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Kursad Turksen *Editor*

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Tissue Differentiation, Repair in Health and Disease

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Editor

Cell Biology and Translational Medicine, Volume 18

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Preface

In this next volume in the Cell Biology and Translational Medicine series, we continue to explore the potential utility of stem cells in regenerative medicine. Amongst topics explored in this volume are regulatory aspects of stem cells, differentiation, maintenance and repair in both health and disease. One goal of the series continues to be to highlight timely, often emerging, topics and novel approaches that can accelerate stem cell utility in regenerative medicine. Amongst, CRISPR, COVID-19-related regulation and editing of stem cell function and potential stem cell-mediated therapeutic options are such timely topics included here.

I remain very grateful to Gonzalo Cordova, the Publishing Editor of the series, and wish to acknowledge his continued support.

A special thank you goes to Shanthi Ramamoorthy and Anju Baskar for their outstanding efforts in the production of this volume.

Finally, sincere thanks to the contributors not only for their support of the series, but also for their willingness to share their insights and all their efforts to capture both the advances and the remaining obstacles in their areas of research. I trust readers will find their contributions as interesting and helpful as I have.

Ottawa, ON, Canada — Kursad Turksen

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Mitochondrial Permeability Transition in Stem Cells, Development, and Disease

Sandeep P. Dumbali and Pamela L. Wenzel

Abstract

The mitochondrial permeability transition (mPT) is a process that permits rapid exchange of small molecules across the inner mitochondrial membrane (IMM) and thus plays a vital role in mitochondrial function and cellular signaling. Formation of the pore that mediates this flux is well-documented in injury and disease but its regulation has also emerged as critical to the fate of stem cells during embryonic development. The precise molecular composition of the mPTP has been enigmatic, with far more genetic studies eliminating molecular candidates than confirming them. Rigorous studies in the recent decade have implicated central involvement of the F_1F_o ATP synthase, or complex V of the electron transport chain, and continue to confirm a regulatory role for Cyclophilin D (CypD), encoded by *Ppif*, in modulating the sensitivity of the pore to opening. A host of endogenous molecules have been shown to trigger flux characteristic of mPT, including positive regulators such as calcium ions, reactive oxygen species, inorganic phosphate, and fatty acids. Conductance of the pore has been described as low or high, and reversibility of pore opening appears to correspond with the relative abundance of negative regulators of mPT such as adenine nucleotides, hydrogen ion, and divalent cations that compete for calcium-binding sites in the mPTP. Current models suggest that distinct pores could be responsible for differing reversibility and conductance depending upon cellular context. Indeed, irreversible propagation of mPT inevitably leads to collapse of transmembrane potential, arrest of ATP synthesis, mitochondrial swelling, and cell death. Future studies should clarify ambiguities in mPTP structure and reveal new roles for mPT in dictating specialized cellular functions beyond cell survival that are tied to mitochondrial fitness including stem cell self-renewal and fate. The focus of this review is to describe contemporary models of the mPTP and highlight how pore activity impacts stem cells and development.

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Keywords

Adenine nucleotide translocator · ATP synthase · Calcium signaling · Cyclophilin D · Cyclosporin A · Differentiation · Mitochondrial permeability transition pore · NIM811 · Oxidative phosphorylation · Reactive oxygen species · Stem cells

Abbreviations

AHS	Alpers-Huttenlocher Syndrome
ANT	Adenine nucleotide translocator
CsA	Cyclosporin A
CypD	Cyclophilin D
ETC	Electron transport chain
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
MCU_{cx}	Mitochondrial calcium uniporter complex
mPT	Mitochondrial permeability transition
mPTP	Mitochondrial permeability transition pore
NIM811	(Melle-4)cyclosporin
OMM	Outer mitochondrial membrane
OSCP	Oligomycin sensitive conferring protein subunit
OXPHOS	Oxidative phosphorylation
Pi	Inorganic phosphate
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TCA	Tricarboxylic acid cycle
VDAC	Voltage-dependent anion channel

1 Introduction

The mitochondrial permeability transition (mPT) is a process wherein strict barrier function of the inner mitochondrial membrane (IMM) is abruptly lost. Under homeostasis, the IMM is impermeable, comprised of a specialized system of transporters and exchangers that restrict and tightly regulate the movement of molecules, ions, and metabolites between matrix and intermembrane space (Zorov et al. 2009). Mitochondrial function depends upon the electric insulation provided by the IMM to maintain a proton gradient sufficient for generation of mitochondrial membrane potential ($\Delta\Psi_m$) and ATP production (Mitchell 1966). Although precise molecular composition remains debated, an entity referred to as the mitochondrial permeability transition pore (mPTP) is implicated in regulating the mPT and is sensitive to endogenous molecules, such as inorganic phosphate, adenine nucleotides, ROS, fatty acids, nitric oxide, H^+, Mg^{2+}, and Ca^{2+} (Antoniel et al. 2018; Chernyak and Bernardi 1996; Costantini et al. 1996; Furuno et al. 2001; Halestrap and Pasdois 2009; Halestrap et al. 2004; Haworth and Hunter 1979; Hunter and Haworth 1979a, b; Kowaltowski et al. 1996, 1998; Więckowski et al. 2000). Opening of the pore permits the passage of any small molecular weight solutes <1.5 kDa between the matrix and intermembrane space (IMS), resulting in depolarization of the IMM. Dissipation of $\Delta\Psi_m$ caused by pore opening uncouples electron transport from the phosphorylation required for ATP synthesis and also disrupts other $\Delta\Psi_m$-dependent activities such as mitochondrial protein import (Bonora et al. 2013; Halestrap et al. 2002). Various mitochondrial matrix metabolites, such as Ca^{2+}, NAD^+, glutathione, and ROS, are released into the intermembrane space. This breakdown of barrier function can lead to mitochondrial swelling and Bax/Bak-dependent OMM herniation that permits extrusion of matrix contents into the cytosol (Karch et al. 2013; McArthur et al. 2018). Release of matrix proteins and other metabolites into the cytoplasm can destabilize cellular homeostasis and amplify oxidative damage to proteins, nuclear DNA, ion channels, transporters, and membrane phospholipids (Zorov et al. 2014). Unrestrained, mPT can initiate cell death, but mPT is reversible and can produce transient flickering of IMM permeability when balance within the cellular environment is tipped toward negative regulation of mPT (Boyman et al. 2019).

Modulation of the mPTP is documented in embryonic development, normal physiology, disease, and injury. In the context of disease and

injury, pathological opening of the mPTP can be triggered by an excess of endogenous molecules that cause oxidative stress and mitochondrial Ca^{2+} overload (Biasutto et al. 2016; Zoratti and Szabò 1995). If conditions favorable to mPT are sustained, imbalance in matrix-IMS composition leads to mitochondrial swelling, breakdown of the outer mitochondrial membrane (OMM), and release of mitochondrial solutes that induce cell death and other pathologies (Karch and Molkentin 2014; Suh et al. 2013). Substantial evidence supports that opening of the mPTP worsens outcomes following ischemia-reperfusion injury of cardiac and neural tissues (Bonora et al. 2020; Halestrap and Richardson 2015; Hausenloy et al. 2020). Indeed, a large number of clinical trials have been designed around pharmacological inhibition of the mPTP and/or modulation of mitochondrial functions; yet, despite success in preclinical models, the vast majority have failed to produce improvement in clinical outcomes (Bonora et al. 2022; Carrer et al. 2021; Singh et al. 2021). Pharmacological inhibition of mPT does appear to provide protection in ischemia-reperfusion injuries (Leger et al. 2011; Matsumoto et al. 2018; Rekuviene et al. 2017) and surgery (Chiari et al. 2014). But, true benefit has yet to be definitively demonstrated in clinical trials for many ischemia-reperfusion injuries (Bøtker et al. 2020; Upadhaya et al. 2017). Beyond injury, opening of the pore is implicated in exacerbating metabolic diseases, including diabetes, thus targeting the mPTP could be considered for these disorders as well (Taddeo et al. 2014). Prolonged mPT activity is most frequently associated with pathophysiology, yet transient mPT could also play roles in cardiac development and recovery from injury (Elrod et al. 2010; Hausenloy et al. 2004; Korge et al. 2011), synaptic plasticity and efficacy (Mnatsakanyan et al. 2017), and in homeostasis of physiological Ca^{2+} levels (Bernardi and von Stockum 2012). A growing body of evidence in stem cells indicates that mPTP closure is central to self-renewal, fate commitment, cell survival, metabolic reprogramming, and regenerative response to stress (Pérez and Quintanilla 2017). Several excellent recent reviews cover in great depth the known endogenous and pharmacological inducers and inhibitors that could be leveraged for mPTP drug design, as well as mPTP regulation by post-translational modifications and signaling networks (Alves-Figueiredo et al. 2021; Bonora et al. 2022; Carrer et al. 2021; Morciano et al. 2021). Unclear is how targeting of the mPTP in stem cells could be leveraged for therapeutic use. In this review, we describe current knowledge regarding regulation of this enigmatic pore and focus on its role in stem cells and development.

2 Ca^{2+}-induced Mitochondrial Permeability Transition

Opening of the mPTP is highly dependent upon mitochondrial Ca^{2+}. Shuttling of Ca^{2+} between the extracellular, cytosolic, and mitochondrial compartments is vital to mitochondrial function and can communicate life or death signals via the mPT (Giorgi et al. 2008). Specialized channels in the OMM and IMM control accumulation of Ca^{2+} within the mitochondrial matrix (Nicholls 2005). Flux of Ca^{2+} between the cytosol and the IMS is chiefly mediated by the beta-barrel voltage-dependent anion channel (VDAC) super-family, a high abundance mitochondrial porin localized to the OMM (Zeth and Zachariae 2018). These porins serve as gatekeeper to small ions and high molecular weight metabolites, facilitate the exit of millions of molecules of ATP per second from the mitochondria, and regulate cell death through association with pro- and anti-apoptotic proteins (Choudhary et al. 2014; Noskov et al. 2016). Their role in the formation of the mPTP is thought to stem from transport of Ca^{2+} and ATP/ADP across the OMM rather than as a core component or accessory factor of the mPTP (McCommis and Baines 2012). From the IMS, Ca^{2+} crosses the highly impermeant IMM into the matrix through the selective mitochondrial calcium uniporter complex, MCU_{cx}, and is extruded by Na^+/Ca^{2+} and H^+/Ca^{2+} exchangers (Filadi and Greotti 2021; Garg et al. 2021; Palty et al. 2010; Villa et al. 1998). Within the matrix, Ca^{2+} modulates the activity of enzymes important for ATP synthesis,

making Ca^{2+} a vital messenger between the cell's bioenergetic demand and the mitochondria's ability to supply "free energy". Yet, pathological conditions that favor excess sequestration of Ca^{2+} in the matrix, resulting in Ca^{2+} overload, can initiate mPTP opening and trigger a cascade leading to cell death. The mPTP itself does not appear to be important for Ca^{2+} efflux under stress although evidence supports its role in homeostasis via transient pore opening (Bernardi and Petronilli 1996; Lu et al. 2016; De Marchi et al. 2014).

Fundamental understanding of the mPT was first established from experiments conducted with mitochondrial isolates or permeabilized cells where increasing concentrations of Ca^{2+} (and other modulators) could be loaded to trigger mPTP opening (Bernardi et al. 2006; Rasola and Bernardi 2007). Yet, cells are equipped with sophisticated machinery designed to protect them from excess extracellular Ca^{2+} associated with physiological stresses (Giorgi et al. 2018; Nicholls 2005); thus, experimentation in vivo has required the use of compounds capable of modulating Ca^{2+} transport and the mPTP in intact cells (Carrer et al. 2021). Endogenous molecules, ATP and potassium chloride (KCl), elevate mitochondrial Ca^{2+} by stimulation of efflux of Ca^{2+} stores from the endoplasmic reticulum and influx of extracellular Ca^{2+} via voltage-gated Ca^{2+} channels on the plasma membrane, respectively (Barsukova et al. 2011). The Ca^{2+} ionophore ionomycin (A23187) is also a popular compound used to trigger the mPT because it increases cytoplasmic and mitochondrial Ca^{2+} by formation of lipid-soluble complexes with Ca^{2+} that enable its transport across endoplasmic reticulum and plasma membranes (Pressman 1976). In contrast, phenothiazines, anesthetics, and divalent cations Mg^{2+}, Ba^{2+}, and Sr^{2+}, generally delay or inhibit sensitivity of the mPTP to opening by competing with Ca^{2+} for binding sites or obstructing Ca^{2+} influx (Zoratti and Szabò 1995). Many modifiers of mPT exert their activity via modulation of the sensitivity of mPTP core components and regulatory factors to Ca^{2+} (Bernardi et al. 2015); nevertheless, some cellular states favor mPTP activation independently of Ca^{2+} concentration. For example, because the mPTP is a voltage-gated channel, loss of mitochondrial membrane potential alone can trigger its opening (Bernardi 1992). Thus, precise understanding of the mPT has required careful isolation of the effects of matrix Ca^{2+}, membrane potential, and other mechanisms thought to regulate the conformational change of mPTP components.

3 Reactive Oxygen Species and Other Inducers of mPT

A host of endogenous and pharmacological compounds increase the sensitivity of the mPTP to opening (Zoratti and Szabò 1995). The most well-studied inducers include Ca^{2+} (detailed above), inorganic phosphate (Pi), protonophores, oxidizing agents, and reactive oxygen species (ROS). Respiratory complexes and other mitochondrial enzymes are responsible for the vast majority of oxidative stress in the cell, though reactive nitrogen species produced by nitric oxide synthase are also important sources of oxidative stress that can trigger mPT (Briston et al. 2017; Kaludercic and Giorgio 2016; Vieira et al. 2001; Vorobjeva et al. 2020; Zorov et al. 2014). Indeed, the mPTP is generally sensitized by oxidants and desensitized by antioxidants that directly scavenge free radicals or otherwise increase antioxidant defenses (Chernyak and Bernardi 1996; Costantini et al. 1996; Kaludercic and Giorgio 2016; Kowaltowski et al. 1998; Petronilli et al. 1994). Early studies found that exposure of mitochondria to oxidizing agents activates opening of the pore, as by oxidation of mitochondrial glutathione with the ROS donor *tert*-butyl hydroperoxide (TBHP) or cross-linking of dithiols with arsenite oxide to produce oxidized pyridine nucleotide pools (NAD^+, $NADP^+$) (Chernyak and Bernardi 1996; Connern and Halestrap 1994; Petronilli et al. 1994). In contrast, sensitivity of the mPTP could be reversed by the reducing agent dithiothreitol. More recently, antioxidants have been designed to be targeted to the IMM to improve ROS scavenging and removal. Some of these, including the cardiolipin-binding peptide elamipretide (Szeto-

Schiller 31 (SS-31), MTP-131, or Bendavia) and the compounds astaxanthin and MCI-186, potently inhibit mPT and prevent mitochondrial swelling and depolarization in isolated mitochondria and cell cultures (Baburina et al. 2019; Rajesh et al. 2003; Szeto 2006; Zhao et al. 2004).

Not coincidentally, Ca^{2+} is a major contributor to ROS generation (Kanno et al. 2004). Mitochondrial Ca^{2+} alters activity of several dehydrogenases in the tricarboxylic acid cycle (TCA) and activity/conductance of complexes I, III, IV, and V in OXPHOS (Glancy et al. 2013; Territo et al. 2000). Thus, by perturbation of TCA and electron transport chain (ETC) enzyme activity, excess mitochondrial Ca^{2+} can lead to build up of reduced NADH and greater probability of electrons moving from ETC complexes to O_2 to form superoxide radicals (Bertero and Maack 2018). Several ETC complexes, including complexes I, II, and III, and oxidoreductases of the TCA cycle have been directly implicated in ROS production associated with mPT (Batandier et al. 2004; Bonke et al. 2016; Korge et al. 2017a, 2017b). It is not surprising then that several inducers that trigger mPTP via ROS generation do so in conjunction with elevation of matrix Ca^{2+} and/or alteration of OXPHOS (Baumgartner et al. 2009; Davidson et al. 2012; Gu et al. 2015; Hansson et al. 2008; Hou et al. 2013; Krestinin et al. 2020; Lindsay et al. 2015). Yet, some studies also suggest that ROS generation can occur independently of Ca^{2+} dysregulation, with potential consequences for mPT (see discussion below).

Several studies have also demonstrated feedback between ROS production and mPTP opening, though much debate surrounded whether ROS acted independently of membrane potential to trigger mPTP opening (Zorov et al. 2014). In a seminal study of ROS and mPT, Zorov and colleagues observed a phenomenon they described as ROS-induced ROS release that could be inhibited by blocking mPT (Zorov et al. 2000). Specifically, photoexcitation of TMRM-loaded mitochondria induced production of superoxide anion radical ($\cdot O_2$-) and hydroxyl radical (·OH), which further promoted a burst of ROS production. This secondary burst of ROS was accompanied by a collapse in membrane potential. Importantly, compound-based inhibition of mPTP or complex I of the ETC reduced magnitude of the ROS burst, strongly supporting that ROS was derived from the ETC and depended upon mPT. Likewise, scavenging of ROS or use of superoxide dismutase (SOD) mimetics inhibited mPT, highly suggestive of positive feedback between ROS signaling and opening of the mPTP. In this study, ROS appeared to be wholly responsible for opening of the mPTP, as chelation of Ca^{2+} or inhibition of the MCU_{cx} had no effect on ROS-induced mPT. Subsequent studies corroborated these findings, confirming that opening of the mPTP elevates ROS production (Batandier et al. 2004; Maciel et al. 2001). Interest in this area continues with more recent work showing that enforced opening of the mPTP contributes to elevated ROS production in cultured myofibers, thereby activating caspase-3 and subsequent cell death (Burke et al. 2021). In summary, oxidative stress and Ca^{2+} work in concert with other inducers to sensitize the mPTP to opening.

4 Negative Regulators of mPT

Negative regulators of the mPT have attracted great attention for their potential therapeutic value. Endogenous molecules that desensitize the mPTP and thus generally increase the levels of Ca^{2+} needed to stimulate mPTP opening include divalent cations (Mg^{2+}, Mn^{2+}, Ba^{2+}, Sr^{2+}), K^+, Na^+, protons (H^+)/acidic pH, adenine nucleotides (ADP, AMP, ATP), creatine, and reduced pyrimidines (NADH) (Bernardi et al. 1992; Dolder et al. 2003; Haworth et al. 1980; Hunter and Haworth 1979b; Qian et al. 1997; Szabo et al. 1992). Elevated membrane potential, corresponding with energization of mitochondria, also prevents mPT (Hunter et al. 1976). A number of compounds have been found to inhibit pore opening, some without known mechanism of action (ER-000444793, triazoles TR001 and TR002) and others thought to sequester CypD (CsA and analogs (NIM811, JW47), Debio025,

sanglifehrin A, 7I6, and cyclophilin inhibitor 1), inhibit ANT (bongkrekic acid and cinnamic anilides GNX-4728 and GNX-4975), bind to TSPO (TRO40303), block ETC activity (oligomycin and derivatives), interrupt fatty acid availability (nupercaine and tetracaine), or scavenge ROS (elamipretide, astaxanthin, and MCI-186). Recent reviews describe these pharmacological compounds in detail, along with disease applications and known targets/mechanism of action (Bonora et al. 2022; Carrer et al. 2021; Morciano et al. 2021). Identification of negative regulators of the mPTP remains an area of intense interest as development of inhibitory molecules of mPT could offer new therapeutic options for treatment of ischemic-reperfusion injuries and will no doubt be aided by better understanding of the proteins that comprise and regulate the channel.

5 Molecular Composition of the mPTP

The precise molecular composition of the mPTP has been enigmatic, with far more genetic studies eliminating molecular candidates than confirming them. Rigorous studies in the recent decade continue to confirm a chief regulatory role for Cyclophilin D (CypD) in modulating the sensitivity of the pore to opening and have implicated central involvement of the F_1F_o ATP synthase in classical mPTP high-conductance behavior. Recently suggested is that members of the adenine nucleotide translocator (ANT) family serve as a pore-forming component in what may be an independent low-conductance channel, raising the possibility that two distinct mPTP channels comprised of different structural components mediate response to Ca^{2+} and other inducers (Bround et al. 2020; Karch et al. 2019) (Fig. 1). Full conductance channels are thought to produce long-lasting mPTP permeability resulting in mitochondrial swelling and cell death; whereas, the low conductance channels transiently release Ca^{2+} and rapidly restore IMM barrier function. Several other proteins have been tested for their contributions to mPT but have largely been excluded as core components. These include the PiC in the IMM, mitochondrial creatine kinase (mtCK) in the IMS, and hexokinase (HK), translocator protein of 18 kDa (TSPO), and VDAC in the OMM. Current models incorporate them as regulators or accessories to transport of mPTP modulators (Baines et al. 2007; Dolder et al. 2003; Gutiérrez-Aguilar et al. 2014; Krauskopf et al. 2006; Kwong et al. 2014; Šileikyte et al. 2014; Vyssokikh and Brdiczka 2003; Whittington et al. 2018). Recent expert reviews of contemporary models of the mPTP are available that provide comprehensive discussion of the critical roles that upstream signaling pathways and post-translational regulation of mPTP core components play in the modulation of pore activity (Alves-Figueiredo et al. 2021; Bonora et al. 2022; Morciano et al. 2021). Indeed, the dynamic conformational change of proteins required to control opening and closing of the pore appears to depend upon post-translational modifications, such as phosphorylation, acetylation, S-nitrosylation, S-glutathionylation, sulfenylation, nitration, deamination, succinylation, and ubiquitination (Alves-Figueiredo et al. 2021). Thus, composition of key components will be only briefly summarized here.

CypD is a mitochondrial matrix protein encoded by *Ppif* that serves as a chief regulatory component of the mPTP (Elrod and Molkentin 2013). CypD is poised to orchestrate a host of activities as scaffold to a large number of signaling and structural proteins and through its effects on ETC complex activity and regulation of synthasome assembly; yet, it is most widely appreciated for its regulation of the mPTP (Beutner et al. 2017; Porter and Beutner 2018). CypD is not a core component of the channel nor does it fundamentally alter pore properties but it does sensitize the pore to opening (Baines et al. 2005; De Marchi et al. 2006). CypD was the first protein identified that firmly established mPTP as a bona fide molecular entity and not simply an artifact of IMM breakdown due to lipid phase, as part of early work identifying CypD as the target of an immunosuppressant capable of suppressing Ca^{2+} and ROS-induced mPT, cyclosporin A

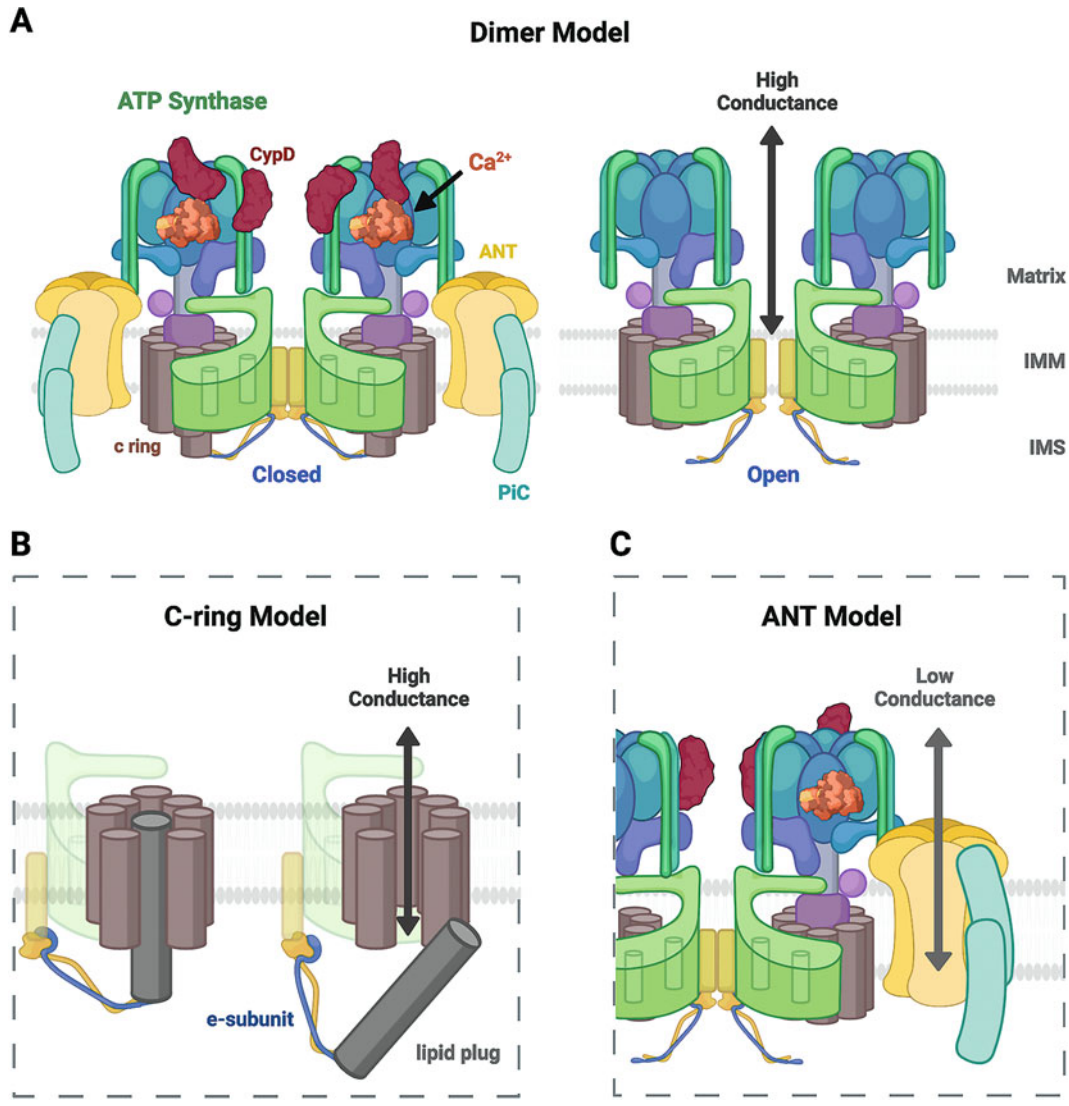

Fig. 1 Models of mPTP structural composition and conformational change. Current working models of the mPTP include a high-conductance channel formed by (**a**) ATP synthase dimers or (**b**) the c-ring component of monomeric ATP synthase. (**c**) In addition, literature suggest that an independent low conductance channel is formed by ANT

(CsA) (Broekemeier et al. 1989; Crompton et al. 1988; Halestrap and Davidson 1990; Halestrap et al. 1997; Tanveer et al. 1996). Although CypD's mechanism of action is still largely unknown, it has been shown that CypD-binding partners include components of the mPTP, including the F_1F_o ATPase oligomycin sensitive conferring protein subunit (OSCP) (Giorgio et al. 2009). Also proposed is that CypD normally occupies and thus masks an inhibitory phosphate-binding site within the mPTP (Basso et al. 2008). This site is exposed when CypD is displaced by CsA treatment or by *Ppif* knockout, thereby decreasing mPTP sensitivity to inducers such as Ca^{2+} and ROS. Indeed, a large body of research has leveraged the *Ppif* knockout mouse and CsA's ability to modify mPTP sensitivity to understand mPTP composition, modulators, and

participation in Ca^{2+} handling and cell death. In fact, the first studies of *Ppif* knockout mice provided some of the most compelling support for the contribution of CypD to regulation of mPT (Baines et al. 2005; Nakagawa et al. 2005). Ablation of CypD conferred mitochondria with resistance to depolarization and protection from cell death following Ca^{2+} overload or H_2O_2-induced oxidative stress. Further, research from the Molkentin group found that cardiac-specific transgenic mice overexpressing CypD were more susceptible to mitochondrial swelling and cell death, two hallmarks of mPT (Baines et al. 2005). These studies also demonstrated that CsA-sensitive mPT and CypD were responsible for regulating necrotic cell death induced by ROS and Ca^{2+} overload but that apoptotic cell death remained intact in *Ppif* null cells. Additionally, some of the first in vivo studies to suggest a physiologic role for the mPTP in maintenance of Ca^{2+} homeostasis came from investigation of the *Ppif* null mouse. With sustained exercise, *Ppif* knockout resulted in heart failure, which was attributed to impaired Ca^{2+} efflux from the mitochondrial matrix and associated activation of Ca^{2+}-dependent dehydrogenases (Elrod et al. 2010). This increased dehydrogenase activity caused by elevated matrix Ca^{2+} impaired the heart's ability to utilize fatty acids as a mitochondrial fuel source, thus limiting metabolic flexibility necessary to adapt to increased bioenergetic demand under exercise-induced stress. These observations were significant to the mPTP field and pointed to a possible role for mPTP in permitting efflux of Ca^{2+} out of the mitochondria to prevent Ca^{2+} overload via the "flickering" or transient opening of the mPTP, which was first described a decade earlier (Petronilli et al. 1999). Interestingly, these data are also consistent with early reports of CsA's effect on respiration, noting that CsA caused a large accumulation of Ca^{2+} in the mitochondrial matrix (Fournier et al. 1987; Jung and Pergande 1985). Importantly, CypD is implicated as a regulator in most models of the mPTP, and its role in mPTP sensitization has served as a unifying litmus test for interrogating mPTP activity since the earliest observations of mPT.

ATP synthase, or mitochondrial complex V of the electron transport chain (EC 7.1.2.2), is a conserved enzyme of the IMM, and its primary function is synthesis of ATP from adenosine diphosphate (ADP). Beyond its role in conversion of electrochemical potential of the mitochondrial membrane to ATP, ATP synthase is now also believed to serve as a key structural component of the high-conductance mPTP (Morciano et al. 2015). ATP synthase consists of the water-soluble F_1 domain that extends into the mitochondrial matrix and the highly polar F_o domain integral to the IMM (subunits c, e, f, and g) (Spikes et al. 2020). The F_1 domain is comprised of a catalytic portion of alpha-beta trimers and a regulative OSCP. Though several models for assembly of an ATP synthase pore exist, two of these warrant comment here. The first model hypothesizes that channel formation at the interface between two ATP synthase monomers serves as the mPTP conducting core (Fig. 1a). The first study to suggest this model demonstrated that ATP synthase dimers, but not monomers, conducted current activated by Ca^{2+} and oxidizing agents when introduced into artificial planar lipid bilayers (Giorgio et al. 2013). mPTP activity dependent upon this dimer model has since been corroborated by other studies, including extension of the concept to pinpoint a critical interface between subunit g and subunit e of two interacting monomers (Carraro et al. 2018; Giorgio et al. 2017). Yet, other studies have argued that monomeric ATP synthase is sufficient to produce mPTP activity (Mnatsakanyan et al. 2019). Evidence support another model in which subunit c of F_o ATP synthase is the core (Alavian et al. 2014; Azarashvili et al. 2014; Bonora et al. 2013) (Fig. 1b). In this model, mPTP forms at sites of ATP synthase dimers but requires dissociation of the dimers to open (Bonora et al. 2017). Detachment of F_1 domains is thought to trigger a conformational change within ATPase. A possible mechanism is that subunit e on the IMS aspect of the IMM interacts with a polar ring-like pore formed across the IMM by subunit c (Pinke et al. 2020). Deformation of the e subunit is proposed to cause the release of a lipid plug that moves out of the barrel-like c-ring, thereby enabling passage

of solutes from the matrix into the IMS. This model is still debated, however, as genetic disruption of the c subunit has produced conflicting results. For example, depletion of the three genes that encode the c subunit, *ATP5MC1*, *ATP5MC2*, and *ATP5MC3* (previously *ATP5G1*, *ATP5G2*, and *ATP5G3*), was shown to reduce OMM rupture, loss of membrane potential, and cell death by mPTP inducers like ionomycin and H_2O_2 (Bonora et al. 2013). Consistent with a role for the c subunit, transient overexpression of *ATP5MC1* amplified mPT response. An independent group also deleted *ATP5MC1*, *ATP5MC2*, and *ATP5MC3,* but observed intact mPTP activity, arriving at the conclusion that the c subunit (and other F_o subunits involved in proton translocation, A6L and a) were dispensable (He et al. 2017). Moreover, simulations of c-ring structure from two species, *S. cerevisiae* and *B. pseudofirmus*, predicted that the lumen of the c ring is highly hydrophobic, rendering it unlikely to be capable of permitting passage of cations typical of the mPT (Zhou et al. 2017) though more recent cryo-EM may address some of these concerns as the ring appears to distort and widen upon ATPase exposure to Ca^{2+} (Pinke et al. 2020). Neither model is invulnerable to criticism, and some incongruency could be due to complexities of independent mPTP channels. For instance, the observation that loss of the c subunit produces CsA-sensitive channel activity distinct from classical mPT lends support to the notion that CypD-regulated pores with differing conductance exist (Neginskaya et al. 2019).

The adenine nucleotide transporter (ANT) was one of the first components proposed to serve a structural role in the mPTP and has since emerged as a key candidate for the core of an independent low-conductance channel (Fig. 1c). Four ANT isoforms have been identified in human (ANT1–4) and three in mice (Ant1, Ant2, and Ant4) (Ellison et al. 1996; Lim et al. 2011; Rolland et al. 2011; Schiebel et al. 1993). ANT isoforms are encoded by distinct loci in the genome, and their unique expression profiles depend upon tissue type, developmental stage, and cell cycle status (Brower et al. 2007; Cozens et al. 1989; Karch et al. 2019; Ku et al. 1990; Li et al. 1989; Lunardi et al. 1992; Schiebel et al. 1993; Stepien et al. 1992). ANT2 (*SLC25A5*) and ANT3 (*SLC25A6*) are ubiquitously expressed in many cell types (Karch et al. 2019; Stepien et al. 1992; Torroni et al. 1990). ANT1 (*SLC25A4*) is expressed in the heart, muscle, and brain; whereas, ANT4 (*SLC25A31*) is restricted to embryonic stem cells, germ cells, embryonic ovaries, and testis (Karch et al. 2019; Li et al. 1989; Lim et al. 2011; Rolland et al. 2011). Early studies implicated ANT in mediating the mPT and indicated that ANT regulates contact sites between the IMM and OMM, positioning ANT to mediate flux across mitochondrial membranes (Bücheler et al. 1991; Halestrap and Davidson 1990; LêQuôc and LêQuôc 1988). ANT later appeared in protein fractions that could generate complexes in artificial proteoliposomes containing mPTP activity (Beutner et al. 1996, 1998). Importantly, pull-down assays designed to identify CypD-binding proteins revealed that ANT and VDAC bound to CypD and, when these fractionated complexes were reconstituted into liposomes, they recapitulated properties of the mPTP in a CsA-dependent manner (Crompton et al. 1998). Yet, the first study of ANT knockout mice showed that Ant1;Ant2-doubly deficient cells retained functional mPTP, leading to exclusion of ANT as an essential unit of the pore (Kokoszka et al. 2004). Important work by Molkentin's group has shown that loss of one isoform results in upregulation of the others, demonstrating that ANT isoforms can compensate for one another and express in multiple tissue types even when atypical for the isoform, with the exception of Ant4 (Karch et al. 2019). Their recent triple knockout of all mouse ANT isoforms, Ant1, Ant2, and Ant4, has since invited speculation that ANT contributes to low-conductance mPTP activity (Karch et al. 2019). This study demonstrated through comparisons of membrane depolarization, Ca^{2+} retention, and mitochondrial swelling that mitochondria lacking all isoforms of ANT were desensitized to Ca^{2+}-induced mPTP opening but that mPTP activity was still present at high levels of Ca^{2+}. Triple-null mitochondria treated with CsA showed no mPTP activity, as measured by

mitochondrial swelling, suggesting that remaining pore activity was dependent upon CypD. Indeed, quadruple deficiency of Ant1, Ant2, Ant4, and CypD *(Ppif*$^{-/-}$*)* confirmed that all mPTP activity could be accounted for by the ANT family members and CypD. Patch clamping currents of the IMM further revealed that the conductance properties of Ant-triply deficient mitoplasts (mitochondria stripped of the OMM), contained fewer pores that were relatively unresponsive. These data were the first indication that two separate pores might comprise total mPTP activity, one of which was composed of ANT family members and another highly dependent upon CypD (likely representing a complex with ATP synthase depicted in Fig. 1).

6 mPTP in Stem Cells and Development

A growing body of literature has reported the critical importance of metabolism in stem cell biology (Shyh-Chang and Ng 2017). The mPT is implicated in modulating metabolism during cellular differentiation of various stem cells, including pluripotent stem cells and those of the cardiac, neural, hematopoietic, endothelial, and hepatic lineages. Diversity in metabolic requirements of different tissue lineages and cell types makes universal conclusions about how the mPTP dictates stemness and fate choice unlikely, but generally an open state correlates with greater multi−/pluripotency (Fig. 2). Mechanistically, several studies demonstrate that the mPTP shapes stem cell bioenergetics by altering mitochondrial network maturation, inner membrane complexity, OXPHOS, matrix Ca^{2+}, and superoxide flashes. Reports detailed below suggest that closing of the mPTP promotes the mitochondrial maturation necessary to meet elevated OXPHOS demands typical in a variety of differentiating stem cells. Still other studies suggest that regulatory components of the mPTP promote glycolysis as the dominant bioenergetic pathway. For example, when *Ppif* was deleted to desensitize the mPTP, matrix Ca^{2+} was elevated and TCA activity was amplified, favoring glycolytic glucose metabolism over mitochondrial OXPHOS (Tavecchio et al. 2015). CypD is known to exert direct effects on metabolism via transcription of mtDNA-encoded dehydrogenases and ETC enzymes, modulation of oxygen consumption, and regulation of metabolic pathways; thus, some differing conclusions regarding the effects of mPTP inactivation on metabolism could be due to mPTP-independent mechanisms and should be considered with caution (Menazza et al. 2013; Radhakrishnan et al. 2015; Tubbs et al. 2014). The vast majority of studies reveal roles for closing of the mPTP in cellular differentiation, many of which are detailed below. Importantly, one study has shown the reverse, that reprogramming of somatic cells to a pluripotent state can be enhanced by transient enforced mPTP opening (Ying et al. 2018, 2021). Opening of the mPTP triggers a mitochondrial ROS/miR-101c pathway that leads to epigenetic alteration (demethylation) at promoters of pluripotency genes, leading to greater chromatin accessibility. Thus, in addition to playing roles in stem cell differentiation, self-renewal, and survival, the mPTP appears to impact mechanisms of chromatin remodeling that dictate stemness.

Many compelling reports of the critical role that mPT plays in stem biology come from studies of the developing heart. Adaptations in bioenergetics and mitochondria occur during differentiation of cardiac stem cells and reflect normal developmental changes within the maturing embryonic heart (Beutner et al. 2014; Chung et al. 2007, 2008; Porter et al. 2011). Immature cardiac stem cells have fragmented, perinuclear mitochondria that are relatively inactive by comparison to mature cardiomyocytes (Beutner et al. 2014; Porter et al. 2011). Undifferentiated myocytes rely instead upon anaerobic glycolysis. Several reports from George Porter's group show that the mPTP is open at early stages of cardiomyocyte differentiation (Hom et al. 2011; Lingan and Porter 2016; Lingan et al. 2017). Age-associated remodeling in differentiating cardiomyocytes of the embryo and neonate produces longer mitochondria with tightly packed cristae, a more-polarized mitochondrial membrane, elevated OXPHOS, and greater energy

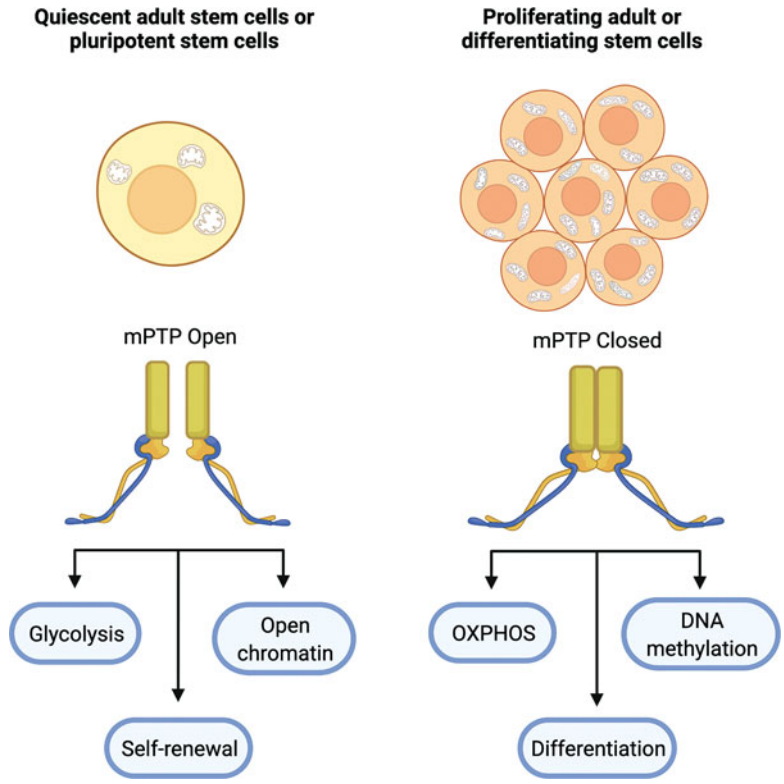

Fig. 2 Stem cell fate is modulated by the state of the mPTP. Common themes among the most multi-/pluripotent stem cells is that they generally prefer glycolysis for energy production, have less active mitochondria, exhibit an open-state mPTP, and have greater chromatin accessibility especially at genetic loci important for multipotency. For adult stem cells, this bioenergetic metabolism protects their quiescence and limits oxidative stress. As stem cells proliferate and differentiate, they typically undergo metabolic reprogramming associated with greater oxygen consumption. Closing of the mPTP promotes maturation of mitochondrial machinery and increased utilization of mitochondrial fuels, which differentiating stem cells need to meet elevated demands for ATP generation and other mitochondrial metabolites. Epigenetic modifications reinforce lineage-specific gene expression programs

efficiency marked by lower output of ROS (Hom et al. 2011; Porter et al. 2011). These same adaptations could be accelerated in utero and in vitro by inhibition of mPT with CsA or NIM811, thereby improving cardiomyocyte differentiation and function (Hom et al. 2011; Lingan and Porter 2016; Lingan et al. 2017). Independent studies have corroborated the role of mPTP closure in cardiomyocyte differentiation and the importance of mitochondrial maturation in fate determination from mouse and human pluripotent stem cells (Cho et al. 2014; Fujiwara et al. 2011; Yan et al. 2009). Inhibition of the mPTP by CsA or NIM811 increased expression of genes required for mitochondrial activity and improved several metrics of mitochondrial function, including OXPHOS activity, membrane potential, and ATP generation (Cho et al. 2014). Interestingly, authors observed synergistic benefit of quenching ROS in differentiation cultures such that concurrent treatment with CsA and antioxidants Trolox or NAC enhanced the fraction of cells committing to the cardiomyocyte fate (Cho et al. 2014). Enhanced myogenesis was highly dependent upon closure of the mPTP, as antioxidants alone had no effect on frequency of cells expressing markers of the cardiomyocyte lineage.

Literature also strongly support a role for the mPTP in the nervous system. Proper neurological development relies upon tight regulation of metabolism in neural stem and progenitor cells. As newly formed neurons mature, they undergo dynamic changes in metabolic pathway utilization and fuel source (Mattson et al. 2008). Indeed, neural progenitors switch from anaerobic glycolysis to aerobic OXPHOS during differentiation (Candelario et al. 2013). These findings are consistent with increases in mitochondrial superoxide flashes observed during neuronal differentiation, which appear to be critical for promoting fate commitment and exit from the cell cycle (Hou et al. 2012). Hou and colleagues found that limiting mitoflash frequency with ROS scavengers or mPTP inhibitors elevated progenitor proliferation; whereas, increasing superoxide flashes promoted neural differentiation. These data demonstrate that transient flickering of the mPTP, producing brief superoxide flashes, negatively regulates self-renewal of neural progenitor cells in conjunction with activation of differentiation programs and signaling that inhibits proliferation. The mPTP can also cause the death of neural stem cells in response to stressors, such as anesthetics or other toxic compounds. The neurotoxicity of anesthetics is poorly understood but, consistent with typical triggers of mPTP opening, is predicted to be linked to elevated Ca^{2+} signaling, ROS, and neuroinflammation (Bai et al. 2013; Orrenius et al. 2003). For example, neurons derived from human embryonic stem cells have been shown to undergo mPTP-dependent cell death upon response to supratherapeutic doses of anesthetic (Twaroski et al. 2015). Propofol at high doses induced activation of fission protein Drp1 (phospho-Ser616) and its respective regulator cyclin-dependent kinase 1, leading to death of neurons (Twaroski et al. 2015). Treatment with an inhibitor of mitochondrial fission, mdivi-1, prevented cell death in conjunction with delay of mPTP opening and reduction of mitochondrial depolarization and fragmentation. Similarly, chronic activation of macrophages of the central nervous system, known as microglia, can cause death of healthy neurons and progressive degenerative neurological disorders through production of ROS, inflammatory cytokines, and chemokines. In a study highly suggestive of mPTP regulation in neural stem cells, overexpression of Hsp75 decreased apoptosis and preserved mitochondrial membrane potential. It was found that Hsp75 overexpression inhibited formation of CypD-dependent mPTP, thereby suppressing activation of a mitochondrial mediated cell death cascade initiated by microglia (Wang et al. 2015). Together, these studies suggest that, in addition to determining fate selection and proliferation, regulation of the mPTP is critical for cell survival of neural stem and progenitor cells.

Metabolic adaptation is also critical for differentiation of blood lineages and development of the immune system. A number of studies have found that mPT activity can modulate self-renewal of blood stem cells and subsequent maturation of their progeny. Throughout adulthood, blood cells are replenished by hematopoietic stem and progenitor cells that reside in the bone marrow. Any perturbation in the balance of self-renewal, proliferation, or maturation of these cells can result in hematologic malignancies, cytopenias, anemia, and infections. Hematopoietic cell transplantation remains the most common and curative stem cell therapy in the clinic; yet, collection and culture of donor hematopoietic stem cells can be problematic due to the detrimental effects of oxidative stress on stem cell self-renewal. Studies led by Hal Broxmeyer's group found that exposure of hematopoietic stem and progenitor cells to normoxia outside the body produced superoxide flashes that impaired regenerative function. Reports describe loss of regenerative potential of adult hematopoietic stem cells exposed to normoxia in a process termed extra physiologic oxygen shock/stress (Broxmeyer et al. 2015). These findings are complemented by studies showing that the damaging effects of ambient air could be forestalled by processing of adult mouse bone marrow or human cord blood with CsA. Donor cells processed with CsA exhibited enhanced numbers of phenotypically identified hematopoietic stem cells and functional competitive repopulating activity (Broxmeyer et al. 2015;

Mantel et al. 2011, 2015). The mechanism of enhanced engraftment with CsA treatment is believed to derive from temporary protection from ROS and/or genotoxic stress associated with hours of handling in normoxia. Interestingly, even earlier studies pointed to a role for the mPTP in the blood lineage. The receptor tyrosine kinase c-Kit is a prototypical surface marker of hematopoietic stem cells, but is also expressed on stem cells of the gut, germline, nervous system, and melanocytes. Hallmarks of mPT, including depolarization of membrane potential and ROS production, could be induced in an erythroleukemia cell line engineered to activate p53-induced apoptosis (Lee 1998). Apoptosis, loss of membrane potential, and ROS generation could all be suppressed in these cells by stimulation of c-Kit with its cognate ligand SCF, suggesting that signaling downstream of c-Kit is important for protecting hematopoietic cells from mPT-dependent cell death. CsA has been used in a vast number of studies of the immune system and as a therapy to suppress graft-versus-host disease after transplantation, largely due to its inhibition of T-cell receptor signaling through calcineurin (Flores et al. 2019; Shevach 1985). Conclusions from studies using CsA for its immunosuppressive activity must be made with caution, but it is worth noting that collective evidence supports a role for mPT in B-cell lymphopoiesis. During B-cell development, CsA interrupts differentiation of B-1 lymphocytes, a highly specialized subset of B cells, but promotes production of conventional B-2 lymphocytes (Arnold et al. 2000). Similar enhancement by CsA has been observed in NK cell differentiation, though mechanism is not fully understood (Flanagan et al. 1999; Kosugi and Shearer 1991). Genetic models have also been used in isolation, but can be suggestive of a role for mPT. One likely component of the low-conductance mPTP, ANT, appears to be required for development of B lymphocytes and red blood cells in embryogenesis. Curiously, hypomorphic deletion of *Slc25a5* which encodes Ant2 in these lineages was accompanied by opening of the mPTP, reduction in respiration capacity and ATP production, elevated ROS, and increase in death of B cells and erythroid cells (Cho et al. 2015). Data supporting a role for mPTP in maturation of developing red blood cells have been corroborated by other independent genetic studies in mouse and human. Deletion of the mitochondrial chaperonin Hsp60, encoded by *Hspd1*, in hematoendothelial precursors of the mid-gestation mouse embryo causes anemia and other vascular defects (Duan et al. 2019). Authors found reduction in mitochondrial membrane potential and decreased expression of VDAC in erythrocytes emerging from the yolk sac. CsA was able to significantly decrease mPTP-dependent cell apoptosis and partially restored VDAC expression. In another study, it was shown that human hematopoietic stem cells that overexpress the fission factor FIS1 undergo arrest in maturation toward the red blood cell lineage, resulting from fragmentation of the mitochondrial network, impairment in mitochondrial membrane potential, decreased ETC complex abundance, and elevated ROS production (Gonzalez-Ibanez et al. 2020). Treatment of these cells with CsA rescued mitochondrial morphology and restored the cells' ability to properly differentiate and synthesize hemoglobin, lending additional support to the notion that the mPTP could regulate metabolic reprogramming required for maturation of blood lineages.

Endothelial cells are key constituents of blood vessels, and circulating endothelial progenitor cells have attracted great attention for their ability to populate and repair damage to the vascular system (Yoder 2012). From a regenerative medicine perspective, differentiation of mature endothelium from human induced pluripotent stem cells is attractive, yet remains challenging due to incomplete development of functional features critical to flow sensing in the vasculature. A recent study showed that one of these serious maturation defects – failure to synthesize a glycocalyx – was caused by incomplete mitochondrial maturation due to a constitutively open mPTP (Tiemeier et al. 2019). Generation of this "hairy" polysaccharide surface of the apical surface of the endothelium could be stimulated by enforced closure of the mPTP with CsA. mPTP closure improved mitochondrial

function and enabled production of functional glycocalyx capable of flow sensing typical of mature vascular endothelial cells from the body. Other studies generally corroborate an important role for cyclophilins and possibly mPTP opening in endothelial cells through examination of the effects of CsA and its analogs on vascular and endothelial cell development. Whether the mPTP must be open or closed for proper regulation of these processes is unclear, but a body of evidence suggests that some mPTP activity during the course of cell maturation could be important. For example, an early study of embryonic stem cell differentiation showed that CsA shifted fate commitment away from the endothelial cell ($CD31^+$) lineage in favor of cardiomyocyte progenitors (Yan et al. 2009). Additionally, angiogenic sprouting and proliferation of endothelial cells and their progenitors are impaired by CsA and an analog that does not inhibit calcineurin/NFAT signaling, N-Methyl-valyl-4-cyclosporin A (Davies et al. 2005; Nacev et al. 2011). Further, CsA treatment in utero causes the collapse of the cardiovascular system in developing embryos (Pandey et al. 2015). CsA treatment results in progressive reduction in blood flow and disappearance of luminal structures in the vasculature. In this study, changes were attributed to CsA's blockade of Jagged 1-induced Notch activation. Important to note is that CsA and its analogs inhibit multiple members of the cyclophilin family and could interfere with mPTP-independent prolyl isomerase functions such as protein folding. Thus, important limitations of many of these endothelial cell studies are that CypD and mPTP were not directly evaluated. Future work should be aimed at careful examination of mPT in endothelial progenitors during fate commitment and maturation.

Lastly, evidence points to roles for the mPTP in progenitor cell survival and regeneration in the liver. In a study of drug toxicity, induced pluripotent stem cells were derived to model a neurometabolic disorder caused by mutations in mitochondrial DNA polymerase gamma (*POLG*), Alpers-Huttenlocher Syndrome (AHS). Patients with AHS commonly receive valproic acid for control of epilepsy, but acute liver failure caused by genotoxic stress can lead to serious morbidity. Authors found that mPTP and superoxide flashes occurred more frequently in hepatocyte-like cells derived from AHS patients. Further, AHS cells undergo cell death in response to treatment with valproic acid, which could be blocked by CsA, highlighting the mPTP as a potential target for preventing hepatotoxicity of this therapy for AHS patients (Li et al. 2015). Stress response and regulation of metabolism in the liver is highly dependent upon management of cytosolic and mitochondrial Ca^{2+}. Deficiency of MICU1, the Ca^{2+}-sensing regulator of the MCU_{cx}, in hepatocytes sensitizes livers to Ca^{2+} overload, impairs bioenergetics and cell functions, increases mPTP opening, and subsequently results in liver failure (Antony et al. 2016; Pan et al. 2013). Importantly, mPTP inhibition by NIM811 is sufficient to accelerate hepatocyte proliferation and rescue liver regeneration in mice challenged by partial hepatectomy (Antony et al. 2016). In summary, a growing body of literature centered on stem cell biology implicates the mPTP as an important component of the stem cell's toolkit for adapting to evolving metabolic demands associated with fate commitment and regeneration.

7 Concluding Remarks

The mPTP is renowned for inducing programmed cell death and necrosis in response to ischemia-reperfusion injuries. Yet, sustained or transient opening of the mPTP is a crucial parameter in deciding cell fate. Long-term opening leads to bioenergetic collapse, release of cytotoxic matrix molecules, oxidative stress, and cell death; whereas, short-term flickering results in variable conductance of the channel and is purported to support protective intracellular signaling by release of matrix Ca^{2+} and ROS. Emerging evidence supports a central role for mPTP modulation in metabolic programming and survival signaling required for stem cell self-renewal and tissue repair. Although stem cell studies represent a small fraction of all mPT research, new clarity regarding the molecular identity and structural

regulation of the mPTP will undoubtedly accelerate this budding area of research and enable new capabilities in stem cell engineering and therapeutics for regenerative medicine.

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Ethical Approval The authors declare that this article does not contain any studies with human participants or animals.

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HSF1, Aging, and Neurodegeneration

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Abstract

Heat shock factor 1 (HSF1) is a master transcription regulator that mediates the induction of heat shock protein chaperones for quality control (QC) of the proteome and maintenance of proteostasis as a protective mechanism in response to stress. Research in this particular area has accelerated dramatically over the past three decades following successful isolation, cloning, and characterization of HSF1. The intricate multi-protein complexes and transcriptional activation orchestrated by HSF1 are fundamental processes within the cellular QC machinery. Our primary focus is on the regulation and function of HSF1 in aging and neurodegenerative diseases (ND) which represent physiological and pathological states of dysfunction in protein QC. This chapter presents an overview of HSF1 structural, functional, and energetic properties in healthy cells while addressing the deterioration of HSF1 function viz-à-viz age-dependent and neuron-specific vulnerability to ND. We discuss the structural domains of HSF1 with emphasis on the intrinsically disordered regions and note that disease proteins associated with ND are often structurally disordered and exquisitely sensitive to changes in cellular environment as may occur during aging. We propose a hypothesis that age-dependent changes of the intrinsically disordered proteome likely hold answers to understand many of the functional, structural, and organizational changes of proteins and signaling pathways in aging – dysfunction of HSF1 and accumulation of disease protein aggregates in ND included.

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Structured Abstracts

Introduction: Heat shock factor 1 (HSF1) is a master transcription regulator that mediates the induction of heat shock protein chaperones for quality control (QC) of the proteome as a cyto-protective mechanism in response to stress. There is cumulative evidence of age-related deterioration of this QC mechanism that contributes to disease vulnerability.

Objectives: Herein we discuss the regulation and function of HSF1 as they relate to the pathophysiological changes of protein quality

control in aging and neurodegenerative diseases (ND).

Methods: We present an overview of HSF1 structural, functional, and energetic properties in healthy cells while addressing the deterioration of HSF1 function vis-à-vis age-dependent and neuron-specific vulnerability to neurodegenerative diseases.

Results: We examine the impact of intrinsically disordered regions on the function of HSF1 and note that proteins associated with neurodegeneration are natively unstructured and exquisitely sensitive to changes in cellular environment as may occur during aging.

Conclusions: We put forth a hypothesis that age-dependent changes of the intrinsically disordered proteome hold answers to understanding many of the functional, structural, and organizational changes of proteins – dysfunction of HSF1 in aging and appearance of disease protein aggregates in neurodegenerative diseases included.

Keywords

Aging · HSF1, Heat shock factor 1 · Hsp, Specific heat shock protein · HSP, heat shock protein family · Intrinsically disordered proteome · Neurodegeneration · Protein homeostasis

Abbreviations

AD1 and AD2	Activation domain
CAT	Chloramphenicol acetyltransferase
CR	Caloric restriction
DBD	DNA-binding domain
HR	Heptad repeat (HRA/B and HRC aka LZ 1–3 and LZ4)
HSE	Heat shock element
HSF1	Heat shock factor 1
HSP	Heat shock protein family
Hsp	Specific heat shock protein
Hsp70	70 kDa Heat shock protein
hsp70	DNA/mRNA of the Hsp70 protein
HSR	Heat shock response
IB	Inclusion bodies
IDP and IDR	Intrinsically disordered protein and intrinsically disordered region
LZ	Leucine zipper
mHtt	polyQ-expanded mutant huntingtin
ND	Neurodegenerative disease
PDSM	phosphorylation-dependent sumoylation motif
PONDR	Predictor of Natural Disordered Regions
PTM	Post-translation modification
QC	Quality control
SIRT1	Sirtuin 1
TAD	Transactivation domain
TD	Trimerization domain

1 Introduction

In order to cope with challenges and adversities, cells have evolved mechanisms – notably induction of the heat shock transcriptional response – to defend, protect, and mitigate the dire consequences of stress. The heat shock response (HSR[1]), defined as the rapid induction of heat shock proteins (HSP), was initially described in 1962 by Ritossa in Drosophila (Ritossa 1962). The HSR is now considered ubiquitous to all organisms and can be elicited by a wide range of noxious stimuli. HSR is also referred to as *stress response* and the terms *stress genes* and *stress proteins* have been introduced to specifically recognize the overall function of the protein products (Lindquist and Craig 1988; Hendrick and Hartl 1995; Morimoto 1998; Hartl et al. 2011; Morimoto 2011). Our focus here is on the heat shock factor 1 (HSF1), a transcription factor that mediates the induction of HSP chaperones in response to heat and other stresses. HSF1 is a member of the family of heat shock factors that include HSF1, HSF2, and HSF4 (Åkerfelt et al. 2010). We begin by surveying the literature to present an overview of HSF1 structure, function, and regulation in healthy cells. Subsequent sections summarize the findings of recent studies that delineate the molecular organization of HSF1

functional domains with particular emphasis on its intrinsically disordered nature. For readers interested in a deeper understanding of HSF1 function and regulation, there are a number of comprehensive reviews on these topics (Åkerfelt et al. 2010; Anckar and Sistonen 2011; Vihervaara and Sistonen 2014; Nakai 2016; Gomez-Pastor et al. 2018). We next address the deterioration of HSF1 function in aging and most notably in neuronal systems, changes that likely contribute to the emergence of age-related ailments as in neurodegenerative diseases (ND). We conclude by discussing intrinsically disordered regions of HSF1 structure and note that many proteins involved in ND are natively unstructured and exquisitely sensitive to changes in cellular environment as may occur in aging. We postulate that changes in cell milieu notably protein hydration may principally affect the structuring and function of IDPs/IDRs and likely hold answers to understanding many of the age-dependent changes in cell function that result in global homeostatic decline and vulnerability to disease pathogenesis.

2 Heat Shock Factor 1: Structure and Function

HSF1 mediates the induction of heat shock protein (HSP) chaperone expression in response to stresses that perturb protein conformation including thermal stress and amino acid analogs (Åkerfelt et al. 2010) (Lindquist and Craig 1988; Hendrick and Hartl 1995; Morimoto 1998; Åkerfelt et al. 2010; Anckar and Sistonen 2011; Hartl et al. 2011; Morimoto 2011). The human HSF1 is a constitutively expressed transcription factor of 529 amino acid residues (Åkerfelt et al. 2010; Anckar and Sistonen 2011; Vihervaara and Sistonen 2014; Nakai 2016; Gomez-Pastor et al. 2018). Under normal conditions, HSF1 exists primarily as a latent and inactive monomer, stabilized by hydrophobic interactions between N- and C-terminus heptad repeats. Whether this latent form of HSF1 is principally a cytosolic or nuclear factor has not been resolved conclusively. There is evidence that HSF1 constitutively shuttles between the cytosolic and nuclear compartments (Vujanac et al. 2005). Stress causes a dynamic and rapid structural change of HSF1, converting the protein from a monomeric intramolecular repressed state to a homo-trimer stabilized by intermolecular heptad repeat interactions.

There are two lines of thought as to how this "de-repression" of HSF1 occurs, namely: (1) HSF1 is an intrinsic "stress" sensor and the conversion from monomer to trimer is stress triggered and thermodynamically favored. The observation that HSF1 can be activated in vitro as a response to increased temperature is consistent with the suggestion that HSF1 functions as an intrinsic "thermosensor" (Hentze et al. 2016); and (2) HSF1 is sequestered in a monomeric state by binding to Hsp90 and perhaps Hsp70. The associated complex is disrupted by stress due to recruitment of the chaperones by misfolded and non-native protein entities, thereby resulting in the release and trimerization of HSF1. Support for the latter arises primarily from experiments using small molecules that target the Hsp90 chaperone and inhibit its function, which results in the trimerization and activation of HSF1 (Taipale et al. 2010). It remains unclear whether this is due to a direct effect of "unleashing" HSF1 from chaperones, or an indirect effect due to simultaneous release of diverse metastable clients of the Hsp90 proteins resulting in an "unfolded" protein response that triggers HSF1 activation. Recent evidence suggests that the binding of HSF1 to Hsp70 directly controls its activation in yeast and the unleashing of HSF1 from Hsp70 control exposes a hyper-stress transcription program (Masser et al. 2019). Presumably, different combinations of these mechanisms are involved depending on the nature and degree of stress as well as the particular model system under consideration (e.g., HSF binds DNA constitutively in budding yeasts versus a strictly stress-induced binding in mammalians).

3 HSF1 Domain Organization and Structural Characterization

The molecular structure of HSF1 is highly conserved with significant sequence homology observed among mammalians and a fair sequence similarity when compared to other eukaryotic species (Gomez-Pastor et al. 2018; Joutsen and Sistonen 2019). While there are variations in size and amino acid sequence, all HSF1 share a number of conserved structural features as illustrated in Fig. 1a. These features include: (1) a conserved *DNA binding domain* (DBD) located near the N-terminus; (2) a leucine zipper (LZ 1–3)-motif designated as the *trimerization domain* (TD) which consists of heptad repeats (HR-A/B) that self-associate with a leucine zipper (LZ-4) in the monomeric state (HR-C); (3) a *regulatory domain* (RD) that is largely unstructured and undergoes inducible post-translational modifications (PTM) during stress processes; and (4) a conserved *transactivation domain* (TAD) near the C-terminus that interacts with a number of protein partners for concerted gene transcription regulation processes. The sequence alignments for mammalian (human, rat, and mouse) and yeast HSF1 are compared in Fig. 1b, revealing a 95 and 39% similarity within mammalian species and between mammals and yeast, respectively.

The human HSF1 domain architecture and organization is presented in the schematic diagram of Fig. 2a and comprised of four primary functional regions. Unless indicated otherwise, the HSF1 sequence and a.a. positions are in reference to the human HSF1. Specifically, the N-terminal DNA binding domain (DBD) composed of amino acids 16–123 is characterized by a "winged" helix-turn-helix DNA-binding motif. The trimerization domain (TD) spanning amino acids 137–212 consists of hydrophobic heptad repeats HR-A/B commonly referred to as LZ 1–3. In the resting state under physiological conditions, TD oligomerization is allosterically inhibited by the C-terminus HR-C heptad repeat designated as LZ-4 of a.a. residues 384–417, which folds back to form intramolecular contacts with HR-A/B as depicted in Fig. 2b. Following activation, this intramolecular heptad repeat interaction is disrupted and HSF1 undergoes trimerization via specific intermolecular coiled-coil interactions of the TD as illustrated in Fig. 2c. In most species including mammalians, HSF1 contains a transactivation domain (TAD) corresponding to a.a. 400–529 of the human HSF1 C-terminus, and within this there are two activation domains (AD1 and AD2). Biophysical and structural studies of full-length HSF1 have been hampered by its dynamic conformational properties that include intrinsically disordered regions in several stretches of the protein sequence. Accordingly, high-resolution x-ray crystallographic data for the full-length protein are not available as DBD is the only structurally-characterized domain to date.

3.1 DNA Binding Domain (DBD)

Upon stress, HSF1 trimerizes to drive the expression of specific genes involved in cell survival including protein chaperones, protein degradation machinery, anti-apoptotic proteins, and other transcription factors [as reviewed in (Jaeger et al. 2014)]. The HSF1 trimer interacts with the major groove of a cognate DNA sequence of inverted pentameric repeats (nGAAn) known as heat shock elements (HSEs) (Vihervaara and Sistonen 2014). X-ray crystallographic studies of the DNA-binding domain of yeast and mammalian HSF1 have yielded important structural insights on the protein-DNA complex (Harrison et al. 1994; Gomez-Pastor et al. 2018). Specific structural features have been deduced from *Kluyveromyces lactis* (Harrison et al. 1994) and *Drosophila melanogaster* (Vuister et al. 1994) as well as co-crystallized DBD-HSE complexes derived from *Kluyveromyces lactis* (Littlefield and Nelson 1999) and human (hHSF1) (Neudegger et al. 2016).

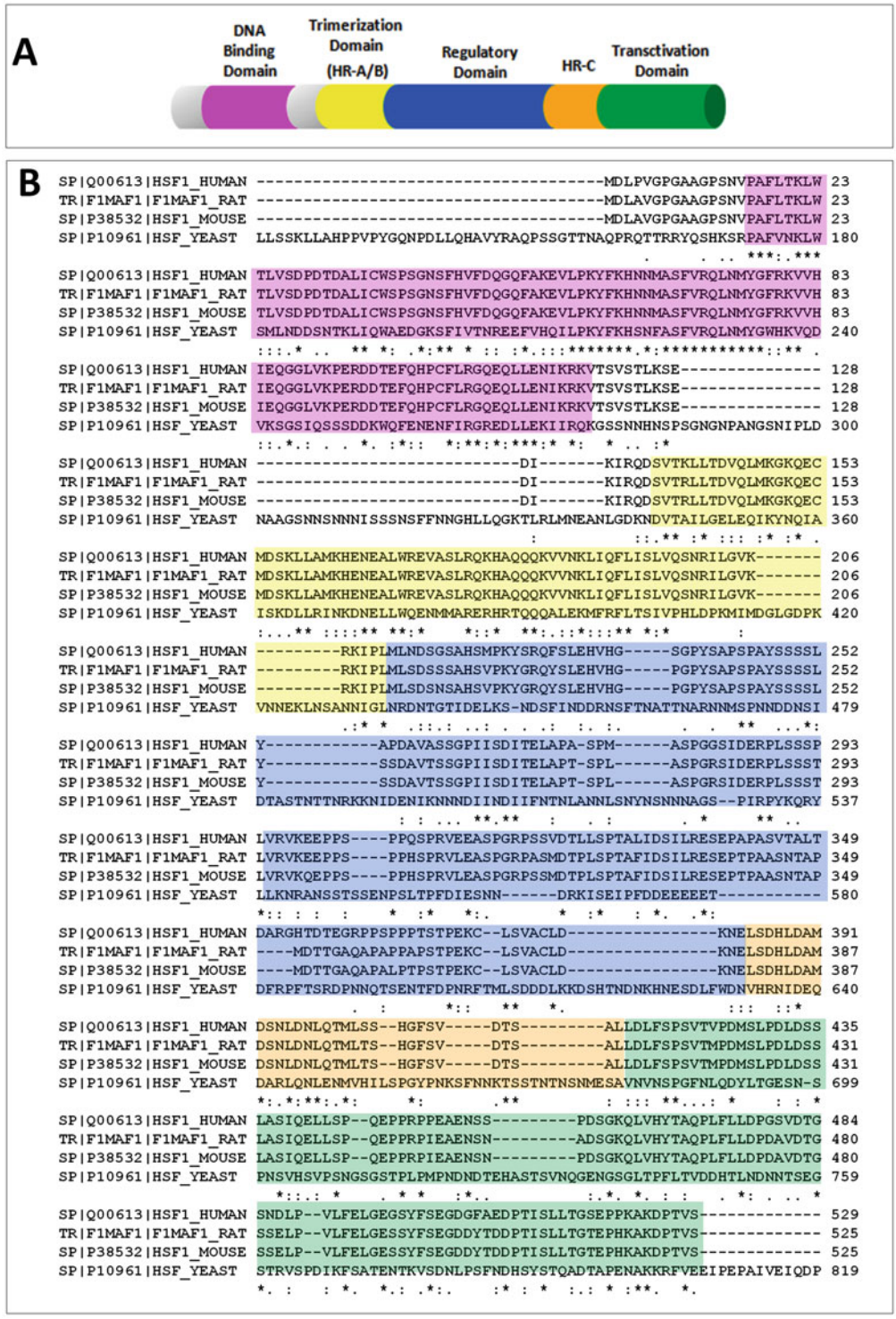

Fig. 1 Domain organization (**a**) and sequence alignments (**b**) for human, rat, mouse, and yeast HSF1. There is no sequence correspondence for the N- and C-termini of yeast HSF1 (i.e., residues 1–120 and 820–833, respectively), and these regions are omitted to improve overall clarity. Sequence alignments are deduced via the UNIPRO FASTA program. (https://www.uniprot.org/align/)

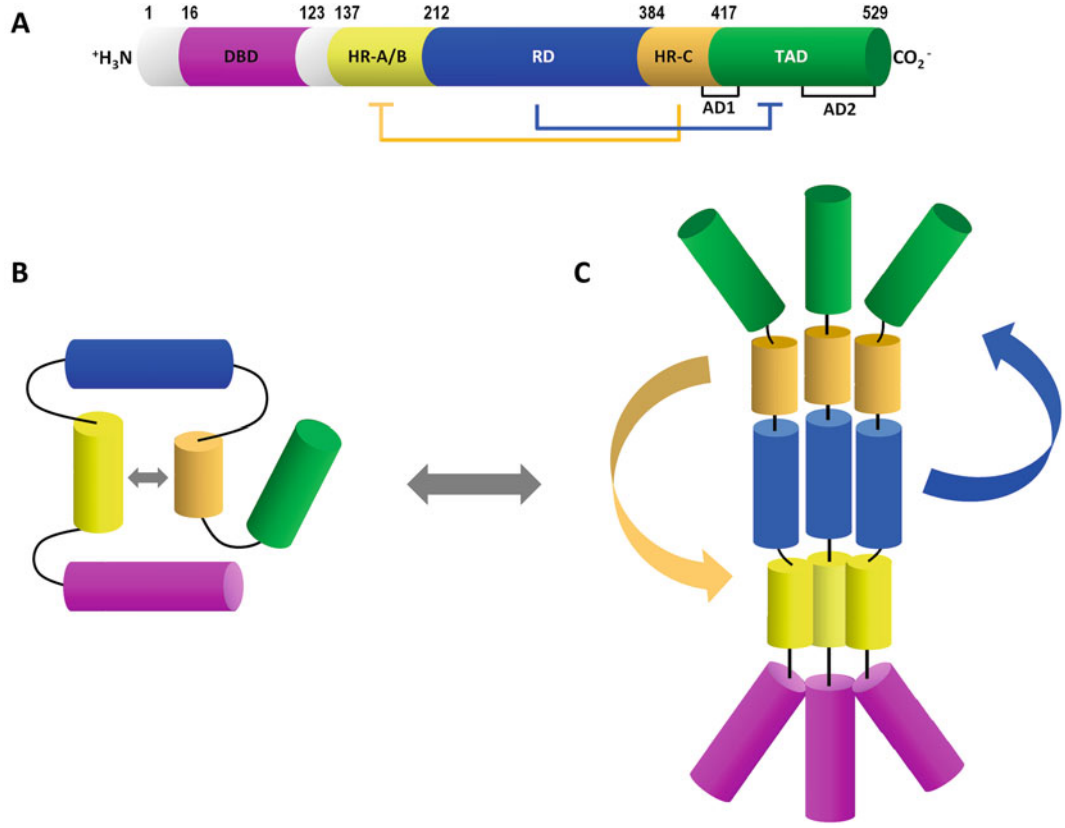

Fig. 2 HSF1 domain organization and Oligomeric states. (**a**) Schematic representation of the human HSF1 sequence with specific domains color coded as follows: DBD (magenta); HR A/B (yellow); RD (blue); HR-C (orange); and TAD (Green). The orange, blue, and black brackets signify relationships amongst the following domains: Orange bracket: HR-C interacts and inhibits HR A/B (trimerization domain); Blue bracket: RD regulates and inhibits TAD; Black brackets: AD1 and AD2 activation domains. (**b**) HSF1 monomeric state in which HR-C interacts with HR A/B inhibiting oligomerization. (**c**) HSF1 trimeric state in which HR A/B domains are released from the inhibiting effects of HR-C (orange arrow) and TAD is restrained by RD (blue arrow)

3.1.1 High-Resolution Structural Properties of HSF1 DBD

Structural analysis of HSF1:HSE complexes reveal direct contacts between DBD and the major groove of nGAAn sequences, whereas the remaining protein-DNA interactions occur via water-mediated hydrogen bonds with the phosphate backbone (Neudegger et al. 2016). Inspection of the hHSF1:HSE complex depicted in Fig. 3 shows a protein-DNA interface that establishes contacts with the DNA major groove, a characteristic feature of DBDs in many transcription factors (Privalov et al. 2007). The DBD:DNA interactions are mediated by helix α3 (residues 66–75) which is highly conserved and inserts into the DNA major groove nearly perpendicular to the double helix, establishing contacts between Arg71 and guanine in the GAA motif. The surrounding conserved residues Ser68, Gln72, Asn74, Met75, and Tyr76 stabilize the side chain conformation of Arg71 and orients helix α3 toward the major groove by forming hydrogen bonds with the phosphate backbone. Additional DBD:DNA interactions involving the side chains of Ser68, Arg71, and Gln72 are observed in the co-crystal structure (Neudegger et al. 2016). On an equally relevant note, Lys80, Lys116, and Lys118 are

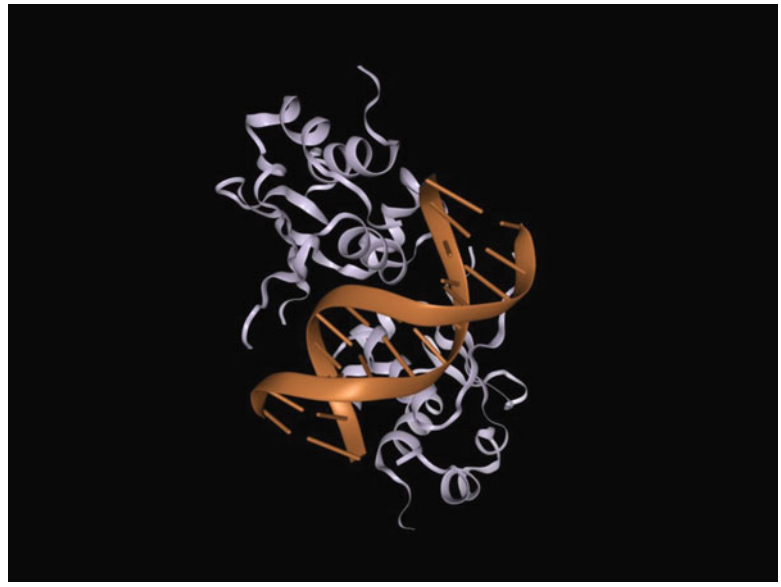

Fig. 3 Ribbon diagram depicting the crystallographic structure of HSF1 DBD in complex with a cognate DNA duplex. Two monomers interacting with the cognate DNA major groove are depicted to improve clarity (PDB:5D5U). The figure is prepared using RCSB PDB tools and NGL Viewer. (Rose et al. 2018)

required for establishing ionic interactions with the DNA backbone. Acetylation of these essential residues effectively abrogates such contacts and negatively regulates DNA binding (Gomez-Pastor et al. 2018).

3.1.2 Energetics of HSF1-Cognate DNA Interactions

Solution studies report association constants for monomeric and trimeric HSF1 DBD:DNA interactions in the micromolar to nanomolar range, respectively (Jaeger et al. 2014). Equilibrium DNAase foot-printing assays of HSF1 trimer interactions with specific 13-bp DNA constructs yields sequence-dependent dissociation constants on the order of 0.2 to $3{\cdot}10^{-9}$ M (Kroeger and Morimoto 1994). Although there is a considerable body of evidence suggesting that HSF1 trimerization is an obligatory requirement for DNA association, monomeric HSF1 can bind DNA albeit with significantly lower affinity. These results are corroborated by mutagenesis studies whereby HSF1 protein ensembles are predominantly shifted to either the monomeric or trimeric state as monitored via size exclusion chromatography (Jaeger et al. 2014) as illustrated in Fig. 4. Specifically, mutations that delete the auto-inhibitory HR-C (LZ-4) sub-domain effectively unleash the inactive HSF1 monomer and facilitate trimer formation. Conversely, deletion mutation of the HR-A/B (LZ 1–3) domain inhibits trimerization, resulting exclusively in the formation of monomeric species (Jaeger et al. 2014). Analytical ultracentrifugation data acquired on HSF1 monomer interactions with DNA cognate sequences are characterized by association-free energies spanning the range of -8 to -10 kcal·mol^{-1} (Kroeger and Morimoto 1994). Temperature-dependent van't Hoff analysis of the resultant DBD binding affinities reveal an enthalpy-driven process (Park and Kim 2012), consistent with observations for other transcription factor-cognate DNA interactions through major groove contacts (Privalov et al. 2007).

HSF1 interacts with the target DNA cooperatively as a trimer, each monomeric DNA-binding domain recognizing a single nGAAn sequence, and three alternately oriented pentamers (i.e., inverted repeats) bind the trimeric HSF1 (Gomez-Pastor et al. 2018). An important issue that remains unresolved to date is whether HSF1 DBD within the trimer assembly undergoes further conformational changes upon DNA binding. Studies on the energetics of HSF1:DNA interactions via analytical ultracentrifugation employing a monomeric species reveal that

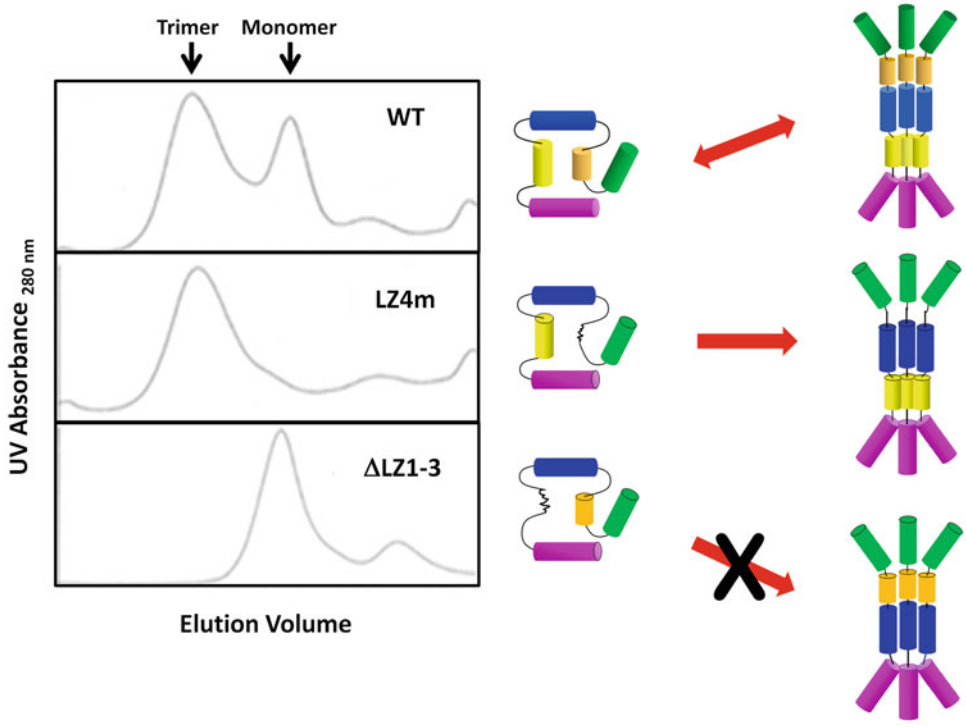

Fig. 4 Size Exclusion Chromatographic (SEC) analysis of HSF1 Oligomeric states. Left: SEC profiles of wild type and mutant HFS1 proteins. [Adapted from (Jaeger et al. 2014)]. Top Panel: Wild-type HSF1 (WT) containing a mixture of inter-convertible monomers and trimers; Middle Panel: HSF1 deletion mutant lacking the HR-C domain (LZ4m) eluting exclusively as a trimer; Bottom panel: HSF1 deletion mutant lacking the HR-A/B domain (LZ1–3) eluting primarily as a monomer. Right: Schematic representations of the monomeric and trimeric species for WT, LZ4m, and ΔLZ1–3 constructs, respectively. The color schemes reflect specific domain regions as described in previous illustrations

HSF1 protein-DNA association is *not* accompanied by major conformational changes (Kim et al. 1994). These findings are corroborated by structural analysis of trimeric HSF1 DBD (Littlefield and Nelson 1999) with the lone exception of a minor shift in the helix-turn-helix motif that accommodates DNA phosphate backbone contacts. Overall, there is a marginal ordering of the DBD in a co-crystal structure with HSE DNA resulting in B-factors that are lower than hydrophobic residues in the DBD core.

It is relevant to note that interactions with HSEs are not solely dependent on direct DBD binding with the target sequence but may also include HSF1 protein-protein contacts that can cooperatively enhance gene transcription events. As deduced from biochemical and structural analysis (Kroeger and Morimoto 1994), HSF1 trimers tend to establish protein-protein interactions in vitro and associate cooperatively along the DNA duplex of tail-to-tail and head-to-head repeats of the nGAAn sequence. Depending on the sequence construct, high affinity interactions are characterized by cooperativity involving trimer-trimer association within the protein-DNA complexes (Kroeger and Morimoto 1994). Biochemical evidence suggests that such intermolecular association operates in a temperature-dependent manner. These findings raise an intriguing proposition that HSF1 trimer interactions might serve to modulate the activation of multiple chromosomal loci and enhance transcription of heat shock promoters in cells (Littlefield and Nelson 1999).

3.2 Trimerization Domain (TD)

Human HSF1 contains three hydrophobic repeats commonly designated as HR-A/B (LZ1, 2/3) and HR-C (LZ-4) [refer to Fig. 2]. Under resting physiological conditions, hHSF1 adopts an inactive monomeric state that is maintained by intramolecular associations among these domains, which are further stabilized by transient interactions with heat shock proteins (e.g., Hsp70) (Tonkiss and Calderwood 2005). Upon exposure to heat stress, these interactions are disrupted and hHSF1 homotrimerizes acquiring an enhanced DNA-binding ability. These findings are consistent with the proposition that HSF1 activation by heat proceeds via conformational changes thereby unleashing the trimerization-prone conformation.

3.3 Regulatory Domain (RD)

The regulatory domain (RD) comprises the longest disordered stretch (residues 212–384) within HSF1 [Refer to Fig. 2] and is a principal target site to modulate the trans-activating function of HSF1. As an example, salicylate induces DNA binding but not the trans-activating activity of HSF1 (Cotto et al. 1996). Phosphorylation of S230 and S236 in the RD [Refer to Fig. 5] is a pre-requisite for transactivation (Gutsmann-Conrad et al. 1998). The notion that RD restrains TAD function is further corroborated by the observation that deletion of RD results in a constitutively active mutant HSF1. Collectively, these observations suggest that the HSF1 RD can sense stress and confers heat inducibility to TAD function.

3.4 Transactivation Domain (TAD)

A disordered segment of residues 401–529 comprising the C-terminal transactivation domain (TAD) (Ravarani et al. 2018) is responsible for regulating the expression of genes to launch the heat shock response. TAD is composed of two distinct regions that are rich in hydrophobic and acidic residues. These activation domains (AD) are designated as AD1 and AD2, spanning amino acids 401–420 and 431–529, respectively (Ravarani et al. 2018). Both regions exhibit equivalent roles in terms of stimulating transcription initiation and elongation, a finding which underscores their importance in guaranteeing the production of HSP for protein homeostasis. TAD functionality may represent an emergent property that depends on a combination of composition (sequence context) and spatial patterning (sequence motif) of particular amino acids within the disordered segments (Ravarani et al. 2018). While AD1 is enriched in hydrophobic residues and includes an alpha-helical segment, AD2 is comprised of hydrophobic, acidic, and proline residues which adopts a non-helical conformation (Åkerfelt et al. 2010).

As in the case of many other transcription activators, the TAD domain of HSF1 is intrinsically disordered (Sect. 5). The preponderance of negatively charged residues in TAD results in a local, extended conformation via intra-chain repulsion, and this structural flexibility drives an assembly of different conformations also known as "fuzzy" complexes for the dynamic interaction of HSF1 with multiple protein partners. The extended TAD provides an appropriate context for exposing aromatic residues and positively charged patches to dock and bind protein partners (Ravarani et al. 2018). The multiplicity of interactions broadens the recognition network (Åkerfelt et al. 2010; Anckar and Sistonen 2011) and expands the cross-talk functionality of HSF1. Proteins tattered to TAD can guide HSF1 to specific target genes and fine-tune the degree and profile of trans-activation (Åkerfelt et al. 2010). In summary, the intrinsically disordered and dynamic nature of TAD allows for a multiplicity of transient interactions that enable a broader network of HSF1 function under physiological conditions. Conversely, promiscuous interactivity can contribute to disease vulnerability due to off-target binding in disease states (Chen et al. 2018).

3.5 Post-translational Modifications (PTMs) Modulate HSF1 Function

3.5.1 Activity Modulation in the DNA Binding Domain (DBD)

Post-translational modifications (PTMs) within various HSF1 domains are identified in Fig. 5. In the DBD, PTMs involving acetylation of Lys80 by P300/CBP disrupt DNA binding ability, whereas deacetylation of Lys80 stabilizes the DNA bound form and thereby extends HSF1 transcriptional activity (Westerheide et al. 2009). The duration of HSF1 binding to DNA can be prolonged by a nutrient sensor and longevity factor designated as Sirtuin 1 (SIRT1), a deacetylase for HSF1 (Westerheide et al. 2009; Gomez-Pastor et al. 2018) specifically targeting Lys80 in the DBD (Refer to Sect. 4.3). As further detailed in Sect. 4.3, the fact that DNA-binding activity of HSF1 is under the control of SIRT1 – a deacetylase known as "the longevity gene" – provides a defining linkage between aging and the HSF1-dependent protein QC system.

3.5.2 Activity Modulation in the Regulatory Domain (RD)

HSF1 undergoes numerous PTMs that are classified as constitutive or functional, operating as transcription inhibitors/stimulators. The RD is subject to various PTMs including phosphorylation and sumoylation, both of which enable RD to control TAD as suggested in studies of HSF1 (Gomez-Pastor et al. 2018) and HSF2 (Yoshima et al. 1998). As noted previously, specific sites of post-translational modifications within the regulatory domain (RD) are identified in Fig. 5. Proteomics analysis employing mass spectrometry has afforded identification and characterization of these PTMs at residue resolution and their relevance to HSF1 function probed via

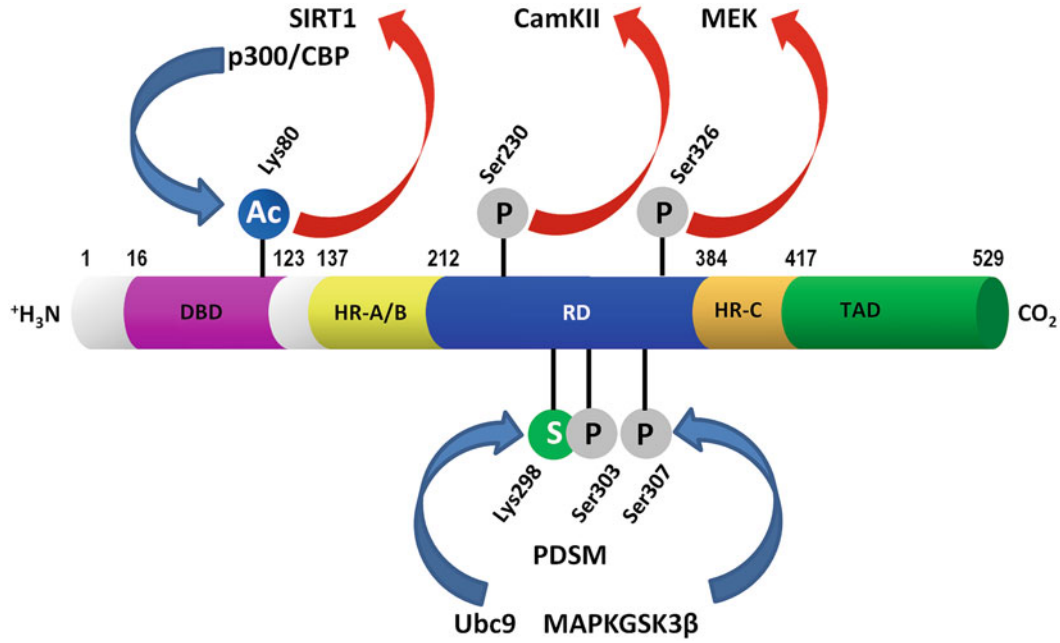

Fig. 5 Representative PTMs in hHSF1 with the corresponding modified amino acid designated in each functional domain. DBD: an acetylation (Ac) site in Lys80 catalyzed by P300/CBP is inhibitory (blue arrow), whereas the reverse deacetylation reaction catalyzed by SIRT1 (red arrow) is stimulatory; activating phosphorylations (red arrows) at Ser230 and Ser326 of RD are catalyzed by CamKII and MEK, respectively. Inhibiting phosphorylations (blue arrows) at Ser303 and Ser307 are catalyzed by MAPKGSK3β. Sumoylation of Lys 298 in the PDSM is catalyzed by Ubc9 and requires Ser307 phosphorylation. The color schemes reflect specific domain regions as described in Fig. 2

Table 1 Representative PTMs identified and characterized in the HSF1 sequence[a]

PTM residue	Domain	Enzyme(s)	Outcome
Ac-K80	DBD	p300/SIRT1	Inhibitory
Ph-S230	RD	CamKII	Stimulatory
Su-K298	RD	Ubc9	Inhibitory
Ph-S303	RD	MAPKGSK3β	Inhibitory (promotes K298 sumoylation)
Ph-S326	RD	?	Stimulatory
Ph-S363	RD	PKC, JNK	Inhibitory

[a]According to (Anckar and Sistonen 2011) and references contained therein

mutagenesis experiments. Mass spectrometric analysis reveals the presence of at least 12 residues that are phosphorylated (Guettouche et al. 2005) as summarized in Table 1. Studies have suggested that phosphorylation at S230 and S326 are important for transcription activation (Gutsmann-Conrad et al. 1998). In addition to titrating the degree of HSF1 trans-activating activity, PTMs can also regulate the strength and duration of HSR. Proteotoxic stress causes HSF1 trimerization and hyperphosphorylation, both of which induce HSF1 sumoylation (Anckar and Sistonen 2007). The phosphorylation-dependent sumoylation motif (PDSM) is responsible for the extent and duration of HSF1 transcriptional activity and in fact eventually suppresses HSF1 transactivating capacity. Sumoylation of the amino acid Lys298 involves a mechanism that is dependent on phosphorylation of an adjacent downstream residue (i.e., S303) as a primer. The regulatory mechanisms of HSF1 are summarized in Table 1 and illustrated in Fig. 5. A schematic representation of the HSF1-HSP pathway appears in Fig. 6.

4 Age-Dependent and Neuron-Specific Attenuation in Induction of the Heat Shock Transcriptional Response

At its core, aging is the decline of homeostatic capacity such that a cell or an organism is no longer capable of repairing damages to macromolecules and organelles to function in an optimal and healthy state. At the organismic level, aging is manifested as increased morbidity and mortality when confronted with challenges or adverse situations (Oechsli and Buechley 1970; Driscoll 1971; Schock 1977; Lakatta et al. 1990). At a cellular level, this may be expressed as a deterioration of various quality control (QC) mechanisms required to maintain the proper structure and function of the proteome resulting in a decreased capacity for adaptive regulation (Adelman 1979). Activation of HSF1 and induction of HSP chaperones serve as the primary QC machinery of the proteome in cells under stress. HSP chaperones assume fundamentally important roles assisting the folding and re-folding of natively structured proteins and ensuring clearance of damaged, dysfunctional, and aggregated species (Gething and Sambrook 1992; Hendrick and Hartl 1993). This QC machinery is necessary for supporting cell survival and recovery after stress (Li and Werb 1982; Hahn and Li 1990; Li et al. 1991). Not surprisingly, there is a body of literature documenting an age-dependent decline in the activation of HSF1 and induction of HSP chaperones in various aging model systems (Liu et al. 1989, 1991, 1996; Choi et al. 1990; Lee et al. 1996, 2008; Lu et al. 2000; Westerheide et al. 2009; Hipp et al. 2019; Santra et al. 2019). The relevance of this diminished capacity to mount a cyto-protective HSR to aging is further discussed below.

4.1 Attenuated Heat Shock Transcriptional Response in Aging: Cell Models

Using human diploid fibroblast as a model of cell aging, our early studies suggested that the heat shock induction of HSP chaperone production is

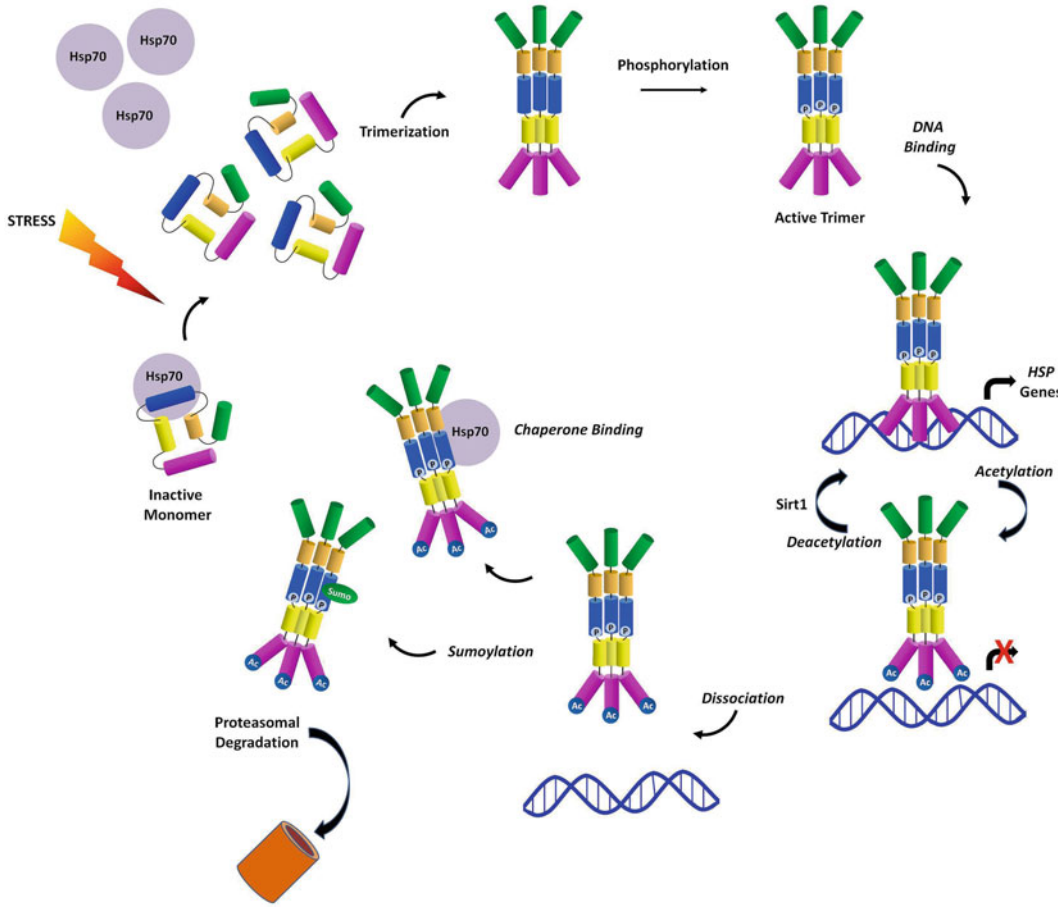

Fig. 6 HSF1 Activation pathway leading to the transcription competent trimer. Top: Inactive HSF1 monomers bound to Hsp70 are exposed to stress. Following trimerization and phosphorylation, the resultant oligomers are DNA binding competent and transcriptionally active. Top to Bottom: The DNA-bound trimers undergo acetylation, abrogating their binding and transcription activity. This process is inhibited by deacetylation which prolongs gene activation. Bottom: Upon post-stress activation, the acetylated trimer is modified via PTMs (e.g., sumoylation) that facilitates dissociation or removal by the proteosomal degradation machinery. The color schemes reflect specific domain regions as described in Fig. 2

progressively attenuated as a function of replicative "age" of the cells (Choi et al. 1990; Liu et al. 1989). Analysis of HSF1 DNA-binding activity, production of $mRNA^{hsps}$ as well as HSE promoter-driven reporter gene expression reveals that the attenuated HSR is due to a decrease in the stress-induced activation of HSF1 DNA-binding activity (Choi et al. 1990). Moreover, the age-related impairment of response to heat shock is multifaceted as we observe a decrease in magnitude coupled with increases in the temperature and time required to trigger the response. It is noteworthy that the ability to mount a heat shock response (HSR) at a physiologically relevant temperature of 39.0 °C is already compromised in cells with approximately 70% of their replicative potential completed as deduced from population doubling levels (PDL) [(i.e., PDL 35 out of an averaged maximal life span of PDL 50–55)] (Byun et al. 2013; Liu et al. 1991). The reduction in stress-induced activation of HSF1 DNA-binding activity is not attributable to a decreased expression of transcription factor as the abundance of HSF1 and mRNA remain

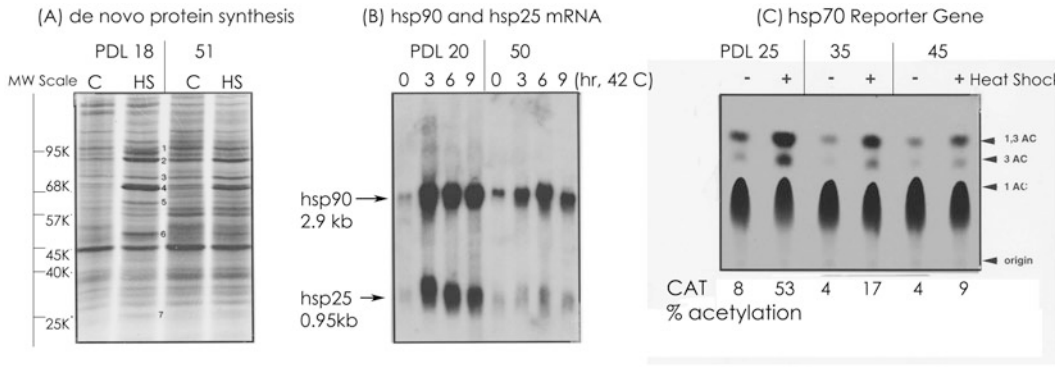

Fig. 7 Attenuated induction of heat shock gene expression in aging human diploid fibroblasts (**a**) Heat shock induction of Hsp synthesis in young (PDL 18) and old (PDL 51) IMR-90 human diploid fibroblasts. Confluent cell culture in 60 mm plates is heat shocked at 42 °C for 6 h with control cells maintained at 37 °C. Cells are labeled with 100 μCi/ml of [35S] methionine during the last hour of incubation followed by harvesting. Aliquots of cell extracts containing 200,000 cpm of radioactivity are analyzed by SDS-gel electrophoresis and autoradiography. The positions on the gel of 98, 90, 78, 72, 64, 50, and 25 kDa Hsp are marked as bands 1–7, respectively. (**b**) Northern blot of hsp90 (2.95 kb) and hsp25 (0.95 kb) mRNA of young and old IMR-90 cells heat shocked at 42 °C for 0, 3, 6, and 9 h. Total RNA was size-fractionated by agarose gel electrophoresis, transferred to membrane, and probed with [32P]-labeled cDNAs. (**c**) hsp70-promoter-driven reporter gene activity. DNA of the hsp70 promoter-chloramphenicol acetyltransferase (CAT) reporter gene is transfected into cells. Twenty-four hours after transfection, the cells are heat shocked at 42 °C followed by recovery. Cells are harvested and CAT activity determined. Activity of the CAT reporter gene expressed as a percent of the substrate acetylated is indicated in the figure. [Adapted from (Lee et al. 1996)]

unchanged in senescent versus young cells (Lee et al. 1996). Representative results of changes in the heat shock transcriptional response in cell aging appear in Figs. 7 and 8. The mechanistic basis of this abatement in HSF1 activation with age presumably involves changes in post-translational modifications including oxidative modification and SIRT1-dependent deacetylation of HSF1 as describe below.

Oxidation and disulfide cross-links of cysteine residues block the trimerization and activation of HSF1 (Manalo et al. 2002). Given the acknowledged role of oxidative damage in aging, one may infer that this could be a mechanism that constrains the activation of HSF1 to result in sub-optimal induction of HSP chaperones in aging cells and organisms under stress. To test this possibility, we treated young human diploid fibroblast cells with H_2O_2 to promote premature senescence and showed that this treatment constrains HSF1 activation in a manner similar to that observed in the "replicative" senescent cells (Lee et al. 2008). This and other considerations suggest that biological readouts can be regulated by both genetic (i.e., transcription and translation) as well as epigenetic (i.e., post-translational modification) mechanisms that change the function, conformation, and localization of regulatory proteins. Our observation that the steady level of HSF1, determined by immuno-Western blot quantitation, remains unchanged with age of the human diploid fibroblast cells would suggest that epigenetic changes in the structural organization and/or post-translational modification of HSF1, including S-glutathiolation and S-nitrosylation, contributes to the age-related dysfunction of HSFs and downstream decline in HSR (Lee et al. 2008).

4.2 Attenuated Heat Shock Transcriptional Response in Aging: Animal Model Systems

Concurrent with studies on age-dependent changes in the regulation of HSF1 and induction

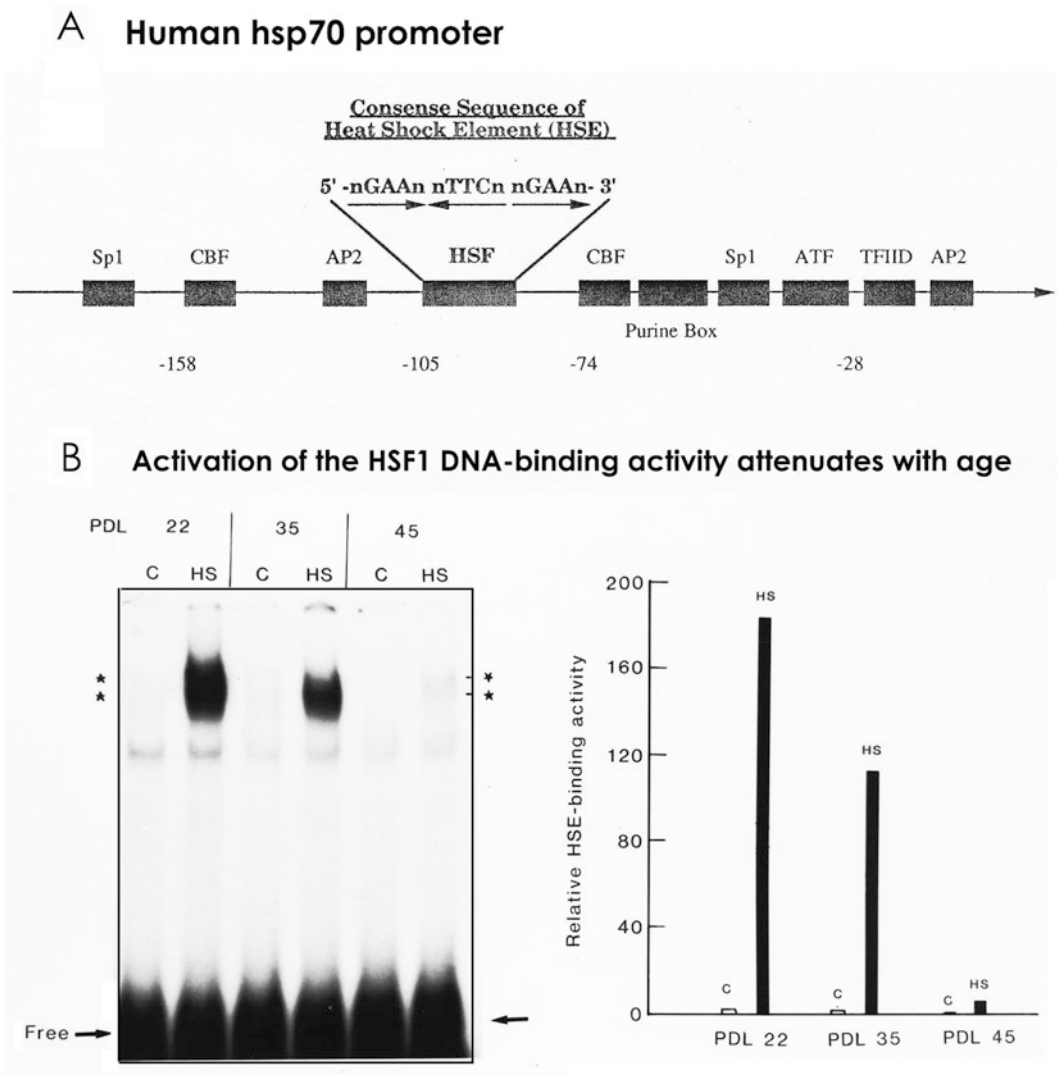

Fig. 8 Heat shock-induced activation of HSF1 DNA-binding activity in Young, Middle-aged, and Old IMR-90 human diploid fibroblasts. (**a**) Schematic diagram of the human hsp70 promoter. A 24-mer oligonucleotide with 4 tandem repeats of the NGAAN sequence is used to assay HSE-binding activity of HSF1. (**b**) Electrophoretic mobility shift assay (EMSA) of HSE-binding activity in extracts of control and heat shocked cells from young, middle-aged, and old IMR-90 cells. The cells are heat shocked at 42 °C for 1 h versus control cells maintained at 37 °C. Aliquots of the cell extracts containing 30 μg protein are used to assay binding of the double-stranded 32P-labeled consensus HSE sequence. Positions of the HSF-HSE complex are indicated by an asterisk while the free [32P] HSE probe is designated by an arrow. Quantitation of the HSE-binding activity appears in the accompanying bar graph. [Adapted from (Choi et al. 1990)]

of HSR in cell models as summarized above, parallel studies from other research laboratories showed similar changes of a diminished response to heat shock in aging animal model systems and in cells derived from these animals. Thus, a reduced expression of the hsp70 mRNA and protein upon heat shock of lung or skin-derived fibroblasts in aged Wistar rats is observed as compared to cells from young adult animals (Blake et al. 1990; Blake et al. 1991). Studies

using liver cells isolated from Fischer F334 rats showed a 40–50% decrease in the heat shock-induction of Hsp70 synthesis, mRNA levels, hsp70 gene transcription rate, and HSF DNA-binding activity in cells of older animals as compared to cells from young adult animals (Gutsmann-Conrad et al. 1998). The relevance of these observations to the biology of aging is supported by the finding that caloric restriction (CR), the only regimen known to retard aging and extend the lifespan of mammals, can reverse this age-related decline in the induction of hsp70 mRNA transcription of hepatocytes (Blake et al. 1990, 1991; Gutsmann-Conrad et al. 1998).

Experiments employing whole-body hyperthermia in animal models as a heat shock paradigm also showed a clear trend of an attenuated heat shock response (HSR) in aging animals; the degree of this attenuation is dependent on the particular cell type, tissue, and organ system evaluated (Blake et al. 1990, 1991). As an example, investigators have analyzed the effects of thermal stress on the regulation of heat shock gene expression in the rat central nervous system and observed that the induction of hsp70 mRNA is highest in a subpopulation of glia, intermediate in dentate gyrus granule cells, and lowest in pyramidal cells of Ammon's horn (Pardue et al. 1992). Studies of whole-body hyperthermia treatment on the induction of hsp70 mRNA in rat organ systems provides further support of an age-related decline in brain, lung, and skin tissues (Blake et al. 1990, 1991).

The HSR is also known as the stress response in that many forms of "stress" other than heat shock can similarly elicit the response. In assessing the relevance of the attenuated HSR in the biology of aging, an important query is whether other forms of physiological and behavioral stresses might also elicit the HSR in a manner that attenuates with age of the organism. Using physical restraint of an animal as a means of activating the hypothalamic-pituitary-adrenal axis, studies show that restraint stress induces the expression of hsp70 mRNA in both the adrenal cortex and vasculature (Blake et al. 1991; Udelsman et al. 1993; Fawcett et al. 1994). The response is rapid and mediated by the activation of HSF1 DNA-binding activity. Moreover, while the adrenal response is adrenocorticotropin-dependent, eliminated by hypophysectomy and restored by exogenous ACTH, the vascular response is under adrenergic control and is selectively inhibited by α1- or β-adrenergic blocking agents. Qualitatively similar results are observed in laboratory animals subjected to surgical stress – anesthesia, laparotomy, hemorrhage, and recovery (Blake et al. 1991). Significantly, both the adrenal and vascular response to restraint stress is down-regulated with age in that induction of hsp70 expression and activation of HSF1 DNA binding are reduced dramatically in older animals as compared to young/adult rats (Blake et al. 1991; Udelsman et al. 1993; Fawcett et al. 1994).

The effect of age in limiting the induction of heat shock gene expression has also been documented in humans using peripheral blood mononuclear cells obtained from donor subjects. Specifically, it has been shown that induction of HSP expression in response to heat and mitogen stimulations is inversely correlated to the age of a donor (Deguchi et al. 1988; Faassen et al. 1989). Importantly, the decreased expression of HSP following mitogen stimulation of lymphocytes from aged donors correlates with a decreased proliferative response of the lymphocytes (Deguchi et al. 1988). Since peripheral blood cells can be obtained from human donors easily as compared to other tissues or cell types, this system may provide an attractive avenue for exploring age-related changes in adaptive stress response in human subjects. Together, these studies on a variety of cell and animal model systems reveal a concerted age-dependent decrease in response to different forms of environmental and biological "stresses." In all cases, aging at the organismic level is associated with a decline in the capacity of cells and organ systems to mount the appropriate HSR for cytoprotection and recovery after stress.

4.3 Regulation of HSF1 by the NAD$^+$-Dependent Deacetylase SIRT1: Implications in the Biology of Aging and Neurodegeneration

SIRT1 is a class III protein deacetylase (HDAC) with an obligatory dependence on nicotinamide adenine dinucleotide (NAD^+) as a cofactor, a requirement that provides a critical linkage of metabolism to gene regulation and cell function. There is evidence that SIRT1 may function as a longevity assurance gene. As an example, calorie restriction (CR) – a regimen that has been implicated in life span extension – can increase SIRT1 activity (Guarente 2000; Grabowska et al. 2017). Indeed, SIRT1-activating compounds have been explored and evaluated as pharmacological alternatives to CR regimens against aging and neurodegeneration.

SIRT1 is a high-networked protein that mediates adaptation to a variety of stresses including HSR (Karvinen et al. 2016). Studies show that HSF1 activity is regulated by SIRT1 (Westerheide et al. 2009). Specifically, deacetylation of HSF1 Lys80 by SIRT1 is necessary to maintain HSF1 DNA-binding activity whereas Lys80 acetylation is an off-switch for DNA binding (Gomez-Pastor et al. 2018), and conversely aging is associated with a decrease in SIRT1 protein expression, HSF1 DNA binding, and HSP induction. Indeed, SIRT1 overexpression enhances the ability of cultured cells to survive prolonged exposure to stress (Westerheide et al. 2009; Gomez-Pastor et al. 2018).

Neurons are post-mitotic cells and their noted vulnerability in age-related neurodegenerative disease may be causally related to deficiencies of the HSR QC mechanism in neuronal cells. Studies on the regulation of HSP induction in regions of the mammalian brain reveal a robust response in glial and ependymal cells relative to the null, delayed, or diminished response in neurons (Manzerra and Brown 1996; Marcuccilli et al. 1996; Nishimura and Dwyer 1996). Studies in various neuronal systems suggest there is a high threshold for induction of stress response, a defect attributed at least in part, to the lack of HSF1 activation (Marcuccilli et al. 1996; Nishimura and Dwyer 1996; Batulan et al. 2003).

In our work, we showed that neural differentiation of primary embryonic neurons and neuroprogenitor cells is associated with a decrease in HSF1 activation and induction of HSP chaperones (Oza et al. 2008; Yang et al. 2008). Moreover, evaluation of pharmacological SIRT1 activators and inhibitors affirms the regulatory role of SIRT1 in Hsp70 induction. In protein cross-linking studies, nuclear SIRT1 and HSF1 form a co-migrating high molecular weight complex upon stress. Experiments employing retroviral vectors to manipulate SIRT1 expression reveal that siRNA-mediated knock down of SIRT1 causes spontaneous neurite outgrowth, coincident with reduced growth rates and decreased induction of hsp70-reporter gene. Conversely SIRT1 over-expression blocks neuronal differentiation. These results show that a decrease in SIRT1 expression is conducive to neuronal differentiation with down-stream events that include a decrease in HSF1 activation and induction of HSP chaperones in differentiated neuronal cells (Liu et al. 2014). Assessment of the sensitivity of undifferentiated and differentiated neuronal cells against stress-induced cell death reveals a significantly greater vulnerability of the differentiated neuronal cells to excitotoxic challenges (Oza et al. 2008; Yang et al. 2008), an observation entirely consistent with the well-known protective role of HSP chaperones against stress-induced pathologies and death.

Collectively, the observation of a diminished capacity to mount the protective HSR in aging cells and organisms in general and, neurons in particular, has relevance and significance to our understanding of the pathogenic mechanism(s) of ND – a class of age-related disease characterized by the presence of non-native aggregates of disease proteins, amyloid-plaque, and Lewy bodies as examples and demise of post-mitotic neurons (Gomez-Pastor et al. 2018; Hipp et al. 2019; Santra et al. 2019). An equally important yet often overlooked function of HSP is that by facilitating protein conformational changes, these chaperones have fundamentally important roles in signal transduction and gene expression.

HSPs are essential for regulating the function, folding, and trafficking of the steroid receptor protein family and transforming tyrosine kinase $pp60^{v\text{-}src}$ (Rutherford and Zuker 1994). In addition to promoting the folding and assembly of nascent proteins, HSP molecular chaperones play a pivotal role in a variety of regulatory processes by facilitating structural transitions of signaling molecules and transcription factors to switch between the "on" and "off" states (Rutherford and Zuker 1994). In summary, sub-optimal HSF1 function of aging organisms in general and of neurons in particular likely will have a plethora of consequences that contribute to their dysfunction and demise under stress.

5 HSF1 and the Intrinsically Disordered Proteome in Aging and Disease

In an effort to better understand the mechanistic basis of the changes in regulation and function of HSF1 in aging as presented above, we turned our attention to the biology of a class of proteins that are intrinsically disordered: proteins that are structurally disordered in their entire length are termed Intrinsically Disordered Proteins (IDPs), and proteins with structurally disordered regions are termed Intrinsically Disordered Region proteins (IDRs). IDPs and IDRs are broadly involved in human disease and the term "disorder in disorders" (or D^2) has been coined to underscore this connection (Kulkarni and Uversky 2019). Indeed, HSF1 and the HSP chaperone proteins are members of the family of intrinsically disordered proteins.

The number of IDPs and IDRs characterized to date has increased dramatically as this class of proteins is generally recognized as challenging the paradigm of a "global minima" in the folding landscape (Uversky 2013). Given their unique sequence features, the development and application of computational tools for identifying and predicting specific regions that are prone to intrinsic disorder represents a priority (Chen et al. 2017). These computer algorithms reveal that IDPs are widespread to account for approximately one third of the eukaryotic proteome (Dunker et al. 2015; Chen et al. 2017; Uversky and Dunker 2010). Moreover, nearly one-half of the eukaryotic genome encodes protein with intrinsically disordered regions (IDRs), a segment of >30 amino acids in length. The malleable and dynamic nature of IDPs as reflected by their binding to different partners and larger interacting surface for functionality per unit peptide length contributes to their selection and prevalence (Uversky 2019).

The importance of IDPs/IDRs is underscored by their disproportionate presence in networks of transcription, chromatin dynamics, RNA translation, signaling, and disease pathogenesis (Uversky 2019). Indeed, proteins involved in ND are overwhelmingly IDPs/IDRs; the compaction and aggregation of these proteins is a common epi-phenomenon in advanced disease states as exemplified by neuritic senile plaques (aka, amyloid plaques) and neurofibrillary tangles in Alzheimer's disease, Lewy bodies in Parkinson's disease, and inclusion bodies in Huntington's disease (Dunker and Kriwacki 2011; Dunker et al. 2015; Uversky 2015, 2016). Some characteristic features of IDPs/IDRs include their structural dynamics, ability to interact with multiple protein partners, and exquisite sensitivity to changes in the physio-chemical environment. The observation that disordered protein is highly over-represented in both transcription and diseases processes is both a remarkable and consequential finding with significant implications on IDR functionality in cell regulation. Moreover, post-translational modifications, a noted feature of IDP/IDR, provides an additional layer to orchestrate and fine-tune the dynamic control of these protein under both physiological and pathological states (Uversky 2019).

5.1 Biophysical Properties of IDPs and IDRs

IDPs and IDRs are characterized by the absence of well-defined secondary and/or tertiary structures under native conditions (Uversky and Dunker 2010). The resultant structural fluidity/

plasticity affords transient and dynamic interactions with protein partners as IDPs may adopt specific conformations upon binding. IDPs are often referred to as *Natively Unfolded, Natively Denatured,* or *Intrinsically Unstructured Proteins*. The disordered nature of IDPs and IDRs derives from the peculiarities of their amino acid composition which is typically populated by a high frequency of charged relative to low occurrence of hydrophobic residues. An intrinsically disordered protein therefore lacks the necessary driving force to fold into a stable and compact three-dimensional structure. The unique amino acid composition and sequence yields large surface areas and hydrodynamic dimensions while resulting in high proteolytic sensitivity (Uversky 2011).

5.2 Role of IDRs in the HSR Machinery

HSP chaperones are IDR-containing proteins, and their intrinsically disordered structure is fundamental in terms of functionality (Pattaramanon et al. 2007; Pujols et al. 2018). Computational analysis suggests that approximately 40% of chaperone residues correspond to IDRs and 10% of the latter comprise long stretches within HSP. Disordered regions in HSP are likely to confer the malleable guardian function of chaperones which act as pliable recognition elements in binding misfolded proteins and promoting the disaggregation/unfolding of non-native species. While IDRs provide a dynamic functionality that enables HSF1 binding and association with different partners, the resultant structure is extremely sensitive to changes in various physicochemical properties including the concentrations of protein, osmolytes, and salts as well as pH and cellular energy levels. It has been suggested that the fluctuation of these parameters with age, driven in part by metabolic decline and a failure of homeostatic control, may trigger numerous events associated with the onset of neurodegeneration. The structural properties of HSF1 have been characterized using computational tools to reveal the extensive network of IDRs that encompass the regulatory and transcription activation domains of the transcription regulator (Pattaramanon et al. 2007; Pujols et al. 2018).

5.3 Predicting and Verifying IDR Content in HSF1

Given the importance of IDP/IDRs in cell regulation, significant efforts have been expended towards developing automated software programs to predict the extent of order-disorder within a particular protein sequence. Specific examples include IUPred (Dosztanyi et al. 2005), PONDR (Obradovic et al. 2003), and an improved meta-predictor designated PONDR-Fit. Employing a selected number of predictive models, the HSF1 sequence has been evaluated in terms of IDR content as illustrated in Fig. 9a. The IDRs are predicted to span regions comprising scores that are equivalent or greater than 0.5. The resultant analysis reveals that human HSF1 is characterized by an extended stretch of disordered residues towards the C-terminus. Experimental data have in fact confirmed the presence of such long stretches of unfolded regions in HSF1 corroborating computational analysis. It has been suggested that these IDRs are fundamentally important in the regulation and function of HSF1 as a master regulator of the HSR (Gomez-Pastor et al. 2018). Interestingly, the isolated HSF1 IDR adopts a more collapsed state upon exposure to acidic pH and undergoes partial folding as a function of temperature or the presence of surfactants as evidenced in circular dichroism studies (Pattaramanon et al. 2007). The ability for IDRs to adopt different conformations as a function of its environment suggests that the IDRs of HSF1 may confer contextual interaction with different partners in physiological versus pathological states.

5.4 IDRs Modulate HSF1 Monomeric State Stability

In the inactive state, HSF1 is predominantly bound to a multi-chaperone complex conceivably

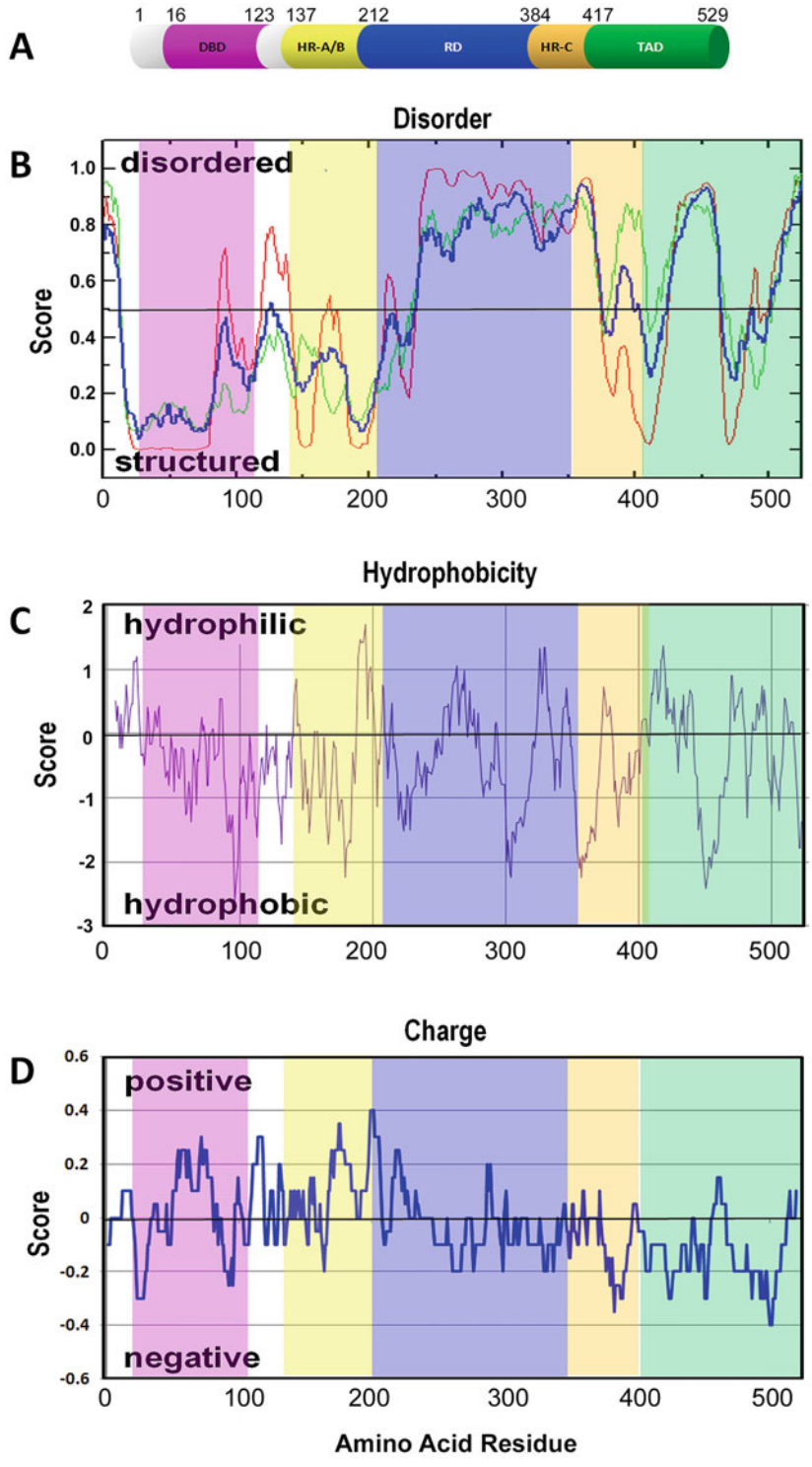

Fig. 9 HSF1 Sequence Analysis Deduced via Predictive Models. (**a**) Structural organization of specific domains and color schemes employed in previous illustrations. (**b**) Intrinsic Disorder evaluated via PONDR in which scores exceeding 0.5 reflect structural disorder. (**c**) Hydrophobicity scale assessed via UniProt in which positive and negative scores correspond to hydrophilic and hydrophobic regions, respectively. (**d**) Net Charge assigned via UniProt in which positive and negative scores reflect the average charge

via IDRs (Guo et al. 2001), thereby stabilizing the HSF1 monomeric state. This serves as an auto-regulatory mechanism afforded by the ability of HSF1 to transcriptionally activate the corresponding chaperone encoding genes. It is important to note that the longest stretches with highest disordered scores (as assessed via PONDR) reside in the regulatory domain (RD) and to a lesser extent in the transactivation domain (TAD). The prediction that both regulatory and transactivation domains are largely disordered implies that these two regions contain highly flexible segments, which is consistent with the difficulty in obtaining X-ray and/or NMR structures of these domains. Employing available proteomic tools, one may visualize specific sequence-dependent properties of the polypeptide chain including hydrophobicity as presented in Fig. 9b (Gasteiger et al. 2005). One characteristic of IDPs is the presence of alternating positively and negatively charged residues throughout the sequence (Zagrovic et al. 2018). In the case of HSF1, there is a predominance of acidic residues toward the C-terminus coinciding with the more disordered regions as depicted in Fig. 9c. In fact, when the isolated C-terminus is exposed to acidic conditions, the polypeptide chain undergoes compaction and ordering due to neutralization of negative charges (Pattaramanon et al. 2007). The overall implication of IDRs in terms of HSF1 regulation and function is enumerated below.

5.5 IDRs Modulate HSF1 Transcription Activation

The intrinsically disordered regulatory domain (RD) and trans-activating domain (TAD) are key regulatory and functional elements of the HSF1 protein (refer to Fig. 9a). An important question is: what functional role does structural disorder play in transcriptional regulation? One line of studies supports the notion that protein disorder might assume a *direct role* in accelerating binding/unbinding and overall DNA search processes, thereby increasing the efficiency of transcription. In line with this suggestion is the observation that the degree of disorder in transcription factors appears to correlate with an organism's level of complexity (Yruela et al. 2017). Perhaps, IDRs in transcription regulators may allow for broader networks and higher efficiency of transcriptional responses.

5.6 Predicting Functional and/or Deleterious Protein-Protein Interactions via Sequence Analysis

The structural dynamics of IDPs/IDRs drive their often transient and facile interaction with multiple protein partners for both functionality and pathogenicity. Due to the exquisite sensitivity to their physical-chemical environment, IDP/IDR can bind and sequester other key regulatory proteins (heterotypic interaction) or undergo phase-transition to form protein aggregates (homotypic interaction). One such structural element that can drive protein assembly and aggregation is a prion-like low complexity sequence (Dosztányi et al. 2009). Using computer algorithms to predict [i.e., ANCHORII (Dosztányi et al. 2009)] and detect such "prion-like" motifs [i.e., Waltz (Maurer-Stroh et al. 2010; Pujols et al. 2018)] in HSF1 yielded a putative amyloidogenic sequence as illustrated in Fig. 10. At this point in time, the functionality of this "prion-like" motif of HSF1 remains to be determined. One possible scenario is that this motif may play a role in both on-target and off-target interaction with other proteins and contribute to the attenuated activation of HSF1 in aging and disease.

5.7 IDPs Undergo Liquid Phase Separation

Recent evidence suggests that an important role of IDRs is to mediate phase transitions and can drive the formation of "membrane-less compartments" with changes in the intracellular environment (Gui et al. 2019). Prominent examples include nucleoli, stress granules, and the formation of nuclear stress bodies composed of HSF1 (Alberti and Hyman 2016). The

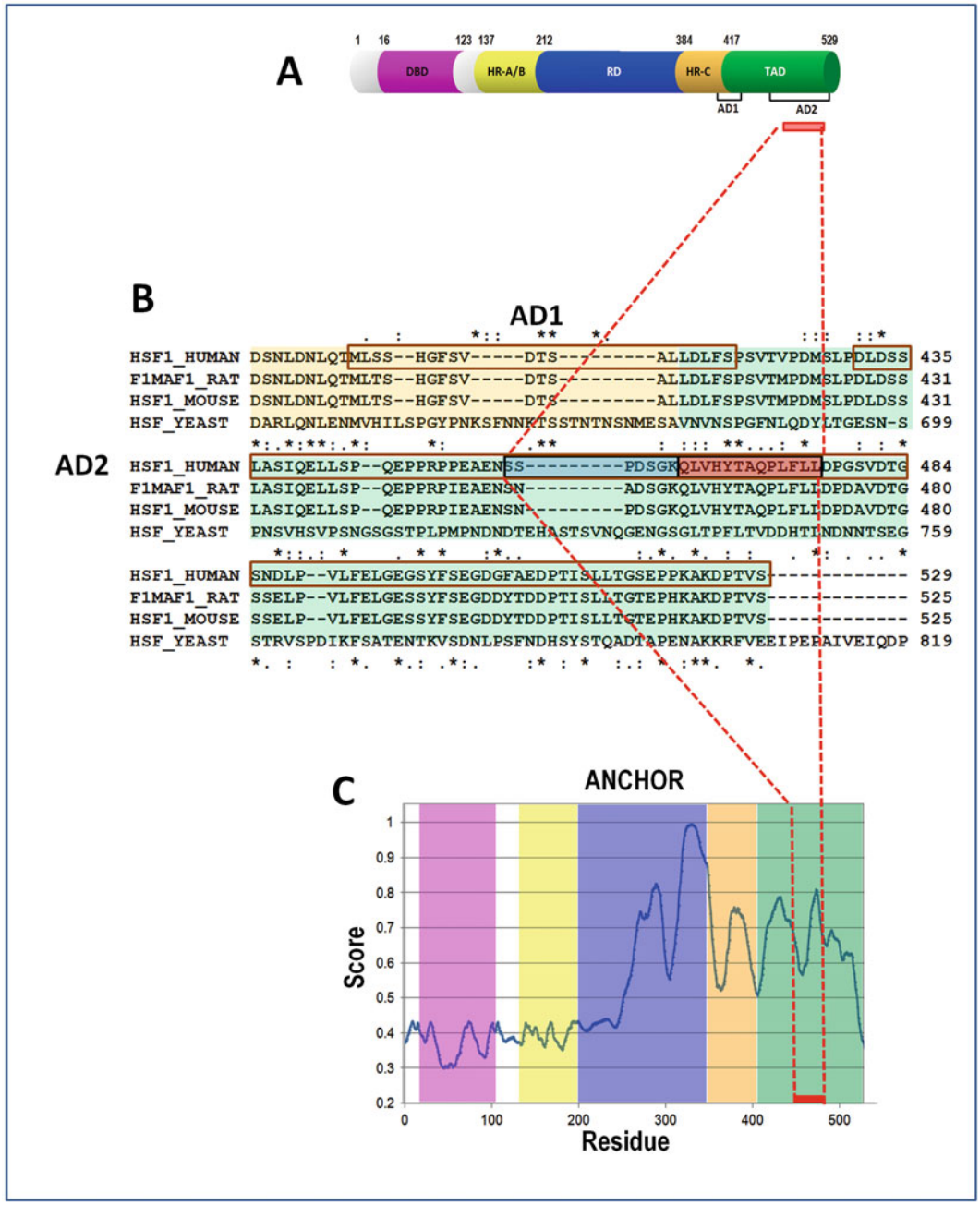

Fig. 10 Predicting Protein-Protein Association Motifs in the HSF1 Sequence (**a**) Structural organization of hHSF1 domains employed in previous illustrations emphasizing TAD AD2 where the presence of several motifs is predicted via ANCHOR, Morf, and Waltz (refer to text for details). (**b**) The C-terminus is comprised of activation subdomains AD1 and AD2, the latter of which contains a contiguous sequence stretch predicted to harbor protein-protein association (residues 457–463) and prion-like (residues 464–476) motifs. (**c**) The corresponding regions are highlighted in an ANCHOR-plot which scores the propensity for a sequence to adopt protein-protein associations. The color schemes reflect specific domain regions as described in Fig. 2

functional consequence of this phase transition includes facilitated reactions, prevention of non-target effects, and sequestration of pathogenic proteins. Since IDP/IDRs are unstructured in their native states with large solvent exposed surface area, their structuring and organization are exquisitely sensitive to environmental changes: protein concentration, pH, osmolarity are among the key factors. Indeed, phase transitions and subsequent separation into microscopically visible protein aggregates are major contributing factors to the formation of protein aggregates in neurodegenerative diseases (Uversky 2015; Babu 2016).

5.8 IDPs, Phase Separation, and Diseases: HSF1 and HSPs Drive the Structuring of Intrinsically Disordered Huntingtin into Forming Aggregates

IDPs and IDRs are broadly involved in human disease (Kulkarni and Uversky 2019). In our research, we have used the Huntington's disease as a model to assess the role of HSF1 and HSP chaperones in the structuring and pathogenicity of the disordered mHtt^{exon1}-EGFP protein. In a recent study, we demonstrated that the heat shock-induced activation of HSF1 and induction of HSP chaperones drives the compaction of diffuse and intrinsically disordered mHtt into forming IBs (Inclusion Bodies) to exemplify the well-known liquid phase separation and condensation of IDPs discussed above (Chen et al. 2018). Moreover, diffuse assemblies of the polyQ-expanded mutant Htt protein quench the activity of HSF1, CREB, and NFκB to repress their reporter gene activity. Conversely, heat shock and induction of HSP chaperones promote the structuring of intrinsically disordered mHtt to form inclusion bodies (IB) that result in de-repression of HSF1 and CREB for improved cell fitness (Chen et al. 2018). These results are in agreement with previous observations of an impaired HSR in mHtt expressing cells (Silva et al. 1998; Chafekar and Duennwald 2012; Kandel 2012) and that the expression of model β sheet proteins leads to deficiency of the normal cytosolic stress response mediated by HSF1 and NFκB (Olzscha et al. 2011). Likewise, a deficiency in striatal neuron proteostasis machinery may contribute to the select demise of these neurons in HD (Margulis and Finkbeiner 2014). Our study adds to a growing stream of evidence that the compaction of IDPs to form aggregates may represent either a consequence of or a coping mechanism in disease states (Arrasate et al. 2004; Bodner et al. 2006; Knowles et al. 2014; van Hagen et al. 2017). Collectively, these considerations suggest that a detailed knowledge base of the structure, function, and regulation of IDPs/IDRs may offer insights into the pathogenic mechanisms of neurodegenerative disease.

6 Perspective: Intrinsically Disordered Proteome, Water, Hydration, Aging, and Neurodegeneration

Liquid water is a universal solvent, a mediator of life's chemical reactions, and its structure is unlike that of any other liquid. Indeed, interactions with water generally govern the folding, structure, stability, and activity of proteins. Herein, we advance a hypothesis that water mobility and protein hydration may hold answers to understanding the many functional and structural changes in aging and the development of age-related diseases. HSF1 is an IDR protein and the intrinsic disorder of its "RD" and "TAD" domains is necessary for the functioning of HSF1 as a transcription activator of heat shock genes. Likewise, many of the proteins implicated in neurodegenerative diseases such as β-amyloid, α-synuclein, and polyQ-expanded huntingtin are IDPs. A unifying feature of IDPs and IDRs is that their native and functional structure is partly or completely unfolded with large solvent exposed surface areas forming more hydrogen bonds with water which continually breaks and reforms as the IDP transitions between conformations. Accordingly, the surrounding water dynamics would have a significantly greater impact on the

structuring and aggregation of IDPs/IDRs as compared to natively structured and folded proteins. The concomitant reduction in water mobility (e.g., increased ions and macromolecular crowding in cells) would reduce the hydration shell of IDPs to drive their compaction and aggregation. Indeed, evidence indicates that the degree of protein hydration and localized water mobility in cells influences the propensity of α-synuclein to aggregate and form fibrils that effect disease outcomes (Stephens and Kaminski Schierle 2019).

Significantly, progressive dehydration occurs during aging in both inbred mice (Hooper et al. 2014; Vashisht et al. 2018) and the human population (Hooper et al. 2014; Vashisht et al. 2018), and this loss of hydration is functionally correlated with deregulated synaptic plasticity (Hooper et al. 2014; Vashisht et al. 2018). Using the parameter of "hydration potential" as an index, studies on inbred mice reveal a progressive ~10% loss of brain water from 6 to 40 weeks of age with corresponding changes in synaptic strength (Vashisht et al. 2018). The authors conclude that "neuronal functions are exquisitely sensitive to dehydration within a specific range that reflects the dehydration levels during aging" (Vashisht et al. 2018). Inevitably, as water activity decreases in the aging brain, all molecular structures and IDPs/IDRs in particular shift toward more dehydrated and compact conformations to drive intermolecular interactions, aggregation, and dysfunction (Stephens and Kaminski Schierle 2019). Collectively, these observations support the notion that progressive and minute changes in the physical-chemical parameters of cells including hydration, water mobility, and molecular crowding may be important contributing factors to the homeostatic and functional decline in aging that result in pathogenic and aberrant phase transitions with a consequent loss of control over intracellular organization and function (Alberti and Hyman 2016).

7 Concluding Remarks

HSF1 plays a critical role in maintaining proteostasis by protecting an organism against stress-induced dysfunctions, counteracting age-associated deterioration, and restrain disease processes such as neurodegeneration. Herein, we present a general overview of the HSF1-dependent HSP quality control mechanism in a healthy cell. We further discuss age-related changes in protein conformation and function that may predispose organisms to pathologies, neurodegenerative diseases included. Research in this particular area has accelerated dramatically over the past several decades with significant advances in cell biology, proteomics, and the development of predictive tools. Despite such achievements on multiple fronts, the causes of aging and the associated development of age-related ailments remain not well understood. Recent studies have furnished evidence that IDPs and IDR-containing proteins are pivotal in biological regulation and their unique sensitivity to cellular milieu changes likely promotes homeostatic deterioration and susceptibility to diseases in aging. Future investigations should therefore focus on identifying the physicochemical parameters that control the structure, function, and organization of IDPs and IDRs in aging cells and organisms. Characterization of these physicochemical parameters can contribute to our understanding of specific changes in protein structure and function as observed for HSF1 that are critical to aging and neurodegenerative disease and lay the intellectual framework for therapeutics development.

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Pathophysiology of Spinal Cord Injury and Tissue Engineering Approach for Its Neuronal Regeneration: Current Status and Future Prospects

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Abstract

A spinal cord injury (SCI) is a very debilitating condition causing loss of sensory and motor function as well as multiple organ failures. Current therapeutic options like surgery and pharmacotherapy show positive results but are incapable of providing a complete cure for chronic SCI symptoms. Tissue engineering, including neuroprotective or growth factors, stem cells, and biomaterial scaffolds, grabs attention because of their potential for regeneration and ability to bridge the gap in the injured spinal cord (SC). Preclinical studies with tissue engineering showed functional recovery and neurorestorative effects. Few clinical trials show the safety and efficacy of the tissue engineering approach. However, more studies should be carried out for potential treatment modalities. In this review, we summarize the pathophysiology of SCI and its current treatment modalities, including surgical, pharmacological, and tissue engineering approaches following SCI in preclinical and clinical phases.

Keywords

Neuroprotection · Neuroregeneration · Scaffolds · Spinal cord injury · Stem cells · Tissue engineering

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1 Introduction

The spinal cord (SC), a highly somatotopically organized structure, is a principal component of the central nervous system (CNS) with a long cylindrical structure that initiates from the medulla of the brain above the C1 (through foramen magnum) and terminates at L1–L2 (as conus medullaris). It conducts sensory, motor, and autonomic information (Khan and Lui 2020). An injury may reduce the ability of SC to partially or completely carry out its primary functions.

Thus, a spinal cord injury (SCI) is an injury to the SC that most often results in everlasting changes in the functioning of the body below the site of the injury (Ahuja et al. 2017). The most prevalent cause of SCI is traffic accidents, which is followed by falls among the elderly (Singh et al. 2014). As per age statistics, the highest incidence of SCI is in people who are less than 30 years of age. Even males are more prone to SCI than females. The prevalence rate of SCI indicates that the occurrence of SCI is geographically distinct and different in different regions. The prevalence rate of SCI (per million population) in Canada (~1289) is more than that of the United States of America (~721–1009), followed by Australia (~681) and other notable regions including Finland (~280), Iceland (~316), South and Southeast Asia (~236–464), and India (~236) (Singh et al. 2014; Furlan et al. 2013; Srivastava et al. 2015; DeVivo 2012; Cripps et al. 2011). The clinical outcomes (loss of motor, autonomic, and sensory functions and paralysis) of SCI may be due to four main characteristic injury mechanisms: persistent compression, transient compression, distraction, and laceration/transaction. SCI is considered a major health problem as the physical, emotional, and economic costs are burdensome for the patient, his family, and society, according to World Health Organization (WHO) (Lynch and Cahalan 2017). After an SCI, many patients exhibit loss of respiratory, autonomic, and sensorimotor functions, posttraumatic stress disorder, anxiety, and even depression which has led to a high mortality rate and a lower quality of life (Ahuja et al. 2017; Lynch and Cahalan 2017).

Currently, frontline treatment option available for SCI is a surgical decompression and drugs that aid to improve the condition and reduce the subsequent injury (secondary injury). In surgical decompression, clinicians stabilize vertebral column and remove bone fragments which are inserted in SC during primary injury. This type of surgical intervention only reduces stress over the SC; evidently, CNS has a low regenerative capacity, and therefore this treatment option alone is unable to heal SCI. Another treatment option currently in practice is pharmacotherapy in which various drugs (methylprednisolone [MP], etc.) are used to reduce complex pathophysiology of SCI. Drugs have common side effects such as headache, vomiting, and so on. Aforementioned treatment options are unable to rebuild the damaged neural network. Currently, there is no validated therapy approach that has shown to improve neurological outcomes successfully.

Neuroprotective and neurorestorative approach by using tissue engineering could be a potential alternative treatment for SCI. It involves the use of growth factor (neural growth factor, brain-derived growth factor, FGF, etc.), cells (neural stem cell [NSC], embryonic stem cell [ESC], mesenchymal stem cell [MSC], iPSC, etc.), and scaffolds (hydrogels, 3D printed scaffold, etc.). Preclinical studies by using tissue engineering significantly improved motor function and ultimately resulted in neural regeneration. This review discusses the pathophysiology of SCI and effect of existing approaches such as surgical and pharmacological for SCI. This review puts insights on tissue engineering approaches using growth factors, cell therapies, and scaffolds for treatment of SCI. New findings from clinical trials are also highlighted.

2 Pathophysiology of SCI

SCI can be attributed to both "traumatic and nontraumatic" etiologies. Traumatic SCI (TSCI) is a tormenting condition that alters the integrity of the SC (David et al. 2019). TSCI may be caused by direct mechanical injury like fall (Ahuja et al. 2017; Chen et al. 2016; Kennedy et al. 2013; Medina et al. 2020), vehicle accidents (Medina et al. 2020; Silveira et al. 2020; Kang et al. 2018; Wang et al. 2016), acts of violence (Watane et al. 2021; January et al. 2018), electrical accidents (Zeb et al. 2019; Delgadillo III et al. 2017; Zhirkova et al. 2020), and recreational activities (Babcock et al. 2018; Li et al. 2021; Wu et al. 2020; Hosaka et al. 2020). A nontraumatic SCI may be the result of a tumor or infection. The most common type of nontraumatic SCI is degenerative cervical myelopathy (DCM) (David et al. 2019). The result of

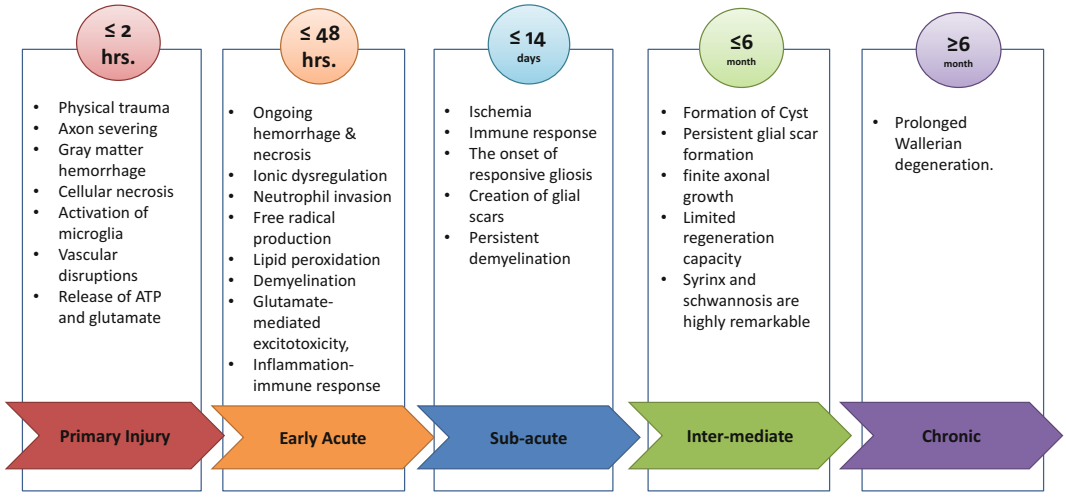

Fig. 1 Timeline of the damage phases and major pathological events following SCI: After any mechanical stress on SC, primary injury occurs with various physiological changes like ischemia, hemorrhage, axon severing, etc. It is followed by early acute and subacute phases which is characterized by evoke immune response, persistent hemorrhage, ischemia, etc. At these phases, most of glial cells aggregate near lesion area to form glial scar which leads to regeneration failure. Injury worsens as it comes to chronic phase ultimately leading to Wallerian degeneration

SCI is the degeneration of motor, sensory, and autonomic functions. After primary injury, secondary injury develops with complex pathological mechanisms and can last for weeks. Timeline of SCI is shown in Fig. 1. There are possible four mechanisms responsible for SCI, viz., hyperflexion, hypertension, axial loading, and penetrating wound. Injury could occur by one of the above mechanisms by one or more biomechanical means, and the characteristics of the tissue damage depend on different aspects of trauma. An in-depth understanding of the pathophysiology and the mechanisms that arise just after SCI are crucial for developing precise therapeutic strategies that can reduce or cure damage. SCI has two phases in its pathophysiology: a main phase, i.e., primary phase (primary injury), and a subsequent secondary phase (secondary injury).

3 Primary Injury

SCI is caused by an early damage to the spine, for example, by mechanical stress. This is referred to as the primary injury. A direct impact seems to be the most prevalent cause of primary damage, and chronic compression is usually caused by bone fragments during fracture–dislocation injuries. Hyperextension injuries, unlike fracture–dislocation injuries, generally arise in quite frequent manner and impact only temporary compression. The other mechanism is distraction damage, which is characterized by a strain and rupture of the SC through its long axis caused by the separation of two neighboring vertebrae. Lastly, laceration/transection injuries are caused by pointed bone remains, severe dislocations, and missile injuries (Alizadeh et al. 2019). Primary injury can range in severity from full to partial, i.e., complete to incomplete. The primary phase is characterized by disruption in blood vessels, axons, and cell membranes, resulting in cellular necrosis within 2 h following injury (Jeong et al. 2021). The breach of the blood–spinal cord barrier (BSCB) causes necrotic cells to discharge DNA, ATP, and K+ in the proximal injured area, resulting in inflammatory responses by cells such as macrophages, microglia, and T-cells. "Inflammatory cytokines" such as interleukin (IL-6), IL-1, and tumor necrosis factor

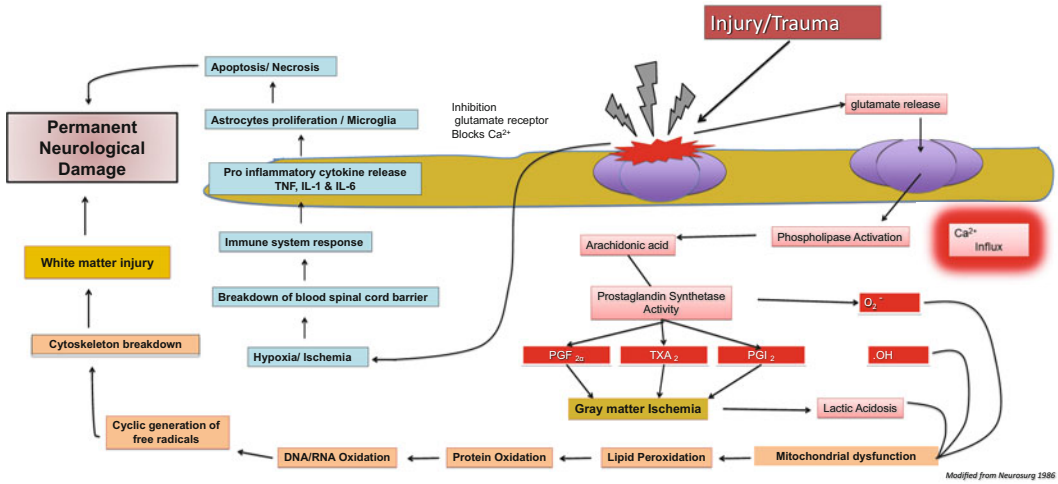

Fig. 2 SCI cascade
Possible pathophysiological changes following SCI. Pathophysiology can be divided into primary and secondary injury. Injury begins with hypoperfusion which results in hypoxia/ischemia to tissue along with excess glutamate release. This leads to initiation of injury cascade consisting of calcium influx, oxidative state, and white and gray matter damage that results in permanent neurological damage to the SC

(TNF) are secreted with optimum levels reaching between 6 and 12 h (Jeong et al. 2021; Ulndreaj et al. 2016). Pathophysiological changes during SCI are shown in Fig. 2.

Primary injury (Fig. 3b) impacts upper as well as lower motor neural connections, leading to a variety of negative consequences on vascular tensions, cardiac output signals, and sensory functions and inhalation, whereas secondary injury worsens the primary injury (Shende and Subedi 2017). Gray matter ischemia causes nerve cell bodies to be destroyed or even connections to be disturbed. Paralysis of muscle can be occurred at injury location due to disruption in motor nerve cell in the ventral horns, but damage to higher nerve cells that cross the injured area causes a loss of efferent impulses to muscles underneath the injury site (Forgione and Fehlings 2013). During trauma, synaptic connections are destroyed and the ability to execute commands by the neural cells is lost due to demyelination and destruction; as a result, neurons die mechanically (Fig. 3c) (Orr and Gensel 2018). The severity of lesion at injury area produces an inhibitory microenvironment that hinders native repair, remyelination attempts, and regeneration (Moriwaki et al. 2016; Liu et al. 2018).

4 Secondary Injury

A variety of pathological processes (more than 20) combine to aggravate the initial damage acquired as a result of primary injury (Jiang et al. 2020; Nakamura et al. 2003). After original trauma, SCIs induce prolonged damage and also the demise of surviving cells. After days or weeks of an injury, a secondary phase begins. Secondary SCI is classified into four stages based on post-injury timeframe and disease pathogenesis, namely, early acute, subacute, intermediate, and chronic (Fig. 1). The early acute phase is defined as the first 48 h after a mechanical trauma on the SC. In this phase, ongoing hemorrhage and ischemia are caused by vascular disruptions. It results in vascular circulation irregularities, swelling, and inflammatory reaction. It is followed by immune reactions, glutamate-mediated excitotoxicity, neutrophil invasion, oxidative stress and free radical production, lipid peroxidation, neurotoxicity

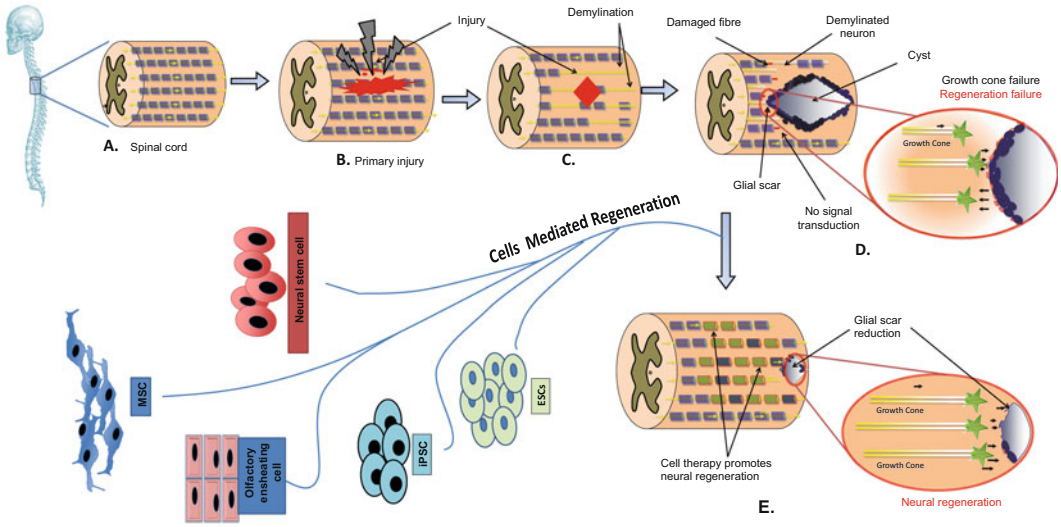

Fig. 3 SCI and cell therapy-mediated SC regeneration and repair
(**a**) Normal SC; (**b**) SCI by mechanical force; (**c**) due to injury, neural tissue is damaged which leads to demyelination of neuron around the injury site; (**d**) immune cell gets activated due to injury; therefore, glial cells migrated toward injury site and aggregate to form glial scar. Due to this, regenerative cell unable to reach injury site leads to no regeneration in that area; (**e**) cell-based therapy will reduce glial scar which ultimately resulted in neuronal regeneration

with changes in regional ionic gradients and Ca^{2++} influx, and apoptosis, which are among the events that contribute to subsequent damage following SCI (Rowland et al. 2008; Ko 2019). Alarmins are released during necrosis, causing resident glia to become reactive and downstream immune cells from the periphery to infiltrate the tissue (Tran et al. 2018). After 48 h to 14 days, subacute phase starts with onset of responsive gliosis in which astrocytes and glial cells are reacted in nonspecific manner in response to injury which results in accumulation of these cells around the injury site and creates glial scar with persistent demyelination (Fig. 3d). Though glial scarring is a restorative action, it is detrimental to axon growth throughout the duration (Silver and Miller 2004). SCI stimulates local astrocytes and peripheral cells, as well as recruiting fibroblasts and invading Schwann cells from periphery, resulting in the formation of lengthy glial scars (cellular) and fibroids (acellular) in the damaged spinal column. The transforming growth factor beta (TGF-β) enhances the activation of astrocytes and the eventual borders of glial scar. ECM molecules can stiffen the surroundings, create a physical obstacle, and provide imprecise topographic cues, all of which can obstruct cell movement (Orr and Gensel 2018). The acute contusions effectively separate gray matter which necrotizes and becomes fluid-like (syrinx) along with the formation of cyst that is highly remarkable in intermediate injury phase ($\leq$ 6 months) (Guest et al. 2018). It is followed by the formation of persistent glial scar which hinders the axonal growth resulting in limited regeneration. If all these pathophysiological cascades continue for more than 6 months, it leads to chronic injury phase. It could cause Wallerian degeneration which is a worsened state of secondary injury. It leads to complete neurological defect, since no regeneration will occur in this region. Therefore, to reduce glial scar is one of the hurdles in neuronal regeneration.

5 Treatment Modalities for SCI

A current SCI therapy focuses on cord stability to avoid additional injury, rehabilitation, non-motor symptom management, and complication prevention.

6 Surgical Decompression

Early surgical intervention is recommended to maximize the healing through neurocompression of remnant partly injured neuronal tissues and to provide early vertebral column stabilization to allow early mobilization for rehabilitation. Posterior and anterior techniques are used in spinal surgery. Excision of the body, open dura technique, open cord technique, anterior fusion, internal fixation, and disc excision are the six types of anterior techniques. Laminectomy, open cord technique, open dura technique, internal fixation, posterior fusion, and disc excision are the techniques categorized for posterior procedures (Duh et al. 1994). The potential for restoring flow of blood and enhancing perfusion while potentially preventing further injury is one of the reasons for immediate surgical decompression (Shank et al. 2019). Within 8 h of a severe SCI, early surgical decompression seems to promote neurological recovery. In addition, partial SCI was found to be more closely linked toward positive neurological recovery as compared to complete SCI (Lee et al. 2018). In individuals with severe traumatic cervical SCI, surgical decompression before 24 h has been more usually linked with neurological recovery of at least two American Spinal Injury Association (ASIA) grades (Eckert and Martin 2017; Ramakonar and Fehlings 2021). Cord edema, epidural hematoma, or bleeding or impinging foreign bodies and bone fragments has already been thoroughly investigated in compressive SCI and their duration during surgical decompression. The degree and duration of cord compression have been shown in preclinical studies to be related to the eventual neurologic deficit (Eckert and Martin 2017). It is hypothesized that parameters such as intramedullary lesion severity may be even more significant than timing of treatments in terms of clinical consequences in SCI (Rouanet et al. 2017). Despite growing acknowledgment that early decompressive surgery is a safe and reasonable therapeutic option, earlier clinical trials have only been suggestive because of low quality of data resulting from small sample sizes, retrospective analysis, and inconsistent methodologies. In incomplete cervical SCI patients, surgery timing had no effect on its neurological outcome (Ter Wengel et al. 2019). Due to operational and logistical reasons, rapid decompression of injured SC is not always possible. Additionally, managing individuals who are clinically fragile as a result of numerous injuries or associated complications may limit immediate spinal decompression. All set of time taking diagnostic procedure may delay the hospitalization of SCI patients. Also, unexpected events, such as global pandemic safety measures, may also pose considerable hurdle in operating early decompressive surgery (Ramakonar and Fehlings 2021).

7 Hemodynamic Management

Following SCI, hemodynamic treatment can be conducted promptly, assisting in the maintenance of adequate SC perfusion and preventing subsequent injury like ischemia. Therefore, practicing to keep constant blood pressure after SCI is a must. Perfusion of the SC is similar to cerebral perfusion (Lee et al. 2021). Hypotension can develop after an SCI even if there is no hemorrhagic shock as sympathetic innervation is interrupted. This is known as "neurogenic shock," and it can lead to SC hypoperfusion and exacerbated injury (Karsy and Hawryluk 2019). It is critical to avoid hypotension and maintain perfusion of the damaged SC by increasing the mean arterial pressure (MAP). Existing medical practice recommendations include keeping MAP in between 85 and 90 mmHg during early 5–7 days after an acute cervical SCI and avoiding systemic hypotension (systolic blood pressure of less than 90 mmHg) (Lee et al. 2021). However, determining a significant link between MAP targets and neurological recovery is difficult due to methodological constraints (Evaniew et al. 2020). Hemodynamic management reduces subsequent ischemia as well as spinal pressure, but, alone, it is unable to heal injured SC. Still there is unclear information about optimizing

hemodynamic management. Further studies on the hemodynamic care of acute SCI are required for enhancing neurological recovery to reduce secondary injury.

8 Pharmacotherapy

On a biological level, current pharmaceutical intervention seeks to decrease the secondary cell death phase. Some common drugs in clinical and preclinical studies are used to reduce additional injury. The use of corticosteroids as a neuroprotective therapy for acute SCI is debatable. MP has emerged as the preferred corticosteroid and a primary treatment option for SCI. MP has been claimed to lessen initial damage along with subsequent harm (Shank et al. 2019; Rouanet et al. 2017). MP has proven to be more effective compared to other corticosteroid substances such as dexamethasone and hydrocortisone. It demonstrates superior antioxidant properties, passes quickly via the cell membranes, and also appears to be more effective in suppressing the neutropenic response and further stimulating the complement components. Naloxone, a nonspecific opioid receptor antagonist, is shown to have anti-inflammatory properties (Tang et al. 2021; Lin et al. 2017). The release of inflammatory factors and microglial activation can be inhibited by naloxone (Tang et al. 2021). Nimodipine (NMD), a calcium antagonist, can improve functional recovery when treated for long period of time following SCI (Leisz et al. 2019; Guo et al. 2021). Tirilazad mesylate has been examined extensively for the protection of neural damage caused by SCI, subarachnoid hemorrhage, head injuries, and stroke (Carratù 2017). Minocycline hydrochloride can have therapeutic benefits resulting in reducing inflammatory microglial, antioxidant, and antiapoptotic activity while improving locomotor activity (Shultz and Zhong 2017; Afshary et al. 2020). Riluzole is a well-known antiglutamatergic agent and appears to be effective in reducing neuropathic pain, improving motor recovery, and reducing abnormal reflexes in initial clinical studies (Srinivas et al. 2019; Meshkini et al. 2018). A potassium channel blocker, 4-AP (4-aminopyridine), has improved motor function recovery, encouraged remyelination, and showed improved axonal region post-injury at a rate too fast to be made clear by axonal regeneration (Noble et al. 2019; Jensen and Shi 2003). Serine protease inhibitors (gabexate mesilate) inhibit NF-B, proinflammatory cytokines, and nitric oxide (Shih et al. 2015; Oh et al. 2020), and rapamycin increases autophagy and provides neuroprotection in a variety of CNS diseases (Li et al. 2018a). N-benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone, a caspase inhibitor, improves motor function and prevents lesion severity significantly after SCI. Caspase 1 and caspase 3 have been recognized as major mediators of apoptosis (Li et al. 2000).

Many drugs are in clinical practice because of their safety and efficiency to minimize pathological outcomes, but they may result in common clinical complains such as hypertension, anxiety, osteoarthritis, osteoporosis, adverse drug reactions, depression, headache, and so on due to low/high dose.

9 SC Tissue Engineering

Tissue engineering (TE) is an interdisciplinary area that combines engineering and life science concepts to create tissue-like structures using live cells, suitable materials, and appropriate biochemical clues (e.g., neuroprotective factors/growth factors) (Figs. 3 and 4). The main goal is the tissue reconstruction by developing grafts for implantation into the body to treat an injury or restore the functionality of a dying organ (Langer 1993; Berthiaume et al. 2011).

Thus, TE can be a viable therapeutic option for people with SCI. (Jones et al. 2001)

10 Neuroprotective Factors

Neuroprotective factors are crucial not only for the survivability and development of differentiating neurons but also for the preservation and

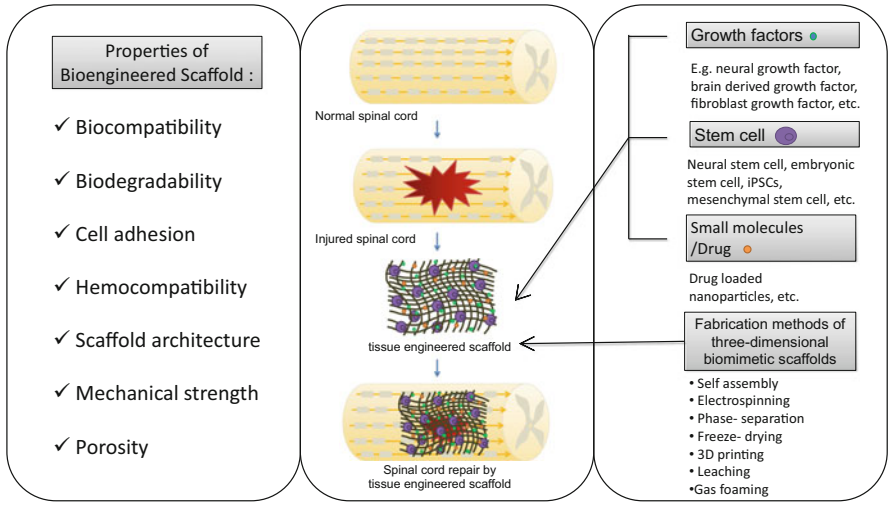

Fig. 4 Tissue engineering approach for repair of SCI: (**a**) Properties of bioengineered scaffold; (**b**) SC regeneration and repair; (**c**) growth factors, drugs or biomolecule stem cells, and scaffold used for tissue engineering of SC

restoration of adult nerve cells during pathological consequences (Solaroglu et al. 2007).

Gangliosides, a plasma membrane component that is thought to have a number of physiological effects on the CNS, such as synaptic plasticity and neuroprotection (Geisler et al. 1993; Chinnock and Roberts 2005), showed increased neuronal mitochondrial activity and promoted the production of neuroprotection genes (Finsterwald et al. 2021). Similarly, CNS-specific neurotrophic factors like nerve growth factor (NGF), neurotrophin-4/5 (NT-4/5) brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) are supposed to control neuronal survival, axonal development, synaptic plasticity, and neurotransmission in the nervous system (Jones et al. 2001; Bregman, and mcatee M, Dai HN, Kuhn PL. 1997). Likewise, fibroblast growth factor (FGF) has been widely studied for nerve regeneration. It might promote axonal development and reduce glial scarring (Ko et al. 2019). aFGF and bFGF are implicated in the regulation of synaptic plasticity and activities in the CNS; therefore, their possible therapeutic impact has been studied extensively (Ko et al. 2019; Reuss and Halbach 2003; Harvey et al. 2015). In acute, subacute, and chronic CNS disorders, the cytokine granulocyte colony-stimulating factor (G-CSF) appears to have powerful antiapoptotic, anti-inflammatory, myelin-protective, antioxidative, and axon-regenerative capabilities (Aschauer-Wallner et al. 2021). Progesterone (PROG) on the other hand showed neuroprotective and promyelinating properties by reducing expression of inflammatory cytokines such as TNF- and iNOS, NOS2, MCP-1, and IL-1, as well as caspase 3 and GFAP (Jure et al. 2019; Ludwig et al. 2017). Thyrotropin-releasing hormone (TRH) therapy has improved motor and sensory function significantly. Thus, TRH or its analogue has neurological effects (Diaz-Galindo et al. 2020). Magnesium has an antinociceptive effect because it inhibits N-methyl-d-aspartate (NMDA) receptors, preventing calcium ions from entering the cells and causing analgesia which results in stimulation of neuronal regeneration (Shin et al. 2020; Wu et al. 2019). Similarly, activated protein C (APC), a physiologic anticoagulant and anti-inflammatory protein, may help to minimize motor impairments caused by SCI (Hirose et al. 2000; Taoka et al. 2000).

Despite the positive outcomes of this neuroprotective approach, it has some challenges in preclinical studies such as selection of suitable animal model and their sex, age, doses of neuroprotection, etc.

11 Stem Cells for SCI

Stem cells are a unique population of cells that are capable of differentiating into different lineages. These cells have an inherent capacity for self-renewal, differentiation, migration, and tissue repair. Preclinical studies for treating SCI with stem cells as a treatment modality have shown tremendous hope. Stem cell therapy is gaining popularity and recognition, as it promises to (a) alleviate nerve tissue degradation, (b) promote tissue regeneration and neovascularization, and (c) assist endogenous cells in regeneration (Fig. 3e) (Coutts and Keirstead 2008). The type of stem cells that are to be used will largely depend on the cell's potency, self-renewal ability, and ease of processing.

12 ESCs

ESCs can be successfully differentiated into neural cells, neural precursor cells, glial cells, low-purity motor neurons, and high-purity oligodendrocyte progenitors (Coutts and Keirstead 2008). Transplantation of ESCs into the acute SCI model showed transplant integration, axonal elongation, tract regeneration, oligodendrocyte-induced remyelination, and restoration of neuromuscular junctions (Jin et al. 2019). ESC cells that overexpress FGF2 showed neuroprotective behavior (Araújo et al. 2017). Definitive neural stem cells (dNSC) were produced from ESCs by clonal expansion method used for the treatment of SCI. There was differentiation of dNSC into oligodendrocytes which leads to axon remyelination and is indicative of motor function recovery in mice SCI model. There were no reports of teratoma formation (Salewski et al. 2015). A comparative study of rat ESCs and autologous bone marrow-derived neurocytes (ABMDN) showed recovery of SC functionally and neurologically. However, ABMDN showed more clinical potential as compared to rat ESCs (Sadat-Ali et al. 2020). It is also very important to address the legal and ethical aspects related to the use of ESCs in cell-based therapies. Additionally, one of the major obstacles that limits their use in clinical settings is teratoma formation, suggesting the disastrous impact of direct usage of undifferentiated cells. Further studies should majorly focus on eliminating the undifferentiated cells that are formed following ESC transplantation and also at designing protocols with guided differentiation (Nussbaum et al. 2007; Thinyane et al. 2005).

13 Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) can be differentiated into neural cells (Cooper et al. 2012; Morizane et al. 2013; D'Aiuto et al. 2014), neural progenitor cells (NPC) (D'Aiuto et al. 2014; Sareen et al. 2014; Nutt et al. 2013), various specific types of neurons like dopaminergic neurons (Nguyen et al. 2011; Chang et al. 2021; Mahajani et al. 2019; Tolosa et al. 2018), and GABAergic interneurons (Iwasawa et al. 2019; Inglis et al. 2020). When undifferentiated iPSCs are directly transplanted at the lesion site, they promoted functional recovery, and there was SC regeneration (Bellák et al. 2020). However, there is a risk of teratoma formation after direct in vivo transplantation of iPSCs, and so they are first differentiated into "subtypes of interest" before any in vivo application. Human-iPSC-derived NS/PCS (hiPSC-NS/PCS) were differentiated into mature oligodendrocytes. After 12 weeks of transplantation of these oligodendrocytes, there was remyelination of the demyelinated axons and functional recovery with no tumor formation (Kawabata et al. 2016). Transplanted iPSC-derived NSCs were survived and differentiated into neurons and glia cells in athymic nude rats with SC lesions. The axons were penetrated into white matter and gray matter of the injured SC, with formation of synapses and improved nerve conduction (Lu et al. 2014). Neural stem/progenitor cells (NS/PCs) with gliogenic potential (GNS/PCs) were differentiated from iPSCs. In vivo results of the transplanted GNS/PCs showed improved motor function and remyelination with no tumorigenic effects (Kamata et al. 2021). iPSC-derived NPCs showed survival of a month and no tumor development or

other side effects in aged SCI rat model (Martín-López et al. 2021). Although iPSCs have immense therapeutic potential for SC repair, still they pose obstacles like designing an appropriate reprogramming protocol, which includes selection of a reprogramming factor and delivery method (Singh et al. 2015). Likewise, genetic instability and the risk of teratoma formation have raised safety concerns for iPSCs for cell-based therapies (Fu and Xu 2012).

14 MSCs

MSCs are an ideal source for tissue engineering and cell-based therapies because of their immunosuppressive, immunomodulatory, and anti-inflammatory and regenerative properties. MSCs can be obtained from various sources like the bone marrow (Pourrajab et al. 2013), arteries and veins (Corselli et al. 2012), amniotic membrane (Alviano et al. 2007), amniotic fluid (Anker et al. 2003), breast milk (Patki et al. 2010), adipose tissue (Wu et al. 2017), synovium (Fan et al. 2009), umbilical cord (El Omar et al. 2014), endometrium (Mutlu et al. 2015), Wharton's jelly (Fong et al. 2011), fetal liver (Joshi et al. 2012), etc. MSCs have been shown to improve bladder function, reduce inflammation, and increase the secretion of trophic factors, all of which contribute to their therapeutic potential after MSC transplantation into SCI animal models (Mukhamedshina et al. 2019).

MSCs can control the extent of secondary injury after SCI by regulating the "macrophage polarization" by secreting factors like IL-4 and IL-13 and chemokines like CCL2 and aid functional recovery (An et al. 2021). BMMSC-derived neuron-like cells showed development of synapse-like structure and evoked action potential in vitro and survived up to 6.5 months at injury site in vivo. These cells were efficient in integrating with host tissue and restored motor function of a paralyzed dog (Wu et al. 2018). Rat cranial bone-derived MSCs (rcMSCs) significantly reduced lesion area and promoted locomotor function as well as electrophysiology recovery (Maeda et al. 2021). When MSCs were transplanted in the vicinity of a spinal cord injury in a rat model, they ameliorated the neuroinflammation and improved the clinical outcomes. There was upregulation of matrix metalloproteinase (MMP) 2 and STAT-3, while there was downregulation of NF-κb p65 and other inflammatory cytokines like IL-1α, TNF-α, and TGF-β (Kim et al. 2019). 3D spheroids of human placenta-derived MSCs (3D-hpMSCs) increased the secretion of anti-inflammatory cytokine and trophic factor such as FGF, VEGF, and PDGF. It minimized lesion area, promoted angiogenic effect, and improved motor function in mice (Deng et al. 2021).

15 NSCs/Neural Progenitor Cell

NSCs are multipotent, self-renewing, highly proliferative, and a heterogeneous population of cells. NSCs have been isolated from the brain (Leong et al. 2013), SC (Curtis et al. 2018), and dorsal root ganglion (Gu et al. 2010). The in vitro stability and viability of NSCs is of particular importance as they retain viability, self-renewability, and differentiation potential even after many freeze–thaw cycles. NSCs are a good choice for repairing injured SC tissue as they are already committed to neurogenic and gliogenic fates (Coutts and Keirstead 2008). When NSCs were grafted at SCI lesion site, they integrated, relayed neuronal signals, and established a neuronal network and synaptic communication between host and the graft (Ceto et al. 2020). Similarly, when human NSCs were grafted into C5 hemisection sites in immunodeficient mice model, the NSCs differentiated into astrocytes and showed long-distance migration. NSC-derived astrocytes formed gap junctions with host cells (Lien et al. 2019). NSCs were isolated from human SC and grafted them in cervical SCI rhesus monkey model. The grafted NSCs survived for almost 9 months and demonstrated functional effects. They integrated themselves in the host tissue (50 mm) and restored neuronal network. Transplantation of NSCs into SCI model reduced the expression of P2X7, P2X4 (expressed in neuropathic pain), and

glial fibrillary acidic protein while increasing the expression of neurofilament proteins. Also, there was axon regeneration and recovery of sensorimotor function (Rosenzweig et al. 2018; Du et al. 2019).

16 Olfactory Ensheathing Cells

In contrast to the SC, which has limited regenerative capacity, the mammalian olfactory system can actively regenerate throughout the organism's life (Coutts and Keirstead 2008). Olfactory ensheathing cells (OECs) are glial cells that wrap around nonmyelinated olfactory axons. OECs aid neural regeneration since they can induce a neuron to cross a glial scar. It has been reported that OECs promote cellular interaction, control neuroinflammation, provide neuro-protection, induce angiogenesis, clear cellular debris, kill bacteria, and release neurotropic and ECM building factors. OECs have been reported to upregulate interleukin-1 receptor antagonist (IL-1Ra) and downregulate (IL-1) and lessen the glial scar. OECs promote regeneration post-intravenous transplantation (Zhang et al. 2021). Meta-analysis data on OEC transplantation shows that OECs are highly capable of neuroregeneration and subsequent functional recovery. Intraspinal transplantation of OECs in rat SCI model showed limb motor recovery and increased motor evoke potential by week 8 (Muniswami and Tharion 2018). Quantum dot-labeled OECs intravenously transplanted into SCI rat showed reduced inflammation and remyelination and improvement in motor function. Intravenous cell transplantation is believed to be more effective than intrathecal or intraspinal cell transplantation (Zhang et al. 2019a). However, the repair of the damaged SC by OECs will require proper isolation, expansion, transplantation, and finally the integration of OECs into the neural circuit (Wright et al. 2018). Further studies are needed to understand the role of OECs in neuropathic pain, their dosages, and their sites of administration (Nakhjavan-Shahraki et al. 2018).

Advancements in stem cell-based therapies are showing tremendous hope for treating conditions with complex pathophysiology like SCI. These therapies are aimed at controlling the extent of secondary injury and restoring lost or damaged tissues. But the possible outcomes of such therapies can be further enhanced by coupling them with various biomaterial scaffolds. Additional knowledge is required to completely validate and ensure the safety of cell-based therapies for treating SCI.

17 Biomaterials for SCI

TE scaffolds made up of biomaterials should possess several properties such as biocompatibility, biodegradability, porosity, mechanical strength, hemocompatibility, noncytotoxic, and so on for being an ideal graft/transplant substrate. A variety of techniques including self-assembly, electrospinning, phase separation, freeze-drying, 3D printing, leaching, and gas foaming has been employed for the synthesis of scaffolds (Fig. 4a, c). Recently used biomaterials and their combinations with cells or other means for neural tissue engineering are discussed in Table 1.

18 Biological Scaffolds/Decellularized Scaffolds

A decellularized (cell-free) neural scaffold can be used as a viable solution for treating SCI. A decellularized matrix as a scaffold has the benefit of being substantially compatible to the tissue being replaced. It is naturally biodegradable and shows fast remodeling in vivo because of its extracellular matrix content (García-Gareta et al. 2020). "The extracellular matrix (ECM) is a non-cellular matrix present in all tissues and organs, which not only provides an appropriate physical framework for intracellular components, but also induces critical physiochemical and physiological cues necessary for tissue morphogenesis, differentiation, and equilibrium" (Frantz et al. 2010). Each tissue and organ's resident cells design and build the ECM, which can be customized in a balanced relationship with its

Table 1 Tissue-engineered materials attempted to promote nerve regeneration

Sr. No	Material	Method of fabrication/modification	Animal experiment	Type of SCI model	Functional recovery	Outcome	References
1	Collagen and stretch-grown tissue-engineered nerve grafts	Encapsulation	Yes	SC complete transections (T10–T11)	Yes	Greater tissue infiltration and less compression No immune reaction Axonal growth	Sadik et al. (2020)
2	Superporous poly (2-hydroxyethyl methacrylate) hydrogel	-Radical polymerization -Plain and MSC-seeded hydrogel	Yes	Underwent transaction (T8)	-	Axon infiltration in gradual manner	Hejčl et al. (2018)
3	Multichannel poly(lactide-co-glycolide) (PLG)	EGFP-progenitor-seeded bridge	Yes	Lateral hemisection (T9–10)	Yes	Reduce inflammation Promote axon regrowth Support spinal progenitors Remyelination	Dumont et al. (2018)
4	Polyethylene glycol (PEG) microspheres/tubes	Free radical polymerization/modified by fibrinogen, thrombin, and cacl2 (Sigma) in tris-buffered saline	Yes	Lateral hemisection (T9–10)	Yes	Supports uniaxial tissue growth Increased regeneration Reduces glial scar	Dumont et al. (2019)
5	Fluorenylmethyloxycarbonyl (Fmoc)- DIKVAV self-assembling peptide hydrogel	Plane and seeded rat mesenchymal precursor cells (rMPC)	Yes	Mild thoracic contusion SCI (T10)	Yes	Anti-inflammatory Axon regrowth Infiltration of astrocyte	Wiseman et al. (2021)
6	Poly(ε-caprolactone) (PCL)	Electrospun, green fluorescent dye, poly (9, 9-dioctylfluorene-alt-benzothiadiazole) (F8BT)	Yes	SC contusion (T9)	-	Provided mechanical support Promotes angiogenesis, neurogenesis, and axon presence	Li et al. (2020)
7	Fibrinogen, thrombin, poly (ethylene oxide), and alginate	Electrospinning	Yes	Dorsal hemisection (T9–T10)	Yes	Scaffold induced the alignment, migration, and proliferation of host cells Remyelination	Yao et al. (2018b)
8	Poly(ethylene glycol) diacrylate (PEGDA)–GelMA	3D printing, NPC-seeded scaffold	Yes	Complete SC transection (T3)	Yes	Axonal regeneration Provide patient specific scaffold	Koffler et al. (2019)
9	Collagen	NSC-seeded, paclitaxel (PTX)-liposome encapsulated collagen scaffold	Yes	Complete transaction (T8)	Yes	Provide instructive microenvironment Axon extension Neural differentiation	Li et al. (2018b)

10	RADA16-RGD peptide and PCL-PEG-PCL-diacrylate (PCECDA)	Photo-cross-linking	Yes	Hemisection SCI model	Yes	Reduced glial scar Neural regeneration	Zhai et al. (2020)
11	Poly(lactide-co-glycolide)/ polyethylene glycol (PLGA-PEG)	Electrospinning	Yes	Complete transection (T10–T11)	Yes	Reduce cavity formation Promote neuronal differentiation	Liu et al. (2015)
12	Chitosan–sodium alginate	Freeze–thaw	Yes	SC hemisection model (T9–T10)	Yes	Reduced scar Motor function recovery Promote regeneration	Yao et al. (2018a)
13	Starch poly-e-caprolactone (SPCL) blend and gellan gum	3D plotting rapid prototyping	Yes	SC hemisection model (T8–T9)	Yes	Noncytotoxic Biocompatible Mechanical property	Silva et al. (2010)
14	Tannic acid (TA) and polypyrrole	–	Yes	Hemisection model (T9–10)	Yes	Mechanical property Good electronic conductivity Promote neurogenesis	Zhou et al. (2018a)

own microenvironment. The native ECM makeup, particularly its tiny components, must nevertheless be preserved, evaluated, and expanded for big tissues and organs (García-Gareta et al. 2020).

Decellularization of the peripheral nerve and SC can be accomplished using a variety of chemicals and enzymes, including triton X-200 (Cerqueira et al. 2018), triton X-100 (Wang et al. 2017; Guo et al. 2010; Jiang et al. 2013), sodium deoxycholate (Wang et al. 2017; Guo et al. 2010; Jiang et al. 2013), and phosphate-buffered saline (PBS) (Jiang et al. 2013). Decellularized scaffold was created by decellularizing sciatic nerves as the SC and had physicochemical qualities similar to autologous neurons and showed low immunological rejection (Gu et al. 2011; Tian et al. 2017). Allogenic rat acellular SC was generated by PBS, triton X-100, and sodium deoxycholate. This acellular SC contained laminin, fibronectin, and collagen contents which induces neural regeneration. 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) cross-linking in combination with chemical extraction techniques enhanced the effectiveness of acellular SC scaffolds and conferred superior biological properties, including improved immunogenicity. These scaffolds promoted the adhesion and differentiation of rat BMMSCs into cells similar to neurons (Xing et al. 2019). Another cross-linker genipin (GP) significantly improves structural stability of acellular rat spinal and promotes cellular adhesion and proliferation in vitro (Jiang et al. 2013). Acellular rat SC seeded with BMMSC reduced inflammation and apoptosis of neural cells. Additionally, it also promotes sensorimotor function recovery in BMMSC-seeded scaffold (Wang et al. 2017). Rat acellular SC scaffolds showed improved bone marrow stem cell survival as well as minimized apoptosis of damaged native neural tissue, conserved the host tissue, and boosted transplant recovery (Chen et al. 2014). Modified acellular peripheral nerve graft was developed by using triton X-100 and sodium deoxycholate followed by freeze–thaw cycle for treating SCI. There was functional recovery after transplantation of acellular nerve graft along with GFP-labeled placental MSCs. It also showed that PMSCs distributed in host SC and differentiated into neuron-like cells and also promoted remyelination (Tian et al. 2017). Acellular rat SC scaffold seeded with rat adipose-derived stem cells (rADSCs) promoted functional recovery in rat SC hemisection model. There was reduction in active gliosis and glial scar formation and increased axon regeneration (Yin et al. 2018). Injectable decellularized peripheral nerve matrix improved efficiency of Schwan cells after transplantation in rat SCI model. It supported Schwan cell survival and axon growth and also promoted locomotor recovery without any immunogenic reaction (Cerqueira et al. 2018). A molded (3D) decellularized SC scaffold is developed by homogenizing bovine SC in 0.1 N NaOH. This method can effectively remove all cells and maintains ECM content. It also showed good cell viability and proliferation (Arslan et al. 2019). Most of decellularized SC scaffold was prepared by using rat SC and few by using bovine SC. Therefore, more research should be carried out to check the efficiency of bovine scaffold in vivo and in vitro for SC repair. The enhanced preparation approach might pave the way for modular acellular biological scaffolds to treat SCIs (Xing et al. 2019).

19 3D Printed Scaffold

The 3D scaffolds provide better cell proliferation and tissue maturation as compared to 2D scaffolds (Kadoya et al. 2016). The main advantage of using 3D printing is that it provides better control over scaffold design and shows improved mechanical properties. 3D scaffolds are highly capable of neuronal regeneration as it guides nerve cells, promotes differentiation, and shows tremendous therapeutic potential. Because of the intricacy of CNS architecture, 3D biomimetic scaffolds provide a way to enhance CNS regeneration via personalized medicine (Koffler et al. 2019).

A novel set of three-dimensional (3D) tubular structures were prepared by using gellan gum and biodegradable blend of starch. In vivo experiments in a rat hemisection SCI model revealed that the structures were effectively

absorbed into the lesion and did not cause persistent inflammation (Silva et al. 2010). Microscale continuous projection printing method (μCPP) can be used to mimic the complex 3D architecture of the CNS organs and even for regeneration of the severed SC. This technique is relatively faster as it can print 2-mm scaffolds in 1.6 s. The polyethylene glycol–gelatin methacrylate (PEG–GelMA) scaffolds prepared by μCPP seeded with neural progenitor cells were transplanted into transection SC rat model. All the 11 rats that received the scaffold retained original scaffold structure even after 4 weeks of implantation. There was no inflammatory response. It supported neuronal growth and modified the astrocyte reactivity to SCI (Koffler et al. 2019). A 3D printed collagen/silk fibroin scaffold seeded with MSCs remyelinated the axon and relayed neural signals (Chen et al. 2022a).

20 Conductive Scaffold

Conductive scaffolds are electrically sensitive scaffolds that regulate signals to electroactive cells for their cellular migration and proliferation. Because neurons respond to electrical signals, conductive scaffolds have a high potential for developing a neural connection within the damaged part. External and internal electrical impulses that convey ECM signals have been demonstrated to be transduced by conductive scaffolds in vitro and in vivo in cardiac and neural scar tissue to enhance organ function and behavior on a macroscopic level (Burnstine-Townley et al. 2020). A conducting polymer hydrogel (CPH) blend with plant-derived polyphenol and tannic acid boosted the differentiation of NSCs and significantly improved locomotor function (Zhou et al. 2018a). Formulating such a conductive scaffold could stimulate the native tissue and provide a bridge to improve the communication between the scaffold and the native tissue. A clinically relevant conductor called "advanced nerve guidance channels (ANGCs)" was developed by using chitosan–gelatin by cryogelation and unidirectional solvent freezing. It showed cellular migration and growth (Singh et al. 2018). This type of study shows it can be an effective tool to guide nerve cells in the scaffold to connect with native cells. An electroconductive and biocompatible nanofiber scaffold was prepared by using polyaniline along with PCL. It provided topographical cues and electrical signals to guide cells in vitro (Garrudo et al. 2019). Polypyrrole–alginate composite incorporated with nanochitosan showed cytocompatibility and promoted neural cell adhesion and proliferation (Manzari-Tavakoli et al. 2020). A novel nanofiber-based fibrin/polyurethane/multiwall carbon nanotube (fibrin/PU/MWCNT) hydrogel was developed. This hydrogel provides a suitable microenvironment that can promote cell adhesion, viability, and their proliferation in vitro. The addition of MWCNT to PU enhanced the conductivity as well as the hydrophobicity of nanofiber hydrogel (Hasanzadeh et al. 2019). 3D printed conductive poly (3,4-ethylenedioxythiophene) (PEDOT):polystyrene sulfonate (PSS) hydrogel with high electrical conductivity retention showed that electrical stimulation of the scaffold can improve neuronal differentiation (Heo et al. 2019). Similarly, polypyrrole/silk fibroin (PPy/SF) electroconductive scaffolds were developed using 3D bioprinting and electrospinning methods. In vitro and in vivo results showed enhancement in axonal regeneration and remyelination when they were electrically stimulated (Zhao et al. 2018). 3D-printed conductive nanocellulose/carbon nanotube scaffolds guided nerve cell attachment, proliferation, and the development of neural networks in vitro (Kuzmenko et al. 2016). Therefore, the development of such scaffold will have more scope in future neural tissue engineering applications.

21 Other Biomaterial Scaffolds

Biocompatible GelMA/ECM hydrogel scaffold developed by decellularization and electrospinning technique promoted NSC differentiation and reduced inflammation and SC regeneration (Chen et al. 2022b). Methacrylated hyaluronic acid (HA)-based hydrogel was modified by the incorporation of ECM components (collagen I and laminin) with suitable mechanical properties. This hydrogel

provided suitable microenvironment for the growth of axon (Spearman et al. 2020). The RADA16-RGD peptide-based hydrogel is prepared by photo-cross-linking of PCL-PEG-PCL-diacrylate (PCECDA). This hydrogel promoted NSC adhesion and proliferation in vitro, while reduction in glial scar enhanced neural regeneration and motor function in vivo (Zhai et al. 2020). An electroconductive, injectable, biocompatible, self-recovering, elastic, and biodegradable hydrogel promoted cell adhesion, proliferation, and differentiation of NSC. The hydrogel showed a unique real-time motion sensing property and a significant gain in motor neuron function and neural regeneration in a zebrafish brain injury model (Xu et al. 2020). The dendritic polypeptides were self-polymerized to form nanofiber scaffolds. It enhanced differentiation of NSCs into functional neurons and eventually increased the motor function (Liu and Li 2018). PLGA-PEG 3D nanofiber scaffold was prepared by electrospinning and showed mouse embryonic fibroblast (MEF) cell adhesion and proliferation. When transplanted into rat SCI model, it regenerated a totally transacted SC (Liu et al. 2015). Chitosan has been extensively used as a scaffolding material as it is biocompatible and shows antimicrobial properties. Similarly, sodium alginate also shows good biocompatibility and stability in vivo. Porous chitosan–sodium alginate scaffolds are prepared by freeze–thaw and transplanted in rat SC hemisection model to examine the locomotor recovery following SCI. After surgery, most of the rats in the control group died, but rats receiving scaffolds showed low death rate. The chitosan scaffold was retained for almost 2 months without any structural change or degradation, which supported to bridge the gap of injury. Additionally, glial fibrillary acid protein (GFAP) expression was very low and the growth of glial scar tissue was inhibited (Yao et al. 2018a).

22 Combinational Tissue Engineering Approach

"Combinational therapy" is the use of two or more therapies in combination. This approach can overcome the disadvantages associated with the use of single therapy. The majority of earlier research has applied drugs or neurotrophic factors along with scaffolds to reduce neuroinflammation and improve axonal regeneration. An endometrial stem cell (EnSC)-seeded biomimetic hydrogel scaffold combined with atorvastatin injection improved locomotion and stopped the progression of secondary injury. Neurotrophic factor combined with collagen-binding domain (CBD) increased the endogenous repair in SCI (Astaneh et al. 2020). A multichannel PLGA scaffold seeded with activated Schwann cells (ASCs) and rat BMMSCs enhanced the survival and differentiation of MSCs into neural-like cells (Yang et al. 2017). A core–shell microfiber scaffold was prepared by using PLGA and FGF 2. This microfiber promoted cellular adhesion and proliferation of PC12 cells and enhanced locomotor recovery after 28 days (Reis et al. 2018). These aforementioned studies (Yang et al. 2017; Reis et al. 2018) were successful in improving neural regeneration and significant gain of sensorimotor function. PLGA surface modified with DOPA-IGF-1, a new recombinant protein, enhanced human umbilical cord MSC (hucMSC) paracrine activity by releasing neurotrophic factors. It showed higher cell adhesion and proliferation (Zhang et al. 2019b). When ASCs and iPSC-derived NSCs were seeded into PCL scaffolds, it promoted functional recovery in rat model (Zhou et al. 2018b). Immunization with neural-derived peptide (INDP), fibrin glue (FG), and dipyridyl (DYP) along with BMMSCs were used for the treatment of acute SCI. It showed significant sensational and functional recovery because of increase in axonal density in tissue (García et al. 2019). Although these results suggest combinational therapy would be beneficial, it is important to note that this is preliminary study, and further investigations are required.

23 Clinical Trials

ESCs have been emerged in clinical settings to treat SCI. Safety and efficacy of hESCs was evaluated in phase 1 trial. It showed functional

recovery in both limbs and bowel and bladder function in five patients with either paraplegia or quadriplegia. No contrary event occurred; therefore, hESCs could be considered effective as well as safe therapy for SCI (Shroff and Gupta 2015). In another phase 1 study, four chronic spinal trauma subjects received intraspinal injection of human SC-derived NSCs (NSI-566) at 12–24 months after spinal trauma. Three subjects out of four showed gains in sensorimotor function without any adverse effect. This study has provided only primary safety data and lacks statistical significance because of small sample size (Curtis et al. 2018). Using autologous mesenchymal stromal cells (MSCs), phase 2 trial was conducted in SCI patients. In this study, three intrathecal 100×10^6 doses of MSCs were given to chronic SCI patients, followed by a follow-up period of 10 months. This study showed variable clinical outcomes including not only gain of sensorimotor function, sphincter dysfunction, sexual function, recovery in neuropathic pain, and sensitivity but also improvement in ASIA grade scale for SCI. All the improvements are regardless of age, time, and severity of SCI. This study does not show any adverse effect in patients. It is important to consider dose of cells and their administration, as there is no standard parameter for MSC administration in SCI patients for better outcomes (Vaquero et al. 2018). Combinational therapy of collagen scaffold (NeuroRegen) along with human umbilical cord MSC (hUBMSCs) was able to treat effectively one thoracic and one cervical SCI patient, respectively. A 1-year follow-up study showed significant improvement in sensorimotor function, bladder–bowel function, and muscle function in both patients; as a result, ASIA impairment scale of both SCI patients improved from grade A to C (Xiao et al. 2018). Therefore, this data provides such combinational therapy would able to integrate with host tissue and have potential to develop neural network which leads to significant functional recovery.

Clinical trials for SCI mainly focus on neuroregeneration by means of cell-based therapy that is mentioned in Table 2, while clinical trials on tissue-engineered scaffold are mentioned in Table 3. All neuro-regenerative experiments or preclinical studies now have been reached to clinical trial phases 1 and 2. To date, various cell-based therapies such as neural precursor cells, NSC, olfactory mucosa ensheathing cells, BMMSCs, WJMSCs, UCMSCs, and adipose-derived MSCs were given to SCI patients for their safety and effectiveness at various dosages. Cells are mainly administrated by intrathecal, intraspinal, epicenter of injury, intramedullary, and percutaneous injection. The completed and ongoing clinical trials for SCI show promising results such as recovery of sensorimotor functions, gain of bowel/bladder function, decreased neuropathic pain, reduced lesion area and inflammation that leads to neurogenesis. However, small sample size may limit statistically significant results of such studies. Further clinical trials on a large number of SCI population must be needed to evaluate results significantly.

24 Future Prospective

SCI causes a slew of issues that must be addressed in order to find a solution for the disease. Apart from many in vivo studies, few fail to be duplicated and transfer therapeutically, which is unsatisfactory to people with SCI who are waiting for treatment alternatives. Various injury models used for SCI in preclinical studies might differ because of targeted damage; thus, the actual situations in clinical setting of human patients with SCI are typically distinctive. We cannot completely rely on the preclinical study results for validating the success of SCI therapies. Considering the difference between regeneration capability and behavioral activities of rats and humans, the clinical trials based on preclinical data may yield unsatisfactory or minimal results (Cofano et al. 2019). Therefore, more emphasis should be given on clinical trials to maximize authenticity of the desired therapeutics. There are many obstacles in the field of neural tissue engineering. Increasing graft survival and tissue regeneration, establishing and maintaining viable connections between neurons, identifying the optimal neurons for enhancing communication,

Table 2 Clinical trial on various cell-based therapies for the treatment of SCI listed on www.clinicaltrials.gov

Sr. No.	Clinicaltrials.gov. Id	Phase of study	Scaffold/cell	Level of injury/ type of injury	Study status	Study period	Study summary	Location
1	NCT04812431	Phases 1 and 2	Neural precursor cells derived from human embryonic cell line	Intrathecal injection	Not yet recruiting	Sept. 2021 to Sept. 2023	The goal of this exercise is to assess the preliminary safety and efficacy of neural precursor cells (PSA-NCAM (+) NPC) obtained from the hESC line for the therapy of paralysis as well as other subacute SCI complaints	Yonsei University Health System, Severance Hospital, Seoul, Republic of Korea
2	NCT02326662	Phases 1 and 2	Autologous NSC	Intraspinal and intrathecal injection	Unknown	Jul. 2014 to Dec. 2018	The use of autologous NSCs in individuals with full traumatic SC damage is being investigated in this study	Federal Research Clinical Center FMBA of Russia, Moscow, Russian Federation.
3	NCT01772810	Phase 1	Human SC-derived NSC	–	Recruiting	Aug. 2014 to Dec. 2022	Study focuses on safety of NSCs derived from human SC for treating chronic SCI	UCSD Medical Center, Division of Neurosurgery San Diego, California, United States.
4	NCT01321333	Phases 1 and 2	Human CNS allogenic stem cell	Intramedullary administration	Completed	Mar. 2011 to Apr. 2015	Study exploring the preliminary efficacy of human CNS allogenic stem cell with thoracic spinal SCI	Foothills Medical Center, Calgary, Alberta, Canada Toronto Western Hospital, Toronto, Ontario, Canada. University Hospital Balgrist- Uniklinik Balgrist, Forchstrasse 340 Zurich, Switzerland.
5	NCT01231893	Phase 1	The olfactory mucosa ensheathing cells	Transplanted into focus of SCI	Unknown	May 2008	The goal of this investigational therapy is to see if transplanting autologous olfactory ensheathing glia and olfactory fibroblasts derived from the olfactory mucosa to patients with full SC damage is safe and feasible	Department of Neurosurgery of Wroclaw Medical University, Wroclaw, Poland.

6	NCT03933072	Phases 1 and 2	Autologous bulbar OEC	Transplant cell by using peripheral nerve graft	Recruiting	Mar. 2016 to Mar. 2023	This trial aims to investigate the safeness and efficiency of transplanted cells in chronic complete SCI	Wroclaw Medical University Wroclaw, Poland
7	NCT02482194	Phase 1	Autologous BMMSCs	Intrathecal delivery	Completed	Jun. 2013 to Mar. 2016	This study focuses on intrathecal transplantation of BMMSC to evaluate safety and their efficacy for treatment of SCI and determination of functional recovery	Armed Forces Bone Marrow Transplant Centre, Rawalpindi, Pakistan
8	NCT03505034	Phase 2	Allogeneic Umbilical Cord MSCs	Intrathecal Transplantation	Recruiting	Sept. 2019 to Dec. 2021	This study tries to find out the best time for cell treatment and their safeness with efficacy to treatment chronic SCI patients	The Third Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China.
9	NCT01694927	Phase 2	Autologous MSCs	Intralesional transplantation	Unknown	Jan. 2012 to Jun. 2014	The purpose of the study is to see if intralesional transplantation of autologous mesenchymal stem cells is a safe and efficient therapy for individuals with SCIs	Clínica Las Condes, Santiago, RM, Chile.
10	NCT03521336	Phase 2	Allogeneic umbilical cord MSCs	Intralesional transplantation	Recruiting	Sept. 2019 to Dec. 2021	This study tries to find out the best time for cell treatment and their safeness with efficacy compared to placebo to treatment in chronic SCI patients	The Third Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China.
11	NCT02574585	Phase 2	Autologous MSCs transplantation	Percutaneous injections	Not yet recruiting	Dec. 2019 to Jan. 2022	Study tries to find out assurance and effectiveness of autologous mesenchymal cell transplantation to treat patient with chronic thoracic and complete SCI	Ricardo Ribeiro dos Santos, Hospital São Rafael, Salvador, Bahia, Brazil
12	NCT01446640	Phases 1 and 2	BMMSCs	Intravenous combined with intrathecal administration	Unknown	Oct. 2011 to Jun. 2014	This clinical trial aims for safety and effectiveness of BMMSC for the treatment of SCI	Guangzhou General Hospital of Guangzhou Military Command, Guangzhou, Guangdong, China.

(continued)

Table 2 (continued)

Sr. No.	Clinicaltrials.gov. Id	Phase of study	Scaffold/cell	Level of injury/ type of injury	Study status	Study period	Study summary	Location
13	NCT02481440	Phases 1 and 2	Allogeneic human umbilical cord MSCs	Intrathecal administrations	Completed	Mar. 2018 to Mar. 2020	This clinical trial aims for safety and effectiveness of repeated subarachnoid administration of hUCMSCs for the treatment of SCI	The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong, China.
14	NCT02152657	Phase 1	Autologous MSCs	Percutaneous injection	Completed	Jan. 2015 to Dec. 2016	This is pilot study to evaluate autologous MSC transplantation through percutaneous injection for their safety and effectiveness in patient with chronic SCI	Hospital São Rafael, Salvador, Bahia, Brazil.
15	NCT02981576	Phases 1 and 2	Autologous BMMSC and AT-MSC	Intrathecal administrations	Completed	Nov. 2016	This is comparative study which evaluates safeness and efficacy of BMMSC and ATMSC for the treatment of SCI patients	Cell Therapy Center, University of Jordan, Amman, Jordan.
16	NCT04520373	Phase 2	Autologous adipose-derived MSCs	Intrathecal injection	Recruiting	Jun. 2020 to Jun. 2024	This study focuses on safety and effectiveness of therapy based on autologous AT-MSCs with patients having paralysis	Mayo Clinic in Rochester, Rochester, Minnesota, United States.
17	NCT01769872	Phases 1 and 2	Autologous adipose tissue-derived MSCs	Intrathecal injection	Completed	Jan. 2013 to Jan. 2016	Study investigates the effect of treatment of AT-MSCs for efficacy and their safety to treat SCI patients	Korea University Anam Hospital, Seoul, Republic of Korea
18	NCT05152290	Phase 1	Allogeneic adult umbilical cord-derived MSCs	Intrathecal injection and intravenous infusion	Recruiting	Jan. 2022 to Jan. 2026	This study aims to investigate effects of intravenous infusion and intrathecal injection of allogeneic UCMSC in patient with SCI	Medical Surgical Associates Center, St. John's, Antigua and Barbuda.
19	NCT01624779	Phase 1	Autologous adipose tissue-derived MSC (AT-MSCs)	Intrathecal injection	Completed	Apr. 2012 to May 2014	This study checks the effect of intrathecal AT- MSC administration in patients with SCI	Korea University Anam Hospital, Seoul, Seongbukgu, Republic of Korea

20	NCT03003364	Phases 1 and 2	Wharton's jelly MSCs	Intrathecal administration	Completed	Dec. 2016 to Feb. 2020	To study the safety data and efficiency of intrathecal administration of expanded WJ-MSCs in patient affected with chronic TSCI	Hospital de Neurorehabilitació Institute GuttmannBadalona, Barcelona, Spain.
21	NCT04288934	Phase 1	Autologous bone marrow-derived MSCs (auto BMMSCs) and Wharton's jelly-derived MSCs (WJ-MSCs)	Spinal medulla	Completed	Aug. 2017 to Sept. 2020	This was comparative study which aims to access safety and effectiveness of auto BMMSCs and WJ-MSCs cell treatment in SCI patients	Cell Therapy Center, University of Jordan, Amman, Jordan.
22	NCT01873547	Phase 3	Umbilical cord MSCs	Subarachnoid infusion by lumbar puncture	Completed	Jun. 2012 to Dec. 2015	This study aims to find out the effective therapy for SCI which includes cell therapy, rehabilitation therapy, and control group	General Hospital of Chinese People's Armed Police Forces Beijing, Beijing, China.
23	NCT01325103	Phase 1	Autologous MSC	Direct transplantation injured area	Completed	Jul. 2010 to Dec. 2012	To evaluate the safety and effect of auto BMMSCs for the treatment of SCI	Hospital São Rafael Salvador, Bahia, Brazil.
24	NCT01909154	Phase 1	MSCs	Subarachnoid and intramedullary	Completed	Mar. 2013 to Mar. 2015	This was a pilot study to check the safety of local delivery of autologous MSCs derived from BM stroma in SC traumatic injuries	Hospital Puerta de Hierro Majadahonda, Madrid, Spain.
25	NCT00816803	Phases 1 and 2	Autologous bone marrow-derived cells	Sites of injury	Completed	May 2005 to Dec. 2008	The goal of this study is to see if autologous BM-derived cell transplantation is safe in chronic SC damage patients	Cairo University School of Medicine Cairo, Egypt.
26	NCT02165904	Phase 1	Adults' autologous MSCs	Subarachnoid administration	Complete	May 2014 to May 2016	Examine the potential therapeutic effectiveness of administering major adult mesenchymal autologous cells that have been grown "in vitro" in individuals with incomplete and chronic SCI	Hospital Puerta de Hierro Majadahonda, Madrid, Spain.

Table 3 Clinical trial on SCI using various types of graft/scaffolds listed on www.clinicaltrials.gov

Sr. No.	Clinicaltrials.gov. Id	Phase of study	Scaffold/cell	Level of injury/ type of injury	Study status	Study period	Study summary	Location
1	NCT02138110	–	Poly(lactic-co-glycolic acid)-b-poly(L-lysine) scaffold	T2–T12 (thoracic acute SCI)	Active, not recruiting	Oct. 2014 to Aug. 2024	In participants with thoracic AIS, multicenter research was conducted to assess the safety and potential utility of the poly(lactic-co-glycolic acid)-b-poly(L-lysine) scaffold	United States, Arizona United States, California United States, New Jersey United States, North Carolina United States, Oregon United States, Pennsylvania
2	NCT02510365	Phase 1	Collagen scaffold	Acute SCI	Unknown	Apr. 2015 to Dec. 2021	The goal of the trial is to determine the safety and effectiveness of a functional neural regeneration collagen scaffold implanted into patients with acute SCIs	Beijing, China Chongqing, China Suzhou, China Tianjin, China Yinchuan, China
3	NCT02688049	Phases 1 and 2	MSCs or NSCs combined with NeuroRegen scaffold	Chronic SCI	Unknown	Jan. 2016 to Dec. 2021	The objective of this study is to see if MSC- or NSC-seeded NeuroRegen scaffold transplantation in patients with SCIs are effective and safe	Tianjin, China
4	NCT02352077	Phase 1	NeuroRegen scaffold TM with BMMSCs or BMMNCs	Chronic SCI	Unknown	Jan. 2015 to Dec. 2021	The objective of this study is to see if bone marrow mononuclear cells or mesenchymal stem cells paired with NeuroRegen scaffold transplantation in patients with SCI are effective and safe	Tianjin, China Suzhou, China Beijing, China
5	NCT03966794	Phases 1 and 2	Collagen scaffold	SCI	Unknown	Aug. 2019 to Dec. 2021	The goal of the study will be how functional neural regeneration collagen scaffold transplantation paired with epidural electrical stimulation affects individuals with SCIs	Tianjin, China

6	NCT02688062	Phases 1 and 2	NeuroRegen scaffold with BMMSC transplantation	Chronic SCI and complete SCI	Unknown	Jan. 2016 to Dec. 2021	The target of this investigation is to check the effectiveness and efficiency of NeuroRegen scaffold with BMMNCs to surgical intradural decompression and adhesiolysis alone for neurological recovery following chronic and total SC damage	Beijing, China
7	NCT03105882	–	Neuro-Spinal Scaffold^{tm}	C5–T1 traumatic cervical acute SCI	Withdrawn (the study did not enroll any subjects)	Mar. 2017 to Mar. 2018	Pilot Study of the Neuro-Spinal Scaffoldtm Clinical Safety and Feasibility in the treatment of complete (AIS A) acute TSCI at the C5–T1 levels.	Toronto, Ontario, Canada
8	NCT03762655	–	Neuro-Spinal Scaffold™ – poly (lactic-co-glycolic acid)-b-poly (L-lysine) (PLGA-PLL)	Complete T2–T12 SCI	Recruiting	May 2019 to Jul. 2028	This study aims to see if the scaffold is safe and effective for the treatment of complete T2–T12 SCI when compared to standard of care open spine surgery	United States, California United States, Colorado United States, Florida United States, Iowa United States, Maryland United States, New Jersey United States, New York United States, North Carolina United States, Oregon United States, Pennsylvania United States, Rhode Island United States, Texas United States, Wisconsin
9	NCT03933072	Phases 1 and 2	Olfactory ensheathing cells (OECs) and olfactory nerve fibroblasts (ONFs)/collagen scaffold and autologous dural nerve grafts	C5 and Th10 complete SC transection	Recruiting	Mar. 2016 to Mar. 2023	The goal of this study is to find if transplanting autologous OECs and ONFs from the olfactory bulb with synchronous reconstruction of the posttraumatic SC gap with peripheral nerve grafts are safe and effective in patients with chronic complete SCIs	Wroclaw, Poland

guiding grafted cells to suitable destinations, and avoiding inappropriate connectivity are all things that must be addressed. There are some crucial considerations in cell treatment such as method standardization, cell potency evaluation, quality management, good manufacturing practice (GMP) and their scale-up, and finally logistics. Clinical translation of the TE products will require testing the biomaterial for its biodegradability as well as biocompatibility. An accurate understanding of its composition, 3D structure, and safety in vivo must also be taken into consideration while using 3D biomaterials to distribute cells. Chronic SCIs to date remain one of the least explored SCIs because more and more focus is given to acute and subacute SCI; it is therefore necessary to increase transplantation studies on the more severe chronic SCI (Duh et al. 1994).

25 Conclusion

In this review, we have discussed the pathophysiology of the SCI and current treatment modalities with their advantages and limitations. It is important to understand that the pathophysiology of SCI is complicated and that a single solution is unlikely to overcome the diverse array of obstacles. Therefore, a combinational tissue engineering therapy might be the future treatment option. More preclinical and clinical studies should be carried out for the most effective treatment modality in order to address the SCI in the near future.

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Application of Biocompatible Scaffolds in Stem-Cell-Based Dental Tissue Engineering

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Abstract

Tissue engineering as an important field in regenerative medicine is a promising therapeutic approach to replace or regenerate injured tissues. It consists of three vital steps including the selection of suitable cells, formation of 3d scaffolds, and adding growth factors. Mesenchymal stem cells (MSCs) and embryonic stem cells (ESCs) are mentioned as two main sources for this approach that have been used for the treatment of various types of disorders. However, the main focus of literature in the

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field of dental tissue engineering is on utilizing MSCs. On the other hand, biocompatible scaffolds play a notable role in this regenerative process which is mentioned to be harmless with acceptable osteoinductivity. Their ability in inhibiting inflammatory responses also makes them powerful tools. Indeed, stem cell functions should be supported by biomaterials acting as scaffolds incorporated with biological signals. Naturally derived polymeric scaffolds and synthetically engineered polymeric/ceramic scaffolds are two main types of scaffolds regarding their materials that are defined further in this review. Various strategies of tissue bioengineering can affect the regeneration of dentin-pulp complex, periodontium regeneration, and whole teeth bioengineering. In this regard, in vivo/ex vivo experimental models have been developed recently in order to perform preclinical studies of dental tissue engineering which make it more transferable to be used for clinic uses. This review summarizes dental tissue engineering through its different components. Also, strategies of tissue bioengineering and experimental models are introduced in order to provide a perspective of the potential roles of dental tissue engineering to be used for clinical aims.

Keywords

Dentistry · Embryonic · Mesenchymal stem cells · Regenerative medicine · Stem cells · Tissue scaffolds

Abbreviations

3D	Three dimensional
ASF-CM	Ameloblast serum-free conditioned medium
a-TDM	Autoclaved treated dentin matrix
BMPs	Bone Morphogenetic Proteins
BMSCs	Bone marrow stromal cells
Ca/P	Calcium phosphates
CAD	Computer-aided design
c-Myc	Myelocytomatosis oncogene
CT	Computerized tomography
DFSCs	Dental follicle stem cells
DP-MSCs	Dental pulp mesenchymal stem cells
DPSCs	Dental pulp stem cells
ECM	Extracellular matrix
ESCs	Embryonic stem cells
FGF	Fibroblast growth factor
G-CSF	Granulocyte colony-stimulating factor
GTR	Guided tissue regeneration
HA	Hydroxyapatite
HDPC	Human dental pulp cell
IFN-γ	Interferon-gamma
IGF	Insulin-like growth factor
iPSCs	Induced pluripotent stem cells
Klf4	Kruppel-like factor 4
MAPK	Mitogen-activated protein kinase
mESCs	Murine embryonic stem cells
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem cells
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
Oct4	Octamer-binding transcription factor 4
PDGF	Platelet-derived growth factor
PDL	Periodontal ligament
PDLSCs	Periodontal ligament stem cells
PEG	Polyethylene glycol
PGA	Polyglycolic acid
PLA	Polylactic acid
PLGA	Poly(lactic-co-glycolic acid)
PLLA	Poly-L-lactic acid
PRHds	Platelet-rich hemoderivatives
RP	Rapid prototyping
SCAP	Stem cells from apical papilla
SHED	Stem cells from human exfoliated deciduous teeth
Sox2	Sex-determining region Y-Box 2
ß –TCP	ß-tricalcium phosphate
TGF-ß	Transforming growth factor-beta
TLR4	Toll-like receptor-4
VEGF	Vascular epithelial growth factor

1 Introduction

Tissue engineering is an interdisciplinary field that applies cells and biomaterials into biological substitutes that are able to repair, replace, or replicate natural tissues (Stevens et al. 2016; Khademhosseini et al. 2006; Aghayan et al. 2021; Goodarzi et al. 2018a). This approach requires three vital parts: identifying suitable cells, making 3d scaffolds, and growth factors to reproduce tissue and reshape the normal function of organs (Anitua et al. 2018). While tissue restoration depends on cellular proliferation, stem cells have been recommended to form a tissue (Kaneko et al. 2018). Stem cells have a special ability to transform into different cells in the body and create various types of tissues as their ability to self-renewal (Zhai et al. 2019; Goodarzi et al. 2018b; Baradaran-Rafii et al. 2020). Another essential component in concept of tissue engineering is scaffolds. Scaffolds maintain chemical stability and mechanical strength of the 3D bioengineered tissue, imitating the in vivo settings (Orti et al. 2018). Scaffolds derived from biomaterials are considered crucial components for successful tissue regeneration by providing an appropriate environment for cell growth, adhesion, and differentiation (Yin et al. 2019). This requires biocompatible scaffolds, which are beneficial to cell adhesion and proliferation. Biocompatible scaffolds are nontoxic and harmless, have good osteoinductivity, avoid immune response in the implant area, and can slowly release an inducer that promotes blood vessels and osteogenesis (Ravindran and George 2015). Recent progress in tissue engineering has caused changes in traditional areas of clinical dentistry. Tissue engineering is being used to fix various dental problems that patients faced frequently. Dental caries is the second most frequent infectious disease after the common cold (Monteiro and Yelick 2017). According to the WHO, approximately 90% of the world's population has experienced dental caries (Monteiro and Yelick 2017). Tooth decay can occur for a variety of reasons, including genetic diseases; microbial problems; trauma; iatrogenic, traumatic, or therapeutic insults; poor oral hygiene; and dental prosthesis failure (Dorri et al. 2017). It also imposes a lot of costs on the individual and society. Furthermore, the discomfort and pain decrease the patient's quality of life (Moussa and Aparicio 2019). As the world's population ages, the need for dental repairs and regeneration increases. Today, due to different options for dental treatments, there are various choices for the restoration of the tooth's function and integrity. Clinical interventions to treat tooth decay range from simple coronal fillings to invasive root canal treatment (Kuboki et al. 2001). Some limitations of the conventional restorative methods are mentioned in papers, such as dependency on an electricity supply, costly handpieces, and highly trained operators (Moussa and Aparicio 2019). Moreover, conventional therapies have been shown to have an increased risk of pulp exposure, postoperative pulpal symptoms, and the weakening of the tooth as a result of more invasive caries removal (Moussa and Aparicio 2019). Since conventional treatments like root canal therapy would make the tooth lose its sensitivity and eventually become dead, dental stem cell therapy is going to be a growing and promising approach for dental disease treatment that can be easily drawn from premolar and wisdom teeth, which could be transplanted for orthodontics purposes (Orti et al. 2018; Monteiro and Yelick 2017). The transplanted cells directly participate in the regeneration. Moreover, they produce building blocks and secrete trophic factors to regulate regeneration procedures (Orti et al. 2018). Regeneration of stem cells in tissue reconstruction and repair should be controlled and supported by biomaterials that act as scaffolds which incorporate biological signals in tissue engineering (Anitua et al. 2018). There are some research that work on the feasibility, outcome, and effectiveness of stem-cell-based tissue engineering in dental fields. The objective of this article is to investigate the potential roles and the perspectives of tissue engineering by using biocompatible scaffolds in order to gain the natural function of a tooth. To this aim, we focused on papers that assess stem-cell-based dental tissue engineering.

2 Tissue Engineering

2.1 Cells

The first component of tissue engineering is cells. Stem cells are types of cells which are in the unspecialized stage and have the capability to self-renew and differentiate into a more specific lineage (Liu and Cao 2010; Rahim et al. 2018; Goodarzi et al. 2014; Larijani et al. 2020). There are two main sources of stem cells that have been usually used in regenerative medicine: adult stem cells and embryonic stem cells (ESCs). Among adult stem cells, mesenchymal stem cells (MSCs) are the most used sources in dental tissue engineering and have been obtained from various tissue of a donor or a receiver himself. Both of these two categories of stem cells have been used to treat many diseases (Alessandrini et al. 2019; Genc et al. 2019; Liu et al. 2020a; Müller et al. 2018; Payab et al. 2018). Also, recent studies introduce another source of stem cells generated from mature differentiated cells, called induced pluripotent stem cells (iPSCs). There are some research that investigated the clinical potential of MSCs, ESCs, and iPSCs in dentistry during the past decade (Gaur and Agnihotri 2021; Amghar-Maach et al. 2019; Ning et al. 2010). Most research focused on the application of dental tissue-derived MSCs in tissue engineering of tooth and periodontal tissue that were reviewed in this article.

2.1.1 Mesenchymal Stem Cells

MSCs are pluripotent somatic stem cells that are present in multiple tissues, such as synovium, muscle, adipose tissue, and bone marrow (Zhu et al. 2013). These cells have been shown to be isolated from a variety of connective tissues. The most common tissue in the body that MSCs are isolated from is the bone marrow and stroma of the spleen and thymus, so it was previously called bone marrow stromal stem cell (BMSCs) (Bianco et al. 2001; Arthur and Gronthos 2020). These cells are derived by the markers that are present in the surface of cells like CD90, CD105, and CD106. MSCs have the ability to self-renew and give rise to different lineages of mesodermal, ectodermal, and endoderm such as bone, tooth, muscle, and neuron under specific in vitro conditions (Ding et al. 2011; Larijani et al. 2015a). BMSCs have been used in various studies and have promising outcomes. Rajan et al. (2014) seeded BMSCs onto b-tricalcium phosphate (b-TCP) as a scaffold, and by 4 months, 80% of the initial jawbone deficit was regenerated with vascularized, mineralized bone sufficient to place oral implants stably. Moreover, intraoral tissue, like the other tissues in the body, has a rich source of stem cells that could be used to regenerate organs in tissue engineering (Han et al. 2014). There are progenitor cells in the dental pulp that can be reproduced when there is an injury in dental tissue and make odontoblasts that produce dentin (Fig. 1). Dental stem cells are a type of MSCs that were isolated for the first time in 2000. Several types of stem cells that participate in tooth formation have been discovered so far: dental pulp mesenchymal stem cells (DP-MSCs) from both stem cells from human exfoliated deciduous teeth (SHED) and dental pulp stem cells (DPSCs), stem cells from apical papilla (SCAP), periodontal ligament stem cells (PDLSCs), and dental follicle stem cells (DFSCs) (Table 1) (Hernández-Monjaraz et al. 2018). Stem cells derived from dental tissue have been shown to be the most suitable cell sources for dental pulp and dentin regeneration due to their ability to form pulp/dentin complex in vitro. SCAP and DFSCs are derived from developing tissue; SCAP is at the tip of developing root, and DFSCs is a loose connective tissue sac surrounding the unerupted tooth (Nada and El Backly 2018; Yao et al. 2004). DFSCs are considered to be a mother cell that has an ability to differentiate into the human periodontal ligament (PDL) fibroblasts, osteoblasts, and cementoblasts when it forms the periodontium (Hernández-Monjaraz et al. 2018). PDL is a highly specialized fibrous connective tissue surrounding and supporting the tooth root and has a significant function in the homeostasis, repair, and nutrition of the tooth (Tomokiyo et al. 2019). The dental tissue-derived stem cells mentioned above are

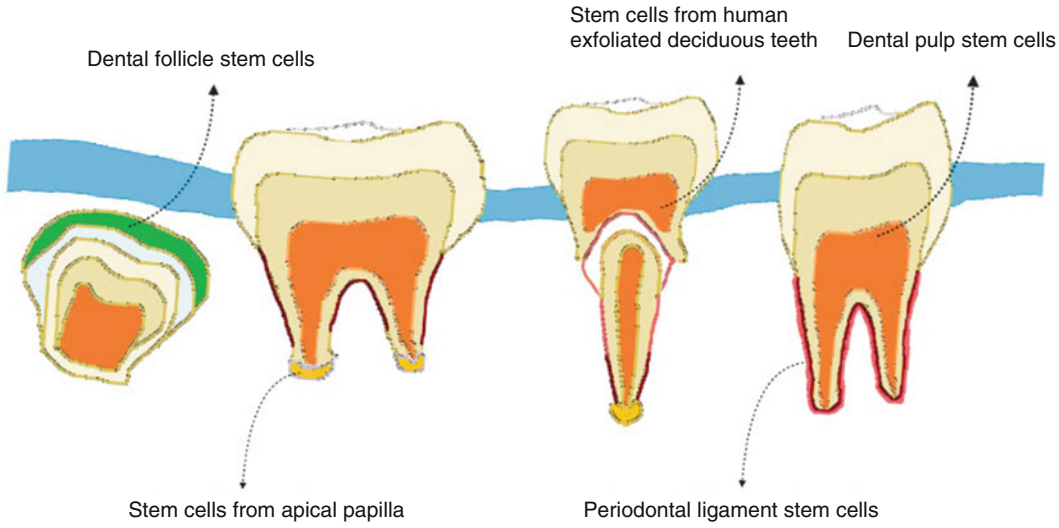

Fig. 1 Different types of human dental stem cells

Table 1 Characteristics of different types of dental stem cells and MSCs

Stem cells	Markers	Tissue formation capacity	Location
DPSCs	CD9, CD10, CD13, CD29, CD44, CD49d, CD59, CD73, CD90, CD105, CD106, CD146, CD166, STRO-1, nestin	Dent (dentin, pulp), neural, bone, muscle, adipose	Permanent tooth pulp
SHED	CD13, CD44, CD73, CD90, CD105, CD146, STRO-1, Oct-4, Nanog, nestin, SSEA-3, SSEA-4	Dent (dentin), bone, microvessel, neural	Immature dental pulp
PDLSCs	CD9, CD10, CD13, CD29, CD44, CD49d, CD59, CD73, CD90, CD105 CD106, CD146, CD166, STRO-1, Scleraxis	Dent (cementum, PDL), tendon, cartilage	Periodontal ligament
SCAP	CD49d, CD51/61, CD56, CD73, CD90, CD105, CD106, CD146, CD166, STRO-1, nestin, Survivin	Dent (dentin, pulp)	Apical papilla of developing root end
DFSCs	STRO-1, CD105, CD90, nestin, notch-1	Dent (dentin, pulp)	Dental follicle of developing tooth
BMSCs	CD105, CD90, CD44, CD73, CD29, CD13, CD34, CD146, CD106, CD54, CD166, STRO-1, Nanog, Sox-2	Bone, microvessel, cartilage, adipose, muscle, alveolar bone	Bone marrow

DPSC Dental pulp stem cells, *SHED* stem cells from human exfoliated deciduous teeth, *PDLSCs* periodontal ligament stem cells, *SCAP* stem cells from apical papilla, *DFSCs* dental follicle stem cells, *BMSCs* bone marrow stromal cells

progenitor cells in dental tissue that extensively proliferate to make osteoblasts, fibroblasts, and tooth cementoblasts that form cementum- and periodontal-like tissues (Hernández-Monjaraz et al. 2018). The application of PDLSCs has been described in recent research. Feng et al. (2010) treated 16 teeth with at least one profound intrabony defect with PDL transplantation and evaluated clinical outcome measures in terms of probing depth, gingival recession, and attachment gain for 32–72 months. The outcome of their trial demonstrated the promising application of dental tissue-derived stem cells in terms of dental tissue engineering in the treatment of human periodontitis.

2.1.2 Embryonic Stem Cells

ESCs are pluripotent cells derived from the inner in vitro fertilized cell mass (ICM) of blastocytes before the first 2 weeks of development with the intrinsic ability to proliferate and differentiate to almost all somatic cell types in the embryo (Orti et al. 2018; Han et al. 2014). Despite their extraordinary capacity for regenerative medicine and tissue engineering, there are serious ethical considerations in the use of human embryo for clinical application. Hence, these restrictions led researchers to redirect their research to adult stem cells (Orti et al. 2018; Jain 2002). However, there are some studies that investigate the role of ESCs in dental tissue engineering. ESCs can be inducible either to osteogenic or chondrogenic cells by the co-culture with PDL fibroblast which aids in the reconstruction of temporomandibular joint (Liu and Cao 2010). ESCs have the ability to proliferate on the surface-extracted tooth root tissue and produce osteogenic factors, such as osteopontin and osteocalcin (Inanç et al. 2009). ESCs have been shown to have the ability to differentiate toward the odontogenic lineage by culturing murine embryonic stem cells (mESCs) in ameloblasts serum-free conditioned medium (ASF-CM) (Ning et al. 2010). To date, ESCs have been used for dental tissue engineering only at experimental results, and there is a long way to go in this field.

2.1.3 Induced Pluripotent Stem Cells

iPSCs are generated from fully differentiated somatic cells by reprogramming and dedifferentiating into cells that possess ESC developmental potential without having any ethical concerns about embryo destruction (Douthwaite et al. 2022). Similar to ESCs, iPSCs have the capacity for self-renewal and differentiate into functional tissues of the three primary germ layers, i.e., ectoderm, endoderm, and mesoderm (Wu et al. 2022). In 2007, iPSC generation was succeeded from adult human skin fibroblasts with ectopic expression of transcriptional factors, including Octamer-binding transcription factor 4 (Oct4), myelocytomatosis oncogene (c-Myc), Kruppel-like factor 4 (Klf4), and sex-determining region Y-Box 2 (Sox2), using retrovirus and virus-free non-integrating techniques (Okano and Morimoto 2022; Wang et al. 2022a). Although iPSCs can be generated from a variety of human tissues, dental tissues (dental pulp cells, human third molar mesenchymal stromal cells, buccal mucosa fibroblasts, gingival fibroblasts, and periodontal ligament fibroblasts) offer an appealing source of precursors as a result of tissue accessibility in order to participate in autologous dental tissues engineering (Radwan et al. 2020; Sunil 2016; Hynes et al. 2015). In spite of significant breakthroughs in iPSCs, there are substantial limitations in iPSC potential for the clinical usage, such as challenges of epigenetic memory of former phenotype, genomic instability, tumor formation risk of retroviruses, and teratoma formation (Chen et al. 2022). Hence, the current efforts should concentrate on fabricating controlled protocols for the induction of iPSCs to produce distinctive pluripotent cells for tissue engineering.

2.2 Growth Factors

Every critical cellular event, such as pulp regeneration and dental morphogenesis in dental tissue engineering, is modulated by growth factors (Hashemi-Beni et al. 2017). So as to achieve functional dental tissue regeneration, there should be a morphogenic signaling molecule that binds to specific receptors on the surface of cells and initiate the cascade of the process (Langer and Vacanti 1993). Growth factors participate in various parts of the process of forming a biological tissue; for instance, they regulate the proliferation rate of stem cells, induce them to differentiate into other cells, or stimulate them to make mineralizable matrices (Chen et al. 2019). There have been some studies that indicate the role of a variety of morphogenic signaling molecules in forming dental tissue. Bone morphogenetic proteins −2, 4, and 7 (BMPs) are the common growth factors in tissue regeneration techniques that have been used in the initiation phase of odontogenesis and morphogenesis. Nerve growth factor (NGF) has a major role in nerve elongation and survival. Granulocyte colony-stimulating factor (G-CSF) mainly has effect in cell homing,

angiogenesis, and neural and antiapoptotic events. Transforming growth factor-beta (TGF-ß) is one of the largest groups of growth factors that influence cells by regulating osteoblast differentiation. Vascular epithelial growth factor (VEGF) and platelet-derived growth factor (PDGF) are the growth factors that impact tissue engineering by the provocation of blood vessel formation. Also, the fibroblast growth factor (FGF) facilitates the differentiation of human dental pulp cell (HDPC) in mineralized tissue (Giannobile 1996; Kaigler et al. 2006; Nakashima and Akamine 2005; Nevins and Reynolds 2011; Rizk and Rabie 2013). These growth factors have been investigated in research for regeneration of diverse dental tissue, including dental pulp, mandibular and maxillary bones, and periodontal tissue (Rizk and Rabie 2013; Giannobile et al. 2001; Khodakaram-Tafti et al. 2018; Acri et al. 2019). The most clinically and preclinically used growth factors for oral and periodontal regeneration have been BMPs, PDGF, insulin-like growth factor (IGF), FGF-2, and TGF-β (Kaigler et al. 2006). BMPs are multifunctional secreted growth factors, which are effective regulators of bone and cartilage formation and repair. BMPs instigate, support, and conserve odontogenesis and osteogenesis, therefore cause dental cell proliferation during embryonic development and tooth homeostasis in adults (Carreira et al. 2014; Ahmad et al. 2020). BMPs −2, −4, −7, and − 12 have been studied to differentiate stem cells toward osteoblast and odontoblast for periodontal and peri-implant bone regeneration (Kaigler et al. 2006; Choi et al. 2002; Ozeki et al. 2017). Numeral studies validate alveolar bone regeneration in various periodontal defects by BMP2-based therapy (Kim et al. 2016; Boda et al. 2019, 2020; Kawai et al. 2018). It also has an ability to provoke cementum formation shown in research (Park et al. 2020; Miyaji et al. 2010). BMP2 can be used to regenerate peri-implant bone or bone height improvement in areas below the maxillary sinus (Kaigler et al. 2006). BMPs are the most and only available growth factor in dental tissue engineering and clinical use compared to bFGF, GM-CSF, IGFs, and PDGF (Carreira et al. 2014). Nevertheless, BMPs have to be coupled to the delivery system in order to apply and preserve their biological activity at the site we want to regenerate, avoiding systemic proliferation. Hence, scaffolds loaded with osteoprogenitor cells and/or growth factors are trending in new studies (Carreira et al. 2014). TGF-β is another principal growth factor that is crucial in tissue development regulation, dentin matrix production, odontoblast differentiation, human dental pulp stem cell proliferation, and differentiation (Liu et al. 2007; Zi 2019). The largest subgroup of TGF-β family that has been used for this purpose is TGF-β1 that existed in the platelets and osseous tissue (Dobie et al. 2002). Odontoblast-like cell differentiation and dentin matrix secretion are initiated by TGF-β1 that send signals to odontoblasts in the human dentin-pulp complex (Dobie et al. 2002). FGF is a major family of signaling protein that has a major role in the many cellular events of mesodermal and neural ectodermal cells (Ornitz and Itoh 2015). In the process of developing tooth, FGF-2 exists on basement membrane between oral epithelium and mesenchymal tissue which regulates differentiation of odontoblasts and ameloblasts (Shimabukuro et al. 2009). FGF-2 has been applied in periodontal regenerative tissue engineering by the effect on odontoblastic differentiation potential of human DPSC (Vaseenon et al. 2020). FGF-2 is included in several scaffolds and induces pulp-dentin complex regeneration by dentin-bridge formation and synthesis of pulp-like tissue (Ishimatsu et al. 2009; Kim et al. 2010). The crucial step in part of dental tissue engineering is supplying blood to the organ that is going to be made. In this part, there are some growth factors that support nutrition, oxygen, and biomolecule to the cells as well as scaffolds which are used in tissue engineering. The most used growth factor that has been applied is VEGF produced by pulp cells and takes action in dentin matrix to enhance angiogenesis and vascularization of the tissue (Rosa et al. 2017). VEGF has a significant role in self-assembling

hydrogel contributes to dental pulp stem cell proliferation, odontoblastic differentiation, and vascularization of connective tissue by itself or together with TGF-β1 and FGF-2 (Galler et al. 2012). Growth factors have a significant role in cell-cell interaction. These factors, especially TGF-β family, accelerate odontoblast differentiation and dentin matrix secretion (Galler and D'Souza 2011). Moreover, recent studies have demonstrated the role of paracrine signaling induced by MSCs as a complementary factor for tissue engineering (Wang et al. 2022b). It was found that the repair effect observed in MSCs grafts on the damaged tissue was primarily due to the secreted factors released by the stem cells rather than cellular engraftment (Wang et al. 2022b; Gonzalez-Fernandez et al. 2022). The principal mechanism in regenerating tissue has been studied as the bioactive factors secreted by the MSCs contributing to the paracrine activity (Rangasami et al. 2021). Although the exact role of the MSC-derived secretome in tissue engineering is still not entirely recognized, there are some research on the dental MSC-derived secretome used in dental tissue engineering (Koosha et al. 2021). The results of DPSC and SHED-derived secretome used in experimental studies have suggested the role of paracrine signaling in regenerating tissues or repairing damaged cells in various diseases (Bousnaki et al. 2021). Dental stem cells stimulate vascularization and angiogenesis by differentiating into mature cells via paracrine angiogenetic factors such as VEGF, PDGF, and FGF, in addition to a variety of stemness-related markers such as SOX-2, Nanog, and Oct-3/4 (Bar et al. 2021). As a result of novel research on MSC-based therapy, cell-free therapy using the MSC-derived secretome and their paracrine activity rather than a cellular replacement or cell-cell interrelation may be a better option as a new therapeutic opportunity in regenerative medicine. By understanding the biological pathways and interaction in tooth regeneration, applying growth factors as one of the essential parts of tissue engineering into the cells and scaffolds will be a part that needs more studies.

2.3 Scaffolds

The third part of tissue engineering is choosing appropriate scaffolds that are crucial in cellular response. Scaffolds have significant roles in different steps of this technique, such as localization of the cells that discussed primarily, preferment of cell adhesion, and supplying nutrition, oxygen, wastes, and biomolecules through its pore that takes part in cell proliferation and differentiation that improve biocompatibility and cellular function (Orti et al. 2018). There are two strategies for periodontal regeneration, including guided tissue regeneration (GTR) and tissue engineering (Needleman et al. 2019). Conventional regenerative approaches, also known as GTR, enhance the proliferation and differentiation of tissue-resident progenitor cells into fibroblasts, cementoblasts, and osteoblasts and inhibit apical migration of the epithelium in the periodontal defect by the application of barrier membranes. In this technique, bone substitutes of autogenic, allogeneic, xenogeneic, or alloplastic origin may be utilized as scaffolds (Table 2) (AlKudmani et al. 2017). A more recent tissue engineering approach aims to combine exogenous progenitor cells, biomaterial scaffolds, and growth factors to produce the sophisticated structure and function of the periodontal tissues (Leyendecker Junior et al. 2018). From previous reviews, different biomaterials which work as scaffolds are used in dental tissue engineering, although for making the ideal one, it should have some special properties to survive in the living tissue and have biocompatibility with the tissue it surrounded. Scaffolds could be classified by their origin, chemical components, physical characteristics, and mode of application. There are two main origins of scaffolds: natural and synthetically engineered polymeric scaffolds. Polymers that have higher surface-to-volume ratio and biodegradability rate are more popular than other polymers in clinical approaches (Dhandayuthapani et al. 2011). Scaffolds used in tissue engineering consist of stem cells and growth factors that incorporate together to form the biological organs, though some cell-free scaffolds have different applications. To classify scaffolds'

Table 2 The components of guided tissue engineering

Guided tissue engineering												
Membrane												
Collagen barriers		Cargile membranes		Polylactic, polyglycolic, and polyglactin copolymer acid barriers			Oxidized cellulose mesh barriers		Laminar bone allograft membranes			
Bone replacement graft												
Autografts				Allografts			Xenografts		Alloplastic grafts			
Cortical bone chips	Osseous coagulum and bone blend	Intraoral cancellous bone and marrow	Extraoral cancellous bone and marrow	Frozen iliac allograft	Freeze-dried bone allograft (FDBA)	Demineralized freeze-dried bone allograft (DFDBA)	Bovine-derived bone replacement grafts	Coralline calcium carbonate	Polymers	Tricalcium phosphate (TCP)	Hydroxyapatite	Bioactive glass

DFDBA Demineralized freeze-dried bone allograft, *FDBA* freeze-dried bone allograft, *TCP* tricalcium phosphate

physical composition, we should put them into four groups: microparticles, hydrogels, macroporous, and nanofibrous materials. The mode of application of scaffolds is divided into two groups: injectable and non-injectable. There are growing interests in recent literature in using biocompatible and biodegradable scaffolds due to their significant advantage of inflammatory response inhibition. Some topics on scaffolds and their ability in dental tissue engineering were reviewed in the following sections.

2.3.1 Scaffold Materials

Natural and Naturally Derived Polymeric Scaffolds

The natural scaffolds are mainly formed from fibrin, collagen, chitosan, alginates, amniotic membrane, glycosaminoglycan, elastin, and dentin matrix (Anitua et al. 2018). Natural polymers are biologically active and are considered to provide better biocompatibility than synthetic polymers (Nakashima and Akamine 2005). The major component of dental pulp and overlying dentin matrix is collagen. High biocompatibility feature of collagen makes it an ideal scaffold for dental tissue engineering (Zhang et al. 2006). Collagen stimulates organization of pre-odontoblasts and adhesion of odontoblasts to the dental pulp, which would finally regenerate biological tissue (Jazayeri et al. 2020). Some studies investigated the application of collagen in synthesis of hard and soft tissue for pulp-dentin reconstruction. These studies demonstrated that collagen-based scaffolds make an appropriate place for DPSCs proliferation and deafferentation into odontoblast (Alraies et al. 2020; Yao and Flynn 2018). Besides the advantages of natural and naturally derived polymeric scaffolds, there are some drawbacks that limit the administration of these scaffold in clinical approaches (Chisini et al. 2019). Control of physiochemical properties of natural polymers is questioned in recent studies that makes synthetically engineered polymeric scaffolds widely used in clinical settings (Sengupta and Heilshorn 2010; Liu et al. 2020b).

Synthetically Engineered Polymeric and Ceramic Scaffolds

Polyglycolic acid (PGA), poly-L-lactic acid (PLLA), poly(lactic-co-glycolic acid) (PLGA), polyglycolic acid, polyethylene glycol (PEG), carbon fiber, Teflon, Dacron, polybutyric acid, bioactive glass, and polycaprolactone are artificial matrixes scaffolds (Anitua et al. 2018; Orti et al. 2018). Synthetically engineered scaffolds may display superior physicochemical properties, such as microstructure and mechanical strength, and allow better control of degradation rate than natural polymers, although they have reduced bioactivity (Roseti et al. 2017). Among this type, polylactic acid (PLA) and polyglycolic acid (PGA) are widely studied in previous research. PLA is a biocompatible polyester that reinforces the adhesion of DPSCs and ex vivo cells (Tatullo et al. 2019). In vitro and in vivo studies have shown that DPSCs can be differentiated to mature odontoblasts by PLA and regenerate tissues that mimic the dentin-pulp complex (Wang et al. 2010; Liu et al. 2011). The interconnected spherical pore structure of PLA scaffolds provides effective mechanical setting for cell proliferation and differentiation (Woo et al. 2009). The favorable mechanical characteristics of PLA scaffolds, besides their controllable degradation rate, make them suitable candidates for pulp tissue engineering (Jazayeri et al. 2020). Similar to PLA, PGA has a wide range of reproducibility and biocompatibility. PGA has various applications for the regeneration of dental tissues, including the bioengineering of whole crowns (Young et al. 2002). The biocompatibility of these synthetically engineered polymeric has been studies in many research (Li et al. 2017a; Song et al. 2021). The chemical properties of these polymers allow hydrolytic degradation through de-esterification. The monomeric components of each polymer are removed by natural pathways when the degradation is completed (Gentile et al. 2014). Ceramic scaffolds, including calcium phosphates (Ca/P) and bioactive glasses or glass ceramics, are natural scaffolds that have been used for hard tissue formation (Zhang et al. 2008).

The Ca/P scaffolds have ß-tricalcium phosphate (ß-TCP) or hydroxyapatite (HA) that can be fabricated synthetically and are used in therapeutic healing processes of bone defects in dental tissue engineering (Hashemi-Beni et al. 2017). Wet methods and solid-state reactions are the techniques for the preparation of HA powder and dense or porous ceramics which are proper applications for artificial teeth and dental regeneration (Hashemi-Beni et al. 2017).

2.3.2 Scaffold Modification

Techniques for Scaffold Fabrication

There are various methods for the fabrication of 3D scaffolds that have been used for several years. These methods can be subcategorized into two main techniques: conventional techniques and rapid prototyping (RP) techniques (Anitua et al. 2018). Conventional fabrication techniques include electrospinning, salt leaching, gas forming, phase separation, and freeze-drying and do not prepare control of the internal architecture of the scaffold or the fabrication of complicated architectures that could be attained by RP techniques (Loh and Choong 2013). Electrospinning method is able to synthesize 3D nanofibrous scaffolds. This method is used for both natural and synthetic scaffolds applied for pulp regeneration (Baylan et al. 2013). Nanofibrous scaffolds fabricated by electrospinning mimic extracellular matrix due to its porosity (Boudriot et al. 2006). Hence, this method is widely used in regenerative endodontics (Baylan et al. 2013; Bottino et al. 2013). Gas foaming method uses high-pressure carbon dioxide gas for the fabrication of highly porous scaffolds. A polymer solution is saturated with carbon dioxide at a high pressure (800 psi) (Li 2017). Since it is difficult to regulate the size and interconnection of pores, this method is commonly applied in industry, not in the fabrication of scaffolds (Nam et al. 2000). Salt leaching is one the most common techniques used to fabricate 3D scaffolds (Kanimozhi et al. 2018). In this method, synthetic or natural polymers are assembled in the existence of the insoluble salt crystals, which are later leached out by incubation in large volumes of water (Chiu et al. 2010). While salt leaching is generally a simple fabrication technique, it is challenging to precisely control the 3D structure of the scaffolds. The conventional techniques are generally used to fabricate highly porous scaffolds resembling the structural pattern of the natural ECM (Maroulakos et al. 2019). Nevertheless, the preciseness and resolution of constructions are low compared to RP methods fabricated by 3D printing. RP, also known as additive manufacturing and solid freeform fabrication, is assisted by imaging data like computerized tomography (CT) and magnetic resonance imaging (MRI) and computer-aided design (CAD) models. Hence, the RP technique can produce precise 3D physical objects layer by layer with distinct physical and chemical properties such as composition, designs, and functions, considered multiphasic, compared with monophasic scaffolds in conventional methods (Yuan et al. 2017). By having an image of the target organ and three-dimensional reconstructions, procedure of mirroring and interpolation of images are used to design the model in real dimensions for the fabrication of small models such as metallic and/or ceramic crowns in dentistry with a minimum of 10 μm resolution (Goiato et al. 2011). Three main categories of techniques involved in 3D bioprinting include laser-assisted, inkjet-based bioprinting, and extrusion-based (Yuan et al. 2018). Laser-assisted techniques have been utilized to produce biocompatible scaffolds with amorphous structures. These techniques have demonstrated exclusive characteristics for micromachining coating, polymerization, and patterning of diverse biomaterials for dental tissue engineering and other fields of regenerative medicine (Yuan et al. 2018). The specific features of laser-assisted bioprinting, such as a nozzle-free, noncontact process, provide cells with high activity and high resolution (<1 μm). Moreover, this technique is consistent with a wide scale of viscosities (1–300 mPa/s) and enables printing tissues with an insignificant impact on cell viability and function (Gu et al. 2022). Inkjet-based bioprinting is another technique based on the conventional inkjet printing process with desktop inkjet printers which exhibit

high resolution of up to 50 μm and cell viability (Su et al. 2021). This technology is a noncontact printing process in which droplets of dilute solutions are dispensed onto a hydrogel substrate or culture dish under computer control (Su et al. 2021). The third and the most frequently used 3D bioprinting technique is extrusion-based printing (Baier et al. 2021). In this technique, cell-laden hydrogels or bio-inks have been extruded onto printing stages, layer-by-layer, to produce 3D structures with lower resolution than inkjet- and laser-based bioprinting around 200 μm (Baier et al. 2021). Acellular bioprinting is a novel type of 3D printing technique which enables the scaffolds to hide from immunological responses (Matai et al. 2020). Deposition and patterning of biological materials in bioprinting are most commonly accomplished with microextrusion, inkjet printing, and laser-assisted printing (Eltom et al. 2019). The techniques that are discussed in this topic have their own advantages and disadvantages. Hence, it is crucial to consider the goal of the study to apply one of these methods in order to have biocompatible scaffolds.

Scaffolds for 3D Cultures of DPSCs

Dental tissue engineering is a growing treatment for tooth loss and decay. For this goal, there should be interaction among stem cells, growth factors, and scaffolds. Cells exist in a 3D niche in their physiological environment (Fig. 2). Thus, 3D cultures have been introduced to fabricate suitable scaffolds to provide the cells exactly the same 3D niche as they have in their physiological environment. This 3D biocompatible scaffold stimulates proliferation, cell adhesion, migration, and differentiation. These scaffolds pose virtually parallel surface topographies like native tissue; hence, they have a more beneficial impact than 2D scaffolds (Ashraf et al. 2018). 3D Scaffolds can generate an environment for the development of stem cells and control their fate both in vitro and in vivo. DPSCs have been recognized as the stem cells with the most potential for dental tissue regeneration. In recent studies, several biomaterials in combination with DPSCs have been published (Ferrarotti et al. 2018; Piva et al. 2017). Bhuptani and Patravale (2016) fabricated porous biocompatible spherical PLGA microscaffolds of particle size range (100–200 μm) and employed them to propagate DPSCs in vitro. DPSCs were adequately proliferated and adhered over the microscaffolds forming a 3D cell-microscaffold construct. Their study demonstrated that the average number of DPSCs grown on PLGA microscaffolds was considerably higher than monolayer 2D culture during the fifth and seventh day; thus, they introduced novel porous microscaffold as a promising scaffold for 3D culture. Other types of scaffolds that have been applied in dental tissue engineering in vitro are hybrid chitosan/gelatin/nanohydroxyapatite (CS/Gel/nHA) scaffolds. Vagropoulou et al. (2021) claimed biocompatible blend of natural polymers (chitosan and gelatin) supported the viability and proliferation of DPSCs over 14 days in culture. The noticeable odontogenic shift of DPSCs, evidenced by upregulation of *DSPP*, *BMP-2*, *ALP*, and the transcription factors *RunX2* and *Osterix*, was revealed by gene expression patterns in this study which is achieved by the combination of CS/Gel/nHA scaffolds prepared by freeze-drying and DPSCs. Moreover, other research have also investigated the in vivo ability of several bioengineered constructs of DPSCs and different scaffolds (Hilkens et al. 2017; Kim et al. 2015). Li et al. (2017b) looked into the effect of dynamic 3D simulated microgravity culture on the proliferation and odontogenic differentiation abilities of hDPSCs in poly(lactic-co-glycolic acid) (PLGA) biocompatible scaffolds in nude mice. Their study revealed that the proliferation and odontogenic differentiation capacities of the hDPSCs organized in the 3D SMG culture system were more remarkable in comparison with those organized in the static culture system. These findings suggested that dynamic 3D SMG culture likely contributes to tissue engineering by improving the proliferation and odontogenic differentiation abilities of hDPSCs in vivo. In another study, nontoxic and nonimmunogenic biomaterials have been used in order to develop dentin pulp-like tissues, enamel dental pulp, and cementum periodontal complexes in vivo (Chang et al. 2020). Chang et al. (2020) explored the

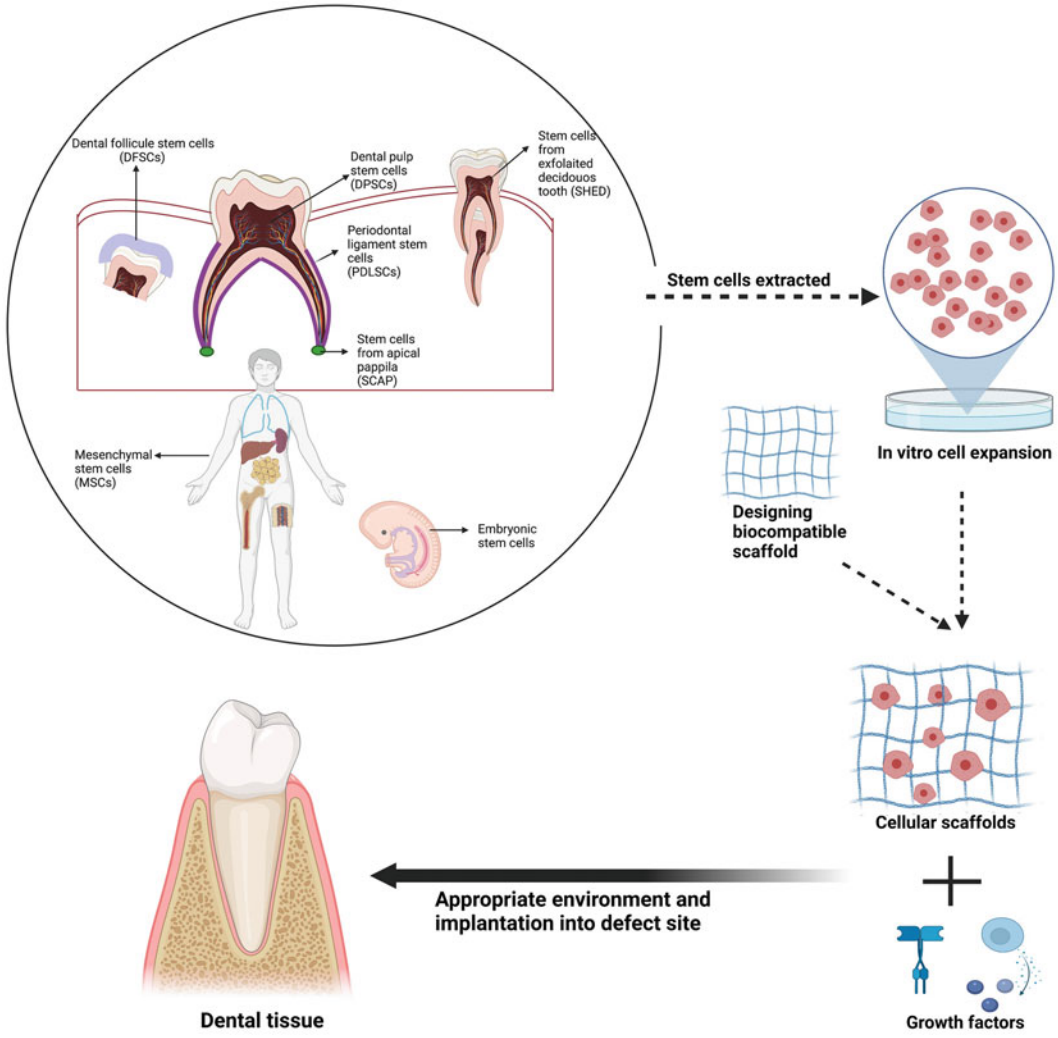

Fig. 2 Diagrammatic representation of biocompatible dental tissue engineering. Stem cells can be derived from different sites in the body and undergo multiple stages to regenerate living tissues. Stem cells that are mainly used in dental tissue engineering are DSCs which are MSC-like populations. *MSCs* Mesenchymal stem cells, *SHED* stem cells from human exfoliated deciduous teeth, *DPSCs* dental pulp stem cells, *SCAP* stem cells from apical papilla, *PDLSCs* periodontal ligament stem cells, *DFSCs* dental follicle stem cells

combination of DPSCs with autoclaved treated dentin matrix (a-TDM) that provided an ideal biomaterial for regeneration of tooth material. In their study, a-TDM was implanted in a mouse model for 6 weeks and was a substrate with DPSCs for tooth reconstruction in a goat animal model in vivo. The author reported that Allogenous a-TDM performed as an applicable scaffold facilitating DPSCs to proliferate and differentiate into a wide ranging of cells, including odontoblasts, fibroblasts, vascular cells, and neural endings. These recent models validate the regenerative potential of DPSCs and biocompatible scaffold combination. These combinations have been shown to support biocompatible scaffolds in stem-cell-based dental tissue engineering by providing cell viability and differentiation and suggest favorable tools for neural tissue applications and bone and dentin restoration.

3 Stem Cell Interactions in 3D Scaffolds

In order to have a dental tissue, it's required to recognize cell-cell and cell-matrix interactions that shape the living tissue by tissue engineering. To simulate an in vivo setting, it is vital to make three-dimensional scaffolds that these interactions take place within it, resulting in proliferation and differentiation of cells. Multiple factors play a role in cell-cell and cell-matrix interaction during dental tissue engineering. Stem cell proliferation and differentiation toward appropriate tissue are controlled by intrinsic factors (transcription factors that express by the cells) and extrinsic factors (extracellular matrix (ECM) and signaling molecules) (Granz and Gorji 2020). Stem cells should have an applicable interaction with their environment that is supplied by scaffolds. To form 3D cell-scaffold structure, scaffolds help cells retain their physiological morphology, adhesion, proliferation, and migration by promoting cell-cell and cell-matrix interaction (Murphy et al. 2013). Naturally derived polymeric scaffolds, such as fibrin, collagen, chitosan, alginates, amniotic membrane, glycosaminoglycan, elastin, and dentin matrix, are extracted directly from ECM. Native ECM interaction with cells regulates cell adhesion and migration by specific integrin ligand. Ligands that are necessary for cell adhesion and migration present on the surface of these natural polymers. Similar to natural ECM, tissue engineering scaffolds resemble the ECM not only in terms of topology but also moderates and promotes cellular behavior including cellular migration, adhesion, proliferation, differentiation, and survival, by producing regulatory signals (Subramanian et al. 2011). Biological activity of scaffolds depends on the density of ligands on its surface that make a place for integrins to bind. The interaction between cells and biomaterial scaffolds is called focal adhesion. Cells adhere to ECM at focal adhesion by transmembrane proteins, mainly integrins, which bind to the ligand on ECM. The other end of the integrin connects to the cell's cytoskeleton. Cell attachment to the surface of a scaffold initiates a chain of physicochemical reactions between cells and the scaffold. These reactions control cell fate, such as adherence, spreading, and contraction. Cell attachment is influenced by multiple factors, such as cell behavior, material surface properties (e.g., hydrophobicity, charge, roughness, softness, and chemical composition of the biomaterial surface itself), and environmental factors (Chang and Wang 2011). Attachment to scaffold surface requires a series of intracellular, transmembranal, and extracellular proteins; hence, it likely involved the adsorption onto the material surface of serum and ECM proteins (Chang and Wang 2011). As the second part of tissue engineering, growth factors are the major player in this interaction that upregulate or downregulate cell reproduction. Growth factors move to the material surface through diffusion, flow, or thermal convection, where they bind via a thermodynamically favorable process (King and Krebsbach 2012). These factors influence cells by transmembrane receptors that transfer signals through the cell membrane to genes (Murphy et al. 2013). Moreover, cellular responses within scaffolds are affected by biomechanical stimuli that support cell differentiation and the production of ECM. Mechano-transduction influences cells in culture by spinner flask bioreactors, flow perfusion bioreactors, dynamic compression bioreactors, and hydrostatic pressure bioreactors. Another important interaction in biocompatible dental tissue engineering is cell responses to dynamic scaffolds (Murphy et al. 2013). Biodegradable scaffolds have different degradation rates which determine properties of the scaffolds. Stem cells which proliferate in higher pace need scaffolds with higher degradation rates than tissues which need more stability. Biodegradable scaffolds change the presentation of ligands on the surface of the polymers, providing a dynamic substrate for cell adhesion, signaling, migration, and differentiation (Chang and Wang 2011). The most applicable technique for dental tissue engineering is the usage of DSCs and 3D scaffolds (Zhang et al. 2013). DSCs produce several immunomodulatory and proliferative mediators, such as IL-6,

IL-10, IL-1β, interferon-γ, TNF-α, TGF-β, hepatocyte growth factor, and VEGF. Also, DSCs mediate immune responses to boost tissue regeneration by not expressing the major histocompatibility complex class II antigen and suppressing T-cell proliferation and lymphocyte activity (Granz and Gorji 2020). DSCs differentiate into odontoblasts, which take part in tooth regeneration by producing the major part of the ECM components of dentin and are necessary in dentin mineralization (Lundquist 2002). Along with collagen (mostly type I collagen, type V, and III), the dentin ECM contains dentin proteoglycans (chondroitin sulfate, dermatan sulfate, keratan sulfate, heparin, and heparan sulfate, hyaluronan), enzymes (MMP-8, MMP-2, MMP-9, MMP-20), growth factors (IGF-I, TGF-β1, PDGF, VEGF), and phospholipids (Orsini et al. 2009). This structure creates an appropriate environment and preserves homeostasis for the proliferation and differentiation of DSCs (Wan et al. 2015). The EphB/ephrin-B signaling pathway reciprocally moderates the attachment and migration of DSCs in 3D scaffolds using the mitogen-activated protein kinase pathway and phosphorylation of Src family tyrosine kinases that lead to living dental tissue as a result (Granz and Gorji 2020). These cell-cell and cell-matrix interactions (that are summarized in Fig. 3) ultimately regenerate a tissue that mimics natural dental tissue

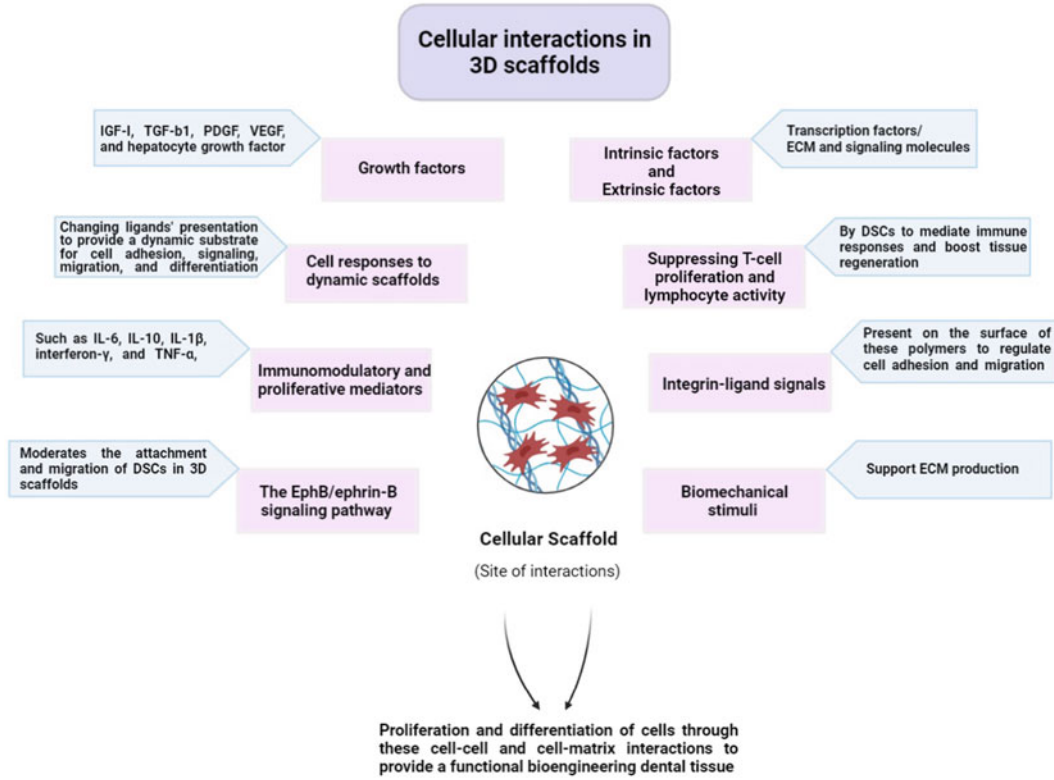

Fig. 3 Cell-cell and cell-matrix interactions. These interactions can provide a tissue that mimics natural dental tissue functionally and morphologically. In this regard, intrinsic factors and extrinsic factors, biomechanical stimuli, cell responses to dynamic scaffolds (Murphy et al. 2013), ligands, regulatory signals (Subramanian et al. 2011), growth factors (King and Krebsbach 2012), suppressing T-cell proliferation and lymphocyte activity, immunomodulatory and proliferative mediators, and the EphB/ephrin-B signaling pathway (Granz and Gorji 2020) have important roles. *ECM* Extracellular matrix, *DSC* dental stem cell, *IL* interleukin, *TNF-α* tumor necrosis factor alpha, *3D* three-dimensional, *IGF-1* insulin-like growth factor 1, *TGF-β* transforming growth factor-beta, *PDGF* platelet-derived growth factor, *VEGF* vascular endothelial growth factor

functionally and in morphological appearance. There are also some other particular bioengineered tooth-based developmental signaling and interactions. For instance, DSCs can be regulated by BMP and Wnt signaling pathways that provide a beneficial treatment approach in the field of tissue regeneration (Zhang et al. 2016). Significant osteoblastogenesis effects of Wnt pathways have been found so far. In this regard, ferutinin (a phytoestrogen from *Ferula* species) can use these pathways to activate DPSCs' differentiation (Rolph et al. 2020). It has been mentioned that Wnt1/β-catenin signaling can be targeted by miR-140-5p (as a microRNAs family member) regulating DPSCs' differentiation (Lu et al. 2019). On the other hand, the interactions of lipopolysaccharides (that are involved in dental infections) with toll-like receptor-4 (TLR4)-mediated nuclear factor-kappaB (NF-κB) and mitogen-activated-protein-kinase (MAPK) signaling pathways have been found in DPSC environment. Herein, the signaling pathways of NF-κB and MAPK have important roles in various stages of cell processes (He et al. 2015). They also affect DPSCs' odonto−/osteogenic differentiation processes that are interferon-gamma (IFN-γ)-regulated pathways. Besides, IFN-γ (at low concentration) can promote proliferation/migration functions of hDPSCs (He et al. 2017).

4 Strategies of Dental Tissue Engineering

Tissue loss (particularly in the craniofacial site) is an important health issue that can impose notable physiological and psychological effects on patients. Thus, it is important to reconstruct the aesthetic and functional features of the craniofacial region. In this regard, tissue engineering has become a potential therapeutic approach for several tissue defects that can be helpful for dental reproduction purposes, too (Abou Neel et al. 2014). The gathering of biological and material sciences together leads to the generation of tissue engineering to overcome the needs for tissue/organ reproduction. As a result, it has been utilized for the regeneration of periodontal tissues successfully even in clinics (Yen and Sharpe 2008). In conventional methods, biocompatible materials are used to fill in roots which do not benefit from any dentin/pulp regeneration. In these treatment strategies, impaired dental tissues are removed and replaced with filling materials with their specific physical/functional features. Disturbances in restorative approaches and impairments of using materials in this line of treatment can cause negative complications which establish the requirement for novel regeneration methods. For instance, it has been shown that endodontic treatment can make teeth devitalized and brittle with other complications post operation. Therefore, studies are working on the regenerating methods regarding endodontic therapeutic approaches nowadays. Herein, stem-cell-based approaches for dentin/pulp regeneration using implantable scaffoldings and an appropriate microenvironment can pave the way for tissue regeneration (Zhai et al. 2019; Ahmed et al. 2020; Moussa and Aparicio 2019). For instance, the regenerative endodontic procedure is mentioned as a beneficial approach that can lead to reinnervation, angiogenesis, and root formation. Indications for this approach include pulp necrosis and inflammation of the preapical structure of immature permanent teeth. Significant tissue destruction makes regenerative endodontic procedure contraindicated. Another contraindication is the lack of patient cooperation (Zbańska et al. 2021). In this regard, we are going to summarize different approaches in the field of dental tissue engineering.

4.1 Dentine-Pulp Complex

The term dentine-pulp complex is derived from the dentine and dental pulp which have the same embryological origin with histological/functional characteristics (Peters et al. 2021). Dentine-pulp complex can affect teeth lifetime notably due to its essential functions including nutrient supplying, acting as a sensory organ, inducing tertiary dentinogenesis, and reacting to the bacterial infiltration through immunological properties (Nakashima 2005). Reparative processes can be

mediated through the interactions of dental pulp cells and dentinal matrix. Thus, dental pulp regenerative strategies may be helpful for healing purposes and root development. Keeping it viable is also important for the aim of maintaining hemostasis, and avoiding some adverse effects belongs to the root canal treatments (Peters et al. 2021). In addition, restoring the dentin-pulp complex is an important step before pulpectomy in order to maintain dental pulp capabilities (Hashemi-Beni et al. 2017). Stem cells, scaffolds, and signaling molecules are three basic components of dentin-pulp regeneration strategies. It is stated that there are two main strategies for regenerating dentin-pulp complex including cell transplantation and cell homing. Cell transplantation can be done through the injection of exogenous stem cells or by being loaded onto scaffolds with or without signaling molecules (Karakaya and Ulusoy 2018). This strategy may benefit from using a vehicle in order to carry and deliver the required materials which can raise the efficacy of the treatment strategy. Peptide and fibrin hydrogels in addition to the biodegradable lactide and glycolid are some of these required materials for cells' encapsulation at dentin-pulp complex regeneration (Abou Neel et al. 2014). Besides injection of stem cells, scaffolds provide superior control for delivering steps, saturation with time-release molecules, modulating stiffness, pore size, and cellular interactions (Huang et al. 2010). Although DPSCs are mentioned to be the cells of choice in dentine-pulp regeneration, some non-dental sources (such as bone marrow-derived MSCs and adipose-derived stem cells) have been also introduced to have roles in this regeneration process (Bakhtiar et al. 2018). As it was mentioned, cell homing is another approach for dentin-pulp complex regeneration. Due to some associated risks of cell transplantation including immune rejection, contamination, and tumorigenesis, cell homing is a suitable alternative for cell transplantation. The high costs and complexity of cell transplantation also make it not being clinically viable (Huang et al. 2013). Studies have shown the equal therapeutic effects of MSC cell-free products to MSCs' with easier generation and more stability in MSC-derived soluble factors (Hu et al. 2020). This cell-free strategy as the active recruitment of stem/progenitor cells might be an easier clinically approach due to its independence of isolation and manipulation of stem cells in vitro (Eramo et al. 2018). Residual DPSCs, SCAP, and bone marrow-derived MSCs are the host endogenous cells that have roles in the cell homing process. The differentiation ability of these cells for angiogenic and odontogenic purposes can rise the positive outcomes of this strategy (Sismanoglu and Ercal 2020). Altogether, stem-cell-free base regenerative medicine strategies contain utilizing of endogenous stem cells, bioactive scaffolds, and growth factors. Endogenous stem cells exist in the remaining periapical or pulp tissue which can populate scaffolds and run regeneration steps. In this regard, incorporated growth factors can be mentioned as chemoattractants (Kitamura et al. 2011). On the other hand, DSC-derived factors can lead to nerve regeneration by promoting neutropenic functions. Different chemoattractants along with extracellular vesicles in DSC secretome can be mentioned as a stable cargo. Indeed, stem-cell-free base regenerative medicine provides a potential therapeutic approach considering the limitations of stem-cell-based therapy (Man et al. 2019; Sultan et al. 2019).

4.2 Periodontium

Periodontium can be damaged by several disorders, traumas, and deep caries. In this regard, tissue engineering can be helpful for the regeneration of the affected tissues. Utilizing PDLSCs can be introduced as a potent cell-based therapeutic approach for the reconstruction of the damaged periodontium (Maeda et al. 2011; Tomokiyo et al. 2019). PDLSCs can mimic MSC features and exhibit putative and perivascular stem cell markers (STRO-1 and CD146) (Benatti et al. 2007). Bone marrow MSCs and adipose-derived

stem cells have also shown probable advantages in the field of dental tissue engineering. (Maeda et al. 2011). Adipose-derived stem cells due to their capacity for differentiation into several cell lineages including adipogenic, chondrogenic osteogenic, and myogenic cells have notable regenerative abilities (Tobita et al. 2008; Larijani et al. 2015b; Goodarzi et al. 2018c). On the other hand, platelet-rich hemoderivatives (PRHds) are also presented as the sources of proteins/cytokines and growth factors that can be involved in wound healing processes of periodontal tissue engineering. Utilizing PRHds can provide a natural system for growth factor release and may avoid the use of xenogenic materials (Babo et al. 2017). Their performance and capacity to be utilized in periodontal therapies may be enlarged through their incorporation into scaffolds (Babo et al. 2017; Oliveira et al. 2015). The periosteum is also mentioned as a plausible source for periodontal regeneration (Chen and Shi 2014). Cultured periosteum in combination with platelet-rich plasma and porous hydroxyapatite granules may lead to better outcomes in periodontal defects (Yamamiya et al. 2008). Cementoblasts also play a role in understanding periodontal regeneration due to their ability in inducing mineralization in periodontal wounds. This capacity was seen in periodontal defects of a rodent model through biodegradable polymer sponges (Chen and Shi 2014; Zhao et al. 2004). The advantages of utilizing MSCs from gingiva in bone regeneration in in vivo studies have been established, too. These cells can give rise to induced pluripotent stem cells which make them another promising source to be used in the clinical application (Egusa et al. 2010; Qiu et al. 2020).

4.3 Bioengineered Whole Tooth

Whole organ bioengineering using 3D cell manipulation can provide a promising option to replace dysfunctional organs. In this regard, there are many studies that focus on whole-tooth replacement in the mouse model (Ono et al. 2017; Chai and Slavkin 2003; Oshima and Tsuji 2015). Indeed, regenerative medicine as an emerging therapeutic strategy in dental-related research can be a helpful approach for the formation of bioengineered whole-tooth treatments that exhibit sensory and functional characteristics of natural teeth. This process can be summarized in four steps including the selection of suitable cell sources and scaffolds, growth factor addition and tooth bud development, implantation of it in tooth loss model, and whole-tooth analysis through functional and anatomical features (Smith and Yelick 2016). DPSCs, SHED, stem cells of the apical pallia, dental follicle precursor cells, PDLSC, and dental epithelial cell rests of Malassez are some of the probable cell sources for tooth regeneration studies (Smith and Yelick 2016; Morsczeck et al. 2005; Shinmura et al. 2008). It should be also mentioned that according to several studies, re-aggravating embryonic germ cells of mouse teeth can lead to the development of tooth replacement with functional characteristics, revascularization, and root formation (Ikeda et al. 2009). Ectodermal dental epithelium in collaboration with neural crest-related mesenchyme can monitor this tooth development (Zhang and Chen 2014). As it was mentioned, the addition of growth factors is another step of tooth bioengineering in which BMP4 can affect tooth morphogenesis and tooth root formation through activation of transcription factors. It also has important roles in ameloblast differentiation. BMP4, BMP2, and BMP7 can regulate tooth patterning and crown shape in combination with each other (Thesleff and Mikkola 2002; Vainio et al. 1993).

Taken together, replacing the missing teeth with dental implants needs particular conditions such as the amount and time of bone loss in addition to the systemic disorders. These conditions can cause some absolute contraindications. In this regard, tissue engineering strategies according to self-regeneration capacity and easy accessibility of their components can be helpful for achieving better results (Sachdeva et al. 2021).

5 Experimental Models for Dental Pulp Regeneration (In Vivo, In Vitro, Ex Vivo)

Besides the critical studies that should be performed on cells and scaffolds, animal models must be developed to be used in preclinical research. So far, many animal and human studies have been used for this purpose (Table 3) (Ono et al. 2017; Kim et al. 2015). About in vivo models for tooth regeneration studies, small animals (including mice, ferrets, rats, and rabbits) are suitable options that provide a large number of samples. On the other hand, they are more cost-effective options than large animals. Breeding management and ethical issues also make small animals better to be used in in vivo studies compared with monkeys, pigs, and dogs as larger animals. Smaller animal models can provide valuable data about interactions between body cells and tissues, particularly in inflammatory and healing processes. They could provide the possibilities of producing transgenic or knockout species which brings important data about a single biological aspect (Fawzy El-Sayed and Doerfer 2017). However, smaller operation sites make it difficult to perform tooth extraction socket/implantation sites. Indeed, larger animal models have more biological complexity with more human resemblance compared with small animal models. Thus, in more advanced research of tooth construct jaw implants, larger animals can be used. Mini-pigs due to their similarities to human dentition are more commonly used in implantation studies. It is stated that implantation sites are important because they can affect outcomes of bioengineering and tooth morphology (Smith and Yelick 2016; Kim et al. 2015; Fawzy El-Sayed and Doerfer 2017). On the other hand, in vitro/ex vivo conditions can provide possibilities of controlling the multipotency capacities of stem cells. Therefore, growth factors can be added which is mentioned to be more difficult in in vivo situations to achieve once the cells are transplanted (Jiang et al. 2015). In vitro experimental models have been developed to minimize the requirements of animal models. However, 3D dentin/pulp complexes can be found in literature since 1998. The customized perfusion chamber and tooth germ models (manufacturing through bioengineering methods) in addition to whole-tooth culture and slice organ culture are also defined as in in vitro options (Hadjichristou et al. 2021; Sakai et al. 2011; Chandrahasa et al. 2011). For instance, the tooth slice/scaffold model can evaluate the effects of biological pathways modulating cellular differentiation on tubular dentin generation, innervation, and vascularization processes. These models are believed to be helpful to develop our knowledge about dental pulp regeneration and the long-term effects of this strategy (Sakai et al. 2011; Demarco et al. 2010).

6 Conclusion and Future Prospect

Regenerative medicine and tissue engineering are mentioned as promising therapeutic approaches for the treatment of degenerative disorders using developmental biology, biomaterials, and stem cell biology. Tissue engineering benefits from utilizing biomaterials in order to provide bioartificial tissues/organs (Horst et al. 2012; Arjmand et al. 2017, 2020; Goodarzi et al. 2015, 2019). As it was mentioned, tissue engineering consists of different approaches developed to restore tissue defects using bioengineering methods or regenerated products of donor/autologous cells. In this regard, biodegradable scaffolds are more commonly used which have been discussed in detail previously (Horst et al. 2012). Hereupon, regenerative therapy in the field of dental tissue bioengineering using biomaterial scaffolds and stem cells has provided a safe and potent strategy for pulp tissue re-establishment. Positive results have been found regarding cell attachment, proliferation, and angiogenesis processes. Also, this strategy can help for tooth restoration, root canal

Table 3 Human clinical studies about dental tissue engineering

Study	Stem cell type	Scaffold	Findings	Study design
Yamada et al. (2006)	BMMSCs	PRP and thrombin-calcium chloride	A significantly greater reduction in VPD and CAL gain	Single-arm and single-center clinical study
Feng et al. (2010)	PDLSCs	Bone grafting material CALCITITE 4060-2	The application of PDLSCs significantly improved clinical parameters of periodontal regeneration	Single-arm and single-center clinical study
Dhote et al. (2015)	huMSCs	β-TCP and PDGF-BB	A significantly greater reduction in VPD and CAL gain	Single-center RCTs
Baba et al. (2016)	BMMSCs	PRP and a composed of PLA resin fibers	All three clinical parameters improved significantly	Single-arm and single-center clinical study
Chen et al. (2018)	PDLSCs	Bio-Oss	A significant increase in the alveolar bone height	Single-center RCTs
Ferrarotti et al. (2018)	DPSCs	Collagen sponge	The application of DPSCs significantly improved clinical parameters of periodontal regeneration 1 year after treatment	Single-center RCTs
Aimetti et al. (2018)	DPSCs	Collagen sponge	The application of DPSCs significantly improved clinical and radiographic parameters of periodontal regeneration	Single-arm and single-center clinical study
Iwata et al. (2018)	PDLSCs	β-TCP	The application of PDLSCs significantly improved clinical and radiographic parameters of periodontal regeneration	Single-arm and single-center clinical study
Brizuela et al. (2020)	huMSCs	PPP	Mineralized tissue formed, defined as tooth remaining in the mouth, no percussion pain, and an apical bone lesion of equal size in the 3 dimensions of space, a decrease in some of them, or no more than a 0.1 mm increase of one of them	Single-center RCTs
Tanikawa et al. (2020)	DDPSCs	Hydroxyapatite-collagen sponge	Mineralized tissue formed Progressive alveolar bone union has occurred in all patients	Single-center RCTs
Sánchez et al. (2020)	PDLMSCs	XBS (bio-Oss collagen)	A significantly greater reduction in VPD and CAL gain	Quasi-randomized controlled pilot phase II clinical trial
Abdal-Wahab et al. (2020)	GF/GMSCs	β-TCP	A significantly greater reduction in VPD and CAL gain and higher concentration of PDGF-BB	Single-center RCTs

BMMSCs Bone marrow mesenchymal stem cells, *CAL* clinical attachment level, *DDPSCs* deciduous dental pulp stem cells, *DPSCs* dental pulp stem cells, *GF* gingival fibroblasts, *GMSCs* gingival mesenchymal stem/progenitor cells, *huMSCs* human umbilical cord mesenchymal stem cells, *PDGF-BB* platelet-derived growth factor-BB, *PDLMSCs* periodontal ligament mesenchymal stem cells, *PDLSC* periodontal ligament stem cell, *PLA* poly-L-lactic acid, *PPP* platelet-poor plasma, *PRP* platelet-rich plasma, *RCTs* randomized controlled trials, *VPD* vertical pocket depth, *XBS* xenogeneic bone substitute, *β-TCP* β-tricalcium phosphate

treatment, maintenance of tooth homeostasis, immunity function, blood supply, as well as the formation of dentin-pulp complex. It also can raise the natural dentition lifelong and can eventually improve the oral health quality (Gong et al. 2016; Galler et al. 2012). These developments require new protocols regarding the utilizing of stem cells, growth factors, and scaffolds that can help regenerate the required tooth and its related supporting tissues (Murray 2012). Indeed, despite the existed benefits of dental tissue engineering, there are also some limitations that should be resolved. For instance, a system of laws is required to answer ethical issues. Human studies should be taken more seriously that need larger periods of evaluation to transfer in clinical uses (Cristaldi et al. 2018). Taken together, there are many in vitro and in vivo research that focus on the development of dental tissue engineering. But more knowledge is still needed to answer the existing questions about odontogenesis and resolve remained obstacles that may help achieve novel therapeutic strategies for dental disorders (Lymperi et al. 2013). Existing technical problems are needed to be solved, and more theoretical mechanisms should be known in order to bring this technology into the clinical setting instead of conventional methods for dental reconstruction. Considerable developments in the knowledge of stem cell technology can accelerate this process (Zhai et al. 2019). Herein, it has been mentioned that genomics, proteomics, and biometrics can help tissue engineering solve biological challenges (Sachdeva et al. 2021). In this review, we defined tissue engineering through its different components. Then, strategies of this approach in the dental field besides introducing some experimental models were discussed.

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Therapeutic Perspectives for the Clinical Application of Umbilical Cord Hematopoietic and Mesenchymal Stem Cells: Overcoming Complications Arising After Allogeneic Hematopoietic Stem Cell Transplantation

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Abstract

This review focuses on the therapeutic features of umbilical cord blood (UCB) cells as a source for allogeneic hematopoietic stem cell transplantation (aHSCT) in adult and child populations to treat malignant and nonmalignant hematologic diseases, genetic disorders, or pathologies of the immune system, when standard treatment (e.g., chemotherapy) is not effective or clinically contraindicated. In this article, we summarize the immunological properties and the advantages and disadvantages of using UCB stem cells and discuss a variety of treatment outcomes using different sources of stem cells from different donors both in adults and pediatric population. We also highlight the critical properties (total nucleated cell dose depending on HLA compatibility) of UCB cells that reach better survival rates, reveal the advantages of double versus single cord blood unit transplantation, and present recommendations from the most recent studies. Moreover, we summarize the mechanism of action and potential benefit of mesenchymal umbilical cord cells and indicate the most common posttransplantation complications.

Keywords

Hematologic diseases · Hematopoietic stem cell · Mesenchymal stem cell · Stem cell transplantation · Umbilical cord

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Abbreviations

ABMI	Autologous bone marrow cell infusion
aGvHD	Acute graft-versus-host disease
aHSCT	Allogeneic hematopoietic stem cell transplantation
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ATG	Anti-thymoglobulin
BM	Bone marrow
BMMSC	Bone marrow mesenchymal stem cells
BMT	Bone marrow transplantation
CAR	Chimeric antigen receptor
cGvHD	Chronic GvHD
CMV	Cytomegalovirus
DCs	Dendritic cells
DFS	Disease-free survival
DL-1	Delta-like ligand 1
DLI	Donor lymphocytes infusion
dUCBT	Double UCBT
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft-versus-host disease
GVL	Graft versus leukemia
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cells
IL-3	Interleukin-3
IPSC	Induced progenitor cells
LC	Liver cirrhosis
LFS	Leukemia-free survival
MSC	Mesenchymal stem cells
MSD	Matched sibling donor
Mtx	Methotrexate
MUD	Matched unrelated donor
NF-kB	Nuclear factor kappa B
PB	Peripheral blood
PBSC	Peripheral blood stem cells
PGE2	Prostaglandin E2
SC	Stem cells
SCID	Severe combined immunodeficient mice
SR-1	Stem Regenin-1
sUCBT	Single UCB
TNC	Total nucleated cells
TRM	Transplant related mortality
UC	Umbilical cord
UCB	Umbilical cord blood
UCBT	Umbilical cord blood transplantation
UM171	HSC agonist pyrimido-indole derivative
VST	Virus-specific T cells

1 Introduction

Allogeneic hematopoietic stem cell transplantation (aHSCT) is a procedure for treating malignant and nonmalignant hematologic diseases, genetic disorders, or pathologies of the immune system when standard treatment (e.g., chemotherapy) is not effective or clinically contraindicated. The usual sources of stem cells for aHSCT are bone marrow (BM) or peripheral blood (PB) (Anasetti et al. 2012). Umbilical cord blood (UCB) cells remain an alternative source, but the advantages and disadvantages of these cells are still highly investigated and discussed. A low risk of graft-versus-host disease (GVHD) and the opportunity to use unrelated transplants suggest that UCB is a desirable candidate for transplantation (He et al. 2005).

The first successful UCB transplantation was performed in 1988 in a child with Fanconi anemia, and since then, it is estimated that over 40,000 UCB transplants have been performed both for adults and children (Gluckman et al. 1989; Mayani et al. 2020). The first cord blood bank was established in 1993, and since that time, five million cord blood units have been banked all over the world (Gupta and Wagner 2020; Dessels et al. 2018). Approximately 800,000 of these are stored in public banks, while others are kept in private banks (Dessels et al. 2018).

This review article mainly focuses on the importance of UCB stem cells as an alternative source for aHSCT and additional therapeutic properties that may facilitate the rate of successful outcomes of aHSCT.

2 Umbilical Cord Stem Cells

The umbilical cord is a narrow tube that connects the developing embryo to the placenta. The outer membrane of the umbilical cord is rich in stem cells (SC) (Anasetti et al. 2012). Mesenchymal stem cells (MSCs) isolated from the arteries, vein, cord lining, and Wharton's jelly have been shown to exhibit phenotypic plasticity, adherence, multipotency, and the capacity to differentiate into many cell types such as osteoblasts, adipocytes, chondrocytes, hepatocytes, and neural and cardiac cells (Molecule boosts numbers of stem cells in umbilical cord blood 2014). While Wharton's jelly has a lower density of MSCs, the large amount of this tissue allows a high number of proliferative cells to be isolated from it. Wharton's jelly-derived MSCs have a high proliferation potency and do not produce teratogenic or carcinogenic effects after subsequent transplantation (Malgieri et al. 2010). Human umbilical cord perivascular cells are almost identical to Wharton's jelly-derived MSCs (Arno et al. 2011).

The cell density of UCB hematopoietic precursors is similar to that of the bone marrow. The main differences are the higher amounts of erythroid and immature granulocyte-monocyte precursors found in the umbilical cord. The UCB contains a mixture of primitive cell precursors that form abundant colonies of rapidly proliferating hematopoietic cells and cells that cannot form colonies in semisolid cultures but can colonize after a few weeks in Dexter-type long-term cultures (Mayani and Lansdorp 1998).

3 Features of Umbilical Cord Blood Hematopoietic Stem Cells

UCB is a rich source of hematopoietic stem cells (HSC) and progenitor cells. UCB cells are distinguished by the higher levels of CD34+ antigen and longer telomeres than found in adult HSC (Mayani et al. 2020; Mayani 2010). In addition, they divide more rapidly as they exit the G0/G1 phase of the cell cycle more quickly (Mayani et al. 2020; Mayani 2010). Higher self-renewal capacity is determined by the overexpression of certain transcription factors (e.g., NF-kB) (Mayani et al. 2020; Mayani 2010). Moreover, UCB cells have the ability to produce cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3) (Mayani et al. 2020; Mayani 2010). Regarding these features, UCB cells can be expanded in vitro in experimental models that in the future may become available in everyday practice.

In contrast to the bone marrow and adult peripheral blood, umbilical cord blood has several potential benefits: the broad availability of cells and less stringent requirements for donor and recipient HLA compatibility reduce the waiting time to receive the transplant. Additionally, the immaturity of UCB cells is related to lower immunogenicity. UCB-derived dendritic cells have lower antigen-presenting activity, which results in lower chances of graft-versus-host disease (GVHD) (Beksac 2016).

The benefits, challenges, limitations, and problems associated with the use of UCB HSCs are summarized in Fig. 1.

4 Therapeutic Peculiarities of UCB Hematopoietic Stem Cells

4.1 Umbilical Cord Blood Stem Cells (UCBSC) Versus Bone Marrow or Peripheral Blood Stem Cells

UCB can be collected noninvasively and ensures prompt availability of stem cells. In countries where public cord blood banks are available, the number of potential donors is higher than bone marrow donors (Malgieri et al. 2010). Procedures for obtaining UCB SC pose no ethical or technical restrictions and are painless (Malgieri et al. 2010). Neither it interferes with the delayed umbilical cord clamping which is defined as 30–60 s after birth (Delayed umbilical cord clamping after birth 2020) and is recommended for vigorous term and preterm infants. Delayed

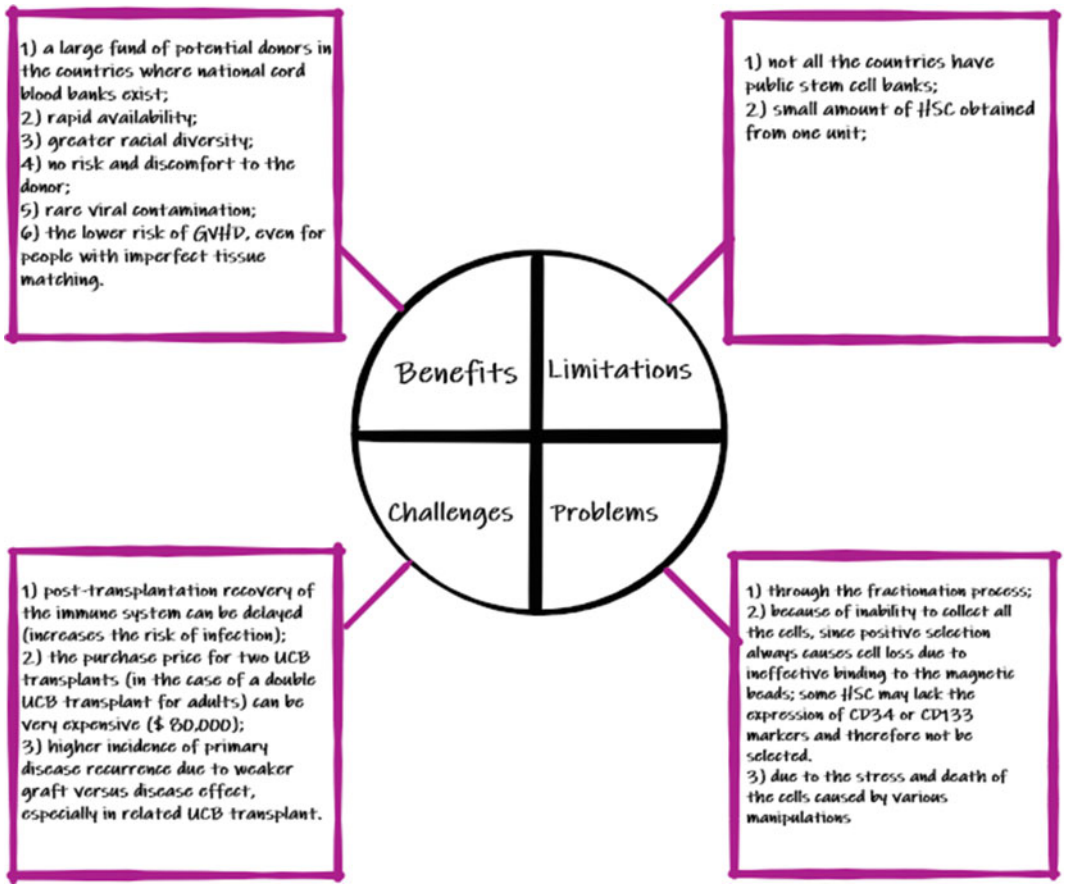

Fig. 1 Benefits, challenges, limitations, and problems related to the use of UCB HSCs (Beksac 2016; Expansion of human cord blood hematopoietic stem cells for transplantation 2010)

cord clamping has many advantages in the early postnatal period as well as in the later childhood (e.g., lower risk of anemia, favorable developmental course). However, there are no evidences that clamping later than 3 min could be more beneficial than 30–60 s (Preterm labour and birth 2020). Thus, after labor, it is possible to reconcile both: delayed umbilical cord clamping and collection of stem cell. The cord blood can be stored in advance. In contrast, bone marrow cells have to be collected directly before transplantation. As a result, there is a potential risk of last-minute consent refusal (Malgieri et al. 2010).

Owing to all the mentioned properties, the waiting time for the transplantation procedure can be shortened by up to 2 weeks compared with 3–4 months for BMT or PBSCT (Gupta and Wagner 2020).

Besides easier and shorter procedures for obtaining stem cells, the most significant advantage of UCB is the possibility of avoiding the most serious complications after aHSCT. Firstly, there is a reduced likelihood of transmitted infections, especially human cytomegalovirus (CMV) (Algeri et al. 2020). CMV reactivation after aHSCT is one of the main clinical challenges to overcome as it often may become lethal during active immunosuppressive therapy, a treatment which is necessary to avoid or to treat acute graft-versus-host disease (aGvHD). Secondly, aGvHD is the main reason for morbidity and mortality after aHSCT. The success of transplantation usually depends on the management of aGvHD. There are many studies analyzing the probability of aGvHD using different sources of stem cells. Most of them state that UCB

transplants have a lower likelihood of aGvHD (Oran and Shpall 2012; Locatelli et al. 2014) since UCB cells are more naive and less alloreactive than adult-type HSC(Algeri et al. 2020). However, the probability of this disease depends on the HLA matching, as one or two incompatibilities increase the rate of aGvHD and chronic GvHD (cGvHD) (Eapen et al. 2017; Gabelli et al. 2020; Rubinstein et al. 1998). Double UCBT (dUCBT) is also associated with greater risk of GvHD (Wang et al. 2019).

The main negative aspects of UCB limiting its use are the delay in engraftment and immunologic recovery. Later after administration, immune T cell reconstitution poses a higher risk of opportunistic infections during the first 3 months after transplantation (Szabolcs and Niedzwiecki 2007; Jacobson et al. 2012). It has been noted that the use of anti-thymoglobulin (ATG) to prevent aGvHD has a negative effect on early CD4+ T lymphocyte recovery (Dahlberg and Milano 2017). In order to keep the balance between controlled aGvHD and immune reconstitution, it is now advised to keep sufficiently high ATG exposure in peritransplantation period of UCB and low exposure during posttransplantation period (de Koning et al. 2017).

To achieve the best outcome of UCB transplantation, the UCBT unit should contain 2,5–3 $\times$ 10^7 total nucleated cells (TNC)/recipient's kg before thawing (Gluckman et al. 2004; Rocha et al. 2009; Hough et al. 2016). However, there are several strategies being developed to overcome this obstacle: UCB expansion ex vivo, improving homing in vivo, selection of optimal UCBT unit, and enhancement of immune recovery (Mayani et al. 2020; Ballen et al. 2013; Dahlberg and Milano 2017).

4.2 Umbilical Cord Blood Transplantation (UCBT) Versus Haploidentical Transplantation (HIT)

Usually, UCB cells are selected as a source for aHSCT when there is no matched related (sibling) donor (MSD) or matched unrelated donor (MUD) available. Only one in four patients may have a MSD for the aHSCT (Algeri et al. 2020; Copelan 2006; Rocha and Locatelli 2008). The possibility of finding a suitable MUD ranges between 16% and 75% depending on the recipient's ethnicity and race(Gragert et al. 2014) and usually is extremely low for racial minorities or mixed ethnicity people (Barker et al. 2010). Thus, UCB transplantation could be the only option as cord stem cells require less HLA compatibility.

Another alternative to UCBT during the last 20 years arises from haploidentical donor selection (Algeri et al. 2020; Passweg et al. 2016). The priority between these two procedures is being intensively discussed. The recent meta-analysis has revealed that UCB and haploidentical transplantation (HIT) are equally effective options for transplantation both in adults and in the pediatric population. But emphasis was made on UCB in children, as UCB ensures lower probability of aGvHD (Locatelli et al. 2014). However, considering that mostly pediatric population was transplanted due to acute myeloid leukemia (AML), UCBT was superior to HIT (Locatelli et al. 2014). It is speculated that UCB transplants could be a second choice after transplantation of material from sibling donors in children.

4.3 UCB Transplantation for Adolescents and Adults

To overcome the small number of cells, and to perform transplantation for adolescents and adults successfully, there has emerged an idea of double unit umbilical cord blood transplantation (dUCBT). However, the latest data has revealed that dUCBT had no advantage to single UCBT (sUCBT) when an adequate amount of TNC (>2,5 $\times$ 10^7/kg) is transfused (Wagner et al. 2014; Michel et al. 2016; Brunstein et al. 2010). dUCBT recipients usually experience higher rates of GvHD (Rocha and Locatelli 2008), but overall survival (OS) and disease-free survival (DFS), as well as time to engraftment, were the same between sUCBT and dUCBT groups (Brunstein et al. 2010; Baron et al. 2017). Interestingly, in almost all the cases of dUCBT, only one unit

dominated owing to competition between two units and the rejection of one of them (Ramirez et al. 2012). However, regarding transplantation with positive MRD before the procedure, dUCBT may have a positive effect – lower relapse rate in comparison with sUCBT (Balligand et al. 2019). This phenomenon may be explained by higher alloreactivity and higher graft versus leukemia (GVL) effect of dUCB (Milano et al. 2016). Interestingly, it is speculated that dUCBT could be a better option than BMT for patients with positive pretransplant MRD (Milano et al. 2016). However, the limitation of this alloreactivity is higher rates of GvHD. It is worth mentioning that the GVL effect depends on the conditioning regimens (e.g., ATG), as ATG may suppress it (Balligand et al. 2019).

When the UCBT unit does not contain enough TNC for adult transplantation, another option can be chosen – the transfusion of UCB with mobilized PB derived CD34+ from a haploidentical donor (Mayani et al. 2020). In this scenario, usually early haploidentical engraftment occurs, and then it is substituted by the durable UCB engraftment (Mayani et al. 2020). This procedure has already shown early hematologic recovery, low incidence of GvHD, and durable remission (Eapen et al. 2007, 2011).

Currently, for adolescent and adult populations, UCB should be considered when there is no MSD or MUD and the transplant is needed urgently (Mayani et al. 2020).

5 Options to Increase UCBC Dosing

Cell dose (e.g., $> 3 \times 10^7$ TNC/kg) has an impact on better engraftment for higher than 5/6 HLA matched grafts; however, it has no effect on less than 4/6 HLA grafts (Eapen et al. 2007). The matching criteria for UCBT were based on low/intermediate resolution of HLA A and HLA B and high resolution of HLA DRB1. Since 2011, the importance of HLA C has been described (Beksac 2016; Eapen et al. 2011). The lower matching of the graft and the higher dose of TNC are necessary. Now it is considered that UCBT should be selected with a higher than 4/6 HLA match and dose of $3–5 \times 10^7$ TNC/kg depending on the results of HLA compatibility. Moreover, the incompatibility of HLA C increases the rates of transplant related mortality (TRM)(Eapen et al. 2011); thus, ideally UCB should match 7/8 or 8/8.

In order to multiply stem cells, several clinical procedures are currently in clinical use. Studies by Horwitz et al. have shown that ex vivo application of nicotinamide can extremely improve outcomes of UCBT (Horwitz et al. 2014). The recent finding by Cohen et al. has revealed that UM171 can be successfully used for stem cell enhancement (Cohen et al. 2020). There are more multiplication boosters that are still under further investigation, such as copper chelator, mesenchymal stromal cells, delta-like ligand 1 (DL-1), Stem Regenin-1 (SR-1), notch-mediated expansion, aryl hydrocarbon inhibition, stem cell renewal agonist, CD3/CD28 co-stimulation, and automated continuous perfusion (Horwitz et al. 2014; Cohen et al. 2020; Wagner et al. 2016; Delaney et al. 2010; de Lima et al. 2012, 2008; Stiff et al. 2018; Jaroscak et al. 2003; Hexner et al. 2016; Anand et al. 2017; Mehta et al. 2017).

Another strategy is to enhance homing of the cells in vivo by manipulating the SDF-1-CXCR4 axis by inhibiting dipeptidyl peptidase 4 (DPP-4), incubating with prostaglandin E2 (PGE2), or enforcing fucosylation of UCB cells (Farag et al. 2013; Cutler et al. 2013; Popat et al. 2015). These agents ensure that more HSC could reach the BM niche.

HSC fucosylation in vitro is one of the perspective methods used to enhance homing of the cells. During inflammation, P-selectin and E-selectin act on leukocyte migration and adhesion to the desired target. After HSCT, P-selectin and E-selectin ensure that intravenously administered HSC migrates to the bone marrow. In many tissues, P-selectin and E-selectin are expressed selectively on endothelial cells, but they are continuously expressed on bone marrow endothelial cells. During transplantation, HSC is injected into a vein and affixes to the bone marrow for multiplying (homing). Homing depends

on the interaction of P- and E-selectin on endothelial cells with their ligands on HSCs. These selectins are membrane-bound C-type lectins, and their relevant ligands should be α1,3-fucosylated to form terminal glycan determinants. Thus, fucosylation of HSC may improve liaison between bone marrow endothelial cells and HSCs(Popat et al. 2015).

In addition, intra-bone transplantation of UCB has demonstrated lower rates of aGvHD, earlier platelet recovery, and lower relapse rate (Brunstein et al. 2009; Frassoni et al. 2010); thus, it is also a promising technique to improve outcomes of UCBT.

5.1 UCB Transplantation (UCBT) for Children

Keeping in mind the small amount of stem cells available in UCB, this transplantation procedure currently focuses predominantly on children as they need fewer stem cells owing to their lower body weight. Acute leukemia (AL) is the most frequent malignancy in the pediatric population; thus, refractory or relapsed AL is the most common indication for aHSCT. Several studies attempted to compare the outcomes of UCB vs. BM/PBSC transplantation for children. The results are similar and state that overall survival doesn't differ between UCBT and BMT, but UCBT has a higher risk of delayed engraftment and immunologic reconstitution, with lower risk of GvHD (Stiff et al. 2018; Rocha et al. 2001; Barker et al. 2001; Dalle et al. 2004). Interestingly, another analysis by Eapen et al. found that matched UCBT has a better leukemia-free survival (LFS) than BMT in children transplanted due to leukemia (Eapen et al. 2007). If the UCBT has one or two HLA mismatches, the LFS is the same in UCBT and BMT groups. Moreover, UCBT could be a better alternative for adult and children's leukemia with positive pretransplant MRD than MUD or mismatched unrelated donor (MMUD) (Milano et al. 2016). UCBT is associated with better CD4+ reconstitution which ensures lower rates of relapse in children with AML (Admiraal et al. 2016a). In vitro studies justify this finding by revealing better GVL effect of UCBT (Horwitz et al. 2014). The recent study by Keating et al. suggests that UCBT could be especially advantageous in treating childhood AML (Keating et al. 2019).

Although unrelated UCB transplants are an important alternative for MUD, it is rarely discussed about the use of UCB and relevance in related cord blood transplantation. As summarized above, the gold standard is aHSCT from siblings due to the genetic and immunological similarities of cells and lower possibility of GvHD. Unrelated UCB cells can also overcome immunologic disparity as they are immunologically naive. UCB transplantation is less reactive because it consists of antigen-inexperienced lymphocytes that hardly react to allo-stimulation. Moreover, UCB transplant has more Tregs that inhibit immunological reactions. These factors may be important for lower possibility of GvHD (Beksac 2016; Kim and Broxmeyer 2011; Kanda et al. 2012). But when we face a related UCB transplantation, both abovementioned features are combined. The comparative analysis of related UCB and MSD transplant showed that the 3-year survival rates were the same between two groups; however, neutrophil engraftment occurred later in the UCB group, but the incidence of aGvHD III–IV degree was higher in sibling group (Stavropoulos-Giokas et al. 2012; Rocha et al. 2000; Cohen and Nagler 2004). The summary of Eurocord has revealed similar results. Children treated with related UCB transplantation due to their hematologic malignancies (mostly acute leukemia) had an engraftment rate of 90% at day 60 and a low percent of aGvHD and cGvHD (12% and 10%, respectively) 2 years after aHSCT (Herr et al. 2010). However, the relapse incidence was high: 47% by 5 years after aHSCT; but it was related to the administration of GvHD prophylactic regimens (e.g., Mtx). Nevertheless, this fact suggests that related UCB may not have such strong GVL effect as unrelated UCB transplantation.

Regarding pediatric aHSCT, it is considered that even unrelated UCB is an important alternative to MUD depending on the urgency of transplantation, HLA matching, and collected cell dose (Delaney and Ballen 2010; Gluckman and

Rocha 2009). Current practice suggests that matched 6/6 or > 6/8 HLA UCB units with adequate TNC count should be considered in the pediatric population, especially for those pathologies where graft versus malignancy effect is not necessary or for high risk with positive pretransplant MRD leukemia (Mayani et al. 2020; Gabelli et al. 2020).

5.2 Alternative Effects of UCBC

UCB is abundant in Tregs cells (CD4 + CD25 + Foxp3+), proliferating in the presence of IL-2. They are the main immunomodulatory cells that ensure maternal-fetal immune tolerance (Leber et al. 2010; Tsuda et al. 2019). Tregs can be successfully isolated and expanded up to 100-fold adding IL-2 (McKenna et al. 2017). These cells could be extremely important in the prevention and treatment of GvHD. As described above, aGvHD is the main cause of posttransplant morbidity and mortality as it is an inflammatory condition that damages tissues and organs. Tregs could inhibit GvHD while preserving the ability of GVL effect (Edinger et al. 2003).

Virus-specific T cells (VST) derived from UCB are an emerging treatment option for viral reactivation after transplantation. Usually patients are faced with cytomegalovirus (CMV), Epstein-Barr virus (EBV), and adenovirus (ADV) viremia, which prompts active aggressive antiviral treatment. Cases where this conventional treatment fails or causes too many toxic reactions may be treated with antivirus-specific T cells (VST) (Gupta and Wagner 2020). Recently, a simplified novel technique has been introduced – a single culture of polyclone VST (Dave et al. 2017).

However, UCBT lacks the possibility after transplantation to use donor lymphocytes infusion (DLI) in order to induce GVL or to prevent graft rejection in mixed-chimerism nonmalignant disorders. But there are some reports of in vitro expanded lymphocytes obtained from small amounts of UCB before transplantation (Berglund et al. 2017).

A very promising field is UCB derived NK chimeric antigen receptor (CAR) therapy. UCB NK cells express checkpoint inhibitory receptors that play the main role in antitumor effect and prevent disease relapse after HSCT. NK cells are characterized by CD16+/CD56+ and are responsible for nonself-antigens without antigen specificity. They are the first cells that reconstitute after HSCT, exerting a positive effect on engraftment, but have less cytotoxic potential (Merindol et al. 2011; Beziat et al. 2009; Verneris and Miller 2009; Wang et al. 2007). The main problem to taking advantage of these effects is the low number of NK cells in UCB; thus, enhancement techniques provide a perspective for anticancer immunotherapy (McKenna et al. 2017; Zhang et al. 2013; Heinze et al. 2019; Nham et al. 2018). The recent conclusions of a phase I/II trial, conducted by Liu et al., have revealed that UCB derived NK-CAR cells for patients with CD19 + malignancies do not cause any cytokine release syndrome, neurotoxicity, or GvHD (Use of CAR-transduced natural killer cells in CD19-positive lymphoid tumors).

6 Therapeutic Properties of Umbilical Cord Mesenchymal Cells

UCB has been valuable mainly for hematopoietic and progenitor stem cells, but the posttransplant outcome may depend on other types of stem cells, e.g., mesenchymal cells, which are also abundant in fetal tissues (cord blood and cord tissue) (Umbilical cord-derived mesenchymal stem cells for hematopoietic stem cell transplantation). We will hence continue with further discussion of the properties and therapeutic use of these cells.

MCSs are multipotent stem cells with self-renewal and high proliferative capacity (Umbilical cord-derived mesenchymal stem cells for hematopoietic stem cell transplantation; Marino et al. 2019). They are distinguished by specific surface markers (negative for hematopoietic or endothelial cells and positive for mesenchymal and cell adhesion molecules) and have mainly a

trilineage differentiation potency: to osteoblasts, chondroblasts, or adipocytes (Dominici et al. 2006).

MSC may be classified as adult-type (e.g., BM, adipose tissue) or perinatal/fetal-type (fetus or extraembryonic – placenta, umbilical cord blood, Wharton's jelly, amniotic membrane) (Marino et al. 2019; Zuk et al. 2002; Abdulrazzak et al. 2010). However, BM remains the main source of MSC. The first experience using MSC to treat aGvHD was reported in 2004 in Sweden (Kelly and Rasko 2021; Le Blanc et al. 2004). Since then, numerous clinical trials have been conducted to determine the benefits of MSCs (Kelly and Rasko 2021). The majority of them includes BM as a MSC source, and some of them analyze cord blood or adipose tissue (Kelly and Rasko 2021).

There are several important differences in MSCs depending on the stem cell source. MSC from fetal tissues are known to have greater expansion and proliferation capacity (Selmani et al. 2008; Galleu et al. 2017; Spees et al. 2016), can be obtained and captured more efficiently (Secco et al. 2008), and express lower HLA class I, and there is absence of HLA DR (Zhang et al. 2009; Deuse et al. 2011) than in adult-type MCS. Thus, fetal-derived MSCs are less immunogenic and may escape recognition of the alloreactive immune system (Umbilical cord-derived mesenchymal stem cells for hematopoietic stem cell transplantation) and have greater immunosuppressive effect (Wu et al. 2011). In addition, fetal-type (e.g., umbilical cord, especially Wharton's jelly) MSCs can be obtained more easily than MSCs from BM. After the delivery, UCMSCs are taken without any interventional procedure or risk for mother and baby (Secco et al. 2008; Lu et al. 2006). Moreover, MSCs from BM are prone to aging and losing their primary features of proliferation and differentiation (Stenderup et al. 2003).

MCSs are significant in improving outcomes after HSCT in several ways. Firstly, MSCs provide a microenvironment for HSC. They ensure expansion of HSC by expressing essential cytokines, adhesion molecules, and extracellular matrix proteins (Lazennec and Jorgensen 2008; Deans and Moseley 2000; Ito et al. 2006). In 2000, the first study that analyzed the results of co-transplantation of HSC and MSCs was performed and revealed better engraftment rates than of HSC transplantation alone (Koç et al. 2000). Interestingly, several studies have shown that faster engraftment was achieved using UCMSC rather than BMMSC (Chao et al. 2011).

Another important feature of MSCs is the possible prevention and treatment of GvHD. GvHD is caused by the graft's T cells attacking the recipient's body. Various inflammation cytokines and activated immune cells dominate in the disease pathophysiology and cause damage of the tissues (e.g., skin, liver, gastrointestinal tract) (Umbilical cord-derived mesenchymal stem cells for hematopoietic stem cell transplantation). The first choice of GvHD treatment remains corticosteroids (Kelly and Rasko 2021); however, around 50% of cases are resistant to these regimens. The 2-year overall survival rates of steroid resistant GvHD is only up to 20% (Westin et al. 2011). There are several options for second line treatment; a wide range of them are under investigation, but no agent has been identified as optimal (Kelly and Rasko 2021). MSCs may decrease the clinical manifestation of GvHD or even prevent it due to immunomodulatory and immunosuppressive features. Secreted cytokines (mainly indoleamine 2,3-dioxygenase – IDO) decrease the proliferation of alloreactive T cells (Galipeau and Sensébé 2018; Harrell et al. 2019; Terness et al. 2002). Moreover, Tregs expansion is induced and then the reactions to alloantigens are suppressed (Wagner et al. 2005). During apoptosis, MSCs release apoptotic extracellular vesicles that also have an immunomodulatory effect (Selmani et al. 2008). In addition, apoptosis induces IDO production in recipient's phagocytes (Galleu et al. 2017). Due to MSCs' paracrine effects, they may limit tissue damage that is caused by GvHD (Spees et al. 2016). These cells also may modulate innate and cellular immune pathways (B cells, NK, monocytes,

dendritic cells) (Umbilical cord-derived mesenchymal stem cells for hematopoietic stem cell transplantation; Weiss and Dahlke 2019).

Lastly, there are ongoing clinical trials on MSCs' antiviral properties after HSCT. The common complication after aHSCT is the reactivation of viruses (e.g., EBV, CMV, ADV, herpes virus (HSV)). In the early phase of viral infection, MSCs produce a strong immune response by releasing anti-inflammatory cytokines, attracting leukocytes to the site of injury and regulating the functions of all immune cells that are involved in the antiviral response (DCs, NK cells, macrophages, B lymphocytes, CD4+ helper, CTL cells) (Harrell et al. 2019; Gazdic et al. 2015). After viral elimination, MSCs produce immunoregulatory cytokines that suppress the excessive activation of the immune system and help to avoid a cytokine storm (Volarevic et al. 2017). Trophic factors support the repair of tissues (Sleem and Saleh 2020; Thanunchai et al. 2015). Nonetheless, there are still safety issues that need to be determined. As MSCs express receptors that are used by viruses (HIV, EBV, herpes) for their interaction with target cells, it is thought that MSCs may transmit viruses for patients undergoing HSCT (Cheng et al. 2013; Rollín et al. 2007; Soland et al. 2014). Though the study of Sundin et al. did not find any DNA derived from CMV, EBV, HSV-1 and HSV-2, or varicella from seropositive healthy donors, intracellular antigens can be found after infection of CMV and HSV type 1 in vitro (Sundin et al. 2006). Thus, this reciprocal interaction of MSCs and viruses needs to be investigated further, in order to make a conclusion about the benefits of MSC therapy for viral infections after HSCT.

To date, there is an agreement that MSCs are safe and well tolerated and usually do not cause any serious side effects except for transient fever (Galderisi et al. 2021; Sharma et al. 2014). It is known that MSCs of the donor may graft in the recipient's BM, but the stroma remains host in origin (Umbilical cord-derived mesenchymal stem cells for hematopoietic stem cell transplantation; Chao et al. 2011; Ball et al. 2007; Villaron et al. 2004; Poloni et al. 2006; Awaya et al. 2002). It means that after some time MSCs are eliminated from the recipient's body. Thus, more than one MSC infusion may be needed to treat GvHD; however, the therapy outcome is not related to the amount of infusions but correlates with the earlier time of MSC therapy (Marino et al. 2019).

As MSCs are a relatively new therapy, more prospective studies are needed to define therapeutic indications, cell sources, optimal dose, and frequency.

7 UCB Cells in Regenerative Medicine

Although UCB is mainly used for transplantation to treat blood disorders, the range of treatable diseases is expanding. Scientists apply umbilical cord blood or tissue in regenerative cell therapy or immune modulation. Purified cell populations could also facilitate possible gene therapy when there is a need to select HSC with the transgene inserted into the desired chromosomal location (Expansion of human cord blood hematopoietic stem cells for transplantation 2010).

UC MSC can replace damaged cells due to their ability to regenerate and differentiate. In addition, paracrine factors can inhibit programmed host cell death, modify immunological functions, and promote endogenous SC proliferation and differentiation (a primary mechanism). The reciprocal interaction between UC MSC and host cells plays a crucial role in current therapeutic approaches (Li et al. 2015).

Host Cell Replacement There are two ways to change cells: transdifferentiation and cell fusion. Transdifferentiation refers to the ability of MSC to differentiate into different cell types. Cell fusion refers to the fusion between a MSC and a host cell that allows nuclear reprogramming: to express specific genes for MSC and apoptosis-protected host cells (Li et al. 2015).

Paracrine Factors UC MSC can secrete a variety of growth factors, cytokines, and chemokines (including hepatocyte growth factor, stromal

factor 1 and monocyte chemotactic protein 1, vascular endothelial growth factor, insulin-like growth factor 1, interleukin-8, brain neurotransmitter glial cell-derived neurotrophic factor). These factors can help to prevent apoptosis of adjacent cells, promote angiogenesis, modulate inflammation, and activate internal SC, which helps to create a favorable environment for internal restorative processes (Li et al. 2015).

Cell-Cell Contacts UC MSCs are in a microenvironment where they can interact with host cells through rigid junctions, gap junctions, and desmosomes to affect host cell proliferation, migration, and differentiation. There is a need for a detailed understanding of the possible reciprocal interaction between umbilical cord MSC and host cells. As a result, it may help us with an alternative concept to decide the fate of transplanted cells and help us elucidate cell therapy strategies (Li et al. 2015).

Today, there are over 180 clinical trials registered on *clinicaltrials.gov*, investigating the effects of UCMSC in regenerative medicine. The application of MSC mainly focuses on the treatment of neurologic, autoimmune, cardiovascular, pulmonary, hepatic, and orthopedic diseases. The most prominent effects are seen in childhood neurology. Autologous cord blood infusion for children with cerebral palsy obviously improves motor and mental function (Sun et al. 2017). Effects of allogeneic cord blood and tissue cells on neurologic functions are also explored, and later phases of clinical trials will reveal more information (Sun et al. 2021).

8 Conclusions and Recommendations for Future Research

UCB SC is an attractive source for HSCT due to its rapid availability, noninvasive collection, lower risk of GVHD, and less stringent HLA matching. UCB is an alternative for MUD for children depending on the urgency of transplantation, HLA matching, and collected cell dose. UCBT should be selected with a higher than 4/6 HLA match and dose of 3–5 $\times$ 10^7 TNC/kg depending on the results of HLA compatibility. Regarding the adult population, UCB should be selected when there is no MSD or MUD available and transplant is needed urgently. dUCB is as effective as sUCB in the adult population However, new techniques of HSC expansion and better homing are emerging that may overcome the issue of small amounts of HSC in one unit.

Special emphasis should be placed on cases with positive pretransplant MRD – UCBC – which have been shown to have better GVL effect and lower risk of relapse than MUD or MMUD in both pediatric and adult populations. The main disadvantages of the UCBC are delays in engraftment and immune recovery, thus higher risk of infections. But now it is also known that recovery is associated with conditioning regimens, especially ATG. Thus, special attention should be paid to ATG exposure during the peritransplantation period in order to avoid GvHD and to restore the immune system more quickly. In the near future, UCBC may be adopted as an important tool to alleviate complications after HSCT, such as Tregs, to suppress GVHD or virus-specific T cells to combat viral infections. A highly promising field is UCB derived NK-CAR therapy, as it shows no cytokine reactions.

In addition, umbilical cords contain not only hematopoietic stem cells but also mesenchymal stem cells. These cells may also facilitate the procedure of HSCT by increasing engraftment rates of HSC. They also may prevent and treat GvHD as well as viral reactivation posttransplantation. There are many ongoing clinical trials that are investigating the properties and therapeutic potential of UC hematopoietic and mesenchymal cells. MSC therapeutic properties for regenerative purposes are expanding rapidly, and it is expected that they will soon be approved by the regulatory agencies and brought into clinical practice.

In summary, the ethical acceptability and clinical features of umbilical cord blood and tissue mean that they may offer a wide range of novel

approaches for the prevention and treatment of disease.

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Conflicts of Interest AM consults for GSK Consumer Healthcare, Kolon TissueGene, Laboratoires Expansciences, Novartis, Orion Corporation, and Sanofi. He is President of the Osteoarthritis Research Society International (OARSI) (May 2019–April 2022) and serves on the Scientific Advisory Boards of Kolon TissueGene, ResearchSquare, and the European Society for Clinical and Economic Aspects of Osteoporosis, Osteoarthritis, and Musculoskeletal Diseases (ESCEO).

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Advanced Nanotechnology Approaches as Emerging Tools in Cellular-Based Technologies

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Abstract

Stem cells are valuable tools in regenerative medicine because they can generate a wide variety of cell types and tissues that can be used to treat or replace damaged tissues and organs. However, challenges related to the application of stem cells in the scope of regenerative medicine have urged scientists to utilize nanomedicine as a prerequisite to circumvent some of these hurdles. Nanomedicine plays a crucial role in this process and manipulates surface biology, the fate of stem cells, and biomaterials. Many attempts have been made to modify cellular behavior and improve their regenerative ability using nano-based strategies. Notably, nanotechnology applications in regenerative medicine and cellular therapies are controversial because of ethical and legal considerations. Therefore, this review describes nanotechnology in cell-based applications and focuses on newly proposed nano-based approaches. Cutting-edge strategies to engineer biological tissues and the ethical, legal, and social considerations of nanotechnology in regenerative nanomedicine applications are also discussed.

Keywords

Nanomedicine · Regenerative medicine · Regenerative nanomedicine · Stem cells · Tissue engineering

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
Ab	Antibody
AuNP	Gold nanoparticle
BM	Bone marrow
CD	Cluster of differentiation
CLNP	Cationic lipid nanoparticle

CNT	Carbon nanotube
CPP	Cell-penetrating peptide
DDS	Drug delivery system
Dex	Dexamethasone
ECM	Extracellular matrix
EMA	European Medicines Agency
ESC	Embryonic stem cell
EU	European Union
EV	Extracellular vesicle
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
GF	Growth factor
GO	Graphene oxide
hASC	Human adipose stem cell
hESC	Human embryonic stem cell
hMSC	Human mesenchymal stromal cell
HA	Hyaluronic acid
HIV-1	Human immunodeficiency virus-1
Tat	trans-activating regulatory protein
iPSC	Induced pluripotent stem cell
IVF	In vitro fertilization
kPa	Kilopascal
MNP	Magnetic nanoparticle
MPI	Magnetic particle imaging
MRI	Magnetic resonance imaging
MACS	Magnetic-activated cell sorting
MSC	Mesenchymal stromal cell
NGF	Nerve growth factor
NKT	Natural killer T cell
NP	Nanoparticle
PA	Photoacoustic
PBAE	Poly(β-amino ester)
PCL	(poly-ε-caprolactone)
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PET	Positron emission tomography
PGA	Polyglycolic acid
PLA	Polylactic acid
PNIPAM	Poly-N-Isopropylacrylamide
PVA	Polyvinyl alcohol
PVP	Polyvinylpyrrolidone
QD	Quantum dot
RM	Regenerative medicine
siRNA	Small interfering RNA
SLNP	Solid lipid nanoparticle
SPION	Superparamagnetic iron oxide nanoparticle
TDN	Tetrahedral DNA nanostructure
VEGF	Vascular endothelial growth factor

1 Introduction

Novel technologies comprised of nanotechnology, tissue engineering, stem cell biology, cell transplantation, small molecules, biotechnology, cell engineering, and genetic manipulations provide a promising breakthrough for regenerative medicine applications (Phinney and Pittenger 2017). Although stem cell-based technologies have been created as a therapeutic revolution to replace pharmaceutical treatments, they still do not provide a practical approach. Stem cells are considered for regenerative medicine because they can generate a wide variety of cell types and tissues that can be used to treat, repair, or replace damaged tissues and organs (Fahy 2002). Stem cells can self-renew for an extended time and differentiate into specialized cellular lineages and tissues on stimulation when given the appropriate microenvironmental cues. There are significant and critical aspects to be considered for stem cell-based therapies, including interaction with the microenvironment, biodistribution, administration route, engraftment and retention, functionality, and survival. Stem cells may not be well tolerated while administered systemically and could become trapped in the vasculature of some organs such as the lungs; therefore, stem cells labeled with nanoparticles and cell tracking could elucidate biodistribution within tissues, the homing process, migration, and engraftment of transplanted cells (Markides et al. 2019).

Nanomedicine may solve many crucial questions on stem cell technology at the nanoscale level (Boulaiz et al. 2011). The intrinsic properties of nanomaterials can be beneficial in cell adhesion, protein adsorption, cell-cell interactions, stem cell growth, cell proliferation, stem cell self-renewal, cell-directed differentiation, and cell migration (Engel et al. 2008). Accordingly, they affect stem cell biodistribution, engraftment and retention, functionality, and survival. In terms of tissue engineering, regenerative nanomedicine impacts tissue regeneration by

providing a supportive role for scaffolds embedded within the extracellular matrix (ECM). Nanoparticles have been shown to regulate the release of some growth factors (GF) and play a significant role in the regeneration of some tissues and organs, notably different from their effects as scaffolds or supporting materials in tissue engineering. In this review, we describe the application of nanotechnology in cell-based technologies, including cell tracking, labeling, genetic manipulation, environmental engineering, differentiation, and cell stimulation, which focuses on newly proposed nano-based approaches to attain the best consequences in regenerative medicine. We also describe cutting-edge strategies to engineer biological tissues and ethical, legal, and social considerations of nanotechnology in regenerative nanomedicine.

2 Nanotechnology in Stem Cell Technologies and Regenerative Medicine

Nanoparticles are 1–200 nm entities with an extraordinary intrinsic property of a high surface-to-volume ratio that makes them potent candidates for regenerative medicine and cellular therapies (Khang et al. 2010). Applications of NPs are in their infancy, making some controversial issues; however, they do not limit their usage. Polylactic acid (PLA), polyglycolic acid (PGA), PCL (poly-ε-caprolactone), and their copolymers are biodegradable polymers that have been widely used due to their ability to form nano-carriers that have tunable scaffolding and release properties. They may encompass a wide variety of nanoparticles and scaffolds for delivery purposes that exhibit "stimuli-responsive" characteristics by undergoing conformational changes in different situations such as swelling or shrinkage in response to variations in temperature, pH, glucose, and magnetic field (Ma 2008; Nair and Laurencin 2007). Nonbiodegradable materials include metals such as gold nanoparticles (AuNP), magnetic nanoparticles (MNP), quantum dots (QD), carbon nanotubes (CNT) in addition to ceramics (nanohydroxyapatite), and nanocomposites that can be used for cell isolation, cell sorting, cell tracking, active targeting, cell differentiation, pharmaceutical/biomolecule delivery, molecular imaging, and in vivo imaging in regenerative medicine. Because of their decreased cost and effectiveness, cell isolation and cell sorting are the highlights of cellular-based therapies. Magnetic-activated cell sorting (MACS) is one strategy based on magnetic separation of the desired cells from a cellular pool based on monoclonal antibody-conjugated MNPs (Ab-MNP). In this method, the cell suspension is incubated with magnetic beads and passed through a column within a magnetic field, enabling the separation of various cell populations according to their surface antigens (CD markers). There are several applications for nano entities, which we intend to discuss in detail (Fig. 1).

2.1 Nanomaterials for Environmental Engineering of Stem Cells

Environmental engineering regulates cellular responses temporarily and permanently. Bioactive molecules are responsible for several tasks within the cells. GFs are a type of bioactive molecule that is crucial for stem cell growth and function. These bioactive molecules are exogenously applied to manipulate migration, proliferation, differentiation, and survival (Park et al. 2017). GFs bind to transmembrane receptors in a specific manner, and phosphorylation of the receptors' cytosolic domains triggers a transduction cascade, regulating a wide range of tasks within the cells. There are significant hurdles in terms of biomolecule delivery to the cells in vitro and in vivo. Stability, administration frequency, dose management, effectiveness, and costs are among the most crucial factors that prohibit the direct use of biomolecules (Mabrouk et al. 2019). Nanotechnology-based drug delivery systems (DDS) have been developed to overcome such hurdles (Mabrouk et al. 2019; Soleymani-Goloujeh et al. 2017). Colloidal micro- and nanospheres provided by using bioavailable and

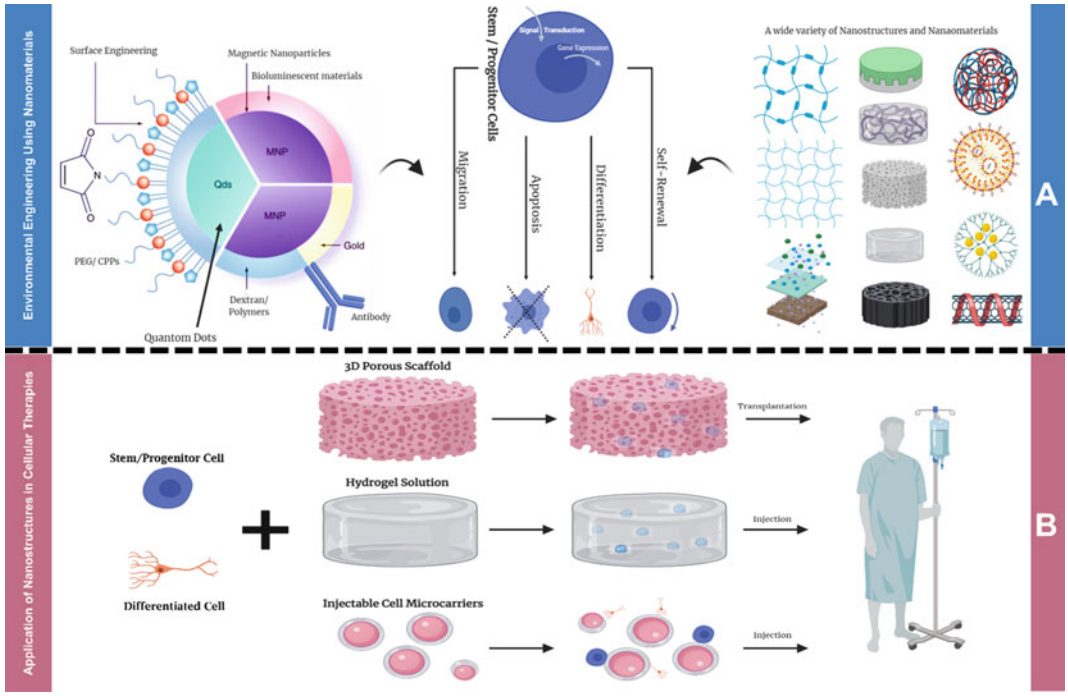

Fig. 1 Schematic illustrations of nanotechnology approaches as emerging tools in cellular-based technologies. (**a**) Environmental engineering use of biomaterials (surface modification and functionalization of nanoparticles for intended applications such as regulation of stem cell fate). (**b**) Application of nanostructures in cellular therapies by combining stem or progenitor cells and differentiated cells with nanostructures/nanomaterials to transplant or inject into patients

biocompatible polymers reduce the biomolecule degradation rate, facilitate controlled release of biomolecules, can be administered less frequently, and have fewer risk errors contaminations (Patra et al. 2018). In this regard, the results of a study showed the efficiency of PNIPAM NPs and VEGF-PNIPAM loaded collagen hydrogel in a VEGF delivery to bone tissue (Adibfar et al. 2018). Recently, researchers have introduced engineered exosomes that contain an RVG peptide for neural targeting and loaded NGF onto the previously mentioned exosomes simultaneously. The resultant exosomes were denoted as NGF@Exo^{RVG}. This delivery system was efficacious in delivery into the ischemic cortex, a burst release of encapsulated NGF protein and de novo NGF protein translated from delivered mRNA. In another study, the delivered NGF reshaped microglia polarization, promoting cell survival, and increased the population of doublecortin-positive cells, a neuroblast marker (Yang et al. 2020), resulting in reduced inflammation (Fig. 1a). The ability of nanoparticles to allow researchers to regulate the proliferation and differentiation of stem cells in a controllable manner is discussed in the next section.

2.1.1 Engineering Stem Cell Microenvironment

The ECM consists of hyaluronic acid (HA), glycosaminoglycans (GAGs), collagens, and elastins. ECM was initially thought to be a supportive structure for tissues and organs (Petreaca and Martins-Green 2019); however, the results of several studies have shown that ECM molecules influence cellular function and behavior. A model introduced by Bissell et al., "dynamic reciprocity," explained how ECM molecules allow in signal transduction across the cell membrane via cell surface receptors and regulate the expressions

of specific genes that affect the ECM composition and interactions with cells (Bhat and Bissell 2014).

Stem cell niches are specific multifactorial extracellular microenvironments where stem cells typically reside and grow (Moore and Lemischka 2006). These specific microenvironments are composed of several soluble and insoluble biomaterials, such as the ECM and other components like GFs and signaling molecules.

Some crucial factors like ECM components and morphogenic signaling molecules can modulate stem cell fate and function. Cadherins, laminins, and morphometric protein families are examples that have been explored in stem cell development and stem cell fate regulation (Moore and Lemischka 2006). Tools such as microcontact printing and dip-pen nanolithography can be used to investigate the effects of the ECM composition and topographic and spatiotemporal effects of ECM components on stem cell differentiation and proliferation, material-based effects on single stem cells, and their function (Salaita et al. 2007). These soft micro- and nanolithographic tools fabricate micro- and nanoscale ECM patterns on different surfaces (Lee et al. 2002). Nanotechnologists have introduced several artificial platforms that mimic the stem cell niche's topological attributes to induce their fate and behavior. In this regard, Park et al. reported that mesenchymal stromal/stem cell (MSC) behavior, including cell differentiation, cell growth, and cell spreading, is tied to the diameter of self-assembled layers of TiO_2 nanotubes (Park et al. 2007).

Several natural or synthetic polymers with tunable properties provide reliable options to regulate stem cell fate (Li et al. 2013). Stiffness is a significant factor that determines cell fate. Natural polymers like HA and chondroitin sulfate show relatively lower stiffness compared with synthetic polymers. Due to their existence in vivo and ability to mimic the physical microenvironment, they are more suitable for regenerative medicine and tissue engineering purposes (Han et al. 2014). Electrospun nanofibrous, ceramics (e.g., calcium phosphate, hydroxyapatite, and bioactive glass), and metal nanocomposites (e.g., titanium) are substrates that provide proper conditions for chondrogenic and osteogenic differentiation of stem cells (Han et al. 2014). Some are naturally found in the bones, and most of them can strongly adsorb proteins, which allows enhanced adhesion, proliferation, and differentiation of MSCs (Kotobuki et al. 2005). MSCs cultured on bioactive glass illustrated osteoblastic phenotype and mineralized ECM, which confirmed osteogenic differentiation of MSCs (Kotobuki et al. 2005). Engler et al. have reported that physical cues could affect stem cell differentiation by culturing hMSCs on hydrogel substrates with various stiffnesses. They said that stem cells expressed neural markers when they were cultured on softer substrates (0.3 kPa).

Culturing them on a rigid substrate (35 kPa) resulted in the expressions of osteogenic markers (Engler et al. 2006). It is well known that stiff substrates affect force sensors (focal adhesion). In turn, these sensors transfer cell-substrate force as a signal within the cell and adjust the cell-ECM interaction by actin-myosin contractions. Gilbert et al. reported that proper stiffness is a crucial prerequisite for maintaining stemness by addressing muscle stem cell pluripotency in cultures with different substrates (Gilbert et al. 2010). Several studies have been conducted to address this issue, and engineered flasks, petri dishes, and plastic culture dishes have been produced that could facilitate investigations on the behavior and fate of stem cells.

It is noteworthy that stem cell niches are not 2D microenvironments; instead, these cells reside in a 3D microenvironment. Thus, the response by stem cells to environmental cues is slightly different from the conditions mentioned earlier, and, in some aspects, it is almost unclear. Since the dynamic properties play an essential role during embryonic development and the whole life period, dynamic changes of the 3D microenvironment could regulate stem cell behaviors (Kirschner and Anseth 2013). Designing desired 3D niches for stem cell growth, direct differentiation, or self-renewal is a newly proposed platform that has opened new avenues in stem cell science and technology (Donnelly et al. 2018). Some nanotechnology-based and other bioengineering

approaches control cell-microenvironment interactions that include hydrogel-, microwell-, and microgel-based technologies. These technologies have been previously reviewed (Donnelly et al. 2018). Unraveling the function of environmental engineering in stem cell differentiation is a hot topic that can be elucidated more in the future.

2.2 Nanomaterials for Molecular and Cellular Imaging

One of the most appealing applications of nanoparticles is their use in molecular and cellular imaging procedures. There are various nanoparticles for imaging purposes such as MNPs (Majidi et al. 2016), semiconductor nanoparticles (e.g., QDs) (Wang et al. 2019), CNTs (Servant et al. 2016), and AuNPs (Meola et al. 2018). They can be functionalized in an interdisciplinary manner by bioorganic, bioinorganic, and surface chemistry with diverse applications. These nanoparticles can be easily synthesized at a large scale from different materials by relatively simple procedures. Here, we focus on the most prominent nanoparticles and their applications in stem cell technologies.

2.2.1 Magnetic Nanoparticles

MNPs are a class of nanoparticles with magnetic properties that can be utilized in biomolecule and drug delivery applications, magnetic cell separation, biosensor applications, mechanotransduction, 3D structured tissues, cell tracking, in vivo imaging, and hyperthermia applications (Hasan et al. 2018). MNPs are the most widely used nanoparticles in regenerative biomedicine and stem cell sciences. The size-to-volume ratio and size-dependent magnetic properties of MNPs differ from their bulk materials and give them unique characteristics (Majidi et al. 2016). MNPs are commonly composed of metals and their oxides that comprise a core-shell structure. Iron oxide nanoparticles with and without core-shell structures are the most common MNPs used in biology and biomedicine.

Iron Oxide Nanoparticles

Iron oxide nanoparticles are the most frequent metal nanoparticles that internalize into the cell cytoplasm or bind to the cell membrane's outer surface. Binding nanoparticles to the cell membrane does not affect cell viability; moreover, these particles can interfere with cell surface molecules and may be easily detached in some cases (Bulte and Kraitchman 2004a). The surface of iron oxide nanoparticles can be modified to maximize the uptake efficiency and minimize side effects on the cells (Bulte and Kraitchman 2004b). Coating superparamagnetic iron oxide nanoparticles (SPIONs) by several synthetic or natural polymers has been shown to enhance their stability and solubility and prevent offered aggregation (Ansari et al. 2019). Surface coating of SPIONs leads to tracking and allows researchers to study stem cells with imaging modalities like magnetic particle imaging (MPI) as well as magnetic resonance imaging (MRI) (Gu et al. 2018). MPI is a technique that directly images iron oxide nanoparticle-labeled cells and can longitudinally monitor and quantify cell administration in vivo. This technique offers depth penetration, near-ideal image contrast, and robustness, making MPI both ultrasensitive and linearly quantitative (Zheng et al. 2016).

MRI works based on the behavior, alignment, and interaction of protons in different body tissues in a magnetic field and gives a tremendous amount of information to generate a detailed image according to the water presence within desired tissue(s). Magnetic iron oxide nanoparticles were introduced instead of conventional gadolinium-based contrast agents in MRI, and new progress has occurred in this area over recent years (Wang et al. 2001). Despite the extensive application of SPIONs for cellular labeling and tracking, various challenges must be overcome to improve the efficacy of cell tracking and the internalization/transfer of these nanoparticles in vivo (Markides et al. 2019). Coating the SPIONs with gold (Nassireslami and Ajdarzade 2018), PEG (Lassenberger et al. 2017), poly(vinyl alcohol) (PVA) (Naqvi et al. 2009), chitosan (Kania et al. 2018), dextran and

poly(vinyl pyrrolidone) (PVP) (Unterweger et al. 2018), albumin (Yu et al. 2016), antibodies (Ab) (Liu et al. 2016), aptamers (Tutkun et al. 2017), fluorescent dyes (Yoo et al. 2012), folate (Mahajan et al. 2013), and other conjugates under different conditions can efficiently promote their uptake without negative effects on cell viability or function (Barrow et al. 2016). We intend to discuss the application of MNPs, especially in vivo cell tracking, in detail.

MNPs for In Vivo Stem Cell Tracking

Transplantation of stem and progenitor cells is a promising therapeutic approach in regenerative medicine. A very prominent strategy is monitoring and tracking in vivo transplanted stem and progenitor cells, which should be noninvasive and not affect differentiation, survival, and proliferation within the desired organs and tissues (Markides et al. 2019). A broad spectrum of choices can pave the way for in vivo imaging studies like multiple photon microscopy, MRI, positron emission tomography (PET), photoacoustic imaging, radioactive cell imaging, optoacoustic imaging, and bioluminescence (Duffy et al. 2020). MRI is widely used because of its accessibility, cost, speed, high resolution, and 3D-reconstruction capabilities (Chemaly et al. 2005). During an MRI, transplanted stem or progenitor cells are labeled with magnetic oxide nanoparticles and traced within the body. MRI also provides information about the transplanted cells and surrounding tissues in these grafted cells (Sykova and Jendelova 2007). The MNPs' various sizes and compositions are synthesized in different ways to gather ultrasensitive and high-resolution images.

SPIONs coated with biomaterials are candidates for biomedical applications. In the past, SPIONs coated with dextran (i.e., ferumoxides, also known as Feridex® in the USA and Endorem® in Europe) were used to label human MSCs (hMSCs) as well as human embryonic stem cells (hESCs) without any requirements for transfection agents (Corot et al. 2006). Despite the use of Endorem® and SPIONs coated with low molecular weight carboxydextran (e.g., ferucarbotran, also known as Resovist®) for several years in clinical trials, they are no longer manufactured because of economic considerations (Bulte 2009). Other contrast agents are coated with dextran and combined with transfection agents (e.g., Fungene™, Superfect™, or Lipofectamine) (Corot et al. 2006). However, transfection agents facilitate cellular uptake but may damage desired stem cells. They are more toxic at higher concentrations and do not show suitable internalization into cells; therefore, one of the most vital requirements for utilizing these agents within contrast agents is dose optimization that may affect the target cell type. Stem or progenitor cells that are labeled with functionalized SPIONs are another strategy under consideration. The HIV-1 Tat peptide, cell-penetrating peptides (CPP), dendrimers, polycationic polymers, and amphiphilic peptides are numerous internalizing ligands that could enhance internalization efficiency. Fluorescent labels, radiolabels, and bioluminescent conjugates are other standard labels combined with MRI that result in valid data. Weissleder and colleagues reported that MNPs (5 nm in size) comprised of monocrystalline superparamagnetic iron oxide cores stabilized with a cross-linked dextran shell could be used as a combined imaging method (e.g., optical and nuclear imaging) (Lewin et al. 2000).

Meanwhile, they increased the size of these nanoparticles to 45 nm (Lewin et al. 2000) and used functionalized nanoparticles with fluorescent labels and internalized ligands (e.g., FITC, conjugated HIV-Tat peptide) for further applications. Another group also labeled these dextran-coated SPIONs with ^{111}In isotope in the case of nuclear imaging modality (Bulte et al. 1999). Lu et al. introduced new dual-modal contrast agent-PLGA/iron oxide microparticles (PLGA/IO MPs) that enhanced both photoacoustic (PA) and MRI functions (Lu et al. 2018). A research work illustrated that the functionalization of ultrasmall iron oxide nanoparticles with glucosamine could be an efficient strategy to increase cellular uptake by MSCs in animal models of cerebral ischemia (Guldris et al. 2017). Several studies have shown that stem cells and stem progenitor cells retain viability,

phenotype, proliferation, and lineage differentiation when labeled with different novel iron oxide nanoparticles (Ramos-Gomez et al. 2015). Hoehn and coworkers validated safe and sensitive novel bimodal iron oxide particles as a substitute for ferumoxides for efficient longitudinal tracking of human neural stem cells in vivo (Aswendt et al. 2015).

Despite reports of the applicability of SPIONs for in vivo imaging, occasional extracellular deposition of SPIONs in tissues has been reported where microphages scavenged these particles and generated noisy signals during MRI studies (Cromer Berman et al. 2013). This group of NPs could induce oxidative stress and cellular damages after direct use in cell tracking (Santoso and Yang 2016). Another drawback is their inability to distinguish between nonviable and viable cells, which is crucial in stem cell therapies (Huang et al. 2015).

Despite the drawbacks, SPIONs are a promising technology to investigate homing, migration, biodistribution, and engraftment of stem cells; therefore, further studies on their long-term effects within the body are needed to shed light on the best option for cell tracking.

2.2.2 Semiconductor Nanomaterials

One of the most commonly used noninvasive live cell imaging techniques is fluorescent microscopy (Pecot et al. 2015). Despite numerous options, organic fluorophores as fluorescent tags are widely used for fluorescent imaging (Kim et al. 2015a). Although there are many advantages for these tags in imaging living cells, their constraints include a short lifetime, low photostability, and low tissue penetration (Park et al. 2019), affecting their routine usage. In addition to MNPs, QDs circumvent many limitations and are extensively used for cell biology applications. QDs or semiconductor nanocrystals have opened avenues to an array of diverse applications in biology and basic sciences, such as cellular imaging (Bakalova et al. 2007), immunoassays (Hoshino et al. 2005), DNA hybridization (Huang et al. 2006), optical barcoding (Han et al. 2001), cell migration (Gu et al. 2007), lineage tracing (Slotkin et al. 2007), in vivo cell tracking (Zhang and Wu 2007), and live monitoring of physiological events that occur in cells (Rosen et al. 2007). These robust semiconductor nanocrystals have broad absorption spectra and narrow emission spectra.

In contrast to their benefits, there are some hurdles in employing these NPs due to intrinsic toxicity that should be considered. QDs are incredibly toxic should they fail. Toxicity issues should be discussed. Their wide applications in regenerative medicine and stem cell technology cannot be ignored according to their extraordinary capabilities. Their wide applications in regenerative medicine and stem cell technology cannot be overlooked according to their outstanding capabilities. Kundrotas et al. have reported that carboxylated QDs can be used as a nonspecific and effective dye for staining BM-MSCs, especially their extracellular structures (Kundrotas et al. 2019). Lei et al. illustrated that conjugation of CdSe/ZnS QDs with Tat peptide and coating with PEG could efficiently track MSCs in vivo, proving their benefit (Lei et al. 2008). QD-labeled cells were injected into the mice's tail vein, and the biodistribution of the cells above was monitored by fluorescent microscopy. Their results revealed that QDs accumulated mainly in the lungs, liver, and spleen, with little or no accumulation in the kidneys, brain, or heart (Lei et al. 2008). Liu et al. reported the ameliorative effects of CdSe/ZnS QD-labeled MSCs in a type 1 diabetes mellitus rat model. They injected labeled cells into the tail veins of rats on the seventh day of diabetes induction. Biodistribution of the QD-labeled MSCs was assessed by fluorescent imaging, and blood glucose levels were set within 8 weeks. According to the results, the pancreases of the diabetic rats had a significantly higher accumulation rate of labeled cells in comparison with the control group. A drastic decrease in blood glucose levels was observed in the MSC-treated diabetic rat group, which indicated therapeutic effects of the labeled cells in this rat model of diabetes mellitus (Liu et al. 2015). Membrane translocation via different endocytosis pathways could be facilitated through surface modification and functionalization of QDs.

Moreover, to avoid the cytotoxic effects of QDs on living cells, additional surface modifications such as coating to protein, PEG addition, and lipids can be applied (Zhang et al. 2019).

Researchers used a complex containing conjugated histidine to β-cyclodextrin with drug lading capability and attached it to QDs (QD-βCD-His) to label human adipose stem cells (hASCs). They monitored this complex biodistribution in a 2D cell culture and a 3D temperature-sensitive chitosan hydrogel scaffold. Dexamethasone (Dex) was loaded into QD-βCD-His nano-carrier (QD-βCD-His@Dex) to induce bone differentiation of labeled adipose stem cells. This group's findings would seem to imply that QD-βCD-His@Dex is a novel appealing dual-purpose nano-carrier for stem cell labeling that has osteoinductive potential in cellular therapy and tissue engineering (Jahed et al. 2020).

2.3 Genetic Manipulation of Stem Cells Using Nanotechnology-Based Gene Delivery Systems

Gene delivery to elicit particular protein/marker production plays a significant role in the potentials for regenerative medicine. Several biomolecules, such as gene vectors (viral and nonviral), siRNA, proteins, and small molecules, have been developed to manipulate essential gene expression levels in stem cells. Genetically engineered progenitor cells and stem cells have been employed to produce immortalized cell lines, invariant natural killer T cells (NKTs) in murine models, immunotherapy, immunoregulation, cancer therapy, and treatment of congenital diseases as well as specific genetic disorders. Due to the undeniable role of transcription factors in the differentiation of stem cells to different lineages, it has been demonstrated that gene delivery could be a handy tool for stem cell differentiation. The development of current gene delivery systems like transduction that utilize viral vectors and electroporation leads to high gene expression levels within stem cells. However, an urgent requirement for implementing novel gene delivery technologies in regenerative medicine is inevitable. Nanotechnology, in collaboration with biotechnology, opens new avenues to deal with complex problems in this area. Nanotechnology-based novel gene delivery systems have been developed for efficient and safe transfection. In this regard, newly proposed nano-vectors and nanosystems are continually being developed and should be considered. Magnetofection, cationic lipid nanoparticles, dendrimers, inorganic nanomaterials (AuNP), silver nanoparticles (AgNP), calcium phosphate, graphene oxide (GO), QDs, MNPs, CNTs, protein and peptide nanoparticles, polymer-based nanomaterials, and exosomes are nanostructures currently employed for this reason (Fig. 2).

2.3.1 Magnetofection

Magnetofection is a technique that employs MNPs coated with a cationic polymer like polyethylenimine (PEI) to transfect desired genes using an external magnetic field. A fundamental limitation to gene delivery is slow vector accumulation and low vector concentration at target tissues; therefore, to overcome this barrier, scientists proposed an appropriate tool called magnetofection. Lin et al. were one of the pioneers using magnetofection for in vitro applications. Wang et al. used magnetic Fe_3O_4 nanoparticles as gene carriers to porcine kidney cells. After surface modification of MNPs with PEI, they applied weight ratios of DNA_{GFP} or DNA_{DsRed} to MNPs lower than or equal to 10:1 or 5:1, resulting in strong binding affinity. Magnetofection resulted in stable and efficient co-expression of the GFP and DsRed in porcine kidney cells (Wang et al. 2014). Pickard et al. reported, for the first time, that oscillating magnetic fields could enhance MNP-based transfection with the reporter and functional genes in monolayered cultures, which yielded high transfection versus neurospheres (Pickard et al. 2015). Surprisingly, Kami et al. reported six- to eight-fold higher GFP expression in designated magnetoplexes prepared from PEI-MNP conjugates in the presence of a magnetic field versus PEI or PEI-MNP conjugates without a magnetic field (Kami et al. 2014). All in all,

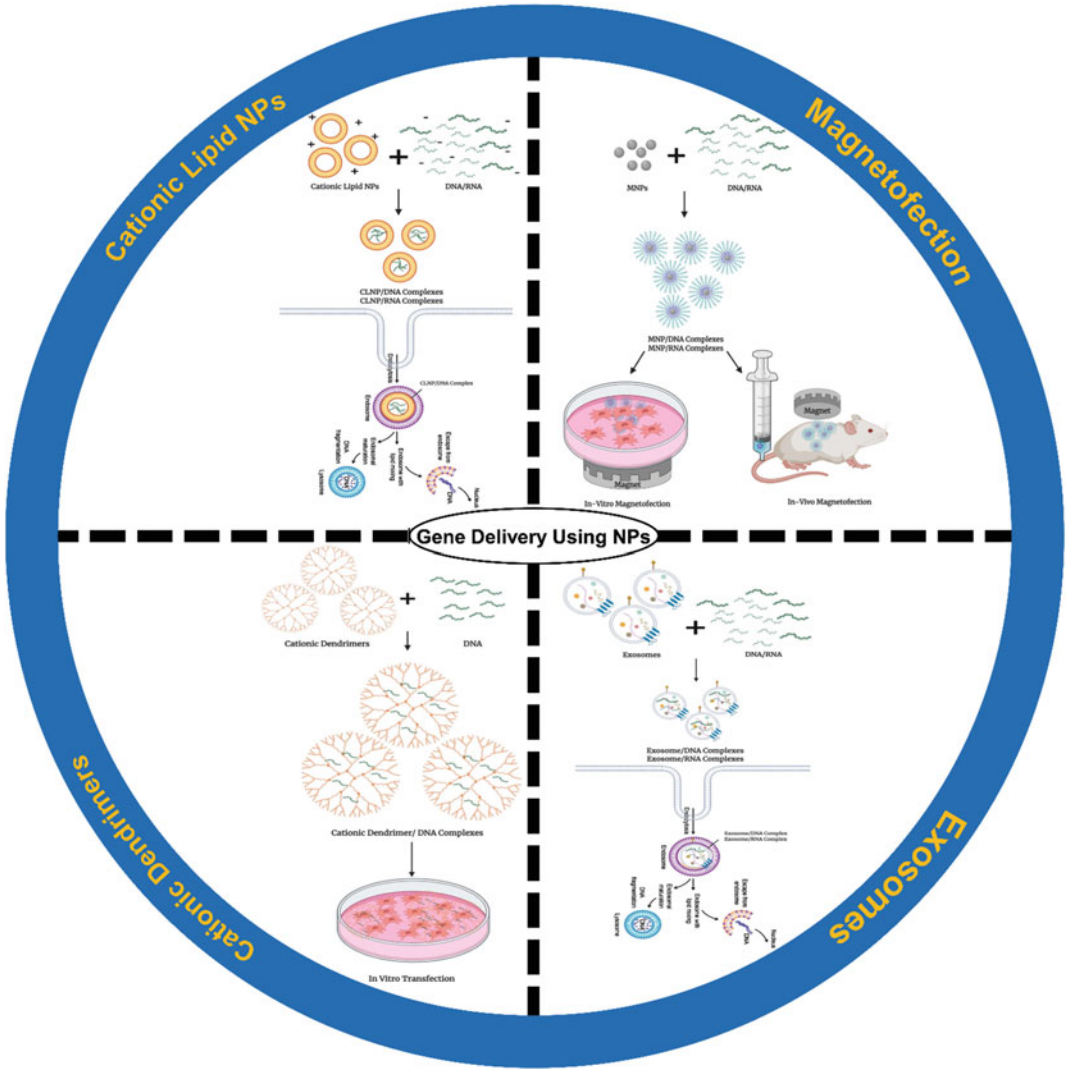

Fig. 2 Nanotechnology-based gene delivery systems for genetic manipulations of stem cells (gene delivery using NPs: magnetofection, cationic lipid NPs, cationic dendrimers, and exosomes)

magnetofection is good ex vivo and in vitro but does not work as an efficient system in vivo in some cases.

2.3.2 Cationic Polymers

Cationic polymers poly(β-amino esters) (PBAEs) are another option on the table for safe nonviral gene delivery but generally show less efficiency than viral gene delivery vectors, and performing some chemical modification is a prerequisite to enable nucleic acid encapsulation and delivery (Kozielski et al. 2014). Due to ease of manipulation, high safety, large cargo capacity, and scale-up conditions, this promising delivery technology facilitates RNAi delivery; however, targeting and stability are the inevitable challenges (Dosta et al. 2018). PBAEs can self-assemble with DNA and form positively charged gene delivery NPs, while they cannot be a good choice in siRNA delivery. Gao et al. investigated the effects of molecular weight and configuration of highly branched PBAEs on gene transfection biocompatibility

and efficiency. Their results showed that preparation of PBAEs in highly branched structures circumvents high molecular weight cytotoxicity compared with conventional PEI, PAMAM dendrimer, and PDMAEMA and converts them as a valuable tool for the development of next-generation gene delivery vectors (Gao et al. 2016).

2.3.3 Virosomes

Virosomes are reconstituted viral envelopes containing virus-derived proteins to allow them to fuse with target cells as vehicles for cellular delivery of antigens, drugs, and nucleic acids.

Ramani et al. reported a site-specific gene delivery in vivo through engineered Sendai viral envelopes. Efficiency and novelty of this gene carrier revealed a stable expression up to 4 mo after a single i.v. administration of transgenes in the mouse chromosomal DNA (Ramani et al. 1998). Nowadays, virosomes are most prevalent in vaccine and drug delivery instead of gene delivery.

2.3.4 Cationic Lipid Nanoparticles

Cationic lipid nanoparticles (CLNPs) are another type of delivery vehicle in gene delivery and transfection. The presence of a permanent positive charge makes them vulnerable vehicles to carry negatively charged nucleic acids based on electrostatic interactions effectively. Interaction between these positive charge carriers and the cellular membrane's negative surface charge could facilitate the cellular internalization of these carriers. The positive amounts of these nanoparticles may cause rapid clearance and unwanted toxic effects. The findings reported by Bondi et al. would seem to imply that solid lipid nanoparticles (SLNPs) fabricated by Compritol 888 ATO (matrix lipid), dimethyldioctadecylammonium bromide (charge carrier), and pluronic F68 (surfactant) could form stable complexes with DNA, as an efficient transfection system, with decreased toxic effects on the cells and provided protection of DNA against DNase I digestion (Bondi et al. 2007).

Limeres et al. aimed to synthesize and characterize cholesteryl oleate-containing solid lipid nanoparticles (CO-SLNs) incorporating protamine (P) to produce P: DNA:CO-SLN complexes as nonviral vectors for gene delivery. The results showed reduced cytotoxicity and increased cellular uptake efficiency (Limeres et al. 2019).

2.3.5 Cationic Dendrimers

Cationic dendrimers are another type of gene delivery tool employed by scientists. Due to their internal cavities, controlled synthesis, and facile functionalization, dendrimers are interested in the scope of drug and gene delivery systems. Different research groups have bonded nucleic acids into dendrimers via electrostatic interactions in the presence of surface amine groups. A new class of gene delivery vectors for MSCs is based upon the generation of a 5 PAMAM dendrimer functionalized with hydrophobic chains. In this regard, peripheral amine groups can bind effectively to DNA and protect it against serum nucleases (Kim et al. 2015b).

Gorzkiewicz et al. synthesized two novel lysine-based dendritic macromolecules (D3K2 and D3G2) characterized by cytotoxicity/genotoxicity and transfection potential in two human cell lines. This approach allowed them to identify an increased cationic D3K2 as a potent delivery agent and increase the intracellular accumulation of large nucleic acid molecules like plasmids (Gorzkiewicz et al. 2020).

2.3.6 Exosomes

Exosomes are a subpopulation of extracellular vesicles (EVs) with a size range between 30 and 150 nm that can be isolated from different body fluids. They can shuttle biological information between other cells and play a vital role in cellular communications. These nanovesicles can be engineered to efficiently deliver their cargo to specific cells due to their active targeting capability. Wood and colleagues delivered exogenous *GAPDH* siRNA to neurons, microglia, and oligodendrocytes in the brain via RVG-targeted exosomes, which resulted in a specific gene knockdown (Alvarez-Erviti et al. 2011). Zhuang et al. conjugated valency-controlled tetrahedral DNA nanostructures (TDNs) with a DNA

aptamer and subsequently loaded these nanostructures onto the EV surface via cholesterol anchoring for active cell targeting. This emerging tool prepares a platform for the directional display of an aptamer on the surface labeling and EV-based Cas9 delivery, which provides a meaningful idea for future cell-selective gene editing approaches like CRISPR/Cas9 (Zhuang et al. 2020).

These technologies are reviewed elsewhere. The synergism of gene therapy and EV communication has opened up new avenues in precision medicine to treat diverse malignancies and disorders.

3 Cutting-Edge Technologies that Use Nanotechnology in Regenerative Medicine

The results gathered from different studies point to the likelihood that cutting-edge technologies that employ nanotechnology in regenerative medicine and cellular therapies will pave the way for establishing novel modalities for early diagnosis, therapies, medical imaging, and theranostics. Cutting-edge studies are used for different purposes. For instance, Wang et al. have reported that a unique multiscale delivery system that encompasses nanoparticles in an alginate hydrogel nano-in-microcapsules co-entrapped with stem cells in a collagen hydrogel could enhance MSC proliferation, maintain their potential for multilineage differentiation, and facilitate perfusion and regeneration of ischemic limbs (Wang et al. 2017).

Microfluidic and nanofluidic technology is another promising technology that can be used for cell isolation, cell differentiation, and diagnosis. Patterning micro- and nanometer range fluidic channels on a substrate can lead to a technology of microfluidic and nanofluidic systems that process and manipulate small (10^{-9}–10^{-18} L) amounts of fluids (Whitesides 2006). We previously mentioned that managing environmental cues is a major factor in stem cell science and technology. Herein microfluidic and nanofluidic devices have been widely used in high throughput screening of a broad range of conditions in addition to simulation of the physiological environment as a prerequisite to stem cell growth (e.g., heterogeneous and 3D growth conditions) (Zhang and Austin 2012).

The microfluidic/nanofluidic technique is an essential tool for single-cell analysis, emphasizing cell trapping, treatment, and biochemical studies. Of note, novel technologies in nanotechnology and regenerative nanomedicine are appealing prospects (Table 1). Table 1 lists some recent cutting-edge technologies that use nanotechnology in regenerative medicine and stem cell technology.

4 Regenerative Nanomedicine: Ethical, Legal, and Social Challenges

The central concept of the legal actions influenced by technology or procedures is the legal certainty of that technology or procedure. Regenerative nanomedicine provides a platform for the convergence of diverse technologies, from interactions between nanomaterials and biological systems to the structural properties of nanomaterials. These interactions result in a double-edged sword with unexpected outcomes, primarily ethical, legal, and social concerns. There are many concerns and questions about the environmental risks, safety, and health effects of nanomaterials.

Translation of nanotechnology from the bench to the bedside imposes a wide variety of challenges, including physicochemical characterizations, process control, biocompatibility, nanotoxicology, scale-up, and reproducibility (Soares et al. 2018). Nanotechnology applications in regenerative medicine, nanomaterial development, manufacturing processes, safety concerns, and ambiguity over classifications are significant uncertainties encountered by scientists (Kelly 2010). The development of nanomaterials is an interdisciplinary area to provide monodispersed nanoparticles that have fewer effects on the immune system and decreased genotoxicity in addition to physicochemical properties (Najafi-Hajivar et al. 2016). In previous years, guidelines

Table 1 Cutting-edge technologies that employ nanotechnology in regenerative medicine and stem cell technology

Cutting-edge technology	Application(s)	References
Nanoparticles, nanofibers, and nanomaterials	Cell isolation, cell differentiation, cell engineering, genetic manipulations, delivery	Xue et al. (2020)
3D bioprinting technologies	Tissue engineering, organ printing	Richards et al. (2017)
Electrospun 3D scaffolds	Tissue regeneration, 3D tissue fabrication, sample holder for facile culture of cells	Jahangir et al. (2018)
Polymer nanocomposites and nanostructures	Tissue regeneration, organ repair	Carola Esposito et al. (2017) and Nejati et al. (2009)
Smart polymers	Nanoengineering of biointerfaces	Arisaka et al. (2016)
Biodegradable polymeric nano-carriers	Immunotherapy, vaccine production, drug delivery	Hsieh et al. (2015)
Electrically conductive biomaterials	Tissue engineering, regenerative medicine (neural repair, wound healing), neural prostheses, biosensors, artificial muscle, drug delivery, photothermal therapy	Baei et al. (2020)
Nanopatterned scaffolds	Neural tissue engineering, regenerative medicine	Yurie et al. (2017)
Organ-on-a-chip (OOC)	3D microfluidic cell culture system, organoid culture system, drug discovery, personalized medicine, precision medicine	Wang et al. (2020)
Tissue-on-a-chip (TOC)	Drug screening, personalized medicine, precision medicine	Haring et al. (2017)
Biomimetic microstructures and biomimetic extracellular matrices	Cell culture simulation, cell migration assay	Huang et al. (2014)
Smart cell culture surfaces	Cell sheet-based regenerative therapy, 3D tissue fabrication	Nguyen et al. (2019)
Self-assembled smart materials (e.g., protein polymers (recombinamers), self-assembled peptides) and cell-penetrating peptides	Protein polymers (recombinamers), cell harvesting, and tissue engineering in the formation of different kinds of 2D or 3D scaffolds, delivery (drug, small molecules, growth factors (GFs), gene, miRNA, lncRNA, siRNA), genetic manipulations	Soleymani-Goloujeh et al. (2017) and Choi et al. (2013)
Nanoimprint-based contact printing technique	Micro-/nanopatterning of proteins	Tran and Nguyen (2017)
Micro- and nanofluidic	Cell microencapsulation, cell isolation, EV isolation, early detection, diseases early detection, precision medicine	Unni et al. (2020) and Zhou et al. (2019)
Magnetic nanoparticles	Cell sorting, cell imaging, hyperthermia, active targeting, single-cell densitometry and weightlessness culture of mesenchymal stem cells	Majidi et al. (2016) and Unni et al. (2020)
Hydrogels	Tissue engineering, drug delivery, growth factor delivery	Kang et al. (2017)
Extracellular nanovesicles	Cell signaling, delivery (drug, small molecules, growth factors, gene, miRNAs, siRNA), genetic manipulations	Ma et al. (2020) and Riazifar et al. (2019)
Nanocapsules	Delivery (drug, small molecules, growth factors, gene, miRNAs, siRNA), cell nanoencapsulation	Musyanovych and Landfester (2014)
Fluorescent nanoclusters	Imaging of cells/stem cells	Molaabasi et al. (2018) and Grady et al. (2019)
Nanoimaging tools	Cell imaging, molecular imaging	Wang et al. (2019)
Theranostics	Monitoring, imaging, and therapy	Choi et al. (2017)

pertaining to general risk assessment have been proposed; however, the importance of this issue and global interests in nanomedicine led to establishing new procedures by the FDA, European Medicines Agency (EMA), other regulatory agencies (Cicha et al. 2018). There is no comprehensive method for manufacturing each nanoparticle, nor are there any gold standards for nanoparticle characterization. It is unclear if nanomedicine should be defined and classified as a medicine or a medical device; therefore, regulatory affairs must use a case-by-case approach to evaluate nanotechnology-derived products. Diverse interactions, reactivity, and effects of nanomaterials can influence decision-making in terms of regulations and laws that pertain to nanomaterials. Nanomedical research rules and regulations will help decision-makers in conventional medicine, and scientists in regenerative medicine apply this new technology (Halamoda-Kenzaoui et al. 2019).

The most systematic research in regenerative medicine is based upon the use of different types of stem cells and nanomedicine, which meant engineering some grafts and tissues to rehabilitate the defects and abnormalities (Traphagen and Yelick 2009). The first ethical challenge in using stem cells for regenerative therapies is the source and type of stem cells. Many controversial moral issues and pros and cons are attributed to different beliefs worldwide using pluripotent stem cells, particularly embryonic stem cells (ESCs) (Munn 2001).

Several attempts were performed in different countries to solve moral and philosophical arguments, which consider that embryos in the blastocyst stage are humans. The federal governments in the USA and Canada decided to use leftover embryos developed by in vitro fertilization (IVF) and obtain informed consent from the donors (Kington 2019). Scientists have produced an alternative cell, induced pluripotent stem cells (iPSCs), to avoid these ethical controversies. iPSCs demonstrated the same expected fundamental properties of ESCs (e.g., pluripotency), but there were no ethical concerns due to their derivation from somatic cells such as skin cells. iPSCs are produced by reprogramming somatic cells and restricting the procurement and use of human embryos (Chan and Harris 2008). The most public concern with the derivation of iPSCs is viruses that are used to deliver reprogramming factors. Therefore, rigorous tests should be conducted to prevent problems in cell reprogramming. Nanotechnology currently helps to circumvent this obstacle. The use of related technologies needs more precautions and investigations to prove that these actions are safe and moral. The ethical considerations, legal actions, and social challenges of stem cells have been reviewed and widely discussed in related books and articles (Soares et al. 2018; Afshar et al. 2020).

Due to the diverse use of nanomaterials in medicine and health, there are numerous concerns in terms of their use in the human body (Allhoff and Lin 2008). It is inevitable to study the impact of nanomaterials on the body; however, time will reveal the long-term effects of nanotechnology-based approaches inside the body and environment nature. Unlimited and uncontrolled nanotechnology in regenerative medicine is a controversial issue that needs additional consideration and should be studied from different aspects.

5 Concluding Remarks and Future Perspectives

In this review, we have summarized the use of nanotechnology-based approaches in cell-based applications, including cell tracking, labeling, genetic manipulation, environment engineering of cells, differentiation, cell stimulation, cutting-edge strategies for engineering biological tissues in vitro and in vivo, cutting-edge technologies in regenerative medicine that use nanomedicine, as well as ethical considerations and legal challenges that focus on newly proposed nano-based approaches to attain the best consequences in regenerative medicine. Unraveling the environmental engineering function in stem cell differentiation is a topic that will be elucidated more in the future. Interactions between material surfaces and biological entities are prerequisites for the structural support of living cells in biomimetic

tissues and scaffolds. Therefore, the intrinsic properties of nanomaterials can be beneficial in several processes, including cell adhesion, protein adsorption, cell-cell interactions, stem cell growth, cell proliferation, stem cell self-renewal, cell-directed differentiation, and cell migration. The application of nanomedicine in the development of structures at the molecular (e.g., subcellular) level enables us to integrate technology with medicine and physiology toward tissue regeneration and optimization of adhesion, proliferation, and differentiation of various cells. Combining nanotechnology with regenerative medicine provides a promising breakthrough due to its applicability in managing nearly unsolvable health complications and could enhance the quality of life.

Addressing future challenges would accelerate nanotechnology approaches toward regenerative medicine and facilitate the therapeutic use of stem cells. Tissue engineering, organ repair, organ 3D bioprinting, nanoengineering of biointerfaces, and OOC/TOC technologies are challenging issues that require more effort and consideration. Paving the way in the scope of up-to-date and efficient delivery systems and theranostics could change the future of biology and medicine.

The use of regenerative nanomedicine is an appealing issue that has answered many questions; however, much research is needed to change the boundaries of science.

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Generation of a Beta-Cell Transplant Animal Model of Diabetes Using CRISPR Technology

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Abstract

Since insulin deficiency results from pancreatic beta-cell destruction, all type 1 and most type 2 diabetes patients eventually require lifelong insulin injections. Insulin gene synthesis could also be impaired due to insulin gene mutations as observed in diabetic patients with MODY 10. At this point, insulin gene therapy could be very effective to recompense insulin deficiency under these circumstances. For this reason, an HIV-based lentiviral vector carrying the insulin gene under the control of insulin promoter (LentiINS) was generated, and its therapeutic efficacy was tested in a beta-cell transplant model lacking insulin produced by CRISPR/Cas9-mediated genetically engineered pancreatic beta cells. To generate an insulin knockout beta-cell transplant animal model of diabetes, a dual gene knockout plasmid system involving CRISPR/Cas9 was transfected into a mouse pancreatic beta cell line (Min6). Fluorescence microscopy and antibiotic selection were utilized to select the insulin gene knockout clones. Transplantation of the genetically engineered pancreatic beta cells under the kidney capsule of STZ-induced diabetic rats revealed LentiINS- but not LentiLacZ-infected Ins2KO cells transiently reduced hyperglycemia similar to that of MIN6 in diabetic animals. These results suggest LentiINS has the potential to functionally restore insulin production in an insulin knockout beta-cell transplant animal model of diabetes.

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Keywords

Beta Cells · CRISPR/Cas9 · Insulin Gene Therapy · Lentivirus

Abbreviations

Cas9	CRISPR-associated system
CRISPR	Clustered regularly interspaced short palindromic repeat

GFP	Green fluorescent protein
GLP-1	Glucagon-like peptide-1
HDR	Homologous recombination repair
KO	Knockout
MODY	Maturity onset diabetes of the young
PACAP	Pituitary adenylate cyclase activating polypeptide
RFP	Red fluorescent protein
T1D	Type 1 diabetes
T2D	Type 2 diabetes
VIP	Vasoactive intestinal peptide

1 Introduction

Diabetes is a disease in which blood glucose levels are too high, largely because of the body's inability to make or efficiently use insulin. There are several recognized mechanisms by which diabetes can develop in humans. Type 1 diabetes (T1D) is characterized by severe insulin deficiency as a result of the destruction of the pancreatic beta cells by the body's immune system (Eizirik et al. 2020; Norris et al. 2020). Some mutations in the insulin gene can also interrupt insulin biosynthesis causing permanent neonatal diabetes (Edghill et al. 2008; Lei et al. 2020). The most common hereditary form of diabetes (monogenic) is the maturity onset diabetes of the young (MODY) caused by autosomal dominant mutations disrupting insulin production (Urbanova et al. 2019). Long-lasting hyperglycemia causes vascular complications associated with multiple organ failures. To circumvent this, proper control of blood glucose is essential to avoid the development of diabetes-related complications in the long run (Laffel et al. 2020). Even in type 2 diabetes (T2D), glycemic control can first be managed with exercise, diet, and oral anti-glycemic drugs; failure to do so generally results in insulin administration (Home and Itzhak 2020). Both fast-acting (as in the case of *lispro*, *aspart*, and *glulisine*) and long-acting (as with *insulin detemir*, *glargine*, and *degludec*) insulin analogs reduce serum glucose levels by facilitating glucose uptake into skeletal muscle and fat tissue, and at the same time, they interfere with glycogenolysis, gluconeogenesis, and lipolysis (Rodbard and Rodbard 2020).

Despite the availability of several insulin analogs, it remains difficult to control blood sugar using these therapeutics, especially when used at high doses (Heise and Pieber 2007). Conversely, low-dose administration of insulin analogs fails to provide 24 h insulin coverage (Ashwell et al. 2006). Despite the existence of numerous fast-acting and/or long-acting insulin analogs, achieving optimal glycemic control remains to be accomplished in diabetic patients. Subsequently, these patients are at increased risk of developing diabetes-related complications over time. Furthermore, the management of diabetes with insulin analogs becomes complicated, considering metabolic variability among individuals. The achievement of proper glycemic control can easily be destabilized by insulin-induced hypoglycemia frequently encountered in diabetic patients (Mohn et al. 2005). Consequently, insulin therapy is almost inevitable for all patients with T1D and most patients with T2D (Aziz 2012). Thus, diabetic patients may require frequent insulin dosage titration to maintain normoglycemia. There has been increased interest in recent years in the development of new methods of insulin therapy to decrease the need for exogenously delivered synthetic insulin (Cernea and Raz 2020). An alternative means to reestablish insulin expression for extending and improving the quality of life in T1D patients is through pancreatic islet cell transplantation (Dirice et al. 2009; Kahraman et al. 2011). Unfortunately, as with any other donor issue, there is a limited supply of pancreas available for transplantation (Sanlioglu et al. 2008). Moreover, life-long immune suppression is required for current islet transplantation protocols (including the Edmonton protocol), and many pancreatic islet recipients require insulin injections again within 5 years because of the high frequency of non-functioning grafts and secondary graft failure (Shapiro et al. 2005). Thus, there is a desperate need to improve insulin therapy, including its delivery method for diabetic patients (Sanlioglu et al. 2013).

Human insulin and insulin analogs do not contain C-peptide, but there is data suggesting that C-peptide can manifest beneficial effects on diabetic patients (Wahren et al. 2012). Consequently, there are differing opinions on whether C-peptide should be included in current insulin preparations. This issue is further complicated by the fact that high levels of C-peptide are found in the blood of early-stage T2D patients, and recent studies have revealed C-peptide can improve kidney function, nerve function, and blood flow to vital organs in diabetic patients (Kamiya et al. 2009). As an alternative to daily administration of human insulin/insulin analogs to moderate blood glucose levels, there is increasing interest in insulin gene therapy to treat diabetes—especially for patients with insulin gene mutations (MODY 10, etc.). Contrary to human insulin and insulin analogs, insulin gene therapy supplies patients not only with insulin but also with the C-peptide (Sanlioglu et al. 2012a). But, disclosure of beneficial effects of C-peptide requires long-term studies. Unfortunately, delayed and/or transient gene expression was blamed for the limited success obtained in recent insulin gene delivery studies (Chan et al. 2003; Han et al. 2011; Lu et al. 1998). Even though continuous insulin gene expression is now viable using current gene delivery vehicles supplying patients with basal levels of insulin, managing postprandial hyperglycemia remains to be a formidable task to achieve due to the inability to control insulin secretion (Elsner et al. 2012).

Owing to the existence of autoimmunity, most insulin gene therapy approaches have targeted tissues other than the pancreas (Gan et al. 2019; Handorf et al. 2015; Nett et al. 2003; Sia et al. 2020). These studies achieved only limited success because none of the tissues targeted for gene delivery possessed the properties of pancreatic beta cells. Assets unique to pancreatic beta cells can be summarized as controlled transcription and translation of proinsulin, the presence of a regulated secretory pathway, inductive secretion, etc. (Sanlioglu et al. 2012a). Beta cells express GLP-1 receptors important in regulating postprandial insulin secretion in response to glucose (Halban et al. 2001). Also, neuronal input is needed to regulate glucose metabolism via PACAP- and VIP-signaling under metabolic stress (Erendor et al. 2021b; Sanlioglu et al. 2012b; Tasyurek et al. 2018b). Because of these reasons, a lentiviral vector (LentiINS) was generated to provide pancreatic beta-cell-specific insulin gene expression to manage daily blood glucose levels and decrease the need for exogenously delivered insulin (Erendor et al. 2021a). To evaluate the therapeutic potential of pancreatic beta-cell-targeted LentiINS, CRISPR/Cas9 programmable nucleases were employed to generate an insulin knockout pancreatic beta cell line (Eksi et al. 2021). Functional properties of genetically engineered pancreatic beta cells were determined following transplantation under the kidney capsule of diabetic animals.

2 Materials and Methods

2.1 Generation of Insulin Gene Knockout Pancreatic Beta Cells Using CRISPR/Cas9

Knockout plasmids containing the CRISPR/Cas9 system for silencing of the mouse Insulin I and Insulin II genes (Insulin I CRISPR/Cas9 KO Plasmid (m2): sc-421,139-KO and Insulin II CRISPR/Cas9 KO Plasmid (m2): sc-421,140-KO) and homologous recombination repair (HDR) plasmids bearing homology regions specific for each gene (Insulin I HDR Plasmid (m2): sc-421,139-HDR-2 and Insulin II HDR Plasmid (m2): sc-421,140-HDR-2) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Lyophilized plasmids were resuspended in 200 μl of ultra-pure, DNase-free water. The transfection procedure included the addition of transfection solutions (Plasmid Transfection Medium: sc-108,062 and Transfection Reagent: sc-395,739, Santa Cruz, CA) to MIN6 cells according to the protocol recommended by the manufacturer. Our assay conditions required extensive passaging and prolonged waiting periods for selection. Because INS-1 rat insulinoma cell line displayed reduced insulin gene expression and glucose response over time,

MIN6 pancreatic beta-cell line (a gift of Prof. Dr. Jun-ichi Miyazaki, Japan) was chosen as the target pancreatic beta cell line for gene editing. Moreover, Min6 cell line has been extensively used in our previous studies including others concerning pancreatic islet beta-cell function (Erendor et al. 2021a, b; Tasyurek et al. 2018a, b). Briefly, MIN6 cells were seeded in six-well plates at a density of 2×10^5 cells and cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO_2. The following day, two different solutions for each transfection (Ins1KO, Ins2KO) were prepared. For Solution A, 1 µg of KO plasmid and 1 µg of HDR plasmid were diluted into the Plasmid Transfection Medium in a total volume of 150 µl and incubated at RT for 5 min. For Solution B, 10 µl of UltraCruz Transfection Reagent was diluted with 140 µl of Transfection Medium and incubated at RT for 5 min. Next, Solution A was added dropwise directly to Solution B using a pipette and incubated for 30 min at RT. Media were replaced with fresh antibiotic-free DMEM medium before transfection, and the Transfection Reagent Complex (Solution A + Solution B) was added dropwise to the wells. No plasmids were added to the negative control group. Cells were monitored for GFP and RFP expressions at 48, 72, and 96 h following transfections. Successful transformants (Ins1KO, Ins2KO) were selected with 1.8 ug/ml puromycin-containing media, refreshed every two days for a total of 14 days.

2.2 Construction of Lentivirus Vector Carrying an Insulin Promotor Connected to a Proinsulin-Encoding Gene

Synthetic double-stranded human insulin promoter (−364/+31) was obtained from Integrated DNA Technologies (IDT, Iowa City, IA, USA). After resuspension of the DNA fragment in Tris–EDTA, an adenylation reaction was performed with dATP and $MgCl_2$ at 70 °C via Taq polymerase. Insulin promoter was then cloned into an entry vector (pENTR5'Ip) using the pENTR 5'-TOPO-TA Cloning kit (Invitrogen K591–20). An entry vector containing the human insulin-encoding gene (pENTRIns; Invitrogen, Ultimate ORF Clone ID IOH7334) was obtained in a bacterial culture. The Virapower Hiperform Promoterless Gateway Expression Kit (Invitrogen, A11145) was used to generate the expression clone (pLentiINS). For this, a MultiSite Gateway reaction was performed overnight at RT with entry vectors pENTR5'Ip and pENTRIns and a target vector pLenti6.4/R4R2/V5-DEST, in the presence of LR Clonase II Plus enzyme. Following proteinase K digestion, the reaction was transformed into *E. coli* and spread on LB agar plates containing ampicillin. Plasmid DNA was isolated by Qiagen Plasmid Mini Kit (Qiagen, Cat. No: 12125) and digested with *AflII* and *XhoI* for identification of the right colonies, followed by confirmation of its DNA sequence using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Cat No: 4337455). Qiagen Plasmid Mega Kit (Qiagen, Cat. No: 12183) was used to isolate larger amounts of plasmid DNA required for transfection. The LentiINS and LentiLacZ viral vectors produced were purified by anion-exchange chromatography, as described previously (Olgun et al. 2019a, b).

2.2.1 Dose Titration of Lentiviral Vectors by qPCR

HT1080 cells (ATCC®CCL-121™) were seeded to 24-well plates at a density of 5×10^4 cells/well. Next, HT1080 cells were transduced with the LentiINS and the beta galactosidase-encoding LentiLacZ vectors at increasing viral doses in the presence of polybrene (6 µg/ml). Cell media were refreshed the next day, and cells were incubated for two more days at 37 °C, 5% CO_2. Media were then removed and replaced with lysis buffer (100 µl/well). Samples were kept at 96 °C, followed by centrifugation at 14,000 rpm for 2 min. Cell lysates were stored at −20 °C before use. Real-time PCR reactions were performed with a Quantitect SYBR Green PCR Kit (Qiagen, Cat No: 204143), as described previously (Tasyurek et al. 2018a). WPRE primers were used for quantification of the integrated lentiviral vector genome, while albumin primers were used as qPCR controls.

2.2.2 In Vitro Gene Expression Analysis of LentiINS Vector

293 T cells (ATCC® CRL-3216 ™), which do not express insulin, and MIN6 pancreatic beta cell lines (kind gift of Prof. Jun-Ichi Miyazaki, Osaka University) were seeded to 24-well plates at a density of 5×10^4 cells/well, for confirmation of the LentiINS vector transgene expression. Both cells were transduced with LentiINS in the presence of polybrene, at increasing MOIs of 0, 5, 25, and 125 for MIN6 cells and an MOI of 125 for 293 T cells. Supernatants were collected 72 h following transduction, and insulin levels were detected with human insulin ELISA kit (Abcam, ab100578).

2.3 Reconstitution of Insulin Gene Expression in Ins2KO Cells by LentiINS

The obtained insulin knockout cells (Ins2KO) were seeded to six-well plates at a density of 2×10^5 cells/well. The next day, LentiINS viral vectors were added to the cells dropwise at various MOIs (1, 10, 25, 50, 100, and 250). On day 3 following transduction, culture media were replaced with fresh media containing 4 μg/ml blasticidin after a PBS wash. Media were replaced with antibiotic-containing fresh media every 2 days, for a total of 14 days.

2.4 Western Blotting Analysis

Cells grown to 80% confluency in 10-cm dishes were lysed in RIPA buffer, and protein concentrations were determined by Bradford assay (BioRad 500–0006). Briefly, Laemmli solution (2×) was added to the cell lysates, which were then boiled for 7 min. Cell lysates (30 μg) were resolved by SDS-PAGE (20%) and electrotransferred to polyvinylidene difluoride (PVDF) membranes (BioRad 162–0177). Membranes were incubated in blocking buffer (5% BSA in PBS-0.1% Tween 20, pH 7.4) for 1 h at RT, followed by three washes of PBS with 0.1% Tween 20. Immunoblotting was carried out at RT for 1 h with mouse monoclonal anti-insulin antibody (Santa Cruz Insulin B (C-12): sc-377,071) or mouse monoclonal anti-GAPDH antibody (Santa Cruz GAPDH Antibody (G-9): sc-365,062). Next, membranes were washed thrice in PBS–0.1% Tween 20, pH 7.4, followed by an hour of incubation with an HRP-conjugated secondary antibody (Abcam Goat Anti-Mouse IgG H&L (HRP): ab205719). Immunoblots were visualized with an enhanced chemiluminescence solution (BioRad 170–5,061) and exposed to X-ray film (Kodak Biomax), followed by development and analysis.

2.5 Induction of STZ-Induced Diabetes in Wistar Rats

Wistar male rats were acquired from Akdeniz University Experimental Animals Unit. Rats were maintained in a humidity and temperature-controlled rodent room on a 12:12 h light:dark cycle with ad libitum access to food and water. Pancreatic beta-cell damage was induced in 6-week-old rats via intraperitoneal injection of streptozotocin (STZ, Sigma-Aldrich; St Louis MO, USA) dissolved in 0.01 M citrate buffer (pH 4.5) in various doses (65, 70, and 80 mg/kg), in a total volume of 300 μl. Blood glucose levels of diabetic rats were measured periodically for 30 days with Accu-Check Compact Glucometer (Roche Diagnostics, Indianapolis, IN, USA, measurement range: 10–600 mg/dl). Animal studies were approved and carried out in compliance with the regulations of Akdeniz University Faculty of Medicine, Institutional Animal Care, and Use Committee.

2.6 Transplantation of Genetically Modified Pancreatic Beta Cells into Diabetic Animals

Pancreatic beta-cell transplantation was performed 10 days after the STZ injections to avoid blood glucose fluctuations and reversion.

Transplantation of genetically modified pancreatic beta cells was performed as described previously (Dirice et al. 2009). Ins2KO pancreatic beta cells were either infected with LentiLacZ or LentiINS at MOI of 100 and transplanted under the kidney capsule of recipient animals at 10^6 million cells/animal. MIN6, LentiINS+Ins2KO, and LentiLacZ+Ins2KO cells were transferred into polyethylene tubing PE-50 (Becton Dickinson, Franklin Lakes, NJ) connected to a 0.5-ml syringe (Hamilton, Reno, NV) at a density of 1×10^6 cells/animal in PBS in a total volume of 30 μl. The left kidney of a rat under intraperitoneal ketamine–xylazine anesthesia was exposed through a lumbar incision. Capsulotomy was performed on the caudal outer surface of the kidney, where the tip of the tubing was inserted and gently advanced under the kidney capsule. After removal of the tubing, the capsulotomy was cauterized with a low-temperature cautery pen. The kidney was placed back into the abdominal cavity, and the incision was closed. Nonfasting blood glucose levels were measured using an Accu-Check Compact Glucometer using blood obtained from tail vein during early afternoon to avoid blood glucose fluctuations.

2.7 Statistical Analyses

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). Data were presented as +/− SEM, and P-value was determined as 0.05.

3 Results

3.1 Generation of Insulin Gene Knockout Pancreatic Beta Cell Line Using Programmable Nucleases

CRISPR/Cas9-mediated gene editing was used to generate insulin-deficient pancreatic beta-cells to produce an insulin knockout beta-cell transplant animal model of diabetes. Specifically, we co-transfected plasmids with three different gRNA/Cas9-encoding genes and two different homology-directed repair (HDR1 or HDR2) plasmids carrying DNA sequences homologous to the targeted mouse insulin gene (Ins1 or Ins2) into Min6 pancreatic beta cell line (Fig. 1a). Detection of green fluorescent protein (GFP) indicated successful transfection of CRISPR/Cas9 KO plasmid, while transfection of HDR plasmid was visually confirmed by detecting red fluorescent protein (RFP) under fluorescent microscopy. As gRNAs/Cas9 complex encoded by CRISPR/Cas9 KO plasmids generates double-stranded DNA breaks, homologous recombination was mediated by HDR plasmids resulting in the insertion of puromycin antibiotic resistance and RFP genes in the targeted insulin gene. Fluorescein signals were examined under fluorescence microscopy 48, 72, and 96 h after the transfection (Fig. 1b). While GFP and RFP signals were both observable 48 h following the transfection, GFP signal intensity decreased 72 h after the transfection. Only the RFP signal remained stable for 96 h following the transfection. Then, transfected pancreatic beta cells were exposed to puromycin at 1.8 mg/ml for 14 d to select for stable transformants defined as RFP-expressing cells (Fig. 1c). Western blotting analysis to detect both Ins1 and Ins2 gene expressions revealed that insulin expression was abrogated only in the Ins2 knockout cell line but not in the Ins1 knockout cells (Fig. 4). Insulin gene knockout was also confirmed by DNA sequencing. Thus, the Ins2 knockout cell line was tested as an insulin-deficient pancreatic beta cell line in the following beta-cell transplantation experiments.

3.2 Construction of a Third-Generation Lentiviral Vector Encoding Insulin Gene (LentiINS)

To provide pancreatic beta-cell-specific insulin gene expression, a lentiviral vector with the insulin promotor connected to an insulin encoding gene was generated. To do this, the DNA

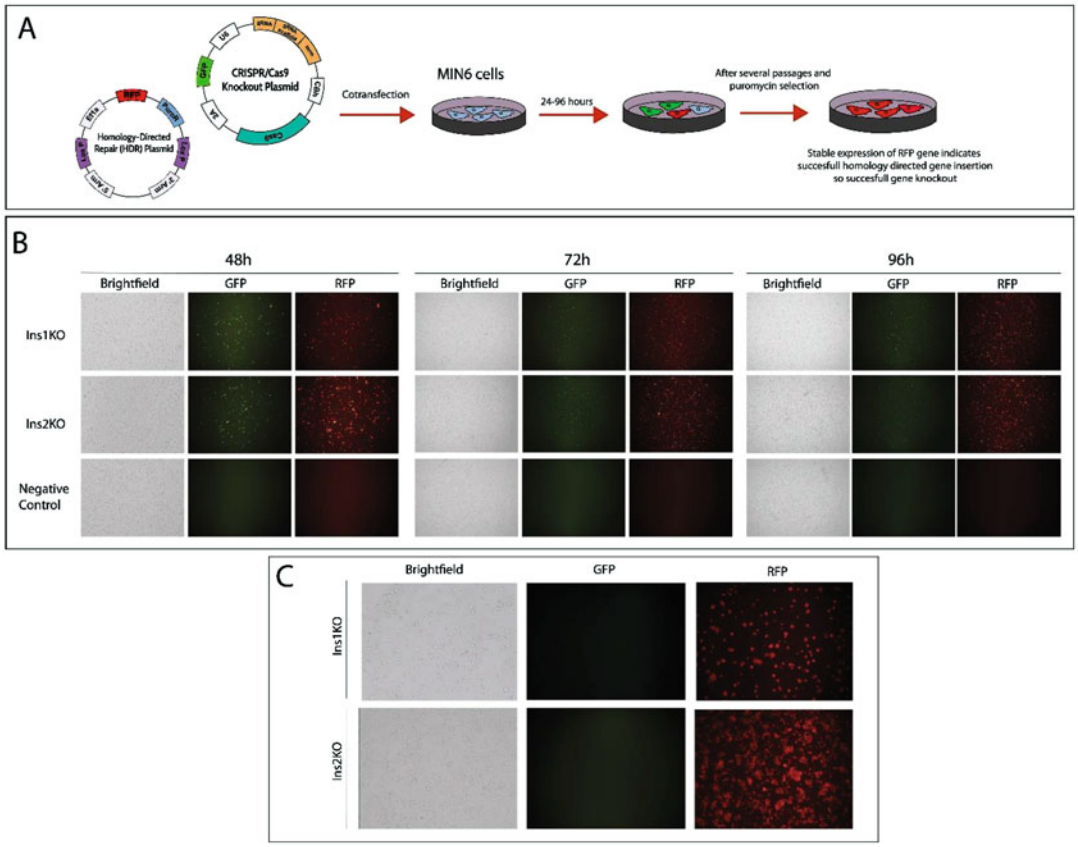

Fig. 1 CRISPR/Cas9-mediated insulin gene knockout process in pancreatic beta cells. (**a**) Experimental strategy for generating an insulin-knockout mouse pancreatic beta cell line using CRISPR/Cas9 and homology-directed repair mechanism. Knockout plasmid encodes sgRNA, Cas9, and GFP reporter genes. Insulin gene DNA fragments (5′–3′ arms) flanked by LoxP sites, RFP, and puromycin resistance genes are located within the homology directed repair plasmid (HDR). (**b**) Fluorescence imaging of MIN6 cells at 48, 72, and 96 h after co-transfection with either insulin1 (Ins1KO) or insulin2 knockout (Ins2KO) plasmids in association with their corresponding HDR (HDR1 or HDR2) plasmids (48 h, 100× magnification; 72 and 96 h, 40× magnification). GFP signal indicates successful transfection with a knockout plasmid, while the presence of HDR plasmid is revealed by an RFP signal as detected under fluorescence microscopy. (**c**) Selection of stable RFP expressing cells, an indication of successful homologous recombination between HDR plasmid and the genome of MIN6 cell line. Puromycin (1.8 mg/ml) was added to the culture medium every other day for 14 days. Site-specific recombination results in the integration of both puromycin and RFP genes to either Ins1 (Ins1KO) or Ins2 (Ins2KO) gene locus depending on the HDR plasmid used

sequence of the insulin promotor was first cloned into the pENTR5'TOPO vector (Fig. 2a), generating a promotor entry vector (pENTR5'Ip). Then, an entry vector carrying an insulin-encoding gene (pENTRIns) and insulin promotor entry vector (pENTR5'Ip) were all combined in a Multisite Gateway LxR recombination reaction with a destination vector (pLenti6.4/R4R2/V5-DEST) to produce the expression clone (pLentiINS; Fig. 2b).

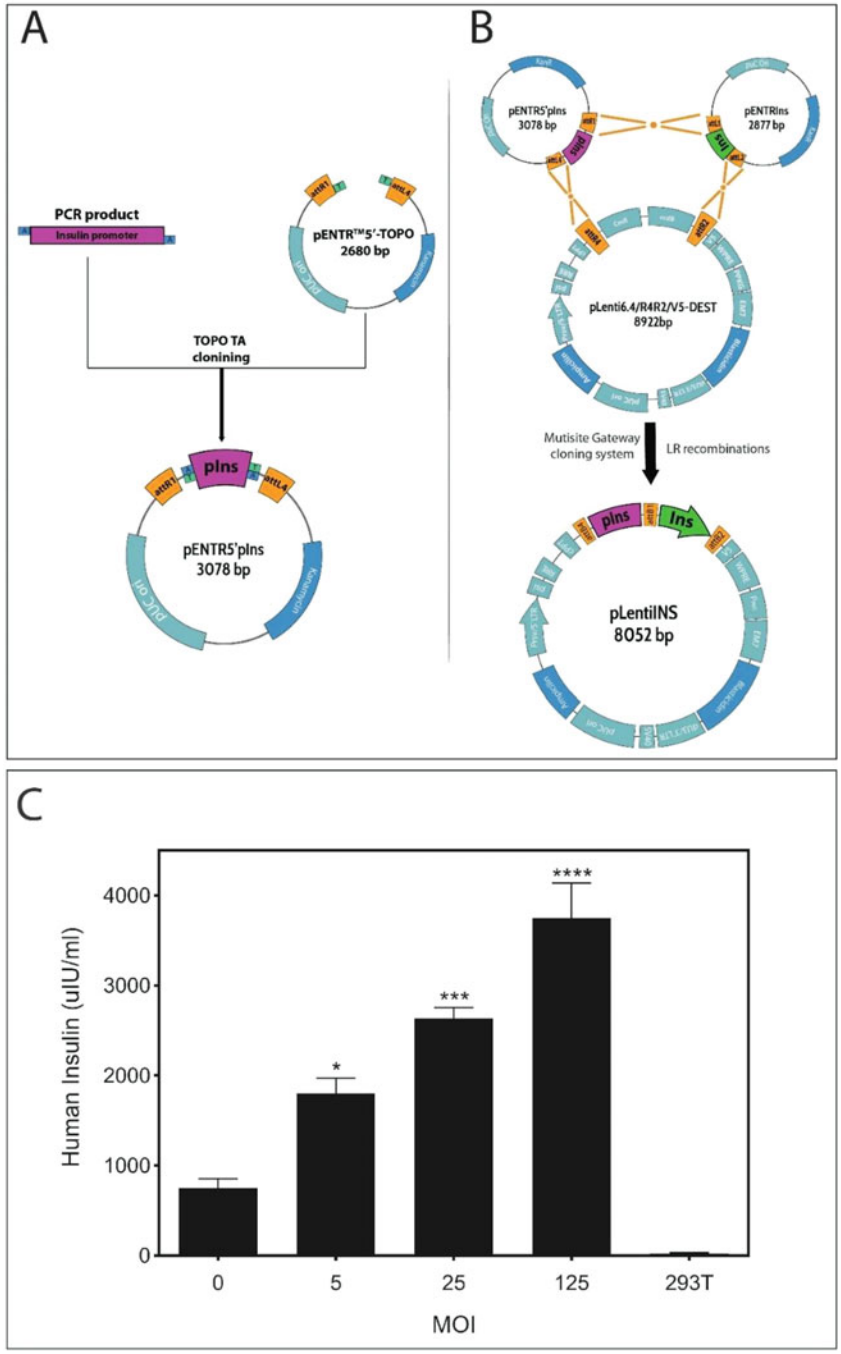

Fig. 2 Generation of third-generation lentiviral vectors encoding insulin gene (pLentiINS). (**a**) Insulin promotor entry vector (pENTR5'Ip) is produced by TOPO/TA cloning of insulin promotor into pENTR5'TOPO plasmid. (**b**). Multisite Gateway LxR recombination between insulin gene entry vector (pENTRIns), insulin promotor entry vector (pENTR5'Ip), and the destination vector (pLenti6.4/R4R2/V5-DEST) yield the expression clone (transfer plasmid-pLentiINS). (**c**) LentiINS transduction of the MIN6 pancreatic beta cell line but not 293 T cells results in insulin gene expression. Lentiviral vector-carrying insulin gene (LentiINS) is produced by transient transfection of 293 T cells with pLentiINS and packaging plasmids as described in Materials and Methods. LentiINS vector was infected into the MIN6 cell line at increasing doses as indicated in the fig. 293 T cells were infected with 125 MOI of LentiINS. ($n = 4$, one-way ANOVA, $^{*}p = 0.01$, $^{***}p = 0.0002$, **** $p < 0.0001$)

3.3 Insulin Promotor-Directed Insulin Gene Expression Via Lentiviral Vectors Is Limited to Pancreatic Beta Cell Lines

Co-transfection of pLentiINS with packaging plasmids (VSV-G, gag-pol, REV) into the 293 T cell line resulted in the production of lentivirus vectors carrying insulin genes (LentiINS). LentiINS was further purified using ion-exchange chromatography, and the viral titer was determined by real-time PCR using genomic DNA isolated from HT1080 cells infected with LentiINS. To assess tissue tropism of the newly generated gene therapy vector, LentiINS was infected into both 293 T cell line and a pancreatic beta cell line MIN6. ELISA assays performed using supernatants obtained from LentiINS-infected cells demonstrated insulin expression was only detectable in the MIN6 cell line but not in the 293 T cell line (Fig. 2c).

3.4 LentiINS Transduction of the Ins2-Knockout MIN6 Cell Line Restored Insulin Gene Expression

Because the Ins2-knockout cell line completely abrogated insulin gene expression in the MIN6 cell line (Fig. 4), this cell line was used as a model for insulin deficiency/complementation experiments concerning MODY-10. To test the therapeutic efficacy of the newly generated insulin-encoding lentivirus (LentiINS), the Ins2KO pancreatic beta cell line was infected with the LentiINS vector at increased doses (Fig. 3). Ins2KO beta cell line infected with LentiINS vector at an MOI of 100 appeared to manifest higher levels of transduction and cell viability compared to other doses used. Stable transformants carrying integrated copies of LentiINS were obtained by blasticidin selection, which lasted 14 days.

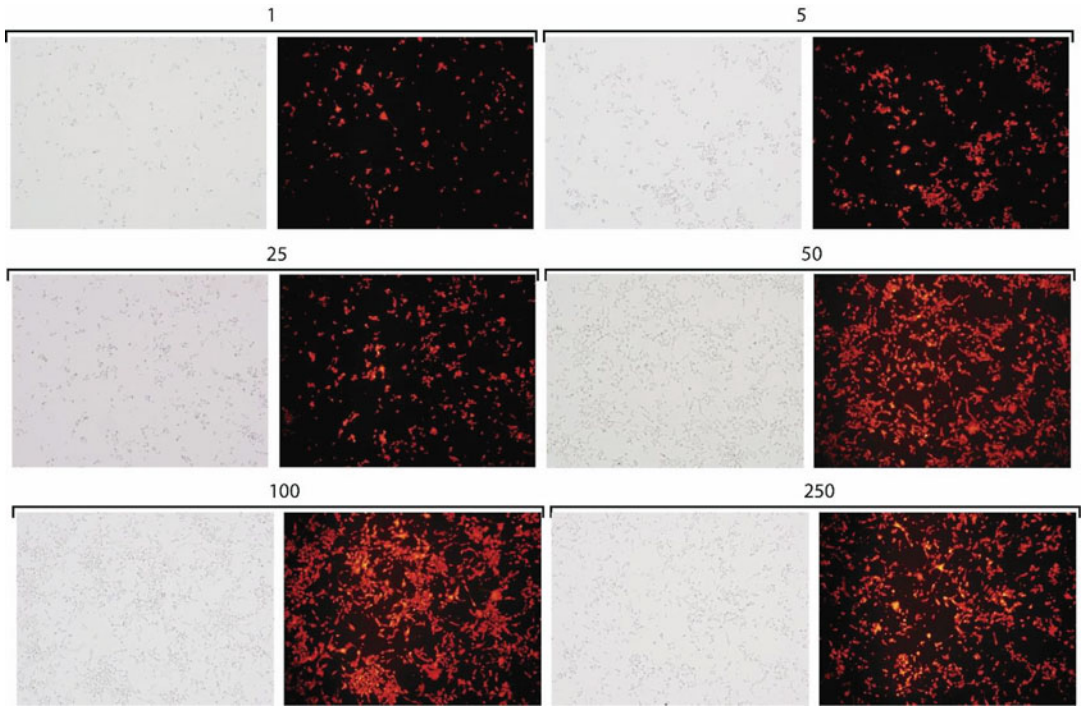

Fig. 3 LentiINS transduction of İns2KO MIN6 cell line. LentiINS vector was injected into the İns2KO MIN6 cell line at increasing doses as indicated in the figure ($n = 3$). Blasticidin application to select stable transformants (LentiINS integration) was carried out at 4 mg/ml by refreshing the cell culture medium every other day for 14 days

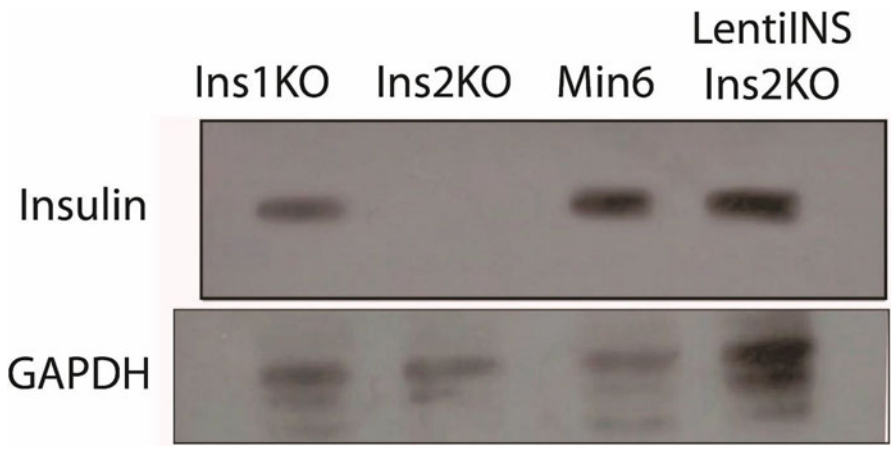

Fig. 4 Western blotting analysis. Western blotting analysis demonstrating insulin expression of MIN6, Ins1KO, Ins2KO, and LentiINS-infected Ins2KO cell lines (LentiINS + Ins2KO). GAPDH expression was used as an internal control

Insulin production by parental MIN6, Ins1KO, Ins2KO, and Ins2KO pancreatic beta cell lines complemented with LentiINS (LentiINS +Ins2KO) was determined by western blotting using antibodies specific for the insulin protein (Fig. 4). As expected, insulin production was readily detectable in the parental MIN6 pancreatic beta cell line. By comparison, there was a slight decrease in insulin production in the Ins1KO cell line and a complete loss of insulin production in the Ins2KO cell line. Importantly, stable LentiINS transduction of the Ins2KO cell line resulted in the restoration of insulin gene expression.

3.5 Generation of STZ-Induced Animal Model Diabetes for Beta-Cell Transplantation

To induce diabetes, we destroyed pancreatic beta cells in the animals. Then, transplantation of genetically modified beta cells produced by CRISPR/Cas9 technology would generate an insulin knockout beta-cell transplant animal model of diabetes. Thus, streptozotocin (STZ) was chosen to destroy pancreatic beta cells to test the functional status of genetically modified pancreatic beta cells. Briefly, male Wistar rats were injected with increasing doses of STZ, and blood glucose levels were followed periodically. All animals became diabetic 3 d after the STZ injections and remained diabetic throughout the follow-up period (Fig. 5a). Mortality rates of animals injected with 80 mg/kg STZ were rather high (Fig. 5b). Since 70 mg/kg STZ-injected animals were slightly lethargic and irritable, 65 mg/kg STZ dose was used as an optimum dose to induce diabetes in animals.

3.6 Testing the Functional Status of Genetically Engineered Pancreatic Beta Cells for Transplantation Purposes

Genetically engineered pancreatic beta cells were transplanted under the kidney capsule of diabetic Wistar rats 10 d after the STZ injections (Fig. 5c). No decrease in blood glucose was detected in diabetic animals transplanted with AdLacZ-infected Ins2KO cells suggesting the successful generation of the insulin knockout beta-cell transplant animal model of diabetes. Blood glucose levels were transiently reduced for a week in animals transplanted with genetically unmodified MIN6 pancreatic beta cells (positive control). In contrast, STZ-injected but sham-operated control animals (STZ control) did not manifest any decrease in blood glucose. Contrary to this

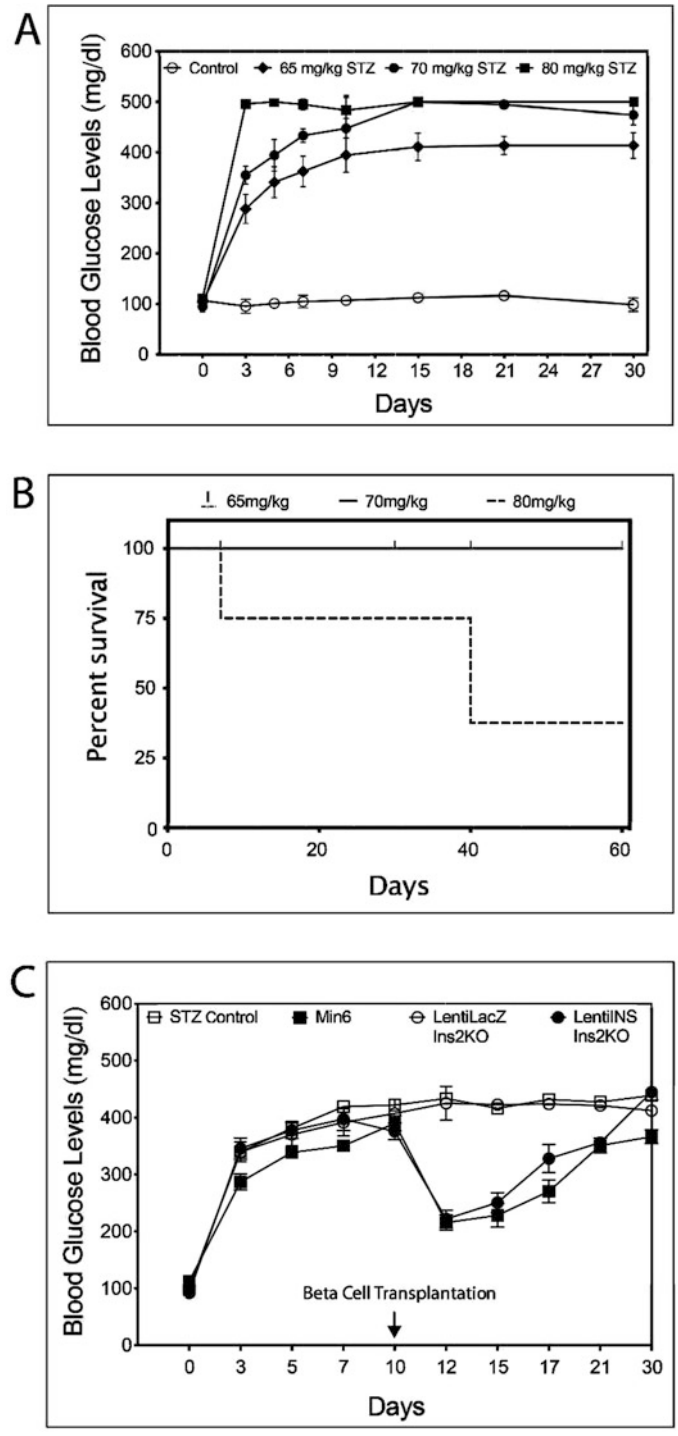

Fig. 5 Functional assessment of genetically modified pancreatic islets in diabetic animals. (**a**) Induction of STZ-induced diabetes leading to absolute insulin deficiency for pancreatic beta-cell transplantation purposes. Wistar rats ($n = 8$/group) were injected with STZ at 3 different doses (65, 70, and 80 mg/kg), and blood glucose levels were periodically monitored (ordinary one-way ANOVA, control vs STZ injections, $p < 0.05$). (**b**) Survival rates of STZ-injected Wistar rats. Control indicates PBS-injected rats in place of STZ. (**c**) Therapeutic efficacy of genetically modified pancreatic beta cells (MIN6) for transplantation. Diabetes was induced by IP injection of 65 mg/kg STZ into Wistar rats. Ten days after the STZ injection, pancreatic beta cells were transplanted under the kidney capsule of diabetic rats ($n = 6$). Blood glucose levels were periodically monitored to assess the functional status of transplanted pancreatic beta cells (two-way-ANOVA, Dunnett's multiple comparison test, STZ vs. LentiLacZ + Ins2KO, not significant (ns); STZ vs. Min6 and LentiLacZ + Ins2KO vs. LentiINS Ins2KO, $p < 0.0001$)

observation, animals transplanted with LentiINS-infected Ins2KO cells displayed a transient reduction in blood glucose similar to what was observed with the transplantation of MIN6 pancreatic beta cells alone. These results suggested that LentiINS infection of insulin knockout pancreatic beta cells successfully complemented insulin deficiency in these cells.

4 Discussion

Pathogenesis of T1D involves immune-mediated destruction of the pancreatic beta cells (Sanlioglu et al. 2008), but not all children with T1D exhibit signs of autoimmunity and are classified as having idiopathic/autoantibody-negative/type 1b diabetes (Edghill et al. 2006). Insulin deficiency is a common denominator of both pancreatic beta-cell destruction and/or functional deficit. Although rare, mutations in the insulin gene (as seen in MODY10 patients) cause overt diabetes (Molven et al. 2008). These patients, like those with true T1D, require insulin injections over time to delay diabetes-related complications (Delvecchio et al. 2020). Pancreatic beta-cell-specific insulin gene therapy might be valuable for early-stage T1D patients who did not lose significant portions of pancreatic beta cells yet. However, unlike type 2 diabetes, late-stage T1D patients possess a limited number of pancreatic beta cells due to immune attack. Expectedly, the residual beta cells in late-stage T1D patients may not be sufficient as host cells for transgene expression. Insulinotropic, anti-inflammatory gene therapy modalities might be needed to induce pancreatic beta-cell proliferation prior to insulin gene delivery to observe a therapeutic benefit, as shown recently (Erendor et al. 2021a). Thus, insulin gene therapy specific for pancreatic beta cells might be valuable not only for T1D but also MODY 10 patients. However, insulin knockout but not deficient mice are available for testing. Despite this, it would be challenging to test in vivo therapeutic efficacy of insulin gene delivery in the insulin gene knockout transgenic mice since these mutant pups die within 48 h after birth (Duvillie et al. 1997).

Considering insulin gene therapy, STZ has widely been used to induce diabetes from rodents to larger animal models (dogs, cats, rabbits, pigs, etc.) (Callejas et al. 2013; Jaen et al. 2017; Jensen-Waern et al. 2009; Robinson et al. 2012; Saleem Mir et al. 2015; Weinstein and Gertner 1971). Although most of the pancreatic beta cells can be destroyed by STZ injection, the remaining beta cells may continue secreting insulin (Wang and Gleichmann 1998). To determine the true potential of pancreatic beta-cell-specific insulin gene delivery, pancreatic beta cells defective in insulin gene synthesis are needed. Since these genetically modified pancreatic beta cells are not commercially available, these modifications have to be introduced using engineered nucleases as described herein. CRISPR/Cas9 programmable nucleases were used in this study to generate insulin gene-mutant pancreatic beta cell line. This was accomplished by the co-transfection of knockout plasmids with different gRNA/Cas9 encoding genes and homology-directed repair (HDR1 or HDR2) plasmids carrying DNA sequences homologous to Ins1 or Ins2 into the MIN6 pancreatic beta cell line. Western blot analysis confirmed insulin expression was knocked out in Ins2KO beta cells, but not in Ins1KO beta cells. Interestingly, we could not detect any insulin expression in Ins2KO cells despite these cells having intact Ins1 genes. This observation is in accordance with previous data showing a lack of Ins1 gene expression in monolayer-grown advanced passages of MIN6 cells (Nakashima et al. 2009; Roderigo-Milne et al. 2002). These data also suggest that Ins2KO pancreatic beta cells might be used to develop a novel insulin-deficient animal model of diabetes involving the transplantation of genetically modified pancreatic beta cells. Nevertheless, transplantation of the Ins2KO cell line failed to reduce blood glucose of STZ-induced diabetic animals, confirming the Ins2 knockout mutant beta cell line is functionally defective in insulin gene expression.

As an alternative to the fluctuating insulin levels provided by repeated bolus insulin injections, a lentiviral vector (LentiINS) carrying an insulin promotor driving the proinsulin gene sequence was generated to better manage daily

blood glucose levels and reduce the necessity for exogenous insulin. Pancreatic beta cell restricted insulin gene expression as demonstrated in MIN6 cell line, but not in 293 T cells, indicating that the LentiINS gene therapy vector exhibited some tissue preference. To evaluate the therapeutic efficacy of the LentiINS vector in an insulin knockout beta-cell transplant animal model of diabetes, the insulin-deficient Ins2KO cell line was infected with LentiINS. Blasticidin selection was utilized to obtain stable transformants with integrated copies of LentiINS and restoration of insulin expression was confirmed via western blotting in the Ins2KO cell line. Transplantation of Ins2KO beta cells infected with LentiINS under the kidney capsule of STZ-induced diabetic animals resulted in the transient reduction of blood glucose similar to what was observed with unmodified MIN6 cells.

There are some basic problems that need to be solved here in order to make this approach an effective treatment. First of all, considering the 3D structures of the islets and the beta-cell content, it is necessary to develop more effective gene delivery methods in order to achieve an effective in vivo gene transfer. Furthermore, in vivo applications concerning genome integrating viral vectors may bring risks such as insertional mutagenesis and oncogene activation (Schambach et al. 2013; Woods et al. 2003; Zhou et al. 2013). This can be circumvented by using integrase deficient lentiviral vectors (Yánez-Munoz et al. 2006; Yew et al. 2022). Despite this, lentiviral vectors are considered to be pretty safe when they are used in ex-vivo human gene therapy studies (Kwiatkowski et al. 2020; Westin et al. 2021). In this scenario, cells isolated from the patient were infected with lentiviral vectors, then these cells are reinfused back into the patient following examination and selection of corrected colonies.

Collectively, these results suggest CRISPR/Cas9 programmable endonucleases could successfully be used to generate insulin knockout mutant pancreatic beta cell lines. Transplantation of the Ins2KO cell line into STZ-induced diabetic animals allowed us to study the functional properties of pancreatic beta cells in the absence of insulin gene expression generating a new insulin knockout beta-cell transplant animal model of diabetes. Transient reduction of blood glucose of diabetic animals transplanted with LentiINS-modified Ins2KO cells suggests that LentiINS gene therapy vector functionally restored insulin gene expression in the insulin knockout beta-cell transplant animal model of diabetes.

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The Link Between Heat Shock Proteins, Renin-Angiotensin System, and the Coagulation Cascade in the Pathogenesis of the Coronavirus-19 Disease

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Abstract

Introduction: Understanding the pathogenesis of COVID-19 is integral for its successful treatment.

Methods: Available literature on the relationship between COVID-19, heat shock proteins (HSP), and the renin-angiotensin-aldosterone (RAAS) system were searched and used to hypothesize how HSP can be targeted in COVID-19.

Results: During SARS-CoV-2 cellular entry, the ACE-2 receptor is downregulated. This leads to the augmentation of angiotensin-2/AT1 receptor axis along with attenuation of the ACE-2/angiotensin$_{1–7}$/Mas axis. Heat shock proteins are key stabilizing molecules in various pathways.

In the heart and vessels, HSP-90 and HSP-60 can facilitate angiotensin-2-mediated myocardial injury and endothelial cell activation. HSP-60-TLR4/CD14 complex formation stabilizes IκB-kinase (IKK) potentiating NF-κB activation. HSPs in lungs and kidneys have antioxidant, vasodilatory, and anti-inflammatory actions and may be protective against the effects of RAAS. Stress-induced HSP-70 has a role in complement-mediated microvascular injury such as has been demonstrated in COVID-19. SARS-CoV-2 can induce autophagy via Beclin-1 and ER (endoplasmic reticular) stress via BIP. These two can be potential targets in the HSP environment.

Conclusion: Various HSP molecules can modulate the effects of the renin-angiotensin-aldosterone (RAAS) system and thus may have a potential role in the pathogenesis of COVID-19.

Keywords

Coagulation · COVID-19 · Heat shock proteins · Renin-angiotensin-aldosterone system · SARS-CoV-2 · Thrombosis

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Abbreviations

ACE1	Angiotensin-converting enzyme 1
ACE2	Angiotensin-converting enzyme 2
Ang	Angiotensin

APD	Aminopeptidase D
APN	Alanyl aminopeptidase N
AT1-R	Angiotensin receptor type 1
COVID-19	Coronavirus 2019 disease
DCT	Distal convoluted tubule
e-NAP	Epithelial NOS-associated protein
e-NOS	Epithelial nitric oxide synthase
G-CSF	Granulocyte colony-stimulating factor
GFR	Glomerular filtration rate
GRP78/BIP	Glucose regulatory protein78/ binding immunoglobulin protein
HOX1	Heme oxygenase 1
HSP	Heat shock protein
IL	Interleukin
MAPK	Mitogen-activated protein kinase
MasR	Mas receptor
MCP1	Monocyte chemoattractant protein1
MCR	Mineralocorticoid receptor
MEK	Mitogen-activated protein kinase kinase
MERS	Middle East respiratory syndrome
MIP1α	Macrophage inflammatory protein
MrgD	Mas-related G-protein-coupled *receptor* type D
MyD88	Myeloid differentiation primary response 88
NF- κB	Nuclear factor-kappa beta
NO	Nitric oxide
PCT	Proximal convoluted tubule
PRR	Prorenin
RAAS	Renin-angiotensin-aldosterone system
SARS	Severe acute respiratory syndrome
SARS-CoV-2	Severe acute respiratory syndrome coronavirus-2
TAL	Thick ascending limb of loop of Henle
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VSMC	Vascular smooth muscle cell

1 Introduction

The coronavirus-19 disease (COVID-19) caused by the SARS-CoV-2 virus has swept through the world as a pandemic. Though a pandemic had been anticipated by the turn of the century (Threats et al. 2004), practically few individuals or even nations had imagined the actual crisis that is unfolding. As it is taking its toll on the weakened health infrastructure, medical supplies, and health workers (Peeri et al. 2020), there is an urgent need to understand how the virus leads to different pathological manifestations.

Genomic sequencing revealed its similarity with few other coronaviruses that were responsible for SARS-2003 (severe acute respiratory syndrome) and MERS-2012 (Middle East respiratory syndrome) outbreaks (Wu et al. 2020). The virus enters the human cells, especially the cells of the respiratory tract via ACE-2 (angiotensin-converting enzyme 2) receptor, where it multiplies inside the cells (Linton et al. 2020). Unlike other respiratory tract infections, it tends to involve multiple systems and thus has many atypical presentations such as acute kidney injury, cardiomyopathy, and thrombotic manifestations (Zaim et al. 2020).

Upon pathogen entry into the human cells, the membrane-bound ACE-2 receptor is internalized and downgraded in the lysosome. This leads to unopposed action of the canonical ACE-renin-angiotensin pathway leading to elevated levels of Ang II (angiotensin 2) and aldosterone. This pathway has the potential to contribute to the pathogenesis of COVID-19 (Verdecchia et al. 2020; Ahmed et al. 2020a).

Heat shock proteins (HSP) have an integral role in regulating the inflammatory response and maintaining cellular homeostasis (Tká 2012). This chapter intends to explore the potential role of various HSPs in orchestrating the events

occurring as a result of activation of the renin-angiotensin-aldosterone system (RAAS).

2 Physiology of the Renin-Angiotensin System

RAAS is one of the most important regulators of blood volume and systemic vascular resistance, responsible for maintaining blood pressure over a long period. It is stimulated in response to (i) decreased blood volume, (ii) decreased sodium, and (iii) adrenergic β-receptor agonism. The net result of RAAS activation is elevated blood pressure and blood sodium concentration.

2.1 The Canonical RAAS Pathway

The canonical or classically known pathway involves renin, ACE, Ang II, and their receptors. (Fountain and Lappin 2019) The juxta-glomerular (JG) cells of the kidney are present around the afferent arterioles. These secrete prorenin (PRR) constitutively. The JG cells are activated in response to lowered sodium delivery (due to poor perfusion or adrenergic β-receptor agonism) causing cleavage of PRR into renin, enabling the entire system. Angiotensinogen is a polypeptide produced by the liver. Renin converts it into an oligopeptide Ang I (angiotensin 1). Ang I is physiologically inactive and is converted to Ang II by ACE-1 (angiotensin-converting enzyme 1), commonly found in the vascular endothelium of kidneys and lungs. Ang II thus generated has many effects, one of those is acting on the AT-R (angiotensin receptors) present in the zona glomerulosa, stimulating the release of aldosterone.

Ang II is a potent vasoconstrictor. It acts directly by binding to the G-protein-coupled receptor present in the arterioles and indirectly by increasing the sodium reabsorption from the PCT (proximal convoluted tubule), while it also acts on the central nervous system, by stimulating the thirst centers, as well as by causing the release of ADH (antidiuretic hormone). Ang II is a fast-acting peptide hormone, whereas aldosterone is a steroid hormone. Thus, aldosterone acts at the nuclear level and takes time to show its action. Aldosterone also increases sodium reabsorption, except that it works on the CD (collecting duct) and DCT (distal convoluted tubule).

2.2 Non-canonical Pathway

These pathways have been described comparatively recently in the last two to three decades (Paz Ocaranza et al. 2020). They comprise of the antihypertensive and anti-inflammatory $Ang_{1–7}$/Mas and alamandine/Mrg-D pathways and the pro-inflammatory, canonical pathway involving Ang III (angiotensin 3) and Ang IV (angiotensin 4) that can augment the canonical pathway.

The ACE-2-dependent pathways involve angiotensin (1–9), angiotensin (1–7), angiotensin A, AT-2R (angiotensin type 2 receptor), Mas receptor (MasR), and the Mas-related G-protein-coupled receptor member-D (MrgD). Ang II is acted upon by ACE-2 and Neprilysin to produce Ang (1–9) and Ang (1–7), respectively. Ang (1–7) can also be produced by the action of ACE-2 on Ang II and ACE on Ang (1–9), respectively. Ang (1–7) mediates its vasodilatory, nitric oxide (NO) secretory, natriuretic, antifibrotic, and anti-inflammatory actions via MasR. When acted upon by aspartate decarboxylase, Ang II is cleaved to angiotensin A, a precursor of alamandine. Alamandine exerts vasodilatory, NO secretory, and antiproliferative action via Mrg-D. Thus, the existence of different pathways of RAAS ensures a yin-yang relationship to maintain homeostasis.

3 HSP and the RAAS System

Ang II influences the renal vascular tone by inducing the enzyme, epithelial-nitric oxide synthase (e-NOS), generating NO, which is a potent vasodilator (Liu Ruisheng et al. 2004). The action of e-NOS is strongly influenced by

HSP-90, and the use of an HSP-90 inhibitor (Radicicol) reduces the GFR (glomerular filtration rate) (Ramírez et al. 2008). e-NOS and HSP-90, working in synergy, undergo apical translocation in the thick ascending loop of Henle (TAL) and inhibit the activity of Na-K-2Cl channel, probably in response to the increased tubular flow (Ortiz et al., 2004). HSP-90 is also involved in stabilizing the structure of the MCR (mineralocorticoid receptor). HSP-90 maintains the receptor in its high-affinity form, allowing aldosterone to exert its action (Ramírez et al. 2004). Thus, it acts as a gatekeeper for aldosterone function. Reduction in the GFR in response to increased sodium concentration in the tubular fluid is called tubuloglomerular feedback (TGF). The exact mechanism responsible is not yet fully understood, but Ang II has a role in enhancing the same feedback, by modulating the action of JG cells (Wang et al. 2001). Ang II is also responsible for mesangial cell contraction by causing HSP-25 phosphorylation via p38 MAPK (mitogen-activated protein kinases) pathway. This mechanism may have a role in TGF enhancement (Müller et al. 1999) (Fig. 1).

4 Interaction of RAAS and Heat Shock Proteins in COVID-19

The SARS-CoV-2 infection has shown to deplete the plasma levels of ACE-2 (Hoffmann et al. 2020). This deficiency of the enzyme ACE-2 leads to unopposed activation of the canonical RAAS pathway that can be tied up with pathology in different systems.

4.1 The Respiratory System

Lung injury in COVID-19 is linked to the release of the inflammatory mediators such as interleukin-6 (IL-6), macrophage inflammatory protein (MIP)1α, granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein-1 (MCP-1), interleukin-10 (IL-10), and tumor necrosis factor (TNF)-α (Yuki et al. 2020). However, studies based on the 2003 SARS outbreak revealed that RAAS has a pivotal role to play in causing acute lung injury. Murine studies have revealed that depletion of ACE-2, which was also seen in SARS 2003 by similar mechanisms, correlated with the severity of the lung pathology (Kuba et al. 2005), and different authors stated that ACE-2 could be protective against severe acute lung failure (Imai et al. 2005).

The role of heat shock proteins in acute lung injury is mostly cytoprotective. Although literature illustrating the exact mechanism responsible for conferring cytoprotection is limited, the actions are briefly discussed below:

i. *HSP-27*: Antioxidant and antiapoptotic, probably by modulating the enzymes glutathione reductase, glucose-6-phosphate dehydrogenase, and glutathione transferase.
ii. *HSP-32/HOX1*: Antioxidant action is exerted by the products of heme degradation, biliverdin, along with ferritin.
iii. *HSP-70*: Antioxidant, antiapoptotic, and anti-inflammatory.
iv. *HSP-60*: Data relating HSP-60 and acute lung injury are limited, but HSP-60 is upregulated in the respiratory epithelium of the asthmatic patients (Wheeler and Wong 2007).

4.2 The Cardiovascular System

Ang II and aldosterone have been implicated in various cardiovascular diseases. Ang II is a primary factor driving cardiac remodeling, which involves cardiomyocyte hypertrophy and myocardial fibrosis (Díez 2004). Ang II also is known to have deleterious effects on blood vessels via (i) endothelial dysfunction and (ii) vascular hypertrophy, leading to decreased compliance.

4.2.1 HSP-90

HSP-90 can modulate the effect of aldosterone on the heart. HSP-90, along with other co-chaperones, is responsible for forming a hetero-complex with the mineralocorticoid receptor, which keeps the receptor in high-affinity conformation (Couette et al. 1996), allowing

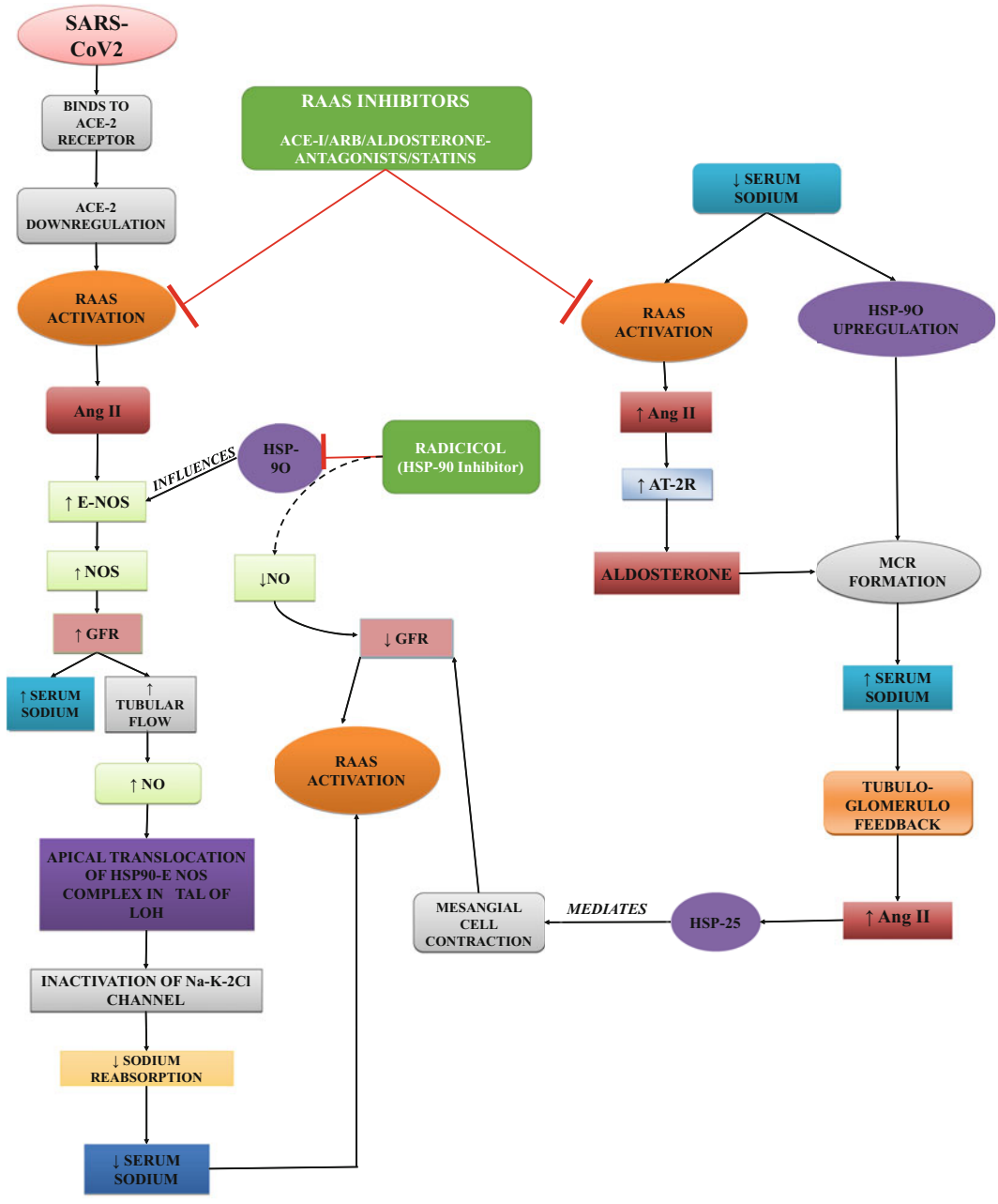

Fig. 1 Link between HSPs and RAAS
Abbreviations; *ENOS* Epithelial Nitric Oxide Synthase, *GFR* Glomerular Filtration Rate, *HSP* Heat Shock Protein, *RAAS* Renin Angiotensin Aldosterone System, *MCR* Mineralocorticoid Receptor, *Ang II* Angiotensin 2, *AT-2R* Angiotensin type 2 receptor, *ACE* Angiotensin converting enzyme, *ACEI* Angiotensin converting enzyme inhibitor, *ARB* Angiotensin receptor blocker, *TAL-LOH* Thick ascending limb of loop of henle

unhindered binding with aldosterone. HSP-90 functions are associated with myocardial injury and heart failure.

Ang II can induce nuclear factor-κB (NF-κB) activation in the cultured cardiac cell (Brasier et al. 2000), and this is responsible for ventricular

myocyte hypertrophy. Also, NF-κB forms a positive feedforward loop, resulting in increased synthesis of angiotensinogen. HSP-90 is required to stabilize the IκB kinase (IKK) that is integral to the activation of NF-κB (Lee et al. 2010). The use of HSP-90 inhibitor geldanamycin results in the suppression of Ang II-induced NF-κB activation. Thus, the actions of both aldosterone and Ang II are dependent on HSP-90 (Fig. 2).

4.2.2 HSP-60

Prolonged stimulation of vascular smooth muscle cells by Ang II causes upregulation of proteins like HSP-60, HSP-70, and calreticulin (Patton et al. 1995). Again, humans, as well as chlamydial HSP-60, can stimulate the macrophages to release metalloproteinase-9 and TNF-α (Kol Amir et al. 1998). This further leads to the activation of human endothelial cells, smooth muscles, and monocyte-derived macrophages to release IL-6. HSP-60 can induce the expression of E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular adhesion molecule on the endothelial cells. Moreover, HSP-60 mediates monocyte adhesion with the endothelium via CD14 in vitro and in vivo (Xu 2002). Toll-like receptor-4 (TLR-4) and CD14 both act as receptors for soluble-HSPs. In vitro studies have revealed that binding of TLR-4 or CD14 with exogenous HSP-60 leads to vascular smooth muscle cell proliferation, mediated by MAPKs (mitogen-activated protein kinases) (Sasu et al. 2001).

Thus, the upregulated HSP-60 can lead to inflammatory changes in the vascular endothelium and may as well as precipitate the IL-6-induced "cytokine storm" in patients suffering from severe COVID-19 infections (Zhang et al. 2020) (Fig. 2).

4.2.3 HSP-70

Similar to HSP-60, HSP-70 is also upregulated by prolonged exposure to Ang II (Patton et al. 1995). Although the role of HSP-70 in endothelial inflammation is controversial, it may have a protective role against atherosclerosis by reducing inflammation, apoptosis, and oxidative stress induced by the unfolded proteins response (Bielecka-Dabrowa et al. 2009). However, exogenous HSP-70 can activate monocytes, upregulating the expression of pro-inflammatory cytokines TNF-α, interleukin (IL)-1β, and IL-6 (Asea et al. 2000). Also, similar to HSP-60, HSP-70 can also bind to TL-4/CD14 receptor activating the MyD88 (myeloid differentiation primary response 88)-dependent NF-κB signaling pathways, responsible for inflammation (Bulut et al. 2002) (Fig. 2).

4.2.4 HOX/HSP-32

HSP-32, also known as HOX-1 (heme oxygenase), is an enzyme that catalyzes the oxidation of heme, releasing iron, biliverdin (an antioxidant), and carbon monoxide (a vasodilator) (Maines 1997). Murine studies have revealed that exposure to Ang II for a prolonged period leads to downregulation of HOX-1 in the vascular smooth muscle cells (Ishizaka and Griendling 1997). As a result, the antioxidant activity of biliverdin is reduced, which, along with Ang II-mediated vascular superoxide production (Rajagopalan et al. 1996), adds to the oxidative stress. Apart from that, vasoconstriction may be augmented due to reduced production of carbon monoxide (Fig. 2).

4.3 The Renal System

Even in the kidneys, HSPs may have a protective role by modulating the harmful effects of RAAS activation.

4.3.1 HOX-1/HSP-32

As opposed to its action on vascular smooth muscle cells, Ang II causes upregulation of the HOX-1 in the murine kidney (Aizawa Toru et al. 2000). Increased HOX-1 activity augments antioxidant response via biliverdin and renal vasodilation via carbon monoxide (Fig. 2).

4.3.2 HSP-90

AT-2R (angiotensin type 2 receptor) has a low prevalence in adult humans. It is only found upregulated in response to certain pathological conditions like heart failure, myocardial injury,

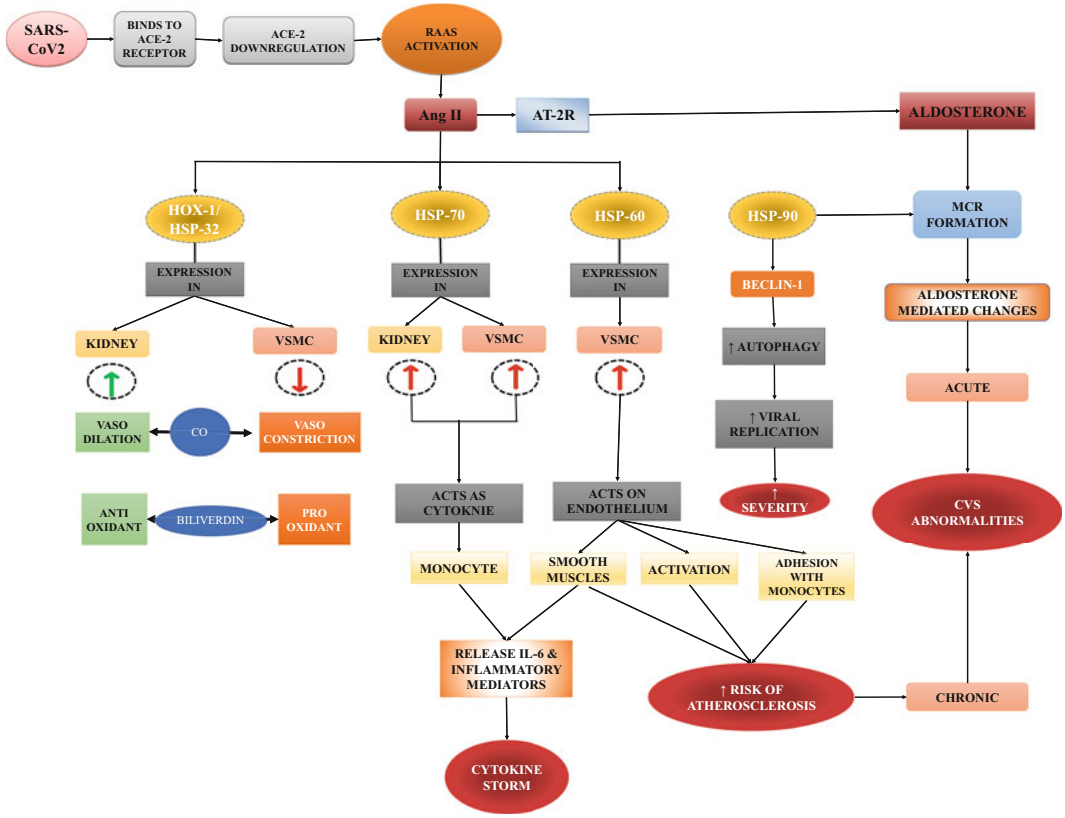

Fig. 2 Role of HSPs in modulating the effects of RAAS Abbreviations*: SARS-CoV2* Severe acute respiratory syndrome-Corona virus 2, *RAAS* Renin angiotensin aldosterone system, *ACE* Angiotensin converting enzyme, *AT* Angiotensin, *AT2R* Angiotensin 2 type receptor, *HOX-1* Heme oxygenase-1, *HSP* Heat shock protein, *MCR* Mineralocorticoid receptor, *VSMC* Vascular smooth muscle cell, *CO* Carbon monoxide, *IL-6* Interleukin 6

and vascular injury (Searles and Harrison 1999). The AT-2R mediates vasodilation via bradykinin and e-NOS in the endothelium. The functioning of e-NOS is dependent on HSP-90, also known as ENAP-1 (eNOS-associated protein 1) (Harris et al. 2000). As mentioned above, HSP-90 is a gatekeeper for aldosterone function. Thus, aldosterone-induced renal damage may be dependent on HSP-90 (Greene et al. 1996; Hostetter and Ibrahim 2003) (Fig. 2).

4.3.3 HSP-70

Murine studies reveal that Ang II is responsible for the upregulation of HSP-70 in the kidney and renal vessels (Ishizaka et al. 2002). HSP-70 has a cytoprotective role on the kidney by tempering the Ang II-induced proinflammatory nicotinamide adenine dinucleotide phosphate oxidase (Manucha 2014) (Fig. 2).

5 Heat Shock Proteins and their Role in Coagulation Cascade

Coagulopathy in patients of COVID-19 stems from inflammatory origins. There is increased incidence of thrombosis in COVID-19 (Ahmed et al. 2020b). There are elevated levels of D-dimers and fibrinogen degradation products (FDPs), sometimes leading to disseminated intravascular coagulation (DIC) (Connors and Levy 2020).

Heat shock proteins can influence the coagulation cascade via the following mechanisms by modulating the inflammatory response (Fig. 3):

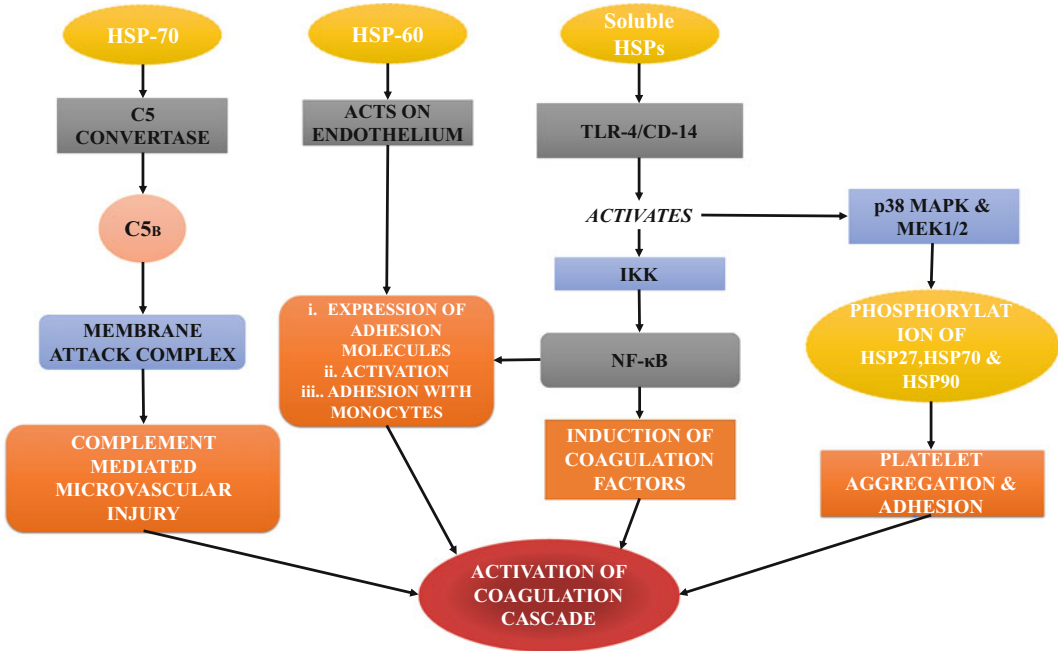

Fig. 3 HSPs and their role in coagulation cascade Abbreviations: *HSP* Heat shock proteins, *TLR* Toll like receptor, *IKK* IκB-kinase, *NF-κB* Nuclear factor-κB, *MAPK* Mitogen-activated protein kinases, *MEK* Mitogen-activated protein kinase kinase

I. *NF-κB mediated*: Soluble HSPs bind to TLR-4/CD14 receptor leading to activation of NF-κB, via IKK pathway (Levi and van der Poll 2005; Kojok et al. 2019), contributing to activation of endothelium and induction of coagulation factors such as TF (tissue factor), factor VIII, u-PA (urokinase plasminogen activator), and PAI-1 (plasminogen activator inhibitor) (Mussbacher et al. 2019).

II. *Complement mediated*: Specimens obtained from lungs and purpuric skin lesion of COVID-19 patients have revealed the presence of deposits of terminal complement components C5b-9 and C4d, which indicates the possibility of complement-mediated microvascular injury leading to thrombosis (Magro et al. 2020). Stress-inducible forms of HSP-70 activate complement, especially C5 convertase enzyme, which generates C5a and C5b. C5a is responsible for increased expression of tissue factor on leukocytes, whereas C5b forms the membrane attack complex (MAC) and activates platelets. Tissue factor activates the intrinsic pathways for coagulation, while activated platelets accentuate clot formation (Afshar-Kharghan 2017).

III. Via *expression of adhesion molecules*: As mentioned earlier in the chapter, HSP-60 induces expression of various adhesion molecules that can take part in coagulation and thrombosis:
   i. Induction of ICAM-1, E-selectin, and vascular adhesion molecules on the endothelial surfaces.
   ii. Activation of endothelial cells, smooth muscles, and macrophages, as well as by mediating the release of IL-6 from the same cells. IL-6, along with some other cytokines, is known to initiate the coagulation cascade (Bester and Pretorius 2016).
   iii. Monocyte adhesion with the endothelium via CD14.
   iv. *HSP phosphorylation mediated*: HSP-27 phosphorylation activates the platelets,

causing its granules to release active substances, responsible for platelet aggregation, whereas phosphorylated complexes of HSP-70 and HSP-90 mediate platelet adhesion (Polanowska-Grabowska and Gear 2000). Protein kinases such as p38 MAPK and MEK1/2 (mitogen-activated protein kinase kinase) are responsible for phosphorylation (Kato et al. 2008), the same enzymes that are activated by the soluble HSP-TLR-4 complex formation (Xu 2002).

6 Therapeutic Targeting of Heat Shock Proteins in COVID-19

Currently, there are no FDA-approved drugs targeting HSPs. However, the following may have a role in abrogating the pathogenesis of COVID-19:

I. Modulation of HSP-90: Geldanamycin is an antibiotic which acts by inhibiting the ATP/ADP binding site of HSP-90. HSP-90, as part of its chaperone function, stabilizes Beclin-1, a key molecule in autophagy. SARS-CoV-2, like few other viruses, is believed to promote the formation of autophagosome, which can be used by the pathogen, as a safe house for further multiplication (Choi et al. 2018). Thus, targeting the pathway involving autophagy can be promising.
   Other drugs with possibly similar actions are chloroquine, estrogen antagonizing drugs, and antidiabetic drugs such as pioglitazone and metformin (Sultan et al. 2020).
II. Modulating Binding Immunoglobulin protein (BIP): BIP or Glucose Regulating Protein-78 (GRP78) is a chaperone protein that is normally present in the endoplasmic reticulum (ER) and translocate to the cell membrane under conditions of stress. BIP is a potential binding site for the SARS-CoV-2 spike protein, facilitating its entry into the cell. Therefore, inhibition of BIP may hamper viral entry (Ibrahim et al. 2020).
   Moreover, exposure to Ang II also increases BIP expression as a part of the ER stress and contributes to atherosclerosis. Targeting BIP may help reduce Ang II-induced ER stress response (Yang et al. 2017).

7 Conclusion

The search for an effective treatment for COVID-19 depends on understanding the exact underlying pathophysiological mechanisms. The role of RAAS is integral in the pathogenesis of COVID-19, perpetuating both end-organ damage and coagulopathy. HSPs mediate and modulate the actions of the RAAS and, thus, can be important targets to modulate the RAAS system. Both roles of RAAS and HSP in COVID-19 warrant further exploration in basic studies as well as with therapeutic randomized control trials.

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Conflict of Interests No conflict of interest exists for either author.

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Aptamer-Based Tumor-Targeted Diagnosis and Drug Delivery

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Abstract

Early cancer identification is crucial for providing patients with safe and timely therapy. Highly dependable and adaptive technologies will be required to detect the presence of biological markers for cancer at very low levels in the early stages of tumor formation. These techniques have been shown to be beneficial in encouraging patients to develop early intervention plans, which could lead to an increase in the overall survival rate of cancer patients. Targeted drug delivery (TDD) using aptamer is promising due to its favorable properties. Aptamer is suitable for superior TDD system candidates due to its desirable properties including a high binding affinity and specificity, a low immunogenicity, and a chemical composition that can be simply changed.

Due to these properties, aptamer-based TDD application has limited drug side effect along with organ damages. The development of aptasensor has been promising in TDD for cancer cell treatment. There are biomarkers and expressed molecules during cancer cell development; however, only few are addressed in aptamer detection study of those molecules. Its great potential of attachment of binding to specific target molecule made aptamer a reliable recognition element. Because of their unique physical, chemical, and biological features, aptamers have a lot of potential in cancer precision medicine.

In this review, we summarized aptamer technology and its application in cancer. This includes advantages properties of aptamer technology over other molecules were thoroughly discussed. In addition, we have also

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elaborated the application of aptamer as a direct therapeutic function and as a targeted drug delivery molecule (aptasensor) in cancer cells with several examples in preclinical and clinical trials.

Keywords

Aptamer · Aptasensor · Cancer cell · Drug side effect · Targeted drug delivery (TDD)

Abbreviations

Ap-DC	Aptamer-Drug Conjugates
Au-NP	Gold Nanoparticles
CEA	Carcinoembryonic Antigen
CRC	Colorectal Cancer
CTC	Circulating Tumor Cancer
DOX	Doxorubicin
EIS	Electrochemical Impedance Spectroscopy
EpCAM	Epithelial Cell Adhesion Molecule
ERK	Extracellular Receptor Kinase
FU	Fluorouracil
GBM	Glioblastoma
HRP	Horseradish Peroxidase
HTS	High-Throughput Screening
IL	Interleukins
MEK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
NFkB	Nuclear Factor kappa B
NGS	Next-Generation Sequence
NSE	Neuron-Specific Enolase
PDGFR	Platelet-Derived Growth Factor Receptor
PTK	Protein Tyrosine Kinase
QD	Quantum Dots
RAF	Rapid Accelerated Fibrosarcoma
RTK	Receptor Tyrosine Kinases
SELEX	Systemic Evolution of Ligands by Exponential Enrichment
STAT3	Signal Transducer and Activator of Transcription 3
TDD	Targeted Drug Delivery
THI	Thionin
VEGF	Vascular Endothelial Growth Factor

1 Introduction

Cancer is a condition in which aberrant cells proliferate uncontrollably and have the potential to spread. It is induced by a variety of factors such as physical inactivity, gene mutation, chemical exposure, and so on. Early cancer identification is critical for preventing organ damage and improving the prognosis of cancer patients. Tumor imaging, biopsy analysis, and cancer biomarker identification have all become common tools for cancer diagnosis in recent decades. Quantitative detection of cancer-specific biomarkers is critical in these strategies for implementing preventive interventions and monitoring prognosis and repetition outcomes. Currently, numerous antibody-based approaches for detecting cancer biomarkers, such as enzyme immunoassays, are widely employed. Despite the high specificity of the antigen-antibody interaction, antibody application in cancer monitoring is severely limited due to high immunogenicity and production costs, low stability, and limited adjustment. In this regard, aptamers have been touted as possible replacements to antibodies for early detection of cancer biomarkers (Zhao et al. 2021; Lacombe et al. 2019).

Aptamers are short single-stranded and stable deoxy ribonucleotide/DNA/ or ribonucleotide/RNA/nucleotide oligomers folding into complex three-dimensional structures. Aptamers may target a wide range of compounds, from chemical dyes and amino acids to antibiotics, proteins, and whole-cell surfaces. Many researches focus on aptamer-targeted proteins for therapeutic purposes (Beier et al. 2014; Lee et al. 2010). The term aptamer was derived by merging the Latin word *aptus* ("to fit") with the Greek word *meros* (part) (Zhuo et al. 2017). The discovery of nucleic acids, such as DNA or RNA, as ligands and target proteins came from studies on viruses, particularly the human immunodeficiency virus (HIV) and adenovirus. Thus, the first aptamer study was published by Sullenger et al. (1990) that a viral protein-binding RNA aptamer hindered viral RNA-protein binding, inhibiting viral multiplication (Nimjee et al. 2017; Kaur et al. 2018).

It was originally reported in 1999 that L-selectin-immunoglobulin merging protein could be purified using aptamer affinity purification method. Similar DNA aptamer-based approaches have been developed for a variety of different proteins, containing Taq-polymerase, thyroid transcription factor, lysozyme, and histidine affinity tag. A number of aptamers for human vascular endothelial growth factor (VEGF) have been also discovered, since 1994 (Lönne et al. 2015). OSI Pharmaceuticals licensed pegaptanib aptamer (in 2000) which is specific for VEGF, known as Macugen, used for the handling of age-related macular degeneration (Ng and Adamis 2006).

1.1 Application of Aptamer

Over the past few years, there has been significant growth in applications of aptamer technology for diagnostic and therapeutic purposes. To date, thousands of aptamers have been widely applied. This includes analytical detection, therapeutics, and cell-based engineering (Zhuo et al. 2017; McKeague et al. 2015). Similarly, they have also been employed in a variety of molecular biology, biotechnology, and biomedical applications due to their ease of synthesis, modification, and stability (Zhang et al. 2019b). Aptamer nanomedicine (therapeutic and nanocomplex aptamer) has been used in drug therapy, gene therapy, and tumor imaging. This field of study has shown significant promise in cancer treatment (Lao et al. 2015).

Furthermore, in life science, aptamers have a wide range of functions, including the potential to inhibit receptor–ligand interactions or activate the stimulation of target receptors, as well as acting as promising targeting carriers for selectively delivering therapeutic medicines to target cells. They can be employed in medication delivery systems and can diminish or limit the effects of bacterial toxins, as well as inhibit pathogen infiltration of immune cells (Afrasiabi et al. 2020; Zhong et al. 2020). The overall purpose of aptamer-based oncology treatments is to inhibit protein-protein or receptor-ligand interactions as an antagonist. As a result, aptamers have been utilized as adversaries against oncoproteins or their ligands in multivalent treatments, directing moieties for drug delivery (Morita et al. 2018; Haßel and Mayer 2019). In this review, the therapeutic capabilities of aptamers under clinical development for anti-cancer therapy are specifically discussed.

1.2 Advantages of Aptamer Over Other Biomolecules

The major drawbacks of antibodies are its larger size (~150–180 kDa) that restrict the access to small biological fragments and the bioavailability nature. Antibodies are frequently immunogenic; thus, they cannot be employed once they have been denatured. Antibody production is a difficult process to scale up and is sensitive to bacterial or viral contamination (Beier et al. 2014). They are less stable and have a shorter shelf life than aptamers. In contrast, the density of immobilized aptamers on solid support can be increased due to their lower size (12–30 kDa), which increases its capacity. A controlled environment with pH, ionic strength, and temperature can be used to perform aptamer selection technique (Zhao et al. 2012; Ali et al. 2019). Aptamers have a great affinity and specificity for attaching to marked molecules. In comparison to other molecular recognition components, it may be simply tagged or replaced with a diversity of reporter molecules at exact sites. The production cost and generation time of aptamer is lower than antibodies. Aptamers can also undergo chemical modification to control their pharmacokinetic profile. This exceptional property may aid in the elution of trapped targets in affinity chromatography (Zhuo et al. 2017; McKeague et al. 2015).

Nucleic acid aptamers, like antibodies, have a good attachment to their targets, with dissociation constants (Kd) extending from nanomolar to picomolar. Apart from having strong affinity for their targets, aptamers also have high specificity, similar to small molecules, allowing them to discriminate between target proteins with identical structural epitopes (Nimjee et al. 2017; Odeh

et al. 2019). As a result, aptamers are becoming more important in molecular diagnostic and therapeutic tools. Furthermore, the application of novel nanomaterials has substantially increased the performance of aptasensors, and nanotechnology's ongoing development also opens up new possibilities for aptamer-based detection (Wang et al. 2019b).

Aptamers are interesting bioreceptors in analytical applications because of their small size, animal-free and low-cost manufacture, excellent stability (particularly DNA aptamers), target variety, high binding affinity, and selectivity for their target molecules. Aptamer technology offers a lot of potential as a novel lead compound source as well as a big role in the biomedical studies and satisfying many requirements needed for diagnosis and precision medicine (Wang et al. 2019a; Modh et al. 2018; Nuzzo et al. 2019).

2 Aptamer Design and Selection Technology

SELEX (Systematic Evolution of Ligands by Exponential Enrichment) was employed in 1990 to select nucleic acid oligomers based on their capacity to bind specific molecule with high affinity (Lönne et al. 2015). Nucleic acids are sometimes referred to be life's genetic blueprint. These molecules can even fold into three-dimensional structures called ribozymes, which catalyze processes, regulate gene expression, convey cellular responses, and mediate physiological responses. Solid-phase syntheses and PCR technologies are utilized to create oligonucleotides and amplify individual membranes. Functional nucleic acid molecules that bind to a selected target or catalyze a chemical reaction can be isolated using these approaches (Dunn et al. 2017). SELEX is a method that select an aptamer by in vitro procedure. In the past years, SELEX is the gold-standard methodology for developing specific aptamers. It regulates binding parameters and selection under non-physiological settings than antibody production. Consequently, high-affinity and chemically stable aptamer probes are produced for targets that are very hazardous or do not provoke an immune response in humans (Sun and Zu 2015; McKeague et al. 2015; Bayat et al. 2018). A number of fluorophores, electrochemical or Raman reporters, or functional groups like primary amine, thiol groups on either the 5′ or 3′ end, on the nucleotides, or on the backbone of DNA can all chemically modify aptamers. As a result, aptamer probes are being created to target for those that are extremely toxic or do not elicit an immune response in vivo (Lee et al. 2010).

Incubation, partitioning, and amplification are the three major steps of SELEX (Fig. 1) (Zhu et al. 2015). The initial library (oligonucleotide pool) contains 10^{14} to 10^{15} different sequences. It starts by incubating with the target molecules, where some of the sequences bind to them. The amount of weakly bound or non-binding sequences is reduced. Partitioning is the process of separating bound and unbound sequences. The eluted oligonucleotide are amplified by PCR or reverse transcription PCR (RT-PCR) to enrich the library (Bayat et al. 2018).

2.1 Next-Generation Sequencing and Bioinformatics

Cloning, colony selection, and Sanger sequencing of a small number of groups using conventional SELEX method often provides interaction to the most common clones. Identical sequences are counted and then aligned to group of comparable sequences. The complete library typically contains thousands of sequences, making it challenging to determine which aptamer is best. Additionally, the sequences with the highest affinity and specificity in the last round of selection are not always the most common. To avoid this difficulty, SELEX approach was updated to use High-Throughput Sequencing (HTS) technology. The greatest features of HTS-SELEX are the ability to sequence libraries throughout all selection rounds. As a result, enhanced sequences are evident considerably earlier in the process, which keeps time. Over-selection can lead to PCR bias, which can be avoided by reducing the number of selection rounds. Furthermore, robust bioinformatics methods can permit thorough aptamer

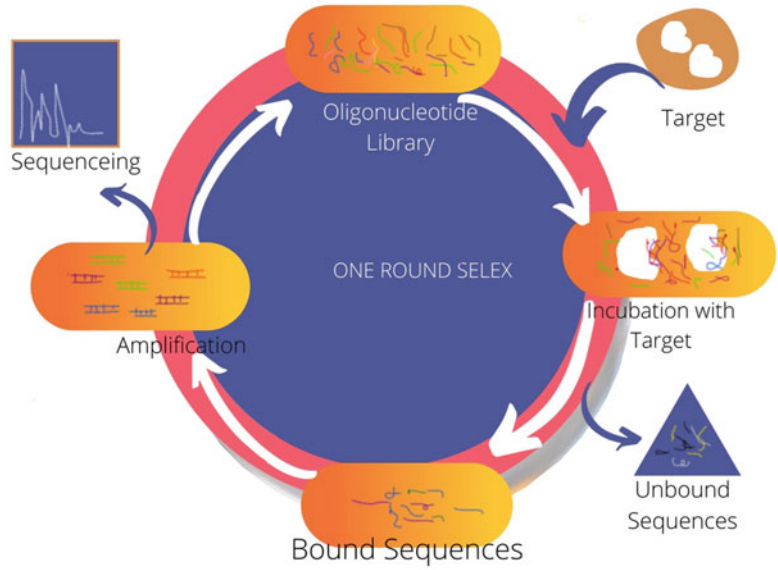

Fig. 1 Oligonucleotide library containing up to 10^{15} different sequence incubated with targets. After incubation, the unbound sequences are separated and the bound sequences are eluted, amplified, and sequenced

characterization, including binding affinity and/or specificity, structure prediction, abundance measurement, and aptamer-target communications, by analyzing massive sequence datasets globally (Blind and Blank 2015; Zhuo et al. 2017; Ortega et al. 2019).

Aptamers were synthesized on living cells such as bacteria, immune cells, and cancer cells utilizing the whole cell SELEX approach. However, the method is difficult, time-consuming, and intricate. As a result, Gijs et al. (2016) developed a methodology that included HT-NGS and bioinformatics investigation rather than standard cloning and sequencing. The method reduces the amount of repetitive selection rounds required to pick just high affinity sequences, while reducing the introduction of interrelated sequences via PCR products (Gijs et al. 2016; Takahashi et al. 2016). A number of SELEX approaches for aptamer selection have been established previously, and Table 1 shows a list of some of them.

3 Challenges in Tumor Diagnosis and Drug Delivery

Early cancer detection and treatment are extremely challenging undertakings that play a critical role in cancer treatment. Traditional chemotherapy medications frequently lack selectivity, leading to significant side effects. The archetypal anthracycline antibiotics, doxorubicin (Dox) and daunorubicin, have been used to treat a variety of malignancies. However, because of the non-selective intercalation to the double-stranded CG sequences of DNA and RNA, they may produce serious adverse effects (Zhuo et al. 2017). According to a study, current chemotherapy lacks specificity, and as a result, the majority of chemotherapeutic medications induce undesirable side effects, genetic and phenotypic abnormalities, as well as tumor chemoresistance and regression. The progressive accumulation of specific genetic and epigenetic abnormalities in cancer cells may promote acquired drug resistance. This difficulty overwhelmed with the introduction of aptamers that endows nanoscale vehicles with specificity, reducing "off-target" effects. Nanoparticles (NPs), Quantum dots (QDs), liposomes, hydrogels, and other self-assembled contracts have been used to transport a diversity of tumoricidal agents (e.g., chemotherapeutic medicines, cytotoxins, and siRNAs) to cancer cell lines related with almost all clinical malignancies. Next-generation sequencing (NGS) technologies have shown cancer's inherent heterogeneity, explaining why patients respond to the same medication in various ways

Table 1 Common SELEX approach for aptamer selection

	Methods	Introduced/modified by	Uses	Reference
1	Negative SELEX	Ellington and Szostak (1992)	To avoid false positive result	Zhuo et al. (2017); Ali et al. (2019)
2	Counter SELEX	Jenison et al. (1994)	Adds additional steps to discriminate aptamers to effectively discriminate from non-specific oligonucleotide	Zhuo et al. (2017)
3	Capillary electrophoresis (CE) SELEX	In 2004	Reduces selection round (1–4)	Bowser and Yang (2013)
4	Microfluidic SELEX	Hybarger et al. (2006); modified by Leu et al. and Park et al. (2009)	After a single round of selection, an enriched aptamer pool can be obtained	Liu et al. (2021)
5	Cell SELEX	Morris et al. (1998), Daniels et al. (2003), Hicke and Marion (2001)	Improve success rate of aptamer screening	Wu et al. (2014); Chen et al. (2016)
6	In vivo SELEX	Mi et al. (2010)	To generate aptamer using live animal models	Cheng et al. (2013)
10	High-throughput (HT) SELEX	Cho et al. (2010)	Reduces the number of iterative selection and chooses only high-affinity sequences	Zhuo et al. (2017); Hoinka et al. (2015)

(Zagidullin et al. 2019; Zhu et al. 2014; Nikolaou et al. 2018; Vasan et al. 2019).

Furthermore, chemotherapeutics target cells that proliferate rapidly without distinguishing between cancer cells and normal cells that reproduce quickly, resulting in significant toxic side effects. Furthermore, they are linked to poor transport to tumor tissues and low intratumoral accumulation, necessitating substantial dosages to achieve therapeutic efficacy. Significant advances in understanding of the molecular pathways that drive malignant growth have revolutionized cancer treatment. Cancer precision medicine that target the unhealthy state, while reducing drug uptake by non-malignant cells, are making great improvement, thanks in part to the progress of active targeting agents (Cerchia 2018).

Traditional analytical approaches for detecting cancer cells, such as PCR, immunohistochemistry, and flow cytometry, were established. However, they lack sensitivity and necessitate expensive equipment and lengthy treatment periods. Biosensors are analytical equipment that can detect cancer cells early in their development. They are effective, sensitive, economical, and quick. Furthermore, conjugating a recognition element with a cancer cell or biomarker improves the detection method's selectivity and sensitivity (Kordasht and Hasanzadeh 2020).

Previous research has shown that nanoparticles can more efficiently encapsulate and deliver anticancer medicines to tumor tissue. Nanoparticles, on the other hand, are not cancer cell specific. They preferentially aggregate in cancer areas due to the tumor tissue's increased permeability and retention (EPR) effect. If nanoparticles can be functionalized with ligands that recognize cancer cells specifically, they will be able to target and transport payloads to cancer cells preferentially, dramatically increasing the therapeutic index (Fu and Xiang 2020; Hu et al. 2019). Therefore, nanoparticle-conjugated aptamer is more effective for drug-targeted cancer due to specificity property of aptamer.

4 Aptamer in Diagnosis and Drug Delivery of Tumors and Different Strategies

In the recent decade, significant progress in cancer therapy has been made as evidenced by a continual decline in the cancer-related death rate of roughly 1.5%. Chemotherapy side effects are still a major concern. The development of cancer-

cell-targeting medicines is one possibility for solving this challenge (Reshetnikov et al. 2018). Targeted drug delivery (TDD) is a method of delivering a drug moiety to a specific body location, with improved drug encapsulation capability, higher and controlled drug stability, and so on. This helps to overcome a specific toxic impact of traditional drug administration, resulting in a reduction of the amount of drug needed for therapeutic efficacy. TDD is proving to be an effective cancer therapy technique and dormant cancer cell due to an improved allocation of drugs to a tumor site with shield from the extracellular environment. Many particular inhibitors, usually small compounds and antibodies, are now in clinical usage for many malignancies. Aptamers are the most specific type of targeted therapy available now (Pan et al. 2018; Recasens and Munoz 2019; Nimjee et al. 2017; Tewabe et al. 2021; Madhusudana Rao et al. 2018).

Aptamer's attractive features make it a good choice for superior tailored medication delivery systems (Khodadadi et al. 2021; Zhuo et al. 2017). Aptamers are used to associate chemotherapeutic medicines, siRNA, nanoparticles, and solid phase surfaces for medicinal and diagnostic reasons because of their chemical modification flexibility (Morita et al. 2018). In recent years, a variety of aptasensors have been developed in conjugate with nanoparticles and other electrochemical and fluorescence detectors. The use of aptamers as identification elements may significantly increase the use of comparative biosensor system (Atapour et al. 2022).

4.1 Aptamer as Direct Therapeutic Molecule

Targeted cancer medicines are a fundamental cornerstone in the period of precision medicine. Targeted therapies can be used to boost the therapeutic index by delivering or targeting a specific molecule (Yoon et al. 2019). Several investigational studies have been performed on various aptamers and were tested in various phases (Ismail and Alshaer 2018). Aptamers have showed early promise as medicines, with hundreds in pre-clinical investigations and only a few in clinical trials (Zhou et al. 2012). They have been employed in the treatment of pathogenic infections, immunological disorders, neurological illnesses, anti-coagulation medicines, inflammatory targeting, and clinical trials. In the next sections, we will look at a few specific instances, with a focus on aptamers that have been produced for cancer treatment (Ismail and Alshaer 2018).

4.1.1 Therapeutic Aptamers in Cancer

The methodologies for developing anti-cancer aptamers varies depending on the target's function and cellular location. These are as follows:

I. Through direct contacts, aptamers can act as antagonists against extracellular targets in the blood stream or on cell surfaces.
II. Intracellular molecules are antagonized by aptamers. The majority of investigation on aptamers were developed to targeting extracellular marks due to its applications in tumor biology. In addition, it is easier to approach, as the extremely polar aptamers do not need to pass the lipid bilayer of cellular membranes. Such problem has no effect on the search for aptamers in contradiction of intracellular targets, such as transcription factors, enzymes, and coenzymes, as well as signal transduction and apoptotic proteins.
III. Furthermore, aptamers have been linked to the stimulation of cellular signaling pathways as agonists (Ismail and Alshaer 2018).

The aptamer directly attaches to the target in therapeutic aptamer methods, modulating downward signaling pathways. Adherence factors, immune system modulators, receptor tyrosine kinases (RTKs), and cell growth modulators are all targets for aptamer-based cancer therapy. Therapeutic aptamers can influence downstream signaling pathways on a cell's surface. They block signaling by inhibiting structural deviations in the target molecule and dimerization with other molecules, or phosphorylation of downstream proteins (Fig. 2) (Zhou et al. 2012).

AGRO100 is the first aptamer to be tested in humans for treatment of cancer. This anticancer aptamer is now known as AS1411, which belongs

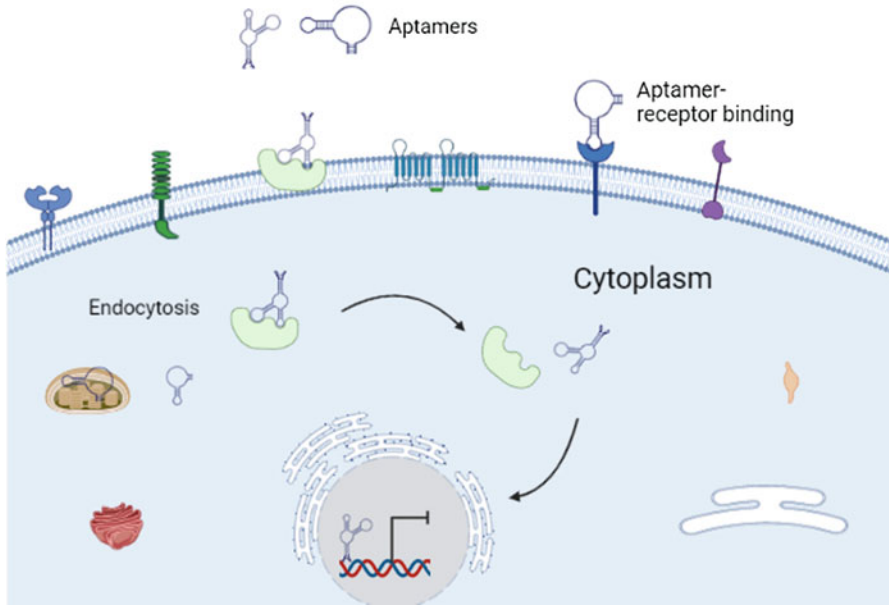

Fig. 2 Schematic illustration of therapeutic potential of aptamers targeted to intracellular molecules in cancer that inhibit proliferation

to the class of molecules referred to as guanosine-rich oligonucleotides (GRO) (Kaur and Roy 2008). Glioblastoma (GBM) is the most frequent principal CNS malignancy marked by aberrant RTK pathway activation. Among RTKs, platelet-derived growth factor receptors (PDGRFs) are overexpressed in a substantial subset of malignant gliomas. Thus, the PDGFR pathway is generally recognized as a driver of GBM, making PDGFRs interesting targets for GBM treatment discovery (Yoon et al. 2019). A study reported that 20 F-Pyrimidines (20 F-Py) nuclease-resistant RNA 33-mer aptamer, the Gint4.T, which fixes and antagonizes the action of PDGFRb. In addition, a new aptamer-siRNA chimera (Gint4.T-STAT3) was established to target STAT3, resulting in STAT3 delivery and silencing of PDGFRb+ GBM cells. In a subcutaneous xenograft mouse model, the mixture inhibits malignant tumor progress and angiogenesis in vivo while also inhibiting cell survival and migration in vitro. Thus, Gint4.T STAT3 conjugates are used as a promising aptamer that can be applied for GBM therapy (Esposito et al. 2018).

Aptamer-based advanced glycation end product (RAGE) was also designed in another study, to disrupt downstream NFkB-mediated signal transduction by blocking the interaction between RAGE and S100B. Apt-RAGE successfully reduced both S100B-dependent and S100B-independent RAGE/NFB initiation in colorectal HCT116 cancer cells in vitro, reducing cell proliferation and migration. Thus, VEGF-A expression and secretion was also inhibited. The decrease of RAGE/NFB/VEGF-A signaling was positively linked with Apt-RAGE-induced angiogenesis inhibition. Thus, Apt-RAGE antagonist is a promising therapeutic drug for colorectal cancer (CRC) (Zheng et al. 2021). A DNA aptamer has also been created as a potential CRC therapeutic agent. Through downregulation of the RAS/RAF/MEK/ERK signaling pathways, the aptamer inhibited CRC cell proliferation by interfering with PDGF-BB binding to its receptor (Sae-Lim et al. 2019).

Anti-HER3, anti-RET, anti-EGFR, and anti-VEGF aptamers have been produced to stop tumor growth by inhibiting RTKs. In cell culture, human receptor tyrosine kinase, RET, binds with

anti-RET aptamer and exerts an anti-cancer effect. When mutated RET dimerizes, it phosphorylates transcription factors important for cell proliferation, such as ERK, NF-B, and AKT, causing thyroid carcinomas to form. When RET dimerization is blocked, transcription factors critical for tumor cell development are not phosphorylated. Tumor cells that overexpress the RTK EGFR have a poor prognosis. In cell culture, anti-EGFR aptamers attach to EGFR monomers and heterodimers formed of EGFR and another RTK on the cell surface. This prevents the downstream transcription factors from becoming activated. On account of the tiny size and prevention of numerous types of EGFR found on cell surfaces, aptamers suppress tumor development in mice and then induce apoptosis in tumor cells unaffected to existing antibody and chemical-based anti-EGFR therapies (Zhou et al. 2012).

4.2 Aptasensor in Cancer

Aptasensor has emerged as one of the most promising early detection techniques for cancer. It is a biosensor with an aptamer recognition feature. They are crucial in the delivery of drugs as well as the creation of new biosensors. They were a dependable recognition factor because of their high ability to connect to specific targets. As a result, they have been utilized to detect biomarkers in order to make disease diagnosis more accurate (Eivazzadeh-Keihan et al. 2018; Di Pietrantonio et al. 2019; Hassan and DeRosa 2020).

Aptamers can be utilized to guide the delivery of medicines, other macromolecules, or nanocarriers to malignant cells due to its advantages listed above. As a result, numerous RNA and DNA aptamers against putative cancer cell surface receptors have been successfully selected. The aptamer-targeted immune-modulatory medicines elicited protective antitumor immunity. In terms of efficacy and toxicity, this could outperform existing "gold-standard" therapies, which has been shown in relevant mouse models. For instance, previously two aptamers, 2′-fluorinated-RNA aptamers (A9 and A10) and DNA aptamers, were selected against refined prostate-specific membrane antigen (PSMA) and Mucin 1 (MUC1) cell surface-linked protein (called MA3), respectively. PSMA and MUC1 are overexpressed on some tumors including breast, colon, lung, and prostate cancers (Alshaer et al. 2018; Gilboa et al. 2015).

Aptamers can bind to cancer cell surface biomarkers, allowing for sensitive detection. Some of the aptamers have been generated to bind with distinct cancer cells. For instance, Sgc8, SYL3C, TD05, TD02, AS1411, SEL15-8, VEGF165, S6-aptamer, and so on. Some aptamers and their application are listed in Table 2 (Kordasht and Hasanzadeh 2020).

4.2.1 Detection of Cancer Using Aptasensors

Aptamers can be, covalently or non-covalently, conjugated with other therapeutic nucleic acids or small compounds (Zhou et al. 2012). Three types of aptamer-directed drug delivery, which are distinguished based on the systems, have been modified. These are aptamer drug conjugates (ApDC), aptamer macromolecule conjugated systems, and aptamer nanomaterial delivery systems (Fig. 3) (Ni et al. 2021).

Aptamer's attractive features make it a good choice for superior tailored medication delivery systems (Khodadadi et al. 2021; Zhuo et al. 2017). They are coupled to various chemical entities such as chemotherapeutic drugs, siRNA, nanoparticles, and solid phase surfaces for therapeutic and investigative purposes (Morita et al. 2018). In recent years, a variety of aptasensors have been developed in conjugate with nanoparticles and other electrochemical and fluorescence detectors. The use of aptamers as identification elements may significantly increase the use of comparative biosensor system (Atapour et al. 2022). Given the medicinal potential of aptamers, conjugating them with enzymes, antibodies, chemotherapeutics, nanoparticles, or oligonucleotides could significantly boost their use (Nuzzo et al. 2019).

A variety of diagnostic substances can be physically or chemically conjugated to aptamers to further personalize their activity. Aptamers that

Table 2 Some aptamers that have shown promise potential as cancer therapeutics

Target	Tumor /cell line	Aptamer	Nanocarrier	Load	Ref
Nucleolin	Breast, ovarian cancer, HeLa cervical cancer cell, colorectal cancer, MCF-7, glioma, GI-1, PANC-1 CELL, HeLa cells	AS1411 (DNA)	Liposome, micelles, PEG-PLGA, PEG-PCL, PLGA-lecithin-PEG, PGO, mesoporous silica, AuNPs, PF127-β-CD-PEG-PLA, PLL-alkyl-PEI, PEG-PLGA, M-PLGA-TPGS, chitosan ss PEEUA, BSA	Cisplatin, FluoresceinPaclitaxel, Docetaxel, DiR, coumarin-6, Doxorubicin, Vinorelbine, shRNA	Nimjee et al. (2017); Alshaer et al. (2018); Lopes-Nunes et al. (2020); Xu et al. (2016)
Prostate specific membrane antigen (PSMA)	Lung and prostate cancer, LNCaP cell line, H40-PLA-PEG, PLGA	A10 (RNA)	PLA-PEG-COOH, SPION, liposome, micelles, PLA, super paramagnetic iron oxide, atelocollagen	Doxcetaxel, cisplatin, doxorubicin	Alshaer et al. (2018); Zununi Vahed et al. (2019); Zhang et al. (2011)
MUC1, PDGFR	Colorectal, breast, lung, adenocarcinoma, colon, MCF-7	DNA aptamer,	Hyaluronic/chitosan, silica, PLGA, iron oxide nanoparticle, AU@spions, QD, DNA icosahedra, liposome	Doxorubicin, TSP	Alshaer et al. (2018); Ninomiya et al. (2014)
MUC1	Colorectal, breast, lung, adenocarcinoma, colon	MA3	Hyaluronic/chitosan, silica, PLGA, iron oxide nanoparticle, AU@spions, QD, etc.	Epirubicin	Alshaer et al. (2018)
MUC1	Carcinoma C26 cells, MCF-7, and C-26 cells	5TR1 DNA aptamer	SPION, PLGA-chitosan	Epirubicin	Alshaer et al. (2018); Taghavi et al. (2017)
Protein tyrosine kinase 7 (PTK-7), CCRF-CEM cell	ALL	Sgc8c	Modified liposomes, DNA monomers, polyvalent aptamer system, gold nanoparticles, *mesoporous silica, aptamer DNA*	Doxorubicin, antisense ONT to P-gp	Alshaer et al. (2018); Zununi Vahed et al. (2019)
HER-2	Breast cancer	SE15–8	Au-NPs	?/???	Kordasht and Hasanzadeh (2020)
EGFR,	Breast, osteosarcoma CSAs	E07 aptamer,	Aptamer conjugate	??/??	Alshaer et al. (2018)
EpCAM, PSMA	HT29 cells, non-small lung cancer, MCF-7, LNCaP cell	RNA-aptamer	PLGA-lecithin-PEG, PEI, liposome	Curcumin, doxorubicin, EpCAM siRNA	Alibolandi et al. (2015); Li et al. (2014)

target proteins overexpressed in tumors, in particular, have proven effective tools for the precise recognition of cancer-specific indicators over the last decade. As a result, applying aptamer-based technologies to actual cancer diagnosis procedures could pave the way for significant advancements in cancer detection (Ciancio et al. 2018).

4.2.2 AS1411 Aptamer

AS1411 is a quadruplex-forming guanine-rich (G-rich) DNA aptamer. It is the first aptamer to target nucleolin in clinical trials for cancer treatment (Morita et al. 2018; Lee et al. 2015). A report showed that AS1411 is an ssDNA aptamer with distinctive biological effects. Anti-proliferative action, nucleolin binding ability,

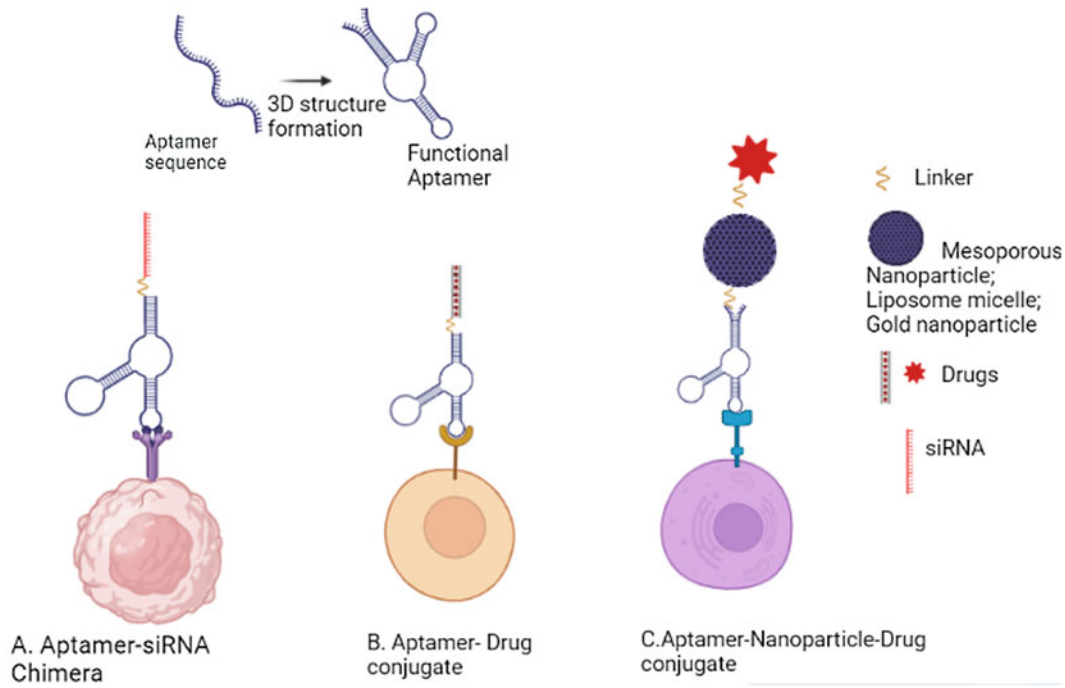

Fig. 3 Schematic illustration of aptamer-based drug delivery in cancer cell

impeding the pro-survival NF-B signaling cascade, blocking DNA replication, inducing cell cycle arrest and apoptosis, and hindering nucleolin binding to Bcl-2 mRNA are only a few examples. In addition, aptamer has a low immunogenicity effect and excellent stability in serum samples. As a result, active targeting ligands exhibit a great capacity to be used in drug delivery systems (Yazdian-Robati et al. 2020). Another study also indicated incubation of B16 melanoma cells with the AS1411-ICG compound accompanying with C8 increase the cytotoxicity of the cell (Lopes-Nunes et al. 2020; Feng et al. 2011).

Likewise, Gold nanoparticles (AuNPs)-based aptamer drug delivery have numerous exceptional characteristics such as high stability, high biocompatibility, low toxicity, and easy modification. Aptamer/hairpin DNA-AuNPs conjugates were developed for targeted drug delivery in the previous years. For example, an AS1411 anti-nucleolin aptamer nano-construct accumulates five times in invasive breast cancer tumors in a tumor-specific way with the absence of noticeable acute injury (Luo et al. 2020; Zhuo et al. 2017).

Study by Su et al. (2019) shows that, the AS1411 aptamer-improved UiO66@AgNCs@Apt was successfully internalized by target cancer cells with high selectivity. To confirm the improved tumor-targeted delivery of DOX, researchers examined in vitro cellular uptake and drug delivery in cancer MCF-7 and normal L929 cells. These findings reveal that UiO-66@AgNCs@Apt@DOX can be imported via AS1411-mediated endocytosis and the released DOX can be transported into nucleus efficiently. This indicated that the aptamer could be employed as an in vitro targeted drug delivery system (Su et al. 2019). In another work, the surface of DOX-loaded bovine serum albumin (BSA) was coated with a tumor-targeting aptamer AS1411. When compared with nanoparticles without aptamer modification, the aptamer-nanoparticle modified complex demonstrates better cellular acceptance and enhanced cell inhibitory activity against malignant MCF-7 cells. This is due to AS1411's particular identification of its receptor,

which is overexpressed on tumor cells (Xu et al. 2018). AS1411 has demonstrated the ability to distinguish cancer cells from normal cells using an electrochemical aptasensor. Using multiple DNA sequences and specificities, this graphene/aptamer-based device may detect protein, small molecules, and nucleic acid targets (Feng et al. 2011). To identify HeLa cancer cells, a label-free electrochemical impedance spectroscopy (EIS) aptamer/graphene-based aptasensor was created in which the modified AS1411 aptamer detect and bind HeLa cancer cells. This has done through the interaction between the overexpressed nucleolins using aptamer attached on the surface of graphene-GCE complex (Kordasht and Hasanzadeh 2020).

4.2.3 Sgc8c-DNA Aptamer

Sgc8c is a 42-nucleotide DNA sequence which has been demonstrated to bind to PTK-7, a cell membrane protein that is overexpressed in CCRF-CEM (human T-cell ALL) cells and a variety of other malignancies, including colon and gastric cancer. Because of its role as a targeted aptamer, sgc8c could be acclimated to make a drug delivery preparation, such as sgc8c-TDN (s-TDN), that specifically targets ALL cells (Liu et al. 2019). Many efforts have been made in recent years to create targeted doxorubicin delivery by intercalate into aptamer. Doxorubicin covalently bonded to the sgc8c aptamer that can precisely target T-cell acute leukemia cells, and, reduces non-target cell damage (Zhuo et al. 2017). Many forms of Sgc8c aptamer-based EC-detecting technologies have evolved and are constantly being developed for the recognition of leukemia cancer cells on account of their remarkable affinity and selectivity for most blood cancer cells (Kordasht and Hasanzadeh 2020; Zhang et al. 2011, 2021; Bagalkot et al. 2006). The sgc8c–Dox conjugates, in comparison to the less efficient Dox-immuno-conjugates, make targeted chemotherapy more viable with medications of varying potencies, according to report by Huang et al. (2009). Zhang et al. (2021) also developed T-ALL targeted sgc8c aptamer as it directly binds the transmembrane PTK7. Aside from having a high selectivity and affinity for PTK7 (Kd = 0.8 nM), sgc8c is small and simple to produce. Therefore, new anti-T-ALL therapeutics include sgc8c-conjugated chemotherapy drugs, and sgc8c-decorated platinum and gold nanoparticles (Zhang et al. 2021). Assembled protein PTK7-sgc8c bind against AUNPs surface. Later the mixture loaded doxorubicin into the hairpin DNA on the AuNPs surfaces in a repeating d(CGATCG) sequence. This increases antitumor activity while lowering toxicity (Luo et al. 2011; Zhuo et al. 2017).

4.2.4 S6-Aptamer

Additional aptamer-based fluorescence probe for carcinomas was recognized using whole cell-based SELEX. In a study, a Cy5-labeled aptamer (named S6) was used to target A549 lung cancer cells. Both in buffer and serum conditions, flow cytometry experiments demonstrated that Cy5-S6 could precisely mark A549 cells. In vivo fluorescence imaging further showed that Cy5-S6 has a good selectivity for detecting A549 cancer. After intravenous injection to nude mice with A549 lung cancer and Tca8113 tongue carcinoma, a significantly extended retention time of Cy5-S6 in A549 tumor was detected (off-target). Two more aptamers (LS2 and ZY8, which were against Bel-7,404 and SMMC-7721 liver cancer cells, respectively) were likewise effective in distinguishing liver carcinomas of diverse subtypes within the same body, demonstrating that this technique is commonly applicable for carcinoma aptamer screening (Dougherty et al. 2015). Through the -SH linkage, cyanine 3 (Cy3)-modified S6 aptamers were connected to magnetic/plasmonic nanoparticles with specific targeting for the SK-BR3 cell line via HER25. The plasmonic shell was modified with aptamer-modified Cy3 for selective breast cancer cell detection and fluorescent imaging with Cy3 as the fluorescence probe (Fan et al. 2014).

A ZnO/G composite and S6 aptamer were also used for sensitive PEC detection of SK-BR-3 cancer cells using a disposable ITO microdevice. The examinations used S6 aptamers with excellent specificity to target SK-BR-3 cells and bio-barcode approach to eliminate cross-reaction, which greatly improved selectivity and sensitivity. These characteristics, together with its high

biocompatibility, make it a good choice for detecting cancer cells early and accurately (Liu et al. 2014a).

4.2.5 NOX-A12-RNA Aptamer

The second therapeutic aptamer, NOX-A12, is a pegylated L-form RNA aptamer introduced to clinical trials for cancer. However, it was only accomplished clinical trials for hematologic malignancies. NOX-A12 can PEGylate at 3-′-terminus to provide additional enrichment of its pharmacokinetic parameters (Morita et al. 2018; Kaur et al. 2018).

A study employed CXCL12-abundant TME heterotypic tumor-stroma spheroids to imitate a solid tumor. NOXA12 increased T and NK cell infiltration in a dose-dependent method. NOX-A12 and PD-1 checkpoint inhibition stimulated T cells in the spheroids in a synergistic manner, demonstrating that the treatments are complementary. In a syngeneic murine model of colorectal cancer, the findings were confirmed in vivo, with the addition of NOX-A12 improving anti-PD-1 therapy (Zboralski et al. 2017).

4.2.6 Other Aptamers

Wang et al. synthesized multiplexed electrochemical paper-based aptasensor, amino functional graphene(NG)-thionin(THI)-AuNPS and Prussian blue (PB)-poly (3,4-ethylenedio xythiophene) (PEDOT)-AuNPS nanocomposite to immobilize carcinoembryonic antigen (CEA) and neuron specific enolase (NSE) aptamers, separately. In a single biological sample, the technology could be able to detect two cancer biomarkers. By way of a label-free electrochemical detection approach, this aptasensor has a fast initial response time, making it an advantageous tool for cancer biomarker testing at the point of care (Wang et al. 2019b).

Zavareh et al. (2020) successfully synthesized a 5-fluorouracil chitosan carbon quantum dot-aptamer (5-FU-CS-CQD-Apt) nanoparticle, which was precisely a targeted delivery of 5-FU anti-cancer medicine utilized in breast cancer treatment. Such method implied that 5-FU-CS-CQD-Apt might be employed as a suitable nanocarrier in breast cancer treatment (Zavareh et al. 2020).

Xu et al. (2020a) developed a colorimetric aptasensor for sensitive and selective finding of circulating cancer-generated exosomes. Latex beads were used to capture the target exosomes followed by bio-recognition with a specific CD63 aptamer, which was biotin-streptavidin bound and coupled to horseradish peroxidase (HRP). This aptasensor has a lot of room for improvement in the future for detecting cancer-derived exosomes for cancer diagnosis in a clinical situation and for development of detection kits (Xu et al. 2020a).

Aptamer liposome delivery system was also developed in 2009. Initially, 12-thymine spacer was attached to 3′ end of anti-nucleolin aptamer sequence and further to a cholesterol tag linked on a PEGylated liposome hydrophobic surface. Finally, it was attached to chemotherapeutic drug, cisplatin. It was found that cisplatin was delivered in to a cancer cell in a specific means (Zhuo et al. 2017). Khodadadi et al. (2021) also developed EpCAM-specific DNA aptamer to supply paclitaxel (PTX) to targeted EpCAM-positive cancer cell. As a result, SPIONs@PTX and SPIONs@PTX-aptamer determined the efficacy of SYL3C aptamer as a target ligand for improving nanoparticle cytotoxicity in MCF-7 and 4 T1 cells. Thus, aptamer-SPIONs@PTX conjugate has a good potential in identifying its target cell (Khodadadi et al. 2021). Aptamer-based PEC method was also developed by Luo et al. (2020) for MCF-7 binding with HCNT (photoactive material). The aptamer shows great specificity for binding mucin 1 protein which is overexpressed on the surface of MCF-7 cell (Luo et al. 2020).

The functionalized affinity matrix phase for VEGF purification was created by covalently coupling the VEGF-binding DNA aptamer V7t1 to magnetic beads. The aptamer is designed to interact to the protein's receptor-binding domain. Because diverse VEGF isoforms have a shared receptor-binding domain, the aptamer can bind to all of them, as has been developed for VEGF121 (Lönne et al. 2015). GSH/ATP dual-responsive florescence enhancement probe by – aptamer modified –QDs were designed to monitor drug release process of liposome (Hu et al. 2019).

Aptamer-conjugated siRNA delivery was also effective in cancer cell. A study developed aptamer-protamine-siRNA nanoparticle (APR) which was specifically targeting for ErbB3-positive MCF-7 cancer cells (Xu et al. 2020b). Using alpha-fetoprotein (AFP) as a paradigm, a new photoelectrochemical aptasensor for cancer biomarkers was developed. The AFP aptamer was improved by AuS bonds on the surface of the Au/GaN photoelectrode and binds to designated protein with high selectivity (Hu et al. 2021). According to a study, the aptamer has a cytotoxic effect on HER2, inhibiting cell proliferation and causing apoptosis. MSN-BM/CD-HApt@DOX allows for HER2-mediated targeting as well as the release of DOX drugs. This allows for better chemotherapeutic efficacy in HERA2-positive cancers (Zhang et al. 2019a).

Several dual-stage EC aptasensors were created in recent years for the primary recognition of two types of breast cancer cells, the MCF-7 and the SK-BR-3. Without any sampling procedure, Sandwich-based EC aptasensors were developed for the detection of cancer cell. Several aptamers are used in this procedure to anchor the sandwich to the electrode, allowing various types of transducer elements that report signal change caused by the target cell binding induction. MUC1-aptamer was labeled to horseradish peroxide (HRP) to improve an unconventional EC aptasensor. Then MUC1 aptamer was bound to the gold electrode's surface to catch MCF-7 cancer cells, while HRP-labeled MUC-1 aptamer (as signal probe) was used to amplify the EC signal (Kordasht and Hasanzadeh 2020).

Amine-functionalized MUC-1 aptamer and lectin-functionalized AuNPs are another sandwich-centered aptasensor. These aptamers were recognized for glycan identification on MCF-7 surface. MUC-1 covalently bonds with carboxylated-MBs via the succinimide coupling (EDC-NHS) approach demonstrating strong MCF-7 cancer cell binding property. A majority of aptasensors use the TLS11a aptamer as a detention mediator for identifying HepG2 cancer cells. Anti-cancer agent, Doxorubicin is conjugated with an aptamer and the complex (TLS11a-GC-Dox) was developed against HepG2 cells (Zhuo et al. 2017; Yazdian-Robati et al. 2020; Kordasht and Hasanzadeh 2020; Liu et al. 2017).

A recent study identified aptamer-based delivery of 5-FU and Dox binding with biosynthesized AuNPs and chitosan(s) for the improvement of glioblastoma treatment. Thus, apt-Dox-CS-Au-5FU NPs complex was internalized into the cell by confirmed using flow cytometer, florescent microscopy, and bio-TEM analysis (Sathiyaseelan et al. 2021). Zhou et al. (2021) investigated a vigorous indication for liver cancer stem-cell, where CD133 was integrated to aptamer (CD133-apt) and was then for liver cancer therapy. Then this aptamer binds to doxorubicin (CD133-apt-Dox) in targeting liver cancer stem-like cells. This complex considerably suppresses the development of liver cancer cells and patient-derived organoids. Consequently, it overwhelms the advancement of xenograft tumors in nude mice which repressed the development of DEN-induced liver cancer in immunocompetent mice (Zhou et al. 2021).

In several forms of solid tumors, detecting circulating tumor cells (CTCs) in peripheral blood has predictive importance, notably for predicting patient survival. A study showed that a new-generation NanoVelcro Chip could capture NSCLC CTCs from blood, while improving nanosubstrate-immobilized NSCLC CTCs after controlling with a nuclease solution. Using the Cell-SELEX technique, two single-stranded DNA aptamers (Ap-1 and Ap-2) were created to substitute traditional antibody-based capture agents. This enabled the exact detection and discharge of NSCCL cells from whole-blood samples using aptamer-grafted NanoVelcro Chips. These properties allow CTC isolation with minimal effect of adjacent white blood cells (WBCs) and minimal impairment of CTC feasibility and roles, arranging the path for molecular and functional investigations of CTCs (Shen et al. 2013). A study also developed aptamer-tethered DNA nanodevice (aptNDs) which can bind to target PTK7. This increases the attention that aptamer-conjugated nanomaterials with various characteristics reveal multifunctional theranostic capability for cancer therapy (Liu et al. 2014b, 2019).

Apt63 is an original aptamer that binds to the beta subunit of F1Fo ATP synthase (ATP5B) on the plasma membrane of some healthy and malignant cells. The aptamer was attached to the plasma membranes of several destructive cancerous breast and prostate cell lines, but not to healthy breast and prostate epithelial cells, and only weakly to non-metastasizing cancer cells. This linkage resulted in a quick cell destruction (Speransky et al. 2019).

The occurrence of Wy5a aptamer on the nanoparticle surface resulted in cancer-targeted release of Docetaxel to PC-3 cells in vitro and in vivo (Fang et al. 2020). 2′-Fluropyrimidine-modified RNA aptamer-doxorubicin physical conjugate specifically targets the prostate-specific membrane antigen (PSMA)-expressing human prostatic adenocarcinoma (LNCaP) cell with high affinity and specificity (Zhuo et al. 2017).

4.2.7 Aptamer-Modified Immune Cells

On top of classic cancer therapies like chemotherapy and phototherapy, experts have found innovative cancer treatments. Aptamer-enhanced immune cells are discovered as new cancer treatment. Immune cells are instructed to attack cancer cells in vivo though preserving normal cells. Natural killer lymphocytes (NK cells), for example, are now being researched for cancer management. Sgc8 targets PTK7 on the cell surface membrane of CCRF-CEM cells and TD05 targets IGHM on the surface of Ramos cells are used in the experiments. The membrane anchor is made of a PEG segment that connects the aptamers to a synthesized diacyl lipid tail containing two stearic acids. Immune effector cells were engineered with aptamers and then demonstrated to detect leukemia cells over non-restricted structures of the major histocompatibility complex (MHC), resulting in increased cancer cell detection and elimination. This was the primary information of aptamer-regulation T-cell redirection to specifically kill cancer cells (Zhu et al. 2015).

Aptamers are also used for immunological disease treatment. The main factor, which stimulates tumor growth factor, STAT3, suppresses CD8+ cytotoxic T cells and activates CD+ regulatory T cells. Thus, the CTLA4apt (aptamer to CTLA4) binds to STAT3 siRNA and the complex (CTLA4apt-STAT3 siRNA) reduces the amount of tumor to connected CD4+ monitoring T-cells. This complex effectively boosts antitumor immune responses in mouse model by depressing STAT3 activation. NA aptamers also bind and support the IL-10 receptor (IL-10R) to overwhelm tumor growth and viral infection (Yasmeen et al. 2020).

5 Conclusion and Future Direction

Several laboratories and companies are working to produce therapeutic compounds selectively to identify the tissues and cells of interest. These compounds have rare/no out of target and side effects, non-immunogenic, and have a powerful activity. Aptamers are intriguing compounds that meet many of the diagnostic and precision medicine needs (Nuzzo et al. 2019). When compared to chemotherapy, aptamer-mediated precision medicine produced better results in clinical applications. Precision medicine, in contrast to traditional chemotherapy, targets the unique characteristics of each patient's cancer phenotypic profile, resulting in dramatically higher therapeutic efficacy and less non-specific toxicity. Aptamer-mediated therapy can target a wide range of targets and can be easily customized by conjugating various anti-cancer drugs. However, because the technology was new and relevant, aptamer-based therapies had limited market success and there were no systematic scholarly investigations on pharmacokinetics and pharmacodynamics (Yang et al. 2018). Several challenges also addressed in aptamer-based targeted therapies which hinders commercialization. These include: nucleic acids are susceptible to in vivo environments, sometimes smaller size of aptamer can be disadvantageous, and SELEX technology is time-consuming and labor-intensive (Yoo et al. 2020). A potential approach for aptamer focused targeted delivery is the unique feature of aptamer and aptasensor that can address above issues. Thus, researches should be focused on commercializing of aptamer in

order to diagnose and treat cancer in an early stage with less or no side effects. More efforts on the investment of aptamer are needed to efficiently use aptamer in clinical purpose. More aptamers are expected to be used for diagnostic and therapeutic reasons in near future.

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Heat Shock Proteins: Central Players in Oncological and Immuno-Oncological Tracks

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Abstract

Heat shock proteins (HSPs) are a group of proteins that promote protein folding, inhibit denaturation of cellular proteins, and maintain other proteins' functional activities when cells are subjected to stress and/or high temperature. HSP classification is generally based on their molecular weights into large and small HSP. The family of small HSPs includes HSPs 27, 40, 60, 70, and 90. The potential roles of HSP27 and HSP70 are quite evident in different solid malignancies, including breast, colorectal, pancreatic, and liver cancers. In this chapter, the authors focus on HSP27 and HSP70 signaling in oncology and their role in different solid malignancies as well as they shed light on the novel role of HSP70 and HSP90 in the immune-oncology field.

Keywords

Breast cancer · $CD8^+$ cytotoxic T-cells · Colorectal cancer · Heat shock protein (HSP) · Hepatocellular carcinoma · Pancreatic cancer

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Abbreviations

AAD	Apoptosis activation domain
AIF	Apoptosis-inducing factor
Apaf-1	Apoptotic protease-activating factor 1
APC	Antigen-presenting cells
Ask1	Apoptosis signal-regulating kinase 1
BID	BH3 interacting-domain death agonist
BT-474	Human breast tumor cell line

CARD	Caspase-recruitment domain
CDK4	Cyclin-dependent kinase 4
Clever	Common lymphatic endothelial and vascular endothelial receptor
CRC	Colorectal cancer
Daxx	Death domain-associated protein 6
DEN	Diethylnitrosamine
DR	Death receptor
EMT	Epithelial to mesenchymal transition
ER	Endoplasmic reticulum
FADD	Fas-associated death domain
FBD	Fas binding domain
GADD153	Growth arrest and DNA damage-inducible gene 153
GRP	Glucose-related protein
HCC	Hepatocellular carcinoma
HER-2	Human epidermal growth factor 2
HSF	Heat shock factor
HSP	Heat shock protein
HUGO	Human genome organisation
JNK	C-Jun N-terminal kinase
LOX-1	Low-density lipoprotein receptor-1
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
NY-ESO-1	New York-esophageal
PES	Phenylethynesulfonamide
PHC	Primary hepatocellular carcinoma
PKC	Protein kinase C
PTEN	Phosphatase and tensin homolog
SPAG9	Sperm-associated antigen 9 protein
SREC-1	Scavenger receptor expressed by endothelial cell 1
SUMO	Small ubiquitin-like modifier
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
WT	Wild type

1 Introduction

1.1 Heat Shock Protein (HSP) History

Heat shock proteins (HSPs) are a group of proteins that facilitate protein folding, inhibit denaturation of cellular proteins, and maintain other proteins' functional activties when cells are subjected to stress or high temperature (Wu et al. 2017). The nomenclature of human HSP is based on the system proposed by the Human Genome Organisation (HUGO) Gene Nomenclature Committee and uses the Entrez Gene database from the National Center for Biotechnology Information (Kampinga et al. 2009). HSP was discovered by Ferruccio Ritossa, who found that small numbers of puffs on polytene chromosomes were induced after exposing the *Drosophila* larvae to temperatures higher than 36 °C (Pardue et al. 1987). The puffing pattern in *Drosophila cells* was correlated with an increase in the synthesis of some selected proteins following the exposure to stresses. One of these proteins was heat shock protein which was discovered in 1974 (Pardue et al. 1987).

1.2 HSP Classifications

Classifications of HSP are based on their molecular weights and they are divided into two main groups either small or large molecular weights. The family of small HSPs includes HSPs 27, 40, 60, 70, and 90 which will be the focus of this chapter. Large HSP includes two major members, glucose-regulated protein 170 (GRP170) and HSP110 (Ciocca and Calderwood 2005). Small HSPs are classified into two families: the first family is the small ATP-independent HSP and their molecular mass is between 8 and 28 kDa, while the second family is ATP-dependent and their corresponding mass ranges from 40 to 105 kDa in which the well-known chaperones of the 70 and 90 kDa families belong to it (Miller and Fort 2018).

1.3 Heat Shock Protein 27 (HSP27)

HSPs have an indispensable contribution to cancer progression, metastasis, apoptosis, and drug resistance (Das et al. 2019). High levels of HSP27 are detected in a myriad of malignancies and can be used as a diagnostic marker in cancer, prognostic marker, as well as a progression marker for the disease (Das et al. 2019; Yun et al. 2019). High expression levels of HSP27 are reported in pediatric acute myeloid leukemia, oral squamous cell carcinoma, thyroid cancer, high-grade

astrocytoma, breast cancer stem cells, hepatocellular carcinoma, invasive ductal breast carcinoma, myelodysplastic syndrome (MDS), and peritoneal metastasis of epithelial ovarian cancer (Yun et al. 2019). However, the immunochemical analysis showed that HSP27 levels decrease in neuroblastoma cells.

1.4 HSP27 Signaling in Oncology

The Fas receptor (CD95/Apo-1) is considered a member of the tumor necrosis factor (TNF) receptor superfamily, in which apoptotic signals are generated upon its activation by the Fas-L ligand (Hayashi et al. 2012). Depending on the adaptor proteins, two different pathways are initiated that interact with its death domain. The interaction between Fas and Fas-associated death domain (FADD) protein is the first pathway. The Fas-FADD interaction results in recruiting and triggering the activity of caspase-8, and consequently caspase-3 is activated as well which leads to the cleavage of crucial signaling proteins (Chang et al. 1998). Regarding the second pathway, the adapter protein Daxx and Fas interact with each other through the Fas binding domain (FBD), triggering apoptosis through its apoptosis activation domain (AAD) (Chang et al. 1998). Apoptosis mediated by Daxx involves the recruitment of the apoptosis signal-regulating kinase 1 (Ask1) and switching on C-Jun N-terminal kinase (JNK) leading to cell death (Chang et al. 1998). Dimeric phosphorylated HSP27 interacts with Daxx both in vivo and in vitro, suppressing its interaction with both Ask1 and Fas and thus blocking apoptosis (Charette and Landry 2000). HSP27 also prevents cytochrome C and dATP-triggered activity of caspase-9 suppressing etoposide-induced apoptosis (Garrido et al. 1999). The AKT activity is enhanced by the inhibition of BH3 interacting-domain death agonist (BID) or protein kinase C delta type (PKC δ) activity contributing to the cell survival (Kim et al. 2007; Parcellier et al. 2003) as shown in Fig. 1.

1.5 Role of HSP27 in Different Solid Malignancies

1.5.1 Breast Cancer

Angiogenesis and cell proliferation are enhanced by HSP27 in peripheral blood vessels by increasing the transcription of vascular endothelial growth factor (VEGF) gene and promoting VEGF receptor type 2 activation in breast cancer cells and subsequently enhancing breast cancer cell growth and metastasis (Wu et al. 2017). The expression of the tumor suppressor gene, phosphatase, and tensin homolog (PTEN) is enhanced by the low expression of HSP27 in breast cancer cells, indicating the negative regulation that HSP27 on PTEN (Cayado-Gutierrez et al. 2013). The downregulation of HSP27 induces chemosensitization to Herceptin and inhibits cancer cell proliferation by decreasing Her2 protein stability (Kang et al. 2008). It activates the EMT process and nuclear factor-κB that contributes to the maintenance of breast cancer stem cells (Wei et al. 2011). The nuclear expression of HSP forms whether the phosphorylated or non-phosphorylated ones can be tumor markers for breast cancer that is accompanied by lymphatic metastasis (Choi et al. 2019).

1.5.2 Colorectal Cancer

HSP27 is upregulated in colorectal cancer and its upregulation increases drug resistance (Yang et al. 2019; Huang et al. 2018). The downregulation of HSP27 leads to an increase in 5-fluorouracil sensitivity, which is why higher survival rates are reported in patients with low HSP27 expression levels than those having higher HSP27 expression rates (Choi et al. 2019).

1.5.3 Pancreatic Cancer

Gemcitabine resistance could be enhanced by the phosphorylation of HSP27. Furthermore, HSP downregulation will regulate the snail expression and increases the sensitivity to gemcitabine in the pancreatic cancer cell lines (Zhang et al. 2015; Taba et al. 2010).

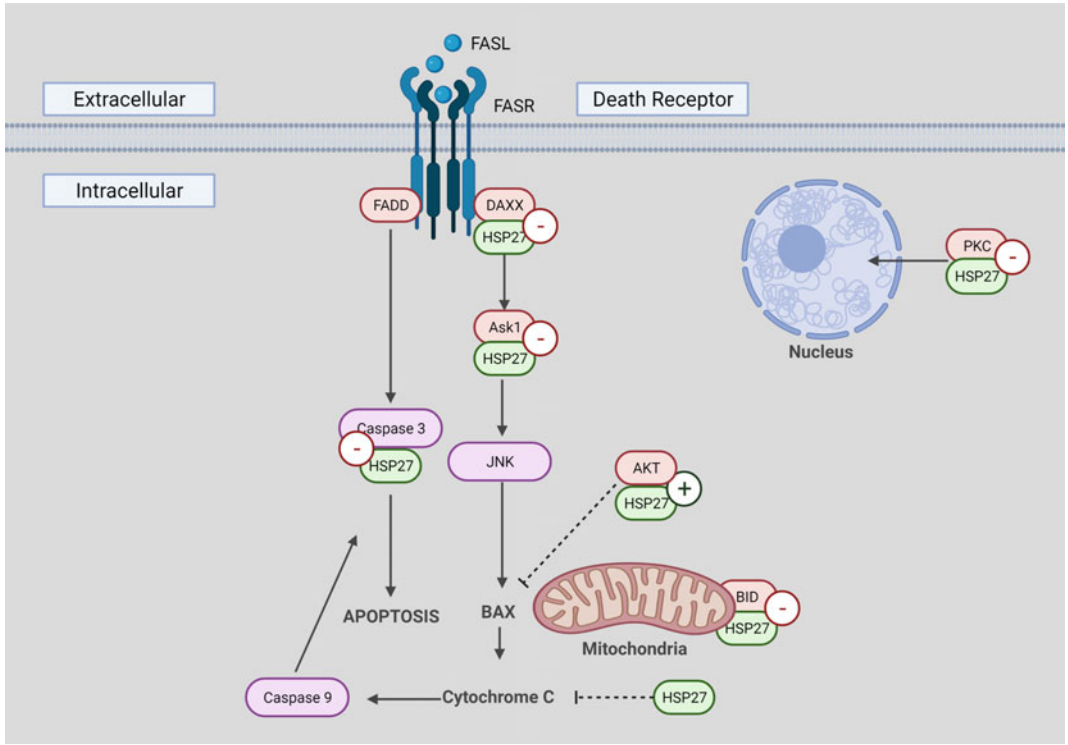

Fig. 1 Role of heat shock protein 27 (HSP27) in different cellular apoptotic processes
HSP27 inhibited apoptosis through the intercorrelation of many signaling pathways – whether intrinsic or extrinsic apoptotic pathways. Bcl-2-associated X protein (BAX) is inhibited by HSP27 through a direct binding to the death domain-associated protein (DAXX) or apoptosis signal-regulating kinase 1 (Ask1) so its function will be suppressed, thus encouraging AKT activity to inhibit BH3 interacting-domain death agonist (BID) or protein kinase C delta type (PKC δ) function. HSP27 also suppresses caspase-3 which contributes directly to cellular apoptosis

1.5.4 Liver Cancer

SUMOylation is an essential post-translational modification accompanied by the development of cancer types including liver cancer (Ge et al. 2017; Eto et al. 2016). Ge and his colleagues found that HSP27 was markedly upregulated in blood samples as well as the tissues of primary hepatocellular carcinoma (pHCC). In addition, they found that when HSP27 is knocked down, this resulted in lower rates of cancerous cell growth and invasion. A worthy notable observation was reported showing the interaction between SUMO2/3 and HSP27 which contributed to HSP27 stabilization inside the cytoplasm and subsequently enhanced the invasion of hepatocellular carcinoma (HCC) cells (Han et al. 2018).

1.6 Heat Shock Protein 70 (HSP70)

The human HSP70 family includes not less than eight members that possess differences between each other in the sequence of amino acids, expression level, and subcellular localization (Chakafana et al. 2019; Takakuwa et al. 2019). HSP70 comprises HSP70-1a, HSP70-1b, HSP70-1t, HSP70-2, HSP70-5, HSP70-6, Hsc70 and, HSP70-9 as shown in Table 1 (Murphy 2013). HSP70-1 protein high expression induces tumorigenicity to fibrosarcoma cells in mice and renders these cells resistant to the killing action of cytotoxic T-cells or macrophages (Jaattela 1995). The induced overexpression of HSP70 in fibrosarcoma enhances drug resistance to topotecan and gemcitabine (Sliutz et al. 1996). Also, transgenic

Table 1 Human HSP70 family (Murphy 2013)

Protein	Alternative names	Locus	Cellular localization
HSP70-1a	HSP70, HSP72, HSP70-1	*HSPA1A*	Cytosol, nucleus, and lysosomes
HSP70-1b	HSP70, HSP72, HSP70-1	*HSPA1B*	Cytosol, nucleus, and lysosomes
HSP70-1t	HSP70-hom	*HSPA1L*	Cytosol and nucleus
HSP70-2	HSP70-3, HSPA2	*HSPA2*	Cytosol and nucleus
HSP70-5	Bib, Grp78	*HSPA5*	Endoplasmic reticulum
HSP70-6	HSP70B	*HSPA6*	Cytosol and nucleus
HSC70	HSP70-8, HSP73	*HSPA8*	Cytosol and nucleus
HSP70-9	Grp75, mtHSP75, Mortalin	*HSPA9*	Mitochondria

mice were found to have enhanced T-cell lymphoma due to the high expression levels of HSP70-1 in T- cells of these mice (Seo et al. 1996). Recently, HSP70-1 was found to exhibit a crucial role in Her-2-induced mammary tumorigenesis (Meng et al. 2011). High levels of HSP70 could act as a marker for the early stages of prostate and hepatocellular cancers and its overexpression in prostate cancer was found to attribute to the resistance toward cisplatin along with advanced disease stages and lymph node metastasis in breast cancer and colorectal carcinoma (Chuma et al. 2003). Malusecka and his colleagues found a positive correlation between the Ki-67 proliferation index which represents a valuable prognostic factor for lung cancer and nuclear HSP70 staining (Malusecka et al. 2001). Moreover, HSP70 expression is also used as a marker for undifferentiated ovarian, uterine, cervical, and oral cancers, malignant cutaneous melanoma, and mediated imatinib resistance in acute myeloid leukemia (Yun et al. 2019; Chatterjee and Burns 2017; Wu et al. 2017; Murphy 2013). It was also found that the poor survival and worse prognosis in breast cancer, acute myeloid leukemia, or endometrial and cervical cancer is associated with the high staining of HSP70 (Murphy 2013).

1.7 HSP70 Signaling in Oncology

1.7.1 Apoptosis

Various pathways of cell death could be suppressed by HSP70 (Yun et al. 2019; Chatterjee and Burns 2017; Wu et al. 2017; Murphy 2013). First, CHOP, also known as GADD153, is incorporated in apoptosis accompanied by ER stress (Krajarng et al. 2015). Apoptosis is induced by the translocation of the proapoptotic Bcl-2 family protein Bax from the cytosol to the mitochondria (Murphy 2013). Gotoh and his colleagues reported the prevention of apoptosis mediated through CHOP by hsp70/DnaJ chaperone pair and this could happen by interacting with Bax along with preventing its translocation from the cytosol to mitochondria (Gotoh et al. 2004). It is also involved directly with the caspase-recruitment domain (CARD) of the cytosolic apoptotic protease-activating factor 1 (Apaf-1) and the interaction with it in an ATP-dependent fashion and the suppression of both the oligomerization of Apaf-1 mediated by cytochrome C/ATP and its subsequent association and activation of procaspase-9 (Saleh et al. 2000). In BCR-ABL-expressing cells, HSP70 binds to death receptors 4 and 5 (DR4 and DR5) and inhibits the activity of the death-inducing signaling complex (DISC). In cells expressing BCR-ABL, HSP70 interacts with both death receptors 4 and 5 (DR4 and DR5), preventing the activity induction of death-inducing signaling complex (DISC). Caspase-independent cell death is suppressed also by HSP70; this is done through the interaction with apoptosis-inducing factor (AIF) and the suppression of AIF-induced DNA fragmentation. Not only that, but also the BH3 interacting-domain death agonist-mediated apoptosis is suppressed by it along with the inhibition of the tumor necrosis factor-α pathway downstream and

several stress-induced kinases as well including C-Jun N-terminal kinase, apoptosis signal-regulating kinase, and p38 mitogen-activated protein kinase as shown in Fig. 2 (Murphy 2013).

Senescence

HSP70 possesses a significant role in controlling the senescence in cancerous cells and against p53-dependent and independent senescence (Murphy 2013). HSP70 is essential for the transformation of the mammary epithelial cells through HER2/neu. It was found that HER2/neu overexpression led to the induction of senescence, specifically when HSP70 is absent as shown in Fig. 2 (Meng et al. 2011).

Autophagy

HSP70 binds to endolysosomal lipid bisphosphate, stabilizes lysosomes, and inhibits lysosomal membrane permeabilization (Yun et al. 2019). It also stimulates autophagy (Wu et al. 2017). Tumor progression can be suppressed by inhibitors of autophagy such as hydroxychloroquine. Leu and his colleagues (2009) found that 2-phenylethynesulfonamide (PES) and their derivatives have a compatible relationship between the inhibition of autophagy and their anti-cancer

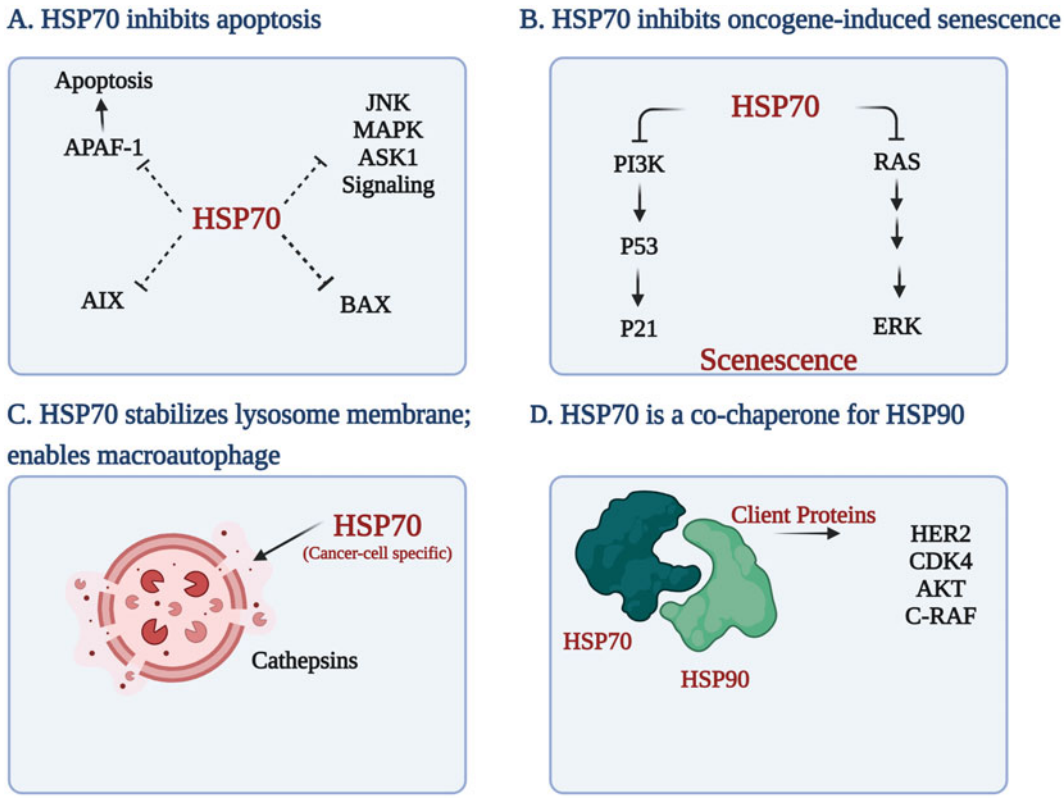

Fig. 2 Role of HSP70 proteins in cancer development and regulation (Murphy 2013)
(**a**) Apoptosis is inhibited by HSP70 through (i) binding to and suppressing the relocation of BAX from cytosol to mitochondria, (ii) forbidding the recruitment and transfer of Apaf-1 to the apoptosome, (iii) binding to and suppressing the kinase activity that is correlated with stress signaling, and (iv) binding to AIF disrupting its activity
(**b**) HSP70 controls cell senescence. HSP70 disrupts cell senescence with the following: (i) disrupting P53-dependent regulation and (ii) antagonizing p-53-independent regulation
(**c**) Lysosome functions are stabilized through binding to endolysosomal lipid bisphosphate and autophagy stimulation
(**d**) HSP70 regulates HSP90 client proteins. HSP70 works as a co-chaperone for HSP90 by delivering the client proteins to HSP90

action as shown in Fig. 2 (Murphy 2013; Leu et al. 2011).

HSP90

HSP70 and HSC70 (its family member) are essential co-chaperones for HSP90 and both participate in the delivery of required proteins to HSP90 (Yun et al. 2019). It was found that when small interfering RNA targets both HSC70 and HSP70, this resulted in proteasome-dependent degradation of HSP90 client proteins which includes also C-RAF and CDK4 as well as tumor apoptosis (Murphy 2013). Massey and his colleagues found that compound Ver-155,008 inhibited HSC70, HSP70, and Grp-78. These inhibitions resulted in the breakdown of the HSP90 corresponding proteins RAF-1 and HER2 and the induction of apoptosis (Massey et al. 2010). HSP90 client proteins such as epidermal growth factor receptor, AKT, and HER2/ErbB2 were sequestered in tumor cells as an insoluble fraction by the HSP70 inhibitor, 2-phenylethynesulfonamide (PES) (Leu et al. 2011). HSP70 inhibitors significantly enhanced the cytotoxicity of the HSP90 inhibitor and inhibited HSP90 machinery.

1.8 Role of HSP70 in Solid Malignancies

1.8.1 Breast Cancer

Jagadish and his colleagues found that HSP70-2 was involved in the development or the progression of breast cancer and was expressed in all breast cancer cell lines (BT-474, SK-BR-3, MCF7, and MDA-MB-231) (Jagadish et al. 2016a). Cellular growth, tumor size, and motility were significantly reduced in the animal models inoculated with MCF7 or MDA-MB-231 cells after HSP70-2 depletion (Jagadish et al. 2016a). It was also found by the same group that the downregulation of HSP70-2 led to not only a decline in the expression levels of cyclins, cyclin-dependent kinase (CDK), and mesenchymal markers, but also it promoted the expression of CDK inhibitors (Jagadish et al. 2016a). A recent study was published showing that the metastatic characteristics of the HeLa, A549, and MCF7 cancerous cell lines were augmented by the downregulation of HSP70 A1A. HSP70 silencing in these cell lines rendered the cells unable to form cadherin-catenin complexes, so they were detached from neighboring cells, and metastasis and EMT were promoted (Kasioumi et al. 2019).

1.8.2 Colorectal Cancer

HSP70-2 is a novel target for colorectal cancer treatments. It was reported by Jagadish and his colleagues that the gene expression of HSPA2 was found in 78% of the specimens of CRC patients disregarding the stages and grades of the disease. HSP70-2 gene expression was prominent in the early stages (I and II) of CRC patients (Jagadish et al. 2016b). There were no differences found between the HSP70-2 gene and the expression of the protein since the protein of HSP70-2 was also found to be expressed in CRC patients in 78% of them. Sperm-associated antigen 9 protein (SPAG9) was produced in 78% of patients having CRC, OY-TES-1 protein was recorded in only 43.3% of the CRC patients, New York-esophageal (NY-ESO-1) protein was found to be present in only 10% of the CRC patients, and the melanoma-associated antigen 3 (MAGE-A3) protein was expressed in 8% of CRC patients (Jagadish et al. 2016b). From these data, the authors found that HSP70-2 could act as a crucial player in multiple stages and diverse grades of CRC patients (Jagadish et al. 2016a).

1.8.3 Pancreatic Cancer

The overexpression of HSP70 in pancreatic cancerous cells led to the inhibition of cell death and the arrest to the response of conventional therapy. Pancreatic cancerous cells and normal ductal cells were compared with each other by Saluja and Dudeja after analyzing the expression level of HSP70; the results indicated a marked increase in the HSP70 protein as well the mRNA levels in the pancreatic cancer cells (Saluja and Dudeja 2008). Moreover, comparing the HSP70 levels in the pancreatic cancer tissue with the normal margins, HSP70 was found to be remarkably high, suggesting the possibility of HSP70 having a role in the resistance of pancreatic cancer cells to apoptosis. Consequently, HSP70

inhibitors could be indicated as therapeutic tools for pancreatic cancer (Saluja and Dudeja 2008).

Heat shock response was found to be inhibited by quercetin in a dose-dependent manner and this could be done by suppressing kinases, the ones which activate the HSP70 expression by phosphorylating HSF1 or reducing the accumulation of HSP70 (Wang et al. 2009). Quercetin was precluded due to its nonspecific toxicity and high dose requirement in a clinical setting (Wang et al. 2009). Lately, triptolide, a new compound, was discovered as a suppressor for the heat shock response (Westerheide et al. 2006). The way it works was by inhibiting HSP70 mRNA and protein expression in a dose-dependent manner within the nM range in pancreatic cancer cells. It was also found to be an activator for caspase-3 and caspase-9, upon which the apoptotic cascade switches on by promoting annexin V and terminal deoxynucleotidyl transferase biotin-dUTP-nick end labeling (TUNEL) which are apoptotic markers (Xia et al. 2012).

1.8.4 Liver Cancer

HSP70 could be potential diagnostic biomarkers for HCC patients (Zaimoku et al. 2019). Furthermore, the potential role of HSP70 in HCC was further investigated in HCC progression (Cho et al. 2019). The authors observed the administration of diethylnitrosamine (DEN) to male mice at postnatally induced primary HCC in 100% of the animal population within 7–8 months. The tumor size and numbers were reduced in DEN-treated HSP70−/− mice in comparison with the wild-type (WT) mice, indicating a strong inhibitory action of HSP70 ablation on tumor development. It was concluded that HSP70 deletion inhibited tumor angiogenesis (Cho et al. 2019).

1.9 Novel Role of HSP70 and HSP90 in the Immune-Oncological Circuits

Both HSP70 and HSP90 can stimulate the anti-tumor immune response whether as natural immunogens or as carriers for antigenic peptides in which major histocompatibility complex (MHC) class I molecules cross-present them (Shevtsov and Multhoff 2016). Antigen cross-presentation on MHC class I is followed by initiation in CD8+ cytotoxic T-cell response (Cruz et al. 2017). The antigen-presenting cell (APC) uptake of HSP and HSP-chaperoned exogenous peptides is done through many receptors such as lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1); alpha-2 macroglobulin receptor CD91; toll-like receptors 2 and 4 (TLRs 2 and 4); scavenger receptor expressed by endothelial cell 1 (SREC-1); fasciclin EGF-like, laminin-type EGF-like and link domain-containing scavenger receptor-1 (FEEL-1); common lymphatic endothelial and vascular endothelial receptor (Clever-1); stabilin-1 (Stab-1); and CD40 (Murshid et al. 2012). The activation of the cytolytic, proliferative, and migratory capacity of natural killer (NK) cells is carried out by HSP70 (HSPA1A) by increasing the expression of NK cell receptors such as the activator C-type lectin receptors CD94/NKG2C and NKG2D. Furthermore, the infiltration of NK cells and CD8+ cytotoxic T-cells is increased upon intra-tumoral infusion of free recombinant HSP70 associated with IFN-γ secretion (Shevtsov and Multhoff 2016).

As previously mentioned, the $HSP70^{+}$ tumor phenotype is associated with highly aggressive tumors that cause invasion and metastasis. However, the pre-activated NK cells with either naturally occurring HSP70 or HSP70-peptide (TKD) combined with IL-2 in low dose (TKD/IL-2) can kill membrane $HSP70^{+}$ tumor cells via apoptosis mediated through granzyme B (Specht et al. 2015). CD94 receptor mediated the interaction between NK cells and HSP70-peptide because this receptor is upregulated after the incubation of NK cells with HSP70 protein or HSP70 peptide together with IL-2, leading to an increase in cytolytic activity of NK cells (Gross et al. 2003). Other receptors are involved in the activation of NK cells including NKG2D and natural cytotoxicity receptor (NCR) (Rahmoon et al. 2017; Youness et al. 2016). HSP70 also enhances dendritic cell

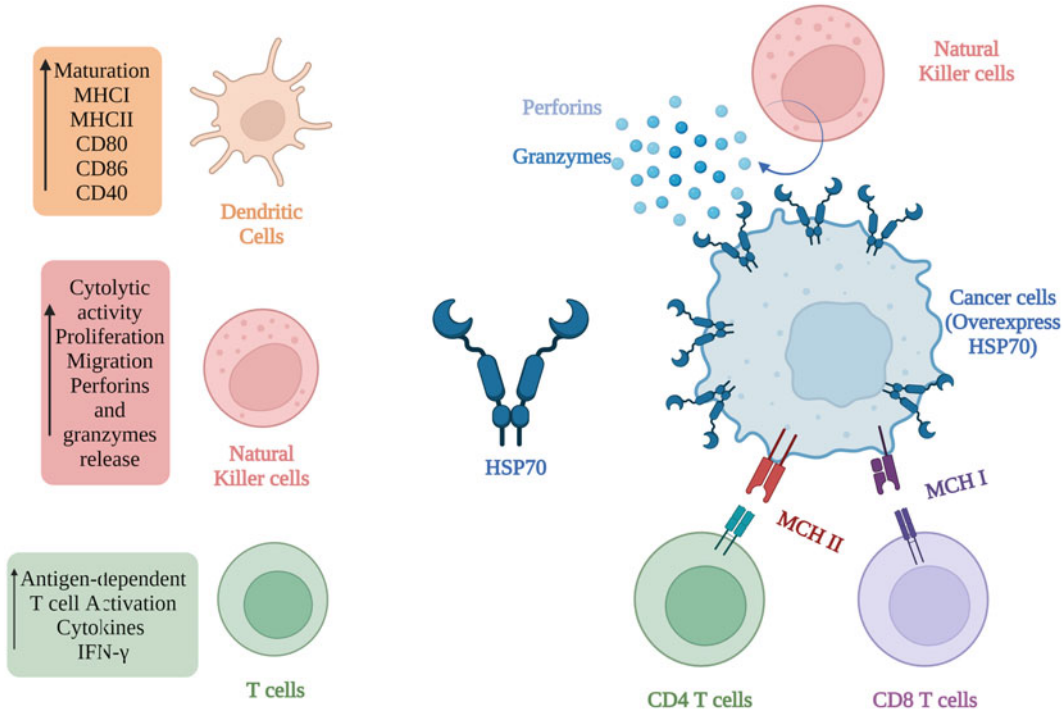

Fig. 3 Role of HSP70 proteins in innate and adaptive immune response
HSP70 affects several immune cells at the tumor microenvironment. For instance, it activates NK cell cytotoxicity and killing capacity through the activation of perforins and granzymes. HSP70 also activates dendritic cells' maturation and its antigen-presenting ability, while for CD8+ T-cells, HSP70 activates T-cell activity and IFN-γ production. Concerning the macrophages, HSP70 increases the expression of several cytokines and chemokines, for instance, TNF-α, nitric oxide and interleukin-1, interleukin-6, and interleukin-12

(DC) maturation, activates the cytolytic and migratory capacity of NK cells, induces the activation of antigen-dependent T-cell, induces the secretion of IFN-γ, and stimulates the release of pro- and anti-inflammatory cytokines (Das et al. 2019). It activates $CD8^+$ T-cell by MHC I molecule and $CD4^+$ via MHC II molecule (Shevtsov and Multhoff 2016) as shown in Fig. 3.

2 Conclusion

In conclusion, HSPs were shown to act as vital players in the oncological circuits. HSP could act as a diagnostic and prognostic marker in different oncological contexts. Nonetheless, HSP plays a fundamental role in switching on several anti-apoptotic and metastatic signals such as the activation of the VEGF signaling pathway and several anti-apoptotic proteins. HSP70 and HSP90 come on the top of the list as aggressive deregulated HSP in many types of solid malignant tumors, for instance, breast, liver, pancreatic, and colorectal cancer. It is also worth mentioning that HSP70 and HSP90 have recently been cast as critical players at the cancer-immune synapse where they, directly and indirectly, affect the cytotoxic immune cells present in the microenvironment of the tumor: CD8+ cytotoxic T lymphocytes and NK cells.

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Conflict of Interest All authors declare they have no conflict of interest.

Ethical Approval for Studies Involving Humans This article does not contain any studies with human participants performed by any of the authors.

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