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Insights in tissue engineering and regenerative medicine 2021: Novel developments, current challenges, and future perspectives

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Insights in tissue engineering and regenerative medicine 2021: Novel developments, current challenges, and future perspectives

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Editorial: Insights in tissue engineering and regenerative medicine 2021: Novel developments, current challenges, and future perspectives

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Editorial on the Research Topic
Insights in tissue engineering and regenerative medicine 2021: Novel developments, current challenges, and future perspectives

The Tissue Engineering and Regenerative Medicine (TERM) pursues a multidisciplinary approach to the development and application of new therapeutic strategies and products for the treatment and repair of tissue defects and disease modulation. Starting at the end of last century, major progress has been made in this field and, especially in the last years, the results obtained by the TERM scientists have been exceptional. This Research Topic outlines recent developments, major achieved accomplishments, and future challenges. The original articles and the reviews highlight some of the latest advancements at the forefront of different aspects of TERM. Hopefully, this article Research Topic will inspire, inform, and provide direction and guidance to a new generation of TERM researchers.

Stem/progenitor cells are key elements in TERM. Among them, the more frequently adopted are Mesenchymal Stem Cells (MSC) from different tissues. The review by Al-Ghadban et al. describes current clinical applications of adipose derived MSC and looks forward to future applications of these cells, including the development of organoids, tissue elements, and organ-on-a-chip systems. Due to the context-sensitivity of MSC to their microenvironment, it is evident that the therapeutic efficacy of these cells is modulated by the pathologically altered tissue environment. Questioning whether the context-sensitive interaction between MSC and specific targets leads to an enhancement, or an inhibition of the therapeutic effects is of crucial significance. The review by Roth et al. presents the author's viewpoint on pathology-related targets of MSC therapeutically applied in tendon and joint diseases, focusing on the equine patient as a valid animal model.

Major progress was made with the transplant of "*ex vivo*" expanded autologous (from the patient) stem/progenitor cells seeded on or associated to carrier biomaterials. The purpose of the review by Selvakumar et al. is to explore the application of regenerative medicine principles into current and future stent designs. The review covers regeneration-relevant approaches emerging in current research and highlights two unique regenerative features of stent technologies: selective regeneration, and stent-assisted regeneration of ischemic tissue as

consequence of an injury. When cells are implanted in association with biomaterials and/or medical devices some weaknesses still hinder the TERM approach. The analysis performed by Adamo et al. proposes a critical overview to identify key aspects to be considered and implemented in designing new tracheal substitutes, thus paving the way towards safer and more effective solutions for treating patients, now incurable.

The increasing knowledge about the physiological body response to injury suggests that the human organism itself could provide all elements needed for tissue repair and regeneration. New strategies, aimed at the stimulation of the body resident stem cells and of the intrinsic endogenous potential of tissues to heal or regenerate, are being developed. Lower limb ulcers represent a major clinical problem for the aging human population, and particularly for diabetic patients. More recently, treatments with allogeneic stem/progenitor cells, cell released microvesicles, or with platelet derivatives, such as Platelet Rich Plasma (PRP), have been proposed as alternative therapies for chronic skin ulcers. In most cases, this resulted in a significant benefit for the patient. The review by Mastrogiacomo et al. summarizes results obtained with these innovative approaches for the treatment of diabetic ulcer patients. The physiological body response to an injury includes major changes in key signaling molecules, such as Reactive Oxygen Species (ROS), that play an important role in the progression of inflammatory disorders. Our understanding of the effects of an enhanced ROS generation on cellular processes has been largely established, but their therapeutic potential is mostly unexplored. The manuscript by Sheppard et al. provides a view of the effects of ROS on skeletal healing. Hopefully, this will allow development of novel strategies to optimize the redox environment for skeletal tissue regeneration.

Advances in imaging techniques are crucial in the progress of the TERM field. The review by Huang et al. is focused on the application of nerve tracer imaging and summarizes current knowledge and mechanisms of action regarding nerve regeneration in organs where transplantation techniques have been widely performed, such as heart, liver, and kidney.

The adoption of suitable preclinical models is a must before considering any human treatment. Given the increasing demand for animal free alternatives in biomedical research, the manuscript by Munzebrock et al. reviews the applicability of some *in vitro* models to mimic the osteoarthritic (OA) joint, focussing on the crosstalk between the different joint tissues. In several of the described models a response to stimuli or drug treatments was observed that mimicked OA *in vivo* processes. In the same line of research, the review by Mainardi et al. analyses existing organs-on-chip platforms used to investigate pathological alterations of intervertebral discs (IVD). The article also proposes the conceptualization of a prospective IVD-on-chip model that could be used for mechano-transduction studies and therapy testing.

Present regulatory laws and ethical concerns recommend that controlled randomized clinical trials be performed before a new tissue engineering and regenerative medicine strategy or tool can be transferred to routine clinical practice and adopted for many patients. Clinical trials with regenerative therapies have shown some limitations, challenges, and uncertainties. However, the field's potential and implications remain great. The review by Petrosyan et al. is an overview of current TERM clinical trials and highlights how regenerative medicine aims to deliver effective patient-specific treatments with lifelong benefits. Looking to the future, the manuscript of Petrosyan et al. presents an update on the recent advances in transplantation that, by combing transplant and regenerative medicine fields, may change the way we think and practice tissue and organ transplantation.

While tissue engineering and regenerative medicine has been progressing over the past two decades, the sub-field of regenerative endodontics is one of the few areas of the field with true application that is currently being implemented in everyday clinical practice. The so called "Minimally Invasive Endodontics" has the goal of conserving tooth structure during conventional root canal therapy to enhance tooth integrity and survival thus addressing naturally inducing regeneration of what has been lost (Elnawam et al.).

Although, in principle, the whole world's population could benefit from these new technologies, at present there are very significant differences in their adoption in different regions and countries of the planet. To fill the existing gaps, specific strategies could be adopted by governments. The study by Kim and Bae reviews the change made by the Advanced Regenerative Medicine and Advanced Biological products act (ARMAB) implemented by the government in South Korea in 2020. We believe that this example could make a significant contribution to other countries which have plans to promote regenerative medicine.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Adipose Stem Cells in Regenerative Medicine: Looking Forward

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Over the last decade, stem cell-based regenerative medicine has progressed to clinical testing and therapeutic applications. The applications range from infusions of autologous and allogeneic stem cells to stem cell-derived products. Adult stem cells from adipose tissue (ASCs) show significant promise in treating autoimmune and neurodegenerative diseases, vascular and metabolic diseases, bone and cartilage regeneration and wound defects. The regenerative capabilities of ASCs *in vivo* are primarily orchestrated by their secretome of paracrine factors and cell-matrix interactions. More recent developments are focused on creating more complex structures such as 3D organoids, tissue elements and eventually fully functional tissues and organs to replace or repair diseased or damaged tissues. The current and future applications for ASCs in regenerative medicine are discussed here.

Keywords: adipose-derived stem cells (ASCs), regenerative medicine, tissue engineering, scaffolds, microfluidic systems

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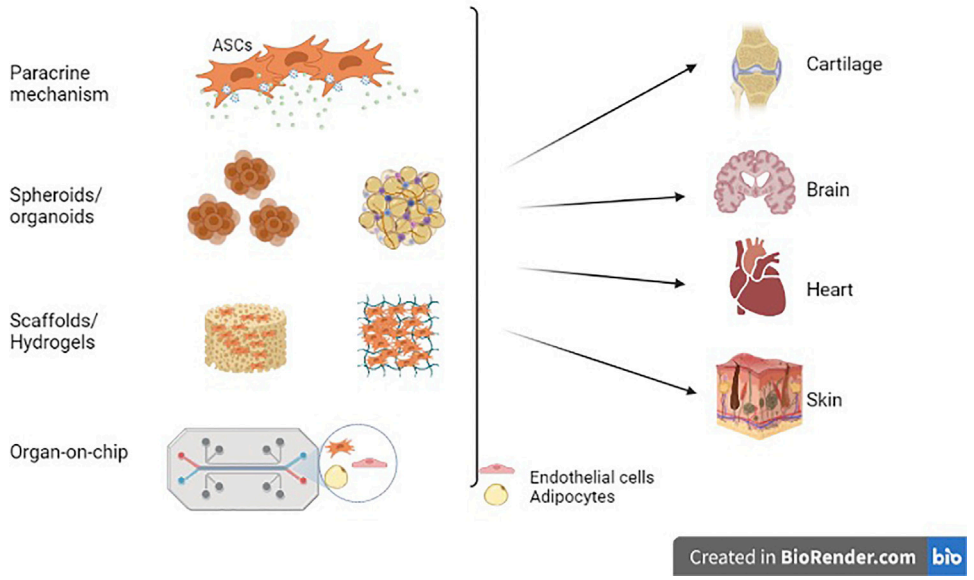
INTRODUCTION

ASCs and ASC-derived extracellular vesicles (ASC-EVs) and ASC conditioned media (ASCs-CM) have been extensively studied and widely used in regenerative medicine. Studies have shown the effectiveness of ASCs and ASCs secretome (Zuk et al., 2001; Giannasi et al., 2020; Trzyna and Banaś-Ząbczyk, 2021) therapy in numerous diseases such as cardiovascular, bone regeneration, osteoarthritis graft versus host disease (GvHD) and autoimmune disorders such Crohn's diseases (Galindo et al., 2017), systemic lupus erythematosus (SLE) and multiple sclerosis (Maria et al., 2017; Al-Ghadban and Bunnell, 2020). In addition, researchers have recently been designing new 3D biomaterials by combining ASCs with biomimetic scaffolds composed of either natural or synthetic materials. These 3D biomaterials have proven effective in tissue repair and organ regeneration (Storti et al., 2019; Gibler et al., 2021). These scaffolds have biological and physical properties that imitate the native ECM niche, which is crucial for stem cell adhesion, growth, proliferation and differentiation along particular lineages. Researchers are also incorporating ASCs into novel microfluidic systems, also known as organ-on-a-chip models, to model diseases in systems that function as intact organs, as substitutes for living organisms for testing of new therapeutic interventions (Zhang et al., 2017; Mofazzal Jahromi et al., 2019; O'Donnell et al., 2020). The content of this review is focused on the current and future applications of ASCs in regenerative medicine. The review also presents strategies and challenges in this field and explores the potential advancement of tissue engineering for clinical applications.

ASC PROPERTIES

Adipose tissue is distributed throughout the body in tissues including the bone marrow, beneath the skin (subcutaneous), within joints (intra-articular) and around internal organs (visceral adipose



GRAPHICAL ABSTRACT |

tissue). Adipose tissue is also located in ectopic sites, including the liver (intra-hepatic) and muscle (intra-muscular). Adipose tissue was viewed as an inactive organ that functioned primarily as an energy reservoir for the longest time (Trayhurn and Wood, 2005; Cawthorn et al., 2012). However, the initial identification of leptin, a cytokine produced by adipose tissue, and subsequently numerous other adipokines, led to the re-classification of adipose tissue as an endocrine organ (Zhang et al., 1994). Adipose tissue can also produce several other cytokines, including pro-inflammatory mediators such as IL-6, TNF-a, IL-1b, IL-8, and MCP-1, that drive inflammation (Coppack, 2001; Caer et al., 2017).

Three distinct types of adipose tissue have been described in humans. White adipose tissue (WAT), localized subcutaneously or in depots within the abdomen, comprises adipocytes and functions as an energy storage depot. Brown adipose tissue (BAT) is distributed throughout the body in interscapular regions, supraclavicular, suprarenal, pericardial, para-aortic and around the pancreas, kidney and trachea. BAT has thermogenic activity induced by shivering and non-shivering mechanisms. The thermogenic activity of BAT is driven by the expression of the mitochondrial membrane protein Uncoupling Protein 1 (UCP1).

In comparison, beige ("brite" or "brown/white") adipose tissue typically localizes with WAT. It serves as an energy storage depot that can express UCP1 and have thermogenic activity. The most common source of adipose tissue used to isolate ASCs is subcutaneous WAT collected from the abdomen, thigh, hips, or buttocks, typically during plastic surgical procedures.

Mature adipocytes are the primary cellular component of adipose tissue; however, adipose tissue represents a heterogeneous population of cells. Adipose tissue is comprised of preadipocytes, pericytes, fibroblasts, smooth muscle cells, endothelial cells, hematopoietic cells, mature immune cells such as B and T-cells, macrophages, myeloid cells and adipose tissue-derived mesenchymal stem cells (ASC).

ASCs are fibroblast-like cells isolated from the stromal vascular fraction (SVF) isolated by processing the adipose tissue (AT) by either enzymatic or mechanical methods. ASCs, comprise roughly 1–10% of the SVF. ASCs adhere to tissue culture plastic, proliferate in cell culture, undergo self-renewal, and effectively differentiate into multiple lineages, *in vitro* and *in vivo*. ASCs have been reported to differentiate into adipocytes, chondrocytes, osteoblasts, cardiomyocytes, skeletal muscle cells, neurons, hepatocytes and tenocytes, at least *in vitro* (Aurich et al., 2009; Vallée et al., 2009; Choi et al., 2010; Harris et al., 2011; Gimble et al., 2017; George et al., 2018; Mohiuddin O. A. et al., 2019; Lee et al., 2020; Harrison et al., 2022).

Flow cytometric analysis of the cell surface cluster of differentiation (CD) antigens is used to characterize ASCs. The majority of CD antigens screened are canonical markers of mesenchymal lineage cells. To date, an ASC-specific CD marker has not been identified that explicitly identifies ASCs. The analysis typically includes screening for both positive and negative CD antigens. Human ASCs are positive for known mesenchymal stem cell surface markers, including the cell adhesion molecules CD29, CD44, CD146, and CD166; the receptor molecules CD90 and CD105; and the GPI anchored enzyme CD73. In addition, ASC should be negative (< 2%) for the hematopoietic cell surface antigens, including CD11b, CD13, CD14, CD19, and CD45. They do not express the endothelial markers (CD31) and the human leukocyte antigen (HLA)-DR

(Tucker and Bunnell, 2011; Al-Ghadban and Bunnell, 2020; Harrison et al., 2022). ASCs are unique in their expression of CD36 (fatty acid translocase) and the absence of vascular cell adhesion molecule (VCAM-1/CD106) (Rajashekhar et al., 2008; Baer et al., 2013). It is worth noting that ASCs isolated from two different subcutaneous adipose tissue depots (abdomen and thigh) express the same CD markers and demonstrated a similar multi-differentiation potential (Choudhery et al., 2015). However, ASCs isolated from subcutaneous adipose tissue showed functional differences such as gene expression, growth factor secretion, proliferation rates and differentiation potential compared to ASCs isolated from visceral AT, suggesting that ASCs isolated from different depots may have unique properties (Hocking et al., 2010; Baglioni et al., 2012; Ong et al., 2014). Despite the differences in their characteristics, ASCs isolated from different depots have been used successfully in both clinical research and tissue engineering applications.

APPLICATION OF ASCS FOR REGENERATIVE MEDICINE

Over the last decade, ASCs have been broadly applied in regenerative medicine applications. ASCs secrete numerous soluble mediators, inflammatory cytokines, angiogenic, trophic and growth factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor (IGF), basic fibroblast growth factor (bFGF), transforming growth factor beta 1 (TGF-β1), stromal cell-derived factor (SDF)-1α, and interleukins. The paracrine factors secreted by ASCs contribute to tissue repair, wound healing and organ regeneration (Kapur and Katz, 2013; Suga et al., 2014; Brini et al., 2017; Li et al., 2019; Trzyna and Banaś-Ząbczyk, 2021). Studies have also shown that ASCs-EVs stimulate the regeneration of damaged tissue similar to the ASCs (Hu et al., 2016; Qiu et al., 2018). Basalova et al., have shown that ASCs-EV reduced fibrosis by stimulating myofibroblast dedifferentiation (Basalova et al., 2020). Other studies have also demonstrated the immunomodulatory effect of ASCs conditioned media in tissue repair and therapy (Yuan et al., 2018). In addition to the 2D monolayer cell cultures and 3D scaffolds extensively used to study the ASCs, an "organ-on-a-chip" technology is emerging as an advanced technique in stem cell-based therapy and will be discussed in this paper.

CURRENT THERAPEUTIC APPLICATIONS OF ASCS

Fat Grafting and Tissue Reconstruction

Fat grafting is a procedure that was initially attempted in 1889. Since then, many attempts have been made to create a standard protocol that will provide ideal outcomes. Cell-assisted lipotransfer (CAL), SVF and ASCs have demonstrated promise in adipose tissue regeneration and augmentation (Zhu et al., 2015; Li et al., 2017; Hong, 2020). The administration of ASCs enhances the outcomes of fat grafting in humans, particularly breast reconstruction, facial reconstruction and cosmetic surgery applications (Shukla et al., 2020b; Fang et al., 2021). This is mainly due to the ability of ASCs to differentiate into endothelial and epithelial cells as well as secrete cytokines and growth factors that promote angiogenesis through paracrine mechanisms and cell-cell interactions in a co-culture system, thus enhancing neovascularization and accelerating wound healing (Nie et al., 2011; Haubner et al., 2013). A study conducted by Kim et al. showed that ASCs intravenously injected in mice migrate to the wound area and promote tissue repair by differentiating into epithelial cells, thus inducing cutaneous regeneration by re-epithelialization of the dermal layer (Kim et al., 2019). Another study by Yu et al. and others demonstrated that transplanted ASC sheets enhance wound healing and reduce scar formation in a nude mice model. In this study, ASCs decreased inflammation and increased cellular viability in damaged tissue (Yu et al., 2018). In addition, fat grafting has shown to be an alternative for patients with wounds that do not respond to traditional therapy. A case report conducted by Vyas et al. demonstrated the ability of ASCs to promote wound healing in a patient suffering from a radiation-induced wound (Vyas et al., 2021). Furthermore, the use of lipoaspirate transplants containing ASCs has been shown to reverse skin necrosis after irradiation-induced degenerative chronic lesions in tissues exposed to oncologic radiotherapy (Rigotti et al., 2007).

Currently, cosmetic surgery represents the highest demand for ASCs, primarily for autologous transfers, with breast reconstruction and facial rejuvenation being the most popular applications (Bora and Majumdar, 2017). Fat reconstruction is typically used in facial soft-tissue repairs and to rebuild tissues subjected to surgical oncological treatments (Li et al., 2017). Nevertheless, the success rate of fat transplants varies between patients, which might be due to several factors, including the methods of ASCs isolation, surgical procedure, and the site of fat transplantation. One of the challenges of autologous fat grafts is fat reabsorption, decreasing the total volume of transplanted fat grafts by 20–70% (Bellini, 2017). However, the use of platelet-rich plasma (PRP), b-FGF, VEGF and estradiol as supplements for ASCs culture has demonstrated the ability to decrease the rate of fat reabsorption and enhanced fat transplant (Yuksel et al., 2000; Pires Fraga et al., 2010; Luo et al., 2013; Li et al., 2017).

Cardiovascular Diseases

ASCs have been demonstrated to mediate improvements after myocardial infarction via their inherent anti-apoptotic, anti-inflammatory, and pro-angiogenic effects. ASCs also contribute to inhibiting fibrosis and cardiac remodeling through the recruitment of endogenous stem cells and influence their re-entry into the cardiovascular lineage cell cycle (Li et al., 2019a). In a rat model of chronic ischemic cardiomyopathy (ICM), a group of researchers sought to identify early changes in cardiac cellular subpopulations and transcription after treatments with a well-characterized and pure cryopreserved allogeneic ASCs isolated from male Lewis (Follin et al., 2021). Treatment with ASCs resulted in altered inflammatory monocyte/macrophage subpopulations, increased CD4/CD8 ratio, and an increased uncharacterized

CD31−CD45−CD90− population. Also, colony-stimulating factor 2, VEGFA and VEGFB were upregulated in the treated animals. These changes are associated with positive chemotaxis, monocyte and macrophage differentiation, and angiogenesis. These results indicate that some of the immediate effects of ASCs were related primarily to monocyte/macrophage regulation (Follin et al., 2021). The first human clinical trial report on the use of cryopreserved off-the-shelf ASCs in ischemic heart disease and heart failure treatment was published in 2017 (Kastrup et al., 2017). The study included ten patients ranging in ages from 30 to 80 years old. The follow-up was done at 1, 2, 3, and 6 months. ASCs were obtained from lipoaspirates from three healthy female donors of ages between 28 and 33 years old. After the 6-months follow-up, overall cardiac function showed a tendency to improve, with left ventricle pump function and a reduction in dilatation of the left ventricle. Interestingly enough, while two patients had donor-specific HLA antibodies at baseline and four patients developed donor-specific *de novo*, this did not affect the efficacy of the treatment, nor were there any significant adverse events post-treatment, suggesting that even with allogeneic ASCs transplantation, immunosuppressant drugs may not be required.

Bone Defects

Bone tissue has an inherent ability to repair and regenerate; however, the "critical-size bone defect" concept limits the extent of this regeneration potential. This concept simply refers to the point at which the extension of the lesion is too great for the effective signal transduction and delivery of growth factors to drive the regeneration. At this point, scaffolds combined with stem cells such as ASCs are required (Mohiuddin O. A. et al., 2019; Storti et al., 2019). Nonetheless, bone-derived grafts are the second most transplanted material, next to blood. Only 28% of patients with open fractures can make a full recovery (Alonso-Goulart et al., 2021) on their own. Bone tissue is composed of three types of cells that maintain its integrity: osteoblast, osteoclast, and osteocyte cells. Bone remodeling and regeneration require the interaction between osteoclasts and osteoblasts (Baddour et al., 2012). Several studies have shown that ASCs can be differentiated into osteoblasts. Adding bone morphogenetic protein 2 (BMP-2), growth factors, PRP, extracellular calcium enhances their differentiation and bone regeneration (Zhang et al., 2014; Yanai et al., 2019). A case study by Mesimäki et al. demonstrated that autologous transplanted ASCs with BMP2 and beta-tricalcium phosphate reconstituted the defect area of the maxillary (Mesimäki et al., 2009). A similar study conducted by Wolff et al. and others showed successful reconstitution of the mandibular defects using tissue engineered constructs of autologous ASCs, beta-tricalcium phosphate (β-TCP) granules, recombinant human BMP-2 in a cohort of three patients (Wolff et al., 2013).

In addition to ASCs therapeutic potential, studies have shown that ASCs-Exo can stimulate bone regeneration through a paracrine mechanism (Zhu et al., 2021). Furthermore, in mouse models, decellularized adipose tissue hydrogels have shown promise, especially when combined with ASCs. The decellularized adipose tissue scaffolds promote bone regeneration and a higher volume of partially mineralized tissue and higher levels of collagen and osteopontin when the scaffolds are treated with either ASCs or osteogenic lineage induced ASCs and hydroxyapatite (Mohiuddin O. A. et al., 2019).

Cartilage Regeneration

In general, cartilage has a narrow potential for regeneration (Tiku and Sabaawy, 2015). Of the different types of cartilage, articular cartilage is the most common therapy target, with joint injury and osteoarthritis (OA) being the main targets of regenerative medicine (Hulme et al., 2021). Clinical trials using autologous and allogeneic ASCs injections, ASCs-Exo, ASCs-CM to treat knee osteoarthritis have been reported to be well-tolerated and yield positive results in pain amelioration, decreased stiffness, and increased physical function with no severe side effects (Chen et al., 2021). A study conducted by Spasovski et al. showed that treating patients with autologous ASCs enhances their clinical scores and reduces pain levels (Spasovski et al., 2018). Another study by Pers and others demonstrated that patients who received autologous ASCs injections in the knee showed improvement due to the paracrine factors released from the cells inducing an anti-inflammatory response (Pers et al., 2018). A recent study conducted by Li and others described the use of ECM/SVF-gel fraction in cartilage defect repair as ASCs migrate from the gel and differentiate in a natural microenvironment, thus increasing the therapeutic potential of ASCs (Li et al., 2020). Furthermore, engineered cartilage is another area that is gaining interest, as ASCs and Poly ε-Caprolactone Scaffolds have successfully been used to regenerate cartilage *in vitro* for later transplantation into a mouse model (Nguyen and Vu, 2021).

Spinal Cord Injuries (SCIs)

While most reports on the use of ASCs to treat spinal cord injuries have been performed in animal models, human clinical trials are underway. Takahashi et al. investigated the outcomes of treating SCIs with either BM-MSCs or ASCs in a murine model (Takahashi et al., 2018). Their data revealed comparable levels of motor function improvement in moderate SCI models. They also observed a higher survival rate after transplantation in the severe SCIs model and improved neuronal and vascular protection in the ASC-treated group (Takahashi et al., 2018). Another group explored using hASCs combined with low-level laser in neuropathic pain in experimental SCI models in rats. Their results demonstrated that combining low-level laser treatment with ASCs improved the level of the motor function recovery and had superior outcomes in alleviating SCI-induced allodynia and hyperalgesia; compared to groups treated with ASCs alone (Sarveazad et al., 2019). The therapeutic potential of mesenchymal stem cells isolated from either bone marrow, adipose tissue, or dental pulp was explored by another group in both a small (rats) and a large (pigs) animal model in spinal cord injury during the spinal contusion subacute period (Mukhamedshina et al., 2019). Applying ASCs in combination with fibrin matrix in the rat model provided significantly higher post-traumatic regeneration results than other MSCs. However, while the application of ASCs embedded in the fibrin matrix at the SCI site in pigs restored neural tissue integrity, no significant

functional improvements were noted. A few case studies utilizing ASCs in human spinal cord injury patients have positive outcomes. The first human trial was performed in South Korea in 14 patients. It employed intrathecal transplantation of autologous ASCs with an 8-months follow-up. While the injury site and degree of impairments were diverse, none of the patients developed severe adverse effects from the transplantation procedure. While there were no differences in the areas of spinal damage as observed in the MRIs before and after the procedure, five patients showed an improved ASIA motor score, ten patients showed improved ASIA sensory score, and two patients that had no control over anal sphincters recovered it after 1- and 4-months post-treatment, respectively (Hur et al., 2016). Finally, the first report from a patient from the CELLTOP study, an ongoing multidisciplinary phase 1 clinical trial conducted at Mayo Clinic, has been released (Bydon et al., 2020). A 53-year-old male patient underwent an autologous intrathecal injection 11 months after a surfing injury., there was a progressive improvement in motor and sensory ASIA scores and the overall quality of life. There were no reports of adverse events within the 18-months follow-up period. Overall, these results highlight that, in general, the potential benefits of using ASCs for spinal cord injury.

Stem Cell Therapy and COVID-19

ASCs are known for their potent immunomodulatory potential, which permits the cells to sense their microenvironment and respond according to the circumstances. Additionally, mesenchymal stem cells from diverse sources have proven effective in impairing viral replication and reducing the viral load (Rogers et al., 2020). Based on that, researchers are currently investigating the possibility of utilizing ASCs to treat COVID-19 infected patients. The primary complications from COVID-19 infection include damage to the lung tissue, excessive inflammation, and progression of lung fibrosis. Shi and others summarized early clinical testing of ASC injections, which highlights the ability of MSCs to reduce infiltration by immune cells and help repair the damaged tissue (Shi et al., 2021). A study conducted by Leng et al. showed that intravenous injection of stem cells was very effective in treating COVID-19 patients with pneumonia by decreasing pro-inflammatory cytokines and increasing the anti-inflammatory cytokines such as IL-10, thus promoting lung repair (Leng et al., 2020). A study by Gentile and Sterodimas investigated the use of autologous or allogeneic ASCs to treat severe COVID-19 cases (Gentile and Sterodimas, 2020). ASC infusions were shown to inhibit the over-activation of the immune system, promoting endogenous repair by improving the lung microenvironment. Although ASCs have shown promising outcomes, the type of stem cells, the dose administered, the interval of time, and the delivery mechanism should be optimized for future clinical applications. Some of these issues may be addressed as results of ongoing COVID-19 clinical trials are published.

FUTURE APPLICATIONS FOR ASCS

Nanotherapeutics: 3D Scaffolds With ASCs

Nanotechnology has opened a new opportunities for novel applications of stem cells for numerous diseases, such as cardiovascular, neurological, vascular diseases, diabetes and inflammation (Masoudi Asil et al., 2020; Dong et al., 2021; Sarathkumar et al., 2021). It is worth noting that NPs are naturally occurring products from ASCs and are secreted as EVs (Wang et al., 2009; Dong et al., 2021). ASC-derived NPs offer novel, non-invasive methods and provide much information about tissue repair and develop a precise method for targeted therapy (Qiu et al., 2018; Dong et al., 2021). Beyond that combining stem cells, with scaffolds is driving the creation of organoids, organ-on-a-chip systems and lab grown organs and tissues.

Scaffolds, synthetic or natural "biologically-derived", to be used in tissue engineering applications, must recapitulate the extracellular matrix (ECM) and imitate an *in vivo* like microenvironment favorable for stem cell attachment and proliferation. Synthetic scaffolds are made up of polyesters, polyethers, polyethylene glycol and polylactic acid (PLLA) (Gibler et al., 2021b; Reddy et al., 2021), while natural scaffolds "commonly used with ASCs" are comprised of collagen, fibrin, gelatin, vitronectin, laminin, alginate, hyaluronic acid, or decellularized materials (DAT) (Sadeghi-Ataabadi et al., 2017; Vinson et al., 2017; Kook et al., 2018; Mohiuddin O. A. et al., 2019; Colle et al., 2020; O'Donnell et al., 2020). The central characteristics of the scaffolds include the composition and porosity necessary to promote cell adhesion, proliferation and differentiation; fibrosity and stiffness; biocompatibility with the tissue and biodegradability with a negligible amount of toxicity or inflammation *in vivo* (Dhandayuthapani et al., 2011; Reddy et al., 2021). Several studies have incorporated ASCs into 3D scaffolds and demonstrated their ability to adhere, migrate and differentiate into specific cell lineages. The choice of nanomaterial used in generating ASC scaffolds should be carefully considered as it might influence the differentiation ability of stem cells. In addition to the ECM-ASC interactions in a scaffold, growth factors (such as VEGF, bFGF and TGF-β) can effectively be integrated to enhance the therapeutic potential by enhancing the proliferation and differentiation of stem cells (Howard et al., 2008; Sell et al., 2011; Sadeghi-Ataabadi et al., 2017). Similarly, studies have demonstrated the function of ASCs seeded on PRP fibrin enriched scaffolds in cartilage repair and tendon regeneration (Drengk et al., 2009; Barbon et al., 2019), as well as skin graft transplantation by inducing tissue angiogenesis (Wang et al., 2019; Gao et al., 2020). Nair and others showed that MSCs incorporated into graphene oxide (GO) scaffolds enhanced osteogenic differentiation. Thus, they may be used for bone regeneration in orthopedic applications (Nair et al., 2015). A similar test of GO scaffolds demonstrated their ability to induce neuronal differentiation of mesenchymal stem cells (Kim et al., 2015). In another study, PLLA and poly 3-hydroxybutyrate scaffolds combined with dental pulp-derived MSCs are a potential treatment of cardiovascular diseases (Castellano et al., 2014). Yin and others demonstrated ASC differentiation into chondrocytes in the context of PLGA gelatin scaffolds supplemented with TGF-β1 (Yin et al., 2015).

Decellularized adipose tissue hydrogels (hDAT) impregnated with stem cells have been widely tested as treatments for spinal

cord and peripheral nerve injury, wound healing, myocardial infarction, cartilage repair, and bone tissue engineering. Mohiuddin and others have shown that DAT hydrogels support ASC proliferation and multilineage differentiation capabilities. Additionally, the ASCs remodeled the microstructure of the hydrogels, making them more compatible for *in vivo*-like adipose tissue regeneration (Mohiuddin O. A. et al., 2019). The use of decellularized scaffolds and ASCs in regenerative medicine is promising. However, more research into the method of preparation, "decellularization and sterilization," should be optimized for each use, as it will affect the quality of stem cells attachment and differentiation. Yang et al. described the different decellularized and sterilization techniques used in the preparation of DAT hydrogels as well as the problems that needs to be resolved before their application in clinicals trials (Yang et al., 2020). The decellularization methods involve a combination of biological (enzymatic digestion DNase/RNase) chemical (isopropanol, Triton X and sodium chloride), and physical (freeze and thaw cycles, homogenization) treatments. The sterilization techniques mainly included 70% ethanol, penicillin and streptomycin, ethylene oxide and UV light (Song et al., 2018; Thomas-Porch et al., 2018; Yang et al., 2020). Although these methods have been proven to be effective in decellularization, the use of certain detergents and enzymes cause the loss of the main ECM components such as collagen, laminin and glycosaminoglycans (GAGs) and destruction of protein-protein interactions. Yang et al. also mentioned that residues of chemical and enzymatic substances or cell debris might affect stem cell adhesion differentiation of stem cells as well as induce an immune response. Thus, optimizing protocols for adipose tissue decellularization will be important to develop 3D biomaterial scaffolds that serve as effective *in vitro* models of the pathophysiology of various diseases and can be used to screen novel therapeutics and reduce the use of experimental animal models.

Microphysiological Systems: "Organ-on-a-Chip" Technology

Microphysiological systems (MPS) are an advanced technology that permits researchers to study cell-cell, cell-ECM and cell-tissue interactions in an in the vivo-like dynamic microenvironment. MPS uses either 3D organoids "assembled from cell-cell interactions in a scaffold-free manner" or on scaffolds to develop "organ-on-a-chip" systems applicable for drug screening and assessment of novel treatments (Zhang et al., 2017; Ronaldson-Bouchard and Vunjak-Novakovic, 2018; Wu et al., 2020; Marrazzo et al., 2021). In MPS, stem cells can be seeded in layers or interconnected cell culture chambers linked by microchannels filled with media (particular to the cell type). They can also incorporate mechanical and biochemical stimuli to replicate human tissues' physiology and biology *in vivo*. The microchannels included in MPS are designed to control the flow of the media between the chambers, and they can be sealed or perfused depending on the type of planned experiments. Although MPS has been widely investigated, only a few published articles presently report using stem cells, including ASCs (Zhang et al., 2017; Paek et al., 2019; O'Donnell et al., 2020; Marrazzo et al., 2021). Kefallinou and others demonstrated the generation of bone marrow-on-a-chip (BMoC), which may be useful as a system to study the stromal niche in diseases such as SLE (Kefallinoua et al., 2020). Another article published by Lavrentieva described a microfluidic gradient system using hASCs and human umbilical cord vein endothelial cells (HUVECs) were embedded in a methacrylated gelatin (GelMA) hydrogel to study the morphological changes and cellular interaction in a stem cell niche and how the stiffness of the gels plays a significant role in developing scaffolds used for clinical applications (Lavrentieva et al., 2020). An MPS combining human ASCs and human adipose microvascular endothelial cells (hAMECs) in a collagen and fibrin hydrogel scaffold to create a 3D microvascular-like network that recapitulates the interaction between the cell types and facilitates the study of vascular inflammation in a dynamic fluid system has been reported (Paek et al., 2019). The data collected using the MPS will provide novel research tools for developing preclinical models representing the physiological niche and potential treatment of vascular and metabolic diseases. However, more research is needed on how mechanical stimulus affects stem cell niche development under physiological conditions. One study has shown that interstitial shear stress in a fluidic system might decrease the ASCs' ability to undergo adipogenesis (Bender et al., 2020).

Challenges and Limitations

Human pre-clinical and clinical trials are underway to provide treatment for multiple diseases, including chronic conditions such as systemic sclerosis, neurological degenerative diseases, non-healing wounds as in diabetic ulcers, or the treatment of fistulas in patients with Chron's Disease, neurodegenerative diseases, atherothrombotic diseases, chronic kidney disease, degenerative osteoarthritis, and enhance recovery after ligament and tendon injuries, and promote the field of reconstructive plastic surgery in breast reconstruction and skin rejuvenation (Shukla et al., 2020). Nevertheless, there are still many hoops to jump ahead and a long way to get these therapies to move from clinical trials to FDA-approved treatments. One of the limitations for the usage of ASCs in regenerative medicine is the donor's age, body mass index (BMI) and health conditions (underlying disease or comorbidities), which might result in reduced immunomodulatory abilities of main regulatory factors (Strong et al., 2013; Barwinska et al., 2018). Thus, ASCs should be fully characterizing and thoroughly screened for *in vitro* aging and inflammatory markers that might affect their regenerative abilities and hinder their usage in clinical application. Further, in-depth research is needed to study the effect of nanomaterial, pore size, stiffness, biodegradability on stem cell proliferation, migration and differentiation while developing scaffolds as a novel therapeutic tool that imitates the *in vivo* microenvironment. One of the limitations for using ASCs scaffolds in an MPS is their proliferation, migration and capability to multi-differentiate into particular lineages in a co-culture system for an extended period.

CONCLUSION

ASCs have therapeutic potential in regenerative medicine and applications in tissue engineering. While the biologic properties of ASCs are not yet fully delineated, the cells are under clinical investigation in human trials for an array of diseases. ASCs have been widely studied for their immunomodulatory effects, anti-fibrotic, anti-apoptotic, and anti-oxidative capabilities in preclinical and human clinical trials. Additionally, the ASC secretome (conditioned media or exosomes) has demonstrated similar effectiveness in regenerative medicine applications. However, the field must be cognizant of the inherent donor-to-donor variation, which can significantly impact the therapeutic potential of ASCs and ASC-derived products regarding their growth and differentiation efficiency, which will directly impact their therapeutic potential as cell therapies their effectiveness in more complex 3D systems.

Furthermore, the emerging technology combining stem cells with scaffolds or microfluidic chip technologies provides novel opportunities to develop more sophisticated and effective treatments with minimal risks and side effects. Therefore, ASC-based scaffolds and "organ-on-a-chip" models represent an advanced technology in regenerative medicine that will provide researchers with new tools to treat numerous diseases that conventional medicine could not effectively cure. However, investigators must address several considerations as the more complex 3D systems develop including, cell types required for their creation, development of standardized methodologies for their generation, criteria for characterization of how effectively it recapitulates an intact organ, and readouts for biologic and efficacy assessments.

AUTHOR CONTRIBUTIONS

Conceptualization and content design by BB; BB, SA-G and MA performed writing, review and editing. All authors have read and agreed to the published version of the manuscript.

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REVIEW
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Intervertebral Disc-on-a-Chip as Advanced *In Vitro* Model for Mechanobiology Research and Drug Testing: A Review and Perspective

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Discogenic back pain is one of the most diffused musculoskeletal pathologies and a hurdle to a good quality of life for millions of people. Existing therapeutic options are exclusively directed at reducing symptoms, not at targeting the underlying, still poorly understood, degenerative processes. Common intervertebral disc (IVD) disease models still do not fully replicate the course of degenerative IVD disease. Advanced disease models that incorporate mechanical loading are needed to investigate pathological causes and processes, as well as to identify therapeutic targets. Organs-on-chip (OoC) are microfluidic-based devices that aim at recapitulating tissue functions *in vitro* by introducing key features of the tissue microenvironment (e.g., 3D architecture, soluble signals and mechanical conditioning). In this review we analyze and depict existing OoC platforms used to investigate pathological alterations of IVD cells/tissues and discuss their benefits and limitations. Starting from the consideration that mechanobiology plays a pivotal role in both IVD homeostasis and degeneration, we then focus on OoC settings enabling to recapitulate physiological or aberrant mechanical loading, in conjunction with other relevant features (such as inflammation). Finally, we propose our view on design criteria for IVD-on-a-chip systems, offering a future perspective to model IVD mechanobiology.

Keywords: intervertebral disc, mechanical loading, microphysiological device design, organ-on-a-chip, mechanobiology, degenerative disc disease (DDD)

1 INTRODUCTION

Low back pain (LBP) is a prevalent health problem, with 80% of people suffering from it at least once in their lifetime (Wieser et al., 2011; Vlaeyen et al., 2018). A major cause of LBP is degenerative disc disease (DDD), an age-related pathology of the intervertebral disc (IVD) (Zhang et al., 2009; Wuertz and Haglund, 2013). While there is also some genetic predisposition (Trefilova et al., 2021), DDD is clearly associated with mechanical risk factors such as spine misalignment (e.g. scoliosis) or excessive IVD loads due to obesity or occupational hazards (e.g. heavy lifting), supporting the relationship between aberrant mechanical loading and IVD degeneration (Adams and Roughley, 2006; Stokes

TABLE 1 | Advantages and disadvantages of IVD models. OoC = organ-on-chip.

	Animal models	Classic *in vitro* models	*Ex-vivo* organ culture	Macroscale bioreactors	OoC devices
• Advantages	• Most accurate recapitulation of the whole IVD	• Operational ease • Low cost • High throughput • repeatability	• Whole IVD recapitulation • Clinically relevant size	• 3D environments • Complex physicochemical stimulation • Whole IVD stimulation • Clinically relevant size	• Mimicking *in vivo* conditions • Possible high throughput • Control of environmental conditions • Imaging and analysis capabilities
• Disadvantages	• Costly and time consuming • Ethical concerns • Animal-human mismatch (size, loading, cells) • Poor control over the experimental conditions • Require dedicated animal facilities	• Largely based on 2D substrates • Low clinical predictivity • Lack of mechanical cues	• Rapid degradation of IVD structures • Poor control over the experimental conditions • Lack of mechanical cues	• Difficult to use • Low throughput • Bulky • Difficult to fabricate	• Not a clinically relevant scale • Challenging classic readouts (e.g. histological stainings) • Difficult to fabricate

et al., 2011; Macedo and Battié, 2019). Despite the proven mechanical nature of DDD, mediators aberrant mechanosensing and mechanotransduction are still poorly understood, thus are not therapeutically targeted. The absence of appropriate preclinical IVD models is one of the reasons hampering these advancements. A better understanding of the molecular mechanisms leading from hyperphysiological mechanical loading to IVD degeneration, inflammation, and nociception, might reveal more effective therapeutic targets.

Mechanobiological responses of the IVD have been traditionally studied using macroscale devices and bioreactors (Molladavoodi et al., 2020). These systems are designed to apply loading patterns with different degrees of complexity (e.g. tension, compression, shear or their combination) to two-dimensional (2D) or three-dimensional (3D) cell-based models, IVD tissue explants, as well as whole IVD organs (Neidlinger-Wilke et al., 2014; Gantenbein et al., 2015; Shan et al., 2017; Peroglio et al., 2018; Pfannkuche et al., 2020). Devices applying mechanical load 2D cell cultures (e.g. capable of stretching (Cambria et al., 2020a)) simulate an oversimplified human body architecture, as cells in 2D rely on adherence to a flat surface thus lacking the support of the surrounding extracellular matrix (ECM) characterizing native tissues. On the contrary, 3D cell-based models utilize biomaterials to mimic the tissue microenvironment (Krupkova et al., 2014; Cambria et al., 2020b). In 3D, IVD cells produce their own ECM and generate an environment responsive to mechanical loading. Macroscale loading bioreactors integrating 3D cell constructs thus provide more physiological conditions over 2D cell cultures and enable the investigation of the mechanobiological response of whole tissues (Peroglio et al., 2018). The most relevant bioreactors for preclinical drug development still remain those capable of dynamic loading of whole IVDs of either human or animal origin (i.e. IVD isolated from large animals as cow, dog or sheep) (Gawri et al., 2011; Gantenbein et al., 2015). However, it is important to consider that such *ex vivo* loading systems mostly do not allow for testing large sample numbers at once. Animal models might mimic better the disease complexity but present inherent differences in loading patterns, genetics, and even cellular composition (e.g. presence of notochordal cells) with respect to human counterparts. **Table 1** summarizes the major advantages and disadvantages of available IVD preclinical models.

Despite large advances in macroscale *in vitro* and *ex vivo* IVD models, it is still challenging to use such systems to investigate fundamental questions on specific cell functions and molecular mechanisms involved in the conversion of mechanical loading to biochemical responses such as inflammation and pain. To improve the understanding of mechanotransduction and predict the success of new therapeutic approaches under loading, new *in vitro* models that enable both 1) recapitulation of the IVD native-like mechanically active environment at a cellular relevant scale and 2) compatibility with high-throughput setups, are warranted. Scalable, easy-to-use, and low-cost *in vitro* 3D loading systems would aid not only in fundamental research but also in advancement of disease-modifying and personalized therapies.

Organs-on-chip (OoC), also referred to as microphysiological systems (MPS), are emerging biomedical research tools derived from microfluidic technologies that allow a precise control over fluid behavior within micrometer-sized channels (Bhatia and Ingber, 2014; Esch et al., 2015). Owing to their three-dimensional scale, resembling the one experienced by cells in the body, cell-based OoCs can attain extraordinary control over cells behavior *in vitro* (Rothbauer et al., 2021). The main advantage of OoCs is their ability to mimic dynamic (even patient-specific) tissue microenvironments, while controlling crucial tissue parameters such as flow rates, molecular gradients, and biophysical cues (e.g. mechanical and electrical) (Zhang et al., 2018a; Ergir et al., 2018; Rothbauer et al., 2021). OoCs can achieve sufficient complexity to recapitulate traits of human pathophysiology, as already demonstrated for various tissues (Huh et al., 2010; Esch et al., 2015; Herland et al., 2020), thus potentially providing more predictable tissue responses than conventional *in vitro* models. Originally, OoCs platforms used the high control over the fluid motion and diffusion at the microscale to obtain finely tuned experimental conditions and mimic blood circulations in tissues

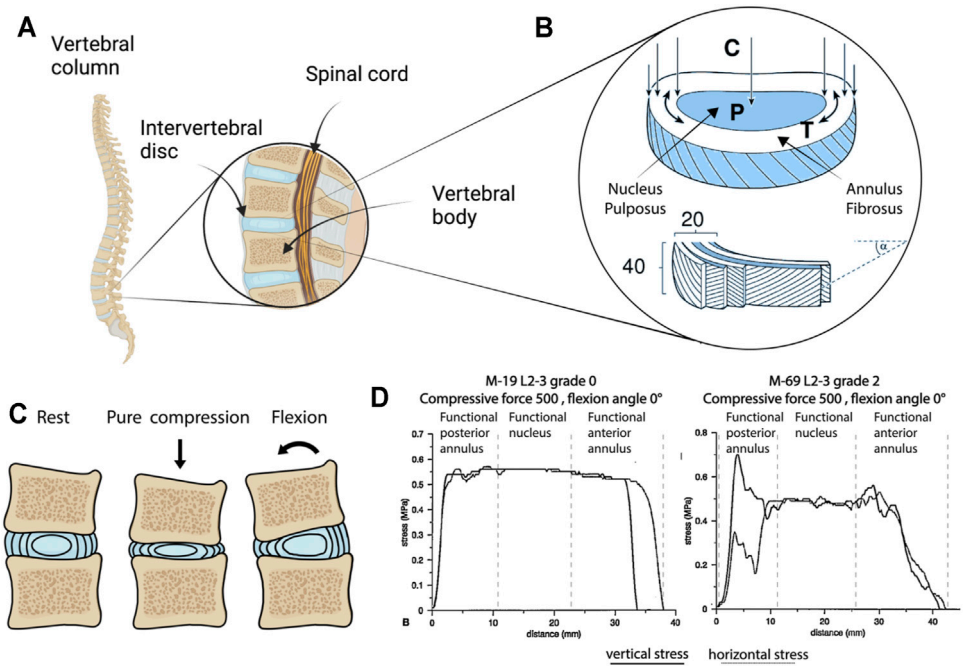

FIGURE 1 | Intervertebral disc (IVD) anatomy and physical stimuli. **(A)** Vertebral column and the IVD (created with BioRender.com). **(B)** Structure, composition and main stresses acting on the components of the IVD. An overall vertical compression (C) of the IVD results in an increase of the hydrostatic pressure (P) in the nucleus pulposus (NP), which in turns tends to expand laterally causing an increment in the circumferential tension (T) experienced by the annulus fibrosus (AF), typically composed of 20 lamellae, each constituted by roughly 40 fibers disposed with a 30° angle (Adams and Roughley, 2006). **(C)** Schematization of the IVD in rest condition (i.e. when no stimuli are applied), following compression leading to a decrease in height and an outward expansion of the AF, and following a flexional slate (e.g. the one arising from someone bending their back). Bending in particular results in a complex stimulation state in the AF with the fibers on one side experiencing compression and on the other side experiencing tension. **(D)** Stress in human AF and in the NP of a grade 0 and a grade II (cadaveric IVD, as determined by stress profilometry) (McNally and Adams, 1992b; Adams, 2004). Images **(B)** adapted from Adams and Roughley (2006), **(D)** from McNally and Adams (1992b) and Adams (2004), reprinted with publisher permissions (SAGE Publications and Wolters Kluwer Health, Inc., respectively).

including lung (Huh et al., 2010), gut, liver, and kidney (Herland et al., 2020). Microfluidic devices have also been extensively used to investigate the effects of shear stress in vasculoendothelial pathologies, both in 2D and 3D models (Chou et al., 2016; Kim et al., 2017). OoCs also represent cost-effective and compact 3D platforms that are compatible with parallelization and automation, thereby facilitating drug screening studies. They can serve as a tool for comprehensive evaluation of various cell types, biomaterials, drugs, and tissue-engineered products, potentially reducing the need for animal testing (following the Replacement, Reduction and Refinement (3Rs) principle). In certain cases, OoCs even demonstrated a higher relevance for predicting human responses, as animal models usually do not fully represent human conditions due to inter-species differences (Zhang et al., 2018a; Ergir et al., 2018; Rothbauer et al., 2021).

Recognizing the importance of mechanical factors in IVD homeostasis and degeneration, multiple loading-based macroscale *in vitro* models have been proposed and reviewed (Pfannkuche et al., 2020). Less attention has however been given to reviewing microscale OoCs aimed at modelling different aspects of the IVD pathophysiology. The main goal of this review is to explore the IVD-on-a-chip technology as a highly relevant tool to mimic and study human IVD pathophysiology in a medium-to high-throughput context. We focus on recent advances in OoCs, with applications in the IVD field together with devices that, while designed for other purposes, could benefit IVD studies (e.g. with the introduction of mechanical loading), and discuss their potential to evaluate new therapies. Finally, we discuss the limitations of the current approaches proposing the conceptualization of a prospective IVD-on-chip model that could be used in future mechanotransduction studies. This comprehensive review is intended for readers with different backgrounds ranging from medical and biological scientists to engineers.

2 INTERVERTEBRAL DISC AND DEGENERATIVE DISC DISEASE

The IVD is a cartilaginous structure located between two adjacent vertebrae in the spinal column (**Figure 1A**). Anatomically, IVDs are constituted by a central nucleus pulposus (NP) encircled by the annulus fibrosus (AF) (**Figure 1B**), and connected to the neighboring vertebrae through hyaline cartilage endplates (CEPs) (Urban et al., 2004; Richardson et al., 2007). The NP is a gel-like structure predominantly composed of a loose network of highly hydrated proteoglycans (PGs) and collagen type II, with a PG/collagen ratio of 26:1 in healthy IVDs (Mwale et al., 2004). The AF

is composed of circumferential lamellae (typically 15–25) formed by closely arranged fibers of collagen type I (**Figure 1B**). The external layer is composed of fibrous collagen type I fibers with a vertical direction connecting the cortical bone annular apophyses. Inner lamellae have fibers with a 30° orientation. Moving from the outside to the inside of the AF, the cellular population changes from fibroblast-like to chondrocyte-like cells responsible for the homeostasis of the fibrocartilaginous inner layers. CEPs, located above and below the IVD and separating the IVD from the vertebral endplates, are approximately 600-μm thick layers of hyaline cartilage rich in collagen type II and PGs. CEPs function as a mechanical barrier between the vertebral bodies and the NP but also as communication channels for nutrient transport from neighboring vascular channels into the IVD (Moon et al., 2013).

The IVD has the necessary mechanical properties to support the body weight and the flexibility to permit spinal movements. The lamellar structure of the AF provides load-bearing function, tensile resistance, and adequate support to maintain the NP pressure (Urban and Roberts, 2003), while the PG-rich composition of the NP mediates resistance to compression. A schematization of the forces to which NP and AF are subjected to, as originally depicted by Adams (2004), and Adams and Roughley (2006) is shown in **Figure 1B**. Compressive forces acting vertically on the IVD result in an increase of the pressure (P) in the NP and of the circumferential tension (T) in the AF.

During IVD degeneration, an imbalance between anabolic and catabolic processes occurs, leading to ECM degradation and functional changes. Degenerative disc disease (DDD) occurs when these changes are accompanied by chronic inflammation and pain. The first signs of DDD commonly manifest as reduced expression/accumulation of aggrecan and collagen type II in the NP, as well as an increase in pro-inflammatory cytokines (e.g. IL-1β, TNF-α) and ECM-degrading enzymes, namely matrix metalloproteinases (MMPs) and A Disintegrin And Metalloproteinase with ThromboSpondin motifs (ADAMTSs) (Wuertz and Haglund, 2013). The microenvironment of the degenerated NP is characterized by low levels of oxygen and glucose, acidic pH, high osmolarity, and complex non-physiological mechanical stress (Gantenbein et al., 2015; Vedicherla and Buckley, 2017), causing a catabolic shift (Johnson et al., 2015; Krupkova et al., 2016). Non-physiological loading and catabolism further reduce ECM turnover, leading to the development of microdamage, clefts, and tears in the AF (Urban et al., 2004). These changes are accompanied by the sensitization of sensory nerves by released nociceptive molecules and/or direct nerve damage, e.g. due to herniation or IVD space narrowing (Peng et al., 2005; Peng, 2013; Wuertz and Haglund, 2013). Furthermore, pro-inflammatory cytokines upregulate the expression of nerve growth factor (NGF), vascular endothelial growth factor (VEGF) and enhance the loss of PGs, which together allow for nerve and blood vessel ingrowth deeper into the IVD (Freemont et al., 2002; Binch et al., 2015). The ingrowth of newly formed nerves and vessels into the IVD was shown to aggravate LBP and to occur in patients with more severe symptoms (Freemont et al., 2001; Aoki et al., 2014a). IVD degeneration is also associated with CEPs becoming sclerotic, losing contact with the vertebral vasculature and exhibiting decreased permeability (Crock and Goldwasser, 1984). This process is considered to contribute to DDD progression by reducing the diffusion of nutrients to the cells of the NP (Ariga et al., 2001), but the correlation between CEPs and NP/AF degeneration was not completely clarified (Grignon et al., 2000).

Discogenic LBP is currently treated symptomatically by physiotherapy and pain medications. In a subset of individuals, these treatments fail and surgery is required. A surgery entails the risk of adverse effects, slow recovery, and high rates of reoccurrence (Henschke et al., 2008; Hoy et al., 2010). Notably, spine fusion (the surgical standard of care for DDD) fails to improve pain and quality of life in 20–30% of patients for various reasons (e.g. adjacent segment disease, implant instability) (Chou et al., 2002; Wei et al., 2013; Kenneth and Pettine, 2019). New approaches to treat DDD include disease-modifying molecular and cellular therapies (Vedicherla and Buckley, 2017; Smith et al., 2018). However, the therapeutic development is hindered by a poor understanding of mechanotransduction mechanisms in IVD degeneration, as well as by a lack of *in vitro* high-throughput drug testing platforms integrating relevant mechanical loading (Smith et al., 2018). In regenerative cell-based approaches, therapeutic cells are expected to support IVD regeneration by differentiating into IVD-like cells and/or by secreting trophic and anti-inflammatory factors to ultimately repair the IVD (Fontana et al., 2015; Sakai and Andersson, 2015; Wang et al., 2015; Meisel et al., 2019; Schol and Sakai, 2019). However, cells often fail to survive in the harsh IVD microenvironment and to adapt to the specific (often non-physiological) mechanical loading typically present in the degenerated IVD. There is therefore a high demand for developing new regenerative strategies compatible with (non-)physiological IVD mechanical loading and new models that recapitulate the altered mechanical environment *in vitro*.

3 MECHANOBIOLOGY OF THE INTERVERTEBRAL DISC

The IVD is commonly exposed to several types of mechanical loading including compression, tension, shear, torsion and their combinations. Spinal loading and PG content play major roles in the mechanobiological responses of the IVD. A schematization of the IVD loading status upon compression and flexion is reported in **Figure 1C.** The IVD experiences a diurnal change in intradiscal pressure according to the variation in day and night activity (Chan et al., 2011a). The majority of fluids in the IVD are absorbed by the negatively charged PGs in the NP that swell and provide compressive resistance. PG fixed charges are electrically balanced by cations in the interstitial fluid, mainly potassium and sodium. Upon application of a mechanical load, the NP loses water (but not ions), while the removal of the applied load causes rapid rehydration due to the osmotic gradient in the NP (Cramer and Darby, 1997; Urban and Roberts, 2003; Galbusera et al., 2014). The increased intercellular osmolality during loading draws water out of the cells, reducing the cell

volume. Mechanical loading thus alters the physical environment of the IVD by causing changes in the water content and the chemical composition of the ECM and the cells (Chan et al., 2011a; Sadowska et al., 2018).

Mechanical stimuli elicit cellular responses in the IVD that depend on the magnitude, frequency, and duration of the loading (Sowa et al., 2011a). Spinal loading causes physiological dynamic compression of the IVD with a frequency between 0.2 and 1 Hz and diurnal variations in magnitude between 0.2 and 0.6 MPa (Chan et al., 2011b). Axial compression and swelling effects in the NP generate bulging and deformation of the AF, resulting in radial and circumferential tension with physiological strains up to 5.5% (Showalter et al., 2016). In general, a physiological level of mechanical loading is beneficial for IVD homeostasis, as it promotes solute transport and cell metabolism (Chan et al., 2011a). However, hyper-physiological mechanical stressors, caused for instance by impact, heavy weight lifting, altered muscle activations, and work/lifestyle factors (e.g. vibration exposure, gait, and posture) contribute to cell death, catabolism, and inflammation leading to IVD degeneration (Chan et al., 2011a; Fearing et al., 2018). Loading in the NP and in the AF changes also during DDD, as quantified in the early nineties by McNally and Adams (1992a) and reported in **Figure 1D**.

Most studies focusing on the role of mechanical loading of the IVD have investigated **c**ompressive stimuli. Static loading was shown to induce detrimental changes including downregulation of ECM genes, protease activation, and cell death both *in vitro* and *in vivo* (Ohshima et al., 1995; Iatridis et al., 1999; Lotz and Chin, 2000; Chen et al., 2004), supposedly via inhibition of nutrient transport and gas exchange (Fearing et al., 2018). Physiological dynamic loading (with a magnitude between 0.2 and 1 MPa) was reported to elicit anabolic responses with the promotion of cell metabolism and maintenance of ECM synthesis depending on magnitude and frequency (Maclean et al., 2004; Neidlinger-Wilke et al., 2005; Korecki et al., 2008). On the contrary, hyper-physiological dynamic compression at high magnitude (>1 MPa), low or high frequency (<0.1 Hz or >1 Hz), or long duration (>8 h per day) causes cell death (Walsh and Lotz, 2004; Wang et al., 2007; Illien-Jünger et al., 2010), decreased expression of anabolic genes as well as increased expression of MMPs, ADAMTS, and pro-inflammatory cytokines (MacLean et al., 2003; Ching et al., 2004; MacLean et al., 2005; Korecki et al., 2009).

Similar patterns were observed in tensile stress studies. Isolated AF cells stretched at low magnitude (1%) and physiological frequency (1 Hz) were shown to maintain proteoglycan production (Rannou et al., 2003). Another study found that low magnitudes (3 and 6%) and frequencies (0.1 and 0.5 Hz) of tensile strain downregulate catabolic mediators, while this effect is lost with higher magnitude (18%), frequency (1 Hz) and prolonged duration (24 versus 4 h) (Sowa et al., 2011b). Outside of the physiological window, stretching can even provoke detrimental biological responses. Cyclic stretching of IVD (mostly AF) cells at a high strain of 8–20% and at either hypo-physiological (0.001 and 0.01 Hz) or physiological frequencies (0.1–1 Hz) was shown to induce downregulation of anabolic markers (aggrecan, collagen II) (Wang et al., 2018) and upregulation of catabolic (MMP 1, 3, 9, 13, ADAMTS 4, 5) (Sowa et al., 2011a; Wang et al., 2018) and pro-inflammatory mediators (cyclooxygenase-2 (COX2), prostaglandin E_2 (PGE2), interleukins (IL) 1β, 6, 8, 15, toll-like receptors (TLR) 2, 4, NGF, tumur necrosis factor alpha (TNF-α), monocyte chemoattractant protein (MCP) 1, 3, and monokine induced by gamma interferon (MIG)) (Miyamoto et al., 2006; Gawri et al., 2014; Pratsinis et al., 2016; Wang et al., 2018).

3.1 Mechanosensing and Mechanotransduction

Mechanosensing is defined as the process by which cells detect mechanical signals, while mechanotransduction is the process used by cells to convert mechanical signals into biochemical responses. While many studies have reported biological responses to mechanical signals in the IVD, the investigation and knowledge of the underlying mechanosensing and mechanotransduction mechanisms are limited. The response of cells to mechanical loading depends on cell morphology, cell-cell interactions, and cell-ECM interactions (Fearing et al., 2018). Mechanosensing occurs via surface receptors that activate intracellular mechanotransduction signaling pathways (Fearing et al., 2018).

Integrins are transmembrane heterodimers composed of α and β subunits that can bind specific ECM ligands depending on their subunits (Fearing et al., 2018). NP cells express the integrin subunits α1, α2, α3, α5, α6, αv, β1, β3, β5, β6, and β8, while AF cells express α1, α5, αv, β1, β3, β5, and β6 (Nettles et al., 2004). There is currently little information on mechanotransduction pathways through integrin binding. Interestingly, integrin-mediated mechanotransduction was shown to be altered in degenerated IVD cells compared to healthy controls (Le Maitre et al., 2009).

Integrins are part of focal adhesions, which connect the ECM to the cytoskeleton. In NP cells, F-actin is expressed as short dispersed filaments mainly at the periphery of the cell, while AF cells display organized fibers throughout the cytoplasm, especially in the outer AF (Fearing et al., 2018). Outer AF cells also express higher levels of β-actin compared to NP cells (Li et al., 2008). On the contrary, NP cells have a higher expression of tubulin compared to outer AF cells (Li et al., 2008; Fearing et al., 2018). Cytoskeletal reorganization is one of the mechanisms by which IVD cells respond to mechanical signals. It was shown that IVD cells stretched in silicone chambers realign perpendicular to the direction of stretching (Abbott et al., 2012) and express more actin filaments compared to controls (Li et al., 2011). Actin formation and organization are mediated by the RhoA/Rho-associated kinase (ROCK) signaling pathway (Amano et al., 2010). Interestingly, ROCK inhibition abolished cell-cell interactions and the formation of clusters in NP cells (Hwang et al., 2014). Cytoskeletal regulation of NP and AF cells has further been associated with the yes-associated protein (YAP) and transcriptional coactivator PDZ-binding motif (TAZ) signaling (Fearing et al., 2019; Wang et al., 2021). Cytoskeletal reorganization mediated by F-actin remodeling occurs also in

response to hypo- and hyper-osmolarity resulting in cell volume changes (Fearing et al., 2018).

Mechanical stress or cell volume change caused by osmotic stress provokes conformational deformations of the cellular membrane that might open mechanosensitive ion channels (Liu and Montell, 2015; Fearing et al., 2018). Candidate ion channels that are expressed differentially between NP and AF cells were identified via proteomic analysis and included sodium, potassium, and calcium channels such as the recently investigated transient receptor potential (TRP) channels. TRP channels are non-selective calcium-permeable transmembrane channels that can be activated by different stimuli, including changes in temperature, pH, osmolarity, as well as oxidative and mechanical stress, either directly by mechanical forces applied to the cell membrane or indirectly via multistep signaling cascades that induce conformational changes, which in turn generate mechanical force on the cell membrane (Walter et al., 2016; Krupkova et al., 2017; Franco-Obregón et al., 2018; Kameda et al., 2019; Sadowska et al., 2019). The TRP vanilloid 4 (TRPV4) ion channel was recently identified as a mediator of stretch-induced inflammation and compression-induced cell damage and degeneration in IVD cells and tissues in the context of hyper-physiological mechanical loading (Cambria et al., 2020a; Cambria et al., 2021).

Another aspect to consider in IVD mechanobiology is its cross-talk with inflammation. During IVD degeneration, inflammation was shown to disrupt the F-actin network and osmotic stress-induced cell volume change in NP cells (Maidhof et al., 2014). Notably, inhibiting actomyosin contractility (with a myosin II inhibitor) mimicked the effects of inflammation on cell biomechanical properties, while increasing actomyosin contractility (using a RhoA activator) protected against the mechanobiological effects of inflammation in NP cells (Hernandez et al., 2020). Furthermore, actomyosin contractility was shown to regulate the nuclear translocation of the pro-inflammatory mediator nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in response to TNFα, controlling the downstream catabolic effects of this pathway (Hernandez et al., 2020). While gaps in IVD mechanotransduction knowledge still exist, these recent findings highlighted that protecting cell mechanobiological integrity is vital for the development of new approaches to prevent or reverse IVD degeneration (Hernandez et al., 2020).

4 ORGANS-ON-CHIP MIMICKING THE INTERVERTEBRAL DISC

Understanding IVD's functional anatomy, mechanobiology, and the changes occurring during DDD progression is instrumental in delineating the conditions to be replicated in OoC systems. The goal of OoCs is defined as "not to build a whole living organ but rather to synthetize minimal functional units that recapitulate tissue- and organ-level functions" (Bhatia and Ingber, 2014). The formulation of a clear experimental hypothesis is therefore paramount in adequately designing and exploiting this technology. Recently, the first attempts at developing microphysiological IVD models emerged in the OoC field. Here we present them grouping the proposed devices in 1) cytokine-based DDD models, 2) IVD-on-a-chip devices providing physical stimuli, and 3) devices that, initially designed to model mechanotransduction in other tissues, could be adopted for IVD studies.

4.1 Cytokine-Based IVD-on-a-Chip Models

While DDD-related pain may be caused by nerve root compression as a result of IVD protrusion, LBP also occurs in patients without nerve compression in MRI images (Loibl et al., 2019). This, not yet completely understood phenomenon, is possibly related to matrix alarmins and cytokine-mediated irritation of the dorsal root ganglia (DRG) pain channels and nerve endings located in the IVD (García-Cosamalón et al., 2010). Pro-inflammatory cytokines released by IVD cells and infiltrating macrophages promote the continuous breakdown of ECM components enabling the invasion of endothelial cells (ECs) and neurons into deeper IVD regions (García-Cosamalón et al., 2010). However, the effects of molecular gradients of inflammatory mediators, metabolic waste products or trophic factors on IVD pathophysiology are still not sufficiently explored. Hwang et al. (2017) developed a microfluidic device able to 1) generate gradients of pro-inflammatory and macrophage soluble factors, and 2) co-culture three different cell types, e.g. neurons, endothelial cells, and NP/AF cells. The device layout is reported in **Figure 2A** (Hwang et al., 2017). The conceptual functioning principle of the device and the achievement of molecular gradients are reported in **Figures 2B,C**, respectively. Exposure to either IL-1β (0–1 ng/ml) or to macrophage secreted factors (generated by THP-1 cells upon their exposure to phorbol myristate acetate) resulted in human AF cells upregulating the expression of inflammatory mediators (i.e. IL-6 and IL-8), degradative enzymes, and tissue inhibitors of metalloproteinases (TIMPs). Through the proposed platform it was possible to recapitulate the dose-dependent catabolic responses of primary human AF cells including morphological and kinetic cell alterations commonly found in the degenerated IVD. This model represents the first step towards a basic understanding of gradient-based mechanisms in inflamed IVD cells (Hwang et al., 2017).

It is apparent that numerous cross-talks exist between the IVD and adjacent structures, but how different cell types influence each other during IVD homeostasis and degeneration remains relatively unknown. Despite the potential of the device designed by Hwang et al. (2017) NP cells, neurons and vascular components were not actually used in this study. Neither the native (3D) architecture of the cells nor ECM components were introduced and the culture time was limited to 72 h. Provided such missing features are integrated, this model could also be adopted to elucidate basic pain mechanisms (e.g. through the NGF or VEGF gradients) (Freemont et al., 2002; Aoki et al., 2014b).

More recently, an IVD co-culture OoC was developed to investigate chemotactic invasion and migration of AF/NP cells and ECs in conditions simulating an inflamed IVD (Hwang et al.,

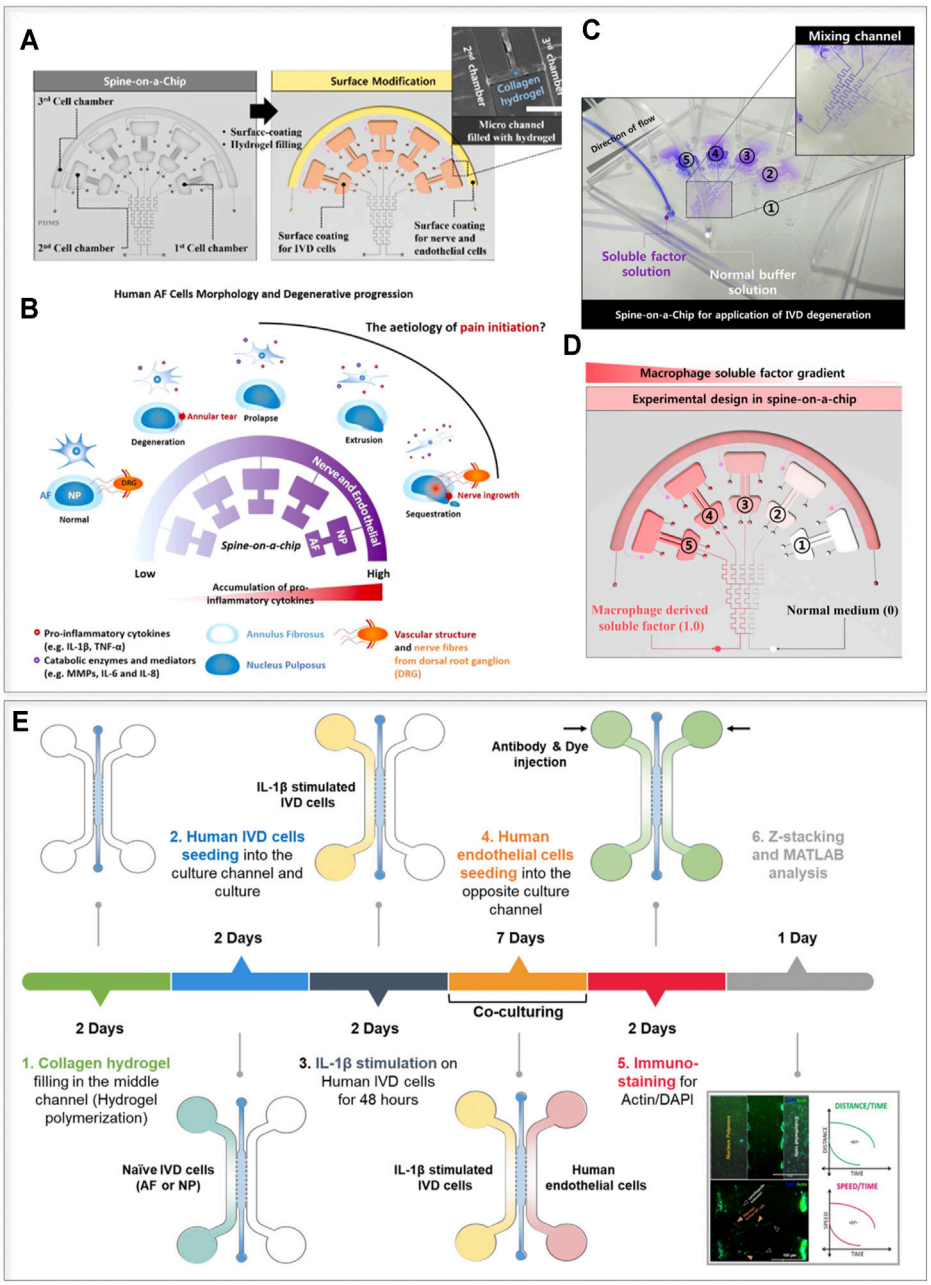

FIGURE 2 | Cytokine-based IVD-on-a-chip models. **(A–D)** The layout and the possible conceptual adoption of the device presented by Hwang et al. (2017). **(A)** The device is composed of three chambers designed to culture AF, NP, and nerve or endothelial cells (Chamber 1, 2, and 3 respectively), and also comprises a gradient generator. Chambers 1 and 2, and 3 were coated respectively with fibronectin and with poly-D-lysine to facilitate cellular adhesion; the small channels connecting Chambers 2 and 3 were filled with a collagen-based hydrogel. **(B)** Conceptual use of the device. Different disease states are mimicked in the model as the cytokine concentration in the chambers increases (as indicated by the purple color). **(C)** Functional validation of the model. The gradient generator allows a concentration gradient in different culture chambers. **(D)** Design of an experiment investigating the effects of a gradient of macrophage-derived factors on IVD cells behavior. **(E)** The device and the experimental timeline of the study performed by Hwang et al. (2020). The authors used a simple device characterized by three channels separated by pillars. The central channel was filled with a collagen hydrogel while the lateral channels were filled with IVD (AF or NP) cells and human endothelial cells respectively. IVD cells were exposed to IL-1β and the effect of the stimulus on IVD cells and on the endothelial compartment in terms of cellular migration was studied. Images **(A–D)** adapted and reprinted from Hwang et al. (2017), **(E)** from Hwang et al. (2020), with publisher (API Publishing) permissions and based on http://creativecommons.org/licenses/by/4.0/, respectively.

2020). The device, schematized in **Figure 2E** together with the adopted experimental timeline, is composed of three distinct chambers separated by two rows of posts. The central chamber was filled with an (initially) acellular collagen hydrogel, while lateral chambers were seeded, respectively, with human, primary, naive or inflamed AF/NP cells, each

cellular population in 2D on one side of the hydrogel. The authors demonstrated that AF/NP cells interact with ECs (immortalized human microvascular endothelial cells, HMEC-1) also suggesting a possible time line: AF cells respond to IL-1β early during the DDD course (resulting in an increased expression of IL6, -8, MMP1, 3 and VEGF family members), while NP cells interact with ECs by responding with higher levels than AF to ECs secreted factors (e.g. production of IL-6 and -8, VEGF, and MMP-3 was significantly higher in NP cells than in AF cells, under the presence of ECs conditioned medium). While the study gave a first insight into intracellular communications in IVD degeneration, paracrine signaling mechanisms involved in cell communication and migration were not characterized (Hwang et al., 2020). More advanced co-culture OoCs could reveal mechanisms modulating the recruitment of non-IVD cells (immune, endothelial, and neuronal cells) and enable precise targeting of pain mechanisms related to disease progression. Moreover, NP cells were still cultured in 2D contrarily to the physiological condition thus possibly disregarding the effect of ECM components (e.g. PGs) in determining cellular migration capacity and tissue chemical permeation. Notably, already existing OoC platforms could be exploited to culture AF/NP cells and also ECs in 3D to better investigate such mechanisms (Huang et al., 2009). Finally, it is worth mentioning that the adopted IL-1β concentrations (i.e. 10 ng/ml) were employed with the aim of obtaining a downstream effect rather than to reflect inflammation *in vivo* (Burke et al., 2002; Andrade et al., 2011; Altun, 2016).

4.2 IVD-on-a-Chip Models Providing Physical Stimuli

Incorporating controlled physical stimuli *in vitro* is essential to mimic human IVD pathophysiology. A key advantage of OoC, as compared to macroscale models, is the possibility of thoroughly controlling fluid flow conditions and parameters. The limited dimension of the channels assures a high laminarity of the fluid flow (the Reynolds number in microfluidic channels can be as low as 1), leading to a higher control of the phenomenon and an easier prediction of experimental conditions. The enhanced control over the fluid flow and consequently over the concentration of solutes and metabolites is also applicable in maintaining *ex vivo* explants, provided their dimensions are compatible with a microscale setup. On this regard, Dai et al. (2019) generated a microfluidic disc-on-a-chip characterized by continuous medium flow designed to accommodate whole mouse lumbar IVDs (8–10 weeks old). The introduction of perfusion chambers allowed an adequate flow exposure and fluid exchange, which improved cell viability and structural integrity in both NP and AF up to 21 days of culture, compared to static controls (Dai et al., 2019). A schematization of the device proposed by Dai et al. (2019) is reported in **Figure 3A**. Four perfusion units, each connected to a syringe (and a pump), were incorporated into the device. Each perfusion unit can host three IVDs thus increasing the experimental throughput. Notably, flow-induced shear stresses have been correlated to an altered response of IVDs in culture (Elfervig et al., 2001; Xia et al., 2015; Chou et al., 2016). Each perfusion unit was dimensioned to assure an adequate nutrient and metabolites exchange while minimizing shear stresses acting on the discs introducing a pressure dropping array of squared pillars at the inlet. Benefits of the constant chemical concentrations as reported by the authors (depicted in **Figure 3B**) include preserved cell viability, IVD structural integrity (i.e. maintenance of alignment and organization of AF lamellae and NP glycosaminoglycan (GAG) content), and conserved low expression levels of ADAMTS4, MMP13, TNF-α and IL-6, otherwise increased in static culture. While mouse IVDs might not be ideal for translational research and drug testing due to major structural and functional differences from human IVDs (Alini et al., 2008; Jin et al., 2018), they could be very useful to uncover the genetic basis of IVD degeneration and aging. As an example, using IVDs from excision repair cross complementation group 1 (ERCC1)-deficient mice provides the context of an aged tissue (Vo et al., 2013). This compact long-term microfluidic organ culture could consequently aid in advancing research on chronic IVD degeneration, mainly if dynamic loading was included in the model (Dai et al., 2019).

Many studies have shown that physiological compression and stretching promote IVD-like ECM formation. However, the effects of shear stress in the IVD are in general less explored. A preliminary assessment of the direct effect of shear stresses on human AF cells in a microfluidic setting was provided by Chou et al. (2016) (**Figure 3C**). The final platform was obtained through two culture passages. First, a polymethylmethacrylate (PMMA) pre-culture chamber (top part of **Figure 3C**) was connected to a fibronectin-coated substrate through double tape. After AF cells adhered to the substrate, this was detached from the culture chamber and assembled to the five-layer device (bottom part of **Figure 3C**). An inlet and an outlet were used for fluid flow while a vacuum inlet was used to apply a negative pressure to unite the culture substrate with the rest of the device. Shear stresses (1 and 10 dyne/cm^2, ~1 and 10 ml/h respectively) were generated in the cell culture chamber of the five-layer microfluidic device by a continuous flow of culture medium injected via a syringe pump. Shear stress distinctly influenced gene expression in AF cells, specifically collagen type I and MMP 1, in a value-dependent manner. The presented study was a first assessment of how AF cells respond to controlled shear. Cells were however seeded in 2D and stimulated with flow rates lower than those associated to physiological motions. Incorporating native 3D architecture could lead to substantial improvements in understanding molecular mechanisms of shear stress/interstitial fluid flow in the pathophysiology of IVD degeneration (Chou et al., 2016). Furthermore, AF cells from patients with IVD-related pathologies might show altered cellular responses, and therefore healthy controls or alternative cellular sources (e.g. derived from iPSCs) should be considered. On a technical note, the usage of vacuum to seal the device is acceptable for short experiments (e.g. a 4 h stimulation as used by the authors) but would not be sustainable for longer culture periods.

These two models provide a proof of concept of the potential of OoC platforms capable of physical stimulation for the study of IVD physiopathology. IVDs *in vivo* are however subjected to a complex strain field constituted by both compression (mainly in

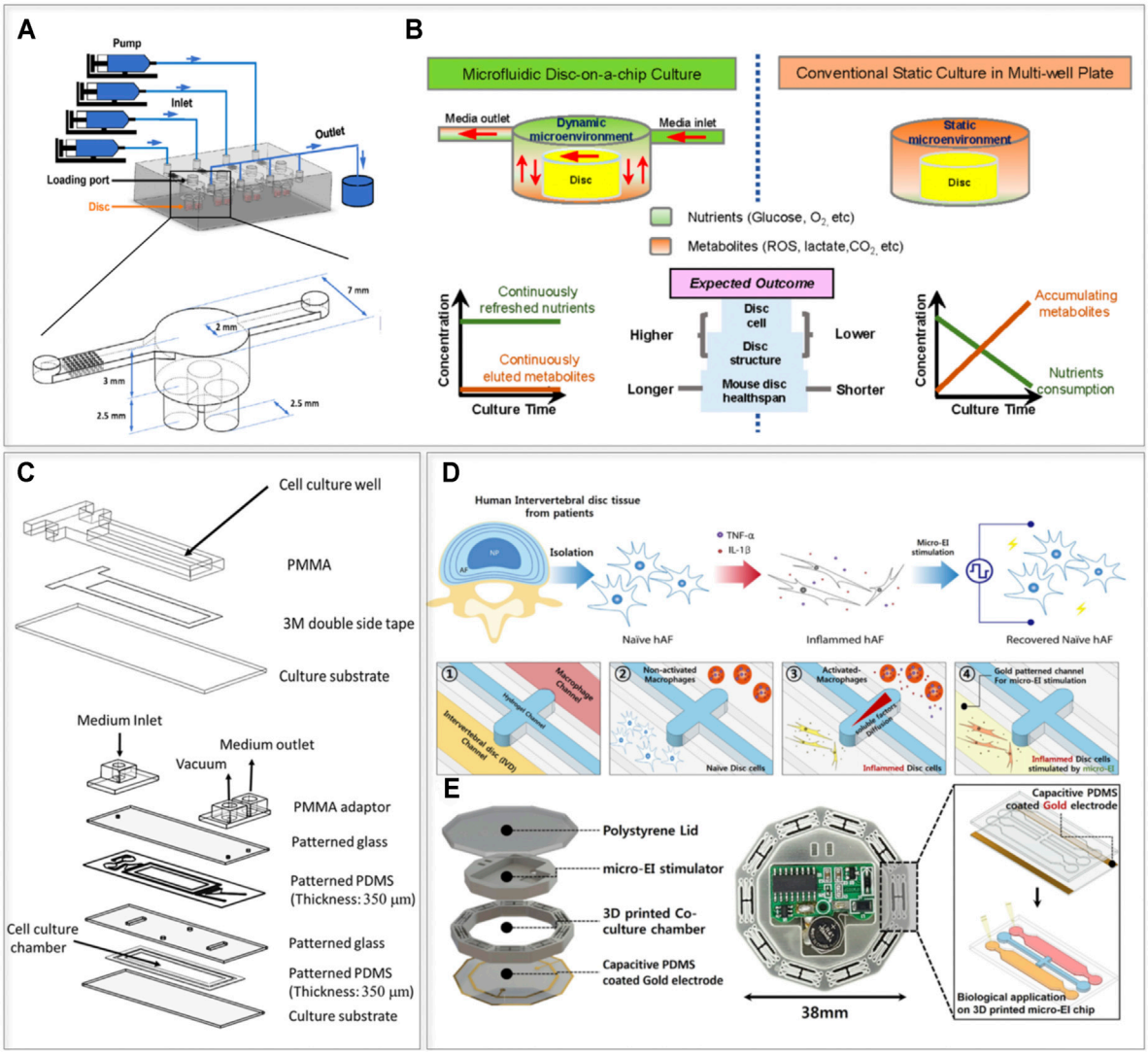

FIGURE 3 | IVD devices providing physical stimuli. **(A,B)** Layout and the conceptual usage of the mouse disc-on-a-chip with controlled flow proposed by Dai et al., 2019). **(A)** The device is composed of four identical chambers, with three IVDs located in each chamber. A matrix of micropillars at the inlet of each chamber reduces the shear flow to which the whole mouse IVDs are exposed. **(B)** The system allows to keep constant levels of nutrients and metabolites resulting in the higher IVD culture times. **(C)** Layout of the shear stress device proposed by Chou et al. (2016). The top part shows the pre-culture chamber, while the bottom part shows the complete PDMS device. **(D,E)** The electrical stimulation device proposed by Shin et al. (2019). **(D)** Description of the experimental procedure from cellular extraction and stimulation to the compartments of the device. **(E)** Depiction of the different layers composing the electrically active device. Images **(A,B)** adapted from Xing et al. (2019) (Dai et al., 2019), **(C)** from Chou et al. (2016), **(D,E)** from Shin et al. (2019), reprinted with publisher (ACS Publications) permissions and based on http://creativecommons.org/licenses/by/4.

the NP) and stretching (mainly in the AF) of 3D structures. A better recapitulation of IVD physiology will therefore be necessary for the establishment of clinically relevant models.

Several OoC platforms include electrical sensors and/or stimulation systems (Maoz et al., 2017; Zhang et al., 2018b; van de Wijdeven et al., 2019). While IVD cells are not excitable by definition, different authors reported a modulatory effect of electrical stimulation (ES) on the expression of degradation markers and a beneficial ES effect on wound healing and inflammation responses (Zhao et al., 2006; Pavesi et al., 2016; Kim et al., 2009). A first attempt to include ES into an IVD microdevice, to recapitulate previously described effects of electrical stimulation on IVD cells (Miller et al., 2016; Wang et al., 2017; Kim et al., 2013), was made by Shin et al. (2019) (**Figures 3D,E**). The device hosted nine culture chambers. Each was constituted by two compartments where primary human AF cells and macrophages (i.e. TPA-activated THP-1 cells) were seeded in 2D and connected through a collagen I hydrogel filled compartment. A micro electrical impulse stimulator lodged in the middle of the device provided cells with ES at different frequencies (i.e. 100, 200, and 300 Hz) leading to beneficial effects on inflammatory mediators (TNF-

α, IL-1β, IL-6, and IL-8) end ECM-degrading enzymes (e.g. MMP1). Similar to previously mentioned devices, this platform is limited by the brief culture time and the 2D configuration of AF cells. Such a microdevice could however be used in preliminary studies to investigate the therapeutic modulation of bioelectrical signaling (McCaughey et al., 2016), for instance in relation to the peripheral nervous system.

4.3 OoC Systems With Mechanical Loading Regimes Suitable for IVD Research

The discrepancy between *in vitro* cell monolayers and native 3D tissue structures results in many cases in altered phenotype, cell morphology and behavior. This renders results from 2D cell-based assays questionable, also in microfluidic based OoC platforms. By cultivating cells in hydrogels, scaffolds, or aggregates, it is possible to implement 3D cell culture systems that allow for an indirect mechanical stimulation. Varying the rigidity and stiffness of the ECM has been indeed shown to modulate cellular behaviors (Ergir et al., 2018). However, both IVD homeostasis and degeneration depend on the complex mechanical stimuli to which the spine is subjected, emphasizing the need for models that directly transmit key dynamic mechanical stimuli. While multiple macroscale bioreactors were designed to study the effect of mechanics, no report of mechanically active IVD OoC platforms is available. Recent developments in OoCs applied to other research fields (Rothbauer et al., 2021) could pave the way for IVD modelling towards more complex systems capable of a fine tuning of the biomechanical cellular stimuli. In this section we describe OoC mechanical devices that, although designed for different biological applications, could be adopted for the investigation of 3D NP and AF microconstructs under loading.

To date, only a few microdevices capable of recreating stretching and/or compression in 3D have been developed (Ergir et al., 2018). Marsano *et al.* (2016) proposed a miniaturized device designed to provide 3D murine and human cardiac cell constructs with controlled and tunable levels of mechanical strain (i.e. monoaxial stretching), which were initially applied to both healthy and pathological cardiac models (Marsano et al., 2016; Occhetta et al., 2018; Visone et al., 2021). A variation of the same device was recently applied in the cartilage field, showing how hyper-physiological confined compression of primary human articular chondrocytes cultured in 3D in a microdevice was sufficient to induce osteoarthritic traits (Occhetta et al., 2019). Specifically, the application of strain-controlled confined 30% compression triggered features of catabolism, inflammation and hypertrophy in the microtissue, similar to those found in clinical osteoarthritis (Occhetta et al., 2019).

The same principle was recently incorporated in a multi-chamber mechanically active OoC device with an increased throughput (Mainardi et al., 2021). The device (**Figure 4A**) is composed of a top cell culture chamber and a compression chamber divided by a thin PDMS membrane. The culture chamber is constituted by a central hydrogel channel divided by two rows of overhanging pillars from lateral culture medium channels, with a gap between the bottom surface of the pillars and the flexible membrane. By applying a positive pressure to the actuation chamber, the membrane bends upwards compressing the 3D hydrogel. The compression level depends exclusively on the relative height of pillars and gap. The posts serve therefore the double function of 1) confining the 3D cell constructs and 2) defining a stroke length controlling a mechanical actuation mechanism. Tailoring the pillars geometrical section makes it possible to achieve a confined compression (e.g. through a T shape, **Figure 4B**) or monoaxial lateral stretching of the cellular construct (e.g. through widely spaced posts with a narrow hexagonal section, **Figure 4C**). Featuring both a 3D environment and mechanical stimulation, these devices could be used as a basis to study AF and NP cell responses to loading. Modulating the loading entity, it would be feasible to verify if these cellular populations respond differently to physiological and/or pathological strain levels.

Most of the mechanically active OoCs in literature (Ergir et al., 2018) focus on uniaxial loading while IVDs are subjected to complex strain fields due to spine flexion and torsion. Gizzi et al. (2017) introduced a microfluidic chip with multi-axial loading capabilities. The device is composed of a central porous membrane coupled with four vacuum chambers and perfused through perfusion channels. These features are evidenced in purple in **Figure 4D**, together with a schematization of the two layers used to close the device and a picture of a PDMS physical platform. While the authors did not report a specific biological application, they demonstrated how, by selectively applying vacuum to the chambers, the device could subject the porous membrane to specific strain fields (i.e. uniaxial, equibiaxial, and biaxial strains). This could be useful in the replication of the complex deformations to which AF fibers are subjected *in vivo*. The displacement field, the strain map, and the von Mises stress obtained on the porous membrane for uniaxial loading, equibiaxial loading and biaxial loading are reported in **Figure 4E.** Notably, the authors adopted a computationally informed design procedure to optimize the achievable strain field. A similar approach could be introduced to replicate the stimulation patterns characterizing IVDs. The main developmental steps towards such mechanically loaded IVD OoCs should thus include device design and *in silico* mechanical characterization, validation of cellular mechanotransduction mechanisms, and subsequent recapitulation of physiological and non-physiological IVD phenotypes upon loading.

5 PERSPECTIVE ON DESIGN CRITERIA FOR IVD-ON-A-CHIP

In the last decade, OoCs have been proven able to recapitulate relevant organs and tissues functionalities *in vitro* by providing native-like biochemical and biophysical cues to 3D cell (co)-culture within biomimetic microarchitectures. Among others, the interest around mechanically active OoC is growing with their ability to recapitulate mechanical stimuli that modulate physicochemical cell and tissue responses.

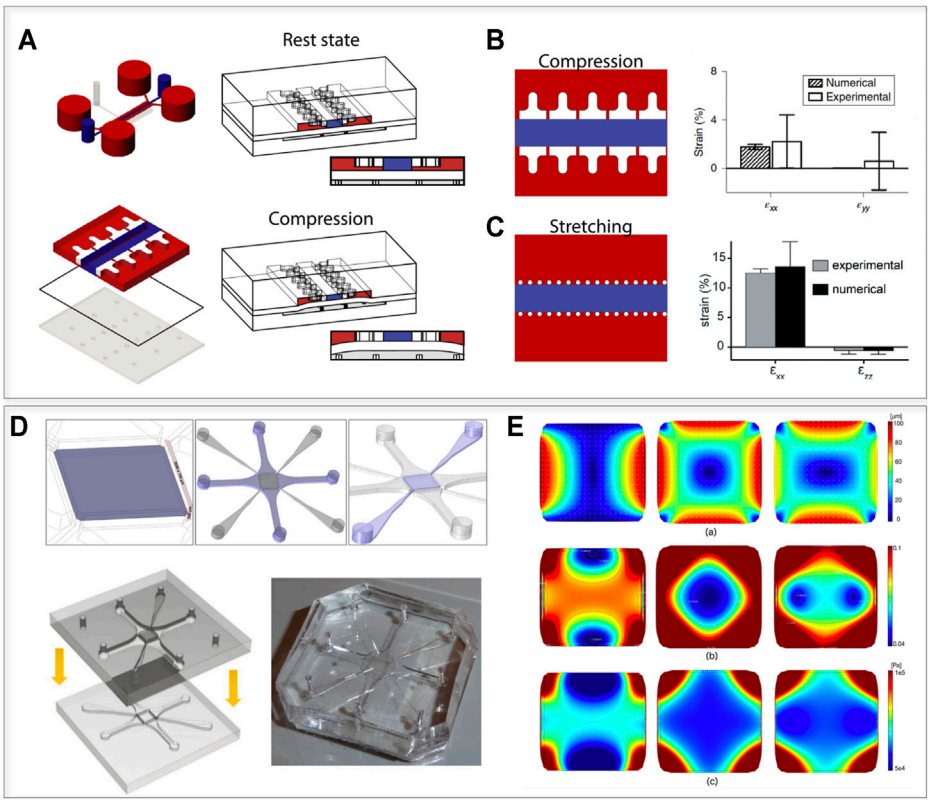

FIGURE 4 | Possible technological transfer, introducing mechanics to IVD-on-chip models. **(A,B)** devices that allow subjection of 3D constructs to defined levels of confined compression or stretching, introduced by Occhetta et al. (2019) and Marsano et al. (2016). **(A)** Device layout and functioning principle. The device contains two chambers (a culture camber and an actuation chamber), divided by a flexible membrane. When a positive pressure is applied to the actuation chamber, the membrane bends upwards until it reaches the mechanical stop provided by two rows of overhanging pillars in the culture chamber. Regulating the distance between the pillars and the membrane, it is possible to apply a defined compression or stretching level. **(B)** Compression device and experimental evaluation of the lateral expansion of the device proposed by Occhetta et al. (2019). The T shaped pillars limit the lateral expansion upon compression resulting in an almost ideal confined compression state. **(C)** Stretching device and experimental evaluation of the lateral expansion of the device introduced by Marsano et al. (2016). Using hexagonal pillars with a wider spacing in between them upon compression the hydrogel in the central chamber expands laterally providing laden cells with a 10% stretching level. **(D,E)** The device layout and the displacement field of the device proposed by Gizzi et al. (2017) allowing complex displacements stimulation states of a porous membrane. **(D)** A central porous membrane is connected by four pneumatic chambers (that can be actuated electively to produce complex strain fields) and to four perfusion channels (highlighted in purple from left to right). The final device is obtained by bonding of two halves. **(E)** Evaluation of the strain field: (E.a) Displacement field induced on the porous membrane (PM) under uniaxial (left), equibiaxial (center) and biaxial 3:5 (right) loading patterns for a maximum pressure $p = -500$ mbar. (E.b) Color map and isolevel contours of the first invariant of deformation for the corresponding loading patterns. (E.c) Color map of the von Mises stress distribution for the three loading patterns. Images **(A–C)** adapted from Occhetta et al. (2019), **(D,E)** from Gizzi et al. (2017), reprinted with publisher (Springer Nature) permissions and based on http://creativecommons.org/licenses/by/4.

OoCs are an ideal tool to capture mechanobiological interactions, with the capacity of 1) providing a fine control over spatiotemporal organization of *in vivo*-like tissue architecture, 2) precisely controlling the magnitude, duration, and frequency of the biomechanical stimuli, and 3) monitoring in real time the effects of applied mechanical forces on cell, tissue, and organ functions [84]. In order to investigate load-associated mechanisms in the human IVD, an ideal microscale device should arguably be capable of recapitulating the IVD anatomy/physiology and, at the same time, allow precise *in situ* analysis of at least the two major IVD cell types (NP and AF) experiencing specific physiological loading stimuli in 3D (e.g. compression and stretching). Here we discuss the features and the parameters that should be considered in a foreseeable IVD-on-a-chip which could be useful in IVD pathophysiology investigations.

It is necessary to note that OoCs are almost exclusively strain regulated (i.e. the parameter set by the user is the strain amount) and not load based, in contrast to macroscale bioreactors. Physiological variations and frequency can easily be obtained at the microscale but measured variations in IVD pressure (0.2–0.6 MPa) (Chan et al., 2011a; Chan et al., 2013; Rosenzweig et al., 2016) are difficult to achieve since actuation pressures in OoC are in the order of 10^{-2} MPa. Moreover, forces acting on the IVDs are orders of magnitude higher than those that can be replicated at the microscale and with the adoption of soft hydrogels as a substrate. It should therefore be kept in mind that while a stricter control on the mechanical stimuli is achievable, it is paramount to properly translate a macroscale stimulus into a microscale one. The ideal IVD-on-a-chip might therefore depend on the specific downward effect to be replicated or the parameter

to be evaluated. In patient-oriented research, an ideal IVD-on-a-chip could be designed aiming at replicating all conditions that characterize a DDD (e.g. altered compression levels, inflammation, low pH) to determine if a given therapeutic option, like the supplementation of cells with restorative capacities (Gryadunova et al., 2021), could withstand the cited conditions.

5.1 Device Concept

The device could be constructed taking inspiration from the described works of Hwang et al. (2020). Featuring compartments for the co-culture of different cellular populations, but adapting the designs that allow the 3D culture of different cellular populations (i.e. NP cells in the middle and AF cells in lateral compartments), together with channels for medium supplementation (Huang et al., 2009). With the use of pillars positioned in between the compartments and/or different actuation chambers, it could also be feasible to apply controlled mechanical stimuli differentiating between the NP (subjected to confined compression) and AF (subjected also to strain) and/or to apply complex stimuli (e.g. reminiscent of those experienced by IVDs during flexion). Effective device structures, layout, and dimensions will require a careful design procedure that factors in 1) the stimuli of the different compartments *in vivo*, 2) the necessity of an adequate supply of nutrients, but also the possibility of subjecting the compartments to DDD chemical stimuli and 3) an adequate tradeoff between complexity and usability.

The catabolic microenvironment in the degenerated IVD negatively influences cell survival and function, with mechanical loading being a possible aggravating (or conciliating) factor. While the AF and NP are the most evidently affected compartments, DDD also involves the recruiting of endothelial cells and nerves from the DRGs (Hwang et al., 2017). In an ideal IVD OoC, different cell types would be co-cultured in separate chambers connected directly or through microchannels to allow for paracrine signaling and diffusion of compounds. However, including compartments for other cells in the same device might further increase the operational complexity (and introduce possible confounding cross-talks). Herland et al. (2020) recently proposed a methodology to couple different OoC models in an effective and automated way. Cross cellular signaling between IVD cells and other compartments could therefore be achieved by fluidically coupling different devices.

Another aspect concerning the device layout regards the incorporation of on-chip biosensing capabilities (recently reviewed in (Ferrari et al., 2020)). Over the last years, a range of biosensing approaches embedded within microfabricated OoC systems have been reported including biosensors for monitoring cell growth and behavior, electrical and mechanical properties, and environmental parameters such as oxygen, pH, and metabolites. The integration of biosensors enables a continuous and non-invasive monitoring of microtissue evolution and dynamic measurements of cellular responses to diverse stimuli, thus providing detailed information about microtissue behavior at the molecular level (Ferrari et al., 2020). An integrated approach coupling a mechanical actuation compartment and biosensors on-chip would thus not only allow a precise control of the magnitude, duration, and frequency of the biomechanical stimuli but also the real-time monitoring of their effects on cell functions (Ergir et al., 2018). These advanced microphysiological IVD models could therefore be used to gain an in-depth understanding of the molecular mechanisms underlying load-induced IVD homeostasis and degeneration, and the role of mechanotransduction in regenerative feedback loops.

5.2 Biomaterials

Embedding cells in 3D OoC platforms often requires the injection of a cell-laden formulation while it is polymerizing. This procedure leads to the necessity of adopting hydrogels instead of solid scaffolds and demands an accurate evaluation of the gel usability (e.g. ease of handling, polymerization rate, speed) and biological relevance (e.g. analogy with the studied tissue ECM). The structure and composition of a biomaterial are critical for cell mechanosensitivity, as they influence how cells react to applied loads. For example, it is known that the cellular responses to substrate stiffness differ between AF and NP cells. AF cells seeded on a stiff substrate (in 2D) display an elongated morphology and distinct actin fibers, while they are round with less clear actin fibers on soft substrates [96]. NP cells tend to form clusters on soft substrates with cell-substrate and cell-cell interactions mediated by cadherins [96]. Concerning the IVD, hydrogels with non-physiological ECM porosity or non-fibrous matrices are not representative of the cell environment in the real tissue but they might be optimal in terms of handling and homogeneity. A possible circumvention of the obstacle could be the adoption of biodegradable materials that although not initially IVD-like, allow IVD cells to generate their own ECM as they are gradually degraded. This requires a preculture period for the achievement of a healthy IVD model before mechanical loading is applied and its effects evaluated (Occhetta et al., 2019). Hybrid materials combining the stability of synthetic matrixes with the binding motifs necessary for cell-matrix interactions and mechanotransduction signaling activation were already introduced (Ehrbar et al., 2007). Recently, an agarose-collagen hydrogel has been developed to mimic both the non-fibrillar (i.e. PGs) and fibrillar (i.e. collagen fibers) components of the IVD matrix (Cambria et al., 2020b). This composite biomaterial is suited for mechanotransduction studies as it combines the mechanical strength of agarose with the biofunctionality of collagen type I and could be a candidate for the study of loading effects in microphysiological settings.

5.3 Cellular Sources

Most of the cited IVD-related studies adopted either NP or AF cells from patients undergoing elective IVD surgeries. While the effect of different stimuli (e.g. shear stress or cytokine administration) could be determined in these studies, a more complete assessment of the mechanisms leading to DDD would require the application of a defined stimulus to healthy cells. However, the procurement of healthy IVD cells is limited by the low availability of suitable donors, yield, and proliferation of human primary IVD cells. Moreover, the presence of different cells types and spatiotemporal variations of IVD cell phenotype further complicate our understanding of IVD

biology (Pattappa et al., 2012). Young human NP (but not adult NP) were shown to contain notochordal cells, while adult NP, AF, and CEPs contain tissue-specific progenitors with enhanced regenerative properties (e.g. multipotent NP progenitor cells (NPPC) in the NP) (Pattappa et al., 2012; Sakai et al., 2012; Tekari et al., 2016). However, investigation of these cell types has been challenging due to their very low yield and the fact that their numbers might further decrease with age and degeneration (Sakai et al., 2012; Tekari et al., 2016). The scale reduction towards OoCs leads to a consistent decrease in the number of required cells with respect to classic 2D models and to macroscale bioreactors, increasing the number of experiments/conclusions that can be generated from a single healthy IVD biopsy (e.g from cadaveric donors) but also from other often limitedly available cellular sources. These include transfected or CRISPR-edited populations, whose number is reduced by gene editing itself and antibiotic selection (Krupkova et al., 2018). With their ability to differentiate into NP-like cells, induced pluripotent stem cells (iPSCs) could also be considered as a valid alternative adult cell source (Tang et al., 2018). A loaded IVD microtissue generated using cells derived from autologous iPSCs could possibly be employed in research on patient-specific DDD mechanisms and personalized drug development. IVD studies performed so far largely disregard the involvement of CEP chondrocytes in the DDD pathogenesis. The incorporation of CEP chondrocytes in OoC IVD models could give new insights into their crosstalks with NP/AF cells. Co-culture OoC IVD models could aid in revealing interactions between different cell types, uncover mechanisms responsible for regenerative functions of tissue-specific progenitors, and/or investigate strategies to enhance load-induced paracrine functions of specific IVD cell types.

6 CONCLUSION

OoCs are *in vitro* microscale models that by recapitulating the cell-cell and cell-ECM functional architecture, the tissue-tissue interfaces, and the physicochemical environment of human tissues and organs, produce levels of analogy not achievable with classic *in vitro* cultures (Bhatia and Ingber, 2014). These functionalities are made possible by the versatility and the ease of prototyping of microfabrication techniques that allow the introduction of gradient generators, multiple culture chambers, mechanical actuators, and biochemical sensors within coin sized devices. In the context of IVD in general or the DDD pathology specifically, OoC systems could thus be configured to study aspects of the disease such as the effect of different mechanical stimulation levels and the exposure to various concentrations of pro/anti-inflammatory factors.

In this review we briefly delineated the IVD anatomy and the mechanosensing and mechanotransduction mechanisms responsible for pathophysiological IVD responses, and described how different microscale *in vitro* models were used as instruments for IVD/DDD research. In particular, we reported devices that 1) permit the co-culture of different cells involved in the DDD pathogenesis (i.e. NP cells, AF cells, neurons and endothelial cells); 2) allow the long-term preservation of mice IVDs; and 3) expose IVD cells to physical stimuli such as shear stresses or electrical impulses. While various OoC models are available for other musculoskeletal tissues (for instance bone-on-chip models as reviewed by Mansoorifar et al. (2021)), the application of this technology to the IVD field is still relatively budding and progress will be needed to develop representative IVD models. New mechanically active OoCs can be designed, or existing OoCs developed for other purposes could be repurposed for the replication of IVD loading. Mechanically active OoCs will allow the determination of stimuli that elicit degeneration in specific cell types, and if a given compound (e.g. mechanotransduction inhibitors such as TRPV4 agonist (Cambria et al., 2020a)) can reduce this phenotype while the injurious mechanical stimulus persists.

Increasing evidence highlights the involvement of other spine components in DDD symptomatology, e.g. detrimental age-related biological changes in CEPs (Fields et al., 2018). A full recapitulation of the architecture of all three tissues is challenging at the microscale but fluidically connected chambers could be adopted, for instance to determine how CEP chondrocytes exposed to altered mechanical stimuli effect NP and AF cell phenotype. Similarly, recent advances involving the incorporation of neuronal cell components and gradients of pain signals into OoC devices could enable progress towards analyzing discogenic pain-on-a-chip.

Little is known about *in situ* interactions between therapeutic and resident IVD cells under loading. Mechanically simulated OoCs could facilitate the optimization of strategies to increase adaptation and integration of cell-based grafts upon implantation (Huang et al., 2014; Sakai and Grad, 2015) in an easily controllable environment. Numerous IVD studies made use of mechanically active macroscale bioreactors, which are more suitable to accommodate whole IVDs, where specific injury characteristics (e.g. annular tears) can be recapitulated *ex vivo*. The concept of OoC does not intent to recapitulate whole IVDs but rather exploits the high experimental parameters control for mechanistic cause-effect investigations.

Overall, numerous further advances are required to achieve a representative IVD-on-a-chip. With the aim of augmenting our understanding of the mechanisms that lead to painful IVD degeneration, OoCs allow a higher throughput and require minimal (in the range of microliters) volumes of solutes and cell numbers while still retaining the capacity to generate 3D microtissues with chemical and mechanical stimuli, and even integrated biosensors.

AUTHOR CONTRIBUTIONS

AMA, PO, and OK contributed to conception and design of the study. EC and OK wrote the first draft of the manuscript. AMA generated figures and tables and wrote sections of the manuscript. AB, IM, SS, OK, and AME provided funding and contributed to manuscript revision. All authors read, and approved the submitted version.

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REVIEW
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Understanding Reactive Oxygen Species in Bone Regeneration: A Glance at Potential Therapeutics and Bioengineering Applications

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Although the complex mechanism by which skeletal tissue heals has been well described, the role of reactive oxygen species (ROS) in skeletal tissue regeneration is less understood. It has been widely recognized that a high level of ROS is cytotoxic and inhibits normal cellular processes. However, with more recent discoveries, it is evident that ROS also play an important, positive role in skeletal tissue repair, specifically fracture healing. Thus, dampening ROS levels can potentially inhibit normal healing. On the same note, pathologically high levels of ROS cause a sharp decline in osteogenesis and promote nonunion in fracture repair. This delicate balance complicates the efforts of therapeutic and engineering approaches that aim to modulate ROS for improved tissue healing. The physiologic role of ROS is dependent on a multitude of factors, and it is important for future efforts to consider these complexities. This review first discusses how ROS influences vital signaling pathways involved in the fracture healing response, including how they affect angiogenesis and osteogenic differentiation. The latter half glances at the current approaches to control ROS for improved skeletal tissue healing, including medicinal approaches, cellular engineering, and enhanced tissue scaffolds. This review aims to provide a nuanced view of the effects of ROS on bone fracture healing which will inspire novel techniques to optimize the redox environment for skeletal tissue regeneration.

Keywords: reactive oxygen species, redox signaling, bone healing, skeletal biology, regenerative medicine, tissue engineering, biomaterials

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1 INTRODUCTION

The influx of skeletal biology research over the past 20 years has improved our understanding of how bones develop, remodel, and repair via very complex mechanisms that requires the interaction of cells from different lineages (General 2004; Hadjidakis and Androulakis 2006). These cells (including osteoclasts, osteoblasts, osteocytes, lymphoid cells, neurons, and vascular cells) each respond to a myriad of signaling cascades and external factors to regulate bone homeostasis and repair; thus, any dysfunction of this system can lead to bone pathology or suboptimal repair (Hadjidakis and Androulakis 2006; Jeon et al., 2016; Lopes et al., 2018). Reactive oxygen species (ROS) are increasingly being recognized as a key component of the bone repair paradigm. Are they "good", or are they "bad"? There is much debate over the role ROS play in the entire bone repair process, as some studies show they are necessary for bone repair and others say they are

detrimental to the process. This review provides a general understanding of how ROS interact and influence key regulators in the bone healing process. Given that there are limited studies specifically investigating the role of ROS in bone healing, this first section compiles the existing literature from *in vitro* and pre-clinical studies to suggest their potential role. The second half reviews the existing evidence for using supplements, drugs, and bioengineering techniques to harness (or dampen) ROS levels to improve fracture healing. For better therapeutics and bioengineering approaches to be developed, we need to understand how they influence the oxidative balance during skeletal tissue healing.

1.1 Bone Healing After Initial Injury

Typically, the first phase in injury healing involves the disruption of local blood vessels, resulting in inflammatory hematoma formation (Schlundt et al., 2015; Sivaraj and Adams 2016). Blood cells, resident bone macrophages (osteomacs), local mesenchymal stem cells (MSCs), and damaged endothelial cells all contribute to directing inflammatory signals to the damaged site (Lopes et al., 2018). These signals are released in a controlled manner to further recruit osteoprogenitor cells, inflammatory cells, and platelets to the area (Mountziaris and Mikos 2008; Lopes et al., 2018). Peak inflammation occurs 24 h after injury, and the entire inflammatory response is usually complete after 1 week (Mountziaris and Mikos 2008). MSCs respond to signals from the damaged area and are recruited to the site where they can begin the process of forming bone tissue. MSCs first cluster together in the hypoxic, avascular area (Percival and Richtsmeier 2013). In fact, it is believed this zone of slight hypoxia and avascularity is necessary for this first mesenchymal condensation to initiate (M. Yin and Pacifici 2001). These MSCs respond to a variety of signaling molecules, mechanical stresses, and oxygen tension to decide whether to differentiate towards osteoblasts or chondrocytes (Hadjidakis and Androulakis 2006; Percival and Richtsmeier 2013; Bahney et al., 2019). As an oversimplified explanation, the MSCs in the inner mass of the MSC condensation favor chondrogenesis, while MSCs in more close contact with inflammatory signals and blood supply favor osteogenesis (Percival and Richtsmeier 2013; Bahney et al., 2019). As the outer MSCs differentiate and the neovascularization encroaches on the hypertrophic chondrocytes, the entire mass is eventually ossified into woven bone (Claes et al., 2012; Bahney et al., 2019). The woven bone formed at the fracture site bridges the two fractured segments and is later remodeled to laminar bone (Hadjidakis and Androulakis 2006; Raggatt and Partridge 2010).

Much is known about how specific signaling cascades, inflammatory markers, the extracellular matrix (ECM) environment, and mechanical stress affect bone repair mechanisms. However, less is known about the role of reactive oxygen species (ROS) in bone repair. At physiologic levels, ROS play important physiological roles in a variety of cell types; however, when ROS levels exceed physiologic levels, they can cause cellular damage and contribute to disease pathogenesis (Kobayashi and Suda 2012; Kalyanaraman 2013; Huang and Li 2020; L.; Zhang et al., 2019; Y.; Zhang et al., 2020; Brown and Griendling 2015). With the more recent discoveries that elevated ROS levels stimulated osteoclast differentiation and influenced osteoblast formation, it is evident that ROS play an important role in regulating the human skeleton via redox signaling pathways (Hyeon et al., 2013, 2; Arakaki et al., 2013).

2 REDOX SIGNALING

Free radicals were first described in biology 1954, and since then, the harmful effects of ROS (a broader term encompassing both oxygen molecules with free radicals and non-free radical intermediates) on a wide variety of cellular processes have been well studied (Commoner et al., 1954; Finkel and Holbrook 2000). ROS include superoxide ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$). More recently, these highly reactive molecules are not just thought to be reapers of havoc, but they are showing to be important molecules regulating downstream signaling cascades. While previously a point of contention among the scientific community, ROS are now widely recognized as second messengers because of their regulated production, the existence of ROS elimination systems, and their target specificity (Raaz et al., 2014). Redox signaling is the general term given to describe the phenomenon of how these molecules mediate signal transduction pathways (Kalyanaraman 2013).

2.1 Reactive Oxygen Species Production Machinery

Intracellular ROS are generated by enzymes and exist primarily in the forms of $O_2^{\bullet}$, H_2O_2, and $\bullet OH$ (Lambeth 2004; Lambeth and Neish 2014; Bedard and Krause 2007). Transmembrane NADPH oxidases (NOXs) and the mitochondrial electron transport chain (ETC) are two significant endogenous enzymatic sources of $O_2^{\bullet}$ and H_2O_2 (Parascandolo and Laukkanen 2019; Murphy 2009). These sources, along with others, are illustrated in **Figure 1**. These molecules have a wide range of downstream targets, and H_2O_2 acts as a second messenger that can integrate environmental stimuli, quickly diffuse through membranes, and activate downstream signal transduction cascades (Bedard and Krause 2007; Kobayashi and Suda 2012). Through the dismutation reaction mediated by either potassium iodide or superoxide dismutase (SOD), $O_2^{\bullet}$ can be reduced to H_2O_2, which can be further reduced to oxygen and water (Dröge 2002; Panday et al., 2015). Enzymes such as glutathione peroxidase (Gpx), catalase, and peroxide redox proteins (PRX), as well as other antioxidants (i.e. Vitamin E), are important ROS scavengers that ultimately convert ROS to oxygen and water to help maintain redox homeostasis (L. Zhang et al., 2019; Y. Zhang et al., 2020; Brown and Griendling 2015; Hu et al., 2018). ROS-induced protein modifications can also be reversed by specific antioxidant defense proteins, including glutaredoxins, thioredoxins, and thioredoxin reductases, making them

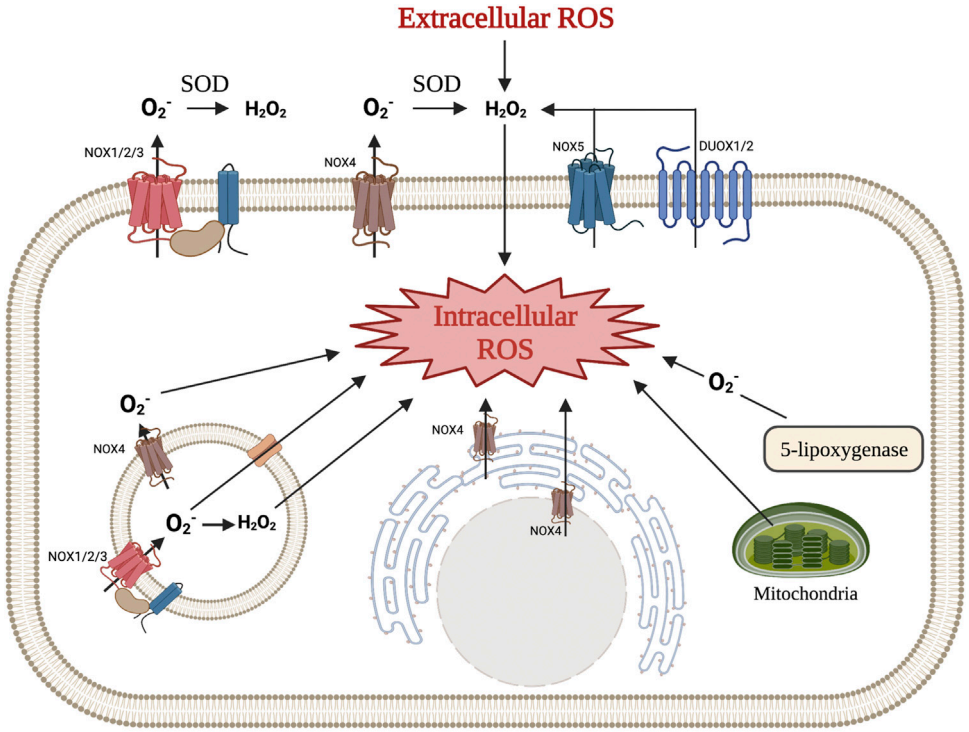

FIGURE 1 | Various mechanisms of intra- and extracellular ROS production. NOX1, NOX2, NOX3, and NOX4 produce superoxide, which is then converted to hydrogen peroxide in the extracellular space. NOX5 and DUOX1/2 produce hydrogen peroxide directly. Hydrogen peroxide produced by these enzymes, along with ROS from other cells, can diffuse across the cellular membrane to function as an intracellular signaling molecule. NOX enzymes can also be found in intracellular membranes (i.e., rough endoplasmic reticulum, endosomes, and nucleus) and produce ROS that can immediately effect signaling pathways. Other mechanisms for intracellular ROS production include the mitochondrial respiratory chain and a byproduct of 5-lipooxygenase. (Illustration created with BioRender.com).

important in the prevention of ROS-induced cellular damage (Drazic and Winter 2014).

In bone tissue, redox signaling has an important role in bone remodeling and bone repair (Garrett et al., 1990; Lean et al., 2003; X.; Sun et al., 2020, 2; Deng et al., 2019), and research has shown that oxidative stress, which leads to aging and estrogen deficiency, may be one of the most critical factors contributing to bone loss (Almeida et al., 2007b; Manolagas and Almeida 2007). Of more focus in this review, redox signaling is also triggered following bone fracture to regulate bone healing and regeneration by targeting resident stromal cells, osteoblasts, osteoclasts and endothelial cells (Ito and Suda 2014; K.-M.; Kim et al., 2019; Kang and Kim 2016; Yamamoto et al., 2020).

3 REDOX SIGNALING AND FRACTURE HEALING

3.1 Role of Reactive Oxygen Species in the Inflammatory Phase and Neovascularization

From distraction osteogenesis and other models fracture healing, the process of osteoblast differentiation and bone mineralization is closely coupled to neovascularization (Percival and Richtsmeier 2013). In fact, the invasion of new blood vessels into the mesenchymal condensations is necessary for the mineralization process to begin (Percival and Richtsmeier 2013; Tomlinson et al., 2013). The decision whether to pursue intramembranous ossification–rather than endochondral ossification–seems to be dependent on closeness of the nearest vascular supply (Percival and Richtsmeier 2013). In particular, NOX1 contributes to the Akt pathway's activation and downregulation of the anti-angiogenic nuclear receptor peroxisome proliferator activated receptor (PPAR), resulting in an event associated with capillary tube formation (Manea 2010; Bir et al., 2013; Huang and Li 2020). On the other hand, NOX2 plays a slightly different role in angiogenesis; it has been shown to promote endothelial cell migration, mobilize endothelial progenitor cells, and exert pro-angiogenic functions in response to vascular endothelial growth factor (VEGF) (Thakur et al., 2010; Schröder et al., 2012). The proliferation and migration of endothelial cells (ECs) could be enhanced by the Upregulation of the Nox4 expression, which results in the activation of receptor tyrosine kinases and the Erk pathway. Kim et al. found a novel positive feed-forward ROS-induced ROS release mechanism in which H_2O_2 (derived from NOX4) partially activates NOX2, thereby promoting mitochondrial ROS (mtROS)

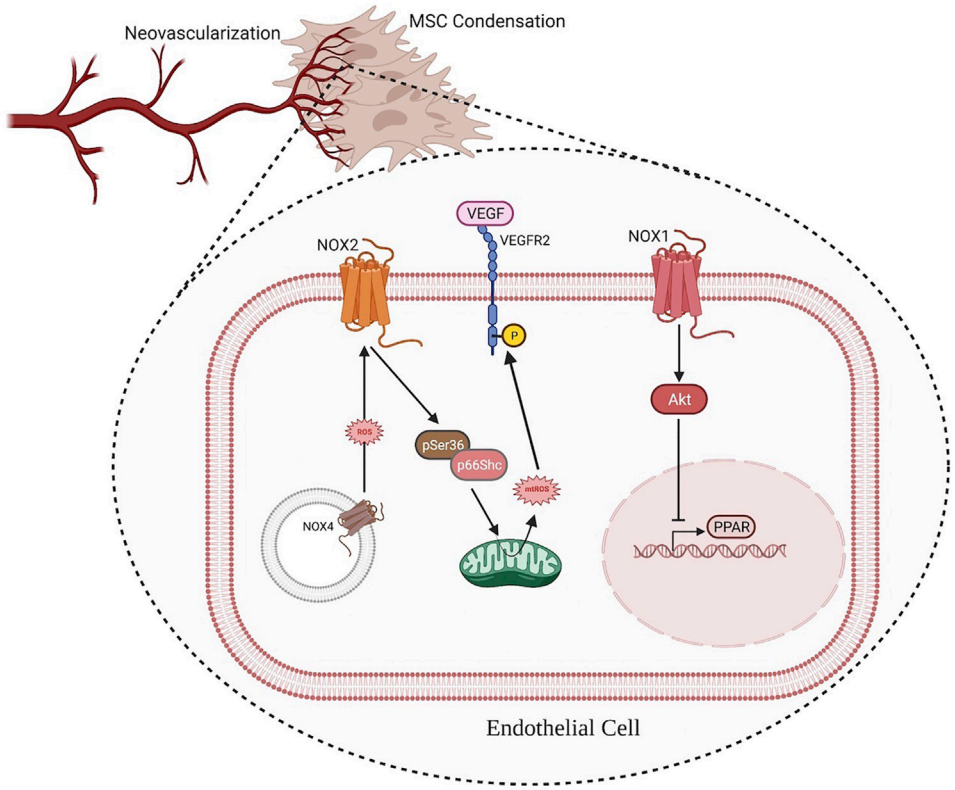

FIGURE 2 | ROS produced by NOX1 contributes to the activation of the Akt pathway and downregulation of the anti-angiogenic nuclear receptor PPAR, resulting in an event associated with capillary tube formation. Additionally, intracellular membrane-associated NOX4 produces ROS, which activates NOX2 and the down-steam factor pSer36-p66Shc to promote mtROS production. The ROS produced by mitochondria further enhances VEGF signaling, thus improving angiogenesis. (Illustration created with BioRender.com).

production through pSer36-p66Shc, which further enhances the ROS-dependent VEGFR2 signaling pathway in ECs. Through this mechanism, the Nox4/Nox2/pSer36-p66Shc/mtROS axis drives an angiogenic switch (Y.-M. Kim et al., 2017) (**Figure 2**).

The role of mitochondria in redox signaling and VEGF signaling has been recently more elucidated. UQCRB (a subunit of complex III in the mitochondrial respiratory chain) can positively regulate VEGFR2 signaling by increasing levels of mtROS as a key regulator of VEGF signaling in ECs. By the above approach, UQCRB also regulates the migration of ECs *in vitro* (Jung and Kwon 2013).

3.2 Effect of Reactive Oxygen Species on Mesenchymal Stem Cells Function

While damaged blood vessels and the hypoxic environment itself kickstarts angiogenesis, MSCs play a large part as well. For intramembranous ossification, it is important that the condensation of MSCs signal in a timely manner to the vascular cells so that osteogenic differentiation and mineralization can occur. MSCs can directly signal to assist angiogenesis in fracture healing by interaction with a variety of factors.

3.2.1 Vascular Endothelial Growth Factor

As previously mentioned, angiogenesis and intramembranous bone formation are closely coupled, such that angiogenesis proceeds osteogenesis (Ko and Sumner 2021). VEGF is the primary signaling molecule allowing osteoprogenitors to both signal to ECs and initiate the release of growth factors needed for the differentiation process (Ko and Sumner 2021). In a mouse model, when VEGF activity was blocked in osterix-positive cells, postnatal intramembranous ossification was significantly impaired (Ko and Sumner 2021; Buettmann et al., 2019). ROS play an important role in regulating VEGF secretion in MSCs. When preconditioned to hypoxic environments, MSCs enhance their angiogenic effect by upregulating VEGF (Black et al., 2008). Increased levels of ROS stabilize hypoxia-inducible factor 1 α (Hif-1 α), preventing its degradation, and allowing it to transcribe a host of hypoxia-related proteins, including VEGF (**Figure 3**) (Black et al., 2008). This ROS/Hif-1α/VEGF signaling pathway is observed in cells involved in osteogenesis, and the disruption of ROS can prevent normal VEGF signaling (F.-S. Wang et al., 2004; V. T. Nguyen et al., 2020). For example, when ROS-scavenging enzymes are inhibited, osteoblasts drastically reduce their

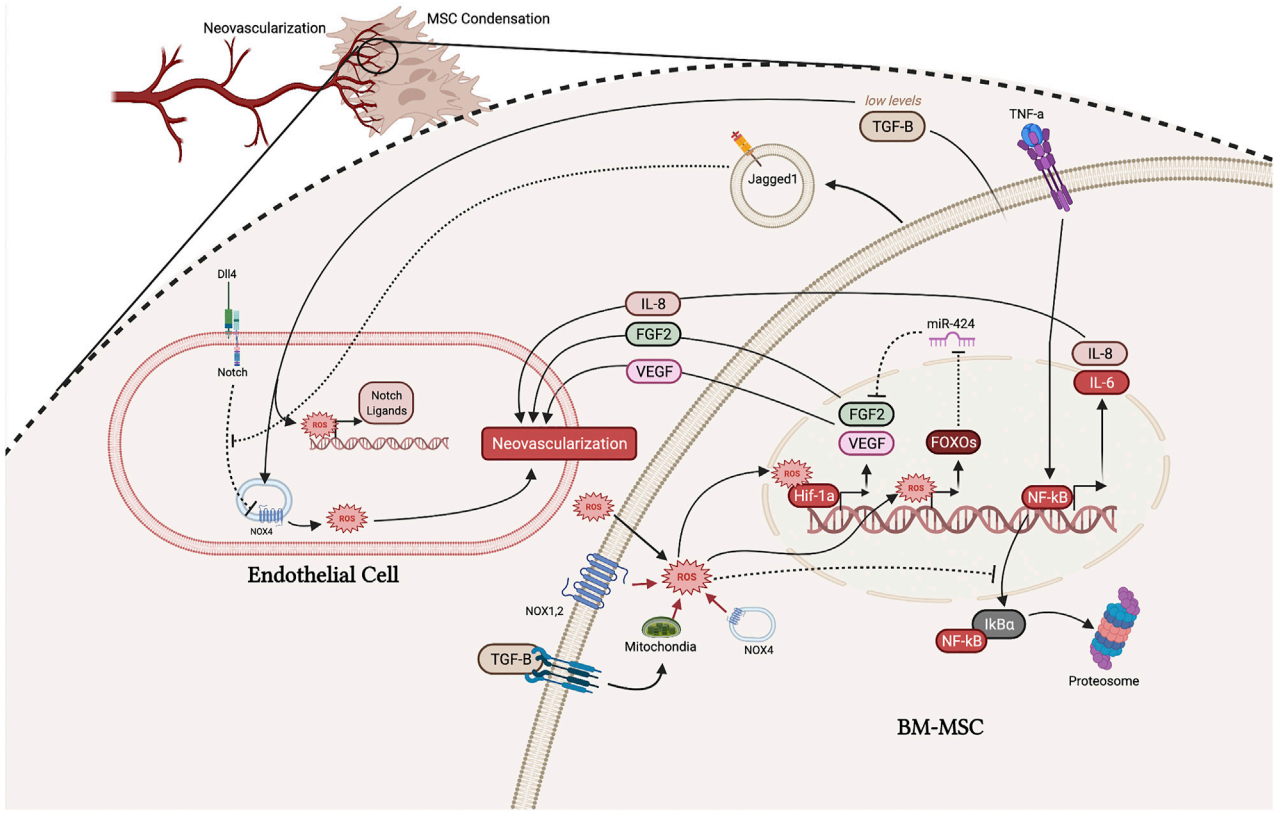

FIGURE 3 | Interaction between ECs and MSCs and in the early microfracture environment. ROS from a variety of sources, including the mitochondria, NOX enzymes, TGF-β signaling, and the extracellular environment, can stabilize Hif-1α resulting in upregulation of FGF-2 and VEGF, which promote neovascularization. ROS also directly upregulate FOXOs which inhibits miR-424, further promoting the functioning of FGF-2. Increased intracellular ROS promotes NF-κB signaling, thus upregulating IL-8 which promotes neovascularization but potentially inhibits intramembranous ossification. Jagged1 and TGF-β secreted from MSCs also function to promote neovascularization by promoting NOX4-generated ROS. (Illustration created with BioRender.com).

transcription of VEGF, thus impairing angiogenesis during bone repair (F.-S. Wang et al., 2004).

3.2.2 Fibroblast Growth Factors

Fibroblast growth factors (FGFs) are a large family of signaling factors that function in all stages of fracture healing and have been shown to be important in MSC differentiation, skeletal vascularization, and osteoblast recruitment (Schmid et al., 2009). FGF2 is the principle FGF expressed in distraction osteogenesis and is known to enhance EC survival, migration, and proliferation (Schröder 2019; Pacicca et al., 2003; Schmid et al., 2009). After FGFs bind the FGF receptor on ECs, phosphoinositide 3-kinase (PI3K) acts to increase ROS via NOX2. ROS produced from the FGF-NOX2 pathway has then been shown to inactivate phosphatases, allowing pro-angiogenic signaling pathways to be active longer (Schröder 2019). In vascular smooth muscle cells, FGF-2 has been shown to stimulate its own expression via a ROS-dependent mechanism (Black et al., 2008). ROS produced through the FGF-PI3K-ROS pathway functions to stabilize and increase expression of Hif-1α, which directly increased the activity at the FGF-2 promoter (**Figure 3**) (Black et al., 2008). Therefore, FGF-2 uses ROS as a key signaling molecule to upregulate its own expression.

FGF-2 secreted from MSCs, as well as other cell types, functions to stimulate angiogenesis in ECs and vascular smooth muscle through a ROS-dependent process. Given that the FGF receptor 1 and 2 are known to signal through the PI3K-AktMDM2 pathway, it is likely that FGF-2 can also stimulate its own expression in MSCs, though no studies have been done to show this possibility (Coutu et al., 2011). Li et al. showed that MSCs in fact have ROS sensing mechanisms to regulate the expression of FGF-2 by suppressing the microRNA miR-424 (L. Li et al., 2017). miR-424 has been shown to negatively regulate bone formation under oxidative stress by inhibiting FGF-2. However, this group found that MSCs upregulate forkhead box O 1 transcription factor (FOXO1) in response to ROS, and FOXO1 then inhibits the production of miR-424 (**Figure 3**) (L. Li et al., 2017; Siqueira et al., 2011, 1). In sum, MSCs can sense ROS levels and subsequently enhance the release of FGF-2; both ECs and MSCs can sense their oxidative environment and upregulate FGF-2 to improve neovascularization of the microfracture environment.

As discussed, low levels of ROS can enhance the early neovascularization of the fracture site; however, high levels of ROS in MSCs can be detrimental and induce rapid senescence (Ludin et al., 2014). To combat the high oxidative stress of the

fracture microenvironment, MSCs possess numerous mechanisms to reduce and manage ROS levels, including high levels of antioxidants, glutathione, and other ROS scavengers (Ludin et al., 2014). One study found that FGF-2 may promote low levels of ROS and maintains stemness of MSCs (Ludin et al., 2014). In a mouse model, FGF-2 *reduced* ROS levels in a PI3K-Akt-MDM2 dependent manner, thus increasing proliferation and self-renewal of MSCs (Coutu et al., 2011). Therefore, low levels of FGF-2 and abundant ROS scavenging mechanisms help MSCs maintain optimum levels of ROS to promote proper MSCs function. Interestingly, FGF plays a different role in the microfracture environment, and both ECs and MSCs can use ROS to upregulate FGF-2 to favor the neovascularization of the fracture site. MSCs seem to favor the expression of FGF-2 in response to ROS, however they may not use an autoregulatory positive feedback loop like ECs. Instead, FGF-2 may in fact still use PI3K, but to reduce levels of ROS so that they can function properly. More rigorous *in vitro* and *in vivo* studies need to be done to further investigate the role of FGF-2 and ROS in the oxidative microfracture environment.

3.2.3 Notch

Notch signaling is one of the most representative pathway in regulating tip cell specification (Suchting and Eichmann 2009). Recent study showing the increase in notch ligands are dependent on Hif-1α, a upstream of Notch pathways (Ciria and Nahuel, 2017). Hif-1α is activated by elevated ROS levels leading to the increase of Notch pathway and subsequent angiogenesis. However, given that there are 4 known Notch receptors that can be activated by 5 different Notch ligands, its role is extremely complex (Ciria and Nahuel, 2017). For example, Notch Ligand Delta-like 4 (Dll4) and Jagged1 have distinct spatial expression patterns and opposing functional roles in regulating angiogenesis (Benedito et al., 2009). Dll4, which is activated by VEGF in angiogenesis, contributes to *inhibiting* the sprouting of endothelial tip cells (Benedito et al., 2009). On the contrary, Jagged1 antagonizes the Dll4-Notch signaling, which promotes angiogenesis (Benedito et al., 2009; Hai et al., 2018).

Furthermore, it has been shown that ROS regulate the notch pathway's role in angiogenesis (Cai et al., 2014; Gonzalez-King et al., 2017). Studies have shown that Notch signaling mediates ROS production in Nox4-dependent manner. In ECs, blocking notch signaling could upregulate the expression of Nox4 and increase ROS generation while using ROS scavengers could result in abolished Notch blockade-induced EC proliferation, migration, and adhesion (Cai et al., 2014). Therefore, the stabilization of Hif-1α in MSCs may result in a decrease in EC's intracellular ROS, which would inhibit angiogenesis. In contrast, Jagged1 is a notch ligand that may *enhance* angiogenesis in the hypoxic fracture microenvironment. Gonzales-King et al. made a step towards confirming this notion by showing that MSCs can package and secrete Jagged1 via exomes, which had a pro-angiogenic effect *in vitro* and *in vivo* (**Figure 3**) (Gonzalez-King et al., 2017). However, the mechanism by which notch signaling influences angiogenesis is still unclear since some notch ligands have opposing actions.

3.2.4 TGF-β

Transforming growth factor beta (TGF-β) is an important cytokine in the early initiation of fracture healing. However, its role in neovascularization of the fracture cite is complex, with some studies showing low levels of TGF-β secreted by MSCs stimulates endothelial tube formation while others show the opposite (Kasper et al., 2007; Myoken et al., 1990). From more recent studies, the role of TGF-β in neovascularization of the fracture site may be elucidated by its effects on ROS in ECs and MSCs. For ECs, TGF-β has a positive effect on NOX4; ROS produced by NOX4 then go on to directly stimulate angiogenesis and facilitate the upregulation of Notch ligands, Dll4, Notch1, and Jagged1 (**Figure 3**) (Yan et al., 2014, 4). It is also possible that Jagged1 functions to antagonize the Dll4-Notch pathway, resulting in a positive feedback loop to further stimulate NOX4 (Ciria and Nahuel, 2017; Gonzalez-King et al., 2017). These other notch ligands produced by ECs can then act directly to promote the osteogenic differentiation of MSCs to heal the fracture (Dishowitz et al., 2012; Ciria and Nahuel, 2017; Gonzalez-King et al., 2017). Thus, TGF-β seems to have an overall favorable effect on angiogenesis and early fracture repair.

However, at higher levels, one study found that TGF-β1 has a negative effect on MSCs by upregulating mitochondrial production of ROS and increasing senescence (J. Wu et al., 2014). While this study only investigated the *in vitro* effect of TGF-β on MSCs, these findings may provide some insight to TGF-β's role in the initial fracture healing response. At lower levels, TGF-β -induced production of ROS may facilitate the pro-angiogenic capacity of MSCs by many of the mechanisms mentioned throughout this section (**Figure 3**). However, at higher levels of TGF-β, the overproduction of ROS may switch MSCs toward an apoptotic phenotype (J. Wu et al., 2014). From *in vivo* studies, the levels of TGF-β gradually increases throughout the fracture healing process over the first 14 days (Poniatowski et al., 2015). While this topic is lacking rigorous experiments, it is reasonable to speculate that low levels of TGF-β functions to facilitate proangiogenic levels of ROS, while higher levels of TGF-β are more favorable in later stages of fracture healing, where an increase in apoptosis may be beneficial in producing calcium deposits for bone nodule formation.

3.2.5 NF-κB and IL-8

One of the best described mechanisms by which MSCs initiate angiogenesis is through the upregulation and secretion of interleukin 8 (IL-8) by inflammatory factors such as tumor necrosis factor a (TNF-α) (J. Wang et al., 2015; Kwon et al., 2013). IL-8 is under the influence of the nuclear factor-kappa B (NF-κB) pathway, and NF-κB has been shown to be greatly affected by ROS levels. Increased ROS levels promotes the proteosome degradation of IκBa, which normally functions to inhibit NF-κB from upregulating inflammatory cytokines (G. Li et al., 2019; Bai et al., 2020). Thus, by promoting the degradation of IκBα, ROS can promote the upregulation of IL-8 (**Figure 3**).

While IL-8 functions to initiate angiogenesis, it also is a potent chemoattractant and recruits osteoprogenitor cells to the fracture site (Lin et al., 2019). By both initiating angiogenesis and

recruiting MSCs, IL-8 would seem to play an important role in intramembranous ossification. However, this may not be the case. Recent literature shows that IL-8 induces chondrogenesis and has little ability to promote osteogenesis and mineralization (Lin et al., 2019). While Yang et al. found that IL-8 increased osteogenesis in a large bone defect mouse model after 14 days, another recent study found that this effect may be due to IL-8's ability to rapidly induce chondrogenesis and synergize with bone morphogenic protein (BMP) 2 signaling (A. Yang et al., 2018; Lin et al., 2019). Therefore, IL-8 potentially favors endochondral ossification, seen in larger fracture site, and likely disrupts intramembranous ossification.

As mentioned above, ROS can favor endochondral ossification via the expression of inflammatory cytokines such as IL-8. This effect is well explained by the size of bone defect; for example, the greater oxidative stress seen in larger defects allows intracellular ROS levels to exceed ROS scavenging mechanisms. This increased intracellular ROS then upregulate IL-8 to promote chondrogenesis. Therefore, the decision to pursue intramembranous versus endochondral ossification is greatly dependent on the MSC's ability to regulate and sense ROS levels. More studies need to investigate the levels of ROS at which IL-8 is upregulated. Further, the existing literature utilizes cell culture and large-sized bone defects to investigate the effects of IL-8 on bone healing (A. Yang et al., 2018, 8; Lin et al., 2019), so there is a need for new bone defect models to assess the role ROS and IL-8 in intramembranous ossification.

3.3 Bone Morphogenic Protein Signaling

Vascular cells are also known to upregulate BMPs in response to hypoxia and VEGF, functioning as a sensor of the fracture microenvironment and delivering the needed factors for MSCs to differentiate (Percival and Richtsmeier 2013). BMP2 is necessary for MSCs to initiate fracture repair, thus its release from vascular cells during hypoxia is a vital signal to kickstart intramembranous ossification. Studies show that silencing BMP2 expression results in fractures that do not heal in a mouse model, as a direct result of MSCs not differentiating to osteoblasts (Tsuji et al., 2006).

There is also an important interplay between ROS and BMP signaling in pre-osteoblasts. BMPs have been shown to upregulate NOX enzymes, thus increasing intracellular ROS in MSCs (Mandal et al., 2011; Sánchez-de-Diego et al., 2019). These ROS can then function to upregulate BMPs, notably BMP2 and BMP4 in MSCs, by increasing the activity of NF-κB signaling (Csiszar et al., 2005). This expression of BMPs by MSCs seems to be of most importance in fracture healing since fracture healing was severely impaired by knocking out BMP2 gene expression in an animal model, whereas knocking out BMP2 expression in osteoblasts and chondrocytes only delayed fracture healing (McBride-Gagyi et al., 2015, 2; Muinos-López et al., 2016). Therefore, NOX dysfunction may have deleterious effects on MSC's ability to differentiate by downregulating BMP2 expression.

ROS and BMPs both play an important role in enhancing osteogenic differentiation of MSCs. Muinos-López et al. also showed that hypoxia and the regulation of ROS play a large role in BMP2 production by MSCs (Muinos-López et al., 2016). In their experiment, they found that hypoxia alone was insufficient to induce BMP2 expression in human periosteal-derived MSCs, and hypoxia with inflammatory cytokines greatly increased BMP2 activity, but not greater than just inflammatory cytokines alone. However, when the ROS-scavenger inhibitor PX-12 was added to the hypoxic and cytokines group the levels of BMP2 significantly decreased (Muinos-López et al., 2016). From their work, it seems that ROS are necessary for the inflammatory cytokines to adequately upregulate BMP2 signaling. Further, they also tested this in a fracture healing mouse model and found that PX-12 significantly reduced Bmp2 signaling, resulting in impaired fracture healing and an atrophic-like nonunion (Muinos-López et al., 2016).

However, given that BMPs signals to activate NOX which then further upregulate BMPs, it is easy to see how this positive regulatory loop can get out of hand, leading to high oxidative stress. It has been extensively published that high levels of ROS are damaging to osteoprogenitors, resulting in decreased proliferation, increased apoptosis, and decreased osteogenic differentiation (X. Bai et al., 2004; Benameur et al., 2015; Denu and Hematti 2016). The above findings suggest there is a delicate balance between MSC intracellular ROS and ROS scavenger mechanisms to allow for optimal ROS-induced BMP2 signaling, while also suppressing ROS to manageable levels.

3.4 FOXO and Wnt Signaling

Activation of the Wnt/β-catenin pathway is generally recognized as enhancing the osteogenic and chondrogenic potential of MSCs, thus important during the fracture healing process (Atashi et al., 2015). In fact, studies have shown this pathway is upregulated during the entire fracture process (H. Xu et al., 2014; Chen et al., 2007). To describe briefly, after an extracellular Wnt ligand binds to the frizzled seven pass transmembrane receptor, the degradation of β-catenin is inhibited, allowing its migration to the nucleus to bind T-cell factor/lymphoid-enhancing factor (Tcf/Lef) to transcribe Wnt effector genes known to upregulate osteogenesis (H. Xu et al., 2014). ROS have been shown to interfere with this signaling cascade, hence impairing the osteogenesis of MSCs. Almeida et al. discovered that the decrease in Wnt effector genes, such as *osteoprotegerin* and *Axin2* is associated with the increase in ROS seen in aged mice (Almeida et al., 2007a).

The above finding then led to the discovery that increased ROS upregulates the transcription of the FOXO family of transcription factors. FOXOs, including FOXO1, FOXO3a, FOXO4, and FOXO6, require β-catenin for the transcription of their target genes (Atashi et al., 2015). In other words, FOXOs steals β-catenin from the Wnt/ β-catenin pathway and uses it to increase transcription of a variety of gene, of which include antioxidant and ROS-scavenging enzymes. In one study, osteoblast number and bone mass was increased in mice deficient in FOXOs (Iyer et al., 2013). Furthermore, mice treated with H_2O_2 was shown to increase FOXO association with β-catenin (Almeida et al., 2007a). On the contrary, Ambrogini et al. showed that mice deficient in FOXOs had

elevated oxidative stress and high levels of osteoblast apoptosis, whereas mice with FOXO overexpression had less osteoblast apoptosis and significantly increased bone formation and vertebral bone mass (Ambrogini et al., 2010).

From the studies above, both Wnt and FOXO signaling is important for fracture healing. However, when ROS levels are elevated to pathologic levels, these two signaling pathways interfere with each other. At physiologic levels of ROS, the constitutive level of ROS-scavengers and antioxidants (downstream of FOXO pathway) are sufficient to prevent cellular damage. However, when ROS are elevated, the FOXO pathway is stimulated to provide more ROS-scavenging power. While this is good for cellular health, it inhibits the osteogenic potential of osteoprogenitors through inhibiting the Wnt/β-catenin pathway.

Up to this point, it is evident that ROS are necessary for fracture healing to take place. However, their levels must be tightly controlled, and increased levels can inhibit or delay the fracture healing process.

4 HARNESSING REACTIVE OXYGEN SPECIES FOR BONE REGENERATION

As discussed extensively in the first half of this review, ROS plays an important role in many pathways involved in the early phase of fracture repair. However, it is also clear that pathologically elevated ROS levels contribute to poor skeletal healing, such as in diabetes. Diabetes claims one of the top spots of diseases affecting Americans, and it is one of the most well researched pathologies related to excess ROS, which has been clearly linked to poor outcomes. Not only does the increased oxidative stress add to the vast tissue damage and complications, but the overproduction of ROS also limits therapeutic potential by negatively affecting stem cell production. More recently, the direct effects of ROS in inducing microangiopathy in bone marrow has been highlighted as a mechanism of diabetes in both contributing to the decline of the disease and preventing healing (Mangialardi et al., 2014). Additionally, the oxidative stress environment of diabetes, or any other pathology, contributes to osteoblast and osteoclast dysfunction, resulting in reduced bone mass and impaired fracture healing (Jiao et al., 2015). Bone specific diseases, such as osteoporosis and bone tumors, along with joint inflammatory diseases, including rheumatoid arthritis and ankylosing spondylitis, have also been linked to an increase in ROS (Wauquier et al., 2009).

Many of the pathologies discussed are associated with an overproduction of ROS, which suggest that certain levels above a threshold may exacerbate the disease state or prevent healing. It is acknowledged that some physiological level of ROS must exist for initiation of distinct cell processes, pointing to some minimum amount that may also need to be present for favorable outcomes. Understanding how ROS levels affect pathological mechanisms and healing allows for better optimization of microenvironment conditions and the development of therapies. We discuss below how therapies may be used to either increase or decrease ROS to improve fracture healing.

4.1 Utilizing Exogenous Antioxidants to Improve Fracture Healing

Antioxidants and ROS-scavenging enzymes seem to hold obvious therapeutic promise as targets to decrease ROS and improve fracture healing. However, as previously discussed, ROS are necessary for the bone healing process to occur, while the levels of ROS may determine the bone healing type and quality.

4.1.1 Vitamin C

Vitamin C is known to play a critical in musculoskeletal healing and serves as a cofactor for prolyl hydroxylase and lysyl hydroxylase, two enzymes necessary for proper collagen three-dimensional conformation. Further, Vitamin C is a powerful antioxidant and a stimulator of osteoblast growth and differentiation (Lee et al., 2017). Therefore, it is reasonable to suspect that supplementation with Vitamin C would improve fracture healing. However, clinical evidence does not fully support this statement. In a large systemic review, DePhillipo et al. reported conflicting clinical evidence for use of Vitamin C in enhancing fracture healing (DePhillipo et al., 2018). One study found that patients who were treated with vitamin C after open reduction internal fixation had higher plasma levels of alkaline phosphatase (ALP) and Osteocalcin, suggesting higher BMD and faster healing with antioxidant supplementation (Sandukji et al., 2011). On the other hand, another group found that vitamin C had no significant effect on time to fracture-healing at day 50 post-surgery of distal radial fractures (Ekrol et al., 2014). Given that the size of fracture, injury mode, and fracture management modality all contribute to the level of ROS at the fracture site, it is difficult to tease out the effect of vitamin C in the latter study. For example, vitamin C may have greater effect on fracture healing after an open reduction internal fixation, compared to an external fixation, since the levels of ROS may be drastically different post-surgery. Better controls need to be in place for clinical studies to better understand the effects of vitamin C on fracture healing.

Similar to the previous clinical studies, animal studies also showed conflicting data on the improvement of fracture healing with vitamin C (DePhillipo et al., 2018). Giordano et al. found that vitamin C supplementation had no effect on histological features of fracture healing at week 2, 4, and 6 (Giordano et al., 2012). However, Sarisozen et al. analyzed fracture repair at week 2 and 3 and saw accelerated fracture repair (Sarisözen et al., 2002). Similarly, Yilmaz et al. measured fracture repair at even earlier time points (5, 10, 15, and 20 days post-fracture) and found vitamin C accelerated fracture healing, while having no effect on end quality of fracture healing (Yilmaz et al., 2001). Taken together, vitamin C may play an important role in accelerating bone healing during the inflammatory phase of fracture healing, when ROS levels are the highest. Interestingly, a recent metanalysis by Sun et al. found that increasing dietary vitamin C by 50 mg/day would decrease risk of hip fracture by 5% (Y. Sun et al., 2018). Given that aging is associated with increased levels of ROS, is it

reasonable to hypothesize that vitamin C may reduce intracellular ROS, allowing aged osteoprogenitors to better handle the higher levels of oxidative stress seen in aging. Therefore, vitamin C may be a promising supplement, not only in fracture healing, but also in the prevention of age-associated bone loss and fracture.

4.1.2 α-tocopherol

α-tocopherol (AT) is a vitamin E isomer that has antioxidant properties and effects on various metabolic systems. AT's effect on bone healing is debated, as some studies show that it does not improve fracture repair while others show the opposite (Sarisözen et al., 2002; Durak et al., 2003; Mohamad et al., 2012). More recent insight compares AT to a double-edged sword. At high doses, AT may have prooxidant effects, block entry of other vitamin E isoforms, and interfere with vitamin K metabolism (Chin and Ima-Nirwana 2014). At lower doses however, AT has antiosteoporotic effects and scavenges ROS (Shuid et al., 2011; Chin and Ima-Nirwana 2014). While AT's effect on bone health is relatively unknown, one finding among the literature seems to be consistent: AT is protective of bone and enhances bone healing under stressful conditions.

Smith et al. studied the effect of AT on hindlimb-unloading and found that high doses (500 IU/kg) produced lower trabecular number and decreased bone volume in mice compared to the lower doses (15 IU/kg and 75 IU/kg) (Smith et al., 2005). However, the higher dose of AT prevented the hindlimb-unloading bone loss been in osteoporotic mice (Smith et al., 2005). In another study, high doses of AT prevented bone loss and improved bone structure of aged mice (24 month-old), while it had not effect on bone structure of younger mice (6 month-old) (Arjmandi et al., 2002). The authors also showed AT increased the mRNA expression of insulin-like growth factor 1 (IGF-1), osteocalcin, and type-1 collagen in the bones of both young and old mice (Arjmandi et al., 2002). Additionally, Zhang et al. noted that smokers had a lower prevalence of fractures if they had higher dietary intake of AT (J. Zhang et al., 2006). By increasing bone mass in an osteoporosis model, improving bone health of aged mice, and preventing fractures in smokers, AT seems to provide a profound protective effect in environments of high oxidative stress.

Unlike most lipid-soluble vitamins, AT is inserted in the lipid cell membrane of cells and lipoproteins, as well as acts ubiquitously throughout the body at high concentrations (Miyazawa et al., 2019). Therefore, at higher concentrations of AT, resulting in higher plasma levels, it is possible that AT could be inhibiting the physiological levels of ROS that are required for bone healing (Baxter et al., 2012). Whereas, in environments of constitutively high ROS, this increase in AT provides a net sum benefit when extracellular ROS levels are harmful to the healing process.

To fit with the hypothesis that the most important aspect of fracture healing is to lower intracellular ROS to maintain optimal function of regulation of osteoprogenitors, AT should decrease ROS in osteoprogenitors and MSCs. A more recent study by Bhatti et al. confirmed that AT decreases oxidative stress in both adipose and bone marrow-derived MSCs, supporting the positive effect of AT on fracture healing (F. U. R. Bhatti et al., 2017; F. U. Bhatti et al., 2017). Additionally, AT has been shown to suppress cyclooxygenase activity by preventing the hydrolysis (activation) of phospholipase A_2 (Miyazawa et al., 2019). This inhibition of phospholipase A_2 may aid in the decrease of intracellular ROS of osteoprogenitors and contribute to the protective effects of AT as shown by Smith et al. (2005). All in all, the direct ability of AT to improve fracture healing is complicated and is potentially dependent on dose and level of bone injury. Durak et al. found that AT improves the later stages of bone healing in a rabbit model of fracture healing, and a more recent paper found that AT can improve the osteointegration of stainless steel metal implants by reducing postoperative stress in a rat model (Durak et al., 2003; Savvidis et al., 2020). Therefore, AT holds significant promise in enhancing fracture healing. From the present evidence, its seems that AT is beneficial in suppressing ROS in environments of high oxidative stress, which allows normal bone healing. However, the literature suggests AT may be detrimental to normal fracture repair, likely by suppressing the healthy, physiologic levels of ROS. More well-controlled studies need to be performed to look at AT's direct role in normal fracture repair to see if beneficial effects can be seen at lower doses of AT.

4.1.3 N-acetyl Cysteine

N-acetyl cysteine (NAC) is a small molecular weight, amino acid derivative antioxidant that can be rapidly transported into the cytoplasm (Yamada et al., 2013). These qualities allow NAC to aid in the scavenging of ROS inside the intracellular space. Further, it has been shown that NAC is catabolized in the cytoplasm and leads to the generation of sulfane sulfur species, which likely perform the bulk of the antioxidant and cytoprotective functions after NAC treatment (Ezeriņa et al., 2018). It is unlikely that NAC by itself significantly contributes to ROS-scavenging, given that the NAC reaction has a low rate constant (Benrahmoune et al., 2000; Ezeriņa et al., 2018).

Further, Yamata et al. showed that NAC directly enhances ALP activity, collagen deposition, and other bone-related markers in osteoblastic cells in culture (Yamada et al., 2013). This group also found that by infusing a collagen sponge with NAC could drastically improve bone healing when implanted into a critical-sized cortical bone defect (Yamada et al., 2013). The osteogenesis enhancing effect of NAC is clear; however, it is not clear whether this effect is due to direct enhancement of osteoblastic differentiation or by decreasing the constitutive level of ROS, allowing optimal MSC function and differentiation. Roper et al. used alcohol to delay fracture healing in mice. This group has shown alcohol induced oxidative stress upregulates FOXO production, which antagonizes Wnt signaling to impair fracture healing (Roper et al., 2016). By administering NAC, they found that ROS levels were suppressed to levels that allowed FOXO levels to decrease, restoring quality of endochondral ossification (Roper et al., 2016). Duryee et al. had similar findings, showing that NAC decreased ROS in alcohol-fed mice and restored the normal innate immune response to fracture healing (Duryee et al., 2018). Taken together, NAC has profound antioxidant actions intracellularly and relatively low actions extracellularly, which make it a promising molecule to suppress ROS levels in osteoprogenitors while not significantly effecting the physiologic levels of ROS in the fracture environment. Further studies need to be conducted that more closely investigate whether NAC is acting primarily to

suppress intracellular ROS of osteoprogenitors and immune cells, or if is acting to suppress ROS in the extracellular space of the fracture environment.

4.2 Modulating Intracellular Antioxidants to Improve Fracture Healing

Using antioxidants such as vitamin C, AT, and NAC hold significant promise for promoting healthy fracture and bone healing; however, these broad antioxidants have the potential to interrupt physiologic levels of ROS that are necessary for the healing process. A likely better approach to improving fracture healing and bone health is to target intracellular mechanisms of ROS-scavenging. Nuclear factor erythroid 2-related factor-2 (Nrf2) is a key factor that positively regulates the expression of antioxidants and ROS-scavenging enzymes through binding antioxidant response element (ARE) (Y. Kubo et al., 2019, 2). Nrf2 signaling plays an essential role in the fracture healing response, as Lippross et al. has shown that knocking out Nrf2 in mice significantly retards callus formation (Lippross et al., 2014).

It has been shown that Nrf2 is locally upregulated in the fracture environment and VEGF stimulates Nrf2 activity, suggesting that Nrf2 is a protective mechanism of osteoprogenitors to control intracellular ROS levels during this stressful process (Kweider et al., 2011). However, the exact role of Nrf2 in fracture repair is debated. Several studies have found that overactivation of Nrf2 is detrimental to bone healing by inhibiting osteoblast differentiation (Hinoi et al., 2006; Kanzaki et al., 2013). So, much like the antioxidants discusses previously, too much of a good thing is not necessarily good, and it is likely the regulation of Nrf2 during various stages of fracture healing that is of primary importance. Supporting this statement, the upregulation of Nrf2 signaling has been shown to be more beneficial in models of excessive oxidative stress, such as in models of osteoporosis, heavy alcohol intake, type-2 diabetes, and smoking (H. Li et al., 2018; Y. Kubo et al., 2019; Aspera-Werz et al., 2018; Ibáñez et al., 2014, 2). In a more recent study, Yin et al. found that moderate increases in Nrf2 (using mice heterogeneous for Keap1) significantly improves osteoblast formation and bone mass in male mice (Y. Yin et al., 2020). The latter study, unlike others, is one of the first to show that enhancement of Nrf2 may provide improved bone health in normal, healthy conditions.

Interestingly, Nrf2 signaling can be modulated via numerous mechanisms, which may hold promise for enhancing bone healing. Thalar et al. found that Sulforaphane, a natural inducer of Nrf2 signaling, enhances osteoblast differentiation, reduces apoptosis of osteoprogenitors, promotes apoptosis of pre-osteoclasts, and improves bone volume of both normal and ovariectomized mice (Thaler et al., 2016). This compound is currently being investigates as a potential to mitigate aging by activating Nrf2 in older adult (Northern Arizona University 2021). Unfortunately, few studies have set out to investigate the role of Nrf2 enhancing drugs on the direct effect on bone healing. In an elegant review by Kubo et al., there are other molecules and drugs, such as dimethyl fumarate, bardoxolone methyl, beta-agonists, VEGF, and others, that have positive regulatory effects on the Nrf2/ARE pathway and may improve fracture healing (Y. Kubo et al., 2019; J.-H. Kim et al., 2014; Takahata et al., 2009). Upregulating Nrf2 signaling may be a promising technique to increase bone-forming cells' ability to suppress harmful intracellular ROS. However, these molecules have been largely understudied, and, while it is likely that increasing Nrf2/ARE signaling will improve bone healing in highly oxidative conditions, it is unclear if upregulating this pathway will improve normal physiological fracture repair. There is a great need for future research to investigate the direct role of Nrf2 in each phase of fracture healing and determine clinical situations in which Nrf2 modulation could provide beneficial outcomes.

5 ENGINEERING APPROACHES TO MODULATE REACTIVE OXYGEN SPECIES IN FRACTURE HEALING

Bone tissue engineering (BTE) remains a relatively new yet promising alternative to the use of bone grafts in the treatment of bone disorders and injuries. BTE provides an unlimited supply of grafting resources and prevents disease transmission. Combinations of scaffolds, cells, and specific chemical and physical stimuli are being developed to optimize bone repair, regeneration, and treatment outcomes. The four major components of a successful BTE treatment require a biocompatible scaffold, osteogenic cells, morphogenic signals, and sufficient vascularization (Amini et al., 2012). While most seem to highlight the importance of an appropriate physiological range of ROS for maximal bone healing effects, how to utilize ROS for improved BTE treatment still needs to be clarified.

5.1 Mesenchymal Stem Cell Preconditioning to Improve Bone Healing

The data for the use exogeneous antioxidants to improve fracture healing is promising. One potential use of cellular priming techniques is to "teach" cells to handle oxidative environments. Studies have found that by pre-treating in oxidative environments, MSCs upregulate ROS-scavenging enzymes that result in improved tissue healing. These primed MSCs could then be combined with bio-scaffolds to improve fracture healing.

5.1.1 Hydrogen Peroxide Preconditioning

Kubo et al. found that by simply preconditioning with low-dose hydrogen peroxide (H_2O_2), bone marrow-derived MSCs (BMSCs) had greater survival and enhanced neovascularization after being implanted into ischemic hindlimbs of mice. This study found that after 14 days of implantation, many more H_2O_2-pretreated BMSCs were viable compared to the untreated group. Further, these preconditioned BMSCs were able to significantly upregulate VEGF expression in ECs after only 1 day of implantation (M. Kubo et al., 2007). More recent studies are finding that preconditioning with oxidative

environments are cellular protective and lead to improved viability, likely by the upregulation of antiapoptotic and pro-survival pathways (Nouri et al., 2019; He et al., 2020). Guo et al. further found that 50 μM H_2O_2 is the optimal dose to maximize their proliferation, survival, and migration BMSCs (Guo et al., 2020). This pretreatment upregulated PI3K/Akt/mTOR pathway, as well as cyclin D1, SDF-1, and CXCR4/7 receptors. After implanting these pre-treated cells into full thickness wounds of mice, they noticed improved engraftment and survival compared to untreated BMSCs. Interestingly, these pretreatments also upregulate key ROS-scavenging enzymes such as heme oxygenase 1, catalase, NQO1, and SOD through the Nrf2 pathway (F. Zhang et al., 2019; Yuan et al., 2017). Thus, these preconditioned cells have an increasingly reductive environment, allowing them to better suppress harmful levels of *intracellular* ROS during the fracture healing process. Additionally, as described in **Figure 3**, preconditioning with ROS upregulates IL-8, FGF2, and VEGF, which may prime MSCs to accelerate the inflammatory phase of fracture healing without tampering with the physiologic levels of extracellular ROS needed for healing.

Curcumin (a dietary product with cellular protective effects) has also been shown to suppress H_2O_2-induced oxidative stress in BMSCs (Yagi et al., 2013). Wang et al. found that by preconditioning with curcumin, along with hypoxic conditions, BMSCs had significantly better survival, proliferative capabilities, and mitochondrial function via the upregulation of PCG-1a/SIRT3/Hif-1α signaling. These preconditioned BMSCs also accelerated cutaneous wound healing in a mice model(X. Wang et al., 2020). Due to its low solubility and high liver metabolism, curcumin has poor efficacy as an oral antioxidant (Safali et al., 2019). However, curcumin-coated biopolymers have shown promise. Bose et al. found that human osteoblasts in curcumin-coated biopolymers had significantly improved viability and morphological characteristics. They also found that curcumin-coated 3D-printed scaffolds showed improved bone formation and mineralization in loadbearing and non-loadbearing implants (Bose et al., 2018). These results show promise for using curcumin locally as an antioxidant to improve bone healing.

5.1.2 Hypoxic Preconditioning

Pre-culturing MSCs in hypoxic conditions replicate these protective results as well. Andreeva et al. found that adipose-derived MSCs cultured in hypoxic conditions had an increase in ROS, but also had an increase in superoxide dismutase and Hif-1α (Andreeva et al., 2015). Taken together, it is reasonable to assume that hypoxic conditions can both increase the ROS needed to upregulate FGF2, VEGF, and IL-8, while also increasing the ROS-scavenging mechanisms to control ROS-induced cellular damage. Thus, hypoxic pretreatments may be a promising technique to prime MSCs for accelerated fracture healing. Other studies have confirmed that hypoxic pre-treated MSCs upregulate VEGF and FGF2, as well as upregulating platelet-derived growth factor (PDGF) and polarizing macrophages to an anti-inflammatory M2 phenotype (Fotia et al., 2015; Maruyama et al., 2020). Furthermore, in an elegant series of experiments, Liu et al. discovered that hypoxia preconditioning of MSCs increases exosomal miR-126, which promotes bone fracture healing (Liu et al., 2020, 12).

While limited, the current literature suggests that hypoxic MSCs have improved osteogenic potential. Yu et al. found that hypoxic MSCs had enhanced osteogenic differentiation *in vitro* and accelerated fracture repair *in vivo* by upregulating Hif-1α and STAT3 phosphorylation (Yu et al., 2019). Further, Ho et al. found that MSCs pretreated under hypoxic conditions and then formed into spheroids entrapped in alginate gels significantly improved healing of a critical-sized bone defect, compared to gels with untreated MSC spheroids (Ho et al., 2018). Their preconditioned MSC spheroids were also found to be more resistant to apoptosis. Finally, hypoxia preconditioned MSCs implanted into bone-like bio-scaffolds showed increase collagen deposition when transplanted into subcutaneous tissue of mice. These promising studies clearly suggest hypoxic preconditioned MSCs may be an effective method to improve fracture healing. However, there is a need for more studies that directly study how preconditioned MSCs can improve *in vivo* fracture healing (Maruyama et al., 2020).

There are numerous other compounds that have been explored to precondition MSCs for the oxidative fracture healing environment. Each of the endogenous antioxidants previously discussed could be used as pretreatments to be infused into bio-scaffolds. For example, Watanabe et al. pretreated BMSCs with N-acetyl cysteine (NAC) before incorporating them into a collagen sponge. These sponges were then placed in a critical-sized rat femur defect and found that the NAC pretreated group resulted in significantly increased new bone formation (Watanabe et al., 2018). While not yet tested in a bio-scaffold for fracture repair, sodium ascorbyl phosphate (a vitamin C derivative) has been proposed as a suitable molecule for coating biomaterials and has been shown to both strongly stimulate osteogenesis and scavenge ROS (Okajima et al., 2020). Further, astaxanthin has recently been shown to protect MSCs from oxidative stress by upregulating Nrf2 signaling (Mohammadi et al., 2021).

Additionally, He et al. pre-treated adipose-derived MSCs (ASCs) with salidroside and hypoxic conditions and found these cells were able to resist H_2O_2-induced cell death. These two pretreatments acted synergistically to give MSCs better resistance to ROS. Further, they found that these conditions upregulated autophagy and activated Akt, Erk1/2, and LC3. These pre-treated cells also had enhanced migration and survival in the presence of oxidative stress (He et al., 2020). These results suggest another pretreatment option to prime MSCs for skeletal tissue healing. However, these pretreatment regimens have not been incorporated in a fracture healing model.

5.2 Molecular Targeting of Reactive Oxygen Species to Improve Bone Healing

A newer, promising approach to suppressing intracellular ROS levels is by using RNA interference (RNAi) technology, such as short interfering RNAs (siRNA) or microRNAs (miRNA)

(Cerqueni et al., 2021; L. L.; Wang and Burdick 2017). In short, siRNAs are small, non-coding RNAs designed to degrade specific mRNA sequences before they are translated into proteins. miRNAs are also non-coding RNA molecules that can interfere with signaling pathways; however, they have a much broader effect by influencing a wider range of mRNA targets and can regulate protein expression (Ahmadzada et al., 2018). By using viral vector delivery systems, siRNA or miRNA can be reliably inserted into BMSCs to target relevant ROS-producing or antioxidant pathways. For instance, siRNAs could be delivered to BMSCs to silence the expression of NOX2, potentially dampening harmful intracellular ROS, while not interfering with pro-angiogenic levels of extracellular ROS. However, off-target effects and potential mutagenesis is a major limitation of delivering siRNAs (Cerqueni et al., 2021). Therefore, alternative delivery options are needed.

Promisingly, hydrogel scaffolds have been shown to be a reliable platform to deliver RNAi particles (M. K. Nguyen et al., 2018; L. L. Wang and Burdick 2017). Effectively, hydrogels encapsulate the RNAi (which is likely housed in a nanoliposome) particle within a 3D matrix (Ahmadzada et al., 2018; Cerqueni et al., 2021). This method limits the RNAi delivery to only local cells involved in the healing response and limits systemic effects. In theory, bone-forming cells that integrate into the hydrogel would be transfected with a RNAi particle that would improve the intracellular reducing environment and improve bone mineralization and healing. However, as extensively reviewed above, there are many cells involved in bone healing and, currently, there is no way of preventing the RNAi from being delivered to ECs or immune cells, where ROS inhibition could be detrimental to bone healing. There is great opportunity for further research in this promising area.

5.3 Other Bioengineering Techniques that Modulate Reactive Oxygen Species for Bone Healing

Osteogenic differentiation of MSCs is influenced by the production of ROS. Yang *et al.* emphasizes the importance of removing overproduced ROS to promote osteogenesis and bone healing. Their study uses microfluidic technology to prepare fullerol-hydrogel microfluidic spheres (FMSs) created by fullerol nanocrystals injected into hydrogel microspheres that essentially work as stem cell carriers (J. Yang et al., 2021). *In vitro* validatory studies demonstrated that these antioxidant spheres protect BMSCs from oxidative damage by capturing both intracellular and extracellular ROS, in addition to promoting osteoblast differentiation via activation of FOXO1 signaling. When injected into rats with calvaria defects, the FMNs appear to be the reason for significantly increased bone formation (J. Yang et al., 2021). Varini et al. engineered scaffolds using cerium (III) and (IV)-containing mesoporous glass beads, which have been shown to have antioxidant properties (Varini et al., 2019). This group found that osteoblasts cultured in these scaffolds had dampened ROS, improved cell viability, and increased proliferation (Varini et al., 2019). This new biomaterial has not yet been tested in a fracture healing model, and the mechanism behind reduced ROS and increase osteoblast proliferation is unknown.

Other groups have engineered smart biomaterials that sense oxidative stress and release antioxidants when they are needed (Cerqueni et al., 2021; Q.; Xu et al., 2016). These biomaterials consist of a structural framework that can entrap antioxidant compounds, and possibly nanoliposomes with RNAi particles as discussed previously. When these biomaterials encounter oxidative stress, functional groups change their conformation and the polymer network degrades, releasing the housed antioxidants. Of interest to bone regeneration, the number of antioxidants compounds can be altered proportionally, allowing for scalability depending on defect size or application. These biomaterials have a wide range of applications; however, few studies have tested their ability to improve bone regeneration (Yao et al., 2019; Shafiq et al., 2021). Further, Wu et al. has shown that ROS-sensing hydrogels can be combined with structural polymers to improve cartilage regeneration (X. Wu et al., 2021). This hybrid biomaterial allows for ROS-sensing technology, without the degradation of the structural framework that is needed for musculoskeletal applications.

Rather than focusing on reducing ROS, others have acknowledged that an increase in ROS is necessary for osteogenesis, especially for the formation of blood vessels to adequately vascularize bone. Bai et al. demonstrated the use of low-level laser therapy (LLLT) in promoting coupled angiogenesis and osteogenesis by use of a BMSC and biphasic calcium phosphate graft implanted into mice and an endothelial and bone marrow stem cell coculture (Bai et al., 2021). Both *in vivo* and *in vitro* studies demonstrated a significant increase in angiogenesis and osteogenesis as well as upregulation of VEGF, TGF-β, and Hif-1α. Through further investigation, it was found that these effects were induced through the ROS/Hif-1α signaling pathway, emphasizing LLLT's production of ROS as the key feature in promoting angiogenesis and osteogenesis. While their results highlight the increase in ROS being beneficial to angiogenesis and osteogenesis, the study emphasizes a suitable window for osteogenic differentiation, with higher ROS levels damaging osteogenic differentiation at later timepoints (Bai et al., 2021).

Other studies have investigated the use of nanovibration and electrical stimulation (EStim) to produce physiological levels of ROS (Díaz-Vegas et al., 2015; Leppik et al., 2020; Orapiriyakul et al., 2020). Using a nanovibrational bioreactor, Orapiriyakul *et al.* investigated the effects of vibrational amplitude on osteogenesis in MSCs and the development of tissue engineered MSC-laden scaffold (Orapiriyakul et al., 2020). The increase in osteogenesis with an increased amplitude is found to be attributed to changes in adhesion, tension, and ion channel regulation, along with activation of energetic metabolic pathways that result in production of ROS and inflammation. Specifically, they looked at the increase in ROS induced by

nanovibration and concluded that low-level ROS production and inflammation are rather byproducts of successful osteogenesis rather than drivers of the process, and inhibition of ROS results in small effects on osteogenesis (Orapiriyakul et al., 2020). While their conclusions are valid, this study did not investigate ROS levels on the magnitude present during fracture healing. Of course, the osteogenic differentiation process will increase levels of ROS due to the increase in oxidative phosphorylation and inhibiting this level of intracellular ROS (via NAC in this study) will likely not affect osteogenic differentiation. As described extensively above, the fracture healing environment has high levels of extracellular ROS and signaling molecules (**Figure 3**) that can further increase intracellular ROS to damaging levels. That said, this very new nanovibrational technology may have powerful applications in the future. Given that the various vibrational amplitudes can be transferred to collagen scaffolds, there may be an interesting opportunity to design a biofeedback control system that regulates ROS production depending on extracellular ROS and signaling molecules. Further, studies need to be done to investigate if nanovibration can also activate ROS-scavenging pathways, or if their activation is merely a byproduct of the increased ROS levels.

Additionally, a review on the use of EStim in bone tissue engineering shows increases in bone healing and regeneration via numerous cell response mechanisms, notably including controlled induction of ROS at an appropriate physiological level. This review highlights the numerous *in vitro* and *in vivo* experimentation surrounding EStim's osteogenic benefit and future potential as a bone tissue engineering therapy (Leppik et al., 2020). EStim has shown to increase ROS via a ATP/PKC/NOX2 signaling cascade in skeletal muscle (Díaz-Vegas et al., 2015). Thus, much like the nanovibrational study, EStim may induce physiologic levels of ROS that increase osteogenic differentiation via stimulation of MAPK pathways (Díaz-Vegas et al., 2015; Leppik et al., 2020). Overwhelmingly, more studies are needed to test these technologies on bone healing in environments of high oxidative stress.

6 CURRENT CHALLENGES, AND FUTURE PERSPECTIVES

Our understanding of the effects of ROS on cellular processes has been more firmly established through cell biology research in the past several decades, but the therapeutic potential of how to utilize ROS is less agreed upon in the literature. We have outlined the current state of the limited research on how ROS are involved in the early stages of fracture healing, which consists of a range of approaches and some contradictory findings. Undoubtedly, ROS are necessary during the early phases of fracture healing. Thus, dampening these levels could interfere with normal, healthy skeletal tissue healing. Conversely, pathologically high levels of ROS can be damaging to bone healing cells and interfere with important signaling pathways in osteogenesis.

Numerous antioxidants have been explored to decrease ROS levels during skeletal tissue healing, with the hypothesis that they would improve results. However, the results are mixed and vary depending on a multitude of factors. As reviewed, oral antioxidants, such as vitamin C, vitamin E (α-tocopherol), and NAC are promising; however, their benefits seem to be the most pronounced in high-ROS disease states, such as osteoporosis, diabetes, and rheumatoid arthritis. The effect of these antioxidants on normal fracture healing are contradictory. These mixed results are most likely due to the inhibition of healthy (necessary) levels of ROS. Further, it is reasonable to conclude that in larger fractures, an exogenous antioxidant will prohibit necessary levels of ROS to aid in neovascularization during fracture repair. Whereas using an antioxidant in a stable fracture may improve fracture healing via improved bone cell function.

Although there does appear to be opportunities in combining drugs, scaffolds, and engineering principles to optimize ROS levels for improved outcomes, further studies need to be conducted to explore this potential. The mechanisms in which ROS affect cellular processes is complex, as discussed in detail above, and understanding how they specifically contribute to bone homeostasis and repair is vital to developing more directed research for therapeutic applications.

AUTHOR CONTRIBUTIONS

AS compiled background literature, as well as wrote most of the manuscript and created the illustrations. AB was a major contributor to writing the manuscript, as well as compiling background research. YD assisted in writing and planning the manuscript, as well as making final edits. SB edited the manuscript. All authors contributed to the article and approved the submitted version.

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GLOSSARY

ALP alkaline phosphatase
ARE antioxidant response element
ASC adipose-derived Stem Cell
AT α-tocopherol
BMSC bone marrow-derived mesenchymal stem cell
BPM bone morphogenic protein
BTE bone tissue engineering
Dll4 notch ligand delta-like 4
EC endothelial cell
ECM extracellular matrix
EStim electrical stimulation
ETC electron transport chain
FGF fibroblast growth factor
FMS fullerol-hydrogel microfluidic spheres
FOXO forkhead box O family of transcription factors
Gpx glutathione peroxidase
H_2O_2 hydrogen peroxide
Hif-1 α hypoxia-inducible factor 1 α
IGF-1 insulin-like growth factor 1
IL-8 interleukin-8
LLLT low-level laser therapy
miRNA microRNA
MSC mesenchymal stem cell
mtROS mitochondrial-produced reactive oxygen species
NAC N-acetyl cysteine
NF-κB nuclear factor-kappa B
NOX NADPH oxidase
Nrf2 nuclear factor erythroid 2-related factor-2
O_2• superoxide
•OH hydroxyl radical
PDGF platelet-derived growth factor
PI3K phosphoinositide 3-kinase
PPAR peroxisome proliferator activated receptor
PRX peroxide redox proteins
RNAi RNA interference
ROS reactive oxygen species
siRNA short interfering RNA
SOD superoxide dismutase
Tcf/Lef T-cell factor/lymphoid-enhancing factor
VEGF vascular endothelial growth factor

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MINI REVIEW
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Regenerative Endodontics and Minimally Invasive Dentistry: Intertwining Paths Crossing Over Into Clinical Translation

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Regenerative endodontic procedures have been described for over a decade as a paradigm shift in the treatment of immature necrotic permanent teeth, owing to their ability to allow root maturation with subsequent enhancement of the tooth's fracture resistance in addition to the potential for regeneration of vital intracanal tissues. Concomitantly, minimally invasive endodontics is another rising concept with the main concern of preservation of tooth structure. Stemming from their potential to preserve the original tooth structure, both regenerative and minimally invasive endodontics could be considered as two revolutionary sciences with one common goal. Achieving this goal would entail not only employing the appropriate strategies to recreate the ideal regenerative niche but modifying existing concepts and protocols currently being implemented in regenerative endodontics to address two important challenges affecting the outcome of these procedures; conservation of tooth structure and achieving effective disinfection. Therefore, the search for new biomimetic cell-friendly disinfecting agents and strategies is crucial if such a novel integratory concept is to be foreseen in the future. This could be attainable by advocating a new merged concept of "minimally invasive regenerative endodontic procedures (MIREPs)," through modifying the clinical protocol of REPs by incorporating a minimally invasive access cavity design/preparation and biomimetic disinfection protocol, which could enhance clinical treatment outcomes and in the future; allow for personalized disinfection/regeneration protocols to further optimize the outcomes of MIREPs. In this review, we aim to introduce this new concept, its realization and challenges along with future perspectives for clinical implementation.

Keywords: minimally invasive endodontics, regenerative endodontic procedures, biomimetic scaffolds, biomimetic disinfection protocols, stem cells, immature necrotic permanent teeth, tooth structure preservation

INTRODUCTION

Regenerative Endodontics and Minimally Invasive Dentistry in the Wake of the COVID-19 Pandemic

At the end of the year 2019, the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-COV-2) virus changed the face of humanity (Ather et al., 2020). Interestingly, a newly defined role for regenerative therapies has re-emerged with the potential of tissue engineering and regenerative medicine to play a critical role in improving clinical practices amid a global viral outbreak. This can range from the development of *in-vitro* models for drug testing and disease modeling, designing drug delivery systems to optimizing vaccine delivery platforms (Tatara, 2020).

In particular, the dental community faced unique challenges, leading to the development of treatment strategies changes to reduce the risks of virus transmission to patients/dental practitioners (Bahador et al., 2021). In endodontics, a shift towards minimally invasive endodontic procedures was suggested over more invasive modalities highlighting a new role for these therapies in light of the COVID-19 pandemic (Azim et al., 2020).

The Dental Pulp: A Unique Commodity

While the dental pulp is a unique resilient tissue responsible for the survival of one of the human body's most important organs; the tooth, it is often undervalued in routine dental practice. While vital pulp therapies have been routinely implemented in daily endodontic practice for years, performing a partial/complete pulpotomy for mature irreversibly inflamed permanent teeth has been a procedure fraught with scepticism over the past years. Recent evidence highlights that these treatments may present a paradigm shift in endodontic therapies in the upcoming era (Duncan et al., 2019a). Congruently, REPs which have been employed as a predictable treatment for immature necrotic teeth for years, have slowly seen applications for mature necrotic permanent teeth as well (El-Kateb et al., 2020; Glynis et al., 2021). Indeed, the past 2 years have witnessed an incredible increase in publications about REPs citing procedures supporting single visit procedures over multiple visit ones (Cerqueira-Neto et al., 2021).

Although all REPs have been practiced for some time, seasoned endodontists and dental practitioners were always reluctant to apply these techniques in their routine dental practices, dreading the lack of predictability and due to perceptions, on shortcomings of available materials and restorative options. With the advent of advanced tricalcium-silicate cements and sophisticated bonding and restorative materials, successful long-term outcomes are being increasingly documented with reproducibility and obvious advantages for both the patient and the practitioner. This new direction is currently on its way to embedding firm roots in the field with a mental makeshift from a science of "maybe" to a science that has actually transitioned into routine clinical practice; ironically pushed forward by a global pandemic. In this review, we introduce a new concept amalgamating the sciences of MIEs with REPs in the hope of paving the way to strategies that will not only regenerate lost tooth structures but conserve those that still remain as well.

REGENERATIVE AND MINIMALLY INVASIVE ENDODONTIC PROCEDURES: TWO REVOLUTIONARY SCIENCES WITH ONE COMMON GOAL

Minimally Invasive Endodontics: From Tooth Preservation to Tooth Survival

Minimally invasive endodontics (MIEs) concept is based on the assumption that tooth structure preservation during access cavity preparation/canal instrumentation is a vital parameter to maintain strength and fracture resistance of the tooth. Contracted endodontic cavity (CEC) designs conserve a larger amount of the coronal tooth structure, thus reducing stress concentration on the occlusal and cervical areas of the tooth (Yuan et al., 2016). Moreover, minimal canal preparation with less taper contributes to the preservation of more cervical dentin which in turn could enhance the resistance of the tooth to fracture under masticatory loads (Yuan et al., 2016; Silva et al., 2020a). Previous finite element analysis studies performed on standardized virtual models showed larger stress concentration areas in the cervical region of teeth with traditional endodontic cavity (TEC) compared to CEC (Silva et al., 2020a). Despite the benefits, there are controversies in literature concerning the cleaning effectiveness and difficult exploration of root canal systems through these accesses, affecting the longevity of teeth from a biological point of view (Silva et al., 2020a).

Regenerative Endodontics as a Minimally Invasive Treatment Modality

Regenerative endodontic procedures (REPs) utilize the concept of tissue engineering to restore the root canal system to a healthy state, allowing for the continued development and regeneration of the root and surrounding tissues (He et al., 2017). Indeed, the primary objectives of REPs mainly address the conservation and preservation of the remaining tooth structure as well as enhancing its survival, targeting the resolution of Apical Periodontitis (AP), induction of apical closure and increased root canal wall thickness and length of immature teeth (Endodontistso AAo, 2016). Regarding these objectives, REPs and MIEs endodontic concepts relatively share an equivalent aim of conservation and preservation of teeth in a functional state. In other words, REPs aim to restore the original tooth structure while MIEs aim to preserve it.

A recent suggestion has been to redefine the definition of regenerative endodontics as follows: "The term regenerative endodontics should embrace the repair, replacement and regeneration of dentin–pulp lost due to age, disease, trauma or congenital defects to restore normal function" (Duncan et al., 2019b).

Theoretically, if we can sterilize the canal, and recreate a 3D "biological filling" in the form of regenerated vital tissues this would ensure an optimum outcome. Regenerative strategies targeting mature teeth will most likely differ from those for immature permanent teeth, due to the different challenges encountered in both situations regarding the weak natural architecture of immature teeth, in addition to, the complexity of biofilms, toxins, and antigens which could reside in the larger dentinal tubules in these teeth (He et al., 2017; Verma et al., 2017; Almutairi et al., 2019; Fouad, 2020). Excessive removal of tooth structure accompanying a TEC especially at pulp chamber walls and around the canal orifices, may decrease the tooth resistance to fracture during function. This fact could be more prominent in REPs where the bulk of dentin is initially much less compared to mature teeth (Elkholy et al., 2021).

Furthermore, the preservation of peri-cervical dentin in particular which has an anatomical pattern of a lesser inter-tubular dentin and wider dentinal tubule lumen can justify the evidence of greater sequestered growth factors including TGF-β1, FGF2, and VEGF found in this region (Tziafas et al., 2019; Ferreira et al., 2020). This would thus preserve the physiologic and defensive function of dentin which has been proven to have a defensive mechanism that involves the release of pro-inflammatory cytokines and antibacterial agents (Ayoub et al., 2020; Shah et al., 2020). This coupled with the ability to perform REPs in canals with apical diameters as narrow as 0.3 mm offers the possibility to apply a combined regenerative and minimally invasive clinical protocol without the fear of leaving behind residual infected tissues (El-Kateb et al., 2020).

Therefore, combining MIEs and REPs might be considered a logical and new idea. On the other hand, it may complicate the outcomes of REPs due to the limitation of the cleaning and disinfection of the root canal system which could be encountered through the CEC. Hence, if such a strategy is to be employed, considerations must not only be given to how modification of the access cavity will influence adequate disinfection, but also should encourage single visit treatment modalities via the application of biologically inspired and biomimetic scaffolds to guarantee tissue regeneration in a bacteria-free milieu.

DISINFECTION AND REGENERATION; A CAUSE-AND-EFFECT RELATIONSHIP

How Detrimental Really is Residual Bacterial Biofilm?

The vast majority of failed regenerative endodontic treatment cases have been diagnosed with necrotic pulp with/without some form of apical pathosis which strongly suggests the association between persistent root canal infection and treatment failure (Almutairi et al., 2019). Concomitantly, complete root canal disinfection in regenerative endodontics in immature teeth is quite challenging as mechanical debridement entirely relies on the use of minimal mechanical instrumentation to avoid further weakening of the root. Therefore, it is possible that bacteria are incompletely eradicated. Yet, unlike conventional root canal treatment (RCT), lowering intracanal bacterial threshold might not be sufficient for the success of REPs as the residual bacteria might repopulate leading to treatment failure (Verma et al., 2017; Ruparel et al., 2019). Another barrier to regeneration is the effect of residual infection on the ideal fate of stem cells (Verma et al., 2017; Lui et al., 2020). Bacteria and their antigens could modify stem cell differentiation into osteoblastic phenotype and hinder their mineralizing capacity (Vishwanat et al., 2017). Additionally, remaining pathogens alter the induced release of TGF-β1 from dentin (Cameron et al., 2019). Bacterial lipopolysaccharides could also remain after root canal disinfection and promote pro-inflammatory cytokines production (Kato et al., 2014). Additionally, induction of an inflammatory periapical plug was demonstrated to hinder cell migration into the root canal and subsequently prevent tissue regeneration (Zaky et al., 2020). Hence, a prerequisite for dentin-pulp tissue regeneration is creating a pathogen-free intracanal environment and preserve the survivability and function of residual host stem cells (Montero-Miralles et al., 2018).

Cytotoxic Effects of Chemicals Used for Irrigation and Intracanal Medicaments and Their Effects on Prognosis of REPs

Optimally, the disinfecting agents used in REPs must balance between having a wide spectrum of antibacterial activity and the ability to promote attachment, proliferation, and differentiation of stem cells (Ayoub et al., 2020). The latest protocol for regenerative endodontic procedures published by the American Association of Endodontists (AAE) included irrigation by 1.5% sodium hypochlorite followed by the use of either calcium hydroxide $Ca(OH)_2$ intracanal medication or low concentrations (1–5 mg/ml) of antibiotic pastes, and 17% EDTA is used in the second visit (AAE, 2018). However, these diluted antibacterial agents could still have a detrimental effect on stem cells. It was reported that, after 1 h of exposure, 1% and 5% EDTA significantly reduced the viability of stem cells of apical papilla (SCAP) and periodontal ligament stem cells, while no cell survival was observed with 1% NaOCl (Scott et al., 2018). Concentrations as low as 1 mg/ml $Ca(OH)_2$ and 0.05 mg/ml TAP significantly supressed SCAP proliferation and odontogenic capacity which is coordinated with lack of continued root development and hard tissue deposition (Bi et al., 2018).

Additionally, it was found that 5 mg/ml of double antibiotic paste had significant negative effects on viability, alkaline phosphatase activity, and mineralization nodule formation of human dental pulp stem cells (DPSCs) (McIntyre et al., 2019). On another front, the current regenerative protocol could elicit indirect stem cell cytotoxicity due to failure to completely eradicate root canal pathogens which in turn activate the immune system leading to cell death (Jewett et al., 2010; Verma et al., 2017; Vishwanat et al., 2017).

Influence of Different Irrigants and Intracanal Medicaments on the Architecture and Physical Properties of Radicular Dentin

The biomechanical performance and physical strength of immature teeth after REPs is a prerequisite for their long-term

survival and success of treatment (Ali et al., 2019). Consequently, the potential added weakening of the immature roots by chemical disinfectants should be avoided. Yet, irrigants and intracanal medications used in REPs may adversely affect the mechanical and physical properties of radicular dentin. It has been reported that immature teeth treated with the regenerative disinfection protocol are more prone to fracture, especially at the cervical area, even after tooth reinforcement with adhesive restorations (Ali et al., 2019). Also, the use of EDTA, $Ca(OH)_2$, and the diluted concentrations of sodium hypochlorite and antibiotic pastes currently recommended by the AAE were found to significantly decrease dentin microhardness and cause demineralization (Yassen et al., 2015). From a biological perspective, the impact of these agents on the dentinogenic capacity of stem cells should not be overlooked.

Taken together, the antibacterial efficiency of diluted irrigants/intracanal medicaments recommended by the AAE is still an open question. Moreover, eradication of root canal bacteria could be less attainable when adopting the CEC design in regenerative endodontics which cannot be overcome by increasing the concentration of the already harmful disinfectants. This entails the search for new biomimetic cell-friendly disinfecting agents that exert a broad-spectrum antibacterial action without affecting the stem cells or root dentin. This might be the key to achieving successful clinical outcomes for MIREPs.

ALTERNATIVE "BIOMIMETIC" STRATEGIES COULD DECREASE CYTOTOXICITY AND PRESERVE THE INTEGRITY OF NATIVE DENTAL TISSUES

Alternatives to Conventional Irrigation Protocols Using Nanomaterials to Enhance the Potency and Decrease Harmful Effects

Recent research in antimicrobial strategies are aimed to enhance the potency of disinfection without having detrimental consequences. Novel disinfectants such as ozone (Kustarci et al., 2009; Silva et al., 2020b), photodynamic therapy (Zorita-García et al., 2019) and cold atmospheric plasma (Pan et al., 2013; Li et al., 2015) have been tried with variable outcomes and only limited clinical success in the field of endodontics. On the other hand, nanoscience has been a breakthrough in almost every field of science and medicine, holding special importance as regards to endodontic disinfection.

Notably, nanobubble water (NBW), has recently been introduced as a promising antimicrobial agent that could be used in many medical, dental and pharmaceutical fields. The application of NBW in Endodontics is still in its infancy, where it is recommended as a promising agent to upgrade the antimicrobial activity of root canal irrigants at lower non-cytotoxic concentrations. It exerts a kinetic force to dislodge pre-established intracanal biofilm and enhance the delivery of root canal medicaments, where it can be of beneficial in REPs. NBW was shown to be more effective in removing smear layer and at the same time preserves dentin microhardness when compared to 17% EDTA. Moreover, NBW in combination with 1.5% NaOCl was proven to be as effective as 5.25% NaOCl regarding the disinfection ability with enhanced penetration into dentinal tubules (Aksel et al., 2020; Osman et al., 2020).

Another promising biomimetic irrigant using catalytic iron-oxide nanoparticles (IO-NPs) has been introduced. The concept was to use IO-NPs in combination with hydrogen peroxide showing potent antimicrobial effects with deep penetration into the entire length of dentinal tubules. However, IO-NPs alone had modest antibacterial effects (Bukhari et al., 2018).

Cell/Tissue Friendly Alternatives to Common Intracanal Medicaments

As previously overviewed, the use of either $Ca(OH)_2$ or antibiotic combinations can cause adverse side effects during REPs. The former is responsible for root weakening even after a short period (Bukhari et al., 2018). The latter has been proven to impair functions of DPSCs even when used at the acceptable clinically recommended dosage (Diogenes et al., 2016). Recently, a nitric oxide (NO)-releasing nanomatrix gel has been introduced for controlled intracanal delivery of antibiotics (Moon et al., 2018). It was suggested that a minimal concentration of antibiotics combined with the effects of NO could be promising as an intracanal medicament, favoring root maturation and revascularization potential compared to the conventional protocol of REP (Kaushik et al., 2015; Moon et al., 2018).

Another promising alternative is the use of probiotics as a potential intracanal medicament. Local delivery of probiotics has shown promising outcomes regarding the eradication of common endodontic pathogens (Bohora and Kokate, 2017; Kim et al., 2020). Moreover, probiotics are proven to increase IL-10 release and decrease the release of IL-1 and IL-6 (Cosseau et al., 2008; Pahumunto et al., 2019) which could be beneficial in modulating the severity of apical periodontitis. However, criteria for the selection of probiotics still needs further investigation focusing on optimizing probiotics delivery to tolerate local stress and adhere to the site of application.

Scaffolds With Dual Effect i.e., Antibacterial and Immunomodulatory Effects Could Favour Regeneration Rather Than Repair

Several recent researches have focused on the development of the smart drug delivery scaffolds to both augment root canal disinfection and reduce the risk of stem cell toxicity (Albuquerque et al., 2017; Bottino et al., 2019). An antibiotic-releasing injectable Platelet-Rich-Fibrin (I-PRF) scaffold was tested against a dual-species biofilm colonized inside the root canal, showing significant reduction in the bacterial count. The engineered biocompatible autologous scaffold that contained both antibiotics and growth factors, could favor regeneration rather than repair (Rafiee et al., 2020). Moreover, THP-1 macrophages were seeded on a concentrated growth factors (CGF) scaffold, revealing the immunoregulatory role of CGF

in macrophage functional activities. The abundance of bioactive factors in the CGF extract facilitated M2 macrophage polarization and modulated cytokine secretion. These results shed light on the therapeutic potential and mechanisms of CGF in regulating the macrophage-mediated immune response, essential for tissue remodeling and healing (Luo et al., 2021).

Lately, an *in-vitro* study has evaluated a novel biomimetic scaffold composed of electrospun poly (lactic acid) nanofibers and electro-sprayed polycaprolactone with tannic acid micro-particles. This 3D cone-shaped scaffold was designed to fill the empty root canal mimicking the architecture of natural extracellular matrix (Terranova et al., 2021). Showing promising cell migration, attachment and proliferation potential, this scaffold could be further loaded with antimicrobial agents in future *in-vivo* studies.

Another interesting scaffold would be the use of decellularized tissues namely extracellular matrices (ECMs) extracted from dental pulp and dentin tissues. It was reported that these extracts have an antibacterial effect against *Streptococcus mutans*, *Streptococcus oralis* and *Enterococcus faecalis* (Smith et al., 2012). Moreover, a functional component of ECM based bio-scaffolds was recently identified, the so called, matrix-bound nanovesicles. These extracellular vesicles were found to be associated with favorable properties including enhanced angiogenic potential, stem cell recruitment and modulation of macrophage phenotype (Hussey et al., 2019; Quijano et al., 2020). Furthermore, the presence of antimicrobial peptides within injectable decellularized ECM scaffolds has been documented and might play a significant role in future studies in the field of regenerative endodontics (Jimenez-Gastelum et al., 2019; Wei et al., 2021).

Incorporating Inflammatory Cytokines, Exosomes and Secretomes Into Scaffolds to Trigger Natural Healing/Regeneration Cascades

The parallel use of immunomodulatory and homing factors in scaffold materials has been shown to induce stem cell homing, modulate cell differentiation and indeed induce regeneration. Cathelicidin peptide (LL37) has been reported to activate the ERK signaling pathway boosting the secretion and expression of vascular endothelial growth factor (VEGF), thus promoting pulp wound healing by inducing angiogenesis (Khung et al., 2015). Likewise, interleukin (IL)-4 was incorporated in high-stiffness transglutaminase crosslinked gelatins scaffold promoting the M2 polarization and guide endogenous cell homing which represents a simple and effective strategy that can support high levels of tissue regeneration (He et al., 2019).

The use of cell-type specific exosomes (micro-vesicles) has also recently gained considerable attention in the field of regenerative medicine. These biomimetic cell products have been proven to be a driving force behind the immunomodulatory effects of mesenchymal stem cells due to their direct effect on cytokine release and M2 macrophage polarization (Zhang et al., 2014). Dental pulp-derived exosomes were shown to trigger the expression of odontogenic genes and odontogenic differentiation of Schwann cells (Huang et al., 2016; Li et al., 2021), enhance angiogenesis (Xian et al., 2018) and promote regeneration of dentin-pulp like tissues (Huang et al., 2016; Ivica et al., 2020). When incorporated within injectable hydrogels, pulp stem cell-derived exosomes were found to accelerate healing of apical periodontitis through a macrophage-dependent response (Shen et al., 2020). Another promising approach is the utilization of DPSC-conditioned medium in various regenerative endodontic applications. This secretome contains trophic factors needed to regulate the inflammatory state (Sarra et al., 2021) as well as possessing significant angiogenic, odontogenic and immunomodulatory potential (Zhou et al., 2021). Therefore, personalized utilization of various functionalized scaffolds in a case-specific manner could indeed lead to optimal regenerative outcomes through local modulation of immune response, creation of a favorable intracanal microenvironment for true regeneration and orchestrating healing cascades of periapical wounds.

DISCUSSION

Disinfection is a critical factor to ensure periapical healing in regenerative endodontics where literature has shown that residual infection can lead to either failure or hinder continued root development (Verma et al., 2017). Having said this, most immature permanent teeth indicated for regenerative endodontic procedures have an etiology of trauma and hence the tooth is mainly sound (Koç and Del Fabbro, 2020). Additionally, many of these teeth are subject to multiple traumatic incidents (Koç and Del Fabbro, 2020) increasing the chances of loss of these teeth. Hence a major critical objective for any type of endodontic treatment; conventional or regenerative is to ensure optimal tooth survival and retention (Elkholy et al., 2021). By minimizing tooth structure removal as we elaborated in this review, fracture resistance of such teeth can be maintained (Isufi et al., 2020; Elkholy et al., 2021), at the same time, to avoid sacrificing disinfection for the sake of preserving tooth structure, we suggest an alternative enhanced disinfection protocol that would conserve sound tooth structure while at the same time maintain an appropriate level of disinfection to allow stem cell migration and success of the final regenerative treatment.

Based on the previous review of literature, the current review suggests the possible implementation of a new concept of minimally invasive regenerative endodontic procedures (MIREPs). Starting from the access cavity design, CEC for non-decayed traumatized teeth and caries-driven-access cavity for decayed teeth, would represent more suitable tooth preserving designs, where the volume of dentin and enamel removal in case of CEC designs was found to be less than 15% which was significantly less than enamel and dentin removal in TEC (Isufi et al., 2020). Additionally, according to a recent systematic review and meta-analysis, a significant increase in fracture resistance was noted when CEC preparation was performed with preservation of tooth marginal ridges (Ballester et al., 2021). Thus this approach, accompanied by minimal mechanical preparation of root canal walls (El-Kateb

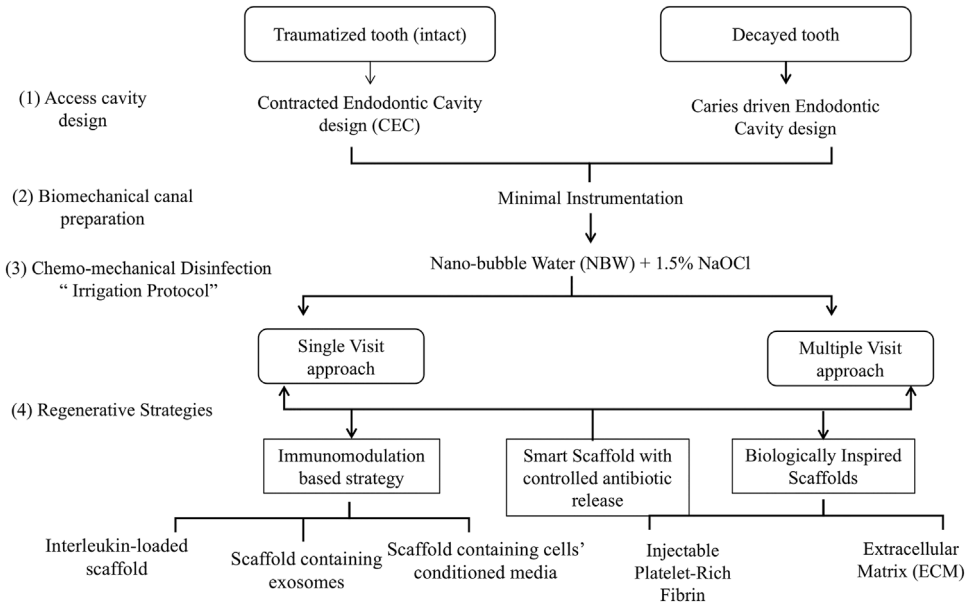

FIGURE 1 | Flow Chart representing possible strategies to implement for "minimally invasive regenerative endodontic procedures (MIREPs)."

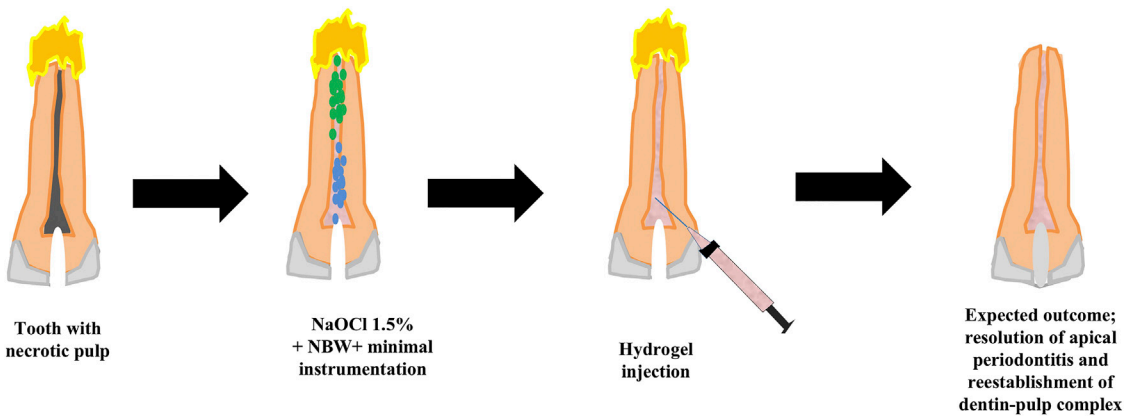

FIGURE 2 | Showing the suggested clinical protocol of MIREPs. A traumatized permanent tooth with a necrotic pulp and periapical disease would be accessed using a contracted Endodontic Cavity design. This would be followed by combined irrigation of 1.5% sodium hypochlorite and nanobubble water to disinfect the canal, dislodge biofilms and release sequestered growth factors. Extracellular Matrix-based hydrogel would then be injected into the pulp space and the tooth finally sealed with bioceramic material and restored by composite resin.

et al., 2020), would offer a chance to preserve the coronal tooth structure and maximize the release of growth factors sequestered within the pericervical dentin (Tziafas et al., 2019).

As it was agreed that proper root canal disinfection through a cell- and tissue- friendly root canal irrigant and intracanal medicament is crucial for a successful outcome of minimally invasive REPs. NBW is hence recommended as an adjunct irrigant to enhance the anti-microbial activity hindered by the lesser accessibility of CEC designs. It has been proved to be efficient against multiple Gram + ve and Gram -ve bacteria with killing time ranging from 2 to 10 min (Osman et al., 2020). A suggested disinfection protocol of combined NBW with 1.5% NaOCl would represent a chemo-mechanical combination through benefiting the mechanical sheared dislodgement of attached biofilms of NBW to a shortly applied chemical disinfection of NaOCl (Aksel et al., 2020).

Possible scenarios for implementation of a MIREP concept as well as a suggested clinical protocol are provided in **Figures 1**, **2** according to the clinical need of each respective case; for example whether the infection is a long-standing one or not and whether a single visit or multiple visits regimen is recommended, there can be several proposed regenerative strategies. The nature/source of the scaffold to be placed could have immunomodulatory function (Khung et al., 2015; Shen et al., 2020), antibacterial action with

controlled antibiotic release (Moon et al., 2018) or a biologically inspired scaffold with dual action (Hussey et al., 2019; Rafiee et al., 2020). One suggested single visit-MIREP protocol for a traumatized permanent maxillary necrotic tooth (**Figure 2**) proposes a contracted endodontic cavity design followed by minimally tapered canal preparation and a disinfection regimen including combined NBW with 1.5% NaOCl followed by the placement of ECM-based hydrogel with/without cell-specific exosomes. Eventually a more optimized treatment outcome might be obtained through this novel biomimetic preservation-regeneration approach and implementing a novel concept of "minimally invasive regenerative endodontic procedures (MIREPs)."

AUTHOR CONTRIBUTIONS

HE: drafted the original manuscript, wrote the section on recent alternatives to medicaments, scaffolds and immunomodulatory agents as well as the discussion section, and designed and executed the figures and edited the final manuscript; MA: drafted the original manuscript, contributed to the discussion section and wrote the section on relationship between minimally invasive endodontics and regenerative endodontic procedures, and edited the final manuscript; AM: drafted the original manuscript, contributed to the section on recent alternatives to medicaments, scaffolds and immunomodulatory agents and edited the final manuscript; MH: wrote the section on effect of biofilms and infection on fate of cells and outcome of regenerated tissues and edited the final manuscript; HA: contributed to the section on relationship between minimally invasive endodontics and regenerative endodontic procedures, and edited the final manuscript; MG: contributed to the section on relationship between minimally invasive endodontics and regenerative endodontic procedures, and edited the final manuscript; RE: substantially contributed to conceptualization, writing, drafting and editing of the entire manuscript. All authors contributed to designing the manuscript as well as revising and approving the final manuscript version.

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REVIEW
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The Added Value of the "Co" in Co-Culture Systems in Research on Osteoarthritis Pathology and Treatment Development

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Osteoarthritis (OA) is a highly prevalent disease and a major health burden. Its development and progression are influenced by factors such as age, obesity or joint overuse. As a whole organ disease OA affects not only cartilage, bone and synovium but also ligaments, fatty or nervous tissue surrounding the joint. These joint tissues interact with each other and understanding this interaction is important in developing novel treatments. To incorporate and study these interactions in OA research, several co-culture models have evolved. They combine two or more cell types or tissues and investigate the influence of amongst others inflammatory or degenerative stimuli seen in OA. This review focuses on co-cultures and the differential processes occurring in a given tissue or cell as a consequence of being combined with another joint cell type or tissue, and/or the extent to which a co-culture mimics the *in vivo* processes. Most co-culture models depart from synovial lining and cartilage culture, but also fat pad and bone have been included. Not all of the models appear to reflect the postulated *in vivo* OA pathophysiology, although some of the discrepancies may indicate current assumptions on this process are not entirely valid. Systematic analysis of the mutual influence the separate compartments in a given model exert on each other and validation against *in vivo* or *ex vivo* observation is still largely lacking and would increase their added value as *in vitro* OA models.

Keywords: osteoarthirits, co-culture models, *ex vivo*, *in vitro*, tissue communication

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1 INTRODUCTION

Osteoarthritis (OA) is a degenerative and progressive joint disease affecting approximately 500 million people worldwide (Hunter et al., 2020). High age, obesity, a previous joint injury or chronic joint overuse are traits often connected with OA (Bijlsma et al., 2011) as is altered load distribution (Abramoff and Caldera, 2020). Within these phenotypes, inflammatory factors are variably involved in disease development and progression (Musumeci et al., 2015). The susceptibility to develop osteoarthritis can be increased by specific genetic alterations of proteins, for instance, involved in inflammatory processes or of components of the cartilage matrix (Sandell, 2012). Although a highly prevalent disease, current treatment options are mainly reducing the symptoms of OA: pain and loss of mobility, with pain management and physiotherapy as the main treatment strategies in primary treatment of OA (Hermann et al., 2018; Skou and Roos, 2019). As they fail to stop or reverse degenerative processes, prosthetic joint replacement is often the last resort in end-stage disease (Abramoff and Caldera, 2020).

OA is a whole organ disease affecting all tissues in and adjacent to the joint. The pathology of OA includes degradation of articular cartilage and ligaments, synovial inflammation (synovitis), malformation of subchondral bone and osteophyte formation. Adjoining muscles and nerves can also be affected by OA (Loeser et al., 2012; Schulze-Tanzil, 2019). The most investigated feature in OA treatment and research is articular cartilage degeneration. In healthy joints, cartilage forms a smooth surface that allows joint movement with very low friction (Johnston, 1997). In OA, the activity and phenotype of the resident chondrocytes are altered, with an increased activity of extracellular matrix degrading enzymes such as ADAMTS and collagenases causing structural and functional changes of the tissue (Goldring, 2000). Influx of immune cells into the synovium which forms the inner joint capsule is assumed to mediate cartilage degeneration by producing inflammatory mediators inducing production of matrix degrading enzymes in the cartilage and reducing synthetic activity (Pessler et al., 2008). The synovium also plays a role in OA pain development by promoting neurogenic inflammation mediated by neuropeptides such as substance P (Stanisz, 2001). The infrapatellar fat pad (IPFP), a tissue directly connected to the synovium, can be involved in OA progression as well. Cytokines and growth factors secreted from immune cells within the IPFP as well as adipokines produced by the adipose tissue increase the content of pro-inflammatory cytokines in the IPFP and in adjoining tissues (Klein-Wieringa et al., 2013). However, the infrapatellar fat pad might also play a beneficial role in joint health. An inverse relation between IPFP size and loss of joint space width in OA patients indicated a potential role of the IPFP as a shock absorber in the joint (Ioan-Facsinay and Kloppenburg, 2013; Han et al., 2014). Finally, a clear interaction between bone and cartilage is present. An increase of TGF-β in subchondral bone, for instance, was found to decrease proteoglycan content in the adjacent cartilage and mediate the OA development in an ACLT mouse model (Zhen et al., 2013). Microcracks and fissures in the subchondral bone are thought to facilitate exchange of molecules between bone and cartilage in human osteochondral explants from OA patients, e.g. by increased hydraulic conductance (Hwang et al., 2008; Yuan et al., 2014). Increased vascularization of the subchondral bone due to OA is also thought to increase the exchange (Yuan et al., 2014). Taken together, the influence as well as the interconnection of different joint compartments on OA pathogenesis has been clearly demonstrated.

Although for final proof of the relevance of these interactions commonly *in vivo* studies are carried out, recently the interest in the use of *in vitro* models to replace *in vivo* studies in joint research has intensified, especially against the backdrop of the societal demand to reduce animal use (Ormandy and Schuppli, 2014). Several types of *in vitro* models are available [elegantly reviewed by Piluso et al. (2019)], of which co-cultures of different cells or tissues are most suited to address the role of interaction between joint tissues in OA pathophysiology. Studying the connection between the different cell types and joint structures could further improve the understanding of OA development and progression. Consequently, the development of therapies for patients suffering from joint diseases would be enhanced. This review will therefore discuss co-culture systems with a focus on their potency to address tissue interaction and their use in drug development in OA. Many co-culture models are used based on the assumption that the mere combination of tissues or cells results in an interaction between the cells in these tissues, and that this interaction reflects OA processes *in vivo*. In our review we therefore limited our overview of co-culture systems to those in which a clear interaction was demonstrated through differential behaviour of tissues or cells caused by the presence of the other tissue(s)/cells, and/or if a culture system was shown to reflect *in vivo* pathophysiological phenomena. Also co-cultures combining cells or tissues as part of regenerative approaches (e.g. MSCs and chondrocytes) were excluded.

2 CELL-BASED TWO-DIMENSIONAL CO-CULTURE MODELS

The interplay of different cell types can be investigated using co-culture. Indirect co-culture models, so culturing one cell type in conditioned medium of another cell type of interest can provide valuable insights. To add more complexity to a monolayer culture, a second cell type is added to form a direct co-culture system. Cells can either be mixed directly and seeded into a monolayer culture or separated using cell culture inserts (**Figure 1**). A benefit of co-culture models is that they are able to demonstrate cell-cell interaction of different cell types involved in OA onset or progression (see **Table 1**). Ideally, cell-based co-culture departs from primary cells. Although cell lines are easier to access and handle, they sometimes lack traits of primary cells, such as the production of inflammatory factors upon stimulation (Santoro et al., 2015).

Conditioned medium of osteoarthritic osteoblasts could increase activity and expression of matrix degrading enzymes such as ADAMTS-4, ADAMTS-5 or various matrix metalloproteases (MMPs) in non-arthritic chondrocytes compared to chondrocytes in conditioned medium from non-arthritic osteoblasts or medium only (Prasadam et al., 2012). The influence of peripheral blood mononuclear cells (PBMC) in osteoarthritic joints was studied by co-culture of human chondrocytes with $CD4^{+}CD127^{dim/-}$ enriched PBMCs. A significant increase of MMP-1 and ADAMTS-5 was found upon co-culture, compared to chondrocytes cultured alone (Platzer et al., 2020).

As rupture of anterior cruciate ligaments (ACL) can result in OA development, the impact of synoviocytes on ligament fibroblasts upon stimulation with TNFα and/or mechanical stress was investigated (Wang et al., 2019). Synoviocytes and ACL fibroblasts obtained from patients undergoing knee replacement after an accident were co-cultured using culture inserts. While TNF-α stimulation slightly increased MMP-1, -2, and -3 and decreased lysyl oxidase (LOX) expression, a marker for ligament healing, these effects were amplified by addition of synoviocytes to the injured ACL fibroblasts. Hence, in this model, the behavior of synoviocytes seems to be mainly inflammatory (Wang et al., 2019). To what extent the synoviocytes of acutely

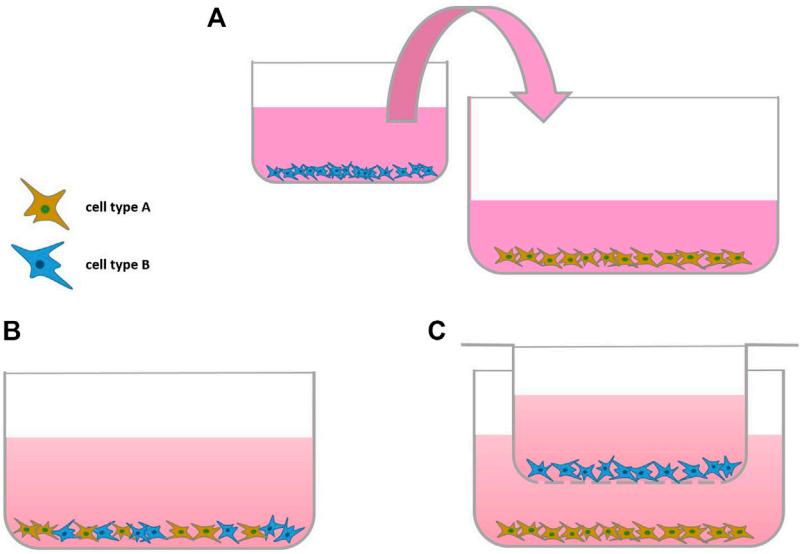

FIGURE 1 | Scheme of Monolayer Co-cultures: **(A)** indirect co-culture, **(B,C)** direct co-culture without and **(C)** with using a cell-culture insert (transwell).

TABLE 1 | Summary of the effects within different co-culture models in monolayer.

Model	Cell	Cell origin	Effect of co-culture	Additional stimulus	Effect on monoculture	Effect on co-culture	References
ACL fibroblasts & synovial fibroblasts	ligament fibroblasts	human	lysyl oxidase ↓	TNFα	MMPs ↑	MMPs ↑↑	Wang et al. (2019)
				TNFα + mech. stress	MMPs ↑	lysyl oxidase ↓↓	
chondrocytes & synovial fibroblasts	chondrocytes	human		IL-1β		H3Ser10 phosphorylation, NFκB activity ↑	Pagani et al. (2019)
chondrocytes & OA osteoblasts	chondrocytes	human	MMPs ↑, ADAMTS-4,-5 ↑ by conditioned medium				Prasadam et al. (2012)
chondrocytes & mononuclear (MN) cells	chondrocytes	human	MMPs ↑, ADAMTS-4,-5 ↑				Platzer et al. (2020)
	MN cells	Human	not described				

injured joints can be considered as diseased and therefore the phenomena observed match those occurring *in vivo* in OA, cannot be verified with certainty, however.

3 CELL-BASED THREE-DIMENSIONAL CO-CULTURE MODELS

2D culture fails to recapitulate the three-dimensional organization of cartilage and other tissues of the joint. Therefore, usually 3D cultures of chondrocytes or cells encapsulated into hydrogels are utilized. Both natural and synthetic hydrogels are used for the bioengineering of tissues (**Table 2**) (**Figure 2**). They are thought to mimic the physiological environment and support viability, proliferation and secretory abilities (Lee and Mooney, 2001; Vinatier and Guicheux, 2016) especially from chondrocytes. Care must be taken in their selection, however, as biomaterials affect cell phenotype (Tsuchida et al., 2013), which may be in part due to hydrogel biomechanical properties and the possibility for material-cell interaction (Engler et al., 2006; Krouwels et al., 2018).

3.1 Chondrocyte and Monocytes/ Macrophages

Co-culture with macrophages reflects the interaction of the type B cells in the synovial lining with cartilage. Upon addition of a LPS-activated murine macrophage cell line to human OA chondrocytes cultured in poly-(ethylene glycol)-diacrylate (PEGDA) using culture inserts, a significant increase of MMP-1, MMP-3, MMP-9, MMP-13, IL-1β, TNF-α, IL-6, IL-8, and IFN-γ compared to OA chondrocytes in monoculture was observed (Samavedi et al., 2017). Conversely, in macrophages, IL-1β and Arginase-1, a macrophage inflammatory marker, were significantly increased by the presence of the

TABLE 2 | Summary of the effects within different cell-based bioengineered co-culture models.

Model	Hydrogel	Cell origin	Cell	Effect of co-culture	Additional stimulus	Effect on monoculture	Effect on co-culture	References
MSOD & HUVEC	GelMA-based	human	MSOD	osteogenic differentiation	a) IL-1β, TNF-α, IL-6 b) cond. chondrocyte medium	ALP↑	ALP↑↑, mineralization ↓ mineralization ↓	Pirosa et al. (2021)
			HUVEC		a) IL-1β, TNF-α, IL-6 b) conditioned chondrocyte medium	VEGF↑ endoth. network formation	VEGF ↑↑, endoth. network maintenance ↑ no network formation	
OA chondrocytes activated macrophages	PEGDA	human	chondrocytes	MMPs ↑, IL-1β, TNF-α, IL-6, IL-8 and IFN-γ ↑				Samavedi et al. (2017)
		murine	macrophages	IL-1β and Arginase-1 ↑				
chondrocytes & activated macrophages	gelatin	porcine	chondrocytes	MMPs ↑, coll II and aggrecan exp.↑; proliferation ↑, coll II GAG content ↓				Peck et al. (2014)
		murine	macrophages	not described				
chondrocytes & osteoblasts	alginate	human	chondrocytes		a) human sclerotic osteoblasts b) IL-1β + IL-6 or OSM		MMP-3, ADAMTS-4,-5 ↑ SOX-9 and coll II ↑, aggrecan ↓ MMPs ↑, aggrecan ↓	Sanchez et al. (2005a), Sanchez et al. (2005b), Lin et al. (2010), Sanchez et al. (2015)
		porcine	chondrocytes	hypertrophy (coll II, aggrecan↓; coll X, bone sialoprotein ↑)	mech. stress		MMPs, ADAMTS-4, -5 ↑, hypertrophy ↑↑	
		a) or b)	osteoblast	not described				
chondrogenic and osteogenic diff. hBMSC	GelMA-based bio-reactor	human	chondrogenic cells		IL-1β	MMPs↑	MMPs↑↑	Lozito et al. (2013), Lin et al. (2014)
chondrocytes and synovial fibroblasts	alginate	murine	chondrocytes		IL-1β		proteoglycan ↓, NO and PGE_2 ↑	Gouze et al. (2004)
			syn. fibroblast	not described				
joint on a chip	fibrin	human	HUVEC		a) TNFα + chemokines b) OA synovium		monocyte extravasation ↑ + chemokines ↑↑ monocyte extravasation ↑↑	Mondadori et al. (2021)
			OA chondrocytes	not described				
			syn. fibroblasts	not described				

chondrocytes. This suggests that changes induced by OA in either cell type intensified response in the neighboring cell type, highlighting the use of such cultures to study the crosstalk of macrophages and chondrocytes in OA (Samavedi et al., 2017). The catabolic marker panel found was similar to that observed *in vitro* OA models, indicating the validity of the model (Blasioli et al., 2014; Samavedi et al., 2017). Whether the effect of OA chondrocytes on activated macrophages was specific for the OA state is not clear, as healthy chondrocytes were not included in the study. Addition of LPS-activated and non-activated murine macrophages to gelatin-embedded porcine chondrocytes increased chondrocyte expression of MMP-1, MMP-3 after 1 week of co-culture (Peck et al., 2014). Activated macrophages also increased cell proliferation as well as collagen II and aggrecan expression, which was claimed to mimic anabolism in early OA. However, at the protein level total GAG and collagen II content were significantly reduced upon co-culture with both unstimulated and LPS-stimulated macrophages. Celecoxib treatment of the co-culture with LPS-activated macrophages was able to significantly reduce MMP-1 and MMP-3 expression after 3 days. PGE_2 levels were significantly reduced after 7 days of

TABLE 3 | Summary of effects within different tissue explant-based co-culture models.

Model	Cell/ tissue origin	Cell/tissue	Effect of co-culture	Additional stimulus	Effect on monoculture	Effect on co-culture	References
cartilage & synovial fibroblasts	equine	cartilage		a) mechanic. stress b) IL-1β	cell clusters & focal cell loss↓	coll II ↑ aggrecan ↑ GAG loss ↓	Gregg et al. (2006), Lee et al. (2013)
	equine	synovial fibroblasts		mechanical stress		ADAMTS-4,-5 ↓, MMP-1 ↑, MMP-3 ↓	
chondrocytes & synovium	rat	chondrocytes		injury on synovium		early OA: aggrecan ↑, late OA: MCP-1 ↑	Lai-Zhao et al. (2021)
	rat	synovium	not described				
damaged ACL & chondrocytes	human	chondrocytes		a) acute damaged ACL b)chronic damaged ACL		coll II ↓ and ADAMTS-4 ↑ periostin ↑ col II ↑ MMP-13 and ADAMTS-4 ↑ periostin ↑ IL-1 ↓	Chinzei et al. (2018)
periosteum & chondrocyte pellets	human	periosteum	COL1A1 ↑, TGF-β↑ IL-6, MMP-2, -7, -13 ↑				Grässel et al. (2010), Rickert et al. (2010), Steinhagen et al. (2012)
	bovine	periosteum	coll II deposition↓ GAG synthesis & release ↓				
	human	chondrocytes	collagen I deposition				
cartilage & synovium	canine	cartilage	maintained proteoglycan content	IL-1β	MMP-13 ↑	proteoglycans↓ gene expression e.g. coll II & MMPs closer to OA patient material	Hardy et al. (2002), Cook et al. (2007), Beekhuizen et al. (2011)
	canine	synovium				COX-2, PGE_2 ↑	
OA cartilage & OA synovium	human	cartilage	MMP-13 ↑, cell viability ↓, GAG production ↓, (GAG release ↑)				
	human	synovium	not described				
cartilage & joint capsule	bovine	cartilage	MMP-13, ADAMTS-4 ↑	mechanical injury		aggrecan digestion↑ MMP-3, ADAMTS-4, -5↑	Lee et al. (2009), Swärd et al. (2017)
	bovine	joint capsule	not described				
osteochondral & synovium	equine	osteochondral explant	collagen II ↑	a) IL-1β	TNF-α, MMP-13 ↑↑	TNF-α, MMP-13 ↑	Byron and Trahan. (2017), Haltmayer et al. (2019)
				b) mech. injury, IL-1β and TNF-α	MMP-1 ↑	MMP-1 ↑↑	
	equine	synovium		b) mech. injury, IL-1β and TNF-α		macrophage shift → M1	
meniscus & OA synovium	human	meniscus	IL-6, IL-8 ↑, MMP-3,-10 ↑, GAG release ↑				Favero et al. (2019)
	human	synovium	not described				
osteochondral	human	osteochondral explant		a-c) respectively a) IL-1 β b) mech. injury c) triiodothyronine d) LPS		MMP-13 & HIF-2α ↑ COL1A1 ↑ senescence markers ↑ COL2A1 ↓ IL-6, MCP-1 ↑	Geurts et al. (2018), Houtman et al. (2021a), Houtman et al. (2021b)
dorsal root ganglia & OA synovium	rat	dorsal root ganglia	neurokinins, neuropeptide Y ↑				Li et al. (2011)
	human	OA synovium	not described				
cartilage & fat	bovine	cartilage	GAG release ↑				Nishimuta et al. (2017); Zhou et al. (2020)
	human	cartilage		OA IPFP cond. medium		collagen & proteoglycan loss MMP-3, COX-2↑	
		chondrocytes		OA IPFP cond. medium		p38MAPK and ERK1/2↑, MMPS, ADAMTS-4 ↑, IL-1β, IL6 and COX-2 ↑	
	either	fat	not described				

TABLE 4 | Summary of effects of drugs in mono and co-cultures.

Model	Species	Cell/tissue	Additional stimulus	Treatment	Treatment effect on monoculture	Treatment effect on co-culture	References
chondrocytes and synoviocytes	human	chondrocytes	IL-1β	NAPA		NFκB pathway activity↓	Pagani et al. (2019)
chondrocytes in gelatin and LPS-activated macrophages	porcine	chondrocytes		celecoxib		MMP1,-3, PGE_2 ↓	Peck et al. (2014)
chondrocytes in alginate and sclerotic osteoblasts	human	chondrocytes osteoblasts		carnosol pre-treated osteoblasts carnosol	IL-6 and PGE_2↓	aggrecan production ↑, MMP-3, ADAMTS-4, -5 ↓	Sanchez et al. (2015)
joint-on-a-chip (fibrin-based)	human	HUVEC	chemokine mix (CCL 2-5)	chemokine receptor antagonist		monocyte extravasation↓	Mondadori et al. (2021)
cartilage and synovium	bovine	cartilage	IL-1α	IL1 receptor antagonist	GAG & collagen loss↘	GAG & collagen loss ↓↓	Mehta et al. (2019)
	bovine	cartilage		ADAMTS-5 targeting nanobody		GAG loss ↓	Siebuhr et al. (2020)
	canine	cartilage	IL-1	hyaluronic acid		MMP-3 ↓, GAG content ↑	Greenberg et al. (2006)
OA cartilage and OA synovium	human	cartilage		MSCs	none	GAG ↑, chondrocyte viability ↑	Topoluk et al. (2018)
		cartilage		triamcinolone acetonide	GAG production ↓	GAG production ↗	Beekhuizen et al. (2011)
		cartilage		amniotic fluid or PRP		ADAMTS-5, TIMP-1 ↓, aggrecan ↑	O'Brien et al. (2019); Osterman et al. (2015)
		synovium	IL-1β			ADAMTS-5, TIMP-1 ↓	
cartilage and joint capsule	ovine	cartilage	LPS	S-(+)-ibuprofen	NO, aggrecan loss ↓↓	NO, aggrecan loss ↘	Bédouet et al. (2015)

treatment indicating that the model might be applicable for drug testing (Peck et al., 2014). The inhibition of chondrocyte ECM synthesis by non-stimulated macrophages however, and the combination of murine with porcine cells should be viewed critically.

3.2 Chondrocytes and Bone Cells

Focusing on the interaction between cartilage and bone, several models incorporating bone cells have been described, many of these based on alginate-encapsulated chondrocytes in culture inserts and monolayers of osteoblasts (Lin et al., 2010; Sanchez et al., 2005a; Sanchez et al., 2005b; Sanchez et al., 2015) (**Figure 2**). Using healthy tissue as source, porcine osteoblasts induced chondrocyte hypertrophy as shown by decreased collagen II and aggrecan expression and increased expression of collagen X and bone sialoprotein (Lin et al., 2010), factors involved in OA pathology (Pesesse et al., 2014). If osteoblasts were subjected to cyclic tensile stress, an even more distinct shift towards hypertrophy and additional

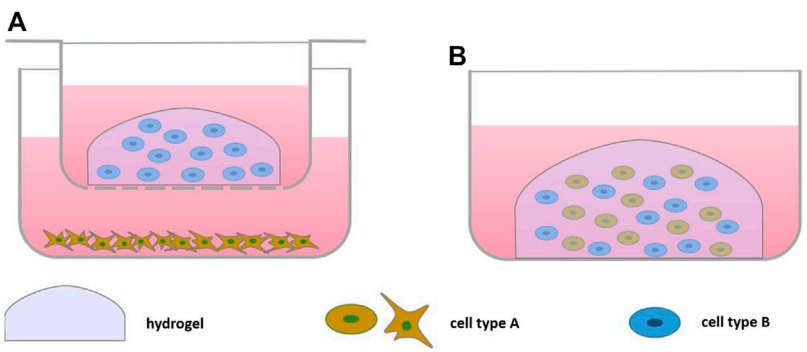

FIGURE 2 | Scheme of Bioengineered Cell-based Co-culture: **(A)** direct co-culture embedding one cell type in hydrogel in a culture insert on top of a monolayer culture, **(B)** direct co-culture embedding both cell types in a hydrogel.

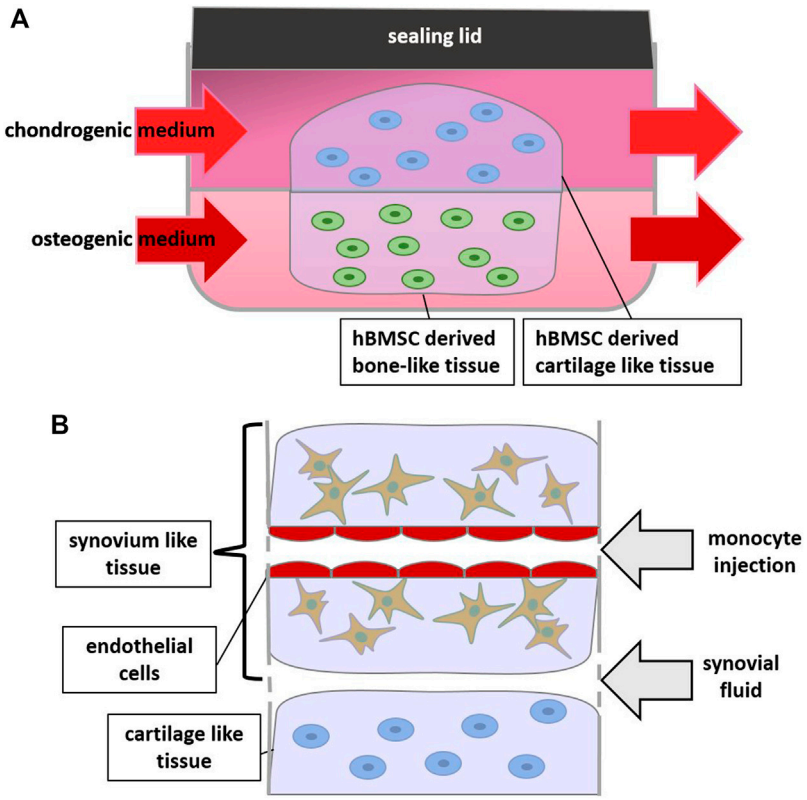

FIGURE 3 | Scheme of Bioreactors and Joint-on-a-Chip: **(A)** 2 hydrogels containing chondrogenic and osteogenic differentiated cells respectively are supplied by different media, **(B)** zoom in on a joint-on-a-chip containing synovium, cartilage and endothelium-like structures which allows for monocyte extravasation experiments.

increase of MMP-1, MMP-3 and MMP-13 expression in chondrocytes was observed, supporting the view that mechanical stress of bone could induce degenerative changes in cartilage. *In vivo,* mechanical stress of bone lead to increased TGFβ signaling, which in turn could promote OA (Zhen and Cao, 2014), matching the findings in this *in vitro* model. TGFβ expression was also increased in the stressed osteoblasts in the model (Lin et al., 2010). Still, the observation that healthy osteoblasts can also induce OA-like changes may indicate less validity of this model.

Human osteoblasts also decreased SOX-9 and collagen II gene expression of human chondrocytes, which was more pronounced by addition of sclerotic compared to non-sclerotic osteoblasts (Sanchez et al., 2005a). Fibroblasts did not induce these changes in chondrocytes, indicating that this influence might be specific to the communication of chondrocytes and osteoblasts (Sanchez et al., 2005a). Pretreating non-sclerotic osteoblasts with an inflammatory stimulus (IL-1β + IL-6 + soluble IL-6 receptor or oncostatin M (OSM)) at levels found in OA synovial fluid upregulated MMP expression and downregulated aggrecan expression to similar extent as the sclerotic osteoblasts (Sanchez et al., 2005b). Although also here healthy cells negatively affected chondrocyte behavior, the more pronounced effects of diseased osteoblasts, in line with *in vivo* observations, suggest its applicability as culture model.

Also in a microsystem bioreactor using microfluidics (Lozito et al., 2013) (**Figure 3A**), the influence of IL-1β stimulation on osseous and cartilage-like tissue components was studied. This model demonstrated that stimulating hBMSC-generated bone-like tissue with IL-1β resulted in a greater inflammatory response (e.g., increased expression of MMPs) in the adjoining cartilage-like component than by stimulating the cartilage component directly, suggesting communication between both joint compartments (Lin et al., 2014).

3.3 Chondrocytes and Synovium Cells

A bioengineered co-culture model of chondrocytes and synovial fibroblasts was used to investigate whether overexpression of glutamine fructose-6-phosphate amidotransferase (GFAT), an enzyme involved in glucosamine production, can influence matrix production. Rat synovial fibroblasts were adenovirally transduced with GFAT cDNA, co-cultured with alginate-encapsulated chondrocytes and stimulated with IL-1β to investigate the influence of GFAT on the response to IL-1β (Gouze et al., 2004). While in the non-transduced co-culture control, a decrease of proteoglycan production in chondrocytes and a simultaneous increase of nitric oxide and PGE_2 in the medium were observed after IL-1β stimulation, GFAT overexpression in synoviocytes prevented these changes (Gouze et al., 2004). Thereby the study demonstrated that a

co-culture model can be utilized to study how gene therapy in one tissue can affect an adjoining tissue in OA.

Complex systems can nowadays easily be reconstructed in organ-on-a-chip approaches. Monocyte extravasation in response to chemokines or the synovial fluid in OA was studied in a joint-on-a-chip-model including OA patient-derived synovial fluid, fibrin hydrogel-embedded OA chondrocytes and OA synovial fibroblasts, as well as perfusable endothelialized channels for monocyte injection (Mondadori et al., 2021) (**Figure 3B**). To mimic shear stress present in the channel, laminar flow of the medium was applied. This induced a shift towards more physiological expression levels of endothelial markers VCAM and ICAM, demonstrating the ability to mimic the *in vivo* situation to a certain extent. Additional TNFα treatment of the endothelial cells synergistically increased ICAM expression. Addition of a chemokine mix (CCL 2, CCL 3, CCL 4, and CCL 5) to the synovial fluid-mimicking compartment further stimulated monocyte extravasation. OA synovial fluid was similarly able to increase monocyte extravasation significantly, suggesting that synovial fluid might be relevant for monocyte extravasation *in vivo* (Mondadori et al., 2021). This model was used to test a CCR2 chemokine receptor antagonist and an antagonist for chemokine receptors CCR2 and CCR5. The antagonists reduced monocyte extravasation, showing that the model could be utilized for drug testing. The effects on the cartilage compartment was unfortunately not further investigated, nor was the added value of any of the other compartments investigated.

3.4 Bone Cells and Endothelial Cells

In order to create an OA model for subchondral bone and its vasculature, a photo cross-linked gelatin methacrylate (GelMA)-based co-culture of a mix of immortalized mesenchymal stromal cell line (MSOD) and HUVEC cells was utilized. MSOD cells underwent osteogenic differentiation by the presence of the HUVEC cells (Pirosa et al., 2021) MSOD mono and co-culture showed signs of mineralization. Cytokine stimulation (IL-1β, TNF-α and IL-6, all at concentrations found in synovial fluid of OA patients) increased endothelial network formation, similar to what is found in OA, in both HUVEC and MSOD-HUVEC culture. However, addition of the MSOD was required to maintain the network. Stimulation also induced demineralization and increase of collagen synthesis similar to changes in OA bone (Pirosa et al., 2021). Moreover, the cytokine-induced increased expression of alkaline phosphatase (ALP) and vascular endothelial growth factor (VEGF), markers for osteogenesis and angiogenesis, respectively, was more pronounced in co-culture. The co-culture was also stimulated with conditioned medium of a cartilage-on-a-chip model which consisted of chondrocytes that were embedded in a PEG-based hydrogel loaded onto a compressible PDMS device (Pirosa et al., 2021). Cells in the chip were supra-physiologically compressed to induce an osteoarthritic phenotype (Occhetta et al., 2019). Stimulation of the MSOD-HUVEC co-culture with the conditioned medium induced demineralization but prevented endothelial network formation, suggesting the pathways triggered were distinctly different from those induced by the abovementioned cytokine panel (Pirosa et al., 2021). Taken together co-culture could clearly demonstrate crosstalk between both cell types and also indicates that bone-like cells influence the phenotype of endothelial cells and vice versa. However, to what extent the MSODs really differentiated into bone cells was not clear.

4 CO-CULTURED TISSUE EXPLANTS

Although cell-based co-culture is a versatile strategy, one of the clear disadvantage is the change in phenotype occurring as a consequence of isolation and expansion of primary cells (Zimmermann et al., 2000; Schnabel et al., 2002; Tsuchida et al., 2014; Rosenzweig et al., 2017). In order to improve translation from model to clinic by studying cell behavior in their natural habitat, tissue explant models have been used in which the physiological or pathological microenvironment of the cells inside is maintained (Geurts et al., 2018). In OA research, these are often derived from cow, but also horse, dog or sheep (Greenberg et al., 2006; Lee et al., 2013; Byron and Trahan, 2017; Swärd et al., 2017; Haltmayer et al., 2019; Mehta et al., 2019), and also human explants gained importance in OA research (**Table 3**) (Hardy et al., 2002; Schwab et al., 2017; Geurts et al., 2018; Topoluk et al., 2018; Favero et al., 2019; Houtman et al., 2021a; Houtman et al., 2021b). Cartilage tissue co-culture models mainly comprise explants of cartilage combined with primary cells or other joint components, such as the attached subchondral bone (Byron and Trahan, 2017; Schwab et al., 2017) synovium (Hardy et al., 2002; O'Brien et al., 2019; Osterman et al., 2015; Araújo et al., 2020) or joint capsule (Swärd et al., 2017), the infrapatellar fat pad (Nishimuta et al., 2017) or nervous tissue (Li et al., 2011) (**Figures 4**, **5A**).

4.1 Co-culture of Explants and Primary Cells

Upon exposure to mechanically injured equine cartilage, primary equine healthy synovial fibroblasts reduced their ADAMTS-4 and 5 expression while increasing expression of MMP-1 as compared to co-culture with normal cartilage (Lee et al., 2013). Vice versa the fibroblasts induced higher collagen II expression in the injured cartilage and also histologically the progression towards an OA phenotype in injured cartilage seemed inhibited, evidenced by decreased cell clusters and focal cell loss (Lee et al., 2013). Also in IL-1β-stimulated equine cartilage, synoviocytes reduced GAG loss and diminished downregulation of aggrecan, although MMP-3 in synoviocytes was upregulated (Gregg et al., 2006). Altogether, synovial cells seem to exert a protective effect on cartilage. To what extent this is found *in vivo* is not clear.

In an elegant study where healthy rat chondrocytes were co-cultured with synovium harvested at different time points after OA induction, addition of synovium initially showed higher aggrecan production of the chondrocytes, suggesting injury induced a transient anabolic effect. With increased OA stage of the isolated synovium, however, this changed into an increase of monocyte chemoattractant protein 1 (MCP-1), a chemokine initiating inflammation (Xu et al., 2015; Lai-Zhao et al., 2021).

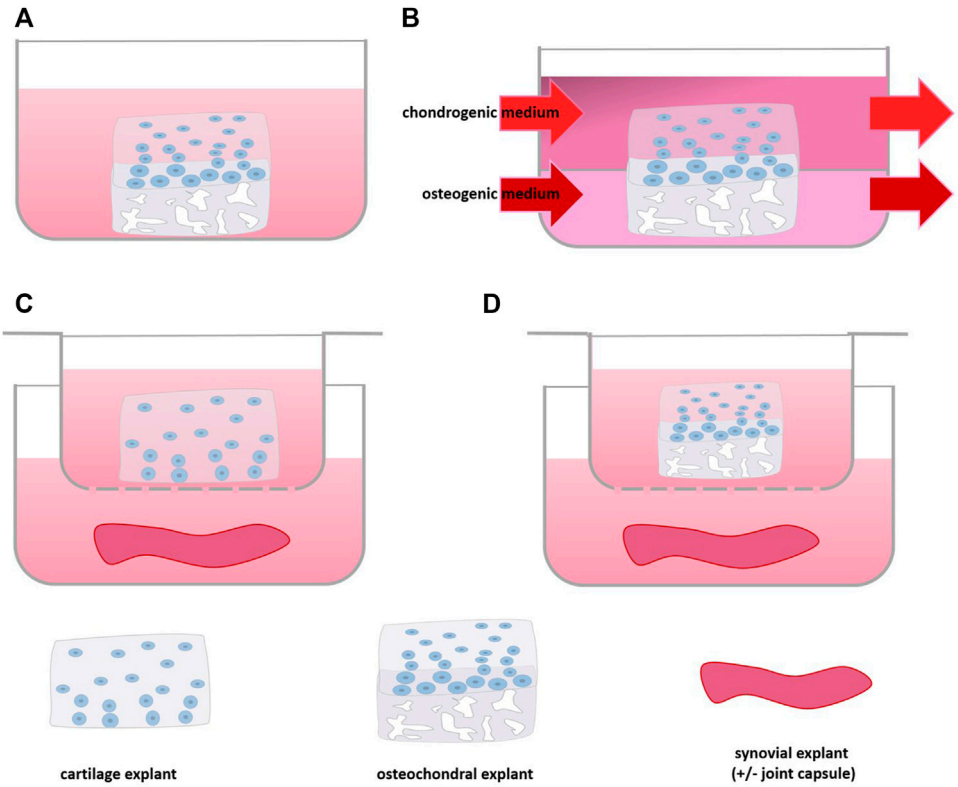

FIGURE 4 | Scheme of Tissue Explant Co-culture: **(A)** culture of osteochondral tissue, **(B)** with separate medium supply for cartilage and bone part, **(C)** co-culture of synovium and cartilage or **(D)** osteochondral tissue using a culture insert.

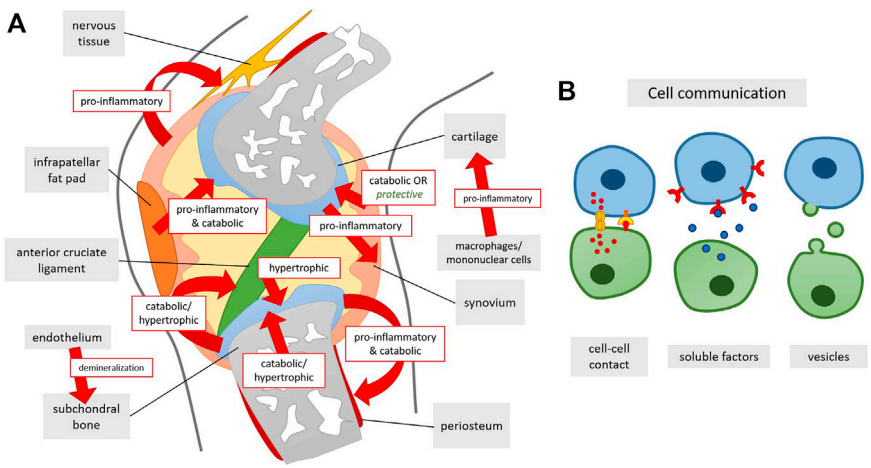

FIGURE 5 | (A) Summary of co-culturing effects on tissue level in the knee joint and **(B)** underlying ways of cellular communication. Arrows indicate the effect of one of the tissues/cell types onto the tissue it is directed at.

The effects did not seem to be related to the OA induction surgery, but rather the injury of the joint, as similar results were observed using synovium from sham surgery controls (Lai-Zhao et al., 2021). A similar approach of determining the effects on joint tissue interaction of early pathological processes involved in OA development was taken by adding human ACL explants from joints with acute and chronic ACL damage to chondrocytes culture. Both types of explants increased human chondrocyte

periostin and ADAMTS-4 expression, chronic remnants reduced Il-1, and increased collagen II and MMP-13 expression, whereas explants from acutely injured ACL decreased collagen II expression. It must be noted that only effects at the mRNA level were studied, and hence may not entirely reflect the metabolic state of the chondrocytes (Chinzei et al., 2018). Although applied as part of the search for cartilage regenerative strategies, a co-culture model of pellets of human chondrocytes from preserved areas in OA cartilage and periosteum explants separated by a culture insert can also be used to mimic the paracrine interaction of these tissues in the joint. Addition of chondrocytes significantly increased TGF-β and COL1A1 gene expression and induced expression of IL-6, MMP-2, -7, and -13 expression in periosteum. The periosteum induced collagen I deposition in pellets (Grässel et al., 2010; Rickert et al., 2010). In contrast, the addition of healthy bovine periosteum increased proliferation and decreased collagen II matrix deposition as well as aggrecan synthesis and release in chondrocyte monolayer, suggesting an overall inhibitory effect of periosteum (Steinhagen et al., 2012).

4.2 Osteochondral Explants

Osteochondral explants consist of cartilage attached to the underlying subchondral bone (**Figure 4A**). Although in this culture system it is not possible to point out the added value of one or the other tissue in the model, it is a frequently used model that has shown to reflect many aspects of *in vivo* disease (Houtman et al., 2021a; Houtman et al., 2021b). A transcriptome wide analysis of mechanically stressed human osteochondral explants demonstrated an increase of MMP-13 gene expression (Dunn et al., 2016; Houtman et al., 2021a). Moreover, increased expression of senescence markers such as FOXO and MYC1 were found after mechanical stimulation, which have also been found to be dysregulated in OA chondrocytes *in vivo* (Matsuzaki et al., 2018; Wu et al., 2018) indicating that the model harbors features of OA. In a study on finding a suitable culture system for OA, conventional stimulation of osteochondral explants by IL-1β was compared to triiodothyronine (T3) stimulation and mechanical stress (Houtman et al., 2021b). On the one hand all three stimuli induced an increase of HIF-2A and MMP-13 and no change in ADAMTS-5 gene expression. On the other hand only IL-1β reduced COL2A1 expression, while T3 stimulation increased COL1A1 expression and hypertrophy, while the mechanical loading only altered mechanical properties of the explant. Thus, depending on the stimulus, but most likely also its dose, different changes in the osteochondral explant can be induced that mimic the different disease aspects of OA (Houtman et al., 2021b).

The effect of Toll-like receptor 4 (TLR-4) agonist lipopolysaccharide (LPS) was tested on human osteochondral explants to see whether it could be used to create an OA model for drug testing. LPS activates TLR-4 in a similar fashion as damage-associated molecular patterns (DAMP) which are thought to play a key role in the inflammatory processes in OA (Geurts et al., 2018; Lambert et al., 2021). In the model, LPS induction increased secretion of the inflammatory markers IL-6 and MCP-1 (Geurts et al., 2018). A similar increase of IL-6 and MCP-1 was also found in LPS-stimulated bone explants, suggesting that the effects seen in the osteochondral explants involved the osseous compartment of the osteochondral explant. Treatment of the LPS stimulated osteochondral explant with SB-505124, a TGF-β receptor type I inhibitor that was shown to attenuate cartilage degradation *in vivo* (Zhang et al., 2018), could reduce IL-6 secretion but increased MCP-1 secretion into the medium (Geurts et al., 2018). Unfortunately, the effects on cartilage integrity were not studied to verify whether these coincided with previous *in vivo* findings. Of note, in the described models the osteochondral explants were cultured in the same culture medium. However, bone cells require other culture conditions than chondrocytes. Using either chondrogenic or osteogenic medium for osteochondral explants precludes optimal culture of both tissue compartments (Schwab et al., 2017). Moreover, the bone tissue may secrete cytokines and other substances into the medium at non-physiological levels (Sanchez et al., 2005a; Iwai et al., 2011). A co-culture platform for osteochondral explants consisting of two separated media compartments was developed to overcome this drawback (**Figure 4B**). A porcine model proved that the platform enabled supply of tissue specific medium and factors. Here, osteochondral explants could be cultured for at least 8 weeks maintaining matrix content and structural and mechanical properties without decreasing viability (Schwab et al., 2017). Combining this culture set up with the aforementioned stimuli could lead to a better OA model where both bone and cartilage could be investigated in a setup closer to the *in vivo* situation.

4.3 Cartilaginous & Synovium Tissue

Also the crosstalk between cartilaginous tissue and synovium, where the release of inflammatory cytokines by the inflamed synovium has been considered a major driver of the production of matrix degrading enzymes by the cartilage, was addressed in co-culture systems (Cook et al., 2007; Lee et al., 2009; Beekhuizen et al., 2011; Topoluk et al., 2018; Favero et al., 2019; Lai-Zhao et al., 2021). Adding healthy synovial tissue to canine cartilage explants (**Figure 4C**) maintained Toluidine blue staining indicative of proteoglycan content to *in vivo* levels, whereas in cartilage monoculture staining was more faint, suggesting proteoglycan loss (Cook et al., 2007). IL-1β stimulation induced a significant increase of both nitric oxide as well as prostaglandin E_2 (PGE_2) in culture medium of co-cultures, but not in cartilage monoculture. Moreover, MMP-13 secretion was increased to a higher extent after IL-1β stimulation in co-culture compared to cartilage monoculture, in line with the postulated role of the inflamed synovial lining *in vivo*. Gene expression of collagens, aggrecan and catabolic enzymes (MMPs, ADAMTS-4 and 5) of canine OA cartilage in co-culture with OA synovial tissue was more in line with tissue directly derived from OA patients than of cartilage in monoculture (Cook et al., 2007). Also in cultures of human OA cartilage and synovial tissue, a series of cytokines were produced that matched the expression profile seen in synovial fluid of OA patients (Zhang et al., 2018). Combining human OA synovium with OA cartilage explant culture increased cartilage expression of catabolic markers such as MMP-13, and

cartilage matrix degradation. Moreover, the addition of human OA synovium to cartilage reduced viability in chondrocytes and progressively reduced GAG content (Topoluk et al., 2018), or decreased GAG production in cartilage. However, in the latter study GAG release was not affected (Beekhuizen et al., 2011). Although this does not match the classical concept of inflammation-induced matrix degradation, it is in line with several *in vivo* studies showing that inhibition of synovial inflammation in OA does not improve cartilage integrity (Tellegen et al., 2018; Rudnik-Jansen et al., 2019). However, using the same type of co-culture in another study the presence of OA synovium did induce an increase of GAG release (Hardy et al., 2002). The catabolic/anti-anabolic cues of OA synovial tissue may be related to the presence of macrophages, as their depletion from human OA synovial tissue not only reduced levels of IL-1β in co-culture, but also reduced catabolic responses such as MMP-13 increase (Topoluk et al., 2018).

Using human OA tissue, also a differential effect of the corticosteroid triamcinolone acetonide (TAA) was shown in co-culture. TAA prevented the decrease in GAG production induced by the presence of synovium, while in cartilage monoculture it decreased GAG production (Beekhuizen et al., 2011). Using such an OA tissue based co-culture system, IL-1β-induced cartilage proteoglycan degradation was shown to correlate with induction of COX-2 expression and PGE_2 production in the synovium. A selective COX-2 inhibitor, SC-236, blocked this degradation in the co-culture, and its effect was reversed by exogenous PGE_2. (Hardy et al., 2002). The relevance of this finding to OA, however, was not entirely clear, as COX-2 protein could not be detected in unstimulated co-culture. Generally, it should be questioned whether additional inflammatory stimulation with IL-1β would be meaningful, as such co-cultures are fully based on OA tissue. Also meniscal tissue was affected by addition of synovium from patients with early stage OA, resulting in higher expression of inflammatory markers such as IL-6 and IL-8, catabolic markers such as MMP-3 and MMP-10 and GAG release (Favero et al., 2019). Thus, co-culture of cartilage or meniscus with synovium demonstrated that co-culturing influences the degenerative and inflammatory state of the cartilaginous tissues involved, mimicking OA *in vivo*. However, using bovine tissue, also healthy synovium induced GAG release and collagen II degradation in healthy cartilage, which suggests that all of these models are equally valid (Siebuhr et al., 2020).

Addition of healthy equine synovium to healthy equine osteochondral explant (**Figure 4D**) culture increased collagen II expression in the cartilage (Haltmayer et al., 2019). While IL-1β stimulation increased secretion of TNF-α and MMP-13 by cartilage in both monoculture and co-culture, the increase was attenuated in the co-culture, suggesting a protective effect of the synovium tissue (Byron and Trahan, 2017). In contrast, a set of stimuli comprising mechanical injury, IL-1β and TNF-α addition induced a significantly higher increase in MMP-1 gene expression in co-culture with healthy equine synovium compared to equine osteochondral explants alone (Haltmayer et al., 2019). Moreover, these stimuli induced a shift towards the more inflammatory M1 type in synovial macrophages, which mimicked the *in vivo* situation closer than the corresponding model of osteochondral explants alone (Haltmayer et al., 2019). The lack of protection by the healthy synovium in the latter study may be explained by the impact of the combined stimuli that may have been too large to overcome.

Also the joint capsule with the fibrous outer layer has been used in co-culture (Lee et al., 2009; Swärd et al., 2017). Adding healthy bovine synovial capsule tissue to healthy bovine cartilage culture increased MMP-13 and ADAMTS-4 expression in cartilage. Mechanical injury of the cartilage additionally increased ADAMTS-5 and MMP-3 expression as well as ADAMTS- and MMP- mediated digestion of aggrecan in the cartilage (Lee et al., 2009; Swärd et al., 2017) only in the presence of the synovial capsule tissue.

Unfortunately, using osteochondral tissue makes it difficult to pinpoint the role of bone and cartilage. Investigating the interplay and assigning changes with processes in one of the tissues may help revealing the pathological mechanisms involved in OA. However, taking into account that the joint is comprised of multiple tissues, it could also be argued that the more tissue types are included the closer is the model to the *in vivo* situation.

4.4 Co-Culture of Other Joint Structures

Apart from bone, cartilage and synovium, other joint structures such as infrapatellar fat pad or innervating tissue are known to influence joint homeostasis, OA onset and progression (Clements et al., 2009; Cai et al., 2015; Kim et al., 2016). While no effect was observed of adding bovine healthy fat pad tissue to bovine meniscus explant culture, it did increase GAG release in cartilage explant culture (Nishimuta et al., 2017). As net GAG content within the cartilage was not altered, GAG release may have been attributed to increased GAG production, suggesting that the infrapatellar fat may also have beneficial effects (Nishimuta et al., 2017). However, addition of OA IPFP-derived conditioned medium induced proteoglycan and collagen II loss in preserved cartilage from OA patients and an increase of MMP-3 and COX-2 positive cells (Zhou et al., 2020). Additionally, conditioned medium from OA-derived IPFP increased p38MAPK and ERK1/2 signaling in human chondrocytes significantly inducing the upregulation of MMPs, ADAMTS-4 as well as IL-1β, IL-6 and COX-2 in chondrocytes. (Zhou et al., 2020). These results indicate the role of IPFP as a causative factor in OA. This is in line with an *in vivo* study in which lipodystrophic mice on a high-fat diet were protected from OA development and lost this protection upon fat transplantation (Collins et al., 2021). Possibly early pathological changes convert the positive effect of the IPFP in a healthy joint to a negative influence.

Clinically, pain is a major burden in OA, but the mechanisms involved in the generation of pain are still poorly understood. To investigate the influence of inflamed synovium on nervous tissue, synovial tissue from healthy human donors or osteoarthritic patients was co-cultured with dorsal root ganglia (DRG) obtained from healthy rats (Li et al., 2011). In co-culture with human osteoarthritic synovium, rat DRGs showed increased expression of the neurokinin substance P and its receptors

NK1 and NK2, indicative of increased nerve stimulation (Li et al., 2011). Moreover, expression of neuropeptide Y receptor, which has been shown to be associated with chronic pain (Upadhya et al., 2009), was increased upon co-culture with OA synovium, but not healthy synovium or DRG culture alone, delineating the role of the synovium in OA pain as already indicated *in vivo* (Im et al., 2010; Li et al., 2011). However, as tissues were from rat and human origin, whether this is observed in human/human co-culture remains to be elucidated.

4.5 Drug Development in Tissue-Co-Culture Systems

Co-culture systems have also been used for drug development. So far, various types of treatments, ranging from drugs to platelet-rich plasma and stem cells to biomechanics-changing additives were investigated in OA models (**Table 4**). Most of the co cultures systems used to this end have been based on the use of synovial and cartilage tissues or cells.

An insert-based co-culture system of IL-1β stimulated human chondrocytes and synoviocytes was used to look at the effect of the N-acetyl phenylalanine glucosamine derivative (NAPA) as a drug targeting the NFκB pathway involved in OA. The addition of synoviocytes to chondrocytes induced increased phosphorylation on serine 10 in histone 3, suggesting higher NFκB pathway activity and a shift to a more inflammatory state (Pagani et al., 2019). NAPA treatment was able to reduce the phosphorylation in the co-culture. Whether the co-culture model better mimics the *in vivo* drug response was not explained as no comparison of NAPA treatment on mono and co-culture was performed (Pagani et al., 2019). Even though chondrocytes and synoviocytes from both healthy and OA tissue were available, both cell sources were used interchangeably. However, most likely the strong stimulation with IL-1β would not have allowed for the detection of any differences. In combination with LPS stimulation, addition of sheep synovial capsule tissue to cartilage weakened the protective effect of S-(+)-ibuprofen on nitric oxide synthesis and aggrecan loss, possibly related to the higher levels of PGE_2 produced by the synovium (Bédouet et al., 2015). In contrast, the combination with synovial tissue eliminated the requirement for continuous presence of IL-1 receptor antagonist in order to prevent GAG and collagen loss and improve chondrocyte viability in IL-1α-stimulated cartilage explants (Mehta et al., 2019). The discrepancy in the role of the synovial tissue may lie in the type of stimulus, with LPS possibly inducing a more general and strong inflammatory response, also in the synovium via Toll Like receptors, compared to IL-1α. Principal component analysis of secretome data from the co-culture in comparison with respective monocultures revealed distinct clustering between the culture setups, which indicated crosstalk between cartilage and synovium (Mehta et al., 2019).

IL-1β stimulated co-cultures of cartilage and synovium from OA patients were also used to study the therapeutic effect of PRP and amniotic viscous fluid. Both significantly reduced ADAMTS-5 and TIMP-1 expression in both cartilage and synovium, back to levels seen in non-stimulated cartilage, and increased aggrecan expression in cartilage, although only PRP also reduced nitric oxide production (Osterman et al., 2015; O'Brien et al., 2019). Unfortunately, the added value of co-culture was not addressed in either study. Several recent clinical trials furthermore failed to show any effect on joint integrity and clinical outcomes of PRP treatment compared to placebo (Hohmann et al., 2020; Bennell et al., 2021). Also hyaluronic acid (HA), a key component of the synovial fluid, has been used for treating inflammation and pain in OA as so called visco-supplementation. Commercially available hyaluronic acid (Hyalgan or Synvisc) added to IL-1-stimulated canine synovium-cartilage co-cultures inhibited the loss of GAG content in cartilage, and reduced MMP-3 expression if Hyalgan was added (Greenberg et al., 2006). Yet, the *in vitro* effects of these biologicals are in contrast with the poor level of evidence of efficacy of HA treatment (Lin et al., 2019). This may further point towards the limited value of IL-1 stimulation *in vitro* models of OA, at the concentrations commonly used, which are between 100–1000 fold higher than those found in OA patients (Calich et al., 2010; Vangsness et al., 2011; Tsuchida et al., 2014). The IL-1 levels in the pg/ml range found in the synovial fluid of these patients, together with the increased concentrations of its natural inhibitor IL-RA may also explain why several clinical trials failing to show an effect of IL-1 inhibition in the treatment of OA (Kahle et al., 1992; Irie et al., 2003; Tsuchida et al., 2014). Another emerging OA treatment is the use of mesenchymal stem cells. Amniotic and adipose tissue derived MSCs were able to increase GAG content and chondrocyte viability and prevent an increase in OARSI score in cartilage, only in synovium cartilage co-culture from OA patients. (Topoluk et al., 2018). It was, however, not clear what processes in the synovial tissue were responsible for this modulating effect.

More recent approaches also investigated the use of an ADAMTS-5 targeting nanobody (Nanobody® M6495), which was able to dose-dependently inhibit the bovine synovium-induced GAG breakdown in bovine cartilage explants and in human OA cartilage monoculture (Siebuhr et al., 2020). This indicated potential as a treatment for OA, although for human OA cartilage proof of effectivity was obtained only in cartilage stimulated with high doses of proinflammatory cytokines.

Non-sclerotic and sclerotic osteoblasts were used in co-culture with chondrocytes to study the effect of carnosol, a polyphenol extracted from rosemary. Pretreatment of sclerotic osteoblasts with carnosol could prevent and even partially reverse the reduced aggrecan production by chondrocytes induced by untreated sclerotic osteoblasts. Carnosol pretreatment furthermore decreased MMP-3, ADAMTS-4 and ADAMTS-5 in chondrocytes compared to co-culture with non-pretreated osteoblasts. However, the reduced collagen II gene expression in chondrocytes could not be mitigated by pretreatment (Sanchez et al., 2015).

5 MECHANISMS FOR INTERACTIONS BETWEEN JOINT TISSUES

Several mechanisms have been postulated to be operational in the crosstalk between joint tissues. Cells within synovium and cartilage secrete cytokines which are also found in the synovial

fluid resulting in inflammation and cartilage degradation (Goldring and Otero, 2011). Indirect evidence for the role of cytokines in this crosstalk is the similarity of cytokine levels in co-cultures of human OA cartilage and synovial tissue to OA synovial fluid (Rutgers et al., 2010) and the observation that an antibody neutralizing oncostatin M in synovial fluid could counteract the inhibition of matrix production in synovial fluid-exposed cartilage tissue (Beekhuizen et al., 2013a). Also the prostaglandins that are derived from unsaturated fatty acids were shown to act as proinflammatory mediators. PGE_2 produced by the human OA synovium correlated with proteoglycan degradation in OA cartilage (Hardy et al., 2002). Extracellular vesicles (EVs) may also play a role in OA progression, by carrying proteins, mRNA and even DNA from one tissue to another (**Figure 5**) (Miyaki and Lotz, 2018). *In vitro*, exosomes produced by IL-1β stimulated human synovial fibroblasts induced increased expression of MMP-13 and ADAMTS-5 and decreased ACAN and COL2A1 gene expression in human chondrocytes, as well as increased proteoglycan release in rat cartilage. Analysis of the exosomes showed differential expression of 50 miRNAs, which may have contributed to the EV induced changes (Kato et al., 2014). Gap junctions are also found in synovium and other joint tissues and there expression is altered in OA joint (Donahue et al., 2018). *In vitro* data also showed gap junction mediated calcium signaling between synovial cells and chondrocytes in co-culture (D'Andrea et al., 1998) or gap junction formation between chondrocytes and osteoblasts. Their contribution to OA, however, remains to be explained. Cell-cell-contacts *via* gap junctions between chondrocytes and osteocytes have not been confirmed *in vivo* so far. Even though it is well established that osteocytes communicate via gap junctions consisting of connexins (Doty, 1981), connexins expressed in chondrocytes are thought to rather function as hemichannels which are not coupled to another cell but fulfil other roles (Plotkin and Stains, 2015).

6 CONSIDERATIONS AND DISCUSSION

Co-cultures of joint cells and tissues have been widely used in OA research and have replaced animal models to a certain extent. Essential to their applicability is the degree to which they reflect the *in vivo* processes in OA, in terms of cartilage metabolism, inflammation and the response of these parameters to pathophysiological stimuli or drugs. However, this is not always easy to define, as our capacity to real-time detect processes occurring in OA is still limited. Crosstalk between co-cultured cells or tissues, evidenced by differential behavior compared to their respective monocultures, may further indicate the relevance of a co-culture system. However, as shown for the degenerative effect of healthy bovine synovial tissue on healthy cartilage tissue, this still may not always reflect the *in vivo* conditions. Several explanations may be given for this phenomenon. First of all, the presence of other joint tissues may be required for maintenance of homeostasis. Also the process of cutting tissue and inserting it into a novel biochemical and mechanical environment may affect behavior, as was shown by the peak in cytokine production by cartilage explants immediately after their isolation (Beekhuizen et al., 2011). Also the ratio of one versus the other tissue may affect the response as was shown in cell and cell/tissue co-culture (Larsson et al., 2011). Achieving the optimal ratio mimicking the *in vivo* joint will pose a challenge that nevertheless may be worthwhile taking. Finally of course it is possible that the assumed *in vivo* interactions do not occur, or to a different extent. The complexity of co-culture models comes with several tradeoffs. Monolayer co-cultures may yield insightful and detailed information on the interplay of cell types. However, cultured cells often lose their *in vivo* phenotype. Bioengineered models, utilizing hydrogel-encapsulated cells may at least offer a more native microenvironment, especially for bone and cartilage cells and can help investigating to what extent neighboring cells and tissues influence each other in a 3D environment. Biomechanical properties of the hydrogel should always be evaluated as stiffness or porosity of the hydrogel might differ from *in vivo* (Bao et al., 2020). Tissue explants provide the native tissue structure to the cells. Patient-derived tissue explants are a good resource for co-culture systems as they reflect these characteristics and are derived from spontaneously degenerated tissue. However, in addition to the limited availability of such tissues, OA is a disease with different phenotypes, where mechanical stress, inflammation and degeneration play roles of varying importance. Hence, tissue properties vary highly between donors. Therefore animal-derived explants have also often been utilized. Here, culture conditioned are better controlled yielding in higher reproducibility. Yet, a clear drawback of animal tissue is that joint physiology might differ from human and that physical characteristics such as joint size and cartilage thickness cannot be matched optimally to human OA joint tissue (Singh et al., 2021). Another distinct disadvantage of this approach is that these tissues originate from young and healthy animals, and therefore OA has to be induced *in vitro*. Stimuli such as cytokines or mechanical stress are frequently used to this end. However, care must be taken that the stimulus is physiological, which currently often is not the case for several proinflammatory cytokines used to induce OA, commonly at supraphysiological concentrations (Beekhuizen et al., 2013b). Using cytokine concentrations of the synovium of OA joints, possibly even in more complex mixes, can help to make the stimulus more pathophysiological (Beekhuizen et al., 2013a) and thereby prevent ineffective treatments being developed such as those based on IL-1 inhibition. Still, any response observed will still be generated using young tissue and cells if animal tissues are used and therefore human tissues remain the source of choice. Possibly the type of stimulus may be adapted to the research question. If anti-inflammatory drugs are tested, the stimulus may be based on inflammatory characteristics. This would imply that depending on the research question, the model does not need to mimic all features of OA. However, several

crossroads in the pathological mechanisms of OA have been described, so care must be taken to thoroughly characterize existing and novel co-culture models based on OA induction by external stimuli. More systematic comparisons of co-cultures with their respective monocultures and their correlation to *in vivo* data will be indispensable here. Finally, most models described in the current review consisted of only two different compartments, while the joint comprises many tissues. Platforms such as joint-on-a-chip systems may enable the combination of multiple joint compartments while allowing to evaluate their individual roles, provided the cell phenotype stays similar to in the joint *in vivo*. Here, any issues concerning human cell availability may be counteracted by making use of induced pluripotent stem cell (iPS) technology, which has advanced recently to the possibility of generating different cell types.

7 CONCLUSION

The utilization of co-culture models is key to reduce the animal use in OA research and to gain more understanding of the interplay in OA joints at the cell and tissue level. This can be a useful tool in drug development, but also in other research questions such as the role of biomechanical loading. The model should reflect respective characteristics of and processes in OA. Better validation of models is key here. Moreover, ideally more complex models incorporating more tissues and cell types are introduced.

AUTHOR CONTRIBUTIONS

Conception and design of the review: KM, VK, JA, JG, and LC, Collection of data/reviewed articles: KM, VK, JG, and LC Drafting the manuscript: KM, VK, JG, and LC Revising the manuscript: KM, JG, JA, and LC Approval of the manuscript: KM, VK, JA, JG, and LC.

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REVIEW
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Applying Principles of Regenerative Medicine to Vascular Stent Development

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Stents are a widely-used device to treat a variety of cardiovascular diseases. The purpose of this review is to explore the application of regenerative medicine principles into current and future stent designs. This review will cover regeneration-relevant approaches emerging in the current research landscape of stent technology. Regenerative stent technologies include surface engineering of stents with cell secretomes, cell-capture coatings, mimics of endothelial products, surface topography, endothelial growth factors or cell-adhesive peptides, as well as design of bioresorable materials for temporary stent support. These technologies are comparatively analyzed in terms of their regenerative effects, therapeutic effects and challenges faced; their benefits and risks are weighed up for suggestions about future stent developments. This review highlights two unique regenerative features of stent technologies: selective regeneration, which is to selectively grow endothelial cells on a stent but inhibit the proliferation and migration of smooth muscle cells, and stent-assisted regeneration of ischemic tissue injury.

Keywords: stent, selective regeneration, reendothelialization, restenosis, bioabsorable

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INTRODUCTION

Cardiovascular diseases, a group of disorders of the heart or blood vessels, are considered to be the leading cause of mortality worldwide. An estimated 17.9 million people died from cardiovascular diseases in 2019 (32% of all global deaths), with 85% due to heart attack and stroke (WHO, 2021). Coronary artery disease is the most common type of cardiovascular diseases; (Virani et al., 2020) about 18.2 million (6.7%) adults age 20 and older in US have this disease.

Angioplasty, a surgical procedure to open vessel blockages along with placement of a vascular stent, is a common treatment for heart attack and an option for stroke treatment or stroke prevention. More than 2 million people get a stent each year for coronary artery disease alone (Medtronic, 2019). Coronary artery stents account for just over two-thirds of all types of vascular stents. The global market of coronary stents was valued at an estimated $7.7 billion in 2019 and is expected to grow at a compound annual growth rate of 4.7% to reach $11.3 billion in 2027 (GVS, 2020), with some predicting even faster growth (9.8%) (DAIC, 2020). This growth is driven by the rising prevalence of cardiovascular diseases, a growing geriatric population, an increased number of angioplasties and rising preference for the procedure. Though stenting is suggested for severe narrowing of coronary arteries according to current clinical guidelines, a recent report questioned the effectiveness of stenting, compared to medications, in many patients with severe but stable heart disease (Reuters, 2019; NIH, 2020). Additionally, restenosis and/or other disease progression, after 4 years of stenting, were found in 26–35% patients in a study (Taniwaki et al., 2014; Cassese et al., 2015). In patients with peripheral artery stents, 18–40% at 12 months were reported to have in-stent

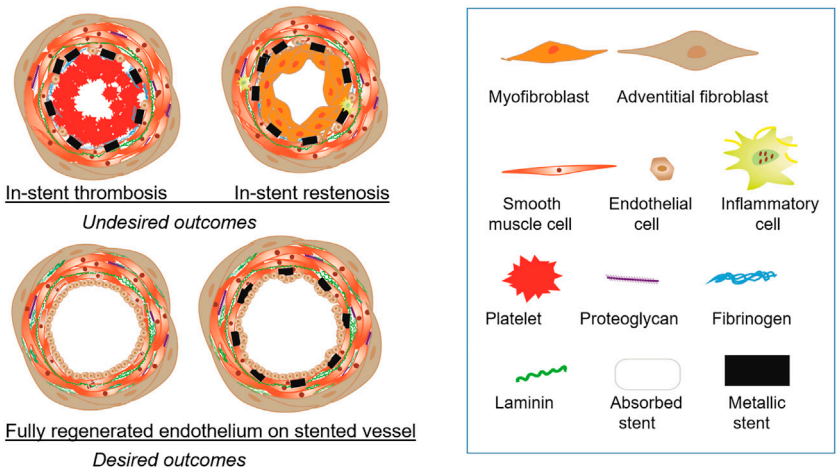

FIGURE 1 | Outcomes of endovascular stents. (Top panel) Stents produce undesired outcomes such as in-stent thrombosis and in-stent restenosis. In-stent thrombosis involves accumulation of platelets and presence of fibrinogen. In-stent restenosis causes narrowing of the lumen greater than 50% due to the overgrowth of smooth muscle cells with neointima formation. (Botton panel) The ideal vascular regeneration with stents will have a fully developed endothelial monolayer lining the lumen with either the stent completely bioabsorbed or permanently encapsulated in the intimal layer.

restenosis (Schillinger et al., 2006; Laird et al., 2010), while 5–10% for patients with coronary stents (Shlofmitz et al., 2019; Lawton et al., 2021). To strategize for improved stent performances, there is a re-surging interest in resorbable biomaterials, surface and biomolecule engineering approaches, capitalizing on the concept of vascular healing and regeneration.

Now is an important moment to examine regenerative approaches for this widely-used device in interventional cardiology and assess their benefits and risks for future stent therapies. We will start with a brief overview of the current status of stent uses in clinic, including stent types and uses in treating various cardiovascular diseases. Then, this review focuses on new methods emerging in recent stent research endeavors related to the regeneration concept. Comparative analyses of these methods include the regenerative effects, therapeutic effects and challenges faced. Lastly, thoughts about future perspectives in this subject area are provided in context of recent developments in relevant regenerative medicine.

CURRENT STATUS OF CLINICAL USE

Vascular stents are a device to treat a partially clogged artery by holding the arterial wall open and preventing it from collapsing. The majority of stents within the coronary stent market are bare metal stent (BMS) and drug eluting stent (DES), with the latter holding a larger share and being accepted as a safer treatment (Jensen and Christiansen, 2019). Nevertheless, the BMS is still widely used due to low cost and low hospitalization rates.

Major clinical issues for stent usage are thrombosis and restenosis. Restenosis, re-narrowing of the stented vessel due to proliferation and migration of smooth muscle cells, is triggered by arterial injury during stenting. **Figure 1** illustrates desired and undesired fates of a stented vessel. BMS shows a higher rate of restenosis than DES, while the risk of DES is often a very late (>12-month post-implantation) thrombosis. In general, the pathology associated with stent thrombosis and restenosis starts with the loss of endothelium due to injury, followed by fibrinogen absorption, platelet activation or neointimal hyperplasia (Nakamura et al., 2016). The contributing factors are numerous, including biological, patient-specific, mechanical, and technical factors. The evolution of stenting technologies is accompanied by the gradually improved understanding of stent-related vascular pathologies such as acute arterial injury, inflammation, extracellular matrix deposition, and negative vascular remodeling. It is only through addressing these relevant pathologies that new stent technology can be developed to balance acute, chronic, and long-term requirements for stability, healing and regeneration of stented vessels. The most important element is a complete regeneration of fully functional endothelium over stented vessels.

DES

Widely-used DESs have a polymer coating blended with a drug or drug mixture that inhibit vascular smooth muscle cell growth. These anti-proliferative drugs are released over several months to help prevent restenosis. After that period, the stent may trigger platelet adhesion initiating thrombotic response. Controlled release of anti-proliferative agents inhibits neointimal growth but also delays arterial healing, thereby predisposing to a late risk of thrombosis. This high rate of late thrombosis (1–12 months post-implantation), demonstrated by the first generation stents, stimulated DES research for new generation stents in the last decade.

Contemporary second or third generations of stents are superior to the first generation in a number of clinical measures, including late stent thrombosis and strut coverage (Otsuka et al., 2014; Wiemer et al., 2017; Migita et al., 2020), showing a significant reduction in the risk of target lesion revascularization compared to early-generation DESs.

Compared to the first generation stents, these stents display thinner stent struts (7–20 μm vs. 75–140 μm) and advancements in coatings including new anti-proliferative drugs and/or new polymers (Bangalore et al., 2018; Zaman et al., 2019). Reduced strut thickness offers greater flexibility and deliverability, while improvements in the drug or polymer realm permit faster vascular healing. Polymer-free stents are also available to decrease inflammatory reactions (McGinty et al., 2015; Worthley et al., 2017). Though newer generation DESs improve the effectiveness and safety profiles of their predecessors, long-term chronic inflammation and very late thrombosis remain serious concerns (Mori et al., 2017). More importantly, the prevention of cell growth by anti-proliferative drugs is counterproductive to vascular healing and regeneration; the coverage of stents with endothelial cells requires growth of these cells.

Covered Stent

Covered stents have a fabric material component that covers a metal stent. The majority of covered stents use polytetrafluoroethylene and have various clinical applications in peripheral arterial disease management. Covered stents offer an effective strategy for the creation of transjugular intrahepatic portosystemic shunts (Geeroms et al., 2017; Cho et al., 2020), as well as for repair endovascular aneurysm (O'Donnell et al., 2019), traumatic arterial lesions, and obstructive vascular disease of the aortoiliac and femoropopliteal sectors. Recently, the application of covered stents have expanded to seal coronary artery perforation (Lee et al., 2016; Wang et al., 2017; Birkemeyer et al., 2021; Wańha et al., 2021) and other perforations in clinic (Rizk et al., 2020). It also has a potential role in the treatment of friable embolization-prone plaques (Dogu Kilic et al., 2016). In the case of transjugular intrahepatic portosystemic shunts, covered stents, compared to BMS, showed higher estimated overall survival in patients (Gupta et al., 2017).

Recently, covered stents have become a platform that offer a minimally invasive, safe, and effective solution for arterial emergencies, such as bleeding, pseudoaneurysm, dissection, or fistula (Geeroms et al., 2017; O'Donnell et al., 2019). During the last few decades, treatment of these potentially life-threatening lesions has shifted from emergency open surgery to a stenting approach. Focusing on iatrogenic arterial injuries, Ruffino et al. gave an insight into the advantages of covered stents (Ruffino et al., 2020). In fact, it was believed that almost all arterial lesions may be treated with covered stents, except for those without anatomic suitability.

Taken together, current stent-assisted therapies—BMS, DES and covered stents—are employed to combat a wide range of vascular diseases and emergencies. Despite low complication rates (approximately 2–3% per year after the first year), stent therapy is still associated with a risk of restenosis and thrombosis, which persists for over 20 years. Recent comparisons of stent therapy and non-stent therapy for treating different clinical conditions such as coronary artery disease and infrainguinal peripheral artery disease showed no difference between stent and non-stent therapies, while the stent procedure is more invasive and has a significantly higher cost (Banerjee et al., 2019; NIH, 2020). Therefore, to demonstrate more competitive advantages over non-stent artery interventions, stent-assisted therapies demand innovations to proactively enhance arterial regeneration and long-term arterial health, in addition to the prevention of disastrous outcomes.

NOVEL DEVELOPMENTS AND CHALLENGES IN STENTS

Similar to the application of regenerative medicine concepts in other tissues or devices, regeneration-relevant approaches emerge in the stent technology in two forms: regenerative surface and biodegradable or bioresorable scaffolding materials. However, due to the presence of diseased artery tissue around a stent, major considerations of all stent technologies must include their therapeutic impact such as the inhibition of arterial recoil and smooth muscle proliferation. To this end, we review the two major types of approaches to stent design by comparing and evaluating their regenerative effects and therapeutic effects.

Regenerative Stent Surface

Continued efforts towards stent surface modifications are seen in the 1990s and 2000s as well as more recently. Earlier efforts focused on therapeutic effects such as reduced restenosis, using 1) *inorganic coatings*, including diamond-like carbon (Airoldi et al., 2004), pyrolytic carbon (Danzi et al., 2004), titanium nitride oxide (Windecker et al., 2005), and carbide (Unverdorben et al., 2003); 2) *gene-eluting coatings*, incorporating mRNA, siRNA, miRNA, or plasmid DNA of therapeutic genes in the coating; (Feldman et al., 2000; Sharif et al., 2012; Wang et al., 2021) 3) *cell-seeded stent,* using endothelial cells or progenitor cells (Zhu et al., 2008; Shi et al., 2010; Jantzen et al., 2011; Raina et al., 2014). The inorganic coatings provided ineffective or inconclusive performance (e.g., restenosis rate), compared to uncoated BMS (Kim et al., 2005; Meireles et al., 2007; O'Brien and Carroll, 2009). The gene-eluting coatings, like DES, incorporate genes for therapeutic moiety, but offer a longer term efficacy and a wider variety of therapeutic strategies beyond suppressing cell proliferation for restenosis inhibition (Yang et al., 2013; Adeel and Sharif, 2016; Fishbein et al., 2017; Hytönen et al., 2018). Nevertheless, the clinical translation of such coatings is still extremely challenging due to significant technical hurdles, demanding improvements over release kinetics, cell-specific transfection, reduced toxicity, and inflammation. Similarly, great difficulty in the clinical translation is faced by cell-seeded stents (Jantzen et al., 2016; Tsukada et al., 2021).

The root cause of stent pathology, as revealed by numerous studies on BMS and DES, (Cornelissen and Vogt, 2019; Bedair et al., 2017; Kobo et al., 2020; Scafa Udriște et al., 2021), is endothelial dysfunction and/or impaired growth of endothelial cells in the stented artery. Healthy endothelial cells naturally prevent the blood from clotting, and produce molecules such as nitric oxide (NO) and prostacyclin for smooth muscle relaxation and growth inhibition. Thus, modifications of the stent surface in the last decade involve

TABLE 1 | A summary of regenerative surface modification approach from the literature.

Regenerative approaches	Examples	Animal model for evaluation	Time points	Regenerative outcome	Therapeutic outcome	Challenges/Questions
Stem cell secretomes	Exosome-eluting stent using MSC-derived exosomes	Bilateral renal ischaemia-reperfusion injury model with rat (Hu et al., 2021)	Short term (28 days)	Accelerated re-endothelialization; promoted muscle tissue repair through increased reperfusion	Decreased in-stent restenosis; regulated macrophage polarization	Isolation of exosomes that are free from harmful contaminants and have a consistent set of functional properties
EPC-capture molecules	Anti-CD133 and Anti-CD34	Rabbit abdominal aorta (Wu et al., 2015)	Medium term (12 weeks)	Better EPC capture by anti-CD133 stent compared to anti-CD34 stent	Anti-CD133 stents accelerate tissue regeneration without excessive neointima	A low number and high heterogeneity of circulating EPC
	Anti-VE-Cadherin and Anti-CD34	Rabbit iliac artery (Lee et al., 2012)	Short term (42 days)	Better EPC capture and re-endothelialization on anti-VE-Cadherin stent compared to anti-CD34 stent	Anti-VE-Cadherin stents reduced restenosis *in vivo*	
	Anti-CD34	Pig coronary artery (van Beusekom et al., 2012)	Medium term (90 days)	Improved early endothelialization	No significant difference in neointimal thickness with anti-CD34 stent	Cannot differentiate between EC produced from circulating cells and remnant EC proliferation
	Anti-CD133	Pig left anterior or circumflex arteries (Sedaghat et al., 2013)	Short term (28 days)	No effective increase in re-endothelialization or neointima reduction	No significant therapeutic outcome	No regenerative or therapeutic benefits. Larger sample size, longer term studies are needed
	Combo® DTS (anti-CD34)	Human patients (Blessing et al., 2020)	Long term (615 days) with median follow-up of 189 days	Augmented EPC recruitment due to anti-CD34 antibody labeling might promote neointima formation.	Combo® DTS showed 40% restenosis rate with low rate of major cardiovascular events in follow-up	Adverse EPC differentiation in the proinflammatory environment and/or enhanced attraction of myeloid cells may cause restenosis.
	Anti-CD133 with chitosan/hyaluronic acid coated stent	Pig coronary artery (Zhang et al., 2015)	Short term (28 days)	Better resistance to blood flow erosion; Targeted capture of hematopoietic stem cells; Inhibited migration and proliferation of smooth muscle cells	Improved re-endothelialization and reduced thrombosis, inflammation and rejection	The role of chitosan and hyaluronic acid on the improved stent performance is unknown to be studied
	VE-cadherin extracellular domain + adhesive protein	Rat (Yang et al., 2020a)	Short term (1 month)	Accelerated endothelialization; Tight junction formation; Improved endothelial barrier function	Good hemocompatibility	Will the regenerated endothelial layer with tight junctions prevent late stage thrombosis?
NO-producing coatings	NO donor: NONOate	Rabbit (Zhu et al., 2020)	Short term (1 month)	Accelerated regeneration of endothelial cells	Anti-restenosis; Good anticoagulation	NO availability is for a limited time; long-term studies are needed
	NO donor: DETA NONOate	Pig coronary artery (Elnaggar et al., 2016)	Short term (28 days)	NO release for 5 days; Induce endothelialization	Reduced inflammation score; Lowered fibrinogen adsorption; Inhibited neointimal hyperplasia	Longer NO release is needed; Identifying appropriate NO release molecule is challenging
	NO-generating SeCA-Dopamine	Rabbit iliac artery (Yang et al., 2018)	Medium term (3 months)	Promoted reendothelialization; NO release supported competitive growth of HUVECs over HUASMCs	Reduced in-stent restenosis and neointimal hyperplasia	The integrity of the endothelial monolayer with sustained NO release must be investigated. The long-term biocompatibility of the Cu- or Se- catalyst is important to the translation of this approach into the clinical practice
	NO-generating: Nano Cu	Rabbit iliac artery (Fan et al., 2019)	Short term (4 weeks)	Promoted re-endothelialization	Promoted anticoagulation and anti-hyperplasia; suppressed thrombosis and stent restenosis	
	NO generating Organoselenium (SeCA)	Rabbit iliac artery (Yang et al., 2020b)	Medium term (12 weeks)	Rapid re-endothelialization; SMC migration and proliferation, EPC recruitment	Inhibition of thrombosis, and effective in-stent restenosis prevention	

(Continued on following page)

TABLE 1 | (*Continued*) A summary of regenerative surface modification approach from the literature.

Regenerative approaches	Examples	Animal model for evaluation	Time points	Regenerative outcome	Therapeutic outcome	Challenges/Questions
Soluble growth factors	rhVEGF	Rabbit iliac artery (Van Belle et al., 1997)	Short term (28 days)	Accelerated endothelialization within 7 days	Reduced in-stent intimal thickness	Formation of mural thrombus should be prevented
	VEGF	Pig coronary artery (Takabatake et al., 2014)	Short term (14 days)	Provided highly selective capture of EPCs, when compared with anti-CD34 antibody-bound stents; Rapid formation of intact endothelium		Results may depend on the form and binding of VEGF. Long-term studies are needed
	Rapamycin-VEGF coating	Pig artery (Wang et al., 2020b)	Short term (42 days)	This combination promoted growth of EC over SMC; efficient re-endothelialization	Suppression of in-stent restenosis	
Surface pattern	Nanotexturing	Rabbit iliac artery (Cherian et al., 2020)	Short term (8 weeks)	Preferential proliferation of endothelial cells over smooth muscle cells; complete endothelial coverage	Reduced neointimal thickening and in-stent restenosis	Unknown mechanism underlying selective cell proliferation on nanostructures
Adhesive Peptides	cRGD	Pig coronary artery (Blindt et al., 2006)	medium term (12 weeks)	Early recruitment of EPC by αvβ3-integrins; Accelerated endothelialization	Reduced neointimal area and percent area stenosis	Attracting the right kind of EPC from the blood stream is a challenge
	RGD and CXCL1	Mice carotid artery (Simsekyilmaz et al., 2016)	Short term (1 week)	Adhesion of early angiogenic outgrowth cells, a type of EPC; Increased re-endothelialization	Reduced neointima and thrombus	Extend this work to long term to firmly establish the efficacy of this process
	REDV	Rabbit iliac artery (Wei et al., 2013)	Short term (28 days)	Random and tightly arranged endothelial cells	Significantly inhibited neointimal hyperplasia	Is REDV a stand-alone peptide in re-endothelialization?
	WKYMVm-HA + sirolimus	Rabbit iliac artery (Jang et al., 2015)	Short term (6 weeks)	Consecutive endothelial lining	Low restenosis rate, similar to commercial DES	Lack of specificity of cell attachment
Other approaches	Epigallocatechin gallate/copper	Rabbit abdominal aorta (Zhang et al., 2021)	Short (1 month) and medium term (3 months)	Upregulated VEGF; *In-situ* re-endothelialization	Anti-hyperplasia; Suppressed SMC proliferation and migration; Enhanced anticoagulation; Alleviated inflammatory reactions	The quality and integrity of the endothelial monolayer needs to improved, which is a major challenge
	Heparin/SeCA	Rabbit iliac artery (Qiu et al., 2019)	Short (1 month) and medium term (3 months)	Created environments that favored the growth of endothelial cells compared to smooth muscle cells	Anti-thrombogenic, anti-restenosis	The intimal hyperplasia and in-stent restenosis parameters soared from 1 month to 3 months. What will be the long term effect on these parameters?
	Biodegradable stent (Polylactic acid)	Pig coronary artery (Lee et al., 2019)	Short term (28 days)	Widest lumen area; Rapid EC proliferation	Less neointimal hyperplasia with no atherosclerosis or thrombosis	The rate of biodegradation matches the rate of formation of neoartery
	CD31-mimic	Pig coronary artery (Diaz-Rodriguez et al., 2021)	Short term (28 days)	Full endothelialization with no activated platelets/ leukocytes	Normal arterial media with no thrombosis	Stability of the coating

HA, hyaluronic acid; SeCA, selenocystamine.

approaches to promote *in situ* re-endothelialization or to integrate critical endothelial functions. This is achieved through cell secretomes, cell-capture coatings, mimics of endothelial products (e.g., NO), surface topography, endothelial growth factor, cell-adhesive peptides and other biologics. **Table 1** provides a brief summary of these functionalization approaches. Details of each approach are described in following paragraphs. As reviewed by Bedair et al. (2017), the immobilization of molecules onto the stent surface involves physical adsorption, encapsulation, ionic bonding or covalent conjugation through various chemical reactions such as carbodiimide chemistry. The covalent conjugation may begin with plasma treatment or a layer of polydopamine for abundant free hydroxyl groups, and then attach a functional polymer such as bioclickable polymer.

The regenerative aspects of a stent are indeed unique. Two prominent features are noted. First, stent-related regeneration is "*selective regeneration*", which is to selectively grow endothelial cells on the stent surface but inhibit the proliferation and migration of smooth muscle cells. Therefore, multifunctional coatings in recent developments exhibit both regenerative effect on the endothelium and therapeutic effects on the smooth muscle. Second, stent-assisted regeneration of ischemic tissue injury is needed in certain applications such as myocardial infarction and renal ischemia, where narrowing of an artery is accompanied by the loss of distal vasculature and thus tissue injury. Stenting provides an opportunity to regenerate the tissues with biologics that regenerate stented artery and distal vasculature (d'Souza et al., 2017). Regenerative biologics can also alleviate the impaired vasorelaxation in nonstented proximal and distal segments of stented arteries, in particular those with DES (Nakamura et al., 2011; van den Heuvel et al., 2010; Lim et al., 2012).

Stent Incorporated With Cell Secretome

Cardiovascular stents incorporated with secretomes from stem cells represents a novel direction for regenerative stents. Hu et al. recently reported exosome-eluting stents for vascular healing and tissue regeneration after ischemic injury (Hu et al., 2021). These stents were coated with exosomes derived from mesenchymal stem cells. The bioactive stents promoted vascular healing and repair of ischemic condition, which usually need additional procedures for regenerative therapy in a patient. Exosomes are nanoscale membranous sacs secreted by most cell types; those from stem cells show great potentials of delivering regenerative and therapeutic benefits (Burke et al., 2016; Eisenstein, 2020; Nikfarjam et al., 2020; Wei et al., 2021). A major regenerative benefit of exosomes is to rejuvenate endothelial cells (Baruah and Wary, 2020), which can not only help to cover the stent with a healthy endothelium but also promote distal revascularization for tissue repair. A prominent therapeutic benefit of exosomes is immune-modulation or immune-suppression (Domenis et al., 2018; Suh et al., 2021). The exosome coating on stents may help camouflage the stent, blocking adverse immune reactions. Another novel aspect of the stent design by Hu et al. is the controlled release of exosomes from the stent coating in the presence of reactive oxygen species, a hallmark of inflammation (Hu et al., 2021). In the long run, stents incorporating cell products like exosomes or other forms of cell secretomes might offer a safer and simpler alternative to cell-seeded stents.

Cell-Capture Stent

Another alternative to cell-seeded stents is to utilize biomolecule coatings to capture circulating cells in the blood for endothelial regeneration (Sethi and Lee, 2012; Pang et al., 2015). To that end, various antibodies recognizing endothelial progenitor cell (EPC), like anti- CD34, VE-cad, or CD133, were loaded to the stent surface (Lim et al., 2011; Leopold, 2013; Wawrzyńska et al., 2019). These antibody-coated stents were compared in terms of their capability of capturing circulating EPCs. All types of antibody-coated stents were completely covered with a cell layer in earlier stage (e.g., one-week post-implantation) than BMS in animals. Animal studies showed that stents coated with anti-CD133 or VE-cad, compared with those with anti-CD34, accelerated re-endothelialization and reduced in-stent restenosis (Lee et al., 2012; Wu et al., 2015).

Preclinical or clinical results using cell-capture stents, however, are not always positive. Those with anti-CD34 were found to improve early endothelialization in swine but not affecting neointimal thickness (van Beusekom et al., 2012). Sedaghat et al., using a porcine model, found no difference in re-endothelialization or neointima formation with the use of CD133-stents compared with BMS (Sedaghat et al., 2013). This is supported by studies showing circulating EPCs or bone marrow-derived cells failed to significantly contribute directly to endothelial regeneration; cell-capture stent might increase restenosis rate in clinic (Blessing et al., 2020; Evans et al., 2021). Such inconsistency among preclinical outcomes of cell-capture stents can be attributed to the low number and the high heterogeneity of circulating EPCs (Medina et al., 2017; Keighron et al., 2018). Antibodies on stents may also increase unspecific binding of mononuclear cells, likely causing complications (Sedaghat et al., 2013).

For all regenerative purposes and translational intents, current and future endeavors utilizing cell-capture mechanism may continue in three directions: 1) combining cell-capture molecules with other prohealing/proregenerative molecules; (Chen et al., 2015; Zhang et al., 2015; Wang et al., 2020a; Yang et al., 2020a) 2) using more selective or specific molecules to recognize a subset of EPCs; (Hirase, 2016; Tang et al., 2016) 3) exploiting EPC-derived secretomes such as those in the form of exosomes (Zeng et al., 2021).

NO-Producing Coatings

NO is one of the most potent molecules produced by healthy endothelial cells that play multiple essential roles in cardiovascular physiology (Napoli et al., 2006; Strijdom et al., 2009). Vascular injury, just like in the case of stent deployment, reduced NO production of endothelium. Incorporating a NO-producing mechanism into stent coatings has a range of benefits, including the prevention of thrombosis through inhibiting platelet aggregation, anti-inflammation through reducing monocyte adhesion, inhibition of SMC migration and proliferation through SMC relaxation, as well as stimulation of endothelial proliferation for stent endothelialization (Rao et al., 2020). A recent review by Rao et al. detailed this topic, in particular the therapeutic effect of NO-producing coatings for the prevention of thrombosis and restenosis (Rao et al., 2020).

There are two types of NO-producing coatings, NO-releasing and NO-generating coatings. NO-releasing coatings involves exogenous NO donors such as N-diazeniumdiolates (i.e., NONOates), S-nitroso-N-acetylpenicillamine, and peptide amphiphiles (Kushwaha et al., 2010; Zhu et al., 2020), immobilized by polymer or liposome (Elnaggar et al., 2016). These donors release NO in limited periods of time. To extend the release time, recent developments involves the attachment of NO donors to polymers (Hopkins et al., 2018) or enzyme-sensitive linkers (Winther et al., 2018; Midgley et al., 2020). NO-generating coatings utilize eNOS gene (Sharif et al., 2012), or immobilize

catalysts such as copper and selenium to convert endogenous NO donors such as S-nitrosothiols into NO (Yang et al., 2018; Fan et al., 2019; Zhang et al., 2019; Yang et al., 2020b). All the studies using stents with either NO-releasing or NO-generating coating show promising results *in vitro* and *in vivo*. This is consistent with the results from recent graft study which employed NO-producing vascular graft on rodents for 12 weeks and found rapid endothelialization and hampered SMC proliferation (Enayati et al., 2021). Despite promising outcomes, long-term preclinical evaluations on NO-generating stents are yet to be performed.

VEGF-Induced Regeneration

VEGF is a most potent, endothelial-specific growth factor to regenerate endothelial cells. Early attempts to incorporate it into stent coatings were made as early as 1990s (Van Belle et al., 1997). However, inconsistent results regarding re-endothelialization and inhibition of restenosis were found in animal studies, which might be derived from different methods of preparing VEGF-eluting stents. Opposite to the finding from an earlier study using VEGF-eluting stent (Van Belle et al., 1997), Swanson et al. found VEGF-eluting stents did not accelerate re-endothelialization or inhibit restenosis, but reduced stent thrombosis (Swanson et al., 2004). Both studies evaluated stents with iliac artery model on New Zealand rabbits. Recent VEGF-bound stents have found more success in the preclinical evaluations. Using a porcine coronary model, Takabatake et al. used VEGF on poly-(ethylene-co-vinyl alcohol) coated stents, and found VEGF-bound stents, compared with anti-CD34 antibody-bound stents, provided highly selective capture of EPCs, followed by a rapid formation of intact endothelium at an early period of stenting (Takabatake et al., 2014). Yang et al. showed DES with VEGF gene completed re-endothelialization and significantly suppressed in-stent restenosis after 1 month compared to commercial DES (Yang et al., 2013). Wang et al. spatially bound VEGF and rapamycin to the base and top of hierarchical capillary coating, respectively, and showed the competitive growth of endothelial cells over smooth muscle cells on the stent surface as well as a high level of re-endothelialization and a very low level of in-stent restenosis using a minipig model (Wang et al., 2020b).

In conclusion, VEGF-bound stents hold great promise in endothelial regeneration but the outcome using this regenerative molecule depends on: 1) co-grafting condition, such as drugs or other molecules coexisting on the stent and collaborating into action at the stent-artery interface; 2) the form of VEGF (e.g., gene, protein, and peptide), and 3) the conjugation method of VEGF, for example eluting vs. polymer-bound.

Surface Pattern

Specialized micro- and nano-patterns have been introduced on the stent surface to reduce the risk of blood clotting, expedite healing and improve endothelialization. The design of textures on the thin struts of a stent includes nanotubes, ridges, pores, diamonds, leafy structure, or even a pattern mimicking the shape of smooth muscle cells (Cherian et al., 2021) (Junkar et al., 2020). Importantly, the textures are assessed by preferential growth of endothelial cells over smooth muscle cells (Cherian et al., 2020). However, the reason for this selective cell proliferation remain elusive. Manufacturing methods can involve anodization (Saleh et al., 2017), atomic layer deposition (Yang et al., 2019), lithography or femtosecond laser. Lee and Desai, for example, altered the anodization conditions to achieve nanotubular coatings with 110 and 70 nm nanotube diameters, and found competitive growth of endothelial cells over smooth muscle cells on nanotubular stents (Lee and Desai, 2016). Nanostructure formation is free of polymers or new chemicals, which might speed up the approval process for the clinical translation.

Other Biologics-Based Coatings

Two major groups of other biologics used in stent coatings, often in combination with other approaches, are adhesive peptides and glycosaminoglycan (GAG) molecules such as heparin and hyaluronic acid or GAG-mimics such as chitosan.

Adhesive peptides are instrumental for the attachment of circulating cells like EPCs. Blindt et al. found that DES with cyclic RGD peptide inhibited neointimal hyperplasia by recruiting EPCs in porcine coronary arteries (Blindt et al., 2006). With improved vascular healing in second-generation DESs, recent approaches employed adhesive peptides in conjunction with other functional molecules such as CXCL1 (Simsekyilmaz et al., 2016), or more specific adhesive peptides such as YIGSR (Alexander et al., 2018) and REDV (Wei et al., 2013). Jang et al. coated DES with WKYMVm peptide and hyaluronic acid, and using rabbit iliac model found the WKYMVm coating promoted endothelial healing but did not reduce restenosis rate compared to commercial DES (Jang et al., 2015). Wei et al. immobilized REDV peptide and phosphorylcholine on BMS and showed the competitive ability of endothelial cell growth over smooth muscle growth *in vitro* and *in vivo*, and reduced restenosis compared to BMS (Wei et al., 2013). The downside of using adhesive peptides to recruit circulating cells, however, is its possibility of increasing unspecific binding of other circulating cells such as mononuclear cells.

Immobilization of GAGs or GAG-mimics onto the stent surface is a strategy to enhance stent anti-thrombogenicity. Heparin, heparin-like molecules and hyaluronic acid are often utilized in combination with other molecules to coat stent surfaces. Heparin was used with NO-producing, DES, or bioresorbable stents (Bae et al., 2017; Lee et al., 2019; Qiu et al., 2019; Zhu et al., 2020; Zhang et al., 2021). Due to its anti-thrombogenic, anti-fouling characteristics, hyaluronic acid often serves as a base adhesive material on stents (Zhang et al., 2015; Kim et al., 2016).

Besides adhesive peptides and GAGs, new signaling molecules such as CD31-mimic are used to stimulate the proliferation of adjacent endothelial cells to regenerate the endothelium (Diaz-Rodriguez et al., 2021).

Stent Coating With a Combination of Biomolecules

There is an increasing interest in researching multifunctional stent surfaces that contain components promoting therapeutic effect, selective regeneration, and/or critical endothelial functions. In particular, coatings are designed to both

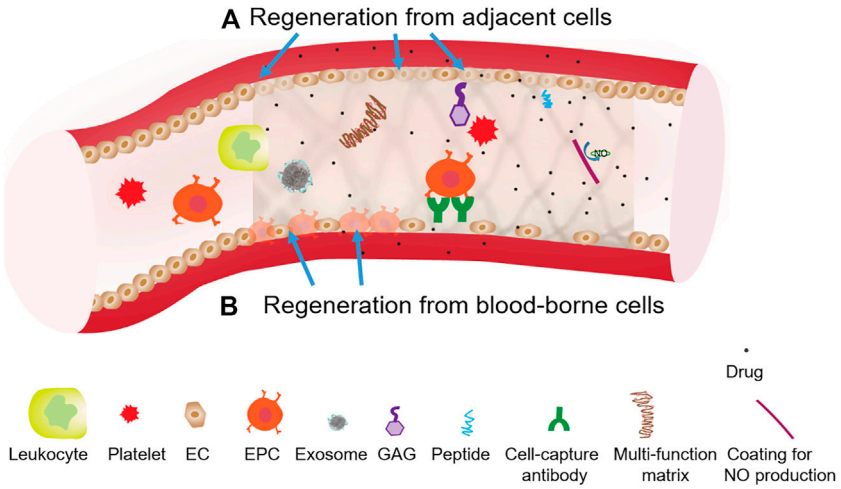

FIGURE 2 | Two generation mechanisms using surface functionalizations to override drug inhition effects on stent endothelialization. **(A)** Regeneration from adjacent cells, which can involve the functionalization of stents with angiogenic peptide or NO producing coating. **(B)** Regeneration from blood-borne cells, which can involve the functionalization of stents with certain cell-capture antibodies, adhesive peptide or growth factor.

counteract endothelial dysfunction and mimic endothelial characteristics. For example, recent studies have combined NO production with cell-adhesive ligands or anti-thrombogenic molecules (Qiu et al., 2019; Yang et al., 2020b; Yang et al., 2020c; Lyu et al., 2020; Zhu et al., 2020; Ma et al., 2021; Zhang et al., 2021). Yang et al. functionalized the stent with NO-generating organoselenium and EPC-targeting peptide through mussel adhesive chemistry and bio-orthogonal conjugation (Yang et al., 2020b). The ratio of the two components on stent the coating was optimized for anti-thrombosis, smooth muscle inhibition and EPC-capture capacity, ultimately leading to rapid re-endothelialization and effective stent restenosis prevention *in vivo*.

Regenerative Strategies for Covered Stents

Few attempts have been made to functionalize or replace the existing polytetrafluoroethylene-covered stent. Among them is the use of polyurethane-covered stent in recent studies, but its clinical efficacy is yet to be demonstrated with comparisons to polytetrafluoroethylene-covered stent (Hernández-Enríquez et al., 2018; Song et al., 2021). A new trend is to develop covered stents using natural-derived or biodegradable materials with regenerative potentials, as illustrated by endothelial progenitor cells-laden coronary stent covered with ECM (Park et al., 2021) and stent covered with silk and elastin proteins (Putzu et al., 2019). Additionally, to replace inert materials used for covering stents, a wide range of vascular-regenerative polymers available for vascular graft applications can also be excellent candidates (Leal et al., 2021). For example, we demonstrated vascular grafts composed of coaxial fibers with a structure of polycaprolactone core and photoclickable, 4-arm thiolated polyethylene glycol-norbornene sheath, which can be conjugated to biomolecules for vascular regeneration (Iglesias-Echevarria et al., 2021). In the future, the platform of covered stent with regenerative covering may evolve into a new type of stent therapy which utilize bioabsorbable materials with regenerative signals to better treat vascular diseases.

In summary, new developments in the stent surface functionalizations focus on the regeneration of the vascular endothelium or the restoration of major endothelial functions such as anti-thrombogenicity and NO production. The endothelium normally controls smooth muscle activities, and provides an efficient barrier against thrombosis and inflammation. However, the endothelium reestablished after the stenting procedure (e.g., percutaneous coronary intervention) is incompetent in terms of its integrity and function, showing poorly formed cell junctions, reduced expression of anti-thrombotic molecules (e.g., thrombomodulin, prostacyclin), and decreased NO production (Otsuka et al., 2012). Drugs from DES further inhibit endothelial regeneration or function restoration. The missing or incompetent endothelium in existing stent products (e.g., BMS, DES, and covered stent) is a root cause of late or very late in-stent thrombosis and restenosis, as well as impaired function of proximal or distal blood vessels. As shown in **Figure 2**, re-endothelialization mechanisms include regeneration from adjacent cells and regeneration from blood-borne cells, both of which require regenerative cues on stents to override the cell inhibition from therapeutic agents (i.e., drugs). Improved understanding of regeneration mechanisms for competently functioning endothelium is essential for long-term stent performances.

Bioabsorbable Vascular Stent

To mitigate the risk of restenosis and thrombosis in stent, it has long been envisioned that a fully bioresorbable polymer is used in replacement of metal. The first-generation, FDA-approved BVS product was created and launched by Abbott as Absorb BVS stent in September 2012. This type of products was characterized by a degradation rate, ranging from 6 months to 2 years, to avoid undesired long-term effects, but demonstrated worse clinical performances including in-stent thrombosis, restenosis, and

target vessel myocardial infarction, when compared to BMS or DES (Capodanno et al., 2015; Lipinski et al., 2016; Serruys et al., 2016). Thus, it was not recommended (Neumann et al., 2019), and pulled off the market in 2017. The first-generation BVS also was found a long-term concern on side branch ostia (Onuma et al., 2017).

Newer BVS technologies and devices mostly loaded with drugs are currently undergoing preclinical and clinical testing (Ellis et al., 2017; Serruys et al., 2017; Omar and Kumbhani, 2019). A promising feature of BVS, shown in a clinical study comparing BVS to DES, is more significant outward remodeling (Serruys et al., 2017). To decrease BVS thrombogenicity, one advancement, like the major improvement made in the second generation of DES, is a progressive reduction of the BVS strut thickness to as thin as 60 μm (Buccheri and Capodanno, 2019). The majority of BVSs on the pre-market consist of polymers such as polylactic acid used in Absorb BVS, but degradable metals such as magnesium (Chen et al., 2019; Hideo-Kajita et al., 2019) or zinc (Fu et al., 2020) are also actively being developed for newer generation of BVS.

To enhance therapeutic and regenerative performances of BVS, bioactive molecules can also coat the BVS surface or be integrated into the bulk polymer. Any regenerative molecules reviewed above (**Table 1**) may be added into or on top of BVS. Besides these molecules, Yang et al. formed a multilayer coating of collagen type III and hyaluronic acid on a polylactic acid stent via layer-by-layer assembly, which enhanced endothelialization and thromboprotection, and inhibited excessive neointimal hyperplasia using a rabbit abdominal aorta model (Yang et al., 2021). Collagen type III does not present binding sites for platelets while retaining the affinity for endothelial cells.

For a more detailed summary of BVS, please refer to additional reviews on the topic (Omar and Kumbhani, 2019; Cockerill et al., 2021; Yuan et al., 2022). In brief, researchers in academia and industry share the dream of a bioabsorbable scaffold that delivers therapeutic and regenerative agents to the vessel, maintains radial strength for a sufficient period of time (>6 months), and then disappears when therapy and regeneration jobs are done. The BVS materials are resorbed over 6 months to 2 years, having a potential of eliminating chronic inflammation and enabling endothelial regeneration, when compared to permanent metallic stents. However, the clinical performances of existing BVS are inferior to DES or BMS. Improved understanding of the failure mechanisms of existing BVS is essential for the design of new generation BVS with more superior clinical performances.

CHALLENGES AND FUTURE PERSPECTIVES

With advancements in stent technologies and implantation techniques, the event-free survival rate for stent patients, in particular those with the new generation DES, has kept improving in the last decade. Based on the overall satisfactory results from the new generation DES, it can be extremely challenging for any new stent design or material to demonstrate better effectiveness while being safe and worth enormous investments into innovation and translation. An additional barrier that hampers the translation of the new design or material to clinical applications lies in the lack of sufficient understanding of mechanisms underlying the materials interactions. Further refinement of existing DES has an excellent benefit-risk ratio. Therefore, future developments of permanent metallic stents such as new functional coating or topography strategies may take the advantage of the current DES platform, based off it for design and performance analysis.

To address the long-term concern about permanent metallic stents—an ongoing risk of restenosis or thrombosis arising from the implant site which persists for at least 20 years (Yamaji et al., 2010; Yamaji et al., 2016; Stone et al., 2019) occurring after the first year at a rate of approximately 2–3% per year—BVS composed of biodegradable polymers or metals holds great potentials. Hypothetically, BVS provides a temporary scaffold for selective vascular regeneration—a complete re-endothelialization and significant outward remodeling. Such regeneration ideally may prevent the adverse events, including strut fractures, loss of vessel compliance, vasomotion, maladaptive vascular remodeling, and development of late neoatherosclerosis (Stone et al., 2019). Currently, this dream of BVS is not close to reality. A step further towards the dream is to innovate biodegradable materials engineering and innovate designs for both stent structure and surface, with the goal of balancing short-term and long-term requirements for BVS in terms of mechanics, therapy and regeneration. In parallel, mechanisms underlying BVS-related vascular pathobiology should be defined in a synergistic manner.

Finally, an amazing array of stent functionalization strategies are available in the literatures. However, translation efforts of them into clinical practices remain very limited if not futile. Future efforts in this area may fall into two categories: 1) Stent design according to specific needs of a patient's cohort (or a specialized preclinical model) for competitive efficacy and superior outcome. For example, the regeneration of distal vasculature is only desired in ischemic conditions. Another example is for cell-capture stents: the EPC quantity, quality and capability can vary greatly among patients, and thus *in situ* stent cellularization results can vary accordingly. In this case, custom design strategies may be necessary for a specific patient's cohort. 2) Multiple signaling strategies with defined spatiotemporal regimes of regenerative signals.

AUTHOR CONTRIBUTIONS

WT drafted the review. PS, MR, and RJ edited it.

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MSC in Tendon and Joint Disease: The Context-Sensitive Link Between Targets and Therapeutic Mechanisms

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Mesenchymal stromal cells (MSC) represent a promising treatment option for tendon disorders and joint diseases, primarily osteoarthritis. Since MSC are highly context-sensitive to their microenvironment, their therapeutic efficacy is influenced by their tissue-specific pathologically altered targets. These include not only cellular components, such as resident cells and invading immunocompetent cells, but also components of the tissue-characteristic extracellular matrix. Although numerous *in vitro* models have already shown potential MSC-related mechanisms of action in tendon and joint diseases, only a limited number reflect the disease-specific microenvironment and allow conclusions about well-directed MSC-based therapies for injured tendon and joint-associated tissues. In both injured tissue types, inflammatory processes play a pivotal pathophysiological role. In this context, MSC-mediated macrophage modulation seems to be an important mode of action across these tissues. Additional target cells of MSC applied in tendon and joint disorders include tenocytes, synoviocytes as well as other invading and resident immune cells. It remains of critical importance whether the context-sensitive interplay between MSC and tissue- and disease-specific targets results in an overall promotion or inhibition of the desired therapeutic effects. This review presents the authors' viewpoint on disease-related targets of MSC therapeutically applied in tendon and joint diseases, focusing on the equine patient as valid animal model.

Keywords: mesenchymal stromal cells (MSC), tendon, joint, osteoarthritis, context sensitivity, target, immunoregulation

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INTRODUCTION

The former idea behind the application of mesenchymal stromal cells (MSC) in injured tissue was to transplant a source of undifferentiated progenitor cells and thereby achieve local effects, which were deemed to be a direct integration of MSC, further leading to restoration and regeneration at the site of injury. In musculoskeletal conditions, this is supported by studies investigating the retention and fate of locally applied MSC and by the repeated proof of MSC differentiation potential into osteogenic, chondrogenic, adipogenic and myogenic cell lines (Forest et al., 2010; Sole et al., 2013; Vieira et al., 2014). However, this assumption is more and more supplemented by findings about the ability of MSC to communicate transcellularly by direct cell-to-cell contact and release of soluble factors and extracellular vesicles (Islam et al., 2012; Liu et al., 2018; Witwer et al., 2019). With the knowledge of these transcellular communication mechanisms, the idea of a locally acting MSC requires a broader

explanation than the plausible cell replacement theory alone. Moreover, locally applied MSC seem to be adaptive and promote context-sensitive cell communication. To predict MSC-target interaction, the cell and matrix composition and immunological state of the target tissue are pivotal. However, due to the complex pathophysiological mechanisms in the course of inflammation, cell and matrix destruction as characteristics at the site of injury are inconsistent, depending on the type of tissue, the grade of cell destruction and the grade of local inflammatory reaction (Ackerman et al., 2021; Scanzello and Goldring, 2021). Furthermore, though not being addressed within the present work, tissue specific resident MSC have already been shown to react context-sensitive in the respective pathophysiological microenvironment and may also be relevant for target interaction of applied MSC (Costa-Almeida et al., 2019).

Animal models play a vital role in providing information about tissue-context-sensitivity of MSC. Particularly the clarification of the MSC mode of action in injured tendons and joints forced scientists to study artificially induced lesions in various species (Delling et al., 2015; Ahrberg et al., 2018; Kwon et al., 2018; Kim et al., 2019; Khan et al., 2020). Nevertheless, all artificial disease models remain an approximation and do not allow to investigate the naturally occurring pathophysiological circumstances. Therefore, we consider it crucial to include animal models based on naturally occurring tendon and joint diseases in basic research strategies, for which the horse is most suitable and repeatedly used (Becerra et al., 2013; Smith et al., 2013; Berner et al., 2016; Broeckx et al., 2019). However, the so far limited number of existing pre-clinical and clinical studies focus on clinically detectable effects and safety, thereby only indirect conclusions about target specific cellular mechanisms of action can be drawn.

Understanding the context-sensitive mode of action of MSC specifically in tendons and joints will form the basis for their future successful application. Here, we discuss selected therapeutic targets and mechanisms of MSC applications in tendon and joint diseases and illustrate their mutual interplay. Due to its high relevance with respect to investigating naturally occurring diseases, we focus on research in the equine model.

TARGETS AND MECHANISMS OF MSC IN TENDON AND JOINT DISEASE

The desired targets of MSC in therapeutic applications can be deviated from the pathophysiological mechanisms driving the respective disease. However, it is important to note that the desired targets are not necessarily the targets that are actually addressed, and even if so, the effect on the target might not be as initially expected. In this line, it must be considered that MSC are highly sensitive to their environment, which will be further addressed below. Nevertheless, aiming at a well-directed cell therapy, the first step must be to identify pathophysiological key players and to observe the effects of MSC after transplantation.

Possible Targets of MSC in Acute and Chronic Tendon Disease

Possible targets of MSC in tendon disease include cellular components on the one hand, such as the resident tenocytes, invading immune cells and endothelial cells, and the extracellular matrix (ECM) on the other hand. Regeneration of the highly specialized matrix must always be the central goal, as it is responsible for tendon function. However, a functional replacement of cells and ECM by the MSC alone is unlikely to be achieved. Rather, the MSC should support a milieu which allows the tendon to regenerate. In this line, the cellular targets could be in the foreground in phases of active inflammation and acute injury, whereas the scarred ECM may need to be focused in chronic tendon disease.

In acute tendon disease or injury, activated tenocytes and invading immune cells, namely macrophages, foster a milieu of inflammation and further matrix degradation. Indeed, in a canine model of acute tendon injury, macrophages could be identified as a target of MSC treatment, which promoted their M1/M2 phenotype switch (Shen et al., 2016; Gelberman et al., 2017). While the identification of the underlying molecular mechanisms and targets is more complex, this effect appeared to be mediated by interleukin (IL)-4 (Shen et al., 2016; Gelberman et al., 2017). The specific interplay of tenocytes and MSC *in vivo* has not been elucidated in much detail so far, yet besides direct tenocyte targeting, a protection of tenocytes *via* macrophage modulation and overall reduction of pro-inflammatory stimuli can be assumed (Manning et al., 2015). Furthermore, neovascularization plays a critical and often controversially discussed role in tendon healing, as it is crucial to successful healing but persistently increased vascularization negatively impacts long-term outcomes (Korntner et al., 2019; Liu et al., 2021). A transiently increased vascularization has repeatedly been observed as a response to MSC treatment of equine tendon lesions (Conze et al., 2014; Ahrberg et al., 2018). This suggests that vascular endothelial cells are targeted in a beneficial way, which likely is related to increased vascular endothelial growth factor (VEGF) levels (Okamoto et al., 2010; Yuksel et al., 2016).

In chronic tendon disease, failed ECM repair has led to scar tissue within the tendon. Its inferior biomechanical properties predispose to re-injury and its altered biophysical and biochemical properties fail to provide the appropriate guidance for regeneration to resident cells. Therefore, it appears advantageous to target the matrix remodeling process, with enzymatic degradation of scar matrix components and promotion of collagen I fibrillogenesis and cross-linking. So far, some insights into MSC-driven matrix remodeling *in vivo* can be deduced from studies dealing with acute equine tendon disease, but suitable models for chronic disease are lacking. Here, it could not only be shown that the tendon matrix has an improved architecture and composition after MSC injection (Schnabel et al., 2009; Crovace et al., 2010; Smith et al., 2013), but also that the treatment reduced collagenase [matrix metalloproteinase (MMP)13] activity (Smith et al., 2013) and upregulated stromelysin (MMP3) gene expression (Romero et al., 2017). This might indicate a selective and beneficial support of

enzymatic tendon matrix remodeling, but data are still scarce and, importantly, these results were obtained after treatment of (sub) acute lesions but not after treatment of chronic disease.

Possible Targets of MSC in Osteoarthritis

Osteoarthritis (OA) was originally understood as a classical "wear and tear" disease, mainly related to degenerative changes in the articular cartilage. However, nowadays OA is referred to as a complex, multisystem disease affecting the whole joint and detailed pathophysiological mechanisms are still not completely understood. Nevertheless, it is beyond doubt that inflammatory processes in the synovial membrane play a crucial role in the initiation of OA and in promoting subsequent cartilage damage and pain at least in the inflammation-driven subset of patients (de Lange-Brokaar et al., 2012). Therefore, the previously suggested differentiation potential of MSC into chondrocytes aiding cartilage regeneration (Satué et al., 2019; Song et al., 2021) was pushed into the background against the ability of the MSC to target local immunocompetent cells.

OA-related synovitis is mainly driven by the innate immune system, which should therefore be targeted by MSC therapies, again with an outstanding role of macrophages. The macrophages potentially targeted by MSC include invading pro-inflammatory monocyte-derived macrophages within the synovial fluid and adjacent joint tissue as well as resident immunomodulatory synovial macrophages consisting of several subgroups of cells (van den Bosch, 2021). Besides their pivotal role in OA-related synovitis, synovial macrophages are considered critical for tissue homeostasis, thus being reasonably involved in the re-establishment of immune homeostasis within the injured joint. Recent studies in the mouse model indicate different roles of invading monocyte-derived and tissue resident synovial macrophages during homeostasis as well as disease. It was shown that locally renewing resident macrophages within the inner synovial lining form membrane-like structures as protective physical barrier between the intraarticular space and the synovial capillary network (Culemann et al., 2019). These resident synovial macrophages maintain their immune-regulatory function even within an inflammatory environment. However, the specific pathogenetic role of these cells has not yet been addressed in OA (Haubruck et al., 2021). Tracking ferumoxytol-labeled murine MSC in a model of induced OA, MSC-treated joints were reported to show a reduced number of pro-inflammatory macrophages in favor of an increased proportion of homeostatic polarized macrophages (Hamilton et al., 2019). In co-culture models, it has been shown that MSC reduce M1-like-activating factors such as IL-1β and tumor necrosis factor (TNF)-α and induce typical M2-like macrophage markers such as IL-10, cluster of differentiation (CD)163 and CD206, partially through the prostaglandin E_2/cyclooxygenase two pathway. Since this was shown in contact as well as in trans-well cultures, the authors suggest different MSC-mediated phases of immunomodulation including firstly an interaction *via* soluble factors and secondly a direct adhesion to the synovium (Manferdini et al., 2017). Yet so far, it remains to be understood whether applied MSC rather support tissue-resident macrophages or regulate primarily the inflammatory phenotype of the invading, mainly pro-inflammatory macrophages. *In vivo* and *ex vivo* models, ideally based on naturally occurring OA, could shed more light on these MSC-macrophage interactions.

Polymorphonuclear cells could represent a further target for MSC applied during OA-related synovitis. In a murine model of induced OA of the knee, locally applied MSC attracted colocalizing polymorphonuclear cells within the synovium. This was likely due to an IL-1β-mediated increased chemokine release of the applied MSC and led to the up-regulation of the phagocytic activity as well as the down-regulation of the pro-inflammatory cytokine release of the locally clustered polymorphonuclear cells (Van Dalen et al., 2019). This upregulation of the phagocytic activity might contribute to the removal of cartilage fragments from the synovial fluid, thus breaking the vicious inflammatory circle in OA.

ADAPTION OF MSC TO THEIR TARGETS IN PATHOPHYSIOLOGICAL CONTEXTS

MSCare highly sensitive to their environment, which entails a mutual interplay between the transplanted cells and their pathologically altered targets. The latter represent a crucial part of the disease milieu that will influence the MSC once transplanted (**Table 1**). It remains a central question whether their adaptation to the disease milieu leads to a promotion or inhibition of the desired effects on the respective targets.

Inflammatory conditions, which are often present in tendon as well as joint disease, are well-known to impact on MSC. MSC harvested from inflammatory environments show a decreased and variable fitness, which we have demonstrated for equine synovial fluid-derived MSC from osteoarthritic joints (Burk et al., 2017). In humans, the inflammatory state of synovial fluid in OA-affected knees modulates not only the proliferation of synovial fluid-derived MSC, but also induced a reduced differentiation potential, which is suggested to result in a lower ability to reverse OA (de Sousa et al., 2019). Additionally, MSC from discarded articular cartilage collected from OA patients during joint replacement therapy showed a rapid and strong mineralization upon chondrogenic induction, while markers of chondrocyte hypertrophy and stem cell osteogenesis were induced. These complex mechanisms demonstrate that MSC differentiation within the inflammatory environment might be coupled with undesired chondrocyte hypertrophy and osteogenesis (Hu et al., 2019).

Subjecting healthy MSC to inflammatory conditions impacts on their mode of action. This includes a decreased differentiation potential but can, up to a certain extent of inflammation, promote MSC immunomodulatory and protective mechanisms. With regard to tenogenic differentiation, we have shown that not only the presence of pro-inflammatory cytokines, namely IL-1β, but also the presence of leukocytes decreased the expression of the tendon transcription factor scleraxis in equine MSC (Brandt et al., 2018). Similarly, chondrogenic differentiation in pellet culture was decreased in the presence of the pro-inflammatory cytokines (Brandt et al., 2018), corresponding to findings in

TABLE 1 | Overview of microenvironmental factors and their effect on MSC potential mechanisms of action in tendon and joint disease.

Microenvironmental factors influencing MSC mode of action in tendon and joint diseases			
Soluble components	**Cellular components**	**Extracellular environment**	
Cytokines, chemokines, enzymes, exosomes	Damaged resident cells, invading leukocytes	Extracellular matrix composition and architecture, oxygen tension	
Putative effects on therapeutically applied MSC in tendon and joint diseases			
Differentiation potential ↓	**Immunomodulatory function ↑**	**Angiogenic effects ↑**	**Matrix remodeling ↓**
• Reduced by inflammation • Possible misrouted osteogenic differentiation	• Stimulated by inflammation • Production of anti-/pro-inflammatory cytokines • Regulatory effects on cells of the adaptive and innate immune system, including macrophages	• Stimulated by hypoxia and inflammation • Growth factor release • Support of endothelial cells	• Reduced by fibrotic extracellular matrix • Altered matrix-degrading enzyme activity

human MSC (Kondo et al., 2013; Liu et al., 2017). On the other hand, interestingly, chondrogenically differentiated equine MSC responded less to IL-1β stimulation than their naïve counterparts (Bundgaard et al., 2020). However, most likely more important than differentiation, pro-inflammatory stimulation has repeatedly been shown to increase MSC immunomodulatory potential. In a co-culture model using equine MSC and stimulated or non-stimulated leukocytes, we could show that the modulatory MSC mechanisms depended on the extent of inflammatory stimulation. Mild inflammatory conditions increased the percentage of MSC synthesizing the anti-inflammatory IL-10, while stronger inflammatory conditions promoted the regulatory effects of MSC on T cells, possibly *via* prostaglandin E2. However, not all effects observed in strong inflammatory conditions were strictly anti-inflammatory (Hillmann et al., 2019). With respect to joint disease, recent data has shown that synovial fluid collected from OA-affected joints influences the immunomodulatory properties of the MSC secretome and thereby promotes an anti-inflammatory subset of immune cells including an enhanced macrophage polarization into the M2-like phenotype (Cifù et al., 2020). Hypoxic conditions strongly influenced the migration and cytokine receptor expression of MSC cultured in synovial fluid collected from OA patients (Manferdini et al., 2020).

Extracellular matrices also have a strong impact on MSC properties and behavior (Li et al., 2021). However, the effects of pathologically altered ECM on MSC are still widely unknown, despite their relevance for treating chronic fibrotic conditions, attempting to specifically target the ECM. We observed that culturing equine MSC on decellularized tendon ECM failed to display synergistic effects with the tenogenic transforming growth factor (TGF)-β3 (Roth et al., 2018). This may be due to inhibitory effects of integrin/Rho/Rho-associated protein kinase (Rho/ROCK) axis activation by the extracellular matrix on canonical TGF-β3/smad2/3 signaling (Melzer et al., 2021), providing an example of cell-ECM interactions that could interfere with assumed mechanisms. Recently, we could also demonstrate that MSC undergo pathological adaptions upon exposure to scarred ECM. When culturing equine MSC on decellularized tendon matrices obtained from tendons with naturally occurred chronic disease, tenogenic differentiation was evident despite the ECM alterations in the tendon matrix, but the gene expression and activity of MMP was decreased (Doll et al., 2021). This effect was transient, but could hamper effective targeting of the scar tissue within the ECM.

DISCUSSION

Our growing understanding of pathophysiology and MSC behavior will promote the development of the next generation of MSC-based therapies. However, investigating target and therapeutic cells should go hand in hand and reflects naturally occurring disease. Deciphering the mutual interplay between MSC and their targets in relevant disease environments could provide the missing link for consistent therapeutic success. Recently, an interesting step in this direction was taken with the development of a bioassay, by which the effect of a patient's OA joint microenvironment on the ability of MSC to support cartilage formation could be deduced. MSC-based cartilage formation was modified by the OA joint microenvironment, which could be useful to predict the therapeutic outcome (Neefjes et al., 2021). Such approaches may help to identify "non-responders" in advance and eventually lead to personalized OA treatments. Nevertheless, further strategies to deal with putative non-responders will still be required.

MSC priming, a promising strategy to enhance MSC potency and efficacy, directly results from the context-sensitive nature of the MSC, and aims to train the cells for their therapeutic task by subjecting them to pathophysiological stimuli. So far, this strategy has mainly been investigated with regard to inflammatory priming or "licensing" to enhance MSC immunomodulatory potential. For example, equine bone marrow-derived MSC primed with interferon (IFN)-γ were similarly activated as by co-culture with M1-polarized macrophages (Cassano et al., 2018). As the effects of IFN-γ were more consistent and also led to a chondroprotective secretome, the authors suggested that MSC priming before transplantation could be more successful than having the MSC activated by the pathological *in vivo* environment (Cassano et al., 2018), i.e., by their cellular targets, alone. In this line, in an equine model of chemically induced OA of the radio-carpal joint, inflammatory priming with TNF-α and IFN-γ led to an increased anti-inflammatory and regulatory effect of applied MSC. However, the repeated intraarticular application of inflammatory primed allogeneic MSC resulted in a slight transient inflammatory response,

possibly indicative for an increased immunogenicity of primed MSC (Barrachina et al., 2018). This must be carefully considered, along with the risk of exacerbation upon excessive stimulation, which might affect therapeutic safety of primed MSC. So far, a therapy with non-primed MSC, which naturally adapt to the disease environment, appears to be sufficient to target the immune cells in most patients and represents the safer option until more knowledge is available. However, MSC priming may help non-responding patients and this concept in general appears highly valuable for future therapies.

Aiming to further improve MSC priming approaches, it will be interesting whether priming regimes can be tailored for specific targeting of certain cell types or ECM components. With respect to tendon and OA therapies, macrophages remain the most promising cell type to modulate or alter their functional phenotype, as key regulatory cells in tendon as well as OA-related inflammation. However, little is known about the conditions of the disease environment in which MSC target inflammation and decrease the pro-inflammatory state of macrophages at their best (van den Bosch, 2021). Therefore, pre-treatment options to improve MSC efficacy by targeted influencing of tendon and joint-associated macrophages should be set on the scientific agenda. This implies optimizing MSC and target macrophage communication applicable for different disease stages, including conditions with an unfavourable environment such as the exacerbated OA-related synovitis.

We conclude that the mode of action of locally applied MSC is influenced by the cellular and molecular microenvironment at the injured site and vice versa. In this context, MSC-mediated macrophage modulation represents a key tool to positively influence inflammation in injured tendons and joints. However, a broad range of additional target cells as well as the ECM also have to be addressed. The best possible outcome for any MSC recipient will be achieved when the target tissue is characterized and the applied MSC, including potential priming, are matched with each other as specifically as possible.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AT, JB, and SR constructed the manuscript. AT, JB, SR, and WB edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Innovative Cell and Platelet Rich Plasma Therapies for Diabetic Foot Ulcer Treatment: The Allogeneic Approach

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Cutaneous chronic wounds are a major global health burden in continuous growth, because of population aging and the higher incidence of chronic diseases, such as diabetes. Different treatments have been proposed: biological, surgical, and physical. However, most of these treatments are palliative and none of them can be considered fully satisfactory. During a spontaneous wound healing, endogenous regeneration mechanisms and resident cell activity are triggered by the released platelet content. Activated stem and progenitor cells are key factors for ulcer healing, and they can be either recruited to the wound site from the tissue itself (resident cells) or from elsewhere. Transplant of skin substitutes, and of stem cells derived from tissues such as bone marrow or adipose tissue, together with platelet-rich plasma (PRP) treatments have been proposed as therapeutic options, and they represent the today most promising tools to promote ulcer healing in diabetes. Although stem cells can directly participate to skin repair, they primarily contribute to the tissue remodeling by releasing biomolecules and microvesicles able to stimulate the endogenous regeneration mechanisms. Stem cells and PRP can be obtained from patients as autologous preparations. However, in the diabetic condition, poor cell number, reduced cell activity or impaired PRP efficacy may limit their use. Administration of allogeneic preparations from healthy and/or younger donors is regarded with increasing interest to overcome such limitation. This review summarizes the results obtained when these innovative treatments were adopted in preclinical animal models of diabetes and in diabetic patients, with a focus on allogeneic preparations.

Keywords: diabetic foot ulcers, PRP, cell therapy, advanced therapy, allogeneic preparations

1 INTRODUCTION

Skin ulcers are open sores often accompanied by the sloughing-off of inflamed tissue. A slow-healing ulcer of the leg (usually the lower leg) is typically associated with complications of poor blood circulation, such as varicose veins, deep venous insufficiency, arterial and peripheral vascular diseases. Other causes of leg ulceration include trauma, bacterial and/or mycotic infections, and neuropathy related to diabetic disease. Pressure ulcers (bed sores) are a very common complication

in elderly hypomobile patients. Cutaneous chronic wounds represent a major global health burden in continuous growth since this pathology is closely linked to the higher incidence in the aging population of chronic diseases, including diabetes. Women experience more pain and have a worse quality of life than men (Bartley and Fillingim, 2013; Rovner et al., 2017). There is a direct correlation between pain and quality of life, which is worse for ulcers with a longer duration and a larger area (Guarnera et al., 2007).

The prevalence of skin ulcers and the cost of treatments are very high. In industrialized countries, it has been estimated that 1%–2% of the population will experience a chronic wound during their lifetime (Sen et al., 2009). Lower limb ulcers represent a major clinical problem particularly for diabetic patients (Nabuurs-Franssen et al., 2005). In Europe 5%–7% of the population suffers from diabetes and this is expected to increase significantly during the next 20 years, especially in the elderly. It is estimated that up to 25% of all diabetics will develop an ulcer (Wu et al., 2007; Armstrong et al., 2017).

Chronic ulcers are difficult to heal because of the diminished blood flow interfering with the healing process. Patient care is concerned with preventing a superimposed infection in the ulcer, increasing blood flow in the deeper veins, and decreasing pressure within the superficial veins. Different treatments have been proposed: biological, surgical, and physical. However, these treatments are mostly palliative and none of them can be considered fully satisfactory. More recently, treatments with allogeneic stem/progenitor cells or platelet-rich plasma (PRP) have been proposed as alternative therapeutic options. This review summarizes the results obtained when these innovative treatments were adopted in diabetic ulcer patients.

2 HEALING DEFECTS IN DIABETES

Skin repairs through a complex process that has been conventionally divided into four sequential and partly overlapping phases: prompt blood hemostasis is followed by inflammation, active cell proliferation, and long-lasting tissue remodeling (Shaw and Martin, 2009). Resident cell populations, together with cells recruited from the bloodstream, contribute to wound healing through a continuous molecular crosstalk as well as *via* interactions with the extracellular matrix (ECM). Platelets entrapped in the provisional fibrin clot release cytokines, primarily the stromal-derived factor-1 (SDF-1, also named CXCL12), and growth factors that promote immune cell recruitment and resident cell activation. Hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), transforming growth factor-β1 (TGF-β1) and epidermal growth factor (EGF) are among growth factors released by platelets (Barrientos et al., 2008). Neutrophils, monocytes, and lymphocytes in sequence invade the wound bed. Together with resident immune cells, they trigger responses necessary for recovering tissue sterility and for promoting skin regeneration. They increase reactive oxygen species (ROS) production, and release cytokines, growth factors and antimicrobial peptides (Barrientos et al., 2008; Brazil et al., 2019). Monocytes differentiate into M1 macrophages releasing several proinflammatory mediators. This reach milieu activates resident cell types that start proliferating and migrating. Pro-angiogenic growth factors released by anti-inflammatory M2 macrophages and by migrating keratinocytes (Brown et al., 1992) promote neoangiogenesis that sustains the high metabolic demand of the regenerative phase. The newformed skin is far from being a functional tissue, as it manifests hypertrophic epidermis, irregularly deposited and tick matrix, high cellularity, and excessive blood vessel number. Apoptotic cell removal and matrix reorganization restore skin homeostasis in the remodeling phase. The wound healing process often ends up with a scar that lacks dermal annexes and manifests reduced tensile strength. Dysregulation of the events guaranteeing wound repair may result in either loss of healing with chronic ulcer formation or excessive healing with aberrant scar development (Eming et al., 2014).

The difficult healing and the evolvement of diabetic wounds to chronic ulcers is multifactorial: wound infection, deregulated inflammatory response, abnormally increased oxidative stress, impaired angiogenesis, cell senescence and aberrant extracellular matrix deposition play major roles (Falanga, 2005). These pathogenetic mechanisms are in large part common to venous and arterial ulcers, in which insufficient oxygen and nutrient supply underlie the impaired healing response. As for diabetes, increased glucose levels elicit a specific pathogenetic response due to molecular glycation. Advanced-glycation end products (AGE) interact with their receptors (RAGE) at the surface of different cell types and, through activation of the NF-kB transcription factor, promote ROS overproduction and release of inflammatory mediators (Wautier et al., 1996; Goldin et al., 2006). Glycation is responsible for vasculopathy and peripheral neuropathy, it affects molecular function, increases ECM deposition and crosslinking and, in general, it impairs the activity of the different cell types involved in the healing process.

It is widely recognized that in diabetic wounds cell proliferation, migration, differentiation, and ability to release growth factors are impaired (Spravchikov et al., 2001; Lerman et al., 2003; Thangarajah et al., 2009; Cianfarani et al., 2013; Gallagher et al., 2015; Berlanga-Acosta et al., 2020; Sawaya et al., 2020). The number of recruited circulating cells is reduced due to decreased release of and/or response to chemotactic factors (Tchaikovski et al., 2009; Sawaya et al., 2020). Angiogenesis is profoundly hampered for nitric oxide (NO) deficit, reduced release of angiogenic factors, decreased recruitment and differentiation of hematopoietic and endothelial precursors (Tepper et al., 2010; Kolluru et al., 2012). Cells of the immune system manifest uncontrolled activity in diabetic wounds: initial inflammatory response is impaired facilitating colonization by pathogens, while persistent presence of inflammatory cells and increased cytokine and protease production strongly contribute to healing failure at later stages. Macrophages are major actors in the wound healing process, as well as in the abnormally prolonged inflammatory phase of diabetic wounds when they fail to polarize from the M1 pro-inflammatory phenotype into the M2 anti-

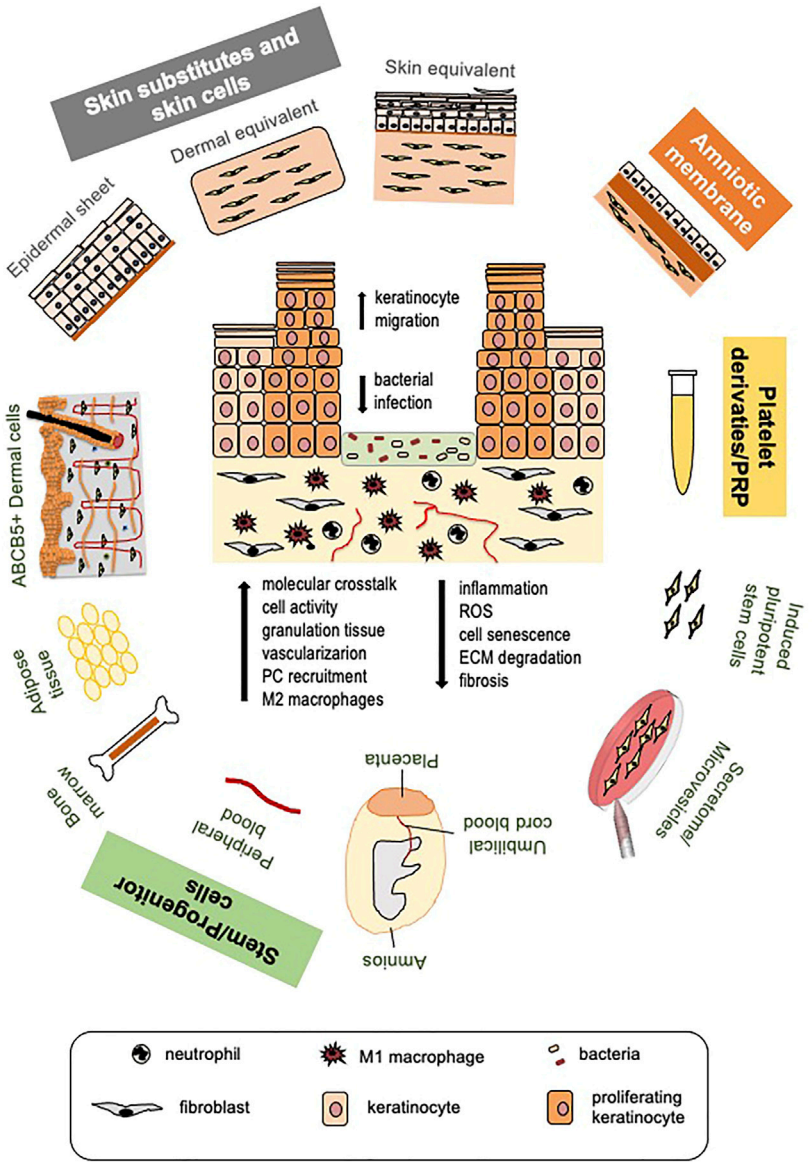

FIGURE 1 | Cell and platelet-derived tools for diabetic ulcer therapy. Different cell or platelet-derived tools with the beneficial effects they elicit on diabetic wound healing (a diabetic ulcer schematic is depicted in the center). Skin cells and skin substitutes, stem cells of different origin and their secretome, platelet derivatives and platelet-rich plasma (PRP) are all potential tools to promote healing of diabetic ulcers. Induced pluripotent stem cells (iPSCs) derived from fibroblasts are promising therapeutic tools for improving autologous and possibly allogeneic cell therapy, not yet tested at clinical level. Therapeutic tools are applied after routine removal of necrotic tissue and antiseptic treatments. Beneficial effects of all approaches are largely due to cell ability to release great amount of growth factors, cytokines and chemokines, and other biomolecules; for this reason, MSC secretome and released microvesicles are being tested as cell-free, safer therapeutic approaches. These therapies increase the molecular crosstalk, promote cell function and, likely, circulating precursor cell recruitment. Re-epithelialization is stimulated due to enhanced keratinocyte migration; granulation tissue formation and vascularization are improved. Mesenchymal stem cells (MSCs), the amniotic membrane and PRP specifically manifest immunomodulatory and immunosuppressive properties leading to reduced wound inflammation and scar tissue formation, allowing allogeneic cell and PRP use without major adverse effects. (PC, precursors cells; ROS, reactive oxygen species; ECM, extracellular matrix).

inflammatory/regenerative one (Mirza and Koh, 2011; Mirza et al., 2014).

Altered cell behavior in diabetes also depends on oxidative stress- and inflammation-driven epigenetic changes which are maintained after reversal to a normoglycemic condition (Cencioni et al., 2014). This phenomenon, named as "hyperglycemic memory" or "metabolic memory," likely plays a role in the high rate of recurrence in diabetic ulcers. Changes in global DNA methylation and deregulation in non-coding RNA expression have been described in diabetic skin and wounds (den Dekker et al., 2019; Ozdemir and Feinberg, 2019). When global microRNA expression was analyzed in non-lesional skin of type 1 and type 2 diabetic mice a general transcriptional impairment affecting the expression of microRNA precursors and biogenesis

gene levels was found (Baldini et al., 2020). This data confirms the complexity of molecular defects in diabetes. Based on this evidence, modification of altered epigenetic marks have been suggested as therapeutic strategies against diabetic complications (Spallotta et al., 2013; den Dekker et al., 2019).

3 INNOVATIVE THERAPIES FOR DIABETIC FOOT ULCERS

Chronic skin ulcers are the most common diabetic complication causing pain and poor quality of life for patients. They frequently develop at foot, being referred as diabetic foot ulcers (DFU). Diabetic neuropathy (damage to the foot's sensory nerves) results in foot deformities and/or ulcers that increase the chance of lower-extremity amputations when no treated. When concomitant peripheral arteriopathy occurs, the risk of DFU development strongly increases (Volmer-Thole and Lobmann, 2016).

A global prevalence of more than 6% was estimated for DFU with consequences on clinical and social costs (Zhang et al., 2017; Raghav et al., 2018). Some amputation is needed in approximately 20% of diabetic patients with an ulcer, and the risk of death increases up to 70% after amputation (Armstrong et al., 2017). Finally, ulcers recur in around 40% of patients within 1 year of remission, and this percentage increases over time, being over 60% after 5 years (Armstrong et al., 2017).

Standard DFU management comprises the removal of necrotic tissue (debridement), interventions on the infection, application of dressings to protect the wound and maintain a moist environment necessary for promoting cell activity, offloading, and strict glycemic control (Everett and Mathioudakis, 2018). Surgical intervention for correcting vascular insufficiency can be considered in ischemic ulcers. In the absence of an active healing response, advanced and innovative therapies are considered. Adoption of skin substitutes, amniotic membrane allografts, stem cell-based therapies, including conditioned medium and released microvesicles, or platelet-rich plasma (PRP) treatments represent the most promising tools to promote healing in diabetes (**Figure 1**).

3.1 Skin Substitutes and Skin Cells

Skin grafts are the most obvious therapeutic approach for replacing the lost tissue of skin ulcers. Cadaveric cryopreserved skin allografts can be used to stimulate healing, particularly for deep wounds with exposed bones and tendons (Snyder and Simonson, 1999). Besides creating a temporary barrier, they act by stimulating re-epithelialization and granulation tissue formation; however, a retrospective comparative analysis on the effectiveness of cadaveric skin allograft against bioengineered living cell constructs revealed that living cells manifest better performance (Treadwell et al., 2018).

Since the pioneering works of Gallico et al. (1984) and Boyce et al. (1995), keratinocytes and/or fibroblasts have been topically applied as epidermal or dermal substitutes or as bilayer engineered constructs of both stratified keratinocytes and matrix-embedded fibroblasts. Engineered bilayer substitutes better reproduce skin structural and functional properties, as the crosstalk between keratinocytes and fibroblasts reinforces the activity of both cell types (Dellambra et al., 2019). Three dimensional bioprinting technology is often used for skin equivalent construction (Tan et al., 2020). 3D-bioprinted skin equivalents may contain endothelial cells and pericytes forming vascular structures that interconnect with host vasculature. These complex constructs were able to promote stable engraftment in a preclinical setting (Baltazar et al., 2020).

Autologous keratinocytes and dermal fibroblasts were found to promote diabetic ulcer healing in few clinical studies. In a small group of patients with deep diabetic ulcers (Wagner degree 3), autologous fibroblast grafts induced complete re-epithelialization, with no recurrence for at least 2 years (Cavallini, 2007). A very good rate of healing was also obtained in a pilot study with keratinocytes (Lobmann et al., 2003). A large observational retrospective trial with autologous fibroblasts and keratinocytes applied to wound with a semisynthetic scaffold (HYAFF 11 system) showed a good rate of complete healing, with low recurrence and excellent safety profile (Uccioli, 2003). This study lacked a control group; when the same system was used in a controlled, randomized clinical study on type 1 and type 2 diabetic ulcers, a significant improvement in healing was observed in dorsal but not plantar ulcers (Caravaggi et al., 2003). A recent meta-analysis on the effectiveness of split thickness skin grafts for diabetic foot and leg ulcers reported a global healing rate of 85.5% after a median time of only 5.35 weeks, with a recurrence rate of 4.2% after 2 years (Yammine and Assi, 2019). These numbers are far better as compared to those obtained with standard treatments.

Allogeneic skin cells from non-diabetic donors were also used to promote healing of chronic DFU without major safety problems being encountered (**Table 1**). Keratinocytes and fibroblasts can be isolated from neonatal foreskin. These cells not only manifest an active phenotype in terms of proliferation, migration and paracrine activity, but also likely promote a scarless healing due to intrinsic properties (Moore et al., 2018). A preliminary study suggested that singularly administered keratinocyte sheets and dermal substitutes are equally effective in promoting healing of diabetic ulcers (Harvima et al., 1999).

Several clinical studies proved the ability of keratinocytes from healthy donors to safely reactivate the healing response in DFU non-responding to conventional treatments. Epidermal substitutes were administered on a hyaluronic acid support (Marchesi et al., 2014; Marchesi et al., 2020), on vaseline gauze (You et al., 2012; Hwang et al., 2019), or loaded on microcarriers (Bayram et al., 2005).

Allogeneic dermal substitutes with normal fibroblasts plated in a spongy matrix of hyaluronic acid and atelo-collagen were used as dressings to promote granulation tissue formation prior to autologous skin grafting (Hasegawa et al., 2005). The allogeneic cryopreserved dermal substitute Dermagraft (Advanced BioHealing Inc., La Jolla, CA, United States), approved by the United States Food and Drug Administration (FDA) for DFU therapy, was tested in a multicenter randomized clinical trial on 245 patients with neuropathic foot ulcers (Marston et al., 2003). It improved the rate of wound closure at 12 weeks, with an incidence of adverse effects like conventional treatments. Despite the observed good results, cryopreserved substitutes manifest high cell death rate and reduced growth factor release

TABLE 1 | Clinical studies on administration of allogeneic cell tools to diabetic ulcers: MSCs, mesenchymal stem cells; Ad-MSCs, adipose tissue-derived MSCs; Pl-MSCs, placenta-derived MSCs; UCB-MSCs, umbilical cord blood-derived MSCs; HSCs, hematopoietic stem cells; ASC, adipose stem cells.

Cell type	Type of study	Administration	N. patients	Effects	References
Keratinocyte epithelium or dermal fibroblasts in gelatin sponge	Case report	Weekly until healing	21, type I and II diabetes (26 ulcers)	All ulcers healed	Harvima et al. (1999)
Primary keratinocyte in a hyaluronic acid scaffold	Clinical case series	Once. Patients observed until 70 days	11, type II diabetes (16 ulcers)	Mean wound area reduction 70%. One ulcer with local severe infection	Marchesi et al. (2014)
Primary foreskin keratinocyte sheet (Kaloderm, Tego Science)	Prospective observational	Weekly (or 2–3 times/week if necessary) until 12 weeks	71, type I and type II diabetes	64.8% of complete healing. No adverse effects	Hwang et al. (2019)
Primary adult keratinocytes attached onto microcarriers	Case-control (double arm)	Every 3 days until healing	40, randomized into two groups of 20	Wound area reduction 92% (treated) vs 32% (controls), at 30 days. Improved wound score	Bayram et al. (2005)
Primary foreskin keratinocyte sheet on vaseline gauze	Case-control (double arm)	Weekly for 11 weeks	59, type I or type II diabetes; 27 cases and 32 controls	Complete healing in 100% of treated wounds and 69% of controls. No adverse effects	You et al. (2012)
Primary fresh dermal fibroblasts from teenagers embedded in fibrin	Case-control (double arm)	Single application	55, type I and type II diabetes; 37 cases and 18 controls	Complete healing in 83.8% of cases and 50.0% of controls at 8 weeks. No adverse effects	Han et al. (2009)
Dermagraft	Randomized, multicenter	Weekly, up to 7 treatments	245, type I or type II diabetes; 130 cases, 115 controls	Complete healing in 30% of treated patients and 18.3% of controls by week 12. Adverse events similar in the two groups	Marston et al. (2003)
Bilayered allografts (Graftskin)	Randomized, multicenter	Weekly, up to 4 weeks (5 times)	208, type I or type II diabetes, 112 treated and 96 controls	Complete healing in 56% of treated patients and in 38% of controls. Adverse effects similar in the two groups	Veves et al. (2001)
Foreskin fibroblasts + UCB-MSCs + HSCs	Pilot study	Single application, follow-up for 12 weeks	4 diabetics with severe PAD	Wound healing from 80 to 90%, reduced rest pains. No adverse effects	Viswanathan et al. (2013)
Pl-MSCs (Cenplacel)	Phase 1, dose-escalation, multicenter	Two applications (day 1 and 8), 24 months follow-up	15, type I or type II diabetes and PAD	7 patients had some degree of healing after 3 months (5 complete healing). ABI was improved in them. No severe adverse effects at all doses	Wu et al. (2017)
AD-MSCs (Allo-ASC-Sheet)	Phase 2, randomized, single-blind	Weekly, 12 weeks follow-up	39, type I and type II diabetes; 22 cases and 17 controls	Wound healing in 82% of treated and 53% if controls, at 12 weeks. Similar HLA levels in the two groups. No serious adverse events	Moon et al. (2019)
Allogeneic Ad-MSCs	Phase I/2, randomized single-blind	Single treatment, mean follow-up 48 months (26-50 months)	20, type II diabetes; 10 cases and 10 controls	Significant acceleration in wound closure in the treatment group	Uzun et al. (2021)

compared with fresh preparations (Mansbridge et al., 1998). On the other hand, working with freshly prepared cells has obvious limitations, such as availability of skin cell donations and maintenance of cell culture standards. A fresh human allograft containing dermal fibroblasts from teenagers strongly promoted healing with no safety complications in a pilot study on diabetic chronic ulcers (Han et al., 2004). A subsequent case-control study using the same allograft confirmed the beneficial effect of this construct, with improved mean healing time and patient satisfaction (Han et al., 2009). No adverse effects were recorded.

Better performances were achieved with allogeneic implants containing both keratinocytes and fibroblasts. Apligraf (Organogenesis, Inc., Canton, MA, United States), approved by FDA for diabetic ulcer therapy, is a cryopreserved, bi-layered allograft, formed by foreskin-derived fibroblasts seeded within a bovine collagen I matrix overlaid by a stratified epithelium of neonatal keratinocytes (Zaulyanov and Kirsner, 2007). A pivotal, randomized multicenter prospective trial showed that Apligraf application to non-infected neuropathic diabetic ulcers significantly increases healing rate, reduces healing time, with no immunological reaction and with long-term reduction of osteomyelitis and lower-limb amputations (Veves et al., 2001).

3.2 Stem/Progenitor Cells of Different Tissue Origin

3.2.1 Mesenchymal Stem Cells

Progenitor cells of mesodermal origin, also named mesenchymal stem cells (MSCs), reside in many tissues, including dermis and skin annexes (Hoogduijn et al., 2006; Hasebe et al., 2011; Ma et al., 2018) (**Figure 1**). It is still debated whether circulating mesodermal progenitors exist that could home to the wound site through a chemokine gradient (Rafii, 2000; Lo Sicco et al., 2018).

MSCs from different tissues share the expression of specific cell surface markers and lack those of hematopoietic and endothelial cells (Maleki et al., 2014; Camilleri et al., 2016).

They manifest high self-renewal and the potential to differentiate into different cell types depending on tissue microenvironment (Pittenger et al., 2019). Although preclinical tracing experiments with labelled MSCs suggested that these cells may differentiate into keratinocytes, endothelial cells and pericytes (Sasaki et al., 2008; Nie et al., 2011), trans-differentiation minimally, if any, contributes to skin regeneration (Phinney and Prockop, 2007). MSC beneficial properties are largely due to paracrine activity and their ability to remodel the tissue through regulating resident cells by releasing growth factors, cytokines, microRNAs, etc. (Mansbridge et al., 1998; Brem et al., 2003; Lazic and Falanga, 2011).

A great number of studies in animal models of diabetes proved MSC efficacy in promoting skin repair (Cao et al., 2017). Stem cell administration reduces inflammation, apoptosis and scar formation, while increases cell proliferation and angiogenesis. Human studies and clinical trials privileged the use of autologous MSCs for safety clues. However, clinical translation gave variable results and was not as effective as hoped.

A critical issue with MSC therapeutic potential is the poor cell survival and engraftment (Baldari et al., 2017). To reinforce MSC function or to enable them to better cope with the hostile microenvironment, several approaches were used (Wiredu Ocansey et al., 2020) (for reviews, Bardali et al., 2017; Shojaei et al., 2018). Scaffolds made by fibrin (Stolzing et al., 2011), collagen (Assi et al., 2016), hydrogels (Wong et al., 2011; Dong et al., 2018) or acellular dermal matrix (Fu et al., 2019) were adopted for MSC delivery and to enhance their therapeutic potential. MSCs cultured in the presence of selective growth factors or engineered to overexpress them showed enhanced key healing functions, particularly angiogenesis and progenitor cell recruitment, in diabetic preclinical settings (Di Rocco et al., 2010; Li et al., 2013; Penna et al., 2013; Yang et al., 2013; Capilla-González et al., 2018; Dhoke et al., 2020; Srifa et al., 2020). Hypoxia pre-treatment also enhanced MSC survival in the diabetic wound environment by minimizing ROS accumulation and improving angiogenesis (Liu et al., 2015). In general, preconditioning MSCs by exposure to physical or environmental shocks promoted their survival (Baldari et al., 2017).

The first and most used autologous MSCs for promoting diabetic wound healing were bone marrow derived (BM-MSCs). BM-MSCs provided promising results in both preclinical and clinical studies (Falanga et al., 2007; Cao et al., 2017). However, bone marrow aspiration and *ex vivo* expansion represent limiting factors in their use. Adipose tissue-derived MSCs (AD-MSCs) manifest properties similar to BM-MSCs (Strioga et al., 2012), but, unlike the bone marrow, adipose tissue is easily accessible and abundant in the body. The therapeutic use of AD-MSCs for DFU treatment has been extensively investigated, at both preclinical and clinical level (Gadelkarim et al., 2018). Additional types of investigated MSCs were derived from fetal or extraembryonic tissues. Among them, umbilical cord blood (UCB) MSCs are easily collectable and manifested efficiency in promoting non-diabetic and diabetic wound healing by exerting multiple beneficial effects (Moon et al., 2017; Zhang et al., 2020). However, privacy concern, and high cost of cord blood preservation may represent limitations in their use (Cao et al., 2017). MSCs from the Wharton's jelly tissue (WJ-MSCs) are emerging as the best umbilical cord-derived MSC population for regenerative medicine. Besides being easily accessible and numerous, WJ-MSCs show features of embryonic stem cells (ESCs) in terms of proliferation, multipotency and low immunogenicity, but do not form teratomas upon transplantation as ESCs do and there are not ethical limitations in their use (Liau et al., 2020). WJ-MSCs also proved to efficiently promote diabetic wound healing in preclinical studies (Jiao et al., 2021; Nilforoushzadeh et al., 2022). Other MSCs that got attention for their ability to promote diabetic wound healing are those obtained from the placenta (Pl-MSCs) or the amniotic fluid (AF-MSCs) (Kim et al., 2012; Kong et al., 2013; Lee et al., 2015; Wu et al., 2017) (**Figure 1**).

A small subpopulation of mesodermal skin cells manifesting MSC features has been recently identified (Riedl et al., 2021). These cells express the ATP-binding cassette subfamily B member 5 (ABCB5+ MSCs). They show strong immunomodulatory properties and better homing to skin wounds as compared to BM-MSCs (Schatton et al., 2015). A preclinical study on a mouse model of venous leg ulcer revealed that ABCB5+ MSCs favor healing by promoting macrophage polarization towards the M2 phenotype, and that this effect is mediated by the release of the interleukin-1 receptor antagonist (IL-1RA) (Vander Beken et al., 2019). Ex vivo-expanded autologous ABCB5+ MSCs were used in a clinical study with few patients affected by chronic venous ulcers (Kerstan et al., 2021). These cells facilitated healing and promoted pain relief with no adverse effects.

When considering the adoption of MSCs as therapeutic agents for DFU, one should be aware that preclinical data showed that the diabetic environment affects the beneficial activity of AD-MSCs and BM-MSCs in promoting wound healing. The number of MSCs is decreased in both type 1 and type 2 diabetes and their proliferative potential, paracrine activity, survival, and recruitment to the wound site are altered (Shin and Peterson, 2012; Cianfarani et al., 2013; Ribot et al., 2017). In a study on type 1 diabetic mice we demonstrated that the allogeneic stromal vascular fraction (SVF) from the adipose tissue of non-diabetic mice was more effective in promoting healing of diabetic wounds as compared to SVF from diabetic mice, in terms of granulation tissue formation, angiogenesis and macrophage recruitment (Cianfarani et al., 2013). Other preclinical studies also showed that the angiogenic response is strongly impaired when MSCs from diabetic animals are transplanted (El-Ftesi et al., 2009; Ribot et al., 2017). By using mouse models, Rennert et al. (2014) found that the AD-MSC niche is altered in diabetes and hypothesized that the reduced angiogenic response of diabetic AD-MSCs depends on the selective ablation of a subpopulation with putative strong angiogenic activity. These preclinical findings were confirmed in humans, where reduced amounts of circulating MSCs, growth factors and anti-oxidant molecules were found in the plasma of type 2 diabetic patients as compared to non-diabetic individuals (Nowak et al., 2014). A selective depletion of a proangiogenic CD271+ AD-MSC

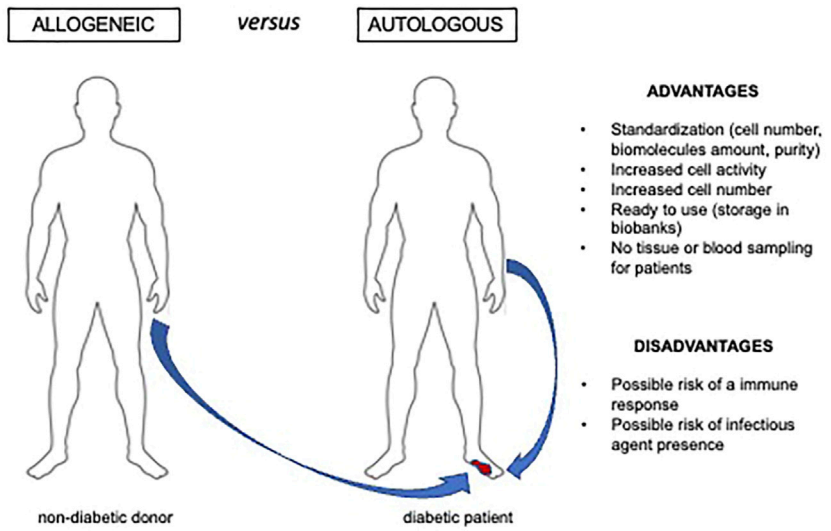

FIGURE 2 | Advantages and disadvantages of allogeneic cell. therapy Allogeneic cells or cell derivatives can be an option for ulcer therapy when harvesting autologous preparations able to reactivate the healing process is not feasible. This is the case of diabetic and/or elderly individuals that manifest impaired cell number and activity.

population was also found in diabetic individuals with cardiovascular diseases (Inoue et al., 2019), and human MSCs exposed to sera of type 2 diabetic patients revealed decreased survival and *in vitro* proangiogenic activity (Rezabakhsh et al., 2017).

Allogeneic MSCs from healthy donors could be a valid therapeutic option to overcome issues of reduced number and activity of diabetic MSCs (**Figure 2**). This approach is feasible as MSCs manifest immune tolerance. Indeed, MSCs express low level of human leukocyte antigen class I (HLA-I) (Wang et al., 2019) and inhibit inflammatory responses. Human MSCs cultured in the presence of different immune cell populations were shown to mitigate immune responses by reducing the release of proinflammatory cytokines (IFN-γ, TNF-α) and by increasing both anti-inflammatory cytokines (IL-4, IL-10), and prostaglandin E2 (PGE2) (Aggarwal and Pittenger, 2005). A recent meta-analysis on clinical studies employing allogeneic AD-MSCs for other pathologies confirmed that these cells are therapeutically effective with no severe adverse effects or evidence of immune response, even though 19%–34% of patients were found to develop antibodies against donors (Gentile et al., 2020). This could represent a potential risk for patients qualifying for a possible future organ transplant.

Following encouraging preclinical results, few preliminary clinical studies with allogeneic MSCs in chronic diabetic ulcers have been published (**Table 1**). A study on diabetic patients with amputation as only option for severe peripheral arterial disease (PAD), evaluated the combination of intramuscular administered allogeneic UCB-MSC and CD34+ hematopoietic stem cells with intralesional implantation of allogeneic foreskin fibroblasts (Viswanathan et al., 2013). After a 3 months therapy, all patients manifested an improvement in wound healing ranging from 80 to 90%, with healthy granulation tissue formation and no local, nor systemic complications. The ankle brachial index (ABI) was improved, and no limb amputation was necessary. Reduced rest pain was also reported, and no ulcer formation was observed after 6 months follow-up.

A phase 1 dose-escalation study aimed at evaluating safety of placental MSCs (Cenplacel) in patients with DFU and PAD showed no adverse effects at all doses tested (Wu et al., 2017). After 3 months, 7 out of 15 ulcers had healed and this effect was durable after 1 year. PAD was improved in patients whose ulcer healed. This therapeutic effect on peripheral vasculature was also reported in a preclinical study with the same therapeutic approach (Francki et al., 2016).

Twelve studies using allogeneic MSCs for diabetic ulcer treatment are presently recorded in the National Institutes of Health (NIH) clinical trial registry (www.clinicaltrials.gov). Of those, eight test the effect of a product containing allogeneic AD-MSCs from healthy donors in a hydrogel sheet (ALLO-ASC-Sheet). A randomized, single-blind, phase 2 clinical trial testing efficacy and safety of this commercial product versus standard therapy has been published (Moon et al., 2019). Despite the relatively small sample size, percentage of healed ulcers after 8 and 12 weeks was increased in the group treated with the ALLO-ASC-Sheet, and mean time to complete healing was significantly shorter in the treatment group. After 2 years follow-up, recurrence was minimal, and no adverse events were recorded. Increased elevation of anti-HLA antibodies was found in the treatment group, but no clear signs of rejections were detected. Among approved clinical trials, there is a phase 1-2a with *in vitro* expanded MSCs of dermal origin (ABCB5+-MSCs) in neuropathic DFU.

3.2.2 Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) are obtained from differentiated adult cells following ectopic co-expression of

genes coding for transcription factors conferring a pluripotent stem cell phenotype (Takahashi and Yamanaka, 2006). Under proper culture conditions, iPSCs can re-differentiate into different cell types, including skin cells (Itoh et al., 2013). This technology allows to obtain large number of rejuvenated, active cells.

Induced PSCs obtained from dermal fibroblasts and subsequently differentiated into smooth muscle cells showed an increased ability to release growth factors and to promote wound healing in diabetic mice when transplanted in a tridimensional scaffold (Gorecka et al., 2020). They improved cell proliferation, neovascularization, extracellular matrix deposition and M2 macrophage polarization.

Dermal fibroblasts from non-healing DFU were found to dedifferentiate to iPSCs as efficiently as fibroblast from non-lesional diabetic skin or from non-diabetic skin (Gerami-Naini et al., 2016). Kashpur et al. (2019) showed that iPSCs derived from non-healing DFU fibroblasts can re-differentiate to dermal fibroblasts that manifest a common gene expression signature with fibroblasts whose parental cells were non-diabetic. This genetic convergence with non-diabetic cells is likely due to epigenetic changes reversing the hyperglycemia-induced "metabolic memory." Reprogrammed fibroblasts showed improved behavior in terms of composition of released extracellular matrix, and ability to migrate and to promote wound healing when grafted in a tridimensional matrix. These cells also manifested changes in microRNA expression, with an increase in microRNAs whose target genes contribute to the healing defects in diabetes and a decrease in those targeting genes that promote skin repair (Pastar et al., 2021).

Since iPSCs from dermal fibroblasts release a richer ECM compared to original fibroblasts, they have been used to develop tissue engineering scaffolds. These scaffolds enhance the activity of DFU fibroblasts that release higher amount of glucosaminoglycans, VEGF and anti-inflammatory cytokines, and reduce the secretion of proinflammatory cytokines (Santarella et al., 2020).

Based on these findings, iPSCs derived from dermal fibroblasts could be considered for a non-invasive cell therapy in DFU. However, strong regulatory constraints exist to their use, the methodology to obtain the cells is highly sophisticated, and, at present, iPSCs are produced only in very specialized laboratories.

3.3 Stem Cell Secretome and Released Microvesicles

Skin substitutes and stem/progenitor cells mainly act not by engrafting (Phillips et al., 2002; Griffiths et al., 2004), but rather by releasing molecules that stimulate the healing process, such as growth factors, cytokines and extracellular matrix proteins (Mansbridge et al., 1998; Brem et al., 2003; Lazic and Falanga, 2011). Moreover, a significant part of the positive effect of the treatments with stem and progenitor cells is most probably due to the release of microvesicles (MVs) carrying different biomolecules, including proteins, coding and non-coding RNAs and lipids (Tsiapalis and O'Driscoll, 2020). This brought scientists and physicians to evaluate the direct use of the cell-conditioned medium and/or of cell-released MVs to induce activation and reactivation of endogenous progenitors and differentiated cells (Ahangar et al., 2020; Bari et al., 2020; De Gregorio et al., 2020; Zhang et al., 2020).

In particular, MSC-secreted exosomes have been quite extensively tested at preclinical level as potential tools for cell-free therapy in cutaneous wound healing (Casado-Díaz et al., 2020). These nanovesicles manifest immunomodulatory and regenerative properties comparable to MSCs. At present, a phase 1 single-arm clinical trial with MSC secretome is registered as completed on the clinical trial registry (www.clinicaltrials.gov; NCT04134676). This study was aimed at testing the therapeutic potential of umbilical cord MSC on healing of chronic ulcers.

3.4 Amniotic Membrane Allografts

Among allogeneic approaches for diabetic foot ulcers, it has to be mentioned the amniotic membrane. This natural dressing is being used for decades to promote tissue regeneration and in skin repair as it releases factors promoting cell function, it is non-immunogenic, reduces pain, inflammation and fibrosis, among other properties (Schmiedova et al., 2021). Amniotic membranes have been widely tested in clinical settings and proved to significantly promote healing of recalcitrant DFU in combination with standard care approaches (Laurent et al., 2017). Commercially available amniotic membrane products are provided with or without chorion, as cryopreserved or dehydrated decellularized tools (Ilic et al., 2016). They can be also used as scaffold for other advanced therapeutic approaches, as assessed in preclinical and clinical studies (Hashemi et al., 2019; Zhou et al., 2019; Xiao et al., 2021).

3.5 Platelet Derivates, Platelet-Rich Plasma

Activated platelets release high amounts of growth factors and active molecules capable to trigger cell proliferation, matrix remodeling, angiogenesis and to modulate inflammation at the wound site (Backly et al., 2011; Greppi et al., 2011; Andia and Maffulli, 2013; Burnouf et al., 2013; Ulivi et al., 2014). Moreover, platelets also release microvesicles and exosomes (Aatonen et al., 2012). Platelet-rich plasma (PRP) or other platelet-derivatives can reactivate latent endogenous regeneration mechanisms.

In vitro experiments from our group showed an initial transient enhancement of the inflammatory response (NF-kB activation, COX-2 induction, and secretion of pro-inflammatory cytokines), followed by the establishment of an anti-inflammatory microenvironment due to prostaglandin E2 (PGE2) production (Ruggiu et al., 2013). At the same time, it was observed an upregulation of proliferative and survival pathways, such as ERKs and Akt, together with induction of Cyclin D1 and the phosphorylation of retinoblastoma protein leading to the re-entry in the cell cycle of quiescent cells. Moreover, PRP promoted recruitment at the wound site of neutrophils and macrophages, which, in turn, stimulated vascularization, and recruitment, activation, proliferation of mesenchymal and epithelial stem/progenitor cells, thus enhancing tissue repair (Pierce et al., 1989).

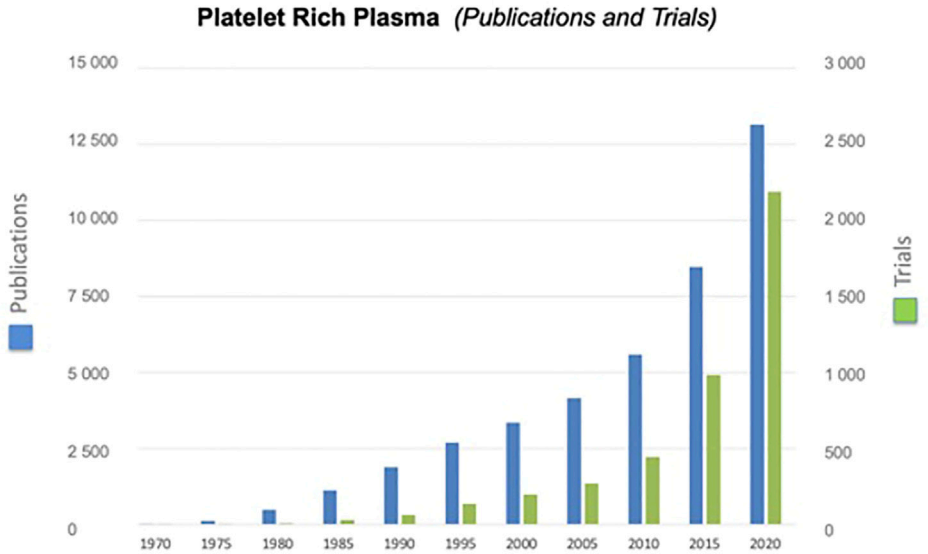

FIGURE 3 | Exponential growth of publications and clinical trials having PRP as subject. Information was derived searching by "Platelet-Rich Plasma" the data base "PubMed" for publications and "Cochrane library (Title, Abstract, and Keywords)" for trials.

Platelet derivatives, primarily PRP, were considered in regenerative medicine to mimic the effects exerted by the platelet clot as wound healing trigger. Given its high availability as an autologous product and the easiness to prepare, PRP is being adopted in different medical fields, in particular in orthopedic and dermatology, for the treatment of chronic inflammatory diseases such as osteoarthritis and diabetic ulcers (Andia and Maffulli, 2013; Picard et al., 2015). Indeed, both the number of scientific publications and the number of clinical trials having PRP as subject is exponentially increasing (**Figure 3**). However, contradictory results are reported in the scientific literature on the outcome of treatments with autologous PRP. The main factor that could explain the different treatment outcomes is the variability of the preparations due to the lack of standardized procedures for PRP production. Moreover, as consequence of the blood recovery from a single donor, parameters, such as platelet concentration, and leukocyte, red blood cells and fibrin, change in different preparations and could be critical for the success of PRP treatment (Weibrich et al., 2002). Further variables arise from the different materials used for the recovery and processing of blood: vacuum blood-collection tubes, anticoagulant, blood collection bags together to time, speed, and number of centrifugations. In general, an important weakness of the clinical studies is the lack of defined parameters to assess the biological quality of the PRP such as the growth factor content and the testing of the product performance before its clinical use. Indeed, only a low percentage of trials included a quality control of the PRP preparations (Alsousou et al., 2009). It is also to note that several of the commercial devices approved by the FDA and other regulatory agencies for the preparation of autologous PRP were checked and approved for the safety, but not for the clinical efficacy of the obtained products (Harm and Fung, 2015).

Despite the above caveats, increasing evidence indicates that PRP is a powerful tool to enhance impaired wound healing. A recent Cochrane review tried to elucidate PRP efficacy on the treatment of recalcitrant ulcers. The meta-analysis is based on ten trials for a total of 442 participants. These studies were heterogeneous in terms of ulcer etiology: venous ulcers, diabetic ulcers, occlusive peripheral vascular disease, vasculitis and/or pressure ulcers. The median wound duration and size were similar at baseline (Stacey et al., 2000; Driver et al., 2006; Anitua et al., 2008). Despite the high heterogeneity in PRP production and application, frozen versus fresh, in almost all cases the PRP used for the treatment was autologous. Substantial variations within trials existed about eligible participants, wound etiologies, trial design, and most trials were judged to be at high risk of bias due to the lack of PRP standardization, different ulcer etiology and disparity between treated and control groups. In summary, four trials treated people with ulcers of mixed etiology reporting good results in comparison to the control group (Driver et al., 2006; Li et al., 2012; Moraes et al., 2014), three trials treated people with venous leg ulcers (Senet, 1990; Stacey et al., 2000; Mj et al., 2016) and three people with diabetes and foot ulcers (Driver et al., 2006; Kakagia et al., 2007; Mj et al., 2016). Nine out of 10 studies compared PRP plus standard care with standard care alone (with or without placebo). One study in people with diabetes evaluated PRP in the context of protease-modulating matrix (Kakagia et al., 2007). Another meta-analysis on clinical trials with a total of 477 diabetic patients compared standard care plus autologous or allogeneic PRP treatment versus standard care and showed consistent, reliable, significant beneficial effect of PRP administration on DFU healing, with no differences in wound complications or recurrences, and a decrease in the risk of adverse events in PRP-treated ulcers (del Pino-Sedeño et al., 2019).

PRP prepared from the blood of a single patient (autologous PRP) can present a significant variability in platelet and other blood component concentrations Moreover, patients with specific health status (diabetics, immune-compromised, and hypomobile) or individuals with age-related problems (neonatal, infant, elderly people) are not retained ideal candidates for the treatment with autologous platelet derivatives (Anitua et al., 2008; Backly et al., 2011; Greppi et al., 2011; Aatonen et al., 2012; Andia and Maffulli, 2013; Ulivi et al., 2014; Picard et al., 2015). A standardized PRP production from pools of human certified buffy coats (allogeneic PRP) has been proposed to reduce PRP variability and to obtain an "off the shelf" product with a well-defined platelet and growth factor concentration (Muraglia et al., 2014, 2015). Indeed, an allogeneic product obtained from a pool of healthy individuals could represent an efficient alternative to the use of autologous PRP. Allogeneic PRP can be stored as blood bank of platelet concentrate with defined cell count and growth factor amount, from donors tested for history of infectious diseases and homologous for ABO and Rh antigens (H-PRP) (Jeong et al., 2010).

An allogeneic PRP or platelet-derived product could reduce the risk of poor standardization. However, the possibility of an immune reaction should be considered. Bottegoni et al. (2016) first published results of a large-scale trial aimed at evaluating the safety and efficacy of allogeneic human PRP on patients suffering from moderate osteoarthritis. Reiterated PRP injections for 2 months showed a statistically significant improvement in all patients within the treatment period. Results worsened during the 4 months of follow-up, particularly in patients older than 80 years, confirming published results reporting PRP decreased efficacy with increasing age (Andia and Maffulli, 2013). Most importantly, this study highlights non-side effects apart from a transitory intra-articular burning. These findings are in line with those obtained by Jang et al. on homologous allogeneic platelet gel treatment of recalcitrant skin ulcers in hypomobile patients (Greppi et al., 2011). Also in this case, no adverse reactions were observed with results that are comparable, in terms of healing, to those obtained with autologous treatment. Analogous results were described by Shan et al. (2013) on DFU treatment with PRP, with an enhancement and acceleration of lower extremity wound healing.

More recently, two articles investigated the effects exerted by an allogeneic non-homologous PRP. In these studies, performed in a mouse model of diabetes, the xenogeneic platelet-rich gel enhanced the healing process without adverse reaction (Spanò et al., 2017). These observations agree with previously reported data where, for the first time, researchers evaluated the immunogenicity of allogeneic non-homologous PRP injection in rabbit large bone defects (Zhang et al., 2013). In this case, no inflammatory effects were observed, but only a slight increase in the amount of peripheral CD4+ T cells. A possible explanation for these findings could be found in the ability of the allogeneic PRP to modify the wound microenvironment. To this regard, we published that allogeneic PRP affects human monocyte differentiation to immature dendritic cells, triggering the generation of a different immune-modulatory subset, able to induce an anti-inflammatory milieu (Papait et al., 2018). We also compared the leukoreduction effect of the PRP on monocyte differentiation, and not significant differences were observed in comparison to standard PRP products.

The aforementioned meta-analysis on selected clinical trials evaluating PRP for DFU treatment (del Pino-Sedeño et al., 2019) included three studies with allogeneic PRP. Authors did not specifically compared efficacy of autologous vs. allogeneic preparations. However, outcomes of the different studies were very consistent in all parameters analyzed, suggesting a similar beneficial effect of the two preparations. A recently published clinical trial evaluating the effect of allogeneic PRP as compared to autologous PRP reported a similar improvement of both treatments with respect to control group, although a better healing rate trend was observed in ulcers treated with the allogeneic preparations. Adverse reactions were not reported for both treatments. These data confirm that allogeneic PRP could be used as a beneficial cell therapy supplement when autologous PRP is not available or difficult to obtain (He et al., 2020).

Clinical evidence, including ours, confirms that the treatment with allogeneic platelet derivatives allows healing also of ulcers hard to treat and slow to heal, which would not benefit of a "standard traditional" treatment. If the efficacy of quality controlled allogeneic PRP is confirmed by additional studies, with this relatively low-cost treatment no longer would patients need to undergo more sophisticated invasive procedures. This would be particularly important for patients who are severely disabled (i.e. bed sores, type 2 diabetes ulcers). Prompt and effective long-term treatment of ulcers and chronic wounds will reduce disease complications (i.e. infections, amputation) and mortality rate.

4 DISCUSSION AND CONCLUSION

Cutaneous chronic wounds, and in particular chronic ulcers in diabetes, represent a major clinical and societal problem in continuous growth because of population aging. Different treatments have been proposed: biological, surgical, and physical. However, most of these treatments are palliative and none of them can be considered fully satisfactory. Cell-based therapies, including stem cell conditioned medium and cell released microvesicles, together with platelet-rich plasma (PRP) treatments, are today innovative therapeutic approaches that share as common goal the reactivation of silent endogenous regeneration processes. In most cases, cells and PRP are obtained from patients as autologous preparations.

Positive preclinical data suggested the adoption of cell therapy on diabetic patients to promote healing of chronic ulcers unresponsive to standard care. Since preclinical data were mainly obtained in rodent models, that only partly recapitulate features of human diabetic wounds, and because preclinical studies often suffer from non-rigorous setting and analysis, so far, the translation to the clinic was often disappointing. Well-structured, large, highly controlled clinical trials are therefore needed to test cell therapy efficacy in diabetic ulcers.

Ex vivo expanded Mesenchymal Stem Cells from different body sites, either of the patient or of a donor individual, manifest high potentialities in promoting healing of diabetic wounds as compared to skin grafts. However, despite the positive results already obtained, due to rules imposed by the regulatory agencies (United States Food and Drug Administration, European Medicines Agency, National Agencies), the need of highly sophisticated cell culture facilities, and the consequent high cost of the procedures, the MSC therapeutic approach cannot be easily applied to patients of largely diffuse pathologies, such as chronic diabetic ulcers. Moreover, for autologous MSCs, the logistics to obtain a biopsy from the patient, expanding the cells in the culture lab, and returning to the patient the final product as a preparation to be immediately used, makes the approach even more difficult to be adopted. It is also to note that in metabolic disorders, such as diabetes or in elderly individuals, cell number reduction and defective paracrine activity could hamper the effectiveness of autologous cell-based therapy.

As for allogeneic therapy, a safety issue related to allo-recognition and rejection may arise as a problem. Preliminary evidence on MSC therapy indicates no major problems following allogeneic cell topical administration. However, a careful evaluation is needed before translation of allogeneic cells to the clinical practice. It is now widely recognized that the therapeutic activity go to head of the transplanted cells strongly relies on their secretome and on released microvesicles. Indeed, secretome and released microvesicles hold great promise as a therapeutic option in regenerative medicine. However, the same regulatory restrictions applying to pharmaceuticals apply to allogeneic stem cells, cell secretome or cell-released microvesicles.

Great promises rely on the use of PRP that could be easily prepared with low invasive techniques and at a relatively low cost. Moreover, at variance with cell and cell-derived products, in most countries PRP is not classified as a pharmaceutical, and it is considered a blood transfusion product. A critical issue with PRP therapy is the lack of standardization in the preparation procedures. This problem is at the root of the discrepancies in results reported in literature. Most of reported PRP treatments have utilized a product obtained from the blood patients (autologous PRP). An allogeneic PRP obtained from a pool of healthy subjects could be a better alternative to the use of autologous PRP. Allogeneic PRP preparations can be quality controlled before their use and can be stored in a freeze-dried form as "off the shelf" product for a future use.

It is expected that allogeneic PRP will have the same, if not better, performance, than autologous PRP. The few published reports seem to confirm this expectation. However, large randomized clinical trials comparing allogeneic PRP to other treatments are still missing. This is mandatory for a reliable, efficient application on a large scale of allogeneic PRP in the clinical practice.

AUTHOR CONTRIBUTIONS

MM conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, financial support; MN bibliographic data, manuscript revision; MC, GF, and CD revised clinical aspects and critically read the manuscript; RC conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; TO conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing.

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REVIEW
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The Growing Medical Need for Tracheal Replacement: Reconstructive Strategies Should Overcome Their Limits

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Breathing, being predominantly an automatic action, is often taken for granted. However, respiratory diseases affect millions of people globally, emerging as one of the major causes of disability and death overall. Among the respiratory dysfunctions, tracheal alterations have always represented a primary challenge for clinicians, biologists, and engineers. Indeed, in the case of wide structural alterations involving more than 50% of the tracheal length in adults or 30% in children, the available medical treatments are ineffective or inapplicable. So far, a plethora of reconstructive approaches have been proposed and clinically applied to face this growing, unmet medical need. Unfortunately, none of them has become a well-established and routinely applied clinical procedure to date. This review summarizes the main clinical reconstructive attempts and classifies them as non-tissue engineering and tissue engineering strategies. The analysis of the achievements and the main difficulties that still hinder this field, together with the evaluation of the forefront preclinical experiences in tracheal repair/replacement, is functional to promote a safer and more effective clinical translation in the near future.

Keywords: tracheal replacement, tracheal surgeries, clinical outcomes, preclinical studies, regenerative medicine, tissue engineering, allotransplantation

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INTRODUCTION

Tracheal and main bronchi dysfunctions represent an unmet medical need in respiratory medicine. Indeed, congenital malformations, malignancies, inflammations, infections, or even traumatic events, including postoperative complications, can alter tracheal and main bronchi structure and function, heavily affecting patients' life. Nowadays, this issue represents an extraordinary leading-edge topic. Indeed, as recently stated by the European Laryngological Society, the heightened number of long-term intubations and the huge tracheostomy rate in critically ill COVID-19 patients might shortly determine an unprecedented increase in laryngotracheal granulomas, stenosis, malacia, tracheal necrosis, tracheo-oesophageal and trachea-innominate fistulae (Alturk et al., 2020; Mattioli et al., 2021; Piazza et al., 2021). The first-choice treatment for managing short tracheal structural alterations is based on tracheal resection and reconstruction by end-to-end anastomosis. However, surgery is inapplicable in longer tracheal injuries exceeding 5 cm in adults and 3 cm in children, and only palliative care can be offered to these patients (Delaere et al., 2019).

To date, a variety of approaches has been proposed and clinically adopted to replace long-segment tracheal defects and reconstruct this vital organ. However, despite the great efforts, the limited results clearly pointed out that a final solution for restoring a functional respiratory system is extremely challenging and far from being addressed (Grillo, 2002; Soriano et al., 2021).

Why is the Trachea so Hardly Replaceable?

Despite its apparently simple shape and structure, the trachea is a complex organ, challenging to be replaced. Indeed, it is a semiflexible tube measuring approximately 5 cm in children under 3 months of age (Lee and Yang, 2001) and reaching 10–13 cm in adults (Cinar et al., 2016). It comprises up to 22 C-shaped hyaline cartilaginous rings posteriorly connected by smooth muscle embedded into a fibro-elastic connective tissue (Brand-Saberi and Schäfer, 2014). This structure gives a peculiar lateral rigidity and longitudinal flexibility, critical for controlling the trachea lumen diameter, preventing its collapse and supporting the trachea during inspiration/expiration (Grillo, 2002; Teng et al., 2012). This architecture confers to the trachea also the ability to resist neck movements and pressure coming from the adjacent oesophagus. Moreover, being in direct contact with the outside world, the innermost part of the trachea is lined by a specialized and pseudostratified respiratory epithelium. This latter plays a central role within tracheal physiology, orchestrating one of the most intricated and sophisticated response mechanisms of the entire human body (Ganesan et al., 2013). Indeed, it not only acts as the primary physical barrier against environmental factors, continuously filtering, warming and humidifying the inhaled air, but it also perceives potential dangers and, in synergy with immune and nervous cells, puts in place the most appropriate response (Davis and Wypych, 2021). The complexity of the respiratory epithelium is reflected by the great heterogeneity encountered at the cellular level, in which each cell type plays a specific role within epithelial physiology (Goldfarbmuren et al., 2020). Thus, every alteration in cell content or selective damage to one or more cell types may contribute to the development of respiratory disorders (Davis and Wypych, 2021). Finally, the trachea is nourished by a multitude of tiny capillaries that branch mainly from the inferior thyroid artery and provide the blood supply to the pseudostratified epithelium and the surrounding tissues (Salassa et al., 1977). It is difficult to recreate or restore this intricated blood supply through anastomosis, therefore it appears as a major challenge in tracheal reconstructive attempts (Delaere et al., 2010).

This review is focused on the main clinical reconstructive strategies for replacing long-circumferential tracheal defects. Procedures relying only on external or internal splint/stent as a mechanical support to the weakened tissue and patch-based reconstructive approaches were not included. According to their manufacturing process, the revised approaches were classified in two macro-categories, namely non-tissue engineering (non-TE) and tissue engineering (TE) attempts (**Figure 1**). Indeed, the former comprises the use of synthetic prostheses or allogenic/autologous tissues directly transplanted *in vivo*, while the latter relies on an *in vitro* step of scaffold manufacturing and/or cell expansion. The clinical outcomes have been analyzed for each strategy to point out the achievements and the weaknesses that still hinder this field. Moreover, non-exhaustive examples of the forefront TE attempts on animal models were described as promising perspectives for the clinical translation. Thanks to this analysis, we identified some critical aspects that must be considered and implemented in designing new tracheal substitutes, thus paving the way towards safer and more effective solutions for treating patients with long-circumferential tracheal defects.

NON-TISSUE ENGINEERING APPROACHES FOR TRACHEAL RESTORATION

In the last century, a plethora of non-TE approaches has been proposed and applied in small series or even single patients to treat long-circumferential tracheal defects. Based on common methodologies, these attempts can be further clustered into three main categories: synthetic prostheses, allotransplantations, and autologous tissue reconstruction. **Table 1** summarizes some of the main non-TE approaches tested so far and still considered as potential tracheal reconstruction strategies.

Synthetic Prostheses

Historically, tracheal reconstructive approaches involving rigid polymer constructs, glass or metal prosthesis, or more flexible materials such as polymeric or metallic wire/meshes, and silicone, have always been unsuccessful in clinical practice (Greaney and Niklason, 2021). Indeed, rigid polymer constructs or prostheses, despite remaining patent, are challenging to be properly sutured and often shifted out. The complication led to airway obstruction with consequent pneumonia or even death (Abbott et al., 1932; Clagett et al., 1948; Longmire, 1948; Cotton et al., 1952; Blades and Beattie, 1986). Moreover, these inert materials do not support the regeneration of the mucosal tissue, essential for a functional tracheal restoration. Similarly, silicone tubes have been used to exploit their durability, low reactivity, and mechanical flexibility. However, the lack of integration with the surrounding tissue, granulation formation and haemorrhages due to graft mobilization strongly limited their clinical diffusion (Neville et al., 1976; Neville et al., 1990; Toomes et al., 1985; Schneider et al., 2001). Finally, polymeric or metallic wire/meshes have also been adopted as tracheal substitutes, displaying unsafe and ineffective outcomes (Greaney and Niklason, 2021). Indeed, besides the usual granulation tissue formation, scaffold migration with erosion of the surrounding vessels and oedema, these prostheses lack adequate mechanical properties, leading to airway narrowing and collapse (Clagett et al., 1948; Kramish and Morfit, 1963). Therefore, to date, prosthetic reconstruction is no longer considered a therapeutic solution for long-circumferential tracheal defects.

Tracheal Replacement With Allogenic Tissues

This strategy relies on the use of human cadaveric allogeneic tissues, among which aortic and tracheal allografts have been widely used.

Aortic Allotransplantation

Aortic allografts have been employed for tracheal repair by different groups, especially in emergencies, due to their wide availability in a variety of sizes (Davidson et al., 2009; Martinod et al., 2017, Martinod et al., 2018; Menna et al., 2021; Wurtz et al., 2006, Wurtz et al., 2010). However, these replacements lack the adequate biomechanical properties of the trachea, as well as the presence of respiratory epithelium. Therefore, to preserve the airway patency, aortic allografts required stenting, often rejected by the patient's body. Moreover, several frequently fatal surgical-related complications were observed, such as severe bilateral pneumonia, anastomotic dehiscence, fungal infection, spinal cord ischemia and even cardiac arrest (Davidson et al., 2009; Wurtz et al., 2006, Wurtz et al., 2010). Additionally, these reconstructive approaches do not consider the need for an effective blood supply reestablishment, and rely only on host cells migration for graft repopulation.

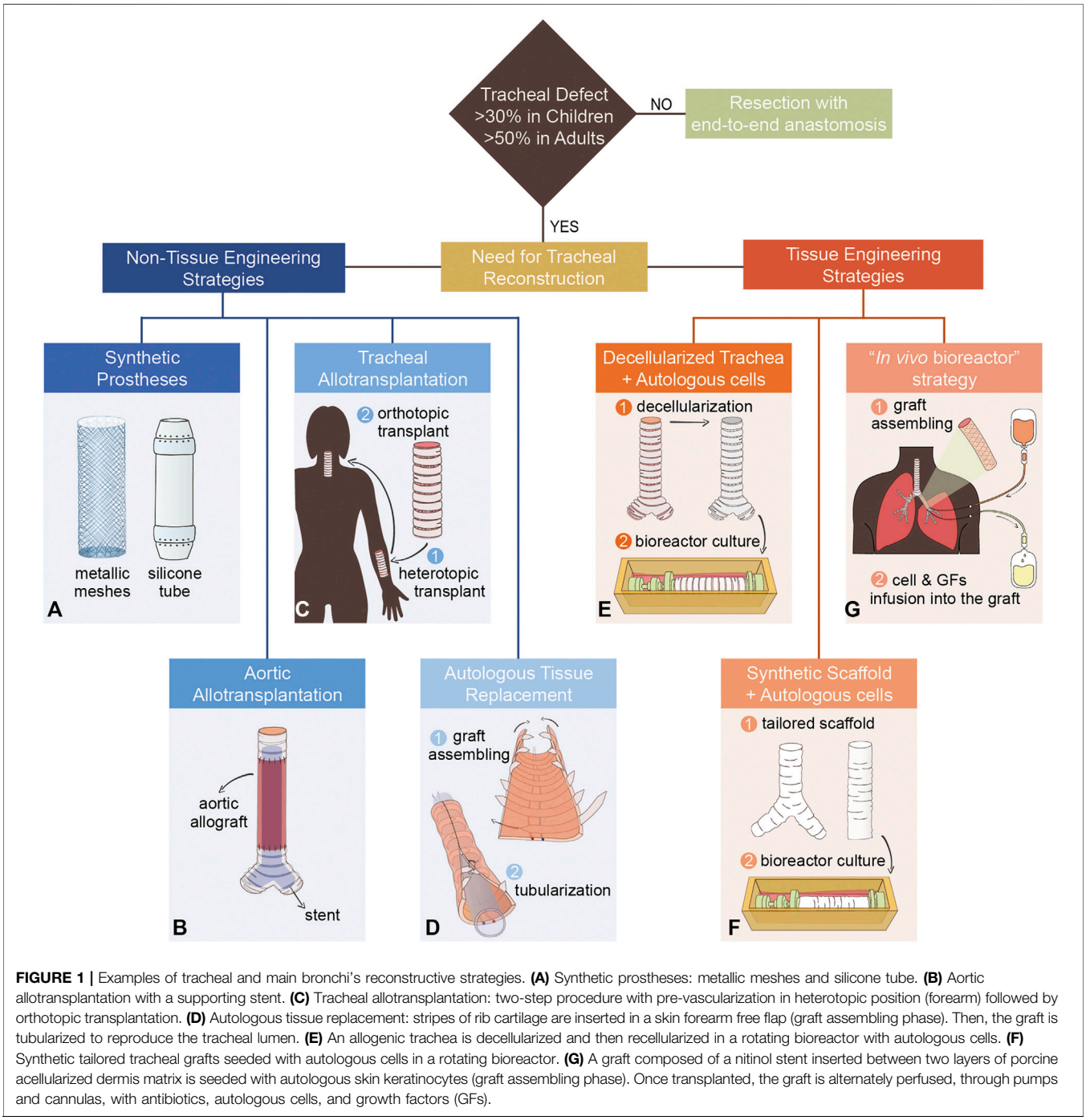

FIGURE 1 | Examples of tracheal and main bronchi's reconstructive strategies. **(A)** Synthetic prostheses: metallic meshes and silicone tube. **(B)** Aortic allotransplantation with a supporting stent. **(C)** Tracheal allotransplantation: two-step procedure with pre-vascularization in heterotopic position (forearm) followed by orthotopic transplantation. **(D)** Autologous tissue replacement: stripes of rib cartilage are inserted in a skin forearm free flap (graft assembling phase). Then, the graft is tubularized to reproduce the tracheal lumen. **(E)** An allogenic trachea is decellularized and then recellularized in a rotating bioreactor with autologous cells. **(F)** Synthetic tailored tracheal grafts seeded with autologous cells in a rotating bioreactor. **(G)** A graft composed of a nitinol stent inserted between two layers of porcine acellularized dermis matrix is seeded with autologous skin keratinocytes (graft assembling phase). Once transplanted, the graft is alternately perfused, through pumps and cannulas, with antibiotics, autologous cells, and growth factors (GFs).

TABLE 1 | Major tracheal and main bronchi clinical reconstructive strategies.

NON-TE APPROACHES

Approach	Patient details (years, gender, pathology)	Material	Cells	Details	Follow-up	Results	Authors
Allotransplantation	Six patients (17–52 yrs, 5 males, 1 female), extensive mucoepidermoid carcinoma (*n* = 1), adenoid cystic carcinoma (*n* = 5)	Stent-supported aortic allograft	N/A	Fresh (*n* = 2) or cryopreserved (*n* = 4) aortic allografts were wrapped with well-vascularized flaps of the pectoral muscle (*n* = 6) and, in two patients, with an additional thymopericardial flap to promote revascularization. A silicon stent was also implanted	26–45 months	Complete tumour resection was achieved in 83% of patients. Three patients suffered from fistulas, while adequate vascularization was observed in all cases. At the end of the follow-up, four patients were disease-free and still alive	Wurtz et al. (2010)
	Twenty patients (24–79 yrs,7 females, 13 males), non-small cell lung cancer (*n* = 11), postintubation tracheal stenosis (n = 3), carcinoid tumour (*n* = 3), thyroid carcinoma (*n* = 2) rhabdomyosarcoma (*n* = 1)	Stent-supported cryopreserved aortic allograft	N/A	A gender-mismatched –80°C cryopreserved aortic allograft was employed for airway reconstruction. A custom-made nitinol stent was inserted into the allograft to prevent airway collapse. No immunosuppressive therapy was required. 7 of the 20 patients were not eligible for transplantation	3–47 months	The overall mortality rate at 3 months was 5%. After a median follow-up of 47 months, 10 of the 13 transplanted patients were alive, with 8 of them showing normal breathing, regeneration of epithelium and *de novo* cartilage within aortic matrix	Martinod et al. (2018)
	1 patient (50 yrs, male), multiple tracheal stenosis and tracheomalacia due to intubation after SARS-CoV-2 infection	Stent-supported cryopreserved aortic allograft	N/A	A non-matched cryopreserved aortic allograft was anastomosed to the cricoid and carina, while a silicon stent was inserted to ensure patency. Both anastomoses were finally carefully covered by omental tissue	2 months	Two months postoperatively, the patient was able to autonomously clear secretions. Neither immunosuppression therapy nor routine bronchoscopy were required	Menna et al. (2021)
	1 patient (24 yrs, female), tracheal stenosis due to idiopathic fibrosing mediastinitis	Tracheal Allotransplantation	N/A	The allograft was wrapped within the omentum. Immunosuppressive therapy was required	12 months	Signs of rejection and necrosis were detected from day 10. A linear silicon endoprosthesis was required to face stenosis. At 1 year of follow-up, the patient was alive and with reduced signs of rejection	Levashov et al. (1993)
	6 patients (17–64 yrs, 3 males, 3 females), long-segment tracheal stenosis (*n* = 5), chondrosarcoma (*n* = 1)	Tracheal Allotransplantation	N/A	Allogenic tracheas were implanted in the forearm to improve vascularization and, in three patients, were repopulated with a patch of buccal mucosa	6–12 months	In three patients, tracheal necrosis and poor vascularization led to a partial loss of the allotransplant. The two patients that received oral mucosal cells and wrapping in the forearm fascia showed normal airways and no adverse events.	Delaere et al. (2010), Delaere et al. (2012)
	1 patient (43 yrs, female), adenoid cystic carcinoma	Tracheal Allotransplantation	N/A	After mechanical decellularization, the donor trachea was revascularized in the forearm of the patient. Seven weeks later, the vascularized allograft was orthotopically implanted	0.7 months	The patient was extubated on day 12. At day 22, a haemorrhage arised from the neotrachea in the mediastinum led to the patient's dead	Iyer et al. (2020)

(Continued on following page)

TABLE 1 | (*Continued*) Major tracheal and main bronchi clinical reconstructive strategies.

NON-TE APPROACHES

Approach	Patient details (years, gender, pathology)	Material	Cells	Details	Follow-up	Results	Authors
	1 patient (56 yrs, female), long-segment cricotracheal stenosis and tracheomalacia	Tracheal Allotransplantation	N/A	In this first single-stage human vascularized long-segment tracheal transplantation, the VCA was placed into the recipient bed. A microscope was used to perform the microvascular anastomoses. Triple immunosuppression was administered	6 months	The restoration of the blood supply was successfully obtained through microvasculature anastomoses. Imaging and bronchoscopic biopsies demonstrate graft vascularization and viable epithelial lining. Six months after transplantation, the patient was able to breathe without the need for tracheostomy or stent	Genden et al. (2021)
Autologous Replacement	1 patient (68 yrs, male), tracheal squamous cell carcinoma	Stent-supported aortic autograft	N/A	A 7 cm abdominal aorta autograft was harvested and replaced with a Dracon graft. A silicon Dumon stent was placed into the aortic graft to avoid aortic wall injury	6 months	An acute respiratory distress syndrome due to granulation required the application of an additional tracheal stent. The patient died at 6 months from septic shock after being treated for pneumonia and a controlateral pneumothorax	Azorin et al. (2006)
	16 patients (37–68 yrs, 7 males,9 females), adenoid cystic carcinoma (*n* = 9), squamous cell carcinoma (*n* = 3), tracheo-oesophageal fistulae (*n* = 2), thyroid cancer with tracheal invasion (*n* = 1), tracheal ischaemic stenosis (*n* = 1)	Fasciocutaneous skin flap reinforced with strips of rib cartilage	N/A	Forearm fasciocutaneous flap vascularized by radial vessels and reinforced through rib cartilages interposed transversally in the subcutaneous tissue. Construct was sutured before implantation	0.8–132 months	Two deaths in the postoperative period due to lung infections and acute respiratory distress syndrome, two deaths for cancer recurrence. Long-term follow-up analysis for 15 patients showed a 65% survival rate at 5 years	Fabre et al. (2013), Fabre et al. (2015)
	5 patients (28–52 yrs, 3 males, 2 females), primary tracheal malignant tumour (*n* = 1), right main bronchial stenosis (*n* = 1), left main bronchus tumour (*n* = 2), adenoid cystic carcinoma (*n* = 1)	Pulmonary tissue flap lined with an elastic metallic stent	N/A	Autologous pulmonary tissue flap lined with an elastic metallic stent to treat extensive tracheal resection	14–84 months	Bronchoscopy after 1 and 2 years detected neither stenosis nor perforation. One patient died at 14 months from severe haemoptysis, while the remaining patients were still alive after 84 months	Zhang & Liu, (2015)
	1 patient (25 yrs, male), postventilation tracheal stenosis	Cutaneous chondromucosal forearm tubular flap	N/A	A 4.5 cm-segment was replaced with strips of costal cartilage sutured around a segment of silicon, previously subcutaneously implanted in the forearm and lined with oral mucosal grafts	6 months	Postoperative analysis at 2 and 6 months revealed normal tracheal calibre, absence of granulation tissue, and a well-vascularized internal mucosal lining	Olias et al. (2005)
	1 patient (38 yrs, male), medullary thyroid carcinoma	Composite skin/omental /oesophageal graft	N/A	Tracheal continuity was restored through a 9 × 6 cm chest wall skin flap sutured to the still viable distal, proximal tracheal stumps and to the lateral oesophageal margins	24 months	After 7 days, a bronchoscopy revealing initial graft stenosis led to the implantation of an Ultraflex stent. 24 months after the operation, the patient was doing well, with no signs of recurrence	Spaggiari et al. (2005)

(Continued on following page)

TABLE 1 | (*Continued*) Major tracheal and main bronchi clinical reconstructive strategies.

NON-TE APPROACHES

Approach	Patient details (years, gender, pathology)	Material	Cells	Details	Follow-up	Results	Authors
	1 patient (43 yrs, female), adenoid cystic carcinoma	Forearm free flap tubed around a stent	N/A	A forearm free flap was harvested and wrapped around an Ultraflex stent before implantation in a 6 cm tracheal defect	16 months	Acceptable function of the neotrachea in the immediate postoperative period. Proximal stricture, sputum retention, and recurrent pneumonia emerged in the following months. Death for malignant hypercalcemia at 16 months	Beldholm et al. (2003)
	1 patient (63 yrs, female), papillary thyroid carcinoma	Forearm free flap with an external mesh support	N/A	The reconstruction of the tracheal defect was obtained through a graft composed of a radial forearm fasciocutaneous free flap combined with a Hemashield vascular graft and reinforced with a PolyMax resorbable mesh	6 months	At 6 months, the patient was symptom-free and has returned to normal activities, with bronchoscopy showing a patent airway	Yu et al. (2006)

TE APPROACHES

Approach	Patient details (years, gender, pathology)	Material	Cells	Details	Follow-up	Results	Authors
Allogenic decellularized tissues	1 patient (30 yrs, female), end-stage bronchomalacia	Decellularized human donor trachea	Epithelial bronchial cells and BM-MSC derived chondrocytes	A donor trachea was decellularized and recellularized with pre-expanded autologous cells. The graft was then used to replace the recipient's left main bronchus	60 months	Continuous reinterventions were needed to remove the different stents rejected by the patient's body and the granulation tissue responsible for stent obstruction	Macchiarini et al. (2008), GonfiOtti et al. (2014)
	1 patient (15 yrs, female), severe congenital malformations (a single left lung and long-segment congenital tracheal stenosis)	Decellularized human donor trachea	Autologous BM-MSCs and epithelial cells from the inferior turbinate	A donor trachea was decellularized with GMP-compliant reagents and recellularized in a bioreactor with autologous cells pre-expanded in a licensed cell therapy facility	0.5 months	Despite promising early results, an acute tracheal obstruction of the posterior wall occurred 2 weeks post-transplantation and led to the young girl's death	Elliott et al. (2017)
	1 patient (10 yrs, male), long-segment congenital tracheal stenosis and pulmonary sling	Decellularized human donor trachea	Autologous BM-MSCs and patches of autologous epithelium	A decellularized trachea was saturated with hematopoietic stem cells, and patches of tracheal epithelium were secured to the graft's lumen via a bioresorbable stent. GFs were administrated as pharmacological support	48 months	Many postoperative interventions were necessary, mainly to clear secretions, granulation, and remove a malacic graft segment. Four years after the transplant, the child was in good health, proving this procedure as lifesaving	Elliott et al. (2012), Hamilton et al. (2015)
	1 patient (38 yrs,1 female), Hodgkin lymphoma	AlloDerm (allogenic decellularized human derma)	N/A	The allogenic decellularized human derma was sutured into a tube and transplanted into the defect. Two different muscle flaps were used to cover and repair the chest and the neck	48 months	Postoperatively, the migration of the graft required its repositioning. Then, nine bronchoscopies (among which two dilatations) were necessary. Four years later, the patient is disease-free and lives a normal life	Bolton et al. (2017)

(Continued on following page)

TABLE 1 | (*Continued*) Major tracheal and main bronchi clinical reconstructive strategies.

NON-TE APPROACHES							
Approach	**Patient details (years, gender, pathology)**	**Material**	**Cells**	**Details**	**Follow-up**	**Results**	**Authors**
Patient-tailored synthetic scaffolds	3 patients (22–37 yrs, 2 males, 1 female), mucoepidermoid carcinoma (n = 1), adenoid cystic carcinoma (n = 1), iatrogenic tracheal injury (n = 1) carcinoma;	Nanocomposite polymer POSS-PCU (n = 1), electrospun polyblend of PET/PU 70%/30% (n = 2), electrospun PET 100% (additional implant, n = 1)	Autologous BM-MNCs	Synthetic tracheal grafts were seeded in a rotating bioreactor with autologous BM-MNCs in combination with locally and systemically GFs (TGF-b3, G-CSF and epoetin)	3,5–55 months	All patients developed graft-related complications and died after multiple surgical interventions. The main problems encountered were anastomotic fistulae, obstructive granulation tissue, absence of graft vascularization and mucosal lining	Fux et al. (2020)
"*In vivo* bioreactor" strategy	1 patient (57 yrs, male), squamous lung cancer	Nitinol stent and two layers of porcine ADM	Autologous skin keratinocytes and TNCs	A nitinol stent was enveloped between two layers of ADM. Skin keratinocytes were seeded on the lumen of the TE substitute. Once transplanted, Ringer's solution and TNCs were injected into the graft	13 months	Bronchoscopy revealed signs of revascularization and biodegradation of the ADM scaffold. After 4 months, a biopsy showed epithelial tissue lining the graft. The patient died of lung cancer relapse 13 months postoperatively	Tan et al. (2017)

The table summarizes some of the main non-tissue engineering (non-TE) and tissue engineering (TE) approaches tested so far and still considered as potential strategies for long tracheal defects repair. For each approach, the number and the type of patients treated, the duration of the follow-up and the clinical results observed were reported. yr, year-old; N/A, not applicable; BM-MSCs, bone marrow-derived mesenchymal stromal cells; GFs, growth factors; GMP, good manufacturing practice; POSS-PCU, polyhedral oligomeric silsesquioxane–poly(carbonate-urea)urethane; PET/PU, polyethylene terephthalate/polyurethane; BM-MNCs, bone marrow–derived mononuclear cells; TGFB-3, Transforming Growth Factor beta-3; G-CSF, Granulocyte-Colony Stimulating Factor; ADM, Acellular Dermal Matrix; TNCs, total nucleated cells; TE, tissue engineering; VCA, vascularized composite allotransplantation.

In 2017, a clinical trial (TRACHEOBRONCART, NCT01331863) evaluated cryopreserved, stented aortic graft for tracheal replacement in patients affected by end-stage airway diseases (Martinod et al., 2017). So far, in the 13 patients reported, Martinod and colleagues showed that the cryopreserved aortic graft promoted the regeneration of new tissue (Martinod et al., 2017, Martinod et al., 2018). However, some stent-related complications such as infection and granuloma were also observed, suggesting that further studies are needed to confirm if this technique is safely applicable for long-circumferential damages or should be restricted to patch repairs only. Recently, a stented cryopreserved aortic allograft has also been used for the first time to treat a post-COVID-19 patient presenting multiple tracheal stenoses, tracheomalacia and ossification (Menna et al., 2021).

Tracheal Allotransplantation

Tracheal segments derived from brain-dead donors have been frequently used as non-vascularized allografts to treat patients with extensive tracheal damages. In 1993, Levashov et al. described a one-stage procedure consisting of direct trachea transplantation from a just passed away donor. Ten days postoperation, despite immunosuppression, signs of rejection appeared, followed by graft stenosis, which required the implantation of a silicone stent (Levashov et al., 1993). The direct tracheal allotransplantations outcomes confirmed these results, coupled with graft's ischemia and necrosis within 2 months post-implantation. Formalin-fixed tracheas have been allotransplanted to reduce tissue immunogenicity, increase its stiffness and be readily available in case of emergency. However, despite early success, the long-term follow up revealed graft stenosis and the need for stenting, reasons why this procedure was almost completely abandoned (Greaney and Niklason, 2021).

In 2008, after studies in animal models and a first human preliminary report in 1979 (Rose et al., 1979; Delaere et al., 1995; Delaere et al., 1996), Delaere's group performed a two-step tracheal allotransplantation in a patient with a long history of tracheal stenosis (Delaere et al., 2010). In this procedure, the trachea obtained from a brain-dead individual was first heterotopically implanted into the recipient's forearm to promote vascularization and then transplanted in its orthotopic position. Additionally, the respiratory mucosa of the donor trachea was replenished by a flap of oral mucosa from the recipient, creating a chimeric graft. The same treatment was applied on six patients leading to several complications, principally related to rejection after withdrawal of immunosuppressant and graft necrosis, which led to a partial loss of the allograft (Delaere et al., 2012; Delaere and Van Raemdonck, 2016). In conclusion, the reconstructed airways were insufficient to sustain respiration, and required tracheostomy in some cases. Recently, the same technology has been applied by Iyer and colleagues to treat a patient affected by tracheal adenoid cystic carcinoma. However, soon after the surgery, a strong haemorrhage arising from the "neotrachea" led to the patient's death (Iyer et al., 2020).

These poor outcomes point out some significant limitations related to this technology, including 1) dependence on donor availability and related blood matching (Delaere et al., 2010), 2) long time requirement for heterotopic pre-vascularization of the graft (inapplicable in emergencies), 3) extended periods of immunosuppression to limit rejection of the graft, which exposes the subject to opportunistic infections and that is inapplicable in case of cancer patients, and 4) altered mucociliary clearance, colonization with abnormal flora and secondary morbidity in case of heterotopic use of skin/oral mucosa flap to replace the necrotic tracheal epithelium (Udelsman et al., 2018).

Only recently, Genden and colleagues, after preclinical *in vivo* studies on animal models (Genden et al., 2002; Genden et al., 2003), tried to overturn a longstanding dogma for which the tracheal microvascular anastomoses is not achievable (Genden et al., 2021). Specifically, Genden performed a single-stage tracheal vascular composite allotransplantation (VCA) involving the thyroid arteries and the muscular wall of the donor's oesophagus that shares blood supply with the donor trachea. The restoration of the blood supply was successfully obtained through microvasculature anastomoses, and a well-perfused continuous airway respiratory epithelium was observed via endoscopy and histological examination. However, even if this approach addresses some of the problems faced in the two-step tracheal allotransplants, the need for immunosuppression still represents a limitation restricting this approach to non-oncologic patients only. Moreover, a longer follow-up and a larger cohort of patients are required to evaluate the safety and the efficacy of this procedure (Randhawa and Patterson, 2021).

Tracheal Replacement With Autologous Tissues

Finally, a different approach involves multiple autologous tissues as a source for tracheal and main bronchi reconstruction, such as aortic autograft (Azorin et al., 2006), pulmonary tissue (Zhang and Liu, 2015), skin (Spaggiari et al., 2005), intercostal artery muscle flap (Bertheuil et al., 2021), forearm flaps and costal cartilage (Beldholm et al., 2003; Olias et al., 2005; Yu et al., 2006; Fabre et al., 2013; Mercier et al., 2018). The common goal of these reconstructive techniques is to generate a tubularized and well-perfused graft to replace the damaged trachea. In many cases, this was combined with a temporary or permanent stent to give structural stability to the transplant (Azorin et al., 2006; Fabre et al., 2013; Zhang and Liu, 2015). In 2013, after preclinical studies on large animal models (Fabre et al., 2009), Fabre and colleagues published the largest series of patients treated with autologous tissue for airway replacement purposes (Fabre et al., 2013, 2015). They used a single-step procedure to generate and transplant a tracheal substitute made by regular intervals of forearm free fasciocutaneous flaps and costal cartilages. Following this procedure, seven out of twelve patients developed acute respiratory distress syndrome, and two became tracheostomy dependent (Udelsman et al., 2018).

Although these innovative approaches allow avoiding immunosuppression, the variable clinical outcomes highlight several issues, such as 1) the need for temporary or permanent stents, 2) the absence of an integer epithelium, which is crucial for a physiologic mucociliary clearance, and 3) the risk of morbidities at the donor site following the withdrawal of autologous tissue.

TISSUE ENGINEERING APPROACHES FOR TRACHEOBRONCHIAL RECONSTRUCTION

Tissue engineering (TE) approaches rely on the combination of cells with an appropriate scaffold for the treatment of significant tissue defects. Indeed, in this interdisciplinary field, principles of biomaterial engineering, genetics, cell biology and clinical science are combined to develop a biological graft to maintain, restore or improve tissues or whole organs (Shafiee and Atala, 2017; Vranckx and Hondt, 2017). This solution can simultaneously address different problems, such as donor shortage (often fatal for patients in critical status, struggling with long waiting lists), immunosuppression therapy and donor site morbidity. In fact, TE strategies take advantage of the body's regenerative potential and, by *in vitro* expansion, allow to obtain enough cells to regenerate extensive body areas from a small biopsy (Gallico, 1985; Corradini et al., 2016). Thus, this field represents a promising strategy for expanding the current reconstructive armamentarium to treat severe unmet medical needs (Vranckx and Hondt, 2017).

Between 2008 and 2017, TE attempts for windpipe and main bronchi's replacement have been tested in compassionate cases, and the trachea has been acclaimed by both the scientific community and mass media as the first tissue-engineered organ (Fountain, 2012). However, this field was impaired by a history of scientific and ethical misconduct (The Lancet, 2018; The Lancet Editors, 2018; Jungebluth et al., 2019), which has led to a general sense of mistrust concerning airway TE potential.

To date, three main strategies have been proposed in tracheal TE, which involves the use of allogenic decellularized human cadaveric donor tissue, synthetic patient-tailored scaffolds, or an "*in vivo* bioreactor" strategy. **Table 1** summarizes some of the main experiences in this field.

Allogenic Decellularized Tissues

Among the different scaffolds used for TE approaches, the extracellular matrix (ECM) obtained through decellularization of allogenic tissues has been used for tracheal replacement. An example of allogenic tissue devoided of the cellular component is the extracellular dermal matrix, recently used as non-vascularized graft for long-segmental tracheal replacement (Bolton et al., 2017). However, this non-resolutive strategy required postoperative refinement due to graft migration. Moreover, this attempt relies only on cell migration from the wound edges, insufficient in case of extensive defects. On the other hand, the ECM obtained from allogenic human cadaveric tracheas has been adopted either with cultured autologous cells or freshly harvested hematopoietic stem cells.

Allogenic Tracheal Extracellular Matrix Plus Cultured Cells

The first patient to receive a TE approach was a 30 year-old woman suffering from end-stage bronchomalacia (Macchiarini

et al., 2008). In this compassionate case, a human donor trachea was decellularized with an extensive protocol to obtain a suitable scaffold to be colonized with autologous cells. Specifically, epithelial cells isolated from a bronchus' mucosal biopsy were cultured under serum-free conditions, while mesenchymal stem cells from bone marrow aspirate were expanded and induced to differentiate in chondrocytes (Macchiarini et al., 2008). Through a perfusion system, a bioreactor with two separate accesses was used to seed epithelial cells onto the internal surface of the decellularized trachea. At the same time, the chondrocytes were injected into the external surface of the matrix. Finally, after surgical removal of the damaged tissue, the avascular graft was shaped and sutured to the remaining native tissue (Macchiarini et al., 2008). In 2014, Gonfiotti et al. reported the patient's 5-year follow-up, describing this TE approach as safe and promising.

However, the postoperative course was characterized by many complications. The development of granulation tissue and cicatricial scar led to the implantation of several endoluminal stents to maintain the airway patent. Continuous reinterventions were necessary to remove the different stents rejected by the patient's body and the granulation tissue responsible for stent obstruction (Gonfiotti et al., 2014). Such graft-related postoperative complications strongly affected the patient's quality of life (Molins, 2019).

Later on, Elliott and colleagues adopted a similar technique to treat a 15 year-old girl born with severe congenital malformations (a single left lung and long-segment congenital tracheal stenosis) after other failed reconstructive approaches (Elliott et al., 2017). In this study, every step of the reconstructive procedure was designed to fulfil good manufacturing practice (GMP) standards. Specifically, a donor trachea was decellularized with GMP-compliant reagents. Meanwhile, autologous bone marrow cells and epithelial cells from the inferior turbinate were expanded in a licensed cell therapy facility and then seeded onto the decellularized matrix in a bioreactor (Elliott et al., 2017). Despite promising early results, an acute tracheal obstruction of the posterior wall occurred 2 weeks post-transplantation and led to the young girl's death. Given this negative outcome, the authors recommended using stents during the first few months postoperatively. Moreover, they highlighted the difficulties in translating a TE reconstructive approach from the preclinical setting to the clinic, even because the *in vivo* models cannot mimic complex clinical scenarios (Elliott et al., 2017).

Allogenic Tracheal Extracellular Matrix Plus Freshly Harvested Cells

To reduce the time needed for producing a TE tracheal substitute, a decellularized trachea was saturated with freshly harvested hematopoietic stem cells. Besides, patches of tracheal epithelium were placed as free grafts within its lumen and secured via a bioresorbable stent. This TE attempt aimed to recreate an *in vivo* microenvironment, recapitulating some of the key stimuli that lead to the physiological post-injury repair. In this respect, several growth factors were systemically administrated both in the preoperative and postoperative periods and locally injected into the avascular TE construct during the implantation. This pharmacological support aimed to mobilize haemopoietic stem cells and endothelial progenitors, improve mesenchymal stromal cells (MSCs) recruitment, induce chondrocyte differentiation, and increase angiogenesis (Elliott et al., 2012). The 4-year follow-up of a 10 year-old child treated with this approach declared the boy's good health, assessing this procedure as lifesaving (Hamilton et al., 2015). Despite this, many reinterventions (more than 25) were needed, especially during the first year of follow-up, to clear secretion, remove granulation tissue and replace the resorbable stent. Difficulties in the re-epithelialization of the decellularized tracheal matrix have been observed. Indeed, the histological analysis of a biopsy from the TE graft at 1 month post-intervention revealed granulation tissue only. Instead, a biopsy of the proximal transplant collected at 42 months, showed an epithelial layer with a mix of squamous and respiratory epithelium and only a few ciliated cells. Probably, the graft vascularization was insufficient to allow the epithelialization of the decellularized matrix from the free graft's patches of tracheal mucosa, and the poor epithelialization occurred from the migration of cells from the wound edges. Nonetheless, this mechanism can cover only a few millimetres of the graft, while the central tissue is left as uncovered granular tissue (Delaere et al., 2014).

Patient-Tailored Synthetic Scaffolds

Since 2011, synthetic tailored scaffolds repopulated by autologous bone marrow cells and supported by growth factors have been proposed to revolutionize regenerative medicine. Those constructs were made of nanocomposite polymer POSS-PCU (polyhedral oligomeric silsesquioxane–poly (carbonate-urea) urethane) or electrospun polyblend PET/PU (polyethylene terephthalate (PET) and polyurethane (PU)) (Fux et al., 2020) and were described as able to integrate within the recipient, generating living and functional grafts covered by epithelium (Del Gaudio et al., 2014; Jungebluth et al., 2011, Jungebluth et al., 2013). However, these papers were retracted for scientific misconduct in the following years. Only recently, Fux and colleagues have unequivocally stated the inadequacy of those constructs for TE purposes. In this retrospective study, the authors presented the first long-term follow-up of three patients, who in total received four synthetic tracheal grafts recellularized with bone marrow–derived mononuclear cells (BM-MNCs). During the postoperative period, all patients developed graft-related debilitating complications. Follow-up analysis showed the formation of anastomotic fistulae and obstructive granulation tissue as well as the absence of vascularization, epithelial lining, or integration within the surrounding tissue. All patients died after multiple surgical reinterventions, revealing the failure of TE synthetic tracheal substitutes as living functional grafts (Fux et al., 2020). Indeed, the "bioengineered" constructs behaved only like an inert scaffold, similarly to synthetic tracheal prostheses (Delaere et al., 2019).

"*In Vivo* Bioreactor" Strategy

To face the challenges related to the delayed revascularization process and infections, Tan et al. proposed the use of the recipient body as a bioreactor for the TE substitute (Tan et al., 2017). This approach aims to combine the commonly separated *in vitro* 3D cell-scaffold culture with the *in vivo* regenerative process. With this purpose, a nitinol stent - providing lateral rigidity and longitudinal flexibility to the TE construct - was surrounded by two layers of a biodegradable porcine acellularized dermis matrix (ADM). One hour before transplant, the luminal side of the scaffold was seeded with epidermal keratinocytes obtained from the digestion of a skin flap. Two catheters were inserted among the two ADM layers and associated with peristaltic pumps during the implantation. Through this cannulation system, the avascular TE graft was perfused for 1 month with Ringer's gentamicin fluid to prevent infection and to keep the epithelial cells alive before final revascularization. Moreover, this system allowed the secondary infusion of total nucleated cells (TNCs) and growth factors to stimulate graft regeneration directly into the transplanted TE construct. Unfortunately, the authors reported the patient's death 13 months postoperatively. Consequently a longer follow-up is unavailable (Tan et al., 2017). Major doubts remain regarding the durability and functionality of this bioengineered construct. In fact, the gradual biodegradation of the ADM scaffold, observed during the postoperative bronchoscopies, and the use of epidermal keratinocytes instead of airway ciliated epithelial cells could be responsible for long-term severe graft-related complications.

FRONTIERS OF TRACHEAL SUBSTITUTES: LESSONS FROM PRECLINICAL ANIMAL STUDIES

Failures and controversies raised by the clinical application of several tracheal substitutes have led to a renewed interest in preclinical studies based on animal models. According to Niermeyer et al., 73% of articles focused on tracheal reconstruction and published between 2015 and 2020 involved an *in vivo* preclinical model. This reveals the need for a more in-depth preliminary analysis of the tracheal substitutes before embarking on new clinical applications (Niermeyer et al., 2020).

This paragraph focuses on the latest *in vivo* preclinical TE attempts, since they stand as one of the most promising approaches to face hurdles that still hinder this field. Indeed, articles concerning tissue-engineered tracheal grafts (TETGs) aim to improve graft integration within the recipient, mimick complex native trachea biomechanics and prevent host adverse responses (Greaney & Niklason, 2021). To give an idea of the possible future clinical applications, a few non-exhaustive examples belonging to the different categories of the current TE approaches are listed below and summarized in **Table 2**.

Concerning the use of synthetic material, several methodologies have been investigated to produce tubular scaffold suitable for tracheal replacement. In 2011, a casting-based manufacturing process was used to create a tracheal-shaped substitute starting from mixtures of rat fibroblast/MSCs and collagen hydrogels. The stiffness of the resulting bioartificial trachea was compared to the one of the native trachea showing no differences. However, when the former was transplanted in a rat model, only three of the nine treated animals survived the implantation, dying within 48 h after surgery. The author suggests that these results could be related to the absence of epithelial cells on the inner layer of the transplanted bioartificial trachea (Naito et al., 2011).

Some years later, Pepper et al. employed a polyethylene terephthalate (PET) and polyurethane (PU) scaffold combined with polycarbonate rings. This structure was seeded with BM-MNCs and transplanted in eight sheep to replace a 5 cm long tracheal defect. Despite promising mechanical tests, all animals showed graft stenosis associated with granulation tissue. Overall, this attempt remarked the centrality of the epithelialization and neovascularization, in the absence of which the outcomes are poor (Pepper et al., 2019).

Using a mouse model, another group compared synthetic non-resorbable PET/PU *vs.* resorbable poly(l-lactide-co-ε-caprolactone)/Polyglycolic acid (PLCL/PGA) scaffolds. Even in this study, graft's stenosis was revealed in both conditions, with no signs of respiratory epithelization in the central part of the grafts (Dharmadhikari et al., 2019).

Several groups also investigated synthetic scaffolds based on polycaprolactone (PCL), given its easy moldability through 3D printers (Gao et al., 2017; Rehmani et al., 2017). To mimic the tracheal structure and mechanical profile, Lee and colleagues used a bellows-designed PCL scaffold reinforced with collagen, silicon rings and seeded with human turbinate mesenchymal stromal cells (hTMSC) sheets (Lee et al., 2018). After implantation in rabbits, the PCL was successfully incorporated within the adjacent tissue and lined by airway epithelium. However, respiratory distress and mild granulation process were observed in all animals. Additionally, the higher levels of interleukin-2 and interferon gamma detected in treated animals, compared to the baseline values, suggested a possible acute rejection (Lee et al., 2018). Two years later, another group used both electrospinning and 3D printing techniques to generate a PCL synthetic scaffold for tracheal reconstruction in a rabbit model. In this study, human bronchial epithelial cells (hBECs) were used to populate the inner layer of the PCL scaffold, while the outer layer was repopulated by either induced pluripotent stem cells-derived mesenchymal stem cells (iPSC-MSCs) or induced pluripotent stem cells-derived chondrocytes (iPSC-Chds). A regenerated epithelium was observed in both conditions at the study endpoint (4 weeks). However, in the iPSC-MSCs group, the epithelium was fully specialized and better organized than in the iPSC-Chds group, suggesting a paracrine effect of iPSC-MSCs in promoting the re-epithelialization process (Kim et al., 2020). To overcome the aforementioned problems related to synthetic tracheal TE grafts, other groups tried to reduce the immunogenicity of the grafts, increasing their survival prospects through pre-implantation vascularization strategies (Soriano et al., 2021). For example, Zhao et al. seeded smooth muscle cells onto a PGA-nitinol stent scaffold to allow the deposition of a collagenous matrix and increase the scaffold angiogenetic properties. The resulting construct was then

TABLE 2 | Preclinical animal studies.

Approach	Animal model, samples size	Scaffold material	Cells	Graft length	Follow-up	Outcomes	Lessons	Authors
Biosynthetic scaffolds	Sheep, *n* = 8	PET/PU reinforced with clinical-grade PC rings	autologous BM-MNC	5 cm	3–16 weeks	Graft stenosis, infections, mechanical failures, and lack of epithelialization were observed in all animals	The lack of epithelization and inappropriate blood supply causes a pro-inflammatory response leading to stenosis and graft failure	Pepper et al. (2019)
	Mice, *n* = 25	nonresorbable PET/PU and resorbable PGA/PLCL polymers	N/A	0.5 cm	1–8 weeks	Stenosis manifested in both groups, leading to premature death with respect to the study endpoint. Lack of respiratory epithelium in the mid-graft region	Graft stenosis was due to new tissue overgrowth in nonresorbable scaffolds and to malacia in resorbable scaffolds	Dharmadhikari et al. (2019)
	Rabbit, *n* = 18	PCL bellows scaffold reinforced with silicon rings and collagen	human turbinate mesenchymal stromal cell (hTMSC) sheets	1.3 cm	4 weeks	The graft lumen was covered by adjacent respiratory epithelium. Mild mucosal granulation was observed	PCL bellows graft could be promising for tracheal replacement, however acute rejection signs were observed	Lee et al. (2018)
	Porcine, *n* = 7	PCL and bovine decellularized ECM	N/A	4 cm	4–12 weeks	Graft lumen was covered by ciliated epithelium along with metaplastic squamous epithelium. Mild granulation tissue was revealed	Possible explanations of the granulation tissue formation could be rapid resorption of acellular scaffold and the absence of an epithelium at the time of implantation	Rehmani et al. (2017)
	Rabbit, *n* = 20	PCL	chondrocytes from rabbit auricle	1.6 cm	2–10 weeks	All animals died for granulation formation, pneumonia, infections, and stenosis. Absence of epithelium on the scaffold lumen surface	The graft had good cartilaginous properties. However, the lack of an epithelial layer and host inflammatory reactions caused stenosis and granulation formation	Gao et al. (2017)
	Rabbit, *n* = 11, Monkey, *n* = 3	PGA and nitinol stent	smooth muscle cells removed through decellularization before transplantation	0.8 cm length in rabbits; 1.8 cm length in monkeys	1–8 weeks	The implanted graft was well integrated, with no signs of collapse or infections. A ciliated epithelium covered its lumen. However, several strictures were observed at different time points	The acellular tissue-stent graft showed good biomechanical properties and proved to be pro-angiogenic *in vivo*, but still affected by stenosis related to delayed epithelialization	Zhao et al. (2016)
	Dog, n = 5	Collagen-coated nitinol frame	N/A	2 cm	4–96 weeks	4/5 dogs survived 18–24 months without signs of tracheal stenosis. Angiogenesis was observed in 3 months, and a good biocompatibility was confirmed	This artificial graft reproduced the physical properties of the native trachea. Regeneration of a ciliated epithelium was revealed, but as a monolayer rather than a pseudostratified columnar epithelium	Sakaguchi et al. (2018)
	Rabbit, *n* = 16	3D printed PLLA scaffold	autologous chondrocytes from rabbit auricle	1.5 cm	8 weeks	Animals in the control group (*n* = 8) whose scaffold was not pre-vascularized died for chronic tracheal stenosis within 1 month. Instead, 6/8 animals whose scaffold was *in vivo* pre-vascularized for 2 weeks survived at 2 months,	Pre-vascularization process supports the regeneration of cartilage tissue and seeded cells' survival, allowing to obtain an epithelialized lumen within the engineered trachea	Gao et al. (2019)

(Continued on following page)

TABLE 2 | (*Continued*) Preclinical animal studies.

Approach	Animal model, samples size	Scaffold material	Cells	Graft length	Follow-up	Outcomes	Lessons	Authors
						showing an open lumen, with little occurred granulation tissue		
	Rabbit, *n* = 23	Electrospun PCL nanofibers covered by 3D printed PCL microfibers	hBECs, iPSC-derived MSC or iPSC-derived chondrocytes	1.5 cm	4 weeks	At the study endpoint of 4 weeks, the engineered trachea appeared covered by epithelium without severe granulation in both groups receiving scaffolds with hBECs and IPSC either derived from MSC or chondrocytes. Moreover, the group receiving IPSC-derived MSC showed fully differentiated epithelium with cilia formation	iPSC-MSCs may have a possible beneficial role in promoting the re-epithelialization process through paracrine mediators	Kim et al. (2020)
	Rat, *n* = 9	Custom-made casting molds of rat fibroblasts and collagen hydrogels	Rat fibroblasts and osteogenically-induced MSC	0.5 cm	24–48 h	3/9 rats died before 48 h, showing some strictures in anastomotic regions, 6 rats died during the operation because they could not be weaned from the respirator because of the impaired bioartificial trachea	The lack of epithelial lining on the lumen of the trachea is a great limitation. Thus, epithelial cells should also be considered within this approach	Naito et al. (2011)
Decellularized scaffolds	Porcine, *n* = 20	Porcine Decellularized trachea	Autologous MSCs-derived chondrocyte and bronchial epithelial cells	6 cm	1.5–8.5 weeks	Only the animals in which the decellularized matrix was seeded with both epithelial and chondrocytes were healthy and without signs of stenosis, infections, and rejection	Matrix seeding with both epithelial and mesenchymal stem cell–derived chondrocytes is required to obtain a functional graft	Go et al. (2010)
	Rabbit, *n* = 16	Decellularized rabbit trachea compared to preserved allograft and synthetic scaffold (POSS-PCU)	N/A	2 cm	1,5–4 weeks	Due to respiratory distress, all animals were early terminated. Graft malacia was observed as well as the absence of epithelization	Stenosis were observed in all groups, suggesting the necessity to evaluate seeded scaffold for tracheal replacement	Maughan et al. (2017)
Scaffold-free constructs	Rat, *n* = 9	Scaffold-free construct supported by a silicone stent	rat chondrocytes, endothelial cells and MSCs	0.48 cm	~ 3 weeks	Vasculogenesis and chondrogenesis were observed. However, the lack of a luminal epithelium and the presence of a stent provoked granulation formation	The graft was sufficiently strong to be transplanted but required stent support to prevent graft collapse	Taniguchi et al. (2018)
	Rat, *n* = 3	Scaffold-free construct supported by a silicone stent	human chondrocytes, MSCs, fibroblasts, and human umbilical vein endothelial cells (HUVECs)	0.38 cm	5 weeks	The presence of epithelial cells from the native trachea and capillary-like structures were confirmed. The strength of the graft was lower than the native trachea	This technique could produce grafts made by human cells only. However, it still presents some limits such as a prolonged culture time to obtain a sufficient number of cells and the need for a stent	Machino et al. (2019)

The table summarizes some of the most recent in vivo *preclinical strategies adopted to improve tracheal and main bronchi reconstruction. For each attempt, the animal model, the number of animals treated, the follow-up duration, the outcomes and the lessons learned have been reported. PET/PU, polyethylene terephthalate/polyurethane; PC, polycarbonate; PGA/PLCL, polyglycolic acid/poly(l-lactide-co-ε-caprolactone); PCL, polycaprolactone; ECM, extracellular matrix; BM-MNC, bone marrow mononuclear cells; POSS-PCU, polyhedral oligomeric silsesquioxane-poly(carbonate-urea)urethane; MSCs, mesenchymal stromal cells; PLLA, poly (L-lactic acid); hBECs, human bronchial epithelial cells; iPSC, induced pluripotent stem cells.*

decellularized, preserving the new extracellular matrix, and implanted in rabbits and nonhuman primates. No graft collapse was observed, while a columnar respiratory epithelium lined the construct lumen. However, mid-grafts stenosis was present (Zhao et al., 2016). In the preclinical study performed by Sakaguchi and colleagues, 80% of dogs transplanted with a pre-vascularized collagen-coated nitinol scaffold survived for more than 18 months. No signs of stenosis were reported, while a monolayer of ciliated cells covered the graft's lumen. These promising results may be due to the pre-vascularization obtained through the graft's heterotopic implantation into the omentum (Sakaguchi et al., 2018). Similar beneficial effects of the pre-vascularization process were observed in a rabbit preclinical study where a 3D printed PLLA (poly L-lactic acid) scaffold seeded with autologous chondrocytes was used to reconstruct a 1.5 cm long tracheal segment. Indeed, the engineered pre-vascularized trachea appeared integrated within the adjacent tissues and covered by respiratory epithelium. Besides, no signs of stenosis were observed with only sporadic granulation tissue formation (Gao et al., 2019).

Besides synthetic scaffolds, decellularized grafts have been employed in preclinical settings. However, when used without the cellular component, they behaved similarly to the polyhedral oligomeric silsesquioxane–poly (carbonate-urea)urethane (POSS-PCU) synthetic scaffolds, mainly developing stenosis (Maughan et al., 2017). Otherwise, when recellularized with epithelial cells and MSC-derived chondrocytes, they become functional and well-tolerated, without signs of rejection or airway collapse (Go et al., 2010). These results remark the need for the cellular component to promote graft re-epithelialization and structural stability essential for preventing host inflammatory response and graft failure.

Finally, new frontiers may arise from scaffold-free constructs. Indeed, through a novel Bio-3D printing system, many groups developed artificial tracheas assembling spheroids composed of various cell types, such as chondrocytes, MSCs and endothelial cells (Taniguchi et al., 2018; Machino et al., 2019). In these studies, to obtain a structure that recapitulates the tracheal shape, the multicellular spheroids were first placed into needle arrays and then underwent a maturation phase inside a bioreactor. Finally, the artificial scaffold-free constructs were implanted in rat models to evaluate the graft performance. At the end of the studies, these artificial tracheas showed signs of chondrogenesis, vasculogenesis, and an epithelium lining the lumen. However, the need for a permanent stent to avoid graft collapse and the presence of granulation tissue represent some limitations of this innovative technique (Taniguchi et al., 2018; Machino et al., 2019).

DISCUSSION

Tracheal and main bronchi dysfunctions represent an unmet and growing medical need, especially in the case of wide circumferential structural alterations where available surgical strategies are ineffective or inapplicable. The long-term clinical outcomes of reconstructive approaches tested so far clearly point out the difficulty of restoring a functional trachea (Grillo, 2002; Soriano et al., 2021).

Since the first challenge of any tracheal replacement's attempt is to reproduce or mimic the native tracheal structural properties, a suitable tracheal substitute must fulfil specific biomechanical requirements. Indeed, it has to be airtight and possess longitudinal flexibility and lateral rigidity to withstand forces arising from respiration, coughing, neck movements and pressure created by the adjacent oesophagus (Grillo, 2002; Boazak and Auguste, 2018). Former approaches not fulfilling these requirements resulted in airway collapse, strictures, graft migration, or haemorrhages which, altogether, stand as the primary cause (68%) of graft-related mortality (Greaney and Niklason, 2021). Consequently, a preliminary assessment of the tracheal substitute's suitability needs to be performed to reduce avoidable life-threatening adverse events on the patients. Indeed, several *in vivo* preclinical studies have been carried out to evaluate biochemical properties of decellularized (Zhao et al., 2016), synthetic (Dharmadhikari et al., 2019) or scaffold-free constructs (Machino et al., 2019). However, since the first requirement for an adequate tracheal substitute is to mimic the tracheal's native biomechanical properties, the definition of standard approaches and biomechanical tests would be extremely helpful to obtain comparable results among different studies and further improve this field (Martínez-Hernández et al., 2021).

Another limitation for a successful reconstructive approach is the lack of studies on the biocompatibility between the tracheal substitute and the host. As previously described, this problem has been reported to trigger acute rejection, granulation tissue formation and graft necrosis, especially in the case of synthetic prostheses (Neville et al., 1976, Neville et al., 1990; Toomes et al., 1985), bioprosthetic substitutes (Hoffman et al., 2001) and tracheal allograft (Levashov et al., 1993; Delaere et al., 2010; Delaere et al., 2012). Before implantation, specific biocompatibility studies should be performed to ensure that the tracheal substitute is made of safe, non-immunogenic material, well-tolerated by the recipient and able to integrate within the body. In order to avoid the aforementioned rejection problem, particularly marked in the case of allograft, also autologous tissues have been clinically applied (Olias et al., 2005; Spaggiari et al., 2005; Fabre et al., 2013, Fabre et al., 2015; Zhang and Liu, 2015; Mercier et al., 2018). However, even this procedure presented some problems, such as donor-site morbidity (Udelsman et al., 2018). In this scenario, TE represents a unique opportunity to rebuild extensive body surfaces, combining cells extracted from a small autologous biopsy with an appropriate scaffold (Shafiee and Atala, 2017; Vranckx and Hondt, 2017).

Regardless of the reconstructive approach, one of the common hurdles in tracheal replacement is graft epithelialization. Indeed, airway functions are critically dependent on the respiratory epithelium, and its absence triggers several complications, including mucous stagnation, infections, graft stenosis, granulation tissue formation, chondromalacia and fibrogenic reaction (Heijink et al., 2014; Paternoster and Vranckx, 2021). Therefore, any tracheal replacement attempt should be aimed at restoring a functional and well specialized respiratory epithelium (Davis and Wypych, 2021). Several aspects must be considered to ensure the presence of a continuous, self-renewing and specialized respiratory epithelium lining the lumen of the tracheal substitute.

First, due to the respiratory tissue's high complexity and cellular heterogeneity, epithelial cells or sheets derived from other autologous districts (i.e., skin or oral mucosa) cannot be used for airway functional recovery (Tan et al., 2017; Udelsman et al., 2018). Second, spontaneous post-surgery reepithelization of the tracheal substitute cannot be taken for granted. In clinical and preclinical studies, several groups relied on the migration of epithelial cells from the recipient's wound edges (Martinod et al., 2017, Martinod et al., 2018; Rehmani et al., 2017), with some successful epithelialization only on short-sized scaffolds (Zhao et al., 2016; Lee et al., 2018; Sakaguchi et al., 2018). Indeed, local epithelial cells can only cover a few millimeteres of the graft, while the central part of the tracheal substitute is often left uncovered, leading to the aforementioned adverse consequences of an absent epithelium (Delaere and Van Raemdonck, 2019). Therefore, a precise and rational reepithelization strategy is mandatory, especially for tracheal substitutes of larger dimensions. On this note, in TE approaches, the culture conditions used for the *in vitro* expansion phase must preserve the proliferative and differentiative potential of the airway epithelial stem cells, preventing their exhaustion. Indeed, the experience gained from regenerative approaches of other human epithelial tissues clearly pointed out that a specific number of stem cells is strictly required to allow the permanent engraftment, renewal, and restoration of the epithelium (Pellegrini et al., 2011; Maurizi et al., 2021). In order to carefully monitor these aspects and have a clear overview of all the process variables, extensive cellular characterization must be performed. This comes through the identification of population-specific molecular markers to be adopted as quality controls during each step of the reconstructive procedure.

To date, there is a huge gap between basic-research knowledge on airway epithelial cells' biology and the application of these insights to TE translational approaches. Indeed, despite the huge work carried out to understand the airway epithelial cells physiology and heterogeneity (Garcıá et al., 2019; Basil et al., 2020; Goldfarbmuren et al., 2020; Zaragosi et al., 2020; Davis and Wypych, 2021), the few groups that clinically employed airway epithelial cells for tracheal TE approaches did not exploit this knowledge, reporting only limited or inadequate cellular characterization (Macchiarini et al., 2008; Elliott et al., 2017).

In order to bridge this gap, preclinical studies—especially those based on *in vitro* models—can be extremely useful to precisely evaluate all the interactions among the different components of the bioengineered construct. Indeed, once in contact with the scaffold, colonizing cells should retain their ability to proliferate and differentiate into their respective specialized cell types, a crucial aspect for re-establishing the system's physiology. Moreover, in the case of TE approaches encompassing cells derived from different tissues (i.e., epithelial cells, chondrocytes, endothelial cells, neural cells etc.), their mutual interaction must be carefully studied to exclude possible acute or chronic cytopathic effects.

Another common issue with all the tracheal reconstructive approaches is the vascularization of the tracheal substitute. Till now, this aspect has deeply hampered the outcomes of treated patients in several clinical studies (Delaere et al., 2012; Delaere et al., 2019). Indeed, efficient and rapid restoration of the blood supply is mandatory to sustain graft survival, allow its integration within the surrounding tissue, avoid necrosis or contamination, and support new cartilage and epithelium regeneration. Some approaches tried to overcome this matter by wrapping tracheal substitutes in a highly vascularized tissue, frequently transposed omentum (Levashov et al., 1993; Elliott et al., 2012; Menna et al., 2021). Although these efforts have been initially interpreted as successful, long-term follow-ups demonstrated that, in most cases, this strategy only temporarily delays the inevitable consequences of wound breakdown at the anastomoses (Delaere and Van Raemdonck, 2014). Alternatively, tracheal substitutes can be *in vivo* vascularized through heterotopic implantation into the recipient's forearm, followed by orthotopic repositioning. Still, such strategies' long-time requirements and invasiveness have strongly limited their application. Once more, a possible solution may arise from *in vitro* or *in vivo* preclinical studies investigating the formation of a vascular network through interaction with endothelial cells or from stimulating the revascularization process through the delivery of pro-angiogenic factors (Lovett et al., 2009).

Alongside vascularization, restoring a functional innervation system within the tracheal substitute should be considered. Indeed, the airway epithelium works conjointly with the immune and nervous systems to guarantee respiratory homeostasis (Davis and Wypych, 2021). So far, this aspect has been neglected, as no significant clinical studies have encompassed the inclusion of this component. Thus, it remains a crucial issue to be addressed in the future.

Finally, another critical point to be mentioned regards the legislative side. Since their proposal in 2001 (Directive 2001/83/EC later expanded in REGULATION (EC) No 1394/2007; https://www.ema.europa.eu/en/human-regulatory/overview/advanced-therapies/legal-framework-advanced-therapies), GMP have further complicated the development of novel tissue-engineered advanced therapeutic medicinal products (ATMPs). These regulations have proven themselves critical to achieving safer and standardized advanced therapies (Aiuti et al., 2017; Hirsch et al., 2017; Ram-Liebig et al., 2017; Barbagli et al., 2018; Pellegrini et al., 2018; Kueckelhaus et al., 2021). Nevertheless, the resulting complexity and high expenses in the manufacturing process strongly discouraged researchers from exploring the TE area of study. These obstacles have led to the predilection of non-TE approaches, which instead fall under less stringent legislation.

To conclude, tracheal reconstruction stands as a huge challenge and has yet to be achieved. In this review, we summarized the main lessons learned from the clinical and most recent *in vivo* preclinical studies, as well as the new frontiers of tracheal TE. We have highlighted the most common issues that still hinders tracheal reconstruction. In our opinion, a strong correlation between basic science and translational medicine is mandatory; careful and extensive preclinical studies are crucial to tackling all the described aspects. Before embarking on new clinical applications, more work on the preclinical side should be done to prevent patients' exposure to avoidable life-threatening consequences. Only by addressing these points reconstructive approaches can become a turning point in

long-circumferential tracheal defects management and affirm themselves as a milestone in regenerative medicine.

AUTHOR CONTRIBUTIONS

DA, GG, VG, FL, and GP contributed to the analysis of literature, writing, correction and to figures design. DA collected and summarized the whole work. GP also provided the final revision. All authors contributed to the article and approved the submitted version.

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Nerve regeneration in transplanted organs and tracer imaging studies: A review

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The technique of organ transplantation is well established and after transplantation the patient might be faced with the problem of nerve regeneration of the transplanted organ. Transplanted organs are innervated by the sympathetic, parasympathetic, and visceral sensory plexuses, but there is a lack of clarity regarding the neural influences on the heart, liver and kidneys and the mechanisms of their innervation. Although there has been considerable recent work exploring the potential mechanisms of nerve regeneration in organ transplantation, there remains much that is unknown about the heterogeneity and individual variability in the reinnervation of organ transplantation. The widespread availability of radioactive nerve tracers has also made a significant contribution to organ transplantation and has helped to investigate nerve recovery after transplantation, as well as providing a direction for future organ transplantation research. In this review we focused on neural tracer imaging techniques in humans and provide some conceptual insights into theories that can effectively support our choice of radionuclide tracers. This also facilitates the development of nuclear medicine techniques and promotes the development of modern medical technologies and computer tools. We described the knowledge of neural regeneration after heart transplantation, liver transplantation and kidney transplantation and apply them to various imaging techniques to quantify the uptake of radionuclide tracers to assess the prognosis of organ transplantation. We noted that the aim of this review is both to provide clinicians and nuclear medicine researchers with theories and insights into nerve regeneration in organ transplantation and to advance imaging techniques and radiotracers as a major step forward in clinical research. Moreover, we aimed to further promote the clinical and research applications of imaging techniques and provide clinicians and research technology developers with the theory and knowledge of the nerve.

KEYWORDS

organ transplantation, nerve tracers, sympathetic nerve, parasympathetic nerve, visceral sensory plexus

Introduction

Human organ transplantation is a very significant development in modern medicine. By removing diseased and necrotic organs and replacing them with healthy and viable ones, patients with life-threatening conditions can be given a second chance at life. As of 2019 epidemiological surveys have shown that more than 100,000 patients worldwide need organ transplants each year (Vanholder et al., 2021). Despite the important role of organ transplantation in extending the life of patients, transplanted organs are exposed to multiple risks in the recipient, including modification of the transplantation technique, immune rejection of the transplanted organ, autonomic innervation after denervation of the transplanted organ, and health monitoring of the transplanted organ (Poole et al., 2019). Current organ transplantation techniques include allogeneic, xenogeneic, and future organ transplantations that target highly differentiated cell and tissue transplantation techniques that can minimize immune rejection in the recipient (Sayegh and Carpenter, 2004; Timsit et al., 2019).

Successful organ transplantation is not only dependent on the histocompatibility between the donor and recipient, but also on the functional recovery of the transplanted organ in the recipient tissue, which is one of the criteria for successful transplantation (Patchell, 1994; Byersdorfer and Turnquist, 2021). Nerve regeneration in organ transplantation is the process by which a patient goes from a completely denervated donor organ to a progressive regeneration of autonomic nerves after organ transplantation (Grupper et al., 2018). The most critical aspect of the nerve regeneration process in transplanted organs is the interaction between the Schwann cells and axons. The process of organs transplantations is a process of immune response (Bolívar et al., 2020). Antigens are present in the Schwann cells of the peripheral nerves of the donor organ, which connect to the nerves of the recipient organ and produce a variety of immune factors, resulting in an immune rejection reaction. It is therefore crucial that sufficient immunosuppressive drugs are given during the organ transplantation process to provide a better environment for nerve regeneration in the organ transplant (Sarker et al., 2018).

Autonomic innervation plays a crucial role in maintaining organ function (Li et al., 2021b) and transplanted organs face transient denervation. By performing peripheral nerve anastomoses during transplantation and giving neurotrophic factor drugs postoperatively, the nerves of the transplanted organ have demonstrated that the repair process can be slow. Several studies have shown that denervated organs have a strong regenerative capacity, and this has led to the widespread use of organ transplantation and saving countless lives (White et al., 2015).

Scientists were able to further observe the neuroanatomical patterns and their normal physiological functions *in vivo* until the advent of conventional tracers in the 1970s, which completely broke this bottleneck and led to a rapid advancement in neuroscience and cognitive science (Kristensson, 1970). Scientists subsequently exploited the physiological properties of rabies virus transmission on nerve axons to develop viral tracers with specificity and high expression (Ugolini, 2010). In conclusion, both traditional neural tracers and viral tracers (Fan et al., 2020; Feng et al., 2020) have been widely used in scientific investigations to reveal neuronal connections between brain regions, neurotransmission between skeletal muscles or visceral organs and the brain, and to further clarify the neural and functional localization of the cerebral cortex (Huang et al., 2022; Li et al., 2021a). With the development of the field of nuclear medicine, radionuclide testing gradually emerged in the 1990s as a clinical application to measure and monitor blood flow, urine, and neurotransmitter expression in patients (Schwaiger et al., 1990). Among other things, radionuclide tracers can be used to assess the functional status of organs and tissues and to determine the progression and prognosis of a patient's disease with the greatest precision and speed in the patient. These are the advantages of radiotracers, such as: ① the simplicity and sensitivity of the means of detection; ② the fact that they are in accordance with the normal physiological conditions of the organism and can be metabolized *in vivo*; and ③ the fact that they can be precisely localized and can provide detection at the molecular and atomic level. We have shown in Table 1 the advantages and disadvantages of conventional tracers, viral tracers, and radioactive tracers. In addition, we have depicted in Figure 1 a timeline for the development of radiotracers.

Currently, with the development of imaging technology, the use of neural tracer techniques to visualize the regenerative processes of autonomic nerves and to label neuronal and neurotransmitter neurotransmission can reveal the sympathetic and parasympathetic reinnervation processes in animals and humans (Bravo et al., 2015; Wang et al., 2018). Positron emission tomography imaging (PET) (Brust et al., 2014), single photon emission computed tomography (SPECT) combined with magnetic resonance (MRI) and computed tomography (CT) (Srinivas et al., 2010), involving radiologically targeted molecules to label the chemical neurotransmitters between synapses, such as ^{11}C-hydroxyephedrine (^{11}C-HED), ^{11}C-epinephrine (Sasano et al., 2008), ^{11}C-phenylephrine (PHEN) (Raffel et al., 1999), 6-^{18}F-fluorodopamine (Goldstein et al., 1993), ^{13}N-ammonia, and flubrobenguane (FBBG) (Zelt et al., 2021) has been found to be useful in presynaptic neuronal transport processes in the cardiovascular system. Our review will demonstrate the use of a radiotracer in the reinnervation of the heart, liver, and kidney after transplantation as shown in Table 2. Numerous studies have illustrated the usefulness of radiotracer applications in probing the neuroanatomy of the cardiovascular system, organ transplantation and the nervous system (Bailey and Willowson, 2013). Therefore, we have summarized the current widespread use of nerve tracers in autonomic disorders in Table 3. We showed the innervation of the heart, liver, and kidney in detail in Figure 2, with sympathetic regeneration of the heart transplant as an example, and we have labelled the commonly used sympathetic tracers in the diagram. The image was created using Biorender.com.

TABLE 1 Describes the advantages and disadvantages of traditional neural tracers, viral tracers, and radioactive tracers currently in use.

Species	Product	Advantages	Disadvantages
Traditional neural tracers	Horseradish peroxidase Cholera toxin B	1 more sensitive	1 Involved in cellular metabolism
	Fluoro-Gold	2 Only fluorescent microscopy and electron microscopy are required for observation	2 Unstable and easily degraded.
	Wheat germ agglutinin		3 Restricted conditions of use
Viral tracers	Adeno-associated viral vector	1 High sensitivity, directionality and selectivity compared to traditional neural tracers	1 High cytotoxicity
	Rabies virus	2 Highly infectious	2 For scientific investigation only
	Herpes simplex virus	3 No attenuation of the signal transmission of neural tracers	
Radioactive tracers	Commonly used radioisotopes are carbon-11, nitrogen-13, oxygen-15, iodine-131 and iodine-135	1 High sensitivity, safe and radiation-free	1 The need for a dedicated laboratory
		2 Simple detection means, suitable for *in vivo* experiments and clinical diagnosis	2 The need for specialist technicians
		3 Non-cytotoxic	3 Protection is sometimes required
		4 Conforms to the normal physiological conditions of the organism and is metabolizable	
		5 More precise localization, widely used for cardiovascular diseases and tissues and organs	

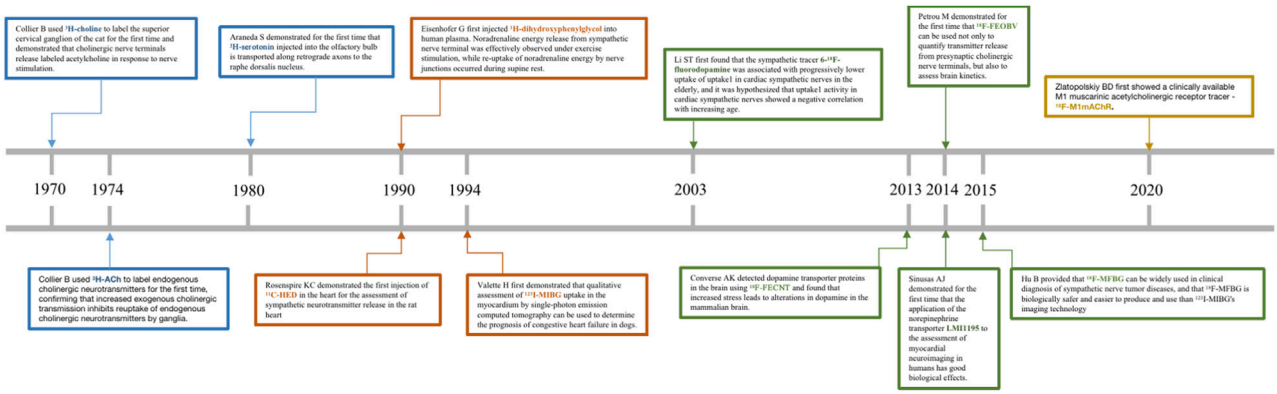

FIGURE 1
Depicts a timeline of the development of radiotracers.

Reinnervation of a transplanted heart

Reinnervation of the sympathetic plexus in the transplanted heart

In 2022, 57-year-old David Bennett became the first patient in history to have a pig heart transplant, but passed away 2 months following the allogeneic cardiac transplantation (Burki, 2022). Back in 1947, Dr Christiaan Barnard set the record for the first in orthotopic heart transplant (Brink and Hassoulas, 2009). The first patient, Louis Washansky, passed away 18 days after the operation, but the second patient, Philip Blaiberg, survived for almost 2 years (Cooper, 2001). We are grateful to the pioneer of heart transplantation, the courageous Dr Christiaan Barnard! While orthotopic heart transplantation is now well established, patients who have undergone cardiac

TABLE 2 The use of clinically available radiotracers in heart and renal transplantation.

Organ transplantation	Nerve tracer	Technology	Nerve reinnervation	Nerve regeneration time	References
Heart transplantation	^{11}C-hydroxyephedrine (^{11}C-HED)	PET/CT	Sympathetic nerve	1 year	Schwaiblmair et al. (1999); Bengel et al. (2001)
Heart transplantation	^{11}C-hydroxyephedrine (^{11}C-HED)	PET/CT	Sympathetic nerve	5 years	Uberfuhr et al. (2000)
Heart transplantation	Iodine-123-meta-iodobenzylguanidine (^{123}I-mIBG)	SPECT	Sympathetic nerve	2 years	Estorch et al. (1999)
Renal transplantation	Iodine-123-meta-iodobenzylguanidine (^{123}I-mIBG)	SPECT	Sympathetic nerve	6 months	Rasmussen et al. (2020)

In clinical studies, Iodine-123-meta-iodobenzylguanidine (^{123}I-mIBG) and ^{11}C-hydroxyephedrine(^{11}C-HED) are the most widely used radionuclide tracers for sympathetic nerve regeneration in cardiac transplants. ^{123}I-mIBG, has also shown significant tracer effects in sympathetic nerve regeneration in renal transplants. We found that cardiac sympathetic reinnervation was observed as early as 1 year after heart transplantation and renal sympathetic reinnervation 6 months after renal transplantation.

TABLE 3 The targeted molecular tracers currently used clinically in autonomic nervous system diseases.

Application	Nerve tracer	Technology	Target	References
Cardiomyopathy	4-^{18}F-fluoro-meta-hydroxyphenethylguanidine (^{18}F-4F-MHPG), 3-^{18}F-fluoro-para-hydroxyphenethylguanidine (^{18}F-3F-PHPG)	PET/CT	Sympathetic nerve	Raffel et al. (2018); Raffel et al. (2022)
Myocardial infarction	^{13}N-ammonia,^{11}C-epinephrine	PET	Sympathetic nerve	Sasano et al. (2008)
Ischemic cardiomyopathy	Flubrobenguane (FBBG)	PET	Sympathetic nerve	Zelt et al. (2021)
Heart failure	*N*-[3-bromo-4-(3-^{18}F-fluoro-propoxy)-benzyl]-guanidine (LMI1195)	PET	Sympathetic nerve	Sinusas et al. (2014); Higuchi et al. (2015); Chen et al. (2018)
Vasospastic angina	Iodine-123-meta-iodobenzylguanidine (^{123}I-mIBG),^{123}I-15-(*p*-iodophenyl)-3-*R*,*S*-menthyl pentadecanoic acid (BMIPP)	PET	Sympathetic nerve	Watanabe et al. (2002)
Alzheimer's disease	(-)-^{18}F-fluoroethoxybenzovesamicol (^{18}F-FEOBV)	PET	Presynaptic cholinergic	Mulholland et al. (1998); Petrou et al. (2014)
Hepatocellular carcinoma	^{14}C-Cho	PET	Choline	Kuang et al. (2010)
Prostate carcinoma, Parkinson's disease	^{11}C-donepezil	PET	Parasympathetic nerve	Gjerløff et al. (2015), Nielsen et al. (2019)

transplantation continue to face a variety of post-operative complications and immune rejection reactions, the causes and mechanism of which deserve to be adequately explored.

Heart transplantation is the treatment of choice for patients with end-stage heart failure (Mehra, 2017). In both allogeneic and orthotopic heart transplantation, the donor heart faces denervation and is vulnerable to a variety of cardiovascular events in the absence of central innervation, such as arrhythmias, abnormal chest pain, sudden atrial fibrillation, sudden cardiac death, and stroke (Joglar et al., 2021; Firoz et al., 2022). With the popularity of the heart transplantation approach and extensive research, it has been reported that the sympathetic nerves of the heart gradually regenerate over time, restoring sympathetic innervation to the heart and participating in the rhythm regulation of the heart and the perfusion of the heart muscle (Jakus et al., 2022). The state of cardiac transplant sympathetic reinnervation varies from one heart transplant patient to another, with some heterogeneity in various regions of the heart (Wilson et al., 1991). The phenomenon of reinnervation after heart transplantation is therefore an innovative point of clinical research!

With the technical support of medical imaging, nerve tracers are used to label the major transmitters of the post-sympathetic adrenergic nerves and thus determine the integrity of the sympathetic nerves (Pandit-Taskar and Modak, 2017). ^{11}C-HED functions as a catecholamine analogue with a neural tracer effect and is used in combination with a presynaptic norepinephrine transporter (NET, uptake-1) to assess the activity of presynaptic sympathetic neuronal transport in the myocardium (Zelt et al., 2021). When assessing the sympathetic integrity of the heart 1 year after heart transplantation using PET, 55% of patients (16) presented with ^{11}C-HED retention in the left anterior descending branch at a rate of 47%. Subsequently, after exercise stress stimulation of cardiac sympathetic fibers, patients

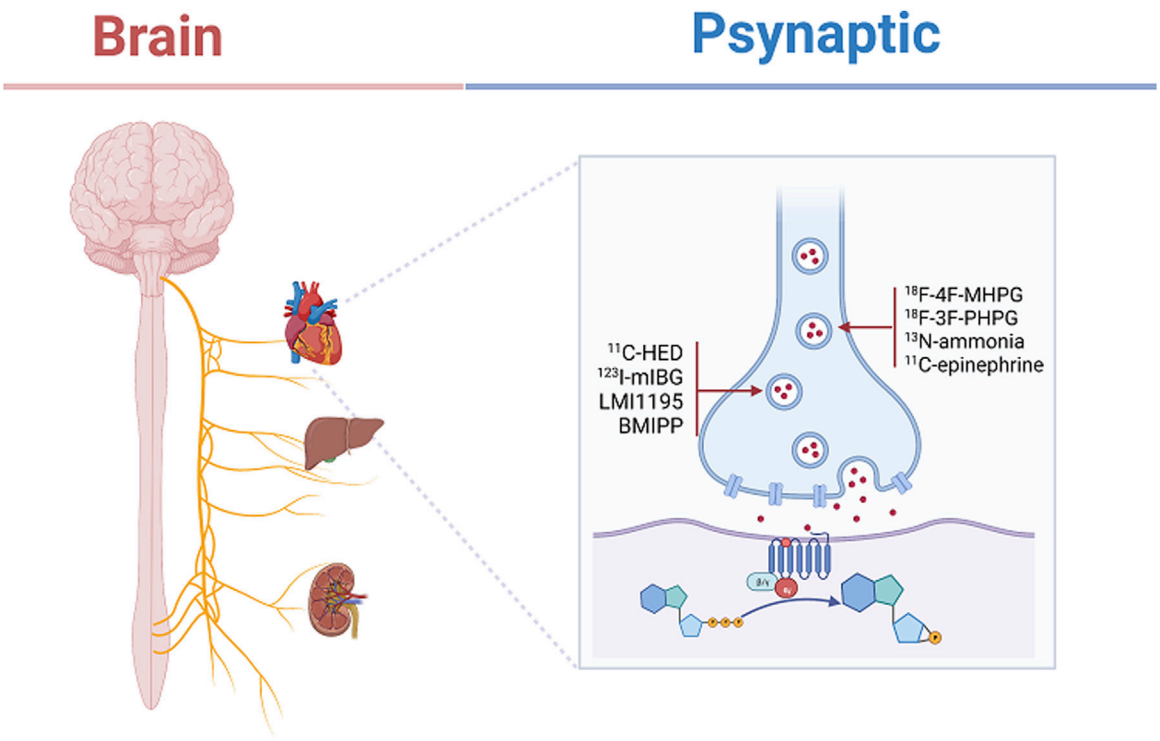

FIGURE 2
Depicts the present state of sympathetic innervation of transplanted hearts. Labeling of commonly used radioactive neurotransmitters in the presynaptic membrane, such as ^{11}C-hydroxyephedrine (^{11}C-HED), Iodine-123-meta-iodobenzylguanidine (^{123}I-mIBG), *N*-[3-bromo-4-(3-^{18}F-fluoro-propoxy)-benzyl]-guanidine (LMI1195), ^{123}I-15-(*p*-iodophenyl)-3-*R,S*-menthyl pentadecanoic acid (BMIPP), 4-^{18}F-fluoro-meta-hydroxyphenethylguanidine (^{18}F-4F-MHPG), 3-^{18}F-fluoro-para-hydroxyphenethylguanidine (^{18}F-3F-PHPG), ^{13}N-ammonia, ^{11}C-epinephrine. This figure was created using the biorender application (Biorender.com).

with cardiac reinnervation present exhibited an 8% increase in left ventricular ejection fraction (LVEF) compared to denervated patients, with the greatest density of reinnervated sympathetic nerves found particularly in the left anterior interventricular region (Bengel et al., 2001). Thus, reuptake of catecholamine analogues by sympathetic nerve endings in the anterior interstitial region of the heart could provide strong evidence of sympathetic reinnervation of the patient's heart.

Cardiac autonomic nerves are involved in the neuromodulation of the sinus node and heart rate variability (HRV) is a widely used non-invasive measure of the cardiac sympathetic and parasympathetic activity and can be used as a tool to assess the recovery of the autonomic nervous system after heart transplantation (Ernst, 2017). In one study, which assessed cardiopulmonary reinnervation in patients after heart transplantation between 2.5 and 12 months, it was observed that low-frequency (LF) fluctuations in HRV of heart transplant patients in the supine position up to 5.7 ms^2 were observed as early as 6 months and was up to 6.0 ms^2 when LF was tested again 1 year later, showing a significant increase in LF variability with sympathetic nerve regeneration (Christensen et al., 2021a). A positive correlation between exercise capacity and peak oxygen uptake and sympathetic reinnervation was observed in patients, with myocardial ^{11}C-HED uptake being twice as high in the reinnervated group compared to the denervated group (Schwaiblmair et al., 1999).

Further labelling of sympathetic nerves using catecholamine analogue tracers revealed a positive correlation between the degree of presynaptic retention of ^{11}C-HED in sympathetic nerves and the HRV produced by sinus node innervation. At 5 years' follow-up of post-heart transplant patients, 58% (22) of patients had a mean ^{11}C-HED retention rate of 10.7% in the left ventricle, while LF in patients with sinus node reinnervation in HRV was up to 5.9 ms^2. This indicates that sympathetic regeneration is a continuous process, with an increase in HRV observed as early as 3.5 months, while 5 years later patients showed a wide distribution of sympathetic reinnervation in the anterior and some lateral regions of the left ventricle (Uberfuhr et al., 2000).

Iodine-123-meta-iodobenzylguanidine (^{123}I-mIBG), a sympathetic blocker guanethidine (Zhang et al., 2014), is a pseudo neurotransmitter analogue released from the presynaptic membrane of sympathetic nerves, bound to uptake-1 at presynaptic terminals labeled and stored in vesicles, and used

to assess presynaptic sympathetic neuronal vesicle storage activity (Mabuchi et al., 2005). In a study, the heart to mediastinal rate ratio (H/M) and the elution rate of the heart were determined using SPECT assessment 4 h after injecting ^{123}I-mIBG into the body (Dilsizian and Chandrashekhar, 2022). In patients with allogeneic heart transplants, increased ^{123}I-mIBG uptake by cardiac sympathetic nerves occurred in 80% of patients 10 years after heart transplantation compared to patients 2 years after heart transplantation. Of these, sympathetic reinnervation occurred as early as 2 years after allogeneic heart transplantation, at which time cardiac sympathetic reinnervation of nerve fibers to ^{123}I-mIBG uptake can be as high as 18% (Estorch et al., 1999). Another study followed up patients after ectopic heart transplantation and found that ^{123}I-mIBG scintigraphy remained absent 6 years after ectopic heart transplantation under the detection of SPECT (Yap et al., 2006). It is hypothesized that non-anatomical heart transplantation may result in limited regeneration of cardiac sympathetic fibers. However, Jenkins et al. found that ^{123}I-mIBG scintigraphy assessed sympathetic regeneration in patients and that sympathetic reinnervation did not correlate with circadian blood pressure regulation in patients. This suggests that ^{123}I-mIBG scintigraphy only confirms the presence of nerve regeneration after cardiac transplantation and that cardiac sympathetic function had not been fully restored (Jenkins et al., 1997).

A recent study reported that quantitative tracers of cardiac sympathetic nerves, 4-^{18}F-fluoro-meta-hydroxyphenethylguanidine (^{18}F-4F-MHPG) and 3-^{18}F-fluoro-para-hydroxyphenethylguanidine (^{18}F-3F-PHPG), can be used to quantify nerve density in denervated regions of patients with ischemic myocardial infraction. It is thus, reasonable to speculate their future application in the exploration of full neurological recovery in patients after heart transplantation (Raffel et al., 2022).

Reinnervation of the parasympathetic plexus in the transplanted heart

In 1995 researchers focused their attention on parasympathetic imaging techniques, using the stimulating effect of meglumine diatrizoate contrast agents on cardiopulmonary chemoreceptors. The heart rate was seen to decrease in heart transplant patients after chemical tracer infusion, which could be inferred from the intact and regenerative parasympathetic innervation of the patients. This study revealed that 6 years after heart transplantation, patients did not only have the parasympathetic reinnervation that the investigators expected, but the rate of residual recipient sinus nodes (RSN) in heart transplant patients showed an increasing trend. This probably occurred without remodeling of the parasympathetic efferent nerves after surgery (Arrowood et al., 1995). The researchers then injected norepinephrine into heart transplant patients to stimulate their pressure receptors to observe changes in cardiac vagal tone, and 4 years after transplantation the patients still did not show increased vagal tone (Arrowood et al., 1997).

Although the results of earlier clinical studies have been unsatisfactory, the latest findings show that cardiopulmonary receptors are reinnervated 1 year after heart transplantation. When Wyller et al. evaluated the autonomic activity of the heart, they found that patients showed an increase in LF in the supine position to 5.7 ms^2 as early as 6 months after transplantation and a trend towards a decrease in heart rate (HR) at rest 12 months after transplantation. During the 20° head-up tilt test the patient showed a decrease in right atrial pressure and a decrease in cardiac output as HR increased. These findings support the idea that cardiopulmonary receptors are subject to cardiac parasympathetic reinnervation 1 year after transplantation (Wyller et al., 2021).

In physiological studies, autonomic transection of the heart transplant leads to vagal denervation of the heart, manifested by the resting tachycardia and variable hourly cardiac malfunction in patients after heart transplantation (Levy et al., 1981). In one study that followed up cardiopulmonary exercise tests in heart transplant patients, cardiac sinus node parasympathetic regeneration could be observed in year two. There was an increase of up to 6.0 ms^2 in high-frequency (HF) fluctuations in the HRV index, and an LF/HF radio of up to 84% plus an increase in tachycardia response during Valsalva exercise in patients in the supine position. It is thus, evident that parasympathetic reinnervation begins to gradually strengthen 2 years after heart transplantation (Christensen et al., 2021b).

With the development of neural tracers, chemical tracers acting on cardiopulmonary receptors are no longer sought singularly but are combined with PET techniques to observe the release of acetylcholine (ACh) transmitters following parasympathetic activation (Khajehali et al., 2018). However, unlike neural tracers of catecholamine analogues, Ach is subject to specific degradation by acetylcholinesterase (AChE), which results in more challenging imaging techniques for observing cardiac parasympathetic nerves. As a result, non-invasive HRV testing is predominantly used clinically for cardiac parasympathetic reinnervation. (-)-^{18}F-fluoroethoxybenzovesamicol (^{18}F-FEOBV) binds to acetylcholine transporter proteins and is prominently labelled at cholinergic nerve endings in the heart. Although ^{18}F-FEOBV has not been explored to date for postoperative parasympathetic regeneration in cardiac transplant patients, it is expected to be a tool for future use in cholinergic radiological studies (Petrou et al., 2014).

Reinnervation of the sensory plexus in the transplanted heart

As a result of heart transplantation, the heart is denervated by sensory fibers. Once a heart transplant recipient develops

ischemic angina, the patient's presentation is highly atypical, making the diagnosis more difficult for the clinician (DeFilippis et al., 2017). Statistics suggest that during the 5-year period after heart transplantation, post-transplant cardiac allograft vasculopathy (CAV) was present in 50% and coronary arteriosclerosis in 10% of patients (García-Baizán et al., 2021).

Research has also shown that heart transplant patients present with pain in the anterior thoracic region after 3 years and stenosis occlusion of the coronary arteries is seen on the coronary computed tomography angiography (CTA). The patient with heterogeneity of coronary stenosis, which occurs mainly in the left anterior descending coronary artery results in complete obstruction of the right coronary vessel. At the same time, post-transplant in the anterior thoracic region has been associated with sensory nerve regeneration in some cases and not in others, and this regeneration often has a negative effect, hence, modulating and reconstructing nerve regeneration in the transplanted heart has great potential for clinical research (Stark et al., 1991)!

Reinnervation of a transplanted liver

Reinnervation of the sympathetic plexus in the transplanted liver

Liver transplantation is one of the treatments for chronic liver failure and hepatocellular carcinoma (HCC) (Tan et al., 2022). The liver has a unique ability to regenerate and the donor liver can return to normal liver morphology after 2 months, but research is still needed on the pattern and mechanism of regeneration and innervation of the liver (Haga et al., 2008). The loss of autonomic innervation of the transplanted liver and the progressive decrease in catecholamine release from the hepatic denervation leads to an increase in hepatic blood flow (HBF) through a reduction in action alpha-adrenergic receptors. However, the change in HBF is not significant and therefore the restoration of HBF cannot be used as a decisive indicator of hepatic nerve regeneration (Kurosawa et al., 2002).

As the liver is denervated, the autonomic nervous system gets out of balance with respect to hepatic glucose uptake, and the net hepatic glucose uptake (NHGU) is no longer inhibited by sympathetic nerves. This process can result in an imbalance between the liver's food uptake function and the body's postprandial glucose regulation, hence the manifestation of the metabolic syndrome in liver transplant patients (Myers et al., 1991; Moore et al., 2012).

Clinical studies have reported that during the 3–5 years following liver transplantation, 50% of patients are susceptible to metabolic syndrome (MetS), obesity and type 2 diabetes as a result of immunosuppression and liver denervation (Becchetti et al., 2020). Thus, monitoring the computed tomography attenuation values (CT-AV) of liver transplant patients 6 months after surgery revealed that patients with values of CT-AV below 60% in the 1-week postoperative group showed signs of impaired liver function at 6 months after surgery due to steatosis or ballooning of the transplanted liver. It was therefore, hypothesized that CT-AV quantification at 1 week following liver transplantation could be used to assess the prognosis and regeneration of liver transplants (Iida et al., 2005). Liver transplant reinnervation is thus, an issue of scientific interest, both from the perspective of exploring the mechanisms of liver transplant nerve regeneration and improving the complications following liver transplant denervation.

Kjae et al. studied sympathetic nerve regeneration after liver transplantation and established two liver groups: a liver transplantation group (n = 13, <30 months after transplantation) and a normal control group (n = 11, normal individuals without liver transplantation). When both groups were subjected to liver biopsy for catecholamine levels, the norepinephrine concentration in the liver transplantation group was found to be only 0.022 nmol/g, and the levels of catecholamines 99% lower compared to normal controls, showing that sympathetic regeneration of liver sympathetic nerves was still not present 2–3 years after live denervation (Kjaer et al., 1994).

In rodent studies, direct observation of liver sections from 3 to 6 months after liver transplantation in rats using immunohistochemistry revealed a positive trend for growth-associated protein 43 (GAP-43) as a marker of neuronal plasticity over time. However, nerve regeneration in the liver portal vein occurred only between 5 days and 3 months after liver transplantation and had ceased in the liver by 6 months. Meanwhile, the ubiquitin hydrolase protein gene product 9.5 (PGP9.5) expression in nerve axons had returned to normal levels (Kandilis et al., 2014).

The results observed from rodents demonstrate that nerve regeneration in the liver is completed at 3–6 months, and in the available human studies reported, some investigators used the same immunohistochemical technique to determine the regeneration of liver nerves in humans by direct observation of nerve regeneration (Boon et al., 1992). Fifteen months after liver transplantation, immunostaining for PGP9.5 was found to be positive on liver sections from transplanted patients, but the expression of nerve regeneration appeared restricted, with positive expression only at portal nerve fibers (Dhillon et al., 1992).

Reinnervation of the parasympathetic plexus in the transplanted liver

Several studies have confirmed that regeneration of Schwann cells wraps around axons after peripheral nerve injury (PNI) and that vagus nerve regeneration is similarly repaired and remyelinated by Schwann cells distal to axons (Clements

et al., 2017). Animal studies have revealed that Netrin-1, a laminar adhesion-associated protein expressed in Schwann cells and axons of motor and sensory neurons, targets and activates the Netrin-1 signaling pathway in Schwann cells to promote the regeneration function of peripheral nerve cells after PNI(Taïb et al., 2022). Wang et al. demonstrated that exogenous supplementation of Netrin-1 in mice after liver transplantation accelerated regeneration of the hepatic vagus nerve. Netrin-1 expression was significantly reduced in the liver tissue of mice after liver resection ($p < 0.05$). Subsequently, exogenous supplementation of Netrin-1 was administered in the tail vein of mice and positive expression of GAP-43 was observed in the liver tissue 1 week after surgery, as evidenced by the nerve regeneration in the liver 1 week after Netrin-1 administration. Subsequent detection of positive anti-choline acetyltransferase (ChAT) in the liver tissue further confirmed that Netrin-1 promotes vagal nerve regeneration in the liver (Wang et al., 2022). It is certainly a revelation to us that the research and development of exogenous targeted neurological drugs for post-liver transplant patients point to pharmaceutical innovation that will help in the rehabilitation of liver transplant patients with neurological regeneration.

It is well known that the central nervous system plays an important physiological regulatory role on the autonomic nerves of the liver (Liu et al., 2021). Neuro-humoral regulation of the liver is mediated through sympathetic afferents to the central nervous system, with projections in the lateral hypothalamus (LH) and ventral medial hypothalamus (VMH) to the dorsal motor nucleus of the vagus (DMV) and subsequent projections to primary neurons involved in hepatic vagal innervation (Berthoud and Neuhuber, 2019). An experiment involving rats showed that partial hepatectomy with concomitant destruction of VMH in a rat's brain resulted in an increase in DNA synthesis in the liver. This probably led to compensatory stimulation of hepatocyte regeneration by the hepatic vagus nerve as a result of disruption of the central nervous system (Kiba et al., 1994). The hepatic vagal branch has an independent innervation function on DNA synthesis in hepatocytes, and once liver denervation has occurred, this results in a delayed effect on DNA synthesis in hepatocytes (Kato and Shimazu, 1983; Kiba et al., 1995). David et al. observed that the hepatic vagus nerve secretes ACh, which acts on muscarinic acetylcholine receptor 3 (mAChR3) in hepatic progenitor cells (HPCs) to promote hepatocyte regeneration. Rats with hepatic vagal branches removed showed impaired proliferation of hepatocytes and bile duct epithelial cells (Cassiman et al., 2002). It is clear that hepatic parasympathetic denervation leads to a decrease in the rate of DNA synthesis in hepatocytes and thus interferes with hepatic regeneration. The rate of DNA synthesis in hepatocytes is reduced by parasympathetic denervation of the liver, thereby interfering with liver regeneration.

In the cytoplasm of nerve cells, presynaptic ChAT acts to synthesize ACh from choline (Cho) and acetyl coenzyme A (CoA), which binds to muscarinic receptors and participates in cholinergic signalling (Sarter and Parikh, 2005). The use of the radiotracer ^{14}C-Cho to label the synthesis of hepatocyte membranes and the process of acetylcholine transmitter synthesis has been found, and it is hypothesized that ^{14}C-Cho could be used as a diagnostic for new-onset HCC and recurrence after liver transplantation (Kuang et al., 2010). In contrast to neural regeneration in heart transplantation, patients who have undergone liver transplantation for HCC are at a risk of HCC recurrence over a 5-year period (Agopian et al., 2015). Despite the temporary lack of studies on hepatic vagal reinnervation, it is suggested that studies using nerve tracers to monitor cancer recurrence in patients after transplantation are more clinically relevant and have a highly complementary diagnostic role for prognosis after liver transplantation (Kim et al., 2020).

Reinnervation of a transplanted kidney

Reinnervation of the efferent nerves in the transplanted kidney

According to literature, as of November 2021, surgeons have transplanted alpha-gal knockout porcine kidneys into brain-dead patients. The results indicate that after 54 h of *in vivo* renal filtration function of the allogeneic kidneys, the patients had yet to experience significant immune rejection, showing the great strides being made today with allogeneic kidney retransplantation (Cooper and Hara, 2021; Dolgin, 2021).

Kidney transplantation is the most effective treatment for end-stage renal disease (ESRD) and is effective in improving the survival of patients with renal failure, with an estimated survival of 19.2 years for kidney transplant patients (Poggio et al., 2021). In anatomical studies, the kidney is innervated by the sympathetic ventral plexus emanating from T12-L2 spine cord, and the nerves of the kidney are involved in innervating water and sodium metabolism and fluid regulation in the kidney (Sakakura et al., 2014). Interruption of renal sensory afferent and sympathetic efferent nerves after renal transplantation leads to inactivation of sympathetic efferent nerve effectors, which in turn activate the renal-renal reflex and inhibit the renin-angiotensin-aldosterone system. This process implies the development of reduced renin secretion, diuresis and pro-sodium excretion, and the lowering of blood pressure (Frame et al., 2016).

In animal experiments, renal denervation was shown to reduce blood pressure in rats with nephrogenic hypertension. Compared with rats with hypertension caused by renal artery ligation, renal denervation restored normal plasma renin secretion, reduced arterial blood pressure by 44 ± 3 mmHg and reduced HR by 33 ± 9 to 61 ± 9 beats per minute, showing that renal denervation improved the symptoms of nephrogenic hypertension (Oliveira et al., 1992). One study

used myocardial uptake of ^{123}I-mIBG to measure autonomic recovery after renal transplantation. They reported a reduced elution rate of ^{123}I-mIBG for cardiac sympathetic nerves assessed 3 months after renal transplantation ($p < 0.05$) and no differential change in the assessment of HRV in patients. It is proposed that sympathetic overexpression due to ESRD was reduced after renal transplantation and that the ^{123}I-mIBG uptake rate could be used as a specific indicator of renal denervation (Kurata et al., 2004).

Given the involvement of renal sympathetic over-activation in the pathophysiology of hypertension, the implementation of renal sympathetic denervation (RDN) is suggested to be an effective treatment for intractable hypertension (Mahfoud et al., 2022; Sarathy and Salman, 2022). In the Global SYMPLICITY Registry project, the Symplicity flex catheter was used to examine ambulatory blood pressure changes in 3,000 patients 3 years after RDN. The results showed a decrease in systolic blood pressure (SBP) starting 6 months after RDN (−11.7 ± 28.6 mmHg, $p < 0.001$) which continued to decrease (−16.6 ± 28.6 mmHg, $p < 0.001$) (Mahfoud et al., 2019). However, experiments in animals have revealed a regeneration of renal nerves after RDN. Findings at 5.5 months after RDN in sheep, showed positive tyrosine hydroxylase (TH) staining in the perirenal tissue, while renal norepinephrine levels were detected reaching 88.9% and 131.0% after 11 months of RDN. These results suggest that nerve regeneration in the perirenal artery 5.5 months after RDN and innervation could return to normal levels after 11 months. But this study is limited to sheep with ductal RDN and may serve as a partial reference for renal denervation in humans (Booth et al., 2015).

In a pathological study, Gazdar et al. used immunohistochemistry to detect renal nerve fibers by observing patients from 5 to 3,012 days after renal transplantation. Positive Bodian staining of regeneration axons were observed as early as 28 days after renal transplantation, and substantial nerve regeneration around the axons after 8 months (Gazdar and Dammin, 1970). Similarly, Rabelink et al. studied renal sympathetic regeneration in allogeneic transplanted kidneys 2 months later, and compared a healthy control group with a renal transplant group for head-out water immersion (HOWI). They found a marked increase in urinary sodium and potassium concentrations in renal transplant patients. It is speculated that 2 months after renal transplantation the kidney is still denervated and that the reduction in renal vascular resistance led to transplant patients exhibiting diuresis and natriuresis (Rabelink et al., 1993).

Mauriello et al. followed up patients for 5 months to 11 years after renal transplantation and found that nerve regeneration could be observed in patients at 5 months of transplantation, with positive staining for the neuronal regeneration and GAP-43 seen in the renal arteries. In contrast, 10 years after transplantation in patients with concomitant hypertension, TH was detected in renal tissue positively labelled in sympathetic nerve fibers. It is hypothesized that the patients experienced regeneration of renal sympathetic nerves in response to hypertension stimulation (Mauriello et al., 2017). Nevertheless, hypertension after renal transplantation is not associated with regenerative innervation of the renal arteries. Grisk et al. reported a remarkable increase in mean arteria pressure 3 weeks after renal transplantation, but TH mRNA levels in sympathetic nerve fibers were not yet significantly altered. This demonstrates that sympathetic nerves were not yet regenerated 3 weeks after renal transplantation and that post-transplant hypertension was not neurogenic (Grisk et al., 2000). In addition, Rasmussen et al. observed a 30% reduction in ^{123}I-mIBG uptake by the transplanted kidney 1 month later compared to ^{123}I-mIBG uptake by the donor kidney within 4 h preoperatively ($p < 0.005$). This illustrates a positive correlation between recovery of renal function in the transplanted kidney and renal reinnervation (Rasmussen et al., 2020).

Reinnervation of the afferent nerves in the transplanted kidney

It has been reported that renal sensory afferent nerve cells originate from the dorsal root ganglion (DRG) of the ipsilateral T10-L2, mostly located in the cortical spinal cord area and the renal pelvic wall (Marfurt and Echtenkamp, 1991; Ma et al., 2002). Increased ureteral pressure stimulates pressure receptors and chemoreceptors in the kidney and increases the release of calcitonin gene-related peptide (CGRP) and substance P (SP) in the renal pelvis tissue. This activates the afferent nerve in the ipsilateral kidney and the renal-renal reflex in the contralateral kidney for contralateral urination and natriuretic response (Kopp and Smith, 1989). As the sensory afferent nerves of the kidney are involved in the neuromodulation of renal chemoreceptors, mechanoreceptors and nociceptive receptors, once renal denervation has occurred, renal denervation of afferent nerves leads to impaired renorenal reflexes in the kidney (DiBona and Kopp, 1997; Barry and Johns, 2015). Based on the importance of renal afferent nerves in physiological studies, it is worth exploring the reinnervation of renal afferent nerves.

Rodionova et al. investigated the regeneration process of unilateral renal denervation by measuring the levels of the neural markers TH and CGRP in rat kidney tissue sections. Compared to 1 week after denervation, rats exhibited a marked increase in CGRP and TH levels 3 months after surgery, which showed some degree of regeneration of afferent and efferent nerves in the kidney after 3 months. Meanwhile, the fluorescent tracer 1,1′-dioctadecyl-3,3,3′,3′tetrameylindocarbocyanine methanesulfonate (Dil) was injected into the ipsilateral DRG cells of the kidney. The results confirmed that ipsilateral renal denervated afferent nerve regeneration is not positively correlated with the innervation of the contralateral afferent nerve (Ai et al., 2007; Rodionova et al., 2016). The same study, which confirmed denervation in sheep, found that CGRP levels in renal tissues returned to normal 11 months after

RDN (Booth et al., 2015). Interestingly, there is species heterogeneity in the reinnervation of renal afferent nerves (Mulder et al., 2013; Booth et al., 2015). While anatomical and biochemical studies currently provide support for renal afferent sensory reinnervation, no definitive human studies have been reported and further clinical trials are needed to address these questions.

Conclusion

Transplanted organs face reinnervation of autonomic nerves. This review on neural regeneration in organ transplantation analyses the use of molecularly targeted imaging techniques and immunohistochemical methods that probe the autonomic nervous system of parenchymal organs such as the heart, liver, and kidney, further supporting the reinnervation of those autonomic nerves in transplanted organs.

In our review of the latest radiotracers for neural regeneration in organ transplantation, we found that the development of radiotracers for sympathetic catecholamines and catecholamine analogues is currently dominated by the development of acetylcholinergic tracers. In addition, the development and application of acetylcholinergic tracers cause a chemical reaction that make the molecules susceptible to decomposition and instability. This could be the reason why the current exploration of neural tracers for parasympathetic nerves is still in the early stages. Despite this negative impact, the use of radiotracers in neuroscience has greatly improved our neurological understanding of the central and peripheral nervous systems. In an environment where diagnostic molecular tracers are showing a spurt in development as the means of imaging technology continue to evolve, it is on the basis of the unique advantages of radioactive tracers themselves in accurately identifying nerve reinnervation that we need to be more refined and quantified in organ transplantation research. Here we find out that the *N*-[3-bromo-4-(3-^{18}F-fluoro-propoxy)-benzyl]-guanidine (LMI1195) tracer is expected to be one of the most promising sympathetic tracers, and existing studies have successfully assessed the heterogeneity of cardiac innervation in patients by quantifying the myocardial uptake of norepinephrine transporter protein in specific regions (Sinusas et al., 2014; Higuchi et al., 2015). Another novel tracer, ^{11}C-GMO, has an excellent kinetic uptake rate and has been shown to have a half-life of 217 h in myocardial sympathetic neurons of rats, 100 times the half-life of ^{123}I-mIBG. It is the stable and prolonged metabolism of ^{11}C-GMO that can be used to finely measure early cardiac sympathetic reinnervation processes (Raffel et al., 2013). We therefore venture to hypothesize that future studies could focus on quantifying the density of regenerating nerves in transplanted organs, which could help predict the prognosis and diagnostic assessment of organ transplant patients. In conclusion, our review elucidates the reinnervation of heart, liver, and kidney transplants with the support of imaging techniques, providing clinicians with diagnostic tools and guidelines in the field of nerve regeneration.

Author contributions

All authors contributed to the manuscript presented methodology, conceptualization, data analysis, and manuscript writing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Regenerative medicine technologies applied to transplant medicine. An update

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Regenerative medicine (RM) is changing how we think and practice transplant medicine. In regenerative medicine, the aim is to develop and employ methods to regenerate, restore or replace damaged/diseased tissues or organs. Regenerative medicine investigates using tools such as novel technologies or techniques, extracellular vesicles, cell-based therapies, and tissue-engineered constructs to design effective patient-specific treatments. This review illustrates current advancements in regenerative medicine that may pertain to transplant medicine. We highlight progress made and various tools designed and employed specifically for each tissue or organ, such as the kidney, heart, liver, lung, vasculature, gastrointestinal tract, and pancreas. By combing both fields of transplant and regenerative medicine, we can harbor a successful collaboration that would be beneficial and efficacious for the repair and design of *de novo* engineered whole organs for transplantations.

KEYWORDS

regenerative medicine, transplant medicine, cell therapeutics, organ regeneration, tissue engineering

Background

In 2011, Transplant International published the first manuscript illustrating how regenerative medicine technologies will impact transplant medicine (Orlando et al., 2011). In the years that followed, the manuscript ranked among the ten most downloaded papers from the website of that journal as a testament to the special interest that regenerative medicine generates in the transplant community. A decade later, regenerative medicine has progressed significantly while hurdles that were unknown at that time have been unveiled. At the same time, the transplant community has started investing significantly in regenerative medicine and has undertaken many initiatives to bridge the two fields. For example, transplant conferences are granting more and more visibility to regenerative medicine topics and are allocating relevant space to regenerative medicine-oriented sessions. In 2016, the International Pancreas and Islet Transplant Association (IPITA) launched in collaboration with the Juvenile Diabetes Research Foundation (JDRF) and the Harvard Stem Cell Institute. This is a one-of-a-kind conference fully dedicated to the application of stem cell technologies to beta-cell replacement; in 2020, this conference series celebrated its third edition despite the COVID pandemic. In January 2021, the American Society of Transplantation (AST) signed a letter of collaboration with the Tissue Engineering and Regenerative Medicine International Society (TERMIS) with the intent to–as explained in the AST website–bringing "*together experts from both fields on the same stage for the first time, in order to share knowledge and ultimately foster progress in organ bioengineering, regeneration and repair which will shape and define the future of both worlds*" (https://www.myast.org/meetings/ast-termis-webinar-joining-forces-shape-our-mutual-future). What has stemmed so far from this collaboration is a new webinar series featuring speakers from both worlds and whose sessions are available on YouTube (the link https://www.youtube.com/watch?v=Qz21se2VSbs&t=212s relates to the very first edition of the webinar series). On the editorial front, transplant journals are publishing more and more regenerative medicine manuscripts. Transplant societies are establishing committees focusing on regenerative medicine-related topics like cell therapy or organ bioengineering. Some examples: In 2014, AST launched the Transplant Regenerative Medicine Community of Practice. The Cell Transplantation Society rebranded its name as the Cell Transplant and Regenerative Medicine Society. At the same time, while the European Society of Organ Transplantation (ESOT) established the European Cell Therapy and Organ Regeneration Section (ECTORS) in 2019.

This manuscript aims at updating the transplant audience on the recent advances in regenerative medicine that may be pertinent to transplant medicine. These advances will be presented separately by organs.

Kidney

Stem cells and their bioproducts for kidney transplant

Mesenchymal stromal cells (MSC) have long been of interest to the kidney transplantation world mainly for their immunomodulatory properties (Podesta et al., 2020). However, aside from their ability to modulate the host immune response, these cells also possess remarkable regenerative, reparative, and angiogenic properties. Their potential medical utility continues to be investigated in about one thousand clinical trials (Pittenger et al., 2019). Initially, MSCs were proposed for cellular therapy, but recently superimposable beneficial effects have been reported using MSC-derived extracellular vesicles (EVs) (Bruno et al., 2019; Correa et al., 2021). These vesicles are nano sized vehicles containing a specific active cargo able to reprogram target cells.

MSCs and their derivatives may have a role in kidney transplantation at mitigating ischemia-reperfusion injury deriving from the stress and tissue damage related to the chain of events donor death>>>procurement surgery>>>organ storage>>>implantation. Strong data demonstrating that MSC may enhance adaptive repair in ischemically damaged human kidneys was provided by Brasile et al. (2019) in an *ex vivo* model of DCD renal allograft preservation. In this study, five pairs of discarded DCD kidneys were treated with 10^8 MSC or placebo, perfused *ex vivo* for 24 h in a proprietary machine perfusion system, and eventually evaluated for DNA synthesis, cytokine/chemokine synthesis, cytoskeletal regeneration, and mitosis. The authors observed that the study group showed increased synthesis of adenosine triphosphate, a reduced inflammatory response, increased synthesis of growth factors, normalization of the cytoskeleton, and mitosis. More recently, a similar study conducted *ex vivo* reported comparable results using 50 million multipotent adult progenitor cells (MAPC) that are inherently similar to MSC (Thompson et al., 2021). In this report, grafts were perfused at 36.5°C for only 7 h and demonstrated improvement in clinically relevant parameters and injury biomarkers. This notwithstanding, two recently published *in vivo* studies have failed in showing beneficial effect of MSC. In a kidney autotransplantation porcine model, the administration of a much lower dose of MSCs (10 million) during *ex vivo* normothermic machine perfusion (Lohmann et al., 2021a) or after cold storage (Lohmann et al., 2021b) was not followed by any beneficial clinical effect within 2 weeks of observation.

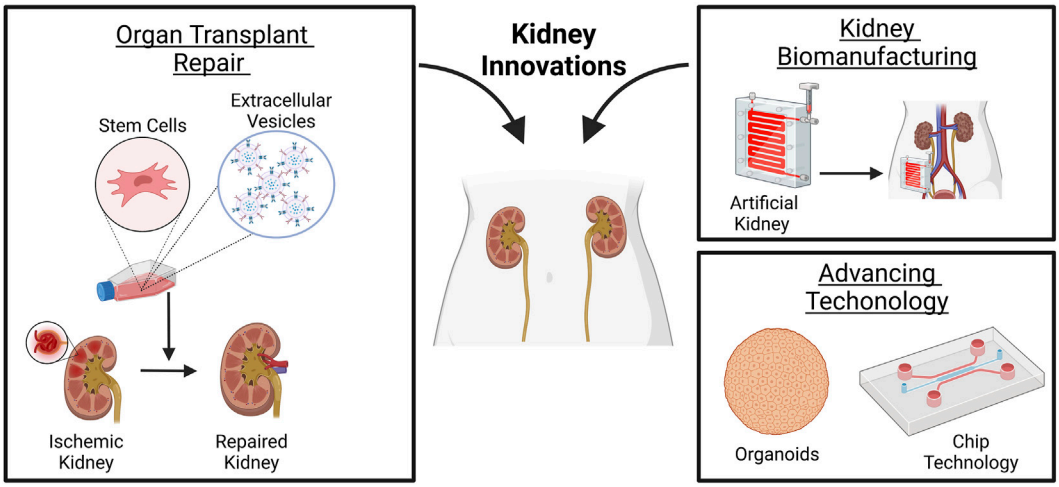

FIGURE 1
Innovations made in renal medicine. Stem cells or derived EVs may mitigate ischemic renal damage before or after transplantation. The design of artificial kidney devices, although limited in fully mimicking kidney function, i.e., secretion of endocrine and immunologic factors, reabsorption, or metabolism, may allow home dialysis and self-care renal replacement therapy for patients waiting for a transplant. Advancing technology in organoids and chip systems may serve as a platform to study disease mechanisms and perform drug screening studies with high reproducibility for the design of patient-specific therapies. Created with BioRender.com.

Few studies have investigated the possibility of using stem cells or derived EVs to mitigate ischemic renal damage after transplantation (Figure 1). Wu *et al.* demonstrated that human Wharton's Jelly MSC-EVs mitigated renal damage, ameliorated function, and improved survival when administered intravenously in post-transplant DCD kidneys in rats (Wu et al., 2018). MSC-EVs reduced cell apoptosis and inflammation, as well as promoted cell proliferation. The effects of MSC-EVs were evaluated 2 weeks post-transplantation and demonstrated reduced renal fibrosis and macrophage infiltration upon EV administration, sustaining their beneficial role in the acute and chronic stages (Wu et al., 2018).

Gregorini et al. (2017) proposed using stem cells or their released EVs to supplement the standard perfusion solution. In detail, they performed 20 min of warm ischemia followed by nephrectomy in rats. The explanted kidneys were perfused for 4 h at 4°C in the hypothermic machine perfusion, with the supplement of 3 million MSCs or EVs, released by the same number of cells. Molecular and histological analyses of kidneys immediately post perfusion, treated with MSC or MSC EVs, revealed significantly lower renal damage than control kidneys and showed an up-regulation of energy metabolism enzymes. Moreover, the evaluation of lactate, glucose, and LDH in the effluent fluid indicated extensive use of energy substrate in the presence of MSC and MSC EVs. More recently, the same group showed that EVs delivered during hypothermic oxygenated perfusion into marginal kidneys significantly reduces ischemia-reperfusion injury (Rampino et al., 2022).

3D kidney biomanufacturing

Tissue engineering (TE) and advances in three-dimensional bioprinting techniques that use a combination of cells, artificial and natural biomaterial, and biologically active molecules to reconstruct or regenerate damaged tissues or whole organs provide a potential solution to the shortage of transplantable kidneys. However, unlike two-dimensional planar tissue, the complex kidney structure is composed of various cell types in specialized locations on the specific composition of extracellular matrix protein for proper kidney function. Thus, making bioengineering of the kidney for transplantation still challenging. Although challenges still exist in whole organ engineering, the development of wearable hemodialysis devices may serve as a viable novel alternative dialysis technology that can enhance a patient's freedom and quality of life (Figure 1). Multiple clinical trials have shown wearable artificial kidneys' benefits and possible pitfalls. Gura et al. (2008) showed in an FDA-approved human trial the design and use of wearable artificial kidneys (WAK). These artificial kidneys were miniaturized, wearable hemodialysis machine, based on dialysate-regenerating sorbent technology. They were designed to be well-tolerated and effective in uremic solute clearance and maintenance of electrolyte and fluid homeostasis for up to 24 h. The University of California, San Francisco, and Vanderbilt University Medical Center have been rigorously developing an implantable artificial kidney (IAK). The group uses a combination of a high-efficiency membrane for hemofiltration with a bioreactor of kidney tubule cells for electrolyte balance

(Salani et al., 2018). Improvements are being made to address the pitfalls of artificial kidneys, such as thrombogenicity, excessive carbon dioxide bubbles, device portability, extended service life, reduced replacement of sorbent cartridges, differentiated phenotype maintenance of cultured tubule cells, and cost-effectiveness. Although these devices are limited in fully mimicking kidney function, i.e., secretion of endocrine and immunologic factors, reabsorption, or metabolism, they do allow home dialysis and self-care renal replacement therapy to be more feasible. They may still serve as a viable option for patients waiting for a transplant.

Organoids and chip technology

In kidney transplant medicine, novel techniques, and technologies such as three-dimensional (3D) cell cultures that incorporate key kidney features can lead to the design of more patient-specific targeted therapies (Figure 1). Kidney organoids, which are 3D cell cultures composed of various cell types, i.e., human pluripotent stem cells (hPSCs) differentiated to kidney cell types, are designed for drug screening, disease modeling, and the generation of tissue for renal replacement. Recently Lawlor et al. (2021) showed that by 3D bioprinting organoids, more manufactured organoids with specific biophysical properties such as size, cell number, and conformation might be generated towards creating uniform patterned kidney tissue sheets. However, limitations still exist in mimicking the filtration barriers and fluid exchange vital for kidney function and responsible for blood filtration with excretion of metabolic waste products and drugs. The use of microfluidic chips, such as glomerulus-on-a-chip (referred to as GOAC) (Petrosyan et al., 2019) and proximal tubule epithelial cells (PTEC) on a chip (Vriend et al., 2018) are shown to recapitulate the functions and structure of the glomerulus. These changes include perm selectivity and active tubular secretions through proximal tubules drug transporters. Current advancements are being made towards addressing the technological limitations regarding the chips' bi-directionality of the flow and the absence of all kidney cell types needed to mimic the full nephron by generating four-lane chips with a unidirectional flow. Nonetheless, the chip systems serve as a platform to study disease mechanisms and perform drug screening studies with high reproducibility.

Heart

Cardiac cell therapy is considered the only cure for cardiovascular disease; current sophisticated long-term treatments (drugs, pacemakers, stents, etc.) are only palliative. However, in the continued absence of standardized criteria, the injection of cells from different sources and stages of differentiation (skeletal myoblasts, embryonic stem cells (ESC), bone marrow-derived mononuclear cells (BMMNCs), mesenchymal stem cells, hematopoietic stem cells, endothelial progenitor cells), have not produced consistent results (Menasche, 2018). All experimental and clinical protocols stem from the same basic concepts to isolate and expand the cells that are going to be implanted. The original tissue is dissolved with enzymes capable of damaging the cell membrane, and isolated cells are cultured using procedures (bidimensional, culture media, etc.) consolidated for mature cells. This approach does not consider that optimal implantable cells are secluded in a specific microenvironment (niche) of the native tissue, where their fate is regulated by specific biological and physical signals (Vining and Mooney, 2017). Current protocols neglect stem cell singularity and yield a suboptimal population of somatic stem cells that retain some level of genetic instability. This genetic instability includes tumorigenic and immunogenic properties. Cultured cells also display inadequate purity that may be responsible for graft-related arrhythmias when transplanted. In most trials aimed at heart repair, MSCs and heart-derived progenitor cells from cardiospheres or c-Kit + resident cells (kit + CPCs) are used despite their poor cardiomyogenic potential. Better results are expected when injecting ECS and iPS, but they are not clinically used. Beneficial cell effects could also be ascribed to mRNA transcripts and/or paracrine signals transferred by stem cell-secreted or directly injected exosomes (Gnecchi et al., 2008). This observation has raised the question of whether injecting selected families of exosomes vs. cells could be more efficient in repairing the cardiac tissue's texture and spur the function of the injured cardiomyocytes. However, recent studies have suggested that the functional improvement in post-MI cardiac function can be attributed to an acute inflammatory-based wound-healing response characterized by the temporal and regional induction of CCR2+ and CX3CR1+ macrophages rather than to the formation of new cardiomyocytes (Vagnozzi et al., 2020) (Figure 2).

Reconstituting the native bio architecture

Current protocols are focused on cell differentiation in the ischemic region but neglect the need to restore the original myocardial bioarchitecture. The unique spiral-like arrangement of the contractile cells in the myocardium represents the anatomical foundation of the heart's functional prowess. Instead, after injection into the injured myocardium, stem cells grow and differentiate without a specific polarization; hence, they contribute to the heart contracting in an uncoordinated and inefficient fashion. The issue of the post-injection cell orientation has been addressed by growing cells on polymeric biocompatible

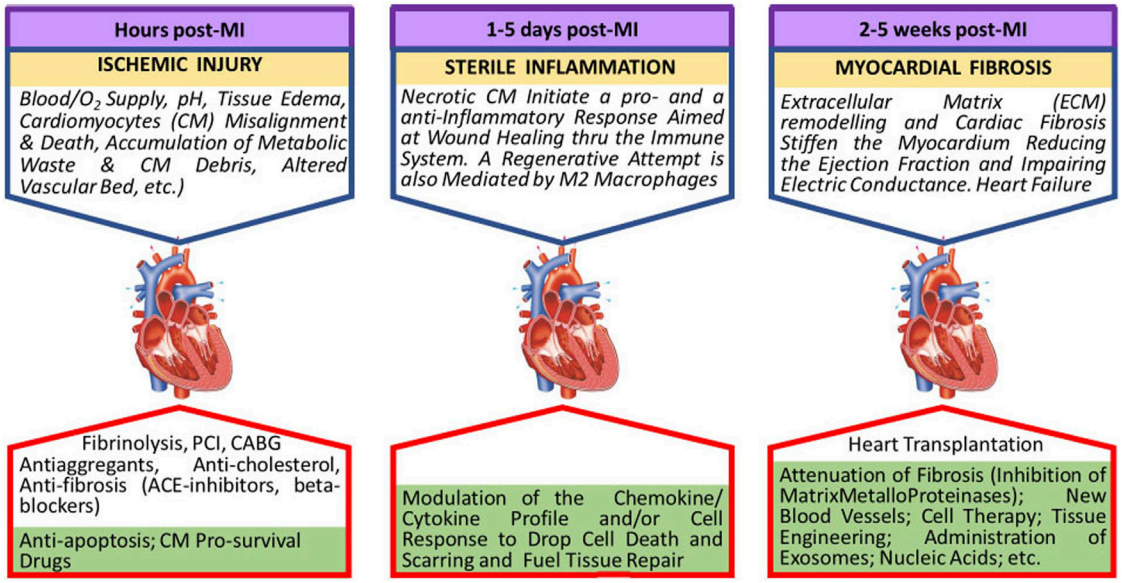

FIGURE 2
Evolution of the myocardial ischemic damage. In lower green boxes future research directions are indicated.

structures (scaffolds). These are made of natural, artificial, and composite materials, characterized by a variegated design providing cells with mechanical support to favor a three-dimensional orientation (Reis et al., 2016). Recent data suggests that the decellularization of the tissue can produce innate ECM scaffolds that supply biological signals to the implanted cells. This was demonstrated through successful repopulation with human iPS-derived cardiomyocytes displaying sarcomere structure and electrical conductivity (Taylor et al., 2018). However, this strategy is affected by the inconsistency of different preparations, the possible transfer of viruses and the potential for rejection. Taken together, all scrutinized solutions do not allow the replication of myocardial architecture.

Further knowledge must be developed on the ECM structure and the complex array of biological and physical signals interlacing scaffolds and cells. In this context, an auspicious research direction is represented by the emulation of the ECM structure (microfibers embedded in a matrix). Experimental scaffolds made of a woodpile structure embedded in hydrogel are already under investigation, and have shown promising preliminary results (Carotenuto et al., 2020). However, the exploitation of different scaffold designs has taught us that cell fate can be addressed through specific signals from the nucleus affecting the stiffness of ECM. Translation occurs via the cytoskeleton and via a biochemical cascade modulated by the TAZ/YAP system (Brusatin et al., 2018). Expanding knowledge on signals that lead to ECM stiffness could be fundamental to designing clinical-grade scaffolds.

Managing the recipient tissue microenvironment

Another factor for successful cell therapy is the modulation of the turmoil microenvironment in damaged tissue. This can hamper its response to humoral/immunostimulant factors or alien cell integration. Deprivation of blood supply modifies tissue pH, while cell debris, tissue edema, and cell misalignment disrupt the signals related to the stiffness of the recipient myocardial tissue. Furthermore, ECM breakdown products, and mitochondrial DNA activate a robust inflammatory reaction, while the invasion of non-myocardial cells creates an ecosystem unfavorable for living cells. In this context, chemokines mobilize monocytes that transdifferentiate into macrophages releasing pro-inflammatory cytokines (TNF, IL-1β, and IL-6) detrimental to surviving cardiomyocytes. These inflammatory factors stimulate fibroblast proliferation, enhancing scar tissue formation to substitute dead cardiomyocytes and prevent ventricular wall rupture. At the same time, the anti-inflammatory M2 macrophages secrete factors that may recruit and activate exogenous or resident progenitor cells. It is crucial to modulate the post-ischemic microenvironment to favor the implant of new healthy cells. An environment in favor of new healthy cells can be achieved by improving the pharmacological treatments currently in use and injecting cell populations able to interact with immune and non-immune cells. MSCs release soluble factors that impair T-cell proliferation and differentiation, cytokine secretion, and cytotoxic potential. They suppress the formation of TH1 and TH17 while enhancing the

formation of TH2 lymphocytes, which produce anti-inflammatory cytokines, such as IL-4 and IL-10 (van den Akker et al., 2013). In addition, MSCs suppress neutrophils, dendritic cells, and natural killer (NK) cells (Raffaghello et al., 2008; Hamid and Prabhu, 2017) which induces the conversion of T cells into T-regulatory cells (Di Ianni et al., 2008). These T cells have cardioprotective and regenerative effects that enhance macrophage differentiation into the M2 subtype. This subtype reduces proinflammatory cytokine production, and stimulates cardiac reparative pathways, anti-inflammatory mediators and angiogenesis (Gore et al., 2015). MSC and CPC-released exosomes can activate post-ischemic modulation of inflammatory and immune responses. Such modifications include the polarization of M1 to M2 macrophages via shuttling miR-182 (Zhao et al., 2019). Thus, exosomes could be used as immunomodulating agents of the myocardial environment to determine post-ischemic conditions more suitable to allow engrafted cells to grow, differentiate and integrate into the recipient surrounding tissue.

Artificial mitochondrial transfer

Mitochondria transfer is one of the biological processes triggered by stress signals, during which mitochondria are transported from healthy donor cells and incorporated into the endogenous mitochondrial network of the damaged recipient cell, in order to repair damage and restore its bio-energetic profile and health (McCully et al., 2022; Wang et al., 2022). As mitochondrial transfer has been found to play a critical role in healing several pathological conditions, AMT has recently emerged as a promising therapeutic approach for numerous disorders characterized by mitochondrial damage, including ischemic injury, which commonly complicates organ transplantation. A first-human clinical study was performed in pediatric patients in critical conditions due to severe myocardial ischemia–reperfusion injury (Emani et al., 2017; Emani and McCully, 2018; Guariento et al., 2021). The patients received autologous mitochondria isolated from their own rectus abdominis muscle. Mitochondria were administered via multiple injections directly in hypokinetic areas of the myocardium. While no adverse side effects were noted, patients receiving AMT had a more rapid and robust return of systolic ventricular function (McCully et al., 2022).

Liver

Cell therapy

Different cell therapies and bio artificial livers have been attempted and used not only for advanced cirrhosis but also for: Acute and acute-on-chronic liver failure, inborn errors of metabolism, chronic cholestatic, autoimmune diseases, and non-alcoholic fatty liver disease (NAFLD, proposed new acronym MAFLD) (Struecker et al., 2014; Qi et al., 2015; Giancotti et al., 2019). Hepatocyte transplantation represents proof of the concept of liver cell therapy. Indeed, clinical observations have demonstrated the procedure's safety, and patients (~100) have shown transitory clinical improvement and/or partial correction of the underlying metabolic defect (Lee et al., 2018). The major challenges associated with hepatocyte transplantation include the limited supply of donor organs to isolate good quality cells, low cell engraftment, cryopreservation difficulties, and the necessity of long-term immunosuppression. Advanced grafting strategies have the potential to improve the outcome of hepatocyte transplantation (Puppi et al., 2012).

MSCs derived stem cells, including bone marrow hematopoietic stem cells (HSCs) (CD34 and CD133) and MSCs (CD105, CD73, and CD90), are autologous, easily sourced, and readily cryopreserved, allowing for transplantation procedures with minimal, if any, complications (Forbes and Newsome, 2012; Moore et al., 2014). However, while clinical outcomes occurred within days to weeks, long-term effects (after more than a few months) were not observed. A recent multicenter phase-II open-label controlled trial of HSCs was completed in which repeated autologous infusions of G-CSF-mobilized CD133 + cells were administered to patients with advanced cirrhosis (versus conservative management or treatment with G-CSF alone) (Newsome et al., 2018). Researchers found no impact on liver function or fibrosis. Most recently, it has been shown that leukapheresis and macrophage infusion were well tolerated (Moroni et al., 2019). Tissues are highly informative, especially when clinical results are weak or absent (Lanthier et al., 2017). Studies have shown that the role of mesenchymal-derived cells does not depend on repopulation but on the production of factors and cytokines with multiple effects (An et al., 2017; Starkey Lewis et al., 2020).

Human fetal and adult livers contain two stem cell niches—the ductal plates/canals of Hering that contain hepatic stem cells (HpSCs) (Schmelzer et al., 2007) and the peribiliary glands that contain biliary tree stem/progenitor cells (BTSCs) (Cardinale et al., 2011). In a controlled trial of subjects with decompensated liver cirrhosis receiving fetal EpCAM + HpSC infusion via the hepatic artery, there was a significant decrease in patient MELD scores in the treated group (N = 25) at the 6-month follow-up (Khan et al., 2010). In Western countries, Pietrosi et al. treated nine patients by intrasplenic infusion of total fetal liver cell population and demonstrated positive effects on clinical scores and encephalopathy (Pietrosi et al., 2015). Preliminary results have been reported for a phase I/II clinical trial consisting of fetal BTSCs transplantation via the hepatic artery in patients with advanced cirrhosis (Cardinale et al., 2014). Remarkably, in all trials employing fetal liver-derived stem cells,

immune suppression was not required even though donors and recipients were not matched for histocompatibility antigens.

Embryonic stem cells evoke ethical concerns. Significant advancements have been made in defining protocols for the differentiation of human-induced pluripotent stem cells (iPSCs) into functional mature hepatocytes, e.g., induced multipotent progenitor cell-derived hepatocytes (Zhu et al., 2014), the direct reprogramming of fibroblasts or MSCs (Huang et al., 2014a; Du et al., 2014; Rezvani et al., 2016), and the utilization of human gastric epithelial cells differentiated into endodermal progenitors (Wang et al., 2016).

Liver engineering using ECM-based scaffolds

In 2009–2010, the first experiments involving whole-liver decellularization in rodents were conducted by Baptista et al. (2009) and successively completed by Uygun et al. (2010). Important further steps include the decellularized vascular network of the rodent liver by Wake Forest (Baptista et al., 2011) and the human liver decellularization by Mazza et al. (2015). Large-scale production of primary liver bipotential adult progenitor cells have been obtained through suspension cultures (Schneeberger et al., 2020). Although, Takebe et al. (2013) and Takeishi et al. (2020) biofabricated human livers for transplantation using human hepatocytes, biliary epithelial cells, and vascular endothelial cells; current challenges involve recreating "admirable" vasculatures (portal and arterial) and the biliary tree framework. An alternative approach to manufacturing the whole organ is the manufacturing of smaller organoids. These organoids can be generated from a growing number of sources, e.g., bile duct-derived organoids (Huch et al., 2015; Tysoe et al., 2019; Rimland et al., 2021), extrahepatic bile duct-derived organoids (Lugli et al., 2016; Sampaziotis et al., 2017), gallbladder-derived organoids (Lugli et al., 2016; Rimland et al., 2021), and hepatocyte-derived organoids (Hu et al., 2018). "Liver bud organoids" were obtained by co-culturing iPSC-derived hepatic endoderm cells, endothelial progenitors, and mesenchymal progenitors (Koike et al., 2019).

Lung

Chronic respiratory diseases are among the leading causes of death after cardiovascular diseases and cancer, accounting for about 5.7% of total deaths in 2017 (Kochanek et al., 2019). The gold standard for treating patients with end-stage lung disease is lung transplantation. However, the availability of donor's lungs is minimal compared to the high demand from patients on the waitlist for lung transplantation. Accordingly, stem cell and tissue engineering aim to address this challenge by manufacturing alternative functional lung grafts for transplantation therapy.

The human lung comprises a sizeable epithelial surface interfacing with the external gaseous environment and a dense vascular network surrounding the epithelium, separated by a thin basement membrane. Accordingly, a prerequisite for lung engineering is to identify and develop expandable, patient-derived sources of lung epithelial and endothelial cells that are necessary to reconstruct the gas-exchange function. The lung epithelium comprises the proximal airways and distal alveol (Franks et al., 2008). The airways are lined by pseudostratified epithelium, including basal, secretory, and ciliated cells (Hogan et al., 2014). Basal cells are the stem cells of airways and can differentiate into secretory and ciliated cells (Hong et al., 2004; Rock et al., 2009). Importantly, basal cells can be conveniently obtained from patients using minimally invasive procedures (e.g., endobronchial brushing), and can be expanded extensively *in vitro* (Mou et al., 2012). Therefore, they are an ideal cell source for reconstituting the proximal airway in bioengineered lungs. The distal alveoli are primarily covered by terminally differentiated alveolar type 1 (AT1) cells, which cover 95% of the gas-exchange surface (Crapo et al., 1982; Williams, 2003), and surfactant-producing alveolar type 2 (AT2) cells, which are alveolar stem cells with the potential of differentiation into AT1 cells (Rock et al., 2009; Barkauskas et al., 2013; Desai et al., 2014). While isolation of primary AT2 has been reported in mice (Demaio et al., 2009; Bantikassegn et al., 2015; Sinha and Lowell, 2016), rats (Bundschuh et al., 1995; Chen et al., 2004; Gonzalez and Dobbs, 2013), and humans (Elbert et al., 1999; Witherden and Tetley, 2001; Wang et al., 2007; Ballard et al., 2010; Fujino et al., 2011), these cells have a very limited proliferative capability *in vitro* (Dobbs, 1990). To generate an alternative source of AT2 cells, by recapitulating embryonic lung development, advances in stem cell engineering has made it possible for directed differentiation of human induced pluripotent stem cells (hiPSCs) into definitive endoderm (D'Amour et al., 2005; Loh et al., 2014), anterior foregut endoderm (Green et al., 2011), and lung epithelial progenitors characterized by NKX2.1 expression (Longmire et al., 2012; Mou et al., 2012; Hawkins et al., 2017). Further development employing 3D hydrogel culture has enabled the derivation of surfactant-producing AT2 cells from the hiPSC-derived lung progenitors (Huang et al., 2014b; Chen et al., 2017; Jacob et al., 2017). Recent progress is being made to finally induce AT1 specification from hiPSC-derived AT2 cells (de Carvalho et al., 2019).

An engineered lung is incomplete without proper vascularization. In sharp contrast to the discontinuous endothelium in the liver sinusoid or the fenestrated endothelium in the small intestine, the pulmonary microvasculature plays essential roles in gas exchange. It features a continuous, non-fenestrated phenotype (Marcu et al., 2018). However, the understanding of pulmonary-specific microvascular phenotype remains limited at the molecular level. There is a lack of understanding of

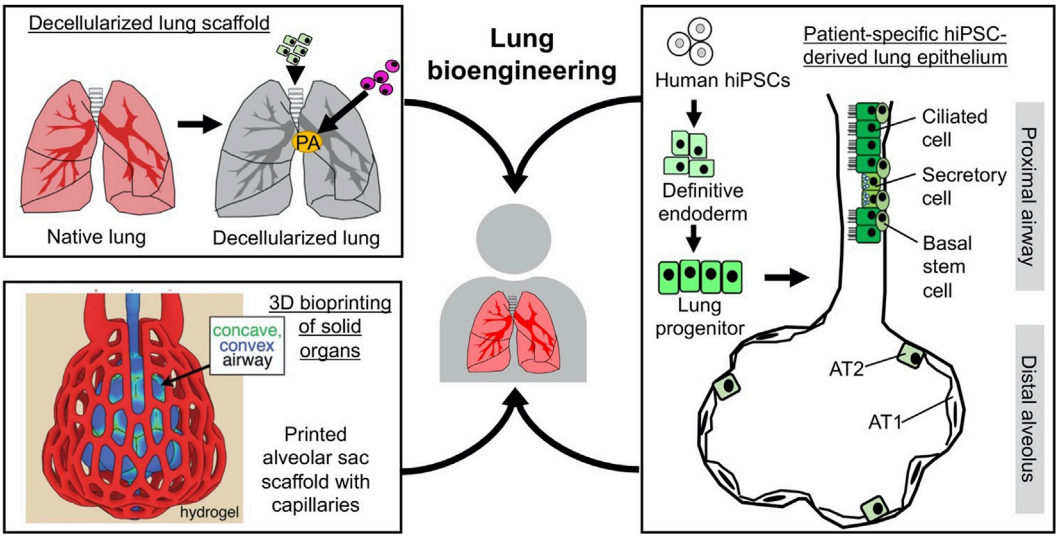

FIGURE 3
Combining regenerative cells and scaffolds for lung bioengineering. The scaffolds can be derived from whole-lung decellularization and 3D bioprinting (adapted from "Multivascular networks and functional intravascular topologies within biocompatible hydrogels" by Grigoryan et al., 2019, Science, 364 (6439), p 461. Copyright 2020 by The American Association for the Advancement of Science. Reprinted with permission). The cells for reconstituting the lung epithelium can be derived from stepwise differentiation of hiPSCs.

signaling that regulates the acquisition of such specialized endothelial phenotype during organogenesis and hiPSC differentiation. Accordingly, most lung bioengineering attempts so far have been focusing on using primary endothelial cells isolated from the lung or other tissues and generic endothelial cells from hiPSC differentiation (Ren et al., 2015; Zhou et al., 2018). Future research should focus on defining the molecular signatures of pulmonary-specific endothelium and developing a strategy for deriving such cells from hiPSCs, as accomplished in deriving blood-brain-barrier endothelium (Lippmann et al., 2012; Neal et al., 2019; Praca et al., 2019).

The structural basis of respiratory function lies in the mutually integrated respiratory epithelial and vascular compartments. Biomaterial scaffolds are usually employed to recapitulate such organotypic tissue organization from a tissue engineering perspective. So far, two scaffolding strategies have shown promise in achieving decellularized and 3D-printed lung scaffolds. Whole-lung decellularization uses detergent to remove all the cellular components while preserving the ECM that outlines internal tissue compartmentalization. The decellularized scaffolds enable compartment-specific delivery of pulmonary epithelial and endothelial cells into the airway/alveolar and vascular compartments, respectively (Figure 3). Such strategy has enabled the bioengineering of functional lung tissues that can provide gas-exchange function *in vitro* and *in vivo* (for short term) in small and large animal models (Ott et al., 2010; Petersen et al., 2010; Ren et al., 2015; Nichols et al., 2018; Zhou et al., 2018). In parallel, advances in 3D bioprinting offer an alternative strategy for manufacturing rationally designed tissue scaffolds. In particular, a new bioprinting technique, StereoLithography Apparatus for Tissue Engineering (SLATE) has recently been reported, which used biocompatible food dye additives as potent photo absorbers. It has also enabled 3D printing of hydrogel into biomimetic alveolar models with both gas and vascular compartments (Grigoryan et al., 2019). Comparing the two scaffolding strategies, decellularized scaffolds offer the advantage of preserving the complex, organotypic ECM composition, while bioprinting has so focused on a limited number of ECM molecules, such as collagen and gelatin. On the other hand, in terms of material availability, 3D printing scaffolds could offer, in theory, unlimited supply, while scaffolds manufactured by native organ decellularization still rely on tissue availability (Figure 3).

Upon proper cellularization and *in vitro* maturation, the engineered lung grafts should be evaluated *in vivo*. Considering the inadequate functionality of the lung tissues engineering with current approaches, complete replacement of native lung function in an animal model is usually not feasible. Accordingly, *ex vivo* lung perfusion (EVLP) and heterotopic transplantation models are being developed to bridge to orthotopic lung transplantation. EVLP is a clinically used procedure for normothermic support of donor's lungs prior to transplantation. Conceived and developed at the Toronto Lung Transplant Program led by pioneering surgeon Shaf Keshavjee (Cypel et al., 2011), the EVLP technology has revolutionized the

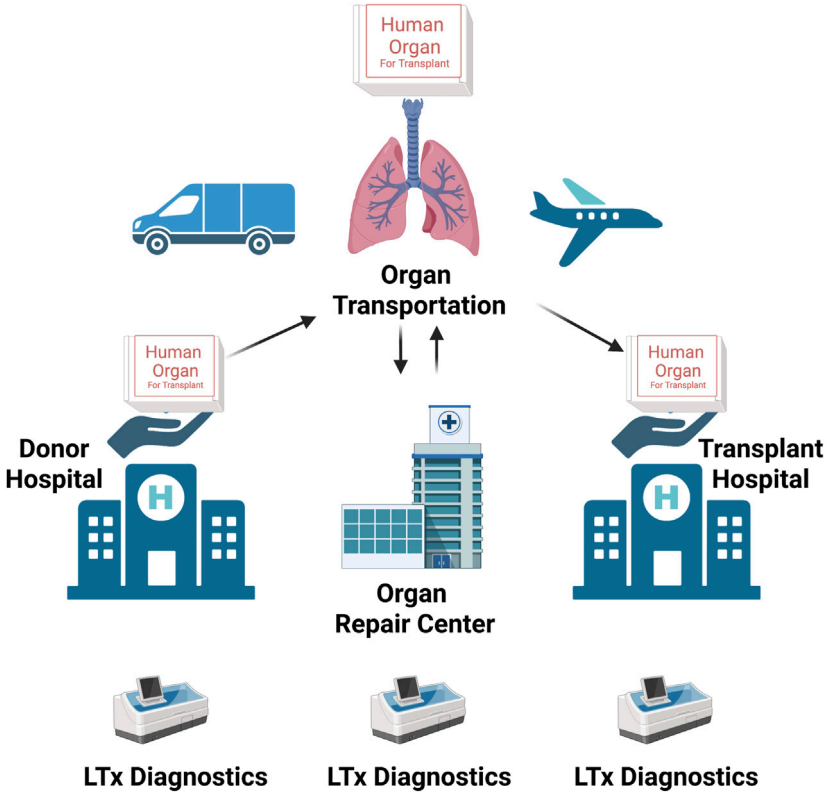

FIGURE 4
The schematic representation of the mode of operation of the modern "organ-management ecosystem." Marginal organs are procured at the donor hospital and then transported and delivered to the "Organ Repair Center." Here, they are subjected to tests to determine whether there is any margin to repair and regenerate them to become transplantable. Once an organ has been repaired and regenerated, it can be transported to the transplant center, where it will be transplanted (adapted from JTCVS Open) (176). Created with BioRender.com.

field of lung transplantation. By repairing and rendering transplantable marginal lung allografts that decades ago would have otherwise been discarded, EVLP has dramatically increased the donor pool and has paved the ground for the development and implementation of a visionary idea, the "Organ Repair Center." This is a highly specialized transplant unit where organs unsuitable for transplant yet with a decent functional reserve are subjected to diagnostic tests and treatments to make them transplantable. (Figure 4).

In a recent study, the EVLP system was adapted to support partial lung decellularization, re-epithelialization, and functional assessment (Dorrello et al., 2017). To bring EVLP closer to lung transplantation, a xenogeneic cross-circulation model has been developed by connecting the pulmonary vessels of human donor lungs to the circulation of a Yorkshire swine. Such whole-blood cross circulation enabled functional and histological recovery of acutely injured human lungs declined for transplantation (Hozain et al., 2020). These EVLP and cross-circulation models offer promising alternatives to conventional orthotopic transplantation for evaluating and potentially further maturing bioengineered lungs.

Gastrointestinal tract

The complex cytoarchitecture of the GI tract presents a challenge to generating tissue-engineered GI organs. GI organs are made of a diverse population of cells that collaborate to regulate organ function. For example, while the epithelial layer is responsible for absorptive and secretory functions, it is regulated by the submucosal plexus of the enteric nervous system (ENS) (Bitar and Zakhem, 2013). Choosing the correct combination of cells and scaffolds to recapitulate these functions is difficult. Cell sources may include donor tissue or pluripotent stem cell (PSC)-derived tissue. While donor tissue is obtained from a finite source and can be challenging to expand *in vitro,* multiple cell types in GI tissue can be isolated. PSCs can differentiate into any tissue type and can be generated from donor tissue or obtained from existing cell lines, thus providing a theoretically infinite source of the material. Individual populations of epithelial cells, smooth muscle cells, and ENS cells have all been isolated from donor tissue and generated from PSCs *in vitro*. The cells must be expanded *in vitro* while maintaining their *in vivo* properties,

which is complex with individually isolated/PSC-generated cell types. *In vivo*, the cells require interaction with other cell populations to maintain and regulate their phenotype and function (Bitar and Zakhem, 2015). Isolated smooth muscle cells (ISMCs) from rat intestines have shown to develop an altered immature phenotype that favors proliferation over differentiation when cultured *in vitro*. However, when intact strips of smooth muscle are isolated, the smooth muscle cells maintain their mature phenotype and undergo period contraction *in vitro*. Enteric neuronal and glial markers were also present in the smooth muscle strips suggesting that they are required to maintain the correct phenotype and function (Walthers et al., 2014).

Multiple sources of scaffold material have been employed to generate tissue-engineered GI tissue. The mechanical properties of the scaffold must be similar to the native extracellular matrix (ECM) *in vivo* to provide the cells with the appropriate mechanical cues and allow for vascular, lymphatic, and neural ingrowth. Synthetic materials such as polyglycolic/poly lactic-co-glycolic acid (PGA/PLGA) or polyglycolic/poly-L-lactic acid (PGA/PLLA) have readily tunable mechanical properties that have been used to generate scaffolds for GI applications (Basu et al., 2012; Maemura et al., 2012; Rego et al., 2016a; Schlieve et al., 2017). Natural scaffolds such as chitosan (Zakhem et al., 2012; Zakhem et al., 2014; Zakhem et al., 2015; Rego et al., 2016a; Rego et al., 2016b) and acellular ECM (Totonelli et al., 2012; Urbani et al., 2018) provide both mechanical and biochemical cues to the cells and maintains the natural architecture of the tissue. The complex 3D tissue models of intestinal epithelium allow for better mimicking cellular interactions of physiology or pathophysiology and applications towards therapeutic drug screenings and regenerative medicine. Bioengineered small intestine epithelium tissue cultured on lyophilized silk protein sponge matrices with macrophages is a novel system for studying the epithelial-immune interactions reflective of inflammatory bowel disease (Roh et al., 2019). While a more physiological model of the small intestine with a functional epithelial barrier was generated using small intestinal submucosa scaffolds seeded with intestinal organoids obtained from intestinal crypts and co-culture with fibroblasts. After 7 days, a subpopulation of cells differentiated into intestinal-specific cell types such as mucus-producing goblet cells or hormone-secreting enteroendocrine cells (Schweinlin et al., 2016). They have also regenerated intestinal and esophageal tissue (Badylak et al., 2011a; Wong et al., 2011; Kitano et al., 2017).

The lack of transplant options and dismal intestinal transplant survival rates present a significant clinical need for tissue-engineered GI organs; however, GI tissue complexity presents a major challenge to achieving this goal. Recent advances in *in vitro* cell culture, such as the development of organoid systems and the generation of scaffolds that recapitulate *in vivo* organ mechanical and biochemical properties, are promising for the generation of tissue-engineered GI organs.

The endocrine pancreas

β cell replacement through either whole pancreas or islet transplantation represents the gold standard for the treatment of longstanding morbid diabetes mellitus (Orlando et al., 2014; Odorico et al., 2018). However, its application is limited by a dramatic organ shortage and the need for lifelong anti-rejection medications whose administration is burdened by high costs and morbidity. Since the advent of the regenerative medicine era, it has become apparent that the field of beta cell replacement offers a formidable platform for the application of technologies aiming at either identifying a potentially inexhaustible source of islets or improving islet (immune)protection, lifespan, viability, and function. The present paragraph will focus on this latter task.

Extensive efforts have been focused on developing effective islet encapsulation approaches to eliminate the need for chronic immunosuppression to prevent allograft rejection and recurrence of autoimmunity, for example by designing novel encapsulation materials, and engineering the site of transplantation to improve graft vascularization and provide local immune modulation, in order to overcome some of the aforementioned challenges. Several strategies, including microencapsulation of islets in hydrogel microcapsules (Scharp and Marchetti, 2014; Cantarelli et al., 2017) as well as retrievable macroencapsulation devices (MEDs) (Weaver et al., 2018), have been developed with the objective of providing an immune protective environment to the islets, with each having its own benefits and limitations (Scharp and Marchetti, 2014). Islet encapsulation in hydrogel microcapsules, including alginate capsules, has been shown to provide immunoprotection to the islets overcoming allogeneic, xenogeneic and autoimmune responses. The spherical shape of the microcapsules also maximizes the surface area to volume ratio, resulting in increased diffusion of oxygen and nutrients. However, the major disadvantage of microencapsulation is that the islet capsules cannot be regarded as a single construct but as a multitude of independent microtissues, and it can be challenging to control their localization as well as practical surgical implantation and retrieval (if needed), thereby raising concerns to the biosafety of this approach (Storrs et al., 2001; Weaver et al., 2018). The MEDs can physically isolate the islets from the surrounding environment by a semipermeable cell containment barrier and provide immunoisolation by preventing direct contact with the host (Scharp and Marchetti, 2014). The larger dimensions of the device also allow for easier retrieval in case of adverse events, overcoming a potential regulatory hurdle associated with cell therapy. However, islets entrapped in the device can agglomerate over time, resulting in a

larger tissue with nutrient and oxygen diffusion limitations, leading to necrosis and loss of function. If they are embedded in a bulk hydrogel (example, alginate sheet), it can result in a reduced surface-to-volume ratio, leading to difficulties in scaling up to a clinically relevant size, without compromising nutrient and oxygen diffusion. Even with thin planar devices providing a larger surface-to-volume ratio, the upscaling to a therapeutic islet dose remains challenging. Finally, the lack of a pancreas-specific biochemical microenvironment or peri-islet niche can adversely affect the long-term viability and function of islets in both microencapsulation and MED platforms. Therefore, research has focused on developing an islet-specific niche by using mammal organs as a source of 3D ECM scaffolds that is inherently biocompatible and provides biochemical cues and 3D support similar to that of the native tissue environment (Mirmalek-Sani et al., 2013; Peloso et al., 2016; Asthana et al., 2021).

Islets have a high oxygen demand, considering the fact that they account for 1%–2% of the pancreatic volume but receive 5%–10% of pancreatic blood flow (Jansson et al., 2016). Therefore, therapeutic islet constructs would require revascularization and integration with the host in order to maintain long-term graft viability and function. In fact, insufficient nutrients and oxygen supply due to the lack of proper revascularization post-transplantation is a major factor for the loss of transplanted islets and insufficient glucose/insulin diffusion delays glucose sensing and insulin secretion (Bruni et al., 2014). The best way to develop vascularized tissues is still through self-vascularization within bioengineered tissue constructs. However, revascularization is a slow process and the time required for the assembly and maturation of a perfusable vascular network throughout the graft may be longer than its survival time, as tissue necrosis often occurs early during the engraftment period due to insufficient oxygen supply. Therefore, incorporation of biochemical factors that favor rapid vascularization could help reduce islet death and loss of graft function after transplantation. Furthermore, a construct architecture and spatial patterning of islets that reduce their distance from the surrounding body fluid/host vasculature would allow for more efficient diffusion. Maximization of graft surface area would also lead to enhanced oxygen diffusion and promote attachment, proliferation and migration of host vascular cells, which would result in rapid vascularization, tissue remodeling, and prolonged islet survival following transplantation. Incorporation of microchannels in the construct containing islets would facilitate sufficient nutrient/oxygen supply with culture media *in vitro* and stimulate accelerated inosculation with the host vasculature *in vivo*. Such an architecture will ensure efficient nutrient and oxygen diffusion (Cabodi et al., 2005; Stachowiak et al., 2005; Ling et al., 2007) by providing an increased surface-to-volume ratio and extending the diffusion limit, compared to a bulk hydrogel (without microchannels), and promote engraftment, thus preserving islet viability and function in larger therapeutic constructs. Traditional biofabrication techniques including, particulate leaching, solvent casting and electrospinning can generate porous scaffolds; however, these techniques have limited compatibility with hydrogels and limited control over construct architecture, including pore/channel size, geometry, and distribution. Moreover, they require application of temperatures, solvents or other conditions that can adversely affect live cells and often rely on post-fabrication cell seeding, which can result in non-uniform cell distribution and poor cell attachment. Additionally, most bioengineered constructs are manually fabricated and assembled, thus lacking a high degree of reproducibility necessary for commercial scale-up, clinical application and regulation. Additive manufacturing technologies that allow for the precise and reproducible fabrication of large 3D constructs with controlled architecture and islet distribution, are therefore being explored to further improve construct prototypes required for making islet-based therapies a reality (Gurlin et al., 2020; Soetedjo et al., 2021). Overall, success in this field will generate only from a combinatorial approach (Figure 5). This will accelerate the translation of current breakthroughs in scientific research to patients and allow islet transplantation to become a widely applicable treatment for morbid, longstanding diabetes mellitus.

Vascularized composite tissue engineering

Since the first successful hand transplant (Dubernard et al., 1999), vascularized composite allotransplantation (VCA) has progressed and been applied to the face (Dubernard et al., 2007), penis (van der Merwe et al., 2017), abdominal wall (Giele et al., 2016), and uterus (Brannstrom et al., 2015). Despite the extraordinary technical advances, the need for immunosuppression remains critical to managing chronic rejection, and difficulties still remain for re-transplantation. Moreover, specifically for body parts such as the hand and face, very narrow morphological criteria, in addition to the classical immunological screening, are responsible for a very limited donor/recipient match. Recent advances in the Vascularized Composite Engineering (VCE) approach in the direct line of solid organs' perfusion-decellularization/recellularization (PDR) is promising towards addressing some underlining concerns. Decellularization of simple and non-vascularized tissues, such as skin and bone, has been extensively described; however, the production of complex and large composite tissue matrices is at its early stage but holds great promise (Badylak et al., 2011b; Orlando et al., 2013; Orlando et al., 2019). The current challenge is to find a correct and versatile decellularizing protocol applicable to each tissue type that

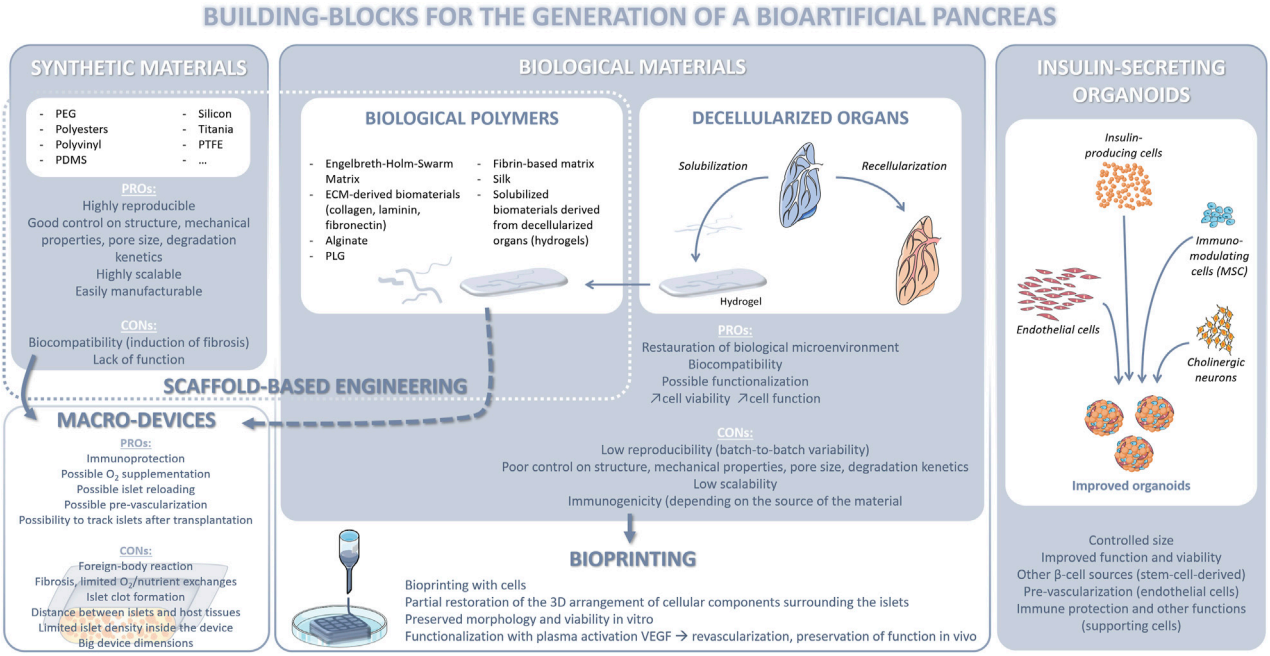

FIGURE 5
Regenerative medicine strategies applied to beta cell replacement.

presents different sensitivity to decellularizing agents. Additional early VCE studies employing PDR techniques have led to encouraging results in the rat limb and face (Jank et al., 2015; Duisit et al., 2018), porcine ear (Duisit et al., 2017a), human face and ear (Duisit et al., 2017b; Duisit et al., 2017c), human hand (Gerli et al., 2018), or sheep uterus (Tiemann et al., 2020). Produced matrices of various origins, sizes, and complexities show preservation of the ECM and their associated vascular tree to allow partial *in vitro* recellularization and *in vivo* transplantation.

For organ engineering, the next crucial step will be the recellularization and transplantation of the scaffolds to generate a functional and sustainable graft. In addition to the number of cells needed to repopulate the ECM, VCE has to address several issues due to tissue types and functions to be restored, i.e., motility, sensation, and the need for very specific bioreactors to be developed, allowing skin/mucosa regeneration and muscular training. The advantage of VCE, compared to SOE, is that a complete *in vitro* recellularization is not obligatory. The organ does not need to be fully functional at the time of implantation, and nearby cell repopulation at the implantation site may also occur, thus highlighting that the recipient can serve as their optimized bioreactor, as advocated by Badylak (Badylak, 2016). Therefore, an adapted approach should find an adequate balance between *in vitro* bioreactor culture and complementary *in vivo* maturation and healing.

Organoids

Organoids are miniature self-organized 3D structures composed of one or more cell types that partially recapitulate the structure and function of tissues and organs. Organoid technology has emerged as a powerful tool for studying organ development, disease progression, and drug screening (He et al., 2020). Organoid cultures represent an essential advancement in tissue engineering and can better recapitulate *in vivo* conditions *in vitro*. Various cell types such as human-induced PSCs, embryonic stem cells (hESCs), cell lines, and primary cells with or without bioreactor or biomaterials/scaffolds such as PLGA or Matrigel are used to generate complex 3D organoids structures that mimic their *in vivo* counterparts. Large and small intestinal, esophagus and stomach organoids have been generated from donor tissue or PSCs (Figures 6A,B) (Spence et al., 2011; Kasagi et al., 2018; Broda et al., 2019; Lau et al., 2019). Organoids are multicellular spheroids with diverse cell populations that mimic their respective organs' organization (Figure 6C). This ability allows the cells to maintain the correct phenotype and function *in vitro*. Organoids can be designed in an organ region-specific manner, such as that of the different brain compartments (Qian et al., 2016). Multipart protocols are also designed to incorporate various growth factors, cytokines, and small molecules to promote sequential differentiation of PSCs. Knowledge of developmental

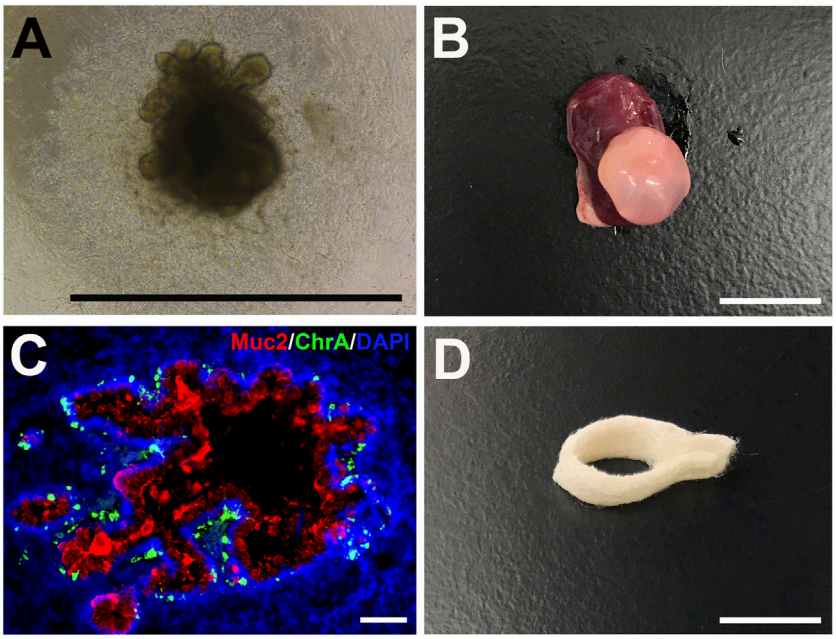

FIGURE 6
Human intestinal organoids (HIOs) and biodegradable scaffolds. **(A)** Brightfield photomicrograph of a PSC-derived HIO after 25 days *in vitro* (scale bar 1 mm) **(B)** Gross photo of a transplanted HIO (tHIO) after 8 weeks *in vivo* (scale bar 1 cm) **(C)** Immunofluorescent staining of an 8 week-old tHIO for goblet cells (Muc2/Cy5) and enteroendocrine cells (ChrA/FITC) (scale bar 100um) **(D)** Polyglycolic acid and poly-L-lactic acid (PGA/PLLA) biodegradable scaffold can be seeded with cells for the generation of tissue-engineered GI organs (scale bar 1 cm).

mechanisms and PSCs differentiation has allowed the development of multilineage organoids such as the kidney. Takasato et al. (2015) show the generation of kidney organoids through the differentiation of PSCs in 2D and 3D cultures using various concentrations of molecules at different durations and time. Organoid cultures are a powerful tool for studying organ development and function in healthy and disease states and potentially generating transplantable tissue. However, since large amounts of organoid tissue are necessary for a transplant, suitable scaffold (Figure 6D) and culture techniques are needed for further advancement (Finkbeiner et al., 2015). Additional limitations of *in vitro* organoids are the inability to generate mature and diverse cellular structures, their inconsistent reproducibility, and the deficiency of surrounding vascular, nervous, and immune systems necessary to recapitulate the *in vivo* tissue interaction. Limitations also exist in generating vascularized and architecturally organized organoids to mimic their *in vivo* organ counterparts precisely and efficiently. 3D bioprinting has been proposed to address and resolve some of these issues and accelerate the generation of complex organoids (Keshavije, 2020; Ren et al., 2021). Thus, in the future, the incorporation of knowledge in stem cell and developmental biology and advancements in material technology such as 3D printing will promote the development of improved organoids for tissue engineering and disease modeling that better mimic their *in vivo* counterparts both structurally and functionally.

Final remarks

In the past few decades, regenerative medicine has provided evidence that technologies like decellularization, 3D bioprinting, cell and organ engineering, and blastocyst complementation may offer platforms for the bioengineering, repair, and regeneration of transplantable organs. Although emerging data is promising, the complexity of solid organs poses a significant challenge, and further research and substantial investments are needed, as well as a synergic collaboration among all stakeholders, namely scientists, academia, industry, funding agencies, and governmental institutions. Advancements in basic research knowledge of stem cells, biomaterial, and developmental biology in combination with various biotechnologies and bioreactors are crucial for fast growth. Development and incorporation of chip technologies, organoids, and 3D bioprinters are opening new avenues and directions for the future. As it is clear to us that "no field in health sciences has more interest than organ transplantation in fostering progress in

regenerative medicine because the future of no other field more than the future of organ transplantation will be forged by progress occurring in regenerative medicine" (Orlando et al., 2019), we therefore infer that transplant medicine should accept the challenge and lead the efforts that will eventually deliver organs manufactured from patient's own cells to the bedside.

Author contributions

GO: Conceptualization, reviewed literature, writing-original draft preparation, and editing. All authors: Reviewed literature, writing-original draft preparation, revised manuscript, review, and editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Impact and challenges of enactment for advanced regenerative medicine in South Korea

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The Korean government has enacted the Act on Advanced Regenerative Medicine and Advanced Biological products (ARMAB) in August 2019, and it has been implemented in 2020. We reviewed the changes made by ARMAB compared to the existing Pharmaceutical Affairs Act and discussed future challenges to accelerate regenerative medicine while ensuring safety and efficacy. This act and regulations focused on the key elements of act as follows: the definition of advanced regenerative medicine (RM), the licensing of related facilities, safety management such as long-term follow-up, clinical research review committee, and establishment of a roadmap. Our study shows that Korea has achieved the second highest number of first approvals for regenerative medicine indications worldwide through expedited approvals encouraging innovation, while maintaining patient safety by mandating long-term follow-up. Additionally, the establishment of an interactive system for retrieval of patients' data and reporting of safety information by manufacturers electronically demonstrates Korea's commitment to innovation for Advanced RM and patient safety.

KEYWORDS

regenerative medicine, enactment, biological products, safety, South Korea

Introduction

With the increase of geriatric syndromes and rare incurable diseases due to aging, the demand for regenerative medicine (RM) is increasing (Cossu et al., 2018). RM or cell therapy products (CTP) have the potential to repair or reconstruct damaged functional cells and tissues for unmet medical needs such as dementia and organ defects (Witten et al., 2015).

Since RM uses live cells and tissues to repair or reconstruct damaged cells and tissues, preventing microbial contamination and maintaining living cells require other processes different from chemical medicines in terms of manufacturing procedures such as sterilization and aseptic testing, validation, collection of the cells, cultivation, production, and follow-up (Giancola et al., 2012). Thus, this process is the key driver for raising the manufacturing cost of RM. In addition, since RM involves personalized

treatment or rare diseases, and thus, there are only a few patients targeted for RMs, it is difficult to secure a sufficient number of patients in clinical trials for regulatory approval (Abou-El-Enein et al., 2016). To compensate for this limitation, post-marketing surveillance for tracking long-term side effects is needed (Hara et al., 2014). Therefore, RM requires a different authorization framework from the current licensing system.

Several countries and the European Union (EU) have established specific legislation for RMs and adopted special requirements for achieving regulatory exemption of RM approval in the EU, the United States, Japan, and Australia (European Medicines Agency, 2007; Azuma, 2015; Food and Drug Administration, 2016; Grounds, 2018; Qiu et al., 2020). In the United States, "the 21st Century Cures Act" contains new legislation affecting cell therapy in 2016, such as the Regenerative Medicine Advanced Therapy (RMAT) designation and "Expedited Programs for RM Therapies for Serious Conditions," including fast track designation, accelerated approval, and priority review designation (Food and Drug Administration, 2016; Kang et al., 2021).

The EU introduced regulation for Advanced Therapy Medicinal Products (ATMPs) in 2007 (European Medicines Agency, 2007). According to Article 28 [the European Commission (EC) regulation of No 1394/2007], each EU member state can apply conditions for the exemption of products from the centralized marketing authorization for ATMPs [referred to as hospital exemption (HE)]. These include the application of specific quality standards, use under the exclusive professional responsibility of medical practitioner and the national traceability, and pharmacovigilance, *etc.* (Cuende et al., 2022).

The Japanese government enacted a suite of laws and amendments specifically designed for the regulation of RM. In 2014, Japan enacted the Pharmaceutical and Medical Device Act (PMD Act) to include a unique regulatory conditional, time-limited market access pathway for RM (Hara et al., 2014; Azuma, 2015; Maeda et al., 2015), and then the Act on the Safety of Regenerative Medicine (RM Act) to establish a framework for RM and clarify measures necessary for ensuring patient safety (Azuma 2015; Tobita et al., 2016).

Globally, the number of companies producing RMs has increased from 772 in 2016 to 1,085 in 2020, and the size of the international RM market in 2020 is calculated to be 27.29 billion USD (Research, 2021). In Korea, the manufacturing of cell therapy has grown at an average annual rate of 29.7%, from 18 million USD in 2014 to 53.57 million USD in 2018, but the number of approved medications in Korea has shown a slowdown since 2015 (Kim et al., 2021). Moreover, the number of clinical trials for RM in Korea increased from 15 in 2010 to 27 in 2012, but the number of clinical trials has been stagnant since 2017.

In Korea, to accelerate R&D on RM as well as CTP, the discussion has been ongoing since 2013, and the Act on the safety of and support for Advanced Regenerative Medicine and Advanced Biological products (ARMAB) was enacted in August 2019 and has been executed since 2020 (Ministry of Health and Welfare, 2020a). This study aimed to describe the history of the act and the details of ARMAB in South Korea, and thereby suggest the landscape for future challenges.

Backgrounds of legislation

First, the legislation was introduced to establish a legal basis for RMs, which were processed with minimal operation/processing. At the time of 2019, the Pharmaceutical Affairs Act only specified the method of approval of CTP manufactured by pharmaceutical companies, and there was no legal basis for collecting and administering somatic cells or adult stem cells to patients in medical facilities with minimal operations. Since somatic cells or adult stem cells were not included in the extent of CTP in the Pharmaceutical Affairs Act, the enactment of the new law has provided the legal basis to enable medical facilities to culture cells and process for RM. Before the legislation, the RMs was not defined as "use a legitimate medical service". In Korea, the payment system for both inpatient and outpatient care is based on a fee-for-service, which defines all of the covered and uncovered medical services in the health insurance benefits scheme. Second, the new law covered RM as well as advanced biopharmaceuticals and integrated all related regulations into one new Act. In the past, regulations related to biopharmaceuticals were scattered in the Pharmaceutical Affairs Act, the Bioethics Act, and the Blood Management Act, but now, they have been unified through the ARMAB Act. The third purpose of enacting the law was for the government to consider biopharmaceuticals as a new industry for future growth and to establish a basis for supporting them. The new act has allowed patients who had no available treatment to receive RM as part of clinical research for RM to be enabled in patients who have no traditional treatment. Nonetheless, it provided the basis for supporting R&D and data governance for RM as well as advanced biopharmaceuticals. By enacting the law, the government should establish a roadmap and action plan, and thereby create the budget for funding of R&D. Based on this law, the government can designate RM support institutions and advanced biopharmaceutical regulatory science centers. Lastly, the new act introduced both the expedited approval and review method and post-market risk management. To strengthen the safety of advanced biopharmaceuticals, a safety management procedure for the entire life cycle from cell collection to final use has been prepared. Long-term follow-up management is legally obligatory.

However, the process of enactment took a long time. The preparation process for the Act started in 2013, after which, several bills were proposed from 2016 to 2018, and opinions from relevant government ministries and the private sector

TABLE 1 Regulations on cellular treatment in South Korea.

Category	Act and regulation	Year
Acts	- Act on the safety of and support for Advanced Regenerative Medicine and Advanced Biological Products (Ministry of Health and Welfare, 2020a) - Enforcement decree on the safety of and support for Advanced Regenerative Medicine and Advanced Biological Products (Ministry of Health and Welfare, 2021)	2019
Regulationss	- Regulations on approval and safety of human cells, etc. and Advanced Biopharmaceuticals (Ministry of Food and Drug Safety, 2020a) - Regulations on the recall and disposal order of hazardous Human Cells, etc. (Ministry of Food and Drug Safety, 2020b) - Rules on safety and support of Advanced Biopharmaceuticals (Ministry of Food and Drug Safety, 2020c) - Regulations on the designation of Advanced RM implementing institution and matters to be observed in the cell processing business (Ministry of Health and Welfare, 2020b) - Regulations on the preparation, submission, and review of Advanced Regenerative Medicine research plans, etc. (Ministry of Health and Welfare, 2020c)	2020
Guideline etc.	- Management Standard for Long-term Follow-up of Advanced Biopharmaceuticals (Ministry of Health and Welfare, 2020d) - Guidelines for evaluating the data integrity of biopharmaceutical manufacturers (Ministry of Food and Drug Safety, 2020d)	2021

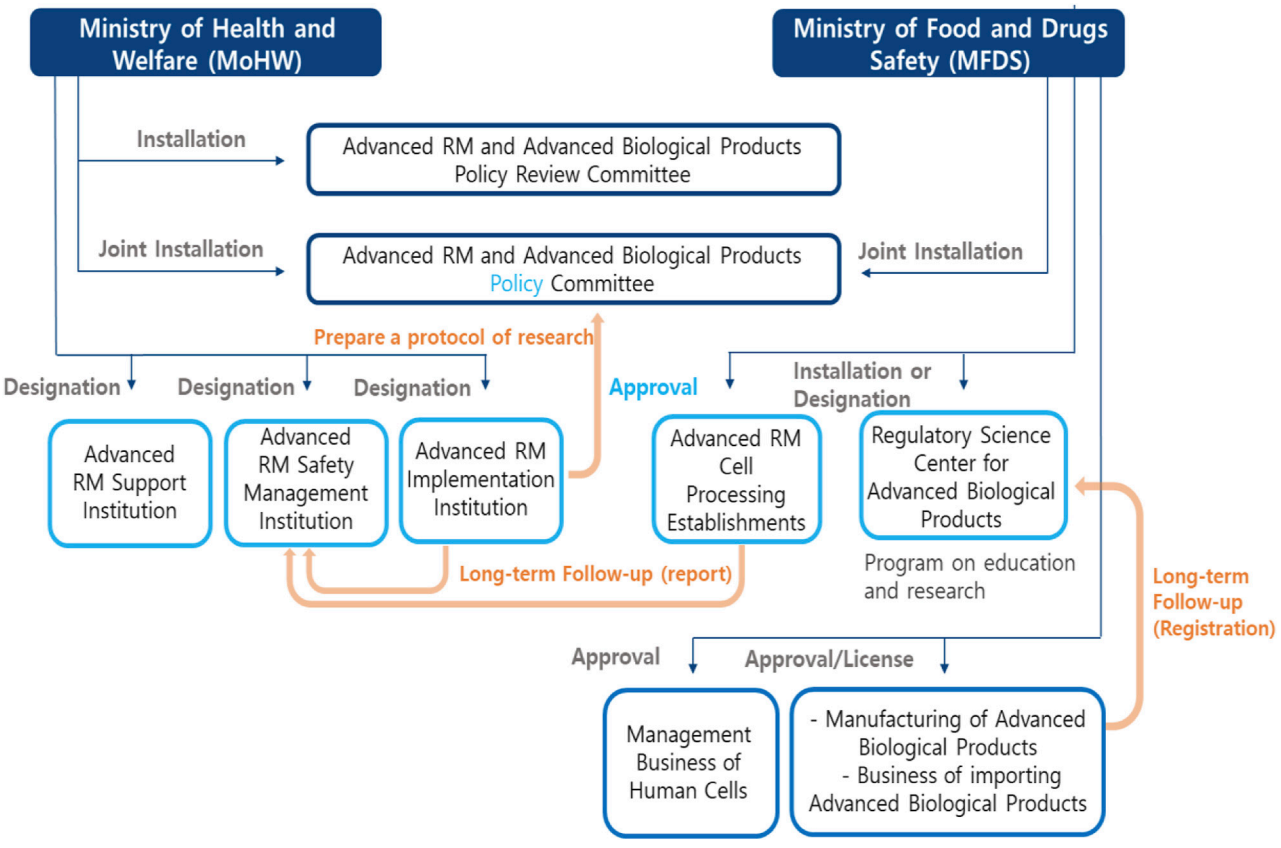

FIGURE 1
Relevant institutions of Advanced RM and Advanced biopharmaceuticals.

TABLE 2 The structure of the ARMAB Act (Ministry of Health and Welfare, 2020a).

Advanced RM	Advanced biopharmaceuticals
- Definition and scope (Article 2 No. 1)	- Definition and scope (Article 2 No. 5)
- Establishment and designation of Advanced RM related Support Agency (Article 9)	- Licensing of pharmaceutical manufacturers (Articles 23, 24, and 25) and import business (Article 27)
- Designation of Advanced RM institution (Article 10)	- The obligations and reporting on the quality control of manufacturing (Article 26)
- Consent for clinical research (Article 11)	- Licensing for the management of human cells (Articles 28 and 29)
- Review committee on the proposal of clinical research (Articles 13 and 14)	- Safety monitoring and Long-term follow-up (Article 30)
- The facility of cell processing on Advanced RM (Articles 15, 16, 17, and 18)	- Establishment and operation of Regulatory Science Center (Articles 32, 33, and 34)
- Safety management agency of Advanced RM (Article 19)	
- Safety monitoring and adverse event reporting (Article 20)	- The designation of products that apply for the expedited approval review (Article 36)
- Long-term follow-up of clinical research patients (Article 21)	

were collected. The final bill was introduced in March 2019, passed by the Legislative Judiciary Committee and the National Assembly plenary session on 31 July 2019, and 2 August 2019, respectively.

After the legislation was passed in 2019, as shown in Table 1, the enforcement ordinance and administrative rules were enacted in 2020 (Ministry of Food and Drug Safety, 2019; Ministry of Food and Drug Safety, 2020a; Ministry of Health and Welfare, 2020a; Ministry of Food and Drug Safety, 2020b; Ministry of Health and Welfare, 2020b; Ministry of Food and Drug Safety, 2020c; Ministry of Health and Welfare, 2020c; Ministry of Food and Drug Safety, 2020d; Ministry of Health and Welfare, 2020d; Ministry of Health and Welfare, 2021). The subordinate statutes included the expedited approval procedure and plan for safety management of advanced biopharmaceuticals reflecting the specificity of RM and advanced biopharmaceuticals. In addition, it includes the establishment of a management system to review and support clinical research on RM at the national level and a strategy to promote clinical research on RM.

The significant change is to divide past RM into regenerative medical treatment and biopharmaceutical medicinal products. Thus, it differs from the regulation in European Union or other countries such as United States and Australia, which involves medicinal products referred to as ATMPs or RMAT in that this law covers both RMs and advanced biopharmaceuticals. This is because, in Korea, all medical services provided by medical institutions, except for cosmetic and cosmetic purposes, must be defined as covered or non-covered in the health insurance benefits list. Also, this law includes long-term follow-up requirements for patient safety.

The role and relationship between each institution is shown in Figure 1. The structure of this law is shown in Table 2.

The key elements of advanced regenerative medicine and advanced biological products act

As shown in Table 3, we classified the key elements of the new act into five categories as follows: 1) terminology of Advanced RM and classification of clinical research, 2) establishment of roadmap and operation of Policy Committee, 3) the regulation on relevant institutions of Advanced RMs (Advanced RM-related Support Agency, Advanced RM institution, clinical research proposal Review Committee of Advanced RM, the facility of cell processing, and safety management agency), 4) the regulation on relevant institutions of Advanced biopharmaceuticals (licensing for the Advanced biopharmaceutical manufacturing or import business, licensing for the collection and supply of human cells, and establishment and operation of Regulatory Science Center), and 5) long-term follow-up and safety management.

Definition and classification of research

Advanced RM is defined as therapies that process human cells that are intended to be used for either the regeneration, repair, and formation of human body structures or functions or the treatment or prevention of human diseases, which include CTP, gene therapy products (GTP), and tissue-engineered products (TEP), whereas Advanced biopharmaceuticals includes advanced bio-convergence products, additionally. Human cells include stem cells, hematopoietic stem cells, somatic cells, immune cells, and xenogeneic cells derived from the human body.

A review committee of a clinical research proposal is established to encourage clinical research for patients without alternative treatment. Clinical research in advanced RM is divided into high-risk, medium-risk, and low-risk groups by

TABLE 3 Key elements of ARMAB Act: Definition, the criteria of the facility (MFDS, 2020a; Ministry of Health and Welfare, 2020a; Ministry of Health and Welfare, 2020b; Ministry of Health and Welfare, 2020c; Ministry of Health and Welfare, 2021)

	Advanced RM	Advanced biopharmaceuticals
Definition and scope	Advanced RM is defined as therapies that process human cells that are intended to be used for either the regeneration, repair, and formation of human body structures or functions or the treatment or prevention of human diseases, which includes cell therapy products (CTP), gene therapy products (GTP), and tissue-engineered products (TEP) Human cells include stem cells, hematopoietic stem cells, somatic cells, immune cells, and xenogeneic cells derived from the human body	Advanced biopharmaceuticals includes cell therapy products (CTP), gene therapy products (GTP), tissue-engineered products (TEP), and advanced bio-convergence products
Player	Medical institutions	Pharmaceutical companies
Type of research	Clinical research in advanced RM is divided into high-risk, medium-risk, and low-risk groups by examining the impact on human life and health and the degree of risk. Clinical research on advanced RM can be conducted after approval by a Review Committee	A clinical trial is according to existing Pharmaceutical Law
Establishment and implementation of the roadmap	The government should establish a basic roadmap every 5 years, including improving regulation, supporting R&D, commercialization, patient safety management, and financing The Policy Committee should be operated by the MoHW, and it consists of 21 members	
Relevant institutions	- Advanced RM related Support Agency (Designation by the Ministry of Health and Welfare (MoHW)) - Advanced RM institution (Designation by the MoHW) - Review Committee on the proposal of clinical research (Operation by the Ministry of Health and Welfare, and MFDS) - The facility of cell processing on Advanced RM (Approval by the Ministry of Food and Drug Safety (MFDS)) - Safety management agency of Advanced RM (Designation by the MoHW)	- The obligation of manufacturing and import, including criteria of quality control - Licensing for the collection and supply of human cells (approval by the MFDS) - Establishment and operation of a Regulatory Science Center (Designation by the MFDS)
Follow-up and safety management	A long-term follow-up investigation for clinical research has been decided by the Review Committee and a long-term follow-up is necessary	In order to strengthen the safety management of the administered patients, targets for "long-term follow-up management" are designated, and serious adverse events are followed up for a certain period or long term after administration

examining the impact on human life and health and the degree of risk. Clinical research on advanced RM can be conducted after approval by a review committee jointly operated by the Ministry of Health and Welfare (MoHW) and the Ministry of Food and Drug Safety (MFDS).

Roadmap establishment and governance

The ARMAB stipulated that the government should establish a basic plan every 5 years, including improving regulation, supporting R&D, commercialization, patient safety management, and financing.

To establish and implement the roadmap, the governance is operated by the MoHW, the MFDS, and the Policy Review Committee operated by the MoHW, which consists of 21 members.

Moreover, this act specified relevant government institutions and agencies of regulations. First, Ministry of Health and Welfare, might establish or designate an Advanced RM-related Support Agency according to Article 9. This agency should conduct research, human resource training, the support of industrial infrastructure, and international cooperation in the field of RMs. Second, according to Article 10, the Advanced RM implementation institution should get the designation of Ministry of Health and Welfare, and be responsible for clinical research with human cells supplied from cell processing facilities. The Advanced RM implementation institution should get the informed consent of the subjects participating in the research and apply for a review of the clinical research proposal, and then obtain approval. Third, the facility of cell processing on Advanced RM should get the approval of MFDS according to Article 15. Fourth, in order to

ensure safety, Ministry of Health and Welfare, should designate the safety management agency of Advanced RM among affiliated government institutions of Ministry of Health and Welfare, according to Article 19. The designated agency must carry out safety monitoring and long-term follow-up of clinical research.

Licensing of pharmaceutical companies and management business

In order to produce and import Advanced biopharmaceuticals, manufacturers, and import businesses should get licensed by MFDS. After obtaining approval, the manufacturing and management obligations should comply with the standards of each step, including facility, equipment, manpower, and quality control of the process from the cell collection stage to the inspection, processing, storage, *etc.*, according to Articles 25, 26, and 27. Nonetheless, the business that collects and supplies human cells should obtain licensing by MFDS according to Articles 28 and 29. In addition, this act includes the establishment and operation of the Regulatory Science Center by MFDS according to Article 32. This agency should conduct research, human resource training, support industrial infrastructure, and international cooperation.

Safety management

The main challenge of the enactment is to ensure safety and protect vulnerable patients from the risk of uncertainty about the evidence of new therapies. For the Advanced RM, the clinical research proposal Review Committee of Advanced RM should review and decide whether the proposal of research is appropriate according to Articles 12 and 14. The safety management agency of Advanced RM should conduct a long-term follow-up for clinical research that the Review Committee recommended a long-term follow-up according to Article 21.

For Advanced biopharmaceuticals, in order to strengthen the safety management, targets for "long-term follow-up management" are designated by MFDS, and serious adverse events are followed up for a certain period or long term after administration according to Article 30. Companies that have obtained approval for clinical trial protocols or medicine products designated as long-term tracking targets must report drug sales and supply details into the Regulatory Science Center system, and any adverse reactions to the MFDS.

The expedited approval review of advanced biopharmaceuticals

According to Article 36, for the designation of the target for fast review, the head of MFDS may designate the applied cell therapies as the subject of "fast review" under certain conditions, such as (1) where there is no alternative treatment and the purpose is to treat serious life-threatening diseases, such as cancer; (2) where the purpose is to treat rare diseases under the Rare Disease Control Act; and (3) where the purpose is to prevent or treat the pandemic of bioterrorist infectious diseases and other infectious diseases under the Act on the Prevention and Management of Infectious Diseases (Fujita and Kawamoto, 2016).

For advanced biopharmaceuticals, commercialization is supported by customized review, priority review, or conditional approval. Each program is as follows: 1) customized review: step-by-step pre-screening by submitting permission data in advance according to the developer's schedule, 2) priority review: priority review over other drugs, and 3) conditional approval: drugs used for serious and rare diseases are approved as data from phase 2 clinical trials under the condition that phase 3 clinical trials will be conducted after marketing. The expedited approval might be expected to reduce the time required for new drug approval by 3.5–4.5 years.

Changes of advanced biopharmaceuticals

In addition, as this law was enacted, changes were made as shown in Table 4. This change was focused on Advanced biopharmaceuticals.

From the cell collection stage to the inspection, processing, storage, *etc.*, it is necessary to manage each step of the process. In addition, the provision of a "record management room" has been made compulsory for manufacturing and import permits. Moreover, a company that collects, imports, inspects, processes, and supplies human cells as raw materials for Advanced Biopharmaceuticals should obtain a license for the management of human cells, and cell and tissue donor suitability assessments are mandatory.

The advanced regenerative medicine and advanced biological's challenge

Achievements

Regarding RM, the Regenerative Medicine Acceleration Foundation in Korea has been designated as the advanced RM support institution, and 34 institutions have been designated as the Advanced RM implementation institution, as of December 2021, which has been increased to 43 institutions in May 2022. Twenty seven medical research proposals for the RM were applied, and five of them were approved as of 1 June 2022. Systems related to the safety of RM have been gradually installed in the Korea Disease Control and Prevention Agency and the Korean National Institute of Health and are scheduled to open

TABLE 4 Changes of advanced biopharmaceutical related regulation before and after ARMAB Act (MFDS, 2019; Ministry of Food and Drug Safety, 2020a; Ministry of Health and Welfare, 2020a).

	Past (pharmaceutical Act)	After the law ARMAB
Aim	The aims were to secure the safety and efficacy of cell therapy products in the application for approval	The aims were to integrate related regulations into one new act and to establish a legal basis for RM. Moreover, the new act introduced both the expedited approval and review method and post-market risk management
		By enacting the law, the government should establish a roadmap and action plan
Definition	Biopharmaceuticals includes biologics, cell therapy products (CTP), gene therapy products (GTP), tissue-engineered products (TEP)*etc.*	Advanced biopharmaceuticals are CTP, GTP, TEP, and advanced bio-convergence products
	However, cases where doctors perform only minimal manipulations such as simple separation, washing, freezing, and thawing of autologous or allogenic cells during treatment are excluded	However, cases where doctors perform only minimal manipulations such as simple separation, washing, freezing, and thawing of autologous or allogenic cells during treatment are excluded
The collection and supply of cell	A pharmaceutical manufacturer collects, processes, stores, and uses living cells by maintaining their specific properties, and the collection is conducted by medical institutions	A company that collects, imports, inspects, processes, and supplies human cells as raw materials for Advanced Biopharmaceuticals should obtain a license for the management of human cells*etc.*
		The requirement of ethics is added to the pharmaceutical manufacturers as follows: cell and tissue donor suitability assessments are mandatory
		(A facility of cell processing on Advanced RMs must obtain a license.)
The quality control of manufacturing	Cell therapy products are managed according to GMP standards in accordance with the same regulations as other pharmaceuticals	From the cell collection stage to the inspection, processing, storage, etc., it is necessary to manage each step of the process. In addition, the provision of a "record management room" has been made compulsory for manufacturing/import permits
Follow-up	A long-term follow-up investigation must be conducted in accordance with the regulations, and a fine of not more than 1.8 thousand USD Won is imposed in the case of violation	In order to strengthen the safety management of the administered patients, targets for "long-term follow-up management" are designated and serious adverse events are followed up for a certain period or long term after administration. Violations are punishable by up to 5 years in prison or a fine of up to 45,000 USD.
The expedited approval review	The expedited approval review program was operated in accordance with the announcement and guidelines	The legal basis for the expedited approval review program has been clarified
		- Customized review: step-by-step pre-screening by submitting permission data in advance according to the developer's schedule
		- Priority review: priority review over other drugs
		- Conditional approval: drugs used for serious and rare diseases are approved as data from phase 2 clinical trials under the condition that phase 3 clinical trials will be conducted after marketing

until 2022. Additionally, 19 cell treatment institutions have been designated so far.

The review system for advanced biopharmaceuticals has been reorganized. Since the enactment of the Act one advanced biopharmaceutical has been approved, which is Kymriah®. 52 biopharmaceuticals had been approved as of December 2021, where 18 of them were advanced biopharmaceuticals (15 CTP, three GTP), and all of them obtained re-approval from the regulatory body. The MFDS has clarified the process for the priority review, and reduced review period from 115 to 90 days. Furthermore, to enhance transparency and consistency of the review, the review results are open to the public within 180 days of the review, and the opinions from the industries are sought within 30 days of the review.

The Korea Institute of Drug Safety & Risk Management (KIDS) has been designated as the Regulatory Science Center for advanced biopharmaceuticals. To lay the foundation for guaranteeing the quality and safety of the post-marketing products, long-term follow-up systems (research plan, sales, and supply) have been planned, and systems for evaluating the quality of the products are being prepared step-by-step.

Also, based on the ARMAB, long-term, electronic follow-up of advanced biopharmaceuticals is established as of November 2021, which allowed patient-centered safety management by letting health care providers and manufacturers to report electronically and retrieve patients' information who had received the advanced biopharmaceuticals.

Challenges to encourage innovation

To activate the conduction of the clinical trials, financial support, providing proper infrastructure should be planned ahead. First, the Korean Government has assigned 30 million

USD for this project, and it has implemented various activities such as constructing GMP facilities and empowerment of the hospitals' cell management capacities. Yet, the deficiencies of the experts capable of conducting research or regulatory affairs are emphasized. Second, the lack of manufacturing infrastructure capable of cell research could be a barrier for small-sized companies. To overcome these problems, the Korean Government has announced the plan for the "Advanced biopharmaceuitlcas-2025" and provided schemes such as joint research and outsourcing manufacturing. Third, after the RMs are approved and produced as commercial products, the reimbursement of the RM is a challenge, because their prices are extremely high since their population size is very limited. If the RM is used as non-listed drugs, then the widespread use of the RMs could be difficult. Finally, patient safety, especially among the vulnerable population, is critical. Various measures to evaluate the safety of the RMs should be followed.

Despite those limitations, in order to maximize the potential value of the RM, an infrastructure for encouraging RM innovation and sharing experience on a global scale is required.

Proposals for foreign countries

According to Qiu et al. (2020), a total of 55 RMs were granted market access (MA) and the number of first approval for regenerative medicine worldwide was highest in the United States, followed by Korea, EU, Japan, and India (Qiu, Hanna et al., 2020). As of 2019, 16 RMs were approved in Korea, thus an accelerated approval program was needed for rapid market access. In other countries, whereas European Union (EU) and Japan enacted specific legislation for RM, the United States comprehensive act contains affecting cell therapy. In accordance with the these act, RMs got accelerated regulatory programs as follows: Regenerative Medicine Advanced Therapy (RMAT) designations in the US by the Food and Drug Administration (FDA), Priority medicine (PRIME) designation by the European Medicines Agency (EMA), and SAKIGAKE (fore-runner initiative) designation by the Pharmaceuticals and Medical Devices Agency (PMDA) in Japan. In addition to the accelerated approval program, the need for financial support was raised to promote research and development. Therefore, Korea has implemented an Act on the safety of and support for ARMAB, which has differences from other countries.

In conclusion, our study provides specific details of the ARMAB act, and we believe the Korean case could be a nice example for other countries which sought to enhance R&D for ARMAB as well as protect patient safety because this law includes long-term follow-up requirements for patient safety. The ARMAB is the first, interactive system in the world, which allowed patients' follow-up information to be retrieved while letting manufacturers report safety information electronically. Also, since the regulation of RM and Advanced biological products is important for the field, related academic society should develop and suggest internationally acceptable guideline including definition and standard of RM. At the same time, further study should be conducted to monitor its impact on patient safety and the innovation of the ARMAB.

Author contributions

D-SK: Design of study, wiring draft paper, writing-review, and editing SB: Writing-original draft preparation and revising manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Regenerative medicine applications: An overview of clinical trials

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Insights into the use of cellular therapeutics, extracellular vesicles (EVs), and tissue engineering strategies for regenerative medicine applications are continually emerging with a focus on personalized, patient-specific treatments. Multiple pre-clinical and clinical trials have demonstrated the strong potential of cellular therapies, such as stem cells, immune cells, and EVs, to modulate inflammatory immune responses and promote neoangiogenic regeneration in diseased organs, damaged grafts, and inflammatory diseases, including COVID-19. Over 5,000 registered clinical trials on ClinicalTrials.gov involve stem cell therapies across various organs such as lung, kidney, heart, and liver, among other applications. A vast majority of stem cell clinical trials have been focused on these therapies' safety and effectiveness. Advances in our understanding of stem cell heterogeneity, dosage specificity, and *ex vivo* manipulation of stem cell activity have shed light on the potential benefits of cellular therapies and supported expansion into clinical indications such as optimizing organ preservation before transplantation. Standardization of manufacturing protocols of tissue-engineered grafts is a critical first step towards the ultimate goal of whole organ engineering. Although various challenges and uncertainties are present in applying cellular and tissue engineering therapies, these fields' prospect remains promising for customized patient-specific treatments. Here we will review novel regenerative medicine applications involving cellular therapies, EVs, and tissue-engineered constructs currently investigated in the clinic to mitigate diseases and possible use of cellular therapeutics for solid organ transplantation. We will discuss how these strategies may help advance the therapeutic potential of regenerative and transplant medicine.

KEYWORDS

regenerative medicine, stem cells, extracellular vesicles, COVID-19, tissue engineering, transplantation, bioengineering

Introduction

Regenerative medicine focuses on replenishing and repairing tissue or organs impaired by disease, trauma, or congenital issues. Cellular therapies, conditioned media, extracellular vesicles (EVs), and seeded cellular patches are promising therapeutic tools to combat various inflammatory conditions and diseases. A large body of pre-clinical research has shown that stem cell therapies can delay disease onset within multiple organs such as the kidney (Sedrakyan et al., 2012; Urt-Filho et al., 2016; Frank and Petrosyan, 2020), lung (Mei et al., 2007; Zhen et al., 2008, 2010; Garcia et al., 2013; Xu et al., 2018), heart (Wang et al., 2015; Galipeau et al., 2016; Miteva et al., 2017), and liver (Gilsanz et al., 2017; Tsuchiya et al., 2019) through immunomodulatory and paracrine mechanisms. Conditioned media and EVs derived from stem cells also demonstrate similar characteristics (Lener et al., 2015; Nassar et al., 2016; Bruno et al., 2017; Riazifar et al., 2017; Sedrakyan et al., 2017; Grange et al., 2019). Mesenchymal stromal cells (mesenchymal stem cells; MSCs), which are used mainly in clinical trials, have a potent self-renewal and differentiation capacity into multi-lineages and may be isolated from various adult tissues such as bone marrow (BM), adipose tissue, and fetal specimens (amniotic fluid and umbilical cord). Cellular therapies are also investigated for transplant medicine with the hopes of repairing marginal organs, minimizing ischemia-reperfusion injury (IRI), and inducing immune tolerance in solid organ transplantation (Leventhal et al., 2016; Sawitzki et al., 2020). In addition to stem cell therapies, immune cell therapies that specifically isolate and enrich anti-inflammatory immune cells are also investigated as a promising regenerative medicine tool towards treating inflammation, promoting tissue regeneration, and enhancing transplant tolerance (Zwang and Leventhal, 2017). Currently, clinicians and scientists have begun providing novel insights into optimizing cellular therapy in the clinical setting to provide a more deliverable, sustained, and impactful clinical benefit to patients (Okano and Sipp, 2020). However, further studies with larger patient cohorts are needed to show the efficacy of cellular therapies, conditioned media, extracellular vesicles (EVs), and seeded cellular patches for regenerative medicine. Here we will review results obtained from current clinical trials and novel cellular therapeutic options investigated towards clinical use. We will discuss how these findings and current novel techniques may help advance the potential therapeutic effects of cellular transplantations, EVs, and tissue-engineered constructs for regenerative medicine and transplantation.

Cellular therapeutics

Promising pre-clinical research studies have shown the potential of multipotent mesenchymal stem cells (MSCs) transplantation as a regenerative medicine therapy option (Vu et al., 2014; Wang et al., 2021). Currently, the U.S. Food and Drug Administration (FDA) has approved a small set of therapies for clinical use (Table 1). Clinical trials have focused on using MSCs immunomodulatory, immunosuppressive, and regenerative potentials with hopes of treating chronic diseases and immune resetting of autoimmune disorders (Table 2). MSCs immunoregulatory properties are attributed to their secretion of numerous cytokines (anti-inflammatory factors: iNOS, IDO, PGE_2, TSG6, HO1 and galectins, cytokines: TGFβ, IL-10, CCL2, IL-6 and IL-7, chemokines: IL-6, CXCR3, CCR5, CCL5, CXCL9-11) and putative angiogenic proteins (VEGF, PDGF, TGFβ) (Shi et al., 2018). The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has set standards to define 'multipotent mesenchymal stromal cells' (MSC) for both laboratory-based scientific investigations and pre-clinical studies (Dominici et al., 2006). Three guidelines must be met for the designation of MSC. Firstly, MSC must be plastic-adherent (tissue culture flasks) in cultured under standard conditions. Secondly, MSC (measured by flow cytometry) must have specific surface antigen (Ag) expression (95% expression of CD105, CD73 and CD90, with absence (5/2%) in expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II). Thirdly, MSC must exhibit differentiation capabilities towards osteoblasts, adipocytes, and chondroblasts under standard *in vitro* differentiating conditions. Not all published clinical trials have adhered to these guidelines, limiting our ability to compare and contrast study outcomes and hindering the field's progression (Table 2).

In current clinical trials, similar to pre-clinical data, clinical administration of cellular therapies has shown angiogenic properties (active secretion of proangiogenic factors) and anti-inflammatory effects (reduced expression of pro-inflammatory markers and T cell proliferation) (Saad et al., 2017; Ye et al., 2017; Zhang et al., 2017). The angiogenic properties of autologous adipose tissue-derived MSCs are attributed to significantly increasing renal tissue oxygenation, cortical blood flow, and stabilizing glomerular filtration rates (GFR) up to 3 months in patients with the atherosclerotic renovascular disease (RVD) (Saad et al., 2017). The anti-inflammatory effects of autologous hematopoietic stem cells are predicted to be beneficial for patients with type 1 diabetes mellitus by lowering the proportion of white blood cells, lymphocytes, T-cell proliferation, and pro-inflammatory cytokine production (Ye et al., 2017). Similarly, anti-inflammatory properties of allogeneic umbilical cord-derived MSCs, show improvements in patients with systemic sclerosis-associated, with better skin thickness scores, lung function, significantly decrease in anti-Scl70 autoantibody titers, and reduction of pro-inflammatory cytokine levels (including transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) levels in serum) (Zhang et al., 2017). Although clinical trials show promising results for MSC use in the clinic, there are limitations in MSCs scalability, interdonor variability, clinical trial outcomes inconsistency, low engraftment rates, variation in immunomodulatory response, and potential regenerative limitations (Tanavde et al., 2015). Recently, induced pluripotent

TABLE 1 A list of cellular and tissue engineered products with the proposed treatments currently FDA approved. All of the approved cellular products are hematopoietic progenitor cell derived from Cord Blood approved for disorders affecting the hematopoietic system. The tissue engineered scaffolds are allowed for the treatment of mucogingival conditions, cartilage defects of the knee, and thermal burns.

Product	Company	Treatment
ALLOCORD (hematopoietic progenitor cell, Cord Blood)	SSM Cardinal Glennon Children's Medical Center	Disorders affecting the hematopoietic system that are inherited, acquired, or result from myeloablative treatment
CLEVECORD (hematopoietic progenitor cell, Cord Blood)	Cleveland Cord Blood Center	Disorders affecting the hematopoietic system that are inherited, acquired, or result from myeloablative treatment
Ducord (hematopoietic progenitor cell, Cord Blood)	Duke University School of Medicine	Disorders affecting the hematopoietic system that are inherited, acquired, or result from myeloablative treatment
GINTUIT (Allogeneic Cultured Keratinocytes and Fibroblasts in Bovine Collagen)	Organogenesis Incorporated	Allogeneic cellularized scaffold product indicated for topical (non-submerged) application to a surgically created vascular wound bed in the treatment of mucogingival conditions in adults
HEMACORD (Hematopoietic progenitor cell, cord blood)	New York Blood Center	Disorders affecting the hematopoietic system that are inherited, acquired, or result from myeloablative treatment
Hematopoietic progenitor cell, Cord Blood	Clinimmune Labs, University of Colorado Cord Blood Bank	Disorders affecting the hematopoietic system that are inherited, acquired, or result from myeloablative treatment
Hematopoietic progenitor cell, Cord Blood - MD Anderson Cord Blood Bank	MD Anderson Cord Blood Bank	Disorders affecting the hematopoietic system that are inherited, acquired, or result from myeloablative treatment
Hematopoietic progenitor cell, Cord Blood - LifeSouth	LifeSouth Community Blood Centers, Inc	Disorders affecting the hematopoietic system that are inherited, acquired, or result from myeloablative treatment
Hematopoietic progenitor cell, Cord Blood - Bloodworks	Bloodworks	Disorders affecting the hematopoietic system that are inherited, acquired, or result from myeloablative treatment
MACI (Autologous Cultured Chondrocytes on a Porcine Collagen Membrane)	Vericel Corp	For the repair of single or multiple symptomatic, full-thickness cartilage defects of the knee with or without bone involvement in adults
STRATAGRAFT	Stratatech Corporation	Treatment of adults with thermal burns containing intact dermal elements for which surgical intervention is clinically indicated (deep partial-thickness burns)

stem cells (iPSCs) derived MSCs (CPY-001) are shown to be safe and well-tolerated in a limited number of patients with steroid-resistant acute graft versus host disease (Bloor et al., 2020). This trial demonstrates for the first time, the possible applicability of iPSC-derived MSCs for a range of other clinical targets that may overcome the fundamental limitations of conventional, donor-derived MSC production processes. Although current clinical trials exhibit similar and limited anti-inflammatory beneficial effects with MSC treatments like previous pre-clinical trials, there is a large variation between each trial. Variations such as cell culture conditions, cell number transplantation, from of transplantation, cell type, and characterization limited the interpretation of each trial. Additional studies with larger cohorts are also needed to address the efficacy of cellular therapeutics in regenerative medicine.

Optimization of cellular therapeutics through modification of dosing, timing, route, and frequency of administration and activation of endogenous cells

MSCs preconditioned with either recombinant proteins, drugs, or *ex-vivo* cell culture conditions and techniques are also investigated to enhance their therapeutic potential before transplantation (Table 2). One form of enhancement strategy applied for cardiac regenerative cell therapy is using a guided cardiopoiesis approach to deliver BM-MSCs expanded and processed for lineage specification to derive cardiopoietic cells. In a Phase III Congestive Heart Failure Cardiopoietic Regenerative Therapy (CHART-1) clinical trial, cardiopoietic stem cells were delivered *via* the endomyocardial route with a retention-enhanced catheter to patients with ischemic heart failure (Bartunek et al., 2017). However, after thirty-nine weeks, the primary outcome was neutral, except for a subset of patients with severe heart enlargement that appeared to have had a consistent beneficial effect. The results suggest that cardiopoietic cell treatment beneficial outcomes may vary depending on the type of cardiac damage present in patients. Another alternative method used to enhance MSC regenerative potential aside from preconditioning the cells with recombinant growth factors, cytokines, or drugs is the use of environmental stimuli, such as hypoxia. Preconditioned MSCs under chronic hypoxic conditions (itMSC) show enhanced immunomodulatory properties when transplanted in non-ischemic cardiomyopathy patients (Butler et al., 2017). After 90 days, the administration of itMSCs was associated with a reduced number of natural killer cells, and the magnitude of this reduction was correlated with improved left ventricular ejection fraction (Butler et al., 2017). However, a single injection of itMSC was not efficient in promoting significant cardiac structural or functional

TABLE 2 A list of clinical trials using regenerative medicine applications. Each trial is identified by disease reference, patient gender, method of treatment, outcome, and International Society for Cellular Therapy Criteria Check (1, 2, 3). 1) MSC must be plastic-adherent when maintained in standard culture conditions. 2) MSC must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules. 3) MSC must differentiate to osteoblasts, adipocytes and chondroblasts *in vitro*. Most clinical trials using MSC appeared to have the 1st and 2nd criteria mentioned, and a large difference was noted between the trials regarding cell number, type, from of transplantation, and culture conditions. Such variation allows for the identification of different forms of effect per experimental group but shows little consistency in the trials performed. Thus, it would be beneficial if clinical trials followed a clearer guideline with minor changes per experimental group to understand better the applicability and efficacy of cellular and tissue engineered therapies.

Diseases reference	Patients male/ Female	Treatment	Outcomes	International society for cellular therapy criteria check (1,2, 3)
Atherosclerotic renovascular disease (Saad et al. (2017))	*n* = 14 (9/5)	Investigational new drug (IND) #15082 and intra-arterial injection of low dose (1.0×10^5 cells/kg) or higher dose (2.5×10^5 cells/kg) autologous adipose-derived MSCs	No side effects. Stimulated angiogenesis and modified immune function. Increased renal tissue oxygenation and cortical blood flow	1) Plastic-adherent, 2) Specific surface antigen (Ag) expression
Newly diagnosed Type 1 Diabetes (Ye et al. (2017))	*n* = 8 3/5	Insulin and intravenous injection of autologous hematopoietic stem cell conditioning with cyclophosphamide (200 mg/kg) and rabbit anti-thymocyte globulin (4.5 mg/kg)	Improved residual C-peptide secretion lowered anti-GAD titers and reduced exogenous insulin dosages. Decreased expansion and function of Th1 and Th17 cells	NA: Cells mobilized with cyclophosphamide (2.0 g/m2) and granulocyte colony stimulating factor (10 mg/kg/day), and then collected from peripheral blood by leukapheresis and cryopreserved
Systemic sclerosis (Zhang et al. (2017))	*n* = 8 3/5	Plasmapheresis and infusion of 1×10^6 cells/kg allogeneic umbilical cord mesenchymal stem cells	Improved mean modified Rodnan skin score, lung function and computed tomography (CT). Decreased anti-Scl70 autoantibody titer and serum transforming growth factor-β and vascular endothelial growth factor	1) Plastic-adherent, 2) Specific surface antigen (Ag) expression, and 3) multipotent differentiation potential
Steroid-resistant acute graft versus host disease (Bloor et al. (2020))	*n* = 15 8/7	Intravenous infusion of CYP-001 (induced pluripotent stem cells-derived mesenchymal stromal cells) two dose 1×10^6 to10^8 cells/kg or 2×10^6 to 2×10^8 cells/kg	Safe and well tolerated, no adverse side effects	1) Plastic-adherent, 2) Specific surface antigen (Ag) expression, and 3) multipotent differentiation potential
Symptomatic ischemic heart failure (Bartunek et al. (2017))	*n* = 107 males	Endomyocardial infusion with a retention-enhanced catheter of 2.4×10^7 bone marrow mesenchymal stem cells expanded and differentiated to cardiopoietic cells	Safe with neutral results. Future clinical trials should consider patient selection based on disease severity markers	1) Plastic-adherent, 2) Specific surface antigen (Ag) expression
Nonischemic Cardiomyopathy (Butler et al. (2017))	*n* = 22 males	Intravenous infusion of ischemia-tolerant human donor allogeneic bone marrow MSCs dosed at 1.5×10^6 cells/kg	Safe, caused immunomodulatory effects, and was associated with improvements in health status and functional capacity	1) Plastic-adherent, 2) Some specific surface antigen (Ag) expression
Ischemic cardiomyopathy (Florea et al., 2017)	*n* = 30 27/3	Transendocardial injection of 2×10^7 or 1×10^8 allogeneic bone marrow-derived human mesenchymal stem cells	Both cell doses reduced scar size, only the 1×10^8 dose increased ejection fraction. Optimal dose and delivery crucial for beneficial results	1) Plastic-adherent
Chronic kidney disease (Nassar et al. (2016))	*n* = 18 9/9	Intravenous and intra-renal arteries injection of 1×10^{10} p/g EVs derived from human cord blood mesenchymal stem cells	Safe and can ameliorate the inflammatory immune reaction and improve the overall kidney function in grade III-IV CKD patients	1) Plastic-adherent, 2) Some specific surface antigen (Ag) expression
Moderate to severe COVID-19 (Meng et al. (2020))	*n* = 9	Three cycles of intravenous infusion of 3×10^7 allogeneic umbilical cord-derived mesenchymal stem cells (manufactured by Vcanbio Cell & Gene Engineering Ltd.)	Safe and well tolerated, reduced IL6	1) Plastic-adherent, 2) Specific surface antigen (Ag) expression, and 3) multipotent differentiation potential
Severe COVID-19 (Shu et al. (2020))	*n* = 12 8/4	Intravenous administration 2×10^6 cells/kg allogeneic umbilical cord-derived mesenchymal stem cells (manufactured by The Jiangsu Cell Tech Medical Research Institute and The Jiangsu Cell Tech Biotechnology Co.)	No adverse reactions, C-reactive protein and IL-6 levels were significantly decreased, lymphocyte count returned to normal range and observed reduced lung inflammation	1) Plastic-adherent, 2) Specific surface antigen (Ag) expression

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Diseases reference	Patients male/ Female	Treatment	Outcomes	International society for cellular therapy criteria check (1,2, 3)
Severe COVID-19 (Shi et al. (2021))	n = 65	Infusion of three doses of 4×10^7 umbilical cord-mesenchymal stem cells (by VCANBIO Cell & Gene Engineering Corp, Tianjin, China)	Safe and showed improvement in whole lung lesion volume	1) Plastic-adherent, 2) Specific surface antigen (Ag) expression, and 3) multipotent differentiation potential
COVID-19-induced acute respiratory distress syndrome (Hashemian et al. (2021))	n = 11 8/3	Three intravenous infusions 2×10^8 cells umbilical cord MSCs (UC-MSCs; 6 cases) or placental MSCs (PL-MSCs; 5 cases)	Improved respiratory distress and reduce inflammatory biomarkers in some. Patients with sepsis or multiorgan failure poor candidates	1) Plastic-adherent, 2) Specific surface antigen (Ag) expression
COVID-19-induced pneumonia (Liang et al. (2020))	n = 1 female	Three intravenous infusions of 5×10^7 umbilical cord mesenchymal stem cells with thymosin α1 and antibiotics daily injection	Safe and well tolerated, showed remission of inflammation symptom	1) Plastic-adherent, 2) Specific surface antigen (Ag) expression, and 3) multipotent differentiation potential
Severe COVID-19-induced pneumonia (Leng et al. (2020))	n = 7 4/3	Intravenous drip of 1×10^6 cells/kg mesenchymal stem cells suspended in 100 ml of saline	Safe and well tolerated, reduced inflammatory response, promoted tissue repair and regeneration	NA: No information classified as clinical grade MSCs
Severe COVID-19 (Sengupta et al. (2020))	n = 27 17/10	Intravenous drip of 15 ml of exosomes (ExoFlo™) derived from allogeneic bone marrow mesenchymal stem cells	Safe and well tolerated, restored oxygenation, downregulated cytokine storm, and reconstituted immunity	NA
COVID-19 pulmonary fibrosis (Wu et al. (2020))	n = 27 19/8	1 or 2 or 3 intravenous transfusion of 3×10^6 cells/kg embryonic stem cell–derived immunity- and matrix-regulatory cells	Safe and well tolerated, with improved clinical symptoms and reduced pulmonary fibrosis	NA
Nonacute stroke ischemic or hemorrhagic (Chernykh et al. (2016))	n = 13 12/1	Intrathecally injection of 2.19×10^7 macrophage type 2 generated from autologous peripheral blood mononuclear cells in 2 ml of saline	Safe and improved neurological recovery possibly by immunomodulatory activity	NA
Living donor kidney transplant recipients (Mathew et al. (2018))	n = 9 6/3	Infusion of 0.5, 1, or 5×10^9 $CD4^+CD25^+$ Tregs isolated from patient's cryopreserved leukopheresis and expanded *in vitro*	Safe and showed no adverse infusion related side effects, infections or rejection events up to 2 years post-transplant	NA
Urethral stricture recurrences urethroplasty (Ram-Liebig et al. (2017))	n = 99 male	Tissue-engineered oral mucosa graft generated from oral mucosa biopsy and manufactured by MukoCell®	Safe and efficient in urethroplasty	NA
Chronic nonhealing venous leg ulcers (Stone et al. (2017))	n = 15 13/2	FDA-approved bilayered living cell construct consists of human foreskin-derived neonatal fibroblasts in a bovine type I collagen matrix below a layer of human foreskin-derived neonatal epidermal keratinocytes	Safe and well tolerated, changed inflammation to acute healing process	NA
Chronic myocardial scar (Bayes-Genis et al. (2016))	n = 5	Autologous pericardial adipose graft (adipose tissue taken from the left or the right side of the pericardium)	Safe procedure that may be efficacious in selected patients	NA
Severe ischemic left ventricular dysfunction (Menasche et al. (2018))	n = 6 5/1	Embryonic stem cell differentiated toward cardiovascular lineage than mixed with fibrinogen and thrombin to form a gel	Safe and showed low risk in short- and medium-term adverse events	NA
Chronic non-ischemic dilated cardiomyopathy (Hare et al. (2017))	n = 34 24/10	Transendocardial injection in ten left ventricular sites by NOGA Catheter of 1×10^8 allogeneic or autologous bone marrow-derived mesenchymal stem cells	Allogeneic MSCs safe and efficacious alternative to autologous MSCs. Therapeutic effects driven by immunomodulation and endothelial restoration	1) Plastic-adherent

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Diseases reference	Patients male/ Female	Treatment	Outcomes	International society for cellular therapy criteria check (1,2, 3)
Type 2 Diabetes Mellitus (Bhansali et al. (2017))	*n* = 20 15/5	Superior pancreatico-duodenal artery injection of 1×10^6 cells/kg of *in vitro* expanded autologous bone marrow-derived mesenchymal stem cells or 1×10^9 autologous bone marrow- derived mononuclear cells (separated by centrifugation)	Both cell types resulted in sustained reduction in insulin doses. MSC showed better improvement in insulin sensitivity while MNC showed an increase in C-peptide response	1) Plastic-adherent, 2) Specific surface antigen (Ag) expression
Dilated cardiomyopathy (Xiao et al. (2017))	*n* = 33 21/12	Intracoronary administration of $(5.1 \pm 2.0) \times 10^8$ bone marrow mononuclear cells or $(4.9 \pm 1.7) \times 10^8$ mesenchymal stem cells	Safe and both show comparable effectiveness. BMSC showed further improvements at 12-month but not BMMC.	1) Plastic-adherent, 2) Specific surface antigen (Ag) expression
Autosomal dominant polycystic kidney disease (Makhlough et al. (2017))	*n* = 6 3/3	Infusion through the cubital vein of 2×10^6 cells/kg autologous cultured bone marrow mesenchymal stromal cells	Safe and well tolerated	1) Plastic-adherent, 2) Specific surface antigen (Ag) expression
Alcoholic hepatitis (Lanthier et al. (2017))	*n* = 28 14/14	Hepatic artery infusion of $0.47 \pm 0.15 \times 10^8$ cells/kg bone marrow derived $CD34^+$ stem cells and mesenchymal stem cells	No clinical efficacy detected	NA: cells mobilized with 5-day course of lenograstim and centrifuged over a Ficoll-Hypaque plus gradient
End-stage liver disease (Rajaram et al. (2017))	*n* = 1 male	Two intrahepatic arterial infusion of 1.2×10^6 autologous bone marrow derived mesenchymal stem cells expanded *in vitro*	Safe with only short-term clinical benefit	1) Plastic-adherent, 2) Some specific surface antigen (Ag) expression
Decompensated liver cirrhosis (Kim et al. (2017))	*n* = 19 9/10	Peripheral vein infusion of 1×10^8/kg bone marrow mononuclear cells	Short term improvement of liver function and volume. High incidence of hepatocellular carcinoma	NA

improvements, highlighting the need to investigate the efficacy of serial dosing of intravenously administered itMSCs to promote a sustained immunomodulatory effect along with structural and functional improvements in the clinic. Thus, clinicians have also carried out studies identifying how different dosages of stem cells and numbers of injections (transplants) may dictate their therapeutic potential. In the TRIDENT Study, Florea et al. have demonstrated in patients with ischemic cardiomyopathy that there are different beneficial outcomes when patients are administered either 20 million or 100 million allogeneic MSC *via* transendocardial injection (Florea et al., 2017). Both groups showed improvement in scar formation; however, improved ejection fraction was noted only in patients receiving 100 million cells. The authors stated that although the two doses of allogeneic MSC are safe for patients, it is crucial to design trials to evaluate optimal dosing for cell-based therapies. Clinical trials have also begun to understand how different cell types produce better results than single-cell transplantation. In pre-clinical studies (Park K.-S. et al., 2019), have recently demonstrated that delivering both cardiomyocytes derived from human induced pluripotent stem cells (hiPSC-CMs) and human mesenchymal stem cell-loaded patch (hMSC-PA) to rats with myocardial infractions can amplify cardiac repair with enhanced vascular regeneration and improved cellular retention and engraftment (Park S.-J. et al., 2019). The combinatory cell delivery can also be applied to organ transplantations to enhance/preserve newly transplanted partial organ engraftment. Benomar et al. demonstrated that patients who were transplanted with pancreatic tissue comprising more than 50% of non-islet cells (likely enriched in ductal, acinar, and MSCs) had a statistically significant lower level of hemoglobin A1c and lower daily requirement of insulin even 5 years after transplantation, compared to those who received islet transplant with more than 50% tissue purity (Benomar et al., 2018); thus, suggesting that non-endocrine cells have a beneficial effect on long-term islet graft metabolic function. The authors identified elevated expression of CA19-9 generally synthesized by pancreatic ductal cells and hypothesized that ductal cells must have been transplanted and continued to proliferate and contributed to the beneficial outcomes. These

findings bring forth an important concept, the need to transplant multiple cell types for better long-term engraftment and function. These results suggest and warrant further investigation into the understanding and application of methods to enhance the therapeutic potential of MSC, either through improved cell culture techniques, the route of delivery, dosage specificity, or a combination of various cell types to further amplify their regenerative potential.

Current therapies are also designed to mobilize patients' tissue-specific progenitor cells using various bioactive molecules such as growth factors, cytokines, and hormones to enhance endogenous regeneration. Activation of endogenous stem cells to promote regeneration or repair holds great promise for the future of translational medicine (Xia et al., 2018). Ansheles et al. demonstrated that using statins (Atorvastatin therapy) in patients with coronary heart disease could significantly increase the pool of endothelial progenitor cells by 72% in 3 months (Ansheles et al., 2017). Patients also displayed a significant decrease in VEGF expression and various metabolic markers such as C-reactive protein, total cholesterol, LDL cholesterol, and triglycerides. Pantin et al. also investigated how to enhance endothelial cell mobilization from patients following allogeneic transplantation to sustain donor-derived hematopoiesis (Pantin et al., 2017). They identified that a high-dose (480 mg/kg) Plerixafor is safe and effective in mobilizing CD31 expressing cells in healthy donors. These studies highlight the use of molecules to enhance tissue regeneration and restoration in disease by activating endogenous resident cells without the need for exogenous cellular infusions.

Extracellular vesicle therapeutics

Cell-to-cell communication is vital to control wound healing and modulate chronic and acute diseases *via* paracrine signaling. Cells communicate *via* the secretion of numerous extracellular vesicles (EVs) which are a heterogeneous population and ranging from 40 nm to a few mm in size under physiological and pathophysiological conditions. EV populations most widely studied and characterized are exosomes (derived from intracellular endosomal compartments and range from 30 to 120 nm in diameter), microvesicles (also known as shedding vesicles are non-apoptotic EVs that originate from the plasma membrane and range from 50 to 1,000 nm in diameter), and apoptotic bodies (originate from cells undergoing apoptosis and range from 50 to 2,000 nm). Multiple pre-clinical studies have demonstrated that conditioned media of cultured stem cells and stem cell EVs show beneficial effects on various diseases (Lener et al., 2015; Bruno et al., 2017; Riazifar et al., 2017; Nguyen et al., 2020). The discovery of exosomes, microvesicles, and apoptotic bodies within the conditioned media has led to a new avenue of research exploring EVs for clinical use. Using EVs, most of the therapeutic effects of stem cells can be achieved with a reduced risk associated with live-cell injection late effects, such as neoplastic transformation and immune response activation (Nassar et al., 2016; Wang et al., 2017; Guo et al., 2020). A limited number of clinical trials have investigated EVs' therapeutic potential in patients with cancer (Morse et al., 2005; Dai et al., 2008) and disease (Nassar et al., 2016). In chronic kidney disease patients (Table 2), EVs isolated from umbilical cord MSCs were shown to be safe and potentially effective in modulating the inflammatory immune reaction (Nassar et al., 2016). Patients who were given two doses of MSC-EVs showed improved eGFR, serum creatinine level, blood urea, and urinary albumin-creatinine ratio, possibly due to a significant plasma level increase in TGF-β1 and IL-10 with a decrease in plasma levels of inflammatory cytokine, TNF-α. Although patients saw a vast improvement after two dosages of therapy at 8 weeks to 9 months, the improvements were not sustained after 9 months, and an additional administration of the EV might be needed (Nassar et al., 2016).

Further studies are also necessary to clarify fundamental questions regarding the generation, origin of isolation (body fluids: plasma, serum, blood, amniotic fluid, cell lines: MSCs, progenitor cells, IPSC's distribution, tissue derived) (Crescitelli et al., 2021) and uptake of EVs and how to scale up to cGMP manufacturing and improve associated quality control and batch tracking methods for the clinic (Riazifar et al., 2017). Another issue brought forth by the International Society for Extracellular Vesicles is the general lack of proper characterization of the different forms of EVs used in pre-clinical and clinical trials as each type contains different cargos and may promote different effects (Théry et al., 2018). There are currently multiple clinical trials initiated and recruiting patients to investigate EVs' application in various diseased organs such as lung, liver, kidney, and heart. The potential use of EVs as a regenerative medicine therapeutic option is vast and promising. There are currently no FDA-approved EV products.

Cellular therapeutics to improve donor organ quality

Cellular therapeutics have also been applied *ex vivo* to improve and recondition donor organ quality before transplantations. Thompson et al. show how *ex vivo* delivery of multipotent adult progenitor cells *via* normothermic machine perfusion in kidneys deemed un-transplantable prompted improved clinically relevant parameters (urine output, decreased expression of injury biomarker NGAL, improved microvascular perfusion) and decreased neutrophil recruitment and pro-inflammatory cytokines (downregulation of interleukin (IL)-1β, upregulation of IL-10 and Indolamine-2, 3-dioxygenase) (Thompson et al., 2020). Brasile et al. also show how 24 h *ex vivo* perfusion of MSC in an Exsanguinous Metabolic

Support tissue-engineering can accelerate the repair of ischemic damage in human kidneys. Promoting regeneration identified by the increased synthesis of ATP (both in the renal cortex and medulla), a reduced inflammatory response (TNF-α, RANTES, IL1-B, IL6), increased synthesis of growth factors (EGF, FGF-2, and TGF-α), normalization of the cytoskeleton (ZO-1 expressed exclusively at the plasma membrane) and increased cellular proliferation (higher expression of PCNA and mitosis) (Brasile et al., 2019). The authors suggest a more prolonged warm reperfusion of a donor's kidney may further improve and repair tubule damages attained from severe ischemic insult. The potential of MSCs to prevent or decrease injuries due to ischemia-reperfusion to further improve organ preservation has also been shown in various organs such as the lung (La Francesca et al., 2014; Lu et al., 2015), liver (Laing et al., 2020), and heart (Yano et al., 2018). Thus, these techniques involving reperfusion using various cell types provide a new avenue to significantly expanding donor criteria to offset current donor shortages. Future studies directed towards identifying the precise reperfusion media, the extent of reperfusion time, and the most suitable cell source can further enhance these techniques' applicability in the clinic.

COVID-19 therapies

COVID-19, the disease attributed to the novel SARS-CoV-2 coronavirus, has given rise to a global pandemic. Although many patients do well, some present fever, dyspnea, hypoxia, and even exhibit moderate-to-severe acute respiratory distress syndrome (ARDS). This group of patients typically require intubation, which is associated with high mortality rates (up to 67%–94%) (King et al., 2020). The detrimental effect of COVID-19 that causes multiple organ failure and even death is correlated with the presentation of a cytokine storm, which is identified as a maladaptive release of cytokines (Brodin, 2021). Elevated expression of inflammatory cytokines such as IL-1B, IFN-γ, IP-10, and monocyte chemoattractant protein 1 (MCP-1) detected in patients with COVID-19 is linked with Th1 cell response (Ye et al., 2020). Currently, MSC and their EVs are considered as a potential therapeutic option against COVID-19 (Table 2). MSC has the innate capacity to promote anti-inflammatory and immune regulatory functions by directly inhibiting abnormal activation of T lymphocytes and macrophages, pro-inflammatory cytokines, and secreting anti-inflammatory cytokines and growth factors such as IL-10 and VEGF to stimulate regeneration and repair. There are currently 16 clinical trials completed with over one thousand studies listed on ClinicalTrials.gov on the use of stem cells or stem cell exosomes to treat coronavirus-related injuries, such as acute kidney and lung injury and various inflammatory processes. Non-randomized case studies, phase 1 and phase 2 clinical trials have shown that human umbilical cord-derived mesenchymal stem cell (UC-MSCs) infusions in patients with moderate and severe COVID-19 pulmonary disease is safe and well-tolerated (Liang et al., 2020; Meng et al., 2020; Shu et al., 2020; Hashemian et al., 2021; Shi et al., 2021). A phase 1 and phase 2 clinical trial with limited patients shows that administration of UC-MSCs or clinical-grade MSCs may help reduce inflammatory cytokines (TNF-α, IFN-γ, IL6, IL8, C-reactive protein) and promote lung recovery in surviving patients (Liang et al., 2020; Hashemian et al., 2021; Shi et al., 2021). Intravenous injection of clinical-grade MSCs (lacking ACE-2 receptor and TMPRSS2) led to increased levels of anti-inflammatory cytokine IL-10, and the normalized presence of immune cells. The patients presented an increase of peripheral lymphocytes, a decrease in C-reactive protein (CRP), a reduced activated cytokine-secreting immune cells (CXCR3+CD4+T-cells, CXCR3+CD8+Tcells, and CXCR3+NK-cells), and a restored levels of regulatory DC cell population ($CD14^{+}$CD11c+CD11bmodregulatory DC cell) (Leng et al., 2020). The use of MSC with the absence of ACE-2 receptor and TMPRSS2 to prevent infection with SARS-Cov-2 may have enhanced the therapeutic effects of MSCs.

There are currently multiple studies listed on ClinicalTrials.gov on the use of EVs to treat COVID-19. Sengupta et al. show that a single dose of intravenous infusion of exosomes derived from BM-MSC (ExoFloTM) in patients presenting moderate-to-severe ARDS helps restore oxygenation, reduces the cytokine storm, to bring back a healthy immune system with no adverse effects (Sengupta et al., 2020). The authors state that exosomes may be used as a preventative measure against progression to invasive oxygen support and mechanical ventilation, which is associated with a high mortality rate. Further studies with randomized controlled trials (RCTs) are warranted to prove efficacy and address what type of EVs and what dosage of EVs are needed to treat COVID-19 patients. A short-term (84 days) Phase 1 clinical trial of twenty-seven COVID-19 patients with pulmonary fibrosis treated with human embryonic stem cell-derived immunity and matrix-regulatory cells, which poses high expression of proliferative, immunomodulatory and anti-fibrotic genes, also show improvements in clinical symptoms (Wu et al., 2020). Additional multicenter randomized placebo-controlled Phase 2/3 trials are underway for further proof. Although these findings are promising, additional studies with larger cohorts are needed to assess the efficacy of MSCs and EVs therapeutic potential to treat and prevent the progression of COVID-19 related injuries in patients. While many clinical trials are listed, not all have begun, and only a few have been completed. Additionally, the completed trials consist of a small sample size, various cellular products, different culture methodology, and need more time for result interpretation. Leading to a discouraging notion that COVID-19 treatment with cellular therapies may not be available soon to treat a significant number of patients. COVID-19 clinical trial moving forward

should focus on clear identification of cellular products used and improve quality of study design to further the future of cellular therapies in treatment of COVID-19.

Immune cellular therapies

Aside from using stem cells, the field of regenerative medicine also investigates the potential isolation and enrichment of specific anti-inflammatory immune cells to treat inflammation, promote tissue regeneration and transplant tolerance (Table 2). In non-acute stroke patients, administration of autologous M2 macrophages is shown to be safe and can modulate inflammatory responses, contributing to angiogenesis and tissue repair (Chernykh et al., 2016). However, the treatment appeared to be more effective in patients with lower endogenous immunosuppressive mechanisms (IL-10, FGF-β, PDGF, VEGF) and increased pro-inflammatory activity (IL-1β, TNF-α, IFN-γ, IL-6). Infusion of autologous Treg cells has also been investigated for kidney transplantation patients to promote transplant tolerance in hopes of avoiding long-term use of toxic immunosuppressive agents that cause increased morbidity/mortality (Mathew et al., 2018). The administration of transplanted polyclonal Tregs ($CD4^+CD25^+$ T cells) derived from the thymus or peripheral tissues of the recipients and expanded *in vitro* into living donor kidney transplant recipients showed a reduction of total CD4+T and $CD8^+$ T cells and a 5–20 fold increased circulating Tregs levels after 90 days. The authors aim to move into a phase II clinical trial to test Treg infusion's efficacy for tolerance induction or drug minimization (Mathew et al., 2018). Chimeric antigen receptor transduced natural killer (CAR-NK) therapy (Liu et al., 2020), and pluripotent stem cell-derived immunosuppressive cells (macrophages) (Tsuji et al., 2020) are also investigated for use in solid organ transplantation as an alternative method of posttransplant management to improve allograft survival and minimize secondary complications. Recently, Tsuji et al. showed the successful generation of immunosuppressive cells from non-human primate ESCs that expressed several immunosuppressive molecules and significantly inhibited allogeneic mixed lymphocyte reaction (Tsuji et al., 2020). The future goal is to move into pre-clinical trials and demonstrate their potential to suppress allogeneic immune reactions against grafts derived from the same donor in transplantation models. Although advancements in surgical technique and immunosuppression regimens have progressed in transplant medicine, many limitations still exist. The chronic use of immunosuppression in transplant medicine promotes several side effects and increases the relative risk of infections, malignancy, cardiovascular morbidity, and organ damage (e.g., liver toxicity, nephrotoxicity, neurotoxicity, and diabetes mellitus). Thus, to further improve solid organ transplantation outcomes, discovering a novel immunoregulating strategy in regenerative medicine using pluripotent stem cells and engineered immune cells to enhance organ survival and tolerance is vital for the growth of transplant medicine.

Tissue engineered grafts

In tissue engineering, a combination of cells, a scaffold, and biologically active molecules are used to reconstruct or regenerate damaged tissues or whole organs. The success of tissue engineering relies on the interplay between multiple scientific disciplines such as cell biology, biomedical engineering, and material science. The identification of proper scaffolds, bioreactors, cell sources, and biomolecules such as growth factors and chemokines are needed to reconstruct or regenerate organs correctly. Currently, contrary to 2D planar tissues, bioengineering solid organs for transplantation is still challenging. Advances have been made towards identifying novel scaffolds, biomolecules, and cells, but protocols towards combining the mixture for solid organs' *de novo* reconstruction are still a limiting factor. Although scientific thinking and approaches towards fully realizing the exciting potential of whole organ engineering are still in their early phases, there have been advances in using novel technology with cell therapy to enhance tissue regeneration and function in the clinic (Table 2).

Tissue engineering is currently applied to creating alternative materials for the reconstruction of multiple organs. Ram-Liebig et al. show that manufactured tissue-engineered oral mucosa graft is safe and efficient in urethroplasty in male patients with surgically unsuccessful pretreated urethral stricture (Ram-Liebig et al., 2017). The procedure involves harvesting a small oral biopsy from the patients and sending it out to a Good Manufacturing Practice (GMP) laboratory manufacturing company, MukoCell®, where the sample is used to create a tissue-engineered oral mucosa graft for the urethroplasty. The transplant success rate was 67.3% at 12 and 58.2% at 24 months and the authors hypothesize that the success rate may be higher if the patients are initially treated with the graft from the beginning. Nonetheless, the authors show that the bulbar and penile urethra reconstruction is feasible, safe, and efficacious in a heavily pretreated population using a tissue-engineered oral mucosa graft. This study demonstrates how current tissue engineering therapies could be successfully standardized and manufactured in a company to provide a constant viable product tailored to everyone.

Clinical studies are also exploring the mechanisms of how tissue-engineered constructs cross-communicate with the diseased milieu to promote healing of a chronic wound. Stone et al. used transcriptomics to understand mechanistically how an FDA-approved bilayer living cell construct (BLCC) promotes the healing of chronic non-healing venous leg ulcers (Stone et al.,

2017). BLCC consists of a layer of the human foreskin–derived neonatal fibroblasts in a bovine type I collagen matrix under a layer of the human foreskin–derived neonatal epidermal keratinocytes. The authors identified that BLCC provides bioactive signals after transplant to the damaged tissue site to promote wound healing *via* modulation of inflammatory and growth factor signaling, keratinocyte activation, and attenuation Wnt/β-catenin signaling. This study identifies mechanistically how tissue-engineered constructs can communicate at the site of injury to promote healing (Stone et al., 2017). The use of a cardiac patch has also garnered much attention, which provides cells a proper microenvironment for tissue development and maturation (Menasché et al., 2018). Bayes-Genis et al. have shown that autologous pericardial adipose graft transplanted within patients treated with coronary artery bypass graft surgery promotes a noticeable improvement in reducing the necrotic mass-sized ventricular volumes after 1 year (Bayes-Genis et al., 2016). The authors used an autologous pericardial adipose graft directly obtained from the patients and surgical glued it in place over the necrotic zone after the coronary artery bypass. The surgeons harnessed the biological regenerative capacity of adipose tissue for patients with a chronic myocardial scar. However, no statistically significant difference was noted in necrosis size, possibly due to the limited patient numbers and the need to refine the surgical procedure (Bayes-Genis et al., 2016). Cardiac patches are also used to address the limitation in the retention and need of large cell numbers for cardiac regenerative therapy. In a phase I clinical trial, Menasche et al. assessed the safety and efficacy of transplanting human embryonic stem cell (hESC)-derived cardiovascular progenitors embedded in a fibrin patch in severe ischemic left ventricular dysfunction patients receiving a coronary artery bypass procedure (Menasché et al., 2018). The cardiac fibrin patch showed no evidence of tumor formation or arrhythmias during the 18 months follow-up. Although the feasibility of producing clinical-grade hESC-CM for transplantation was demonstrated, clinical trials assessing efficacy were not yet conducted due to the small sample size, lack of blinded assessment, and confounding effect of the associated coronary artery bypass grafting. Based on these results, there is still a need to identify the best source of stem or progenitor cells and extracellular matrix or biomaterial to promote tissue regeneration and repair in efficacy and safe manner.

Challenges and hurdles of cellular therapies

Researchers have identified how stem cell heterogeneity, due to differences in source and donor to donor variations, may limit their clinical effectiveness. Autologous (isolated from and transplanted back into the same patient) and allogeneic (isolated from a different patient) stem cells have a different beneficial therapeutic potential based on disease and organ model. Hare et al. demonstrate that although transplantation of both autologous and allogeneic BM-MSCs is safe, feasible, and beneficial when applied in chronic non-ischemic dilated cardiomyopathy (NIDCM), there are slight differences in their beneficial outcomes (Hare et al., 2017). Allogeneic BM-MSCs transplants promote a more significant improvement in functional tests like Ejection Fraction (EF), Minnesota Living with Heart Failure Questionnaire (MLHFQ), Six Minute Walk Test (6MWT), along with the better functional restoration of endothelium and reduction of pro-inflammatory cytokines (TNF-α) 6 months after transplantation compared to autologous BM-MSCs. Similarly, Bhansali et al. also show that autologous bone-marrow or mononuclear cells (MNCs) transplanted in patients with type 2 diabetes mellitus effectively reduce the need for insulin after a year (Bhansali et al., 2017). However, patients with MNC transplants showed a significant increase in second-phase C-peptide response during the hyperglycemic clamp indicating insulin production, while MSC transplanted patients had a significant improvement in insulin sensitivity index and an increase in insulin receptor substrate-1 gene expression. Thus, demonstrating the need for more informative studies to distinguish the differential beneficial effects of different cell cellular therapies. Xiao et al. also compared the efficacy of intracoronary administration of BM-MNCs or BM-MSCs for patients with dilated cardiomyopathy (DCM) (Xiao et al., 2017). After 3 months, both injections showed an improvement in New York Heart Association (NYHA) functional class and left ventricular ejection fraction (LVEF) in patients. However, after 12 months, BM-MSCs transplanted patients continued to significantly improve LVEF and NYHA, unlike BM-MNCs transplanted patients who showed a decrease in LVEF compared to their 3 months follow-up. These results suggest that BM-MNCs provided a temporary improvement in LVEF and NYHA class and only accelerate cardiac function recovery while the improvement observed following BM-MSC therapy is sustained (Xiao et al., 2017). These studies provide novel insights and a comprehensive understanding of how various cell sources and cell types may deliver different therapeutic effects based on disease. Additionally, they highlight the need to tailor stem cell therapies specific to each patient's need to enhance their regenerative potential. Further conformational studies with large, randomized, placebo-controlled clinical trials are needed to clarify the complexity of MSCs (based on origin and application) and their interaction with host tissue.

Although advancements are being made daily in cellular therapy, there are still many challenges in translating pre-clinical results regarding cellular therapy efficacy to promote tissue healing, reduce excessive inflammation, and improve the clinic's survival (Galipeau et al., 2016; Chinnadurai et al., 2018). It has been shown that not all stem cell therapies are initially beneficial. Makhlough et al. show the safety and tolerability of autologous BM-MSC transplanted into six autosomal dominant polycystic kidney disease patients but with no physiological improvement detected after 1 year (Makhlough et al., 2017).

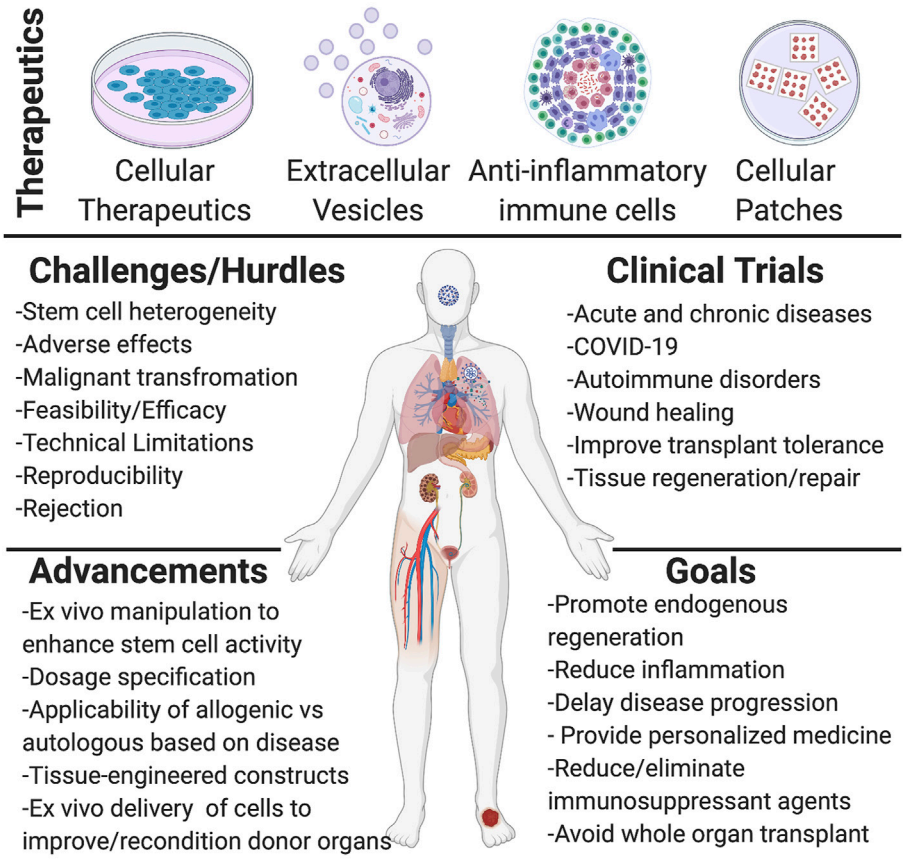

FIGURE 1
Schematic representation of regenerative medicine therapeutics and current knowledge acquired. The first half of the scheme shows the different forms of therapeutics used and investigated for regenerative medicine applications, such as different cellular materials (mesenchymal stem/stromal cells (MSCs), induced pluripotent stem cells (iPSCs), progenitor cells), extracellular vesicles (exosomes, microvesicles, and apoptotic bodies), anti-inflammatory immune cells (M2 macrophages, Treg cells, Chimeric antigen receptor transduced natural killer (CAR-NK)), and cellular patches (tissue-engineered oral mucosa graft, bilayer living cell construct, cardiac patch). The second half of the scheme shows the many challenges and hurdles currently present with cellular therapies, such as efficacy and safety and the various improvements made in regenerative medicine. A list of different targets of clinical trials is listed, along with the different overall goals from such targets. Created with BioRender.com.

Patients exhibited a continuous decrease of GFR with a significant increase in serum creatinine levels. The study was limited to only six patients, and only a single cell transplant was administered, which may partially explain the limited beneficial effects detected (Makhlough et al., 2017). Stem cell therapy's effectiveness may also be limited by the extent of chronic inflammation and fibrosis already present within the patient's damaged tissue. In patients with decompensated (severe) alcoholic liver disease, transplantation of BM-MSCs showed no modification of the disease's progression after 4 weeks (Lanthier et al., 2017) to 8 weeks (Rajaram et al., 2017). Although patients showed an elevation of liver macrophages and upregulation of regenerative liver markers (SPINK1 and HGF), no difference was detected regarding proliferative hepatocyte numbers (Lanthier et al., 2017). There are also potential safety concerns with cellular therapy, such as the potential for malignant transformation of MSCs (Steinemann et al., 2013). A long-term follow-up study of patients with decompensated (severe) alcoholic liver disease transplanted with autologous BM-derived mononuclear cells showed improved liver function and decreased collagen levels in patients' liver transiently 6 months post-transplantation (Kim et al., 2017). Patients also displayed improved biochemical parameters, CP class, and increased liver volume, indicating liver regeneration. Although improved liver function was still evident at the five-year follow-up, patients who had received cell transplantation had an alarming increased risk of developing hepatocellular carcinoma (HCC). This relatively high incidence of HCC within 2 years after autologous bone marrow cell infusion warrants further investigation (Kim et al., 2017). Other studies have also shown that a small group of hematopoietic cell transplant survivors may suffer from not

only solid tumors but also from other significant late effects such as diseases of the cardiovascular, pulmonary, and endocrine systems, dysfunction of the thyroid gland, gonads, liver and kidneys, infertility, iron overload, bone diseases, infection, and neuropsychological effects (Inamoto and Lee, 2017). The leading cause of mortality in adult patients who had received hematopoietic cell transplants includes recurrent malignancy, lung diseases, infection, secondary cancers, and chronic graft-versus-host disease. Thus, long-term risk assessment studies of patients receiving stem cell transplantation are needed to understand the risk of developing cancer and other harmful late effects versus the long-term benefits of stem cell therapy. Another limitation preventing comparison of current clinical trials' and their outcomes, is that not all clinical trials adhered to ISCT criteria in defining the cellular treatments. Moving forward, improved methodological quality, increased sample size, and extended trial duration are needed for a better comparison of clinical trial data and results amongst each study. Cossu et al. and others also emphasized the need for better science, funding models, governance, public and patient engagement to enhance cellular therapy's efficacy and safety in the clinic (Cossu et al., 2018). Regulatory limitations are another hurdle for the application of cell therapies or new technologies. With growing innovations made in regenerative medicine, outdated regulations may not adequately address new challenges posed as technology advances. Thus, new regulations must be designed to protect the patients from unnecessary risk while encouraging investigators, funding bodies, and investors to support research and development and market commercialization of novel products.

Conclusion and future directions

Stem cell and EV therapies, along with tissue engineering, aim to deliver focused, effective patient-specific treatments that provide benefits with a single rather than lifelong intervention. Some major hurdles facing regenerative medicine is the lack of complete characterization, consistency, and standardization of cellular materials and EVs (derivation, cell/EV numbers and method of transplantation) used in clinical trials. Not all published clinical trials adhere to the International Society for Cellular Therapy (ISCT) and International Society for Extracellular Vesicles (ISEV) guidelines (Table 2). This inconsistency limits our ability to compare and contrast study outcomes and hinders the field's progression. Although there remains challenges and uncertainties with these therapies, their potential in regenerative medicine is undeniable, and the implications of this field remain great. Improvements are continually being made to understand and utilize stem cell therapies for regenerative medicine specific to tissue type, disease, and inflammatory state along with understanding of dosage effectiveness, methods for better scalability, and guidelines for improved characterization (Figure 1). Many hurdles and limitations still exist in tissue and organ engineering, hampering researchers from the ultimate goal of whole organ generation *ex vivo*. However, the combination of novel technologies and cell-based therapies to replace, restore, or rejuvenate organ function remains appealing to investigators. The commercialization of tissue-engineered grafts for surgical use is shown to be a viable option for future clinical applications, which 1 day may provide an efficacious and innovative patient-specific therapy towards the treatment of diseases, organ loss, or damage. Thus, we can provide a more consistent and effective form of therapy by identifying and pointing out the limitations and hurdles facing regenerative medicine along with novel technologies and informative studies.

Impact statement

Regenerative medicine aims to deliver focused, effective patient-specific treatments with lifelong benefits. Clinical trials have shown some limitations, challenges, and uncertainties with regenerative therapies; however, the field's potential and implications remain great. Clear identification of the limitation and hurdles of regenerative medicine applications combined with the design and use of novel technologies/techniques, extracellular vesicles, and cell-based therapies to replace, restore, or rejuvenate organ function drives the future toward addressing the limitations of regenerative medicine and designing effective patient-specific treatments.

Author contributions

This manuscript is a work product of the American Society of Transplantation's Transplant and Regenerative Medicine Community of Practice (TRM-COP). AP and GO: Conceptualization, reviewed literature, writing-original draft preparation, and editing. PM, KM, BU, and VG: Revising manuscript, review, and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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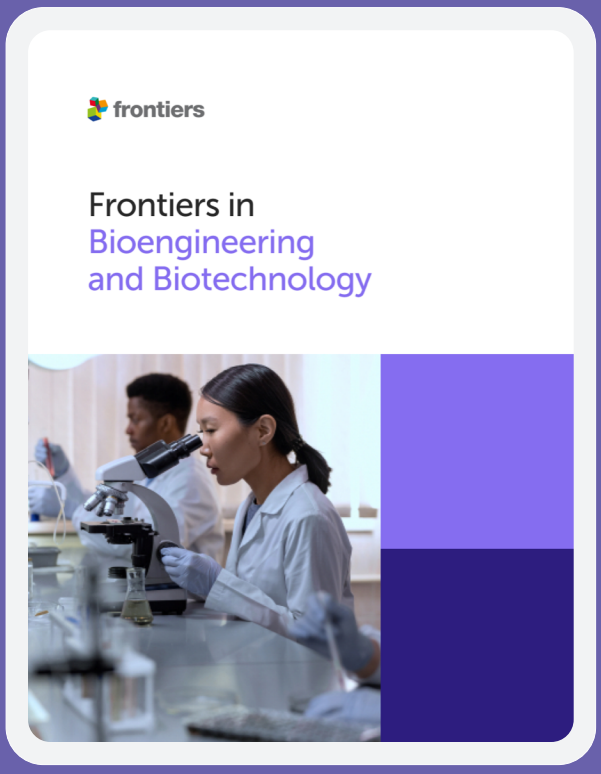

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