 after surgery ($p < 0.01$) and by Day 3, in addition to this there was a significant inhibition of TGF- β mRNA expression in the hepatic tissue ($p < 0.01$). Additionally, the ASC-secretome-treated group showed an increase in significance in Ki67-positive cells (Days 1 and 3: $p < 0.01$; Day 7: $p < 0.05$) when compared to the control. Lastly, Western Blot results indicated that the ASC-secretome-treated group showed a significant increase in PCNA (Days 1 and 3: $p < 0.01$; 7 days: $p < 0.05$), increased p-STAT3 (Days 1 and 3: $p < 0.01$) and significantly inhibited SOCS3 expression (Days 1 and 3: $p < 0.01$) when compared to the controls. These results indicate the secretome of ACS cells promoted liver regeneration after injury.¹⁹⁹ The results from this study are particularly significant as it provides a promising cell-free therapy for the future of liver regenerative medicine.

In another study performed by Jiao et al., 2021, the research team considered whether adipose-derived stem cell (ADSC) secretome would provide protective effects after a hepatic IRI in pigs with partial resection. The results showed that the cellular features of the hepatocytes on electron micrographs namely, the ER, the mitochondria and the nuclei were visually normal preoperatively. However, 1 day post hepatic ischaemia-reperfusion injury (HIRI) noticeable and significant ultrastructural changes to these features, namely ER expansion, mitochondrial swelling, chromatin condensation and nuclear membrane shrinkage, had occurred. These features improved over time and by Day 7, only minimal ER expansion could be noted. Treatment with the ADSC secretome was shown to significantly decrease ER expansion and mitochondrial swelling postoperatively, indicating that the ADSC secretome may enhance the ultrastructural changes observed after HIRI. In addition, the ADSC secretome was shown to significantly reduce the hepatocyte apoptosis rate at Day 1 ($p < 0.01$ in comparison to the IRI group) and Day 3 ($p < 0.001$ in comparison to the DMEM control) postoperatively, thus indicating that the ADSC secretome may alleviate HIRI induced apoptosis in the hepatocytes. Furthermore, treatment with the ADSCs secretome and ADSC non-secretome groups was shown to significantly reduce the activity of caspase 3, 8 and 9 1 Day postoperatively ($p < 0.01$) in comparison to the control groups. This result further indicated the ability of the ADSC secretome to reduce the rate of hepatocyte apoptosis. Lastly, the ADSC secretome was shown to significantly reduce Bax mRNA expression levels on Day 1 ($p < 0.01$) and Day 3 ($p < 0.05$) postoperatively when compared to the DMEM control. Additionally, the Bax/Bcl-2 ratio was also shown to be significantly reduced ($p < 0.01$). Furthermore, P53 was downregulated in the ADSC secretome group when compared to the IRI and DMEM control groups, Day 1 ($p < 0.01$ for both) and Day 3 ($p < 0.05$ and $p < 0.01$), postoperatively. Lastly, Fas and FasL cytoplasmic expression decreased on Day 1 and 3 postoperatively ($p < 0.01$ and $p < 0.05$, respectively) when compared to the HIRI and DMEM control groups.²⁰⁰ This result indicated that the ADSC secretome was able to alter the expression of various programmed cell death-related factors.

In another study using ADSCs, Ma et al., 2022 set out to establish if the secretome of ADSC cells would prevent hepatocyte autophagy

after HIRI in a miniature pig model. The results indicated that on Day 1 post-operation, despite severe liver damage having been sustained, the ADSC secretome-treated group had significant improvements over the HIRI and DMEM control groups. In addition, Transmission electron micrographs indicated that although autophagic structures were noted in all groups, the ADSC secretome-treated group displayed an overall structure of hepatocyte cells, there was reduced mitochondrial and ER swelling, and the nuclear structure was approximately what was expected. These results indicated that the ADSC secretome may protect the pigs from hepatectomy and HIRI. It was further observed that LC3II/LC3I, Beclin-1 and ATG5 levels decreased ($p < 0.01$) and the level of p62 increased ($p < 0.01$) for the ADSC secretome-treated group when compared to the DMEM control, which indicated that the ADSC secretome inhibited hepatic autophagy in hepatic I/R and hepatectomy injury. Additionally, it was shown that the ADSC secretome activated the PI3K/Akt/mTOR signalling pathway as the levels of these signalling molecules were significantly upregulated on Day 1 post operation ($p > 0.05$).²⁰¹ Despite Ma et al., 2022 stating that further research is required due to the limitations of the current study, this study along with the two studies performed by Jiao et al., in 2021 strongly suggest that the secretome of ADSC cells may assist in reducing the current shortfalls in liver regeneration by potentially providing a novel avenue for transferring the secretome from a theoretical possibility to clinical practise.

Whilst all the results presented in this section show extremely favourable outcomes and position the secretome as a potential treatment for improving liver regeneration after severe trauma, further studies are required to assess the safety, efficacy and patient applicability of secretome preparations.

3.4.2 | Pulmonary system regeneration

Currently, there is contradictory research regarding the regenerative and repair capabilities of the lungs. In addition, the cellular complexities required for regeneration and repair are also not well understood.^{202,203} However, the inflammatory response that occurs in the pulmonary parenchyma after the traumatic insult is widely agreed upon. Following lung trauma, activation and recruitment of blood leukocytes and lung macrophages occurs along with the production of immune system mediators such as chemokines, cytokines, arachidonic acid metabolites and free radicals.^{204–206} Lung epithelium toll-like receptors (TLRs), such as TLR 2 and 4, activate neutrophils which lead to a further release of inflammatory mediators from the resident pulmonary cells and alveolar type II cells. Coupled with the initial insult, the molecular cascade impairs the alveolocapillary membrane integrity, in turn increasing cellular apoptosis and necrosis of the epithelium. Consequently, inactivation of the lung surfactant occurs due to the influx of plasma proteins (oedema), which further aggravates the respiratory deficits. In some cases, although not well understood, activation and proliferation of fibroblasts is triggered which leads to the development of fibrosis.²⁰⁴ It seems that fibrosis occurs when the reparative process is disrupted or limited.^{202,207} Even with the influx



of research, supportive therapy and treatment are currently still being utilised as no effective therapy is available to fully repair the lung.²⁰⁸ Therefore, this presents an interesting avenue for the secretome to potentially assist with full functional recovery.

In a study performed by Sala et al., 2023, the research group aimed to investigate whether hyaluronic acid (HA), at different molecular weights and the secretome from umbilical cord mesenchymal stem cells (hUCMSCs) would produce a synergistic effect on lung tissue regeneration. After 21 days of HA and hUCMSCs secretome exposure, an immunofluorescent qualitative increase in SPC marker expression was noted indicating the differentiation of the hUCMSCs into ATII cells in comparison to the DMEM control. This was confirmed in a quantitative ELISA assay when compared to the HA biomaterial alone. This result suggests that the HA and hUCMSCs secretome blend may show potential in lung tissue repair and regeneration. Furthermore, as expected by the researchers, cell viability was maintained over the 21 days in the HA and hUCMSCs secretome group owing to the individual intrinsic properties of both the HA and secretome. Moreover, the HA and hUCMSCs secretome group displayed an increase in the wound closure assay in comparison to the controls, suggesting that the combination enhances wound closure and repair. Lastly, exposure to the HA and hUCMSCs secretome blend appears to exert beneficial anti-inflammatory properties which may assist in supporting regeneration.²⁰⁹ Although the data presented shows promise for aiding tissue regeneration and overcoming the limits of stem cell-based therapies, further research is required to assess the blend's efficacy and safety not only in an *in vivo* model but its clinical applicability to patients.

In a study focussing on acute lung injury (ALI), Kudinov and co-workers assessed the effect of the secretome from placental multipotent mesenchymal stromal cell (MMSC), cultured in either a 2D or 3D arrangement, on the survival, inflammation, regeneration and the deposition of fibrin in a lethal ALI mouse model. Results indicated that the MMSC secretome conveys a survival advantage in ALI all treatment groups exhibited a 0% mortality over the 8-day assessment period in comparison to the sodium chloride control group (NaCl). Whilst the 2D and 3D secretome preparations both resulted in open bronchi and bronchioles, inflated alveoli with clear lumens and thin walls, an absence of oedema and lower exudative interstitial oedema scores, the 3D prepared secretome had lower scores and only a small area of unevenly thickened alveolar walls present. This result suggests that the secretome can confer protection to a similar extent or even better than the resident cells. In addition, although fibrin deposition was lower in both secretome groups than in the control, the difference between them was not significant.²¹⁰ Despite the positive results, this research had its limitations and therefore requires further work to establish the mechanisms that convey the protective effects to elucidate if the secretome could be clinically applicable.

Despite the benefits of the secretome presented in this section, the evidence presented is inadequate, at this stage, to confirm its clinical applicability. Therefore, more research directed towards the regeneration of lung tissue is required before a potential therapeutic can be realised.

3.5 | Cardiac regeneration—myocardial infarction

The heart is a solid organ that exhibits limited regenerative capabilities. After a myocardial infarction, numerous signalling cascades are activated in response to the occlusion of the artery, which results in oxidative stress, impaired membrane metabolism and the mitochondrial activation of apoptotic and necrotic cardiomyocytes (tissue death)^{211,212} as well as the initiation of the inflammatory response by the infiltrating immunocytes. Simultaneously, cardiac fibroblasts metamorphose into cardiac myofibroblasts and deposit ECM to maintain the structural integrity of the heart. Concurrently, endothelial cells form new blood vessels through cellular migration and proliferation to aid in cardiac repair.²¹¹ However, as the collagenous scar matures during the remodelling process, native repair is hindered and heart function decreases.²¹³ Therefore, the secretome presents a novel direction for the generation of a therapeutic to potentially promote regeneration and complete regain of function.

In a study performed by Kompa et al., 2021, they aimed to establish whether the delivery of the secretome subcutaneously from human cardiac stem cells in TheraCyt devices would convey a cardiac repair in a rat myocardial infarction (MI) model. Findings indicated that the subcutaneous delivery of the secretome displayed a cardiac protective effect for MI. At 4 weeks post-MI, there was significant preservation in the left ventricular ejection fraction (LVEF) in the secretome treatment group in comparison to the controls ($p < 0.05$). There was also improvement in the left ventricle (LV) function with lower cardiac hypertrophy ($p < 0.05$) and myocardial vascular density, a reduction in the size of the infarct scar, reduced interstitial fibrosis and lower cardiomyocyte hypertrophy.²¹⁴ This study presents an interesting, novel, non-invasive delivery method of the secretome to drive cardiac protection through secretomic paracrine mechanisms. It provides a means for a targeted and personal therapeutic approach as the device can be removed through a simple procedure once the desired outcomes are achieved.

In a study by Derish et al., 2020, the team set out to determine whether the secretome derived from amniotic stromal mesenchymal stem cells (ASMC) could result in cardiac regeneration. *In vitro* results demonstrated that there was an increase in the metabolic activity of human cardiac microvascular endothelial cells (HCEC) after treatment with the secretome ($p < 0.0001$) as well as an increase in the expression of important cardiac markers namely CXN43, Troponin T and SERCA2 in 3-D ASMC-derived secretome compared to 2-D ASMC-derived secretome ($p < 0.05$). These findings showed that the secretome derived from 3D cell cultures may hold a lot of benefits over their 2D counterparts.²¹⁵ Although the data presented here shows promise, *in vivo* studies are necessary to validate these findings to improve patient outcomes.

In research by Costa and fellow researchers, the team aimed to investigate whether the cell secretome from human amniotic fluid-derived stem cells (hAFS) would be able to encourage cardiomyocyte cell cycle re-entry for cardiac repair and regeneration. After secretome administration after myocardial infarction (MI) in mice, an increase in angiogenesis and reactivation of epicardial progenitors with a

decrease in scarring was noted. Additionally, there was a 2.5-fold ($p < 0.001$) increase in the resident cardiomyocytes when compared to the control. Furthermore, neonatal mouse ventricular cardiomyocytes (mNVCM), isolated from R26pFucci2 mice cardiac tissue, indicated that cardiomyocyte cell cycle re-entry increased over 2-fold when compared to the untreated control ($p < 0.05$).²¹⁶ The research presented here suggests that the secretome from hAFS cells could promote cardiac repair through the support of endogenous regenerative mechanisms. In a recent study by the same researchers, the team investigated whether the secretome, in the form of conditioned medium (CM) or EV, from hAFS could induce cardiomyocyte renewal. In vitro results indicated that the EV treatment produced a significant 2-fold increase in the proportion of mNVCM entering the M phase ($p < 0.05$) whilst there was a positive trend increase for mNVCM re-entering the cell cycle for the CM treatment in comparison to the controls. Interestingly, the secretome derived from mature perinatal hAFSC did not convey a significant cardiogenic effect. In addition, the mitotic checkpoint marker enzyme, Aurora B kinase (AurKB), in the mNCVM cells was expressed and there was a 4.5-fold elevation in the expression of AurKB in the mNCVM cells in response to the EV treatment in comparison to the control ($p < 0.05$). It was also shown that the EV treatment significantly decreased the expression of Cofilin-2 (Cfl-2) which decreased the turnover of actin ($p < 0.0001$) in comparison to the control. At 3 days post MI induction, although no significant effect on the LV cardiomyocytes was noted, a significant increase was noted in the viable LV remote zone of the myocardium in response to the EV treatment in comparison to the control ($p < 0.05$). Furthermore, no regenerative changes were noted between the EV and control groups after 7 days post-MI. Despite this, cardiomyocyte cell cycle progression was sustained in response to the EV treatment when compared to the control ($p < 0.05$). Importantly, the EV treatment exhibited a myocardial response over the control ($p < 0.05$), however, this paracrine effect was not sustained long-term. This study indicated that different components and secretome preparations can confer different advantages.²¹⁷ Despite the benefits, further research is required to understand why the paracrine effects were not observed long-term and before EV treatments for patients can be realised.

In research conducted by Alrefai and researchers, the researchers explored whether the paracrine effects from secretome derived from hMSCs and human induced pluripotent stem cells (hiPSCs) would confer functional cardiac recovery and reduce scar size in rats after MI. In vitro results showed an increase in both VEGF and PDGF in both cell cultures after 60 minutes when compared to the MSC control (VEGF: $p = 0.0016$, PDGF: $p = 0.022$). Since there was no difference of significance between groups after surgery in the LVEF or the fractional shortening readings, by week 2 there was a significant difference in both readings for both secretome treatments in comparison to the control. After week 1 post-MI induction, there was no significant change in group scar sizes ($p = 0.385$), however, between weeks 2 and 4 scar size decreased in both secretome treatment groups when compared to the control. In addition, an increase of significance in angiogenesis in peri-infarct was noted for the two secretome groups

in comparison to the control from 2 weeks post MI, however, no differences were observed between the secretome groups at 2 weeks.²¹⁸ Importantly, this study indicated that secretome treatments may experience a delay in therapeutic benefit and indicates that patience may be required in treatment plans. Further studies are necessary to comprehend the delay in effects and how this could potentially impact patient care.

This section presented research that could result in a potential therapeutic to stimulate cardiac regeneration. This section also highlighted that there may be a delay in secretome effects, which is an important consideration when dealing with severe patient cases. Further studies are required to understand the role of the secretome and confirm its clinical applications.

3.6 | Anti-fibrotic activity

Fibrosis presents massive clinical obstacles for tissue regeneration and repair and often remains unresolved.²¹⁹ Fibrosis does not discriminate and affects all tissues and organs of the body. Fibrosis can be described as the uncontrolled deposition of remodelled ECM tissue in response to a complication during native tissue repair and regeneration.^{219,220} Often patients who have developed tissue or organ fibrosis live with permanent, debilitating or disabling health complications.^{219,221,222} Perpetuating this issue is the unavailability of successful therapeutics to remedy this situation.

3.6.1 | Hepatic system

It is important to note that liver fibrosis has long-lasting and knock-on effects including hepatocellular carcinoma, portal hypertension, liver cirrhosis and hepatic failure and contributes to a large proportion of global mortality and morbidity.²²³ Hepatic fibrosis can be described as the formation of scar tissue from excessive deposition and accumulation of ECM components from an altered wound healing response.²²⁴ Hepatic fibrosis can result from multiple causes including but not limited to chronic alcohol abuse, autoimmune or viral hepatitis, non-alcoholic steatohepatitis and non-alcoholic fatty liver disease to severe liver damage.^{225,226} Whilst there is evidence that when the causative agent(s) responsible for the hepatic fibrosis are removed, fibrosis can be reversed in both patients and experimental models,^{227–229} if left untreated, the fibrosis can progress to end-stages of irreversible liver failure, hepatocellular carcinoma, cirrhosis and even death.²²⁷ Unfortunately, at this stage, treatment options are limited, and the gold standard is to either remove the fibrotic tissue or recommend a liver transplant.²³⁰ This indicates the dire need for a successful therapeutic to improve the lives of patients and reduce their dependency on organ transplants.

Research undertaken by Park et al., 2019, it was hypothesised that miR-214 transfected secretome derived from human ASCs would convey greater antifibrotic activity in a liver fibrosis mouse model in comparison to the natural ASC secretome. The results indicated that the RNA



expression of fibrosis-related markers, for example, TGF- β , MMP-2 and α -SMA was significantly reduced with both secretome treatments ($p < 0.05$). The only significant finding was a significant reduction in MMP-2 expression for the miR-214-secretome in comparison to the natural secretome ($p < 0.05$). Considering the liver function tests 7 days post secretome infusion, serological analyses of alanine transaminase (ALT) and aspartate transaminase (AST), indicated that both were decreased in the secretome treatment groups ($p < 0.05$) and further reduced in the miR-214-secretome group ($p < 0.05$). Similarly, the miR-214-secretome treatment group reduced the levels of IL-6 and TNF- α ($p < 0.05$). The miR-214-secretome group displayed the highest degree of inflammation recovery and the greatest improvement in fibrosis in comparison to the natural secretome ($p < 0.05$). The miR-214-secretome group also indicated an increase in antioxidant activity (SOD) in comparison to the saline infusion ($p < 0.05$). Interestingly, this work showed that the secretome derived from miR-214 transfection improved both the anti-inflammatory and antifibrotic properties *in vivo*.²³¹

A similar study published by Paik and colleagues investigated the potential antifibrotic effects of miR-150-transfected human adipose-derived stromal cell (ASCs) secretome in comparison to the natural secretome. *In vitro* results showed that the miR-150 secretome reduced the expression of TGF- β , alpha-1 type I collagen (Col1A1) and α -SMA fibrosis markers in comparison to the natural secretome ($p < 0.05$). *In vivo* results showed a reduction in the expression of TGF β 1, MMP-2 and α -SMA in response to the miR-150 secretome in comparison to the saline control ($p < 0.05$). A similar result was for noted TGF β 1 and MMP-2 between the secretome groups, however no significant change was noted for α -SMA. In addition, there was an increase of significance in the expression of PCNA in comparison to the natural secretome ($p < 0.05$). Furthermore, a significant reduction in IL-6 and TNF- α expression along with AST and ALT in response to the miR-150 secretome in comparison to the natural secretome was observed ($p < 0.05$). Whilst both secretome groups displayed a decrease in the degree of hepatic fibrosis, the miR-150 secretome displayed a significant amelioration in liver fibrosis in comparison to the natural secretome ($p < 0.05$).²³² These studies highlighted the fact that the transfection of the miRNA could be an important role player in fibrosis through potential post-transcriptional regulation of gene expression and RNA silencing of implicated genes. Despite this, further research is necessary to determine the safety of this potential treatment before clinical translation can occur. The miR-150 secretome group also indicated a reduction in antioxidant activity and α -SMA and an albumin increase compared to the saline infusion. Interestingly, this study indicates that SOD expression was decreased but still enhances antioxidant activity, which contrasts with the work published by Park et al.²³¹

In work performed by Yao et al., 2020, human foetal skin-derived stem cell (hFSSC)-derived secretome would improve hepatic fibrosis in a rat model of liver fibrosis. The findings indicated that there was less collagen deposition (14.9% secretome group vs. 24.3% phosphate buffered saline solution [PBS] group, $p < 0.05$), a significantly lower level of malondialdehyde (MDA), an oxidative stress marker ($p < 0.01$) and reduced TGF- β in the secretome treatment group in comparison to the controls.

Furthermore, results from the biological process of epithelial-mesenchymal transition (EMT) suggested that the secretome group was able to reduce liver fibrosis through suppression of the EMT process. Liver functionality tests indicated that ALT, AST, TBil, γ GT and ALP were all reduced in the secretome treatment when compared to the control group ($p < 0.05$), indicating that the secretome may improve liver functionality. Furthermore, a significant reduction in α -SMA (0.82% α -SMA vs. 5.51% PBS, $p < 0.001$), an increase of significance in PCNA ($p < 0.01$) and increase in hepatocyte nuclear factor 4 α (HNF4 α) area (12.1% HNF4 α vs. 4.5% PBS, $p < 0.05$) was noted for the secretome treatment when compared to the controls, indicating a possible delay in the progression of hepatic fibrosis. Lastly, possible regulation of the TGF- β /Smad signal pathway was noted for the secretome group which could indicate a possible reduction in fibrosis.²³³ The results from this study are very promising for a potential therapeutic to attenuate liver fibrosis.

A study published by Han and co-workers aimed to genetically engineer ASCs to produce a TNF- α inhibitor, etanercept, and then to establish whether the secretome released from the genetically modified ASCs would convey antifibrotic effects. *In vitro* results indicated that thioacetamide (TAA)-treated LX2 hepatic stellate cells (HSCs) exhibited a significantly lower expression of inflammatory (TNF- α and cluster of differentiation 68 [CD68]) and fibrosis proteins (p-SMAD, MMP-2, α -SMA and TGF- β) in response to the etanercept-derived secretome in comparison to the control ($p < 0.05$). *In vivo* results indicated mice with induced liver fibrosis with TAA, had a significantly reduced expression of TNF- α , CD68, F4/80, COL1A1, TGF- β , TIMP-1 and MMP2 in response to the etanercept-derived secretome in comparison to the saline control ($p < 0.05$). In terms of liver function at 7 days post TAA administration, mice treated with the etanercept-derived secretome exhibited a reduced serum expression of ALT ($p = 0.021$) and AST ($p = 0.021$) when compared to the saline control. Systemic inflammation (IL-6, $p = 0.021$; TNF- α , $p = 0.020$) and the percentage of immunoreactive areas (TNF- α , CD68 and MCP-1, all $p < 0.05$) was also decreased in response to the etanercept-derived secretome. Finally, the smallest fibrotic area was observed for the etanercept-derived secretome treatment group ($p = 0.020$) with the lowest collagen deposition ($p = 0.021$).²³⁴ The results indicated that the genetically engineered secretome may present a novel avenue in the quest to overcome liver fibrosis.

3.6.2 | Pulmonary system

Pulmonary fibrosis presents another hurdle in the hospitals for both patients and clinicians. Whilst there are various causes of pulmonary fibrosis, the architectural change to the lungs remains rather consistent.²³⁵ This remodelling, caused by the irregular deposition of the ECM, can disrupt the lung's native functioning by stiffening the lung tissue and severely affecting the alveoli.²³⁶ Over time, these structural changes to the lung tissue can cause lasting scarring and result in dyspnoea and difficulty breathing. Currently, the median survival of patients suffering from idiopathic pulmonary fibrosis (IPF) is a mere 3-5 years due to the limited treatment options available.^{235,236} With this in mind, it is extremely important that researchers develop a

therapeutic for pulmonary fibrosis to benefit patients inflicted with this condition.

In a study performed by Dinh et al., 2020 the research team aimed to see if their hypothesis that lung spheroid cell (LSC) secretome would be able to promote lung repair in pulmonary fibrosis in a manner surpassing MSC secretome in an *in vivo* model. The results indicated that after intratracheal injection of bleomycin (Bleo), the LSC secretome-treated CD1 immunocompetent mice showed a reduction in pulmonary apoptosis by maintaining the alveolar epithelium and reducing the deposition of collagen in comparison to the PBS group. Furthermore, LSC secretome was the only treatment capable of reducing the fibrotic area whilst reversing the damage to the alveolar epithelium back to the healthy state. Furthermore, inhalation of the LSC secretome was shown to promote vascular and alveolar repair through the monitoring of AQP5. Furthermore, the secretome derived from LSC displayed an increase in the proliferation of ProSPC⁺-positive AT2 cells (higher than the MSC secretome), following which the cells divide and differentiate into AT1 cells in comparison to the PBS control. Additionally, only the LSC secretome had improved von Willebrand factor (vWF⁺) expression in the fibrosis model. Additionally, the lung protein levels of ProSPC and vWF increased for both secretome treatments in comparison to the PBS control. Moreover, the levels of α SMA and pro-inflammatory IL-4 expression indicated a decreasing trend whilst MMP-2 expression in the pulmonary tissue indicated an increasing trend for LSC-derived secretome in comparison to the PBS control. In terms of a silica model, the LSC-derived secretome group indicated a significant decrease in fibrotic tissue severity in comparison to the PBS control. In an SD rat model, the LSC secretome-treated group displayed a significant decrease in collagen deposition, fibrosis, SMAD3 expression and MCP-1/CLL2 levels as well as in alveolar epithelial and vascular injury when compared to the PBS control.²³⁷ Interestingly, an endpoint analysis indicated that pulmonary function was only partially improved by the LSC secretome but there was a significant improvement in inspiratory capacity and respiratory compliance. In a similar study performed by Hu et al., 2023 the research team set out to understand the mechanisms and the effects of the secretome derived from human embryonic stem cell (hESC)-derived MSC-like immune and matrix regulatory cells (IMRCs) (hESC-MSC-IMRC) on pulmonary fibrosis in a mouse model of bleomycin (BLM)-IPF. Results indicated an inhibition of NOX4 expression, a decrease in α SMA and a decrease in ROS production coupled with a reduction in oxidative stress markers, HO-1, NOX4 and NRF2, in A549 cells in response to treatment with the secretome derived from hESC-MSC-IMRC when compared to the BLM control. Interestingly, there was an elevation in the activity of the antioxidant enzyme of ROS, glutathione peroxidase (GSH-PX) in the hESC-MSC-IMRC secretome group in comparison to the BLM control group was also observed, suggesting that the secretome could possess antioxidative properties to lower BLM-induced oxidative stress in A549 cells. Furthermore, results from the *in vivo* study, both in the early and late stages of lung fibrosis, indicated that the hESC-MSC-IMRC secretome treatment decreased the progression of pulmonary fibrosis observed by a slight decrease in lung indexes, fibrotic lesions and collagen ECM

deposition. In addition, expression of MDA, an oxidative stress marker, was reduced in mice treated with the secretome in comparison to the BLM control, although the results were not statistically significant. In line with the *in vitro* study, a decrease in α SMA, NRF2, HO-1 and NOX4 was noted in mice treated with the hESC-MSC-IMRC secretome when compared to the BLM control. These results suggest that the hESC-MSC-IMRC secretome may mitigate BLM-IPF at different stages of disease progression. Lastly, treatment with the hESC-MSC-IMRC secretome at different stages of lung fibrosis showed a significant reduction in the expression of TNF- α , Tlr4 and p-NF- κ B and the levels of IL-6, IL-1 β and TNF- α and when compared to the BLM control.²³⁶ Although the cell type of the secretome differs, the mouse model is the same, these findings correlate with the study performed by Dinh et al., 2020,²³⁷ this study is proof-of-concept and requires further research to establish the clinical applicability in pulmonary fibrosis.

This section, particularly the hepatic fibrosis section, highlighted that genetic modification may provide the much-needed avenue in our pursuit of novel therapeutics for overcoming fibrosis. Whilst the data presented in this section shows much promise, further investigation is required to establish if the secretome preparations are safe and effective for patient usage before a commercial pharmaceutical is realised.

4 | THERAPEUTIC POTENTIAL OF THE SECRETOME: CROSS-CONDITION INSIGHTS AND CHALLENGES

From the above section, it is evident that the therapeutic application of the secretome holds great promise across a diverse range of medical conditions. However, when analysed in isolation the true potential of secretome preparations is not realised. However, after an analysis of the major and minor overlaps as well as the contradictory and opposing findings within and across different conditions, an improved understanding of the potential universal benefits of the secretome and condition-specific challenges become apparent.

Of the diverse conditions reviewed, secretome therapies exhibited overlapping benefits particularly associated with anti-inflammatory, anti-apoptotic, angiogenic and anti-fibrotic effects across multiple conditions. These benefits are largely attributed to the various signalling molecules contained in the secretome regardless of tissue or organ targeted. With regards to the anti-inflammatory and anti-apoptotic effects, across the CNS, hepatic, cardiac and pulmonary injuries reviewed MSC-derived secretomes were shown to consistently reduce apoptosis and inflammation. In terms of the CNS injuries discussed MSC-derived secretomes were shown to improve neural tissue preservation and functional recovery due to the reduction in neuroinflammation and apoptosis after TBI and SCI.^{127,129} Likewise, in the cardiac and hepatic models reviewed, despite using different cell types for secretome generation, secretome preparations were shown to significantly increase various growth factors such as VEGF that resulted in increased healing rates and repair.^{141,199} The promotion of angiogenesis was another beneficial factor noted between different conditions. Factors



including but not limited to VEGF and FGF were shown to improve blood vessel formation and enhance tissue repair in cardiac, skin and hepatic models. In comparison, the secretome was shown to increase vascular density and tissue perfusion after myocardial infarction whilst capillary network formation and collagen deposition were supported after an induced skin injury. Thus, taken together, the secretome was shown to drive the neovascularisation required for repair and regeneration after injury across multiple tissue types.^{141,175} It was further demonstrated that the secretome was able to consistently convey anti-fibrotic effects, particularly in hepatic and pulmonary models, where a consistent reduction in fibrotic markers like α -SMA^{231,233,234,237} and TGF- β ^{231,233,234} was potentially responsible for the noted tissue repair and reduced scarring observed. Although success in some pulmonary models was noted, the contradictory results may suggest that the secretome may be utilised in specific injuries. Furthermore, these results may indicate that the secretome factors can balance ECM degradation and deposition to prevent the formation of scars that would ordinarily impair the tissue.

There are some conditions, however, that produced minor variations in outcomes which could likely be due to the origin of the cell type used, the secretome concentration or the administration method utilised. Considering the CNS, different sources of MSC-generated secretome factors resulted in varying results as seen in Tsai et al., 2018 wherein the secretome derived from BMSCs was reported to improve locomotion and conveyed neural protection after SCI,¹²⁷ whilst Vawda et al., 2020 reported limited functional recovery with the secretome derived from HUCMCs.¹²⁶ Despite both secretome batches initially displaying favourable results, BMSCs-derived secretome preparations seemed to improve neuroprotection and regeneration to a greater extent highlighting the fact cell origin may influence which secreted factors are present which may directly influence its CNS repair ability. In a different example wherein different stem cell populations with similar differentiation and regenerative ability were used in hepatic and cardiac models, beneficial effects were noted after treatment with the secretome. However, variations in the site of action, namely lowering fibrotic markers^{199–201} or promoting cell cycle re-entry²¹⁶ were noted for hepatic and cardiac models, respectively. These variations may suggest tissue-specific regenerative requirements and the nuances in understanding the stages of repair and regeneration for individual tissues.

Despite the general therapeutic trends mentioned above, some studies produced contradictory results, particularly in injuries affecting the lungs. Models of pulmonary fibrosis presented inconsistencies where some data recorded only conveyed partial pulmonary improvements,²³⁷ whereas others produced results that were not statistically significant.^{210,236} This suggests that the unique micro-environment of the lungs, characterised by elastic ECM components and complex fibrotic signalling, may require lung-specific factors that some secretomes may lack. This further emphasises the importance of selecting the optimal cell source when generating the secretome to improve therapeutic efficacy.

Whilst secretome factors may provide universal benefits, another important consideration is the fact that secretome efficacy may be tissue dependent. Differences relating to structure and functional

requirements across the different tissues highlight the need for tailored secretome methods and extractions. Interestingly, secretome therapy seemed to be more successful and yield more consistent results in non-neuronal tissue than in CNS injuries. This discrepancy may be due to the presence of the blood–brain barrier which may hinder the entry of all factors required for regeneration, thus limiting its long-term effectiveness. In direct contrast, studies focussing on cardiac and hepatic regeneration, generally reported sustained improvements and functional recovery over the study period indicating that non-neuronal tissues may be more receptive to secretome therapy possibly due to the increased network of blood vessels. Additionally, whilst the liver responded well to the anti-fibrotic factors contained in the secretome, the lungs presented with variable outcomes. Of the studies reviewed, most pulmonary models presented limited responses to some secretome factors whilst showing improved responses to others. This could suggest that the lungs may require a higher concentration of secretome factors or specific secreted factors that may not be present in all secretome preparations. Furthermore, the architectural nature of the alveoli may pose additional challenges for penetration and tissue remodelling.

Lastly, delivery system strategies may also account for the variation observed in the presented studies. The subcutaneous delivery of the secretome in a myocardial infarction model was shown to elicit improved cardiac protection and sustained factor release.²¹⁴ However, wound healing studies emphasised the need for dosing optimisations as excessively concentrated secretomes could elicit toxic effects,²⁰¹ accentuating the need for establishing the correct and optimal dosing window to be efficacious without adverse effects.

5 | DELIVERY SYSTEM STRATEGIES

In the recent past, researchers have designed various strategies to utilise and optimise the dosing of the secretome to achieve improved tissue regeneration and repair. Currently, several delivery systems, such as systemic and direct injections, have been successfully attempted in many different target tissues and sites. It is no surprise that direct local administration has been explored due to its plethora of benefits, including but not limited to the high and sustained drug concentrations at the target tissue whilst reducing potential systemic toxicity.²³⁸ Considering the evolving landscape of regenerative medicine, the emergence of more sophisticated delivery systems, such as micro and nanoparticles, microspheres, hydrogels and scaffolds have begun to emerge.²³⁹ The delivery system of the secretome in the various studies presented in this review is summarised in Table 1.

Of the reviewed research, 78% utilised parenteral routes of administration, particularly IV, subcutaneous and local injection of the secretome. This highlights the preference for these methods possibly due to the convenience,² ease of access²⁴⁰ and ease of monitoring the effect of the secretome on the target tissue. It is no surprise that the bulk of these studies utilised IV administration due to the rapid onset and the almost complete bioavailability (first pass metabolism avoided) of the actives.²⁴⁰ As we focus on the development of a secretome pharmaceutical for the near future, considering the data presented, IV

TABLE 1 Strategies presented to deliver the secretome to the intended tissue for different regenerative tissue engineering applications.

Route of administration	Tissue	Study type	Model	Delivery system	Ref
Intravenous	Spinal cord	In vivo	Rat cervical clip compression/contusion injury model	Intravenously delivered concentrated conditioned media	126
	Spinal cord	In vivo/in vitro	Rat SCI model	Systemic administration of conditioned medium	127
	Brain	In vivo	Experimental TBI rat model	Intravenously infused secretome	129
	Brain	In vivo	Severe TBI rat model	Traumatic injury pre-conditioned injected secretome	138
	Lung fibrosis	In vivo/in vitro	Bleomycin induced pulmonary fibrosis mouse model	Tail vein infusion of secretome	236
	Cardiac	In vivo/in vitro	Myocardial infarction rat model	Injection of secretome after MI	218
	Cardiac	In vivo/in vitro	Myocardial infarction mouse model	Single intra-myocardial injection of conditioned media and EVs	216
	Liver fibrosis	In vivo	Thioacetamide-induced hepatic fibrosis mouse model	Secretome intravenously infused	231
	Liver fibrosis	In vivo/in vitro	Thioacetamide-induced hepatic fibrosis mouse model	Secretome intravenously infused	234
	Liver fibrosis	In vivo	CCL4- induced rat liver fibrosis model	Secretome injected via the tail vein	233
	Liver fibrosis	In vivo/in vitro	Thioacetamide-induced hepatic fibrosis mouse model	Secretome intravenously infused	232
Local	Brain	In vivo	Experimental rat stroke model	Intracerebroventricular conditioned medium administration	142
	Brain	In vivo	Experimental rat stroke model	Infusion of conditioned media into left lateral ventricle	143
	Skin	In vivo/in vitro	Full thickness excision rat wound model	Injection of secretome around the excision	177
	Gingival	In vivo/in vitro	Excisional wound repair mouse model	Secretome delivered to wound margin	182
	Liver	In vivo	Miniature pig model of liver ischaemia-reperfusion with partial hepatectomy	Secretome injected into liver parenchyma	199
	Liver	In vivo	Miniature pig model of liver ischaemia-reperfusion with partial hepatectomy	Secretome injected into liver parenchyma	200
	Liver	In vivo	Miniature pig model of liver ischaemia-reperfusion with partial hepatectomy	Secretome transplanted into liver parenchyma	201
	Cardiac	In vivo/in vitro	Neonatal murine myocardial infarction model	Intraperitoneal administration of EVs	217
Subcutaneous/ sub-dermal	Skin	In vivo	Full thickness excision rabbit wound model	Sub-dermal single dose secretome injection	175
	Skin	In vivo/in vitro	Radiation induced skin injury rat model	Subcutaneous injection of secretome	178
	Cardiac	In vivo	Myocardial infarction rat model	Subcutaneous implantation of secretome in TheraCyt device	214
Intranasal	Lung fibrosis	In vivo	Bleomycin sulfate induced pulmonary fibrosis mouse model	Secretome nebulised	237
	Lung	In vivo/in vitro	Lethal acute lung injury mouse model	Inhalation of lyophilised secretome	210
Polymeric	Bone	In vivo	Critical sized bone defects rat model	Secretome loaded hydroxyapatite around threaded K-wire	155
	Bone	In vivo	Calvarial bone damaged rat model	Atelocollagen suspended condition medium implant	156
	Skin	In vivo	Skin flap viability and wound repair rat model	ECM hydrogel incubated with conditioned medium	176
	Skin	In vivo/in vitro	Cutaneous wound healing mouse model	Secretome or hydrogel-loaded with secretome delivered to wound	179
	Skin	In vivo	Type-2 diabetes mouse model and excisional wound-splinting model	Secretome and growth-factor reduced matrigel delivered to wound	180

Abbreviations: ECM, extracellular matrix; EV, extracellular vesicle; MI, myocardial infarction; SCI, spinal cord injury; TBI, traumatic brain injury.



administration seems to be the chosen route of secretome administration especially in severe and urgent medical conditions. On the other hand, subcutaneous administration for the secretome could confer its own advantages such as slower absorption, dosing of larger actives, bypassing of the liver, with the possibility of increasing absorption of the actives with the hyaluronidase enzyme.²⁴⁰ Whilst there are definite benefits of both IV and subcutaneous injection, one needs to critically evaluate the secretome as the pharmaceutical active. Since the secretome is understood to be a mixture of factors and proteins, it has been noted that providing sustained levels of proteins with IV administration is difficult whilst the rate of absorption from subcutaneous administration cannot be properly controlled.²⁴⁰

Of the remaining studies, a mere 6% utilised the intranasal approach of administration. For pulmonary studies this mode of administration is expected due to the swift delivery of the intended active over the ample surface area of the respiratory tract epithelium as well as the bypassing of first pass metabolism.²⁴⁰ Considering the delivery of the secretome, this mode of administration may in fact not be favourable. Since effective intranasal delivery is largely restricted to a particle size between 1 and 10 μm , the numerous proteins and factors found in the secretome,^{14,240} vary in size and those falling out of this range might not rendered effective or might not reach the desired target site.²⁴¹ Furthermore, due to the severity of pulmonary system damage requiring repair, any changes to the patient's respiratory physiology, which is often the case, will reduce the efficacy of the secretome treatment.²⁴¹ Lastly, compounding issues such as increased nasal secretions will decrease the bioavailability and efficacy of the secretome treatment.²⁴¹

Interestingly, only 16% of studies reviewed utilised a delivery vehicle, in the form of polymers or scaffolds, to deliver the secretome. Whilst the other mechanisms may allow for rapid delivery of the active, clearance from the target site is swift²⁴² and results in multiple dosing regimens. Polymeric and biomaterial scaffolds can render further benefits to the injured tissue as materials, especially aECMs, can further assist in repair and regeneration. Such scaffolds can allow for the delivery of multiple bioactives, in addition to the secretome, whilst being tuneable to the specific tissue mechanics to allow for native cellular processes such as cell migration, invasion and proliferation to aid in the regeneration process.^{243,244}

Despite the above routes of administration being the most utilised, there are other successful routes of administration that have not gained as much attention. Among the other local routes of administration, topical administration of the secretome is predominantly utilised for dermatological applications wherein the secreted factors can directly promote cell migration to initiate regeneration at the site of the wound as observed by Rajesh et al., 2024.²⁴⁵ Deeper tissue administration, particularly intra-articular and intra-tumour injections, have also been explored for targeting specific areas with the added benefit of minimal systemic side effects. Those of particular interest was the administration of human embryonic stem cell-induced mesenchymal stem cell (ESC-MSC) exosomes via an intra-articular injection. Results from this study indicated that the administration of the exosomes alleviated matrix degradation and cartilage destruction in the knee joints of C57BL/6 J mice suggesting the possible balancing of ECM synthesis and degradation.²⁴⁶ Another

notable study found that an intra-tumour injection of an MSC secretome formulation significantly decreased tumour growth and noted a suppression of invasion-related cell types namely, MDR1⁺, PD-L1⁺ and CD44⁺/CD24⁻, suggesting that the MSC secretome may be effective at modulating the growth of triple negative breast cancer (TNBC).²⁴⁷ Lastly, a short-term improvement after mild stroke induction was observed for the secretome delivered intra-arterially from dental pulp stem cells (DPSCs) in a Sprague Dawley model of mild ischaemic stroke.²⁴⁸ Considering the parental routes of administration, the transdermal delivery of hUCMSC-derived exosomes in combination with silver nanoparticles was shown to increase the healing rate of wounds in a diabetic rat model whilst conveying an anti-inflammatory response and improved vascularisation.²⁴⁹ Although intra-muscular and intra-dermal injections as well as a variety of enteral routes (oral, sublingual and rectal) have been explored to a lesser extent due to the likelihood of secretome component degradation, reduced absorption and secretome stability. Especially with these routes, the design and implementation of innovative delivery systems are being considered to overcome these limitations.²⁴⁰

Whilst synthetic materials have been used successfully to deliver the secretome^{250,251} they are plagued with poor cell adherence and the lack of endogenous factors.^{243,252} Natural polymer hydrogels present an exciting avenue for the sustained and controlled delivery of the secretome.² Although injectable hydrogels have shown favourable outcomes in the past,²⁵³⁻²⁵⁵ incomplete *in situ* gelation can result in the loss of the bioactive.²⁵⁶ More advanced approaches such as microparticles with cell-mimicking properties have also been explored. Studies utilising this technology have shown controlled and sustained release of the secretome is possible *in vivo*.²⁵⁷

Whilst all delivery systems are plagued by their own issues, it remains paramount that the secretomes pharmacokinetic and pharmacodynamic (PK/PD) profile²⁴⁰ as well as the predictability²⁴⁰ of the secretome based on the mode of action²⁵⁸ is determined so that the most appropriate delivery system can be selected to elicit the optimal therapeutic effect.

6 | CONCLUSIONS AND CONSIDERATIONS FOR THE USE OF SECRETOMES

As regenerative science continues to broaden, interest into the cell secretome, continues to expand, opening new doors for regenerative therapeutics. These secreted factors are known to contain a plethora of biomolecules that can act in signalling pathways to elicit their effects (regulation of physiological functions) and in turn, potentially act as protagonists to promote regeneration and repair of organs and tissues. Additionally, secretome-based therapies are being recognised as an encouraging alternative to cell-based therapies as they may circumvent the safety and scientific issues associated with cell-based therapies. Secretome-based therapies may present a new chapter in regenerative tissue and organ engineering applications.

For secretome-based therapies to be successful, robust methods in secretome generation and profiling are necessary. For both *in vitro*

and *in vivo* applications, the development and introduction of advanced techniques have enabled researchers to perform high-precision dynamic analysis of cellular secretions to enhance their relative yield and purity whilst improving the relevance of the data collected.²⁵⁹ Considering *in vitro* applications, microfluidic devices have been instrumental in providing real-time, single-cell monitoring of secretions which has allowed researchers to monitor dynamic secretions patterns with high sensitivity.²⁶⁰ Another single-cell, high-throughput approach is droplet-based microfluidics which improves the analysis of secretions at the single-cell level as cells are encapsulated in droplets which assists in minimising sample loss and contamination.²⁶¹ In terms of single-cell isolation, nanowell arrays have become ideal for studies requiring secretion data for individual cells under discrete conditions.²⁶² If increased sensitivity of secreted factors is required, surface-functionalised biosensors are the option to further improve the accuracy of analysed secretions.²⁶³ For applications requiring a more physiologically relevant model, 3D cell culture models have gained a lot of attention and have started replacing conventional 2D cell culture models due to their ability to mimic the native tissue architecture, support native cell behaviour and significantly increase the secretion of cellular factors which has become instrumental in secretome secretion studies. A further advantage of 3D cell culture is the reduced dependence on animal testing.^{263–265} Additionally, 3D cell culture is the reduced dependence on animal testing.²⁶³ To further enhance *in vitro* analysis, the integration of techniques can create powerful multi-modal platforms for improved secretome analysis. For example, combining nanowell technology and surface-functionalised biosensors can assist with highly sensitive secretome detection whilst incorporating 3D cell cultures into such a model can assist in providing a more accurate representation of *in vivo* secretion patterns. For the success of *in vitro* models, some conditions are required to maintain cell viability and the consistent production of secretome secretion. Although different cell types will require different optimal conditions, the most common conditions across cell types are maintaining the temperature of the incubator to 37°C for mammalian cells, cell culture media should be maintained at a pH between 7.2 and 7.4 and the concentration of carbon dioxide should be maintained at 5%.²⁶⁶ Additionally, some studies may require the serum to be removed to avoid protein interference and to improve secretome quantification.²⁶⁷

On the other hand, for *in vivo* applications, continuous extracellular fluid sampling via microdialysis allows for the collection of secreted factors at a defined location at a particular time allowing for an insight into tissue secretion dynamics.²⁶⁸ For particular applications such as in neuroscience, push-pull perfusion allows for focused, region-specific brain secretome collections.²⁶⁹ If tissue-specific secretome samples are required, researchers can use microfabricated probes due to their high-resolution measurements.^{270,271} If secretions from targeted cellular populations are required, optogenetic stimulation and sampling can be utilised as they specialise in cell-type-specific responses.²⁷² In applications where only visualisation of the secretions is necessary, fluorescent reporter imaging can be utilised to provide continuous real-time, non-invasive secretion tracking and

monitoring of cellular behaviour within the chosen organism.²⁷³ As with *in vitro* studies, multiple techniques can be integrated to create multifaceted secretome analysis with improved properties. For example, microfluidic devices can be used in combination with microfabricated probes to either analyse the spatiotemporal dynamics of secretory proteins^{274,275} or to sort and categorise different cell populations to study the secretome from these distinct populations.²⁷⁶ For any of these methods to be successful, optimisations and adjustments to study criteria are required to maximise secretion rates and detection sensitivity.

The usage of the secretome presents an immediate advantage over cell-based therapeutics. Since the secretome is a cell-free preparation, the risk of a rejection reaction is reduced and therefore it is unnecessary to match the donor and the recipient.³⁵ Furthermore, zoonotic disease transmission^{37,277} and immunological reactions from FBS^{278–280} can be avoided if cells are propagated in xeno-free media. Despite the fewer safety concerns, challenges do remain.^{2,281}

Despite cell secretome preparations showing potential in regenerative medicine, numerous challenges and considerations need to be addressed before successful clinical translation can occur as summarised in Figure 5. First, a proper understanding of both the healthy (regulated) and disease signalling pathways needs to be fully outlined and established so that the secretion of all the proteins and factors can be identified and targeted.³⁴ This will assist in understanding how all secreted factors of the secretome exert their effects^{35,37,137,282} and how the secretome from one cell type could directly impact other cell types that express similar receptors.³⁴ This is crucial as variations in secreted factors may obscure findings and influence research outcomes.² Interestingly, clinical trials administering a single component, for example, a single cytokine did not produce favourable and encouraging results. Such research further supports the idea that all factors are required to produce the therapeutic response as the factors may act synergistically.³⁷ Therefore, combination screening should be undertaken to identify synergistic and antagonistic interactions.³⁴ If fully understanding the pathways can be achieved, the design and development of secretome signatures (secretome factors extracted from different cell populations)¹⁴⁸ could be achieved. This could result in a customised, per-patient treatment as a secretome cocktail for specific conditions can be developed. This is based on the premise that the secretome secretions from the chosen cell lines will be in accordance with the patient's needs as the therapeutic end point (dynamic protein concentrations)² will be taken into consideration.^{21,37,283} Furthermore, micro/nanoengineering strategies can be further employed to target central signalling pathways to vary the secretome secreted.² Recently there has been the emergence of 3D printing coupled with the usage of the secretome. Due to its recent emergence, research has been mostly directed towards neural (both SCI²⁸⁴ and TBI^{138,285–287}) and bone^{288,289} regeneration. Results have indicated an improvement in locomotor and cognitive function, electrophysiological activity, tissue regeneration, synapse connectivity, maintenance of myelin, cell viability, cell adhesion, cell growth, angiogenesis, differentiation and mineralisation as well as a decrease in cavity area and apoptosis. Furthermore, a study performed by Bari, 2021

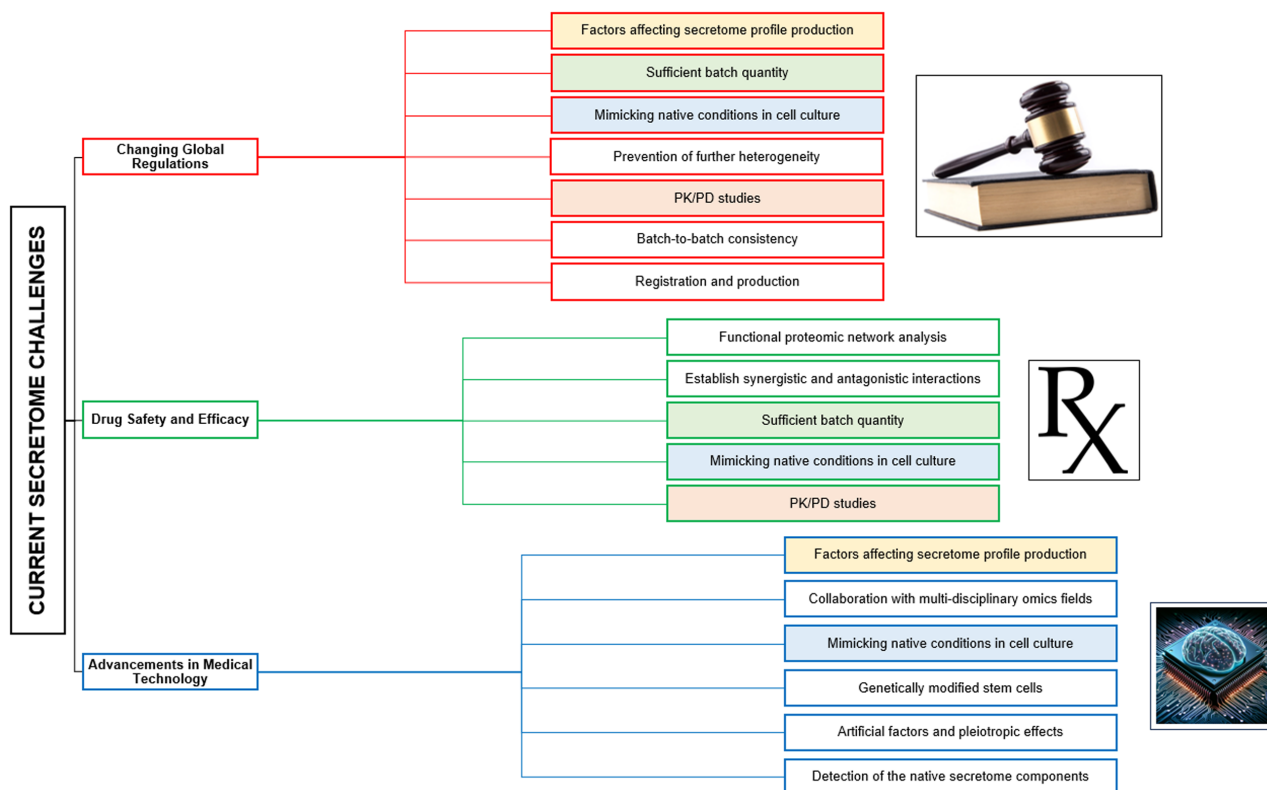


FIGURE 5 Schematic, created using MS Office PowerPoint, illustrating the key regulatory, safety and medical technological challenges associated with the development and clinical implementation of secretome-based therapeutics. These challenges include but are not limited to the standardisation of secretome composition, production scalability, secretome bioactivity consistency, potential immunogenicity, stringent regulatory framework compliance, robust preclinical and clinical validation, long-term safety and effective delivery systems to optimise therapeutic outcomes.

indicated that controlled release of the secretome was possible by altering the polymer properties and combinations to form the 3D printed scaffold.²⁸⁸

Another substantial consideration is the composition of the secretome, which is heterogenic in nature.²⁹⁰ The secretome composition varies largely based on several factors including but not limited to the age of the host, species, tissue source, source of the stem cell, stimuli and microenvironment surrounding the cells, the culture medium and the secretome isolation procedure.^{2,283,290} These abovementioned factors result in different secretome profiles being expressed. For example, MSCs isolated from adipose tissue express a plethora of angiogenic factors whilst bone marrow MSCs consist of factors for angiogenesis-mediated tissue regeneration.² In addition, secretome profiles differ due to different gene expression patterns, and proteomic profiling of secretomes may prove essential in discovering novel druggable targets as well as decoding the secretome factors. For example, utilisation of secretome-based libraries in combination with Phenotypic drug discovery (PPD) can further result in the identification of novel targets and signalling pathways.³⁴ Furthermore, secretome-based screening may assist in identifying proteins secreted by non-encoding genes, which may enhance the understanding of the 'unknown' secretome.^{291,292} Furthermore, combining secretomics with studies of the matrisome, receptome and adhesome may further

broaden the understanding of the signalling pathways and identification of novel druggable targets.

In addition to these challenges, the usage of conditioned media presents additional challenges. Firstly, native cells are exposed to mechanical forces which are converted to signals that affect the secretion of the secretome. Therefore, the therapeutic potential of the secreted secretome may differ in cells that are not exposed to mechanical loading during culturing. However, customisation of the therapeutic profile of the secretome may be possible if mechanical loading can be mimicked during cell culture.²⁹³ Secondly, most of the research presented herein, is performed using conditioned cell culture media. This poses issues as conditioned media cannot reflect all stages in the stem cell life cycle and therefore certain secretions may be limited or absent.³⁵ Furthermore, great care needs to be taken to ensure that the serum and growth supplements added to the cell culture media do not interfere with the detection and analysis of the native secretome components, especially those expressed in the nano- and picogram concentrations.³⁷ To avoid this issue, secretome studies should be performed in serum-free media and then the secretome factors can be concentrated and quantified. Thirdly, if the synthesis of artificial secretome factors is considered, despite potentially lowering the cost of production and reducing the time required for in vitro cell culturing and cell maintenance,³⁵ it is vital to consider that artificial

factors may not exhibit the pleiotropic effects of their native counterparts.³⁷ However, despite this, artificial secretome factors may further allow for customisation of the secretome to achieve regeneration and repair as particular factors can be amplified or suppressed to improve their therapeutic potential.^{2,21,69} Lastly, stem cells may be genetically modified to over- or under-express secretome factors that are required for the regeneration and repair of a certain tissue, further allowing for a tailored biotherapeutic approach.

Probably one of the biggest topics associated with the clinical usage of cell secretomes is the registration and production of the secretome factors. Since secretomes are a pleiotropic mix of components that control pharmacological functions, although currently classified as a biological medicinal product, secretomes cannot be considered a biological medicinal product as they do not contain a single active ingredient such as products containing either a hormone or an antibody. Furthermore, secretomes cannot be classified as Advanced Therapy Medicinal Products (ATMPs) as ATMPs cover tissue engineered products as well as somatic cell and gene therapy medicinal products. Therefore, cell secretomes do not fall exclusively into a normative category and a classification needs to be established.⁵² In addition to the above regulatory issues, before approval of secretome-based therapeutics can be considered, many aspects including but not limited to GMP protocols for batch-to-batch consistency,^{52,258} cell expansion, secretome production, collection, bioprocessing, storage, transport and delivery of secretome factors as well as quality control must be established to ensure patient safety and product efficacy.^{21,35,37,294} Furthermore, secretome usage can only be widely adopted if the pharmacokinetics of all factors post-transplantation are understood, the optimal administration route is determined, correct volume and dosage regimes are established and satisfactory results from clinical trials are obtained.³⁷ Additionally, the biological half-life of each component and its activity needs to be well documented and understood.¹⁷ As previously mentioned, the secretome is heterogeneous in nature, however, care must be taken to prevent the induction of further heterogeneity.^{69,295} To prevent this, bioreactors have been used to ensure batch-to-batch consistency from heterogeneity.²⁹⁶ Lastly, sufficient secretome factors need to be extracted for clinical applications.³⁵

Finally, the exploration of the secreted cellular factors as a novel therapeutic strategy has introduced several possibilities in regenerative medicine. The findings presented in this review, highlight the potential benefits of secretome-based therapies over conventional cell-based treatments, particularly in reducing several risks including immune rejection and tumourigenicity. Furthermore, the cell secretome may provide a safer and more scalable approach to addressing the current regenerative challenges, particularly in tissues with a low regenerative ability. Additionally, the exploration into exosome-based therapies continues to be a critical consideration in facilitating targeted cellular communication to achieve improved repair and regeneration across multiple disease models.

Despite the substantial evidence supporting the potential of the secretome in promoting repair and regeneration after injury, significant obstacles remain which are preventing their immediate clinical

application and usage. These obstacles include but are not limited to, the vast variability in secretome composition, batch-to-batch consistency and predictable therapeutic efficacy—all of which must be resolved before secretome-based therapies can be deemed viable candidates for clinical translation and usage. These challenges highlight the need for extensive research and optimisations, particularly in the production, standardisation and delivery of secretome preparations to ensure reliable and reproducible outcomes.

Whilst promising data has been generated using a variety of preclinical models, future studies focussing on addressing the pertinent biological, technical and regulatory challenges are required to realise the therapeutic potential of the secretome. This may include refining pharmacokinetic and pharmacodynamic profiles, designing and validating clinical-grade production methods compliant with GMP standards and conducting large-scale clinical trials to assess safety and efficacy across diverse patient populations. Increasing collaboration between academia, industry and regulatory bodies will be essential in addressing the translational difficulties and may potentially accelerate the path to market for secretome-based therapies. Such progress may enable the realisation of personalised treatments to offer hope to patients facing limited options for tissue repair and regeneration. Taken together, secretome therapeutics represent a compelling avenue in the design of future treatment options with vast potential to revolutionise regenerative medicine if the current hurdles limiting their introduction can be overcome.

AUTHOR CONTRIBUTIONS

All authors contributed to the conceptualisation and design of this review. The literature search and first draft of the manuscript were performed and written by KDS. PK and YEC provided methodology and scientific and technical assessments as well as editorial input. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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