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Kursad Turksen *Editor*

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Stem Cells in Tissue Differentiation, Regulation and Disease

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Cell Biology and Translational Medicine, Volume 15

Stem Cells in Tissue Differentiation, Regulation and Disease

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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. Among topics explored in this volume are regulatory aspects of stem cells, differentiation, and organogenesis 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. Certainly, COVID-19-related regulation of stem cell function and potential stem cell–mediated therapeutic options for COVID are such timely topics included here.

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

I would also like to acknowledge and thank Mariska van der Stigchel, Assistant Editor, for her outstanding efforts in helping to bring this volume to the production stages.

A special thank you goes to Shanthi Ramamoorthy and Rathika Ramkumar 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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Molecular Mechanisms of SARS-CoV-2/COVID-19 Pathogenicity on the Central Nervous System: Bridging Experimental Probes to Clinical Evidence and Therapeutic Interventions

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic, induced by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has dramatically impacted the global healthcare systems, constantly challenging both research and clinical practice. Although it was initially believed that the SARS-CoV-2 infection is limited merely to the respiratory system, emerging evidence indicates that COVID-19 affects multiple other systems including the central nervous system (CNS). Furthermore, most of the published clinical studies indicate that the confirmed CNS inflammatory

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manifestations in COVID-19 patients are meningitis, encephalitis, acute necrotizing encephalopathy, acute transverse myelitis, and acute disseminated encephalomyelitis. In addition, the neuroinflammation along with accelerated neurosenescence and susceptible genetic signatures in COVID-19 patients might prime the CNS to neurodegeneration and precipitate the occurrence of neurodegenerative diseases, including Alzheimer's and Parkinson's diseases. Thus, this review provides a critical evaluation and interpretive analysis of existing published preclinical as well as clinical studies on the key molecular mechanisms modulating neuroinflammation and neurodegeneration induced by the SARS-CoV-2. In addition, the essential age- and gender-dependent impacts of SARS-CoV-2 on the CNS of COVID-19 patients are also discussed.

Keywords

COVID-19 · Neurodegeneration · Neuroinflammation · Neuroinvasion · Neurosenescence · SARS-CoV-2

Abbreviations

ACE-2	Angiotensin-converting enzyme 2
AD	Alzheimer's disease
ADEM	Acute disseminated encephalomyelitis
ANE	Acute necrotizing encephalopathy
ANM	Acute necrotizing myelitis
ARB	Angiotensin receptor blocker
ATM	Acute transverse myelitis
BBB	Blood-brain barrier
Bmal1	Brain and muscle Arnt-like protein-1 gene
BSG	Basigin
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CJD	Creutzfeldt-Jakob disease
CNS	Central nervous system
COVID-19	Coronavirus disease 2019
CSF	Cerebrospinal fluid
CXCL	C-X-C motif chemokine ligand
DAMPs	Damage-associated molecular patterns
DMT1	Divalent metal transporter 1
DPP4	Dipeptidyl peptidase 4
G-CSF	Granulocyte colony-stimulating factor
HCoV-OC43	Human coronavirus OC43
ICU	Intensive care unit
IFN	Interferon
IL	Interleukin
IVIG	Intravenous immunoglobulin
LETM	Longitudinally extensive transverse myelitis
MCP	Monocyte chemoattractant protein
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metalloprotease
mRNA	Messenger RNA
NF-kB	Nuclear factor kappa-light chain enhancer of activated B cells
NK	Natural killer
NLRP3	NLR family pyrin domain containing 3
NO	Nitric oxide
NOS	Nitrogen oxygen species
NRP1	Neuropilin-1
P2X7	Purinergic receptor P2X7
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PD	Parkinson's disease
PDC	Pyruvate dehydrogenase complex
ROS	Reactive oxygen species
S	Spike protein
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
TLR	Toll-like receptor
TMPRSS	Transmembrane serine protease
TNF-α	Tumor necrosis factor alpha

1 Introduction

Since the emergence of first reported cases of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection (Gorbalenya et al.

2020) at the end of 2019 in Hubei Province, China (Petersen et al. 2020), there has been an unprecedented global effort to describe the virus and the clinical course of the disease (Burki 2021; Happi et al. 2021). Identifying the molecular mechanisms of its pathogenicity has become one of the main goals in understanding and, hopefully, controlling the viral spread worldwide. SARS-CoV-2, the causative agent of the coronavirus disease 2019 (COVID-19), is a positive-sense single-stranded RNA virus, which belongs to the subgenus *Sarbecovirus* of the genus *β-coronavirus*. Its genome is strikingly similar to the bat coronavirus and the receptor-binding domain of the spike (S) glycoprotein of the Malayan pangolin coronavirus (Kadam et al. 2021).

Although in humans the infection usually presents itself with systemic and respiratory manifestations, neurological complications have been reported as well in up to 40% of COVID-19 patients (Jakhmola et al. 2020; Groppa et al. 2020; Silva et al. 2020). Involvement of the central nervous system (CNS) was confirmed by the presence of SARS-CoV-2 in the cerebrospinal fluid (CSF) (Moriguchi et al. 2020; Wu et al. 2020a) and brain parenchyma (Mukerji and Solomon 2021; Serrano et al. 2021), specifically in neuronal and capillary endothelial cells (Baig et al. 2020). It has been postulated that the neurovirulence of SARS-CoV-2 is mediated by its binding to angiotensin-converting enzyme 2 (ACE-2) receptors expressed by endothelial cells of cerebral capillaries and neuronal and glial cells (Ribeiro et al. 2021). The SARS-CoV-2 invasion triggers the innate and adaptive immune responses that drive the neuroinflammation within the CNS compartments and lead to neuronal cell damage and loss (Wu et al. 2020a; Serrano et al. 2021). Neuroinflammation along with accelerated cellular senescence and susceptible genetic signatures might prime the CNS to neurodegeneration and precipitate the occurrence of neurodegenerative diseases (Septyaningtrias and Susilowati 2021; Duarte et al. 2021). Addressing the key mechanisms that modulate the SARS-CoV-2 neuropathogenicity might be translated into the identification of therapeutic targets and development of promising interventions able to counteract the COVID-19-associated CNS injury.

In this review, we provide a summarized update of the pathophysiological mechanisms of SARS-CoV-2-induced CNS tissue damage, with a particular focus on the molecular pathways of neuroinvasion, neuroinflammation, and neurodegeneration. We also cover the emerging spectrum of clinical neuroinflammatory and neurodegenerative disorders associated to COVID-19 and discuss the therapeutic approaches targeting the molecular pathways aimed to minimize the SARS-CoV-2 damage to CNS compartments.

2 The Neurotropism of SARS-CoV-2

2.1 Current Evidence for the Potential Neuroinvasion Routes of SARS-CoV-2

Several recent studies assessing the CNS alterations in fatal COVID-19 cases have provided the first hints into the disease's histopathological tissue morbidities, suggested new data on the presence of viral RNA in the cortical neurons, and revealed the pathological features associated to viral infection with minimal immune cell infiltrate (Puelles et al. 2020; Matschke et al. 2020; Yang and Shen 2020; Solomon et al. 2020; Song et al. 2021). However, recent autopsy reports and postmortem brain magnetic resonance imaging (MRI) scans of COVID-19 patients demonstrated mixed results. Some reports failed to demonstrate the presence of SARS-CoV-2 and any abnormalities in the respiratory center of the brain (Coolen et al. 2020; Kantonen et al. 2020), while one autopsy showed massive microglial activation and T cell infiltration in the medulla oblongata (Schurink et al. 2020), and recent autopsy studies using electron microscopy found viral particles entrapped in dilated vesicles of the neurons in samples from the frontal lobes of COVID-19 patients (Paniz-Mondolfi et al. 2020). Moreover, a study using mice overexpressing human ACE-2 demonstrated that SARS-CoV-2 neuroinvasion,

but not respiratory infection, is actually associated with COVID-19 mortality (Song et al. 2021). Given the multiple reports of CNS invasion, it is reasonable to discuss first the possible ways by which SARS-CoV-2 can invade cells of the CNS.

2.1.1 Hematogenic Route

The hematogenic route of SARS-CoV-2 neuroinvasion involves the circulation of viral particles in the bloodstream and subsequent penetration of the blood-brain barrier (BBB) (Desforges et al. 2014; Zubair et al. 2020). The latter occurs either through the infection of vascular endothelial cells and transcytosis or through migration of virus-infected leukocytes into the CNS, also known as the "Trojan horse mechanism" (Fig. 1a) (Zubair et al. 2020; Kim et al. 2003).

The virus is likely to enter the blood stream and, subsequently, the CNS through the respiratory tract via epithelium-to-capillary or pneumocyte-to-capillary connections, which allow the virus into the pulmonary/systemic circulation (Barrantes 2020). SARS-CoV-2 may also enter the circulation through the gastrointestinal tract via both endocytic and nonendocytic mechanisms (Nash and Buchmeier 1997). The gut-brain axis may be key component involved in disorders that affect the CNS (de Mattos Coelho-Aguiar et al. 2019) since the SARS-CoV-2 infects the human intestinal epithelium, which has a higher expression of ACE-2 receptors than the lungs (Lamers et al. 2020; Xiao et al. 2020). Interestingly, SARS-CoV-2 was detected in COVID-19 patients' rectal swabs (Chen et al. 2020a; Tang et al. 2020) and fecal samples (Wu et al. 2020b) and remains detectable in these samples even after negative results of polymerase chain reaction (PCR) from the nasopharyngeal swab tests. Moreover, COVID-19 patients with concomitant gastrointestinal symptoms have worse clinical outcomes with increased acute respiratory distress and need for mechanical ventilation (Jin et al. 2020).

After SARS-CoV-2 reaches the blood stream, transcytosis to the BBB is due to the presence of the virus in the peripheral circulation paired with the slow blood flow within the microvasculature that enhances binding of the SARS-CoV-2 (S) protein and the ACE-2 receptors in the capillary endothelium, thereby promoting the viral transport across the basolateral membrane (Baig et al. 2020; Dey et al. 2021). Indeed a recent study reported that viral-like particles were actively budding across the brain endothelial cells, suggesting that the hematogenic route is the most probable pathway for SARS-CoV-2 entry into the CNS (Paniz-Mondolfi et al. 2020). After interacting with ACE-2 in the endothelium of cerebral blood vessels, SARS-CoV-2 could alter the BBB permeability and further facilitate viral entry into the CNS (Ibrahim Fouad 2021). This increase in BBB permeability is evidenced by postmortem brain MRI scans of COVID-19 patients showing brain parenchymal abnormalities suggestive of BBB breakdown (Coolen et al. 2020) and can be worsened by the cytokine storm and systemic inflammatory response with remarkable BBB permeability effects triggered by SARS-CoV-2 (Li et al. 2020a; Iadecola et al. 2020a). Furthermore, SARS-CoV-2 can directly reach the CNS through the hematogenic route connecting the nasal mucosa to the anatomically adjacent forebrain (Barrantes 2020).

The "Trojan horse mechanism" is evidenced by the identification of SARS-CoV-2 in circulating monocytes and lymphocytes from infected patients (Gu et al. 2005) and by single-cell sequencing where viral RNA was detected in the macrophages from bronchoalveolar lavage fluid of COVID-19 patients, although it is uncertain whether the immune cells were infected themselves or had phagocytized virus-infected cells (Bost et al. 2020). These infected monocytes can travel to the blood stream, and the peripheral circulation may bring the virus to other organ systems, including the CNS (Gu et al. 2005; Spiegel et al. 2006). Indeed, SARS-CoV-2 can

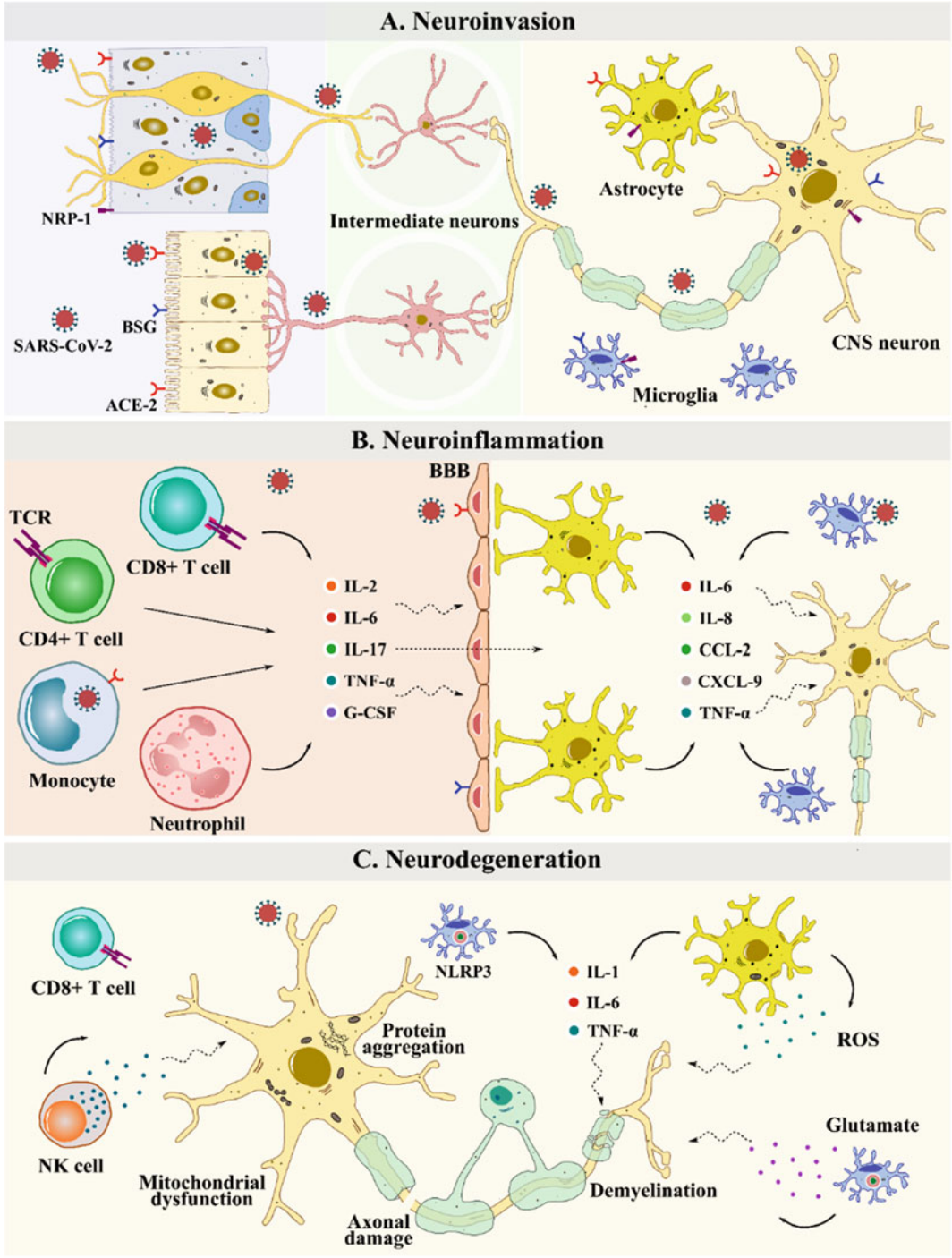

Fig. 1 The SARS-CoV-2-associated mechanisms of neuroinvasion, neuroinflammation, and neurodegeneration. (**a**) Neuroinvasion. The SARS-CoV-2 may gain access into the central nervous system (CNS) via hematogenic and neurogenic routes by binding to the angiotensin-converting enzyme 2 (ACE-2) receptor, basigin (BSG), or neuropilin-1 (NRP1). Through the hematogenic route, SARS-CoV-2 reaches the CNS by infecting the cerebral endothelial cells, through compromised blood-brain barrier (BBB) or via circulating virus-infected leucocytes, the "Trojan horse" mechanism. Through the neurogenic route, SARS-CoV-2 may reach

infect dendritic cells, which are known to migrate to lymph nodes and may be able to disseminate the virus (Spiegel et al. 2006).

2.1.2 Neurogenic Route

The neurogenic pathway, an important route used by neurotropic viruses, involves viral migration to the CNS by infection of the sensory or motor nerve endings and consequent use of the retrograde or anterograde neuronal transport mediated by dynein and kinesin motor proteins to move the virus toward the neuron body (Swanson 2nd and McGavern 2015). The potential neuronal retrograde/anterograde transport and the transsynaptic transfer of SARS-CoV-2 are supported by in vitro studies that detected the virus within neuronal soma and neurites in a human-induced pluripotent stem cell (iPSC)-derived BrainSphere model and in human brain organoids, where SARS-CoV-2 exerted metabolic changes through unique pathways compared to other neurotropic viruses, such as the Zika virus (Song et al. 2021; Bullen et al. 2020). In addition to the retrograde transport and similar to other coronaviruses, SARS-CoV-2 may exploit the axonal endoplasmic reticulum of infected neurons to disseminate within the brain parenchyma (Fenrich et al. 2020).

A clear example of a neurogenic pathway is the olfactory neuronal transport (Fig. 1a). Recently, Meinhardt et al. demonstrated the presence of SARS-CoV-2 RNA and SARS-CoV-2 S protein in anatomically distinct regions of the nasopharynx and brain by in situ hybridization and immunohistochemical staining techniques, which suggest that SARS-CoV-2 could enter the nervous system by crossing the neural-mucosal interface as a result of the close vicinity of the olfactory mucosal, endothelial, and nervous tissues, including the olfactory and sensory nerve endings (Meinhardt et al. 2021). It was also suggested that SARS-CoV-2 might spread from the olfactory epithelium to the olfactory bulb and nerve through endocytosis and exocytosis during transsynaptic transfer (Bulfamante et al. 2020; Pennisi et al. 2020). Furthermore, postmortem brain MRI scans and autopsy of COVID-19 patients also discovered asymmetric olfactory bulbs, microglia activation, astrogliosis, and T cell infiltration in the olfactory bulb, which indicate that the olfactory neuroepithelium may mediate viral entry (Schurink et al. 2020). Supporting evidence previously demonstrated that other coronaviruses are able to enter the brain upon intranasal infection (Netland et al. 2008; Doobay et al. 2007) and that intranasal inoculation of SARS-CoV-2 results in a lethal disease with high levels of viral replication in the brain of mice expressing the human ACE-2 receptor (Kumari et al. 2021). Furthermore, a more recent study proposed that, in humans,

Fig. 1 (continued) the CNS by infecting the epithelial lining of the olfactory mucosa or digestive tract and the supplying nerve endings with subsequent retrograde neuronal transport toward the CNS. (**b**) Neuroinflammation. The SARS-CoV-2-triggered innate and adaptive immune responses may involve the CNS tissue, thereby causing neuroinflammation. Pro-inflammatory cytokines (e.g., IL-6, TNF-α) released by peripheral inflammatory cells (e.g., neutrophils, monocytes, T cells) increase the permeability of the BBB and activate the CNS-resident cells (i.e., microglia and astrocytes). In turn, microglial cells and astrocytes also secrete pro-inflammatory cytokines (e.g., IL-6, IL-8) and chemokines (e.g., CCL-2, CXCL-9), which alter the BBB integrity and recruit peripheral leukocytes into the CNS, ultimately resulting in neuronal cell damage and demyelination. Besides antiviral protection by destruction of the virus-infected astrocytes and microglial cells, the activity of CD8+ T cells might be directed against the neuronal cells and cause cytolysis and demyelination. (**c**) Neurodegeneration. The migrated immune cells and CNS-resident cells may maintain a chronic neuroinflammatory milieu within the CNS that causes neuronal damage, demyelination, and axonal loss. Within the activated microglial cells, the NLR family pyrin domain containing 3 (NLRP3) inflammasome mediates the release of pro-inflammatory cytokines (e.g., IL-6, TNF-α), which exert various deleterious effects on CNS tissue. Activated microglial cells and astrocytes also release reactive oxygen species (ROS) and glutamate, which is responsible for the excitotoxic damage of neuronal cells. Exposure of neurons to SARS-CoV-2 might alter the function of the endoplasmic reticulum and mitochondria and impair the proteostasis, thereby leading to aggregation of misfolded proteins and neuronal apoptosis, altogether translating into neurodegeneration

SARS-CoV-2 CNS entry is due to the infection of supporting, non-neuronal cells, which express ACE-2 receptors and the transmembrane serine protease 2 (TMPRSS2) (Brann et al. 2020).

In addition to the transcribial route and the olfactory nerve, SARS-CoV-2 might also be transferred to the CNS through other peripheral nerves such as the vagus nerve, by which lung and gut afferents reach the brainstem (Yavarpour-Bali and Ghasemi-Kasman 2020; Esposito et al. 2020; Li et al. 2020b). The anterograde and retrograde viral transmission from duodenal cells to brainstem neurons has been reported (Parker et al. 2020), which could be achieved by SARS-CoV-2 infection of enterocytes and further transmission via the vagus nerve to glial and neuronal cells within the enteric nervous system (Esposito et al. 2020; DosSantos et al. 2020). The trigeminal nerve, which innervates nociceptive cells in the nasal cavity and has been successfully tested for drug transportation (Lochhead et al. 2019), may also be a route for SARS-CoV-2 migration to the CNS since it provides sensory nerve endings to the conjunctiva, where SARS-CoV-2 RNA fragments have been found in a patient with conjunctivitis, and to the taste buds, which ascend to the trigeminal nuclei and the nuclei of the solitary tract (Chen et al. 2020b). In addition, the oculomotor and glossopharyngeal nerves are believed to serve the same purpose (Keyhan et al. 2020; Wu et al. 2020c).

2.2 CNS Targets of SARS-CoV-2

2.2.1 Angiotensin-Converting Enzyme 2

Numerous studies have established, beyond doubt, that the membrane-bound metalloprotease ACE-2 is the SARS-CoV-2 host cell receptor (Barrantes 2020). Histochemical and, more recent, messenger RNA (mRNA) transcriptomic studies have dissected its cellular localization in various tissues and proved that SARS-CoV-2 binds to the enzymatic domain of the ACE-2 receptor exposed on the surface of several cell types, including alveolar cells, intestinal epithelial cells, endothelial cells, kidney cells, monocytes/macrophages, neuroepithelial cells, and neurons (Paniz-Mondolfi et al. 2020; Lukiw et al. 2020). Interestingly, ACE-2 is also highly expressed in the two main regions responsible for the regulation of the respiratory cycle, ventrolateral medulla, and nucleus of the solitary tract (Montalvan et al. 2020). After binding of the S protein to the ACE-2 receptor, subsequent cleavage by TMPRSS2, cathepsin L, or furin induces the endocytosis and translocation of SARS-CoV-2 into the endosomes or direct viral envelope fusion with the host cell membrane for cell entry (Millet 2018; Sanclemente-Alaman et al. 2020). In fact, it has been reported that the SARS-CoV-2 S protein can induce the genetic expression and activity of cathepsin L, in vitro (Duarte et al. 2021).

One of the main arguments against SARS-CoV-2 neuroinvasion is that mRNA levels of ACE-2 appear to be very low in the CNS (Chen et al. 2020a; Qi et al. 2020; Sungnak et al. 2020); however, widespread expression of ACE-2 was recently found in both microtubule-associated protein 2-positive neurons and cells in the neural tube-like structures of the human brain organoids, indicating that the mRNA levels of ACE-2 do not accurately reflect the ACE-2 protein expression in the CNS (Song et al. 2021).

2.2.2 Dipeptidyl Peptidase-4

Dipeptidyl peptidase 4 (DPP4), originally known as cluster of differentiation (CD) 26, was identified as a functional receptor for the Middle East respiratory syndrome coronavirus, acting as its main entry pathway (Park et al. 2019). The DPP4 is generally expressed in human bronchiolar and lung epithelial cells (Raj et al. 2013), but it can also be found in the intravascular portion of the endothelial cells and in the CSF (Al-Badri et al. 2018). Furthermore, bioinformatic approaches combining the prediction of human-virus protein interactions and protein docking based on crystal structures have revealed high affinity between the human DPP4 and the S receptor-binding domain of SARS-CoV-2 (Li et al. 2020b). This observation raised the hypothesis that SARS-CoV-2 might also use the DPP4 enzyme as a functional receptor to gain entry into the host cells (Vankadari and Wilce 2020).

However, another study failed to demonstrate the SARS-CoV-2 binding to DPP-4 or the protective role of DPP4 inhibitors against COVID-19 (Fadini et al. 2020).

2.2.3 Basigin

Basigin (BSG) or CD147, a surface molecule belonging to the immunoglobulin superfamily that is a potent inducer of matrix metalloproteases (MMPs) and vascular endothelial growth factor, as well as an important regulator of cell metabolism (Chen et al. 2010), has been reported to be an alternative entry receptor for SARS-CoV-2 (Ulrich and Pillat 2020; Wang et al. 2020). It is widely expressed in epithelial, neuronal, myeloid, and lymphoid cells (Grass and Toole 2016), which might increase the likelihood of infection of multiple organs including those in the CNS. The most robust data supporting the role of BSG as an alternative receptor for SARS-CoV-2 comes from a study using co-immunoprecipitation, surface plasmon resonance, and enzyme-linked immunosorbent assays, which identified BSG as the binding partner of the SARS-CoV-2 S protein that is essential to viral invasion (Wang et al. 2020). Notably, this original finding has already been translated into an open-label clinical trial of a humanized monoclonal antibody against BSG, meplazumab, which reported striking improvements in COVID-19 patients (Bian et al. 2020). However, a recent report was unable to find evidence supporting the role of BSG as a putative binding receptor for the S protein or evidence of direct interaction between the S protein and either of the two common BSG isoforms (Shilts et al. 2021). A recent genomic study investigating gene variants linked to SARS-CoV-2 infection failed to find evidence of BSG variant enrichment in COVID-19 patients despite identifying associations to more established viral entry factors, such as the TMPRSS2 (Latini et al. 2020).

2.2.4 Transmembrane Serine Proteases

In conjunction with the ACE-2 receptor, SARS-CoV-2 uses TMPRSS2 for S protein priming during viral entry (Brann et al. 2020; Hoffmann et al. 2020). TMPRSS2 plays a key role in facilitating the SARS-CoV-2 S fusogenic activity on the host cell plasma membrane and is essential for SARS-CoV-2 entry to the cellular cytoplasm (Zang et al. 2020). The olfactory epithelium cells, mainly sustentacular cells, Bowman's gland cells, and basal cells, highly express the TMPRSS2 receptor, which is necessary for viral binding, replication, and accumulation; however, TMPRSS2 and ACE-2 are, both, absent in the olfactory sensory neurons (Brann et al. 2020; Bilinska et al. 2020; Fodoulian et al. 2020; Butowt and Bilinska 2020). Therefore, a direct connection between TMPRSS2 and neuroinvasion cannot be established.

2.2.5 Neuropilin-1

Neuropilin-1 (NRP1), a transmembrane receptor that lacks a cytosolic protein kinase domain, is also expressed in areas of the CNS, including olfactory-related regions such as the olfactory tubercles and paraolfactory gyri (Davies et al. 2020). The NRP1 receptor has two isoforms: a truncated, secreted form and a transmembrane form, which interacts with SARS-CoV-2 and can serve as an entry factor that potentiates SARS-CoV-2 infectivity, in vitro (Cantuti-Castelvetri et al. 2020; Daly et al. 2020). Its interactions with SARS-CoV-2 and the receptor identification in areas of the CNS suggest the potential role of NRP1 as an additional SARS-CoV-2 infection mediator implicated in the neurological manifestations of COVID-19. While NRP1 alone promotes SARS-CoV-2 entry and infection, its co-expression with ACE-2 and TMPRSS2 markedly potentiates this process (Cantuti-Castelvetri et al. 2020; Daly et al. 2020). Moreover, autopsy studies in COVID-19 patients, as well as mice studies, showed that SARS-CoV-2-infected NRP1-positive cells in the olfactory epithelium, tract, and bulb can mediate the transport of virus-sized particles from the intranasal cavity to the brain (Cantuti-Castelvetri et al. 2020).

2.2.6 Cathepsins

Cell senescence is a pro-inflammatory transformation observed in any cell type due to age- and non-age-related DNA damage (Malavolta et al. 2020). Senescent cells are more susceptible to viral infection and release inflammatory

cytokines of the senescence associated secretory phenotype (SASP) (Malavolta et al. 2020). The cytokines identified in the SARS-CoV-2-mediated cytokine storm are also part of the SASP, which includes cathepsins (Cat) (Borghesan et al. 2020), a group of lysosomal proteases known to be activated by the S proteins from older coronaviruses to facilitate membrane fusion and subsequent release of viral RNA into the host cell (Gierer et al. 2013; Iwata-Yoshikawa et al. 2019; Simmons et al. 2013). A recent animal study demonstrated an age- and sex-related increase in genetic expression and enzymatic activation of CatB, CatL, and CatK by macrophages in response to the SARS-CoV-2 S protein (Duarte et al. 2021). Furthermore, CatL inhibitors have been suggested as targets for COVID-19 treatment by multiple authors (Gomes et al. 2020; Liu et al. 2020; Sacco et al. 2020).

3 SARS-Cov-2-Triggered Neuroinflammation

Inflammation within the CNS compartments after SARS-CoV-2 infection may be elicited by infiltrating peripheral immune cells and their cytokines or by specific responses of the CNS-resident cells (Fig. 1b). Deciphering their roles is of particular importance since, both, peripheral and resident cells initiate and maintain the neuroinflammatory milieu within the CNS tissue.

3.1 Mechanisms of Immune-Mediated Neuronal Damage

3.1.1 SARS-Cov-2-Mediated Cytokine Storm

Systemic inflammation triggered by SARS-CoV-2 may be followed by an exaggerated secretion of pro-inflammatory cytokines known as a cytokine storm, a maladaptive immune response to the infection (Ragab et al. 2020). A wide range of elevated cytokine levels were detected in SARS-CoV-2 patients, including interleukin (IL)-1β, IL-2, IL-7, IL-8, IL-9, and IL-10, granulocyte colony-stimulating factor (G-CSF), granulocyte-monocyte colony-stimulating factor, macrophage inflammatory protein (MIP) 1A, MIP 1B, monocyte chemoattractant protein 1 (MCP1), IFN-γ, and tumor necrosis factor alpha (TNF-α) (Huang et al. 2020a). These cytokines are produced by the cells of innate (macrophages, dendritic cells, and natural killer (NK) cells) and adaptive (cytotoxic T cells (CD8+), T helper cells (CD4+), and B cells) immunity (Costela-Ruiz et al. 2020; İnandıklıoğlu and Akkoc 2020). Although, in patients with SARS-CoV-2 infection, T helper 17 (Th17) cells were recognized as one of the key players in mediating the cytokine storm (Wu and Yang 2020; Xu et al. 2020). High levels of circulating cytokines lead to leukocyte recruitment and increased BBB permeability, allowing the passage of immune cells, cytokines, and chemokines into the CNS tissue (Ragab et al. 2020). In addition, cytokines activate the CNS innate immune signaling pathways, thereby amplifying the neuroinflammatory responses (Iadecola et al. 2020a).

Interleukin 6 and 17 gained much attention, since several studies showed their potential contribution to the emergence of neuroinflammation due to SARS-CoV-2 infection (Najjar et al. 2020; Espíndola et al. 2021). Interleukin 6 is the most frequently reported cytokine increased in patients with SARS-CoV-2 infection (Keyhanian et al. 2020) and is involved in the immunopathogenesis of neuroinflammatory syndromes identified in SARS-CoV-2 patients, such as encephalitis (Bodro et al. 2020a, b), steroid-responsive encephalitis (Pilotto et al. 2020), and acute necrotizing encephalopathy (Virhammar et al. 2020). The mechanisms underlying the effect of IL-6 in the promotion of neuroinflammation are endothelial dysfunction of the BBB, activation of the coagulation and complement system cascades, and modulation of the expression of genes involved in inflammation, apoptosis, and oxidative stress (Erta et al. 2012). Similarly, the cytokine storm reported in patients with SARS-CoV-2 infection includes increased levels of IL-17 (Huang et al. 2020a), which is known to enhance the secretion of other cytokines (G-CSF, IL-1β, IL-6, and TNF-α) and chemokines (MIP 2A, MIP

3A), as well as increase the activation of MMPs and cyclooxygenase-2 (Costela-Ruiz et al. 2020). Furthermore, IL-17 can induce neurovascular endothelial expression of MCP1 and C-X-C motif chemokine ligand (CXCL) 1, which facilitate the traffic of activated Th17 cells into the CNS (Wojkowska et al. 2017). Additionally, IL-17 can cause neuronal toxicity and injury by activation of the nuclear factor kappa-light chain enhancer of activated B cells (NF-kB) (Najjar et al. 2020).

3.1.2 Blood-Brain Barrier Disruption

Several mechanisms have been identified, which might compromise the integrity of the BBB and promote inflammation within the CNS compartments during the onset of SARS-CoV-2 infection. First, the main mediators of the cytokine storm, IL-6, TNFα, and IL-17, were shown to alter the permeability of the BBB under experimental conditions (Najjar et al. 2020). Second, similar to the infection of endothelial cells and inflammation within the peripheral vessels, SARS-CoV-2 might induce the inflammation of the cerebral endothelial cells and, thereby, disrupt the BBB (Teuwen et al. 2020). Third, the barrier functions of the BBB might be altered by activated microglial cells, which can disintegrate the tight junctions of the BBB endothelium by releasing pro-inflammatory cytokines and inducing MMP activity (Bongetta et al. 2020). Following BBB disruption, the interaction between the peripheral immune mediators and the CNS-resident cells translates into impaired neurotransmission, glutamate-mediated neurotoxicity, synaptic alterations, and neuroglial cell death (Septyaningtrias and Susilowati 2021).

3.1.3 Adaptive Immunity Responses

The cytotoxic CD8+ T cells are essential players in antiviral immune protection through IFN-γ, granzyme B, and perforin, which destroy the virus-infected astrocytes and microglial cells (Savarin and Bergmann 2018). Meanwhile, CD4+ T cells boost the cytolytic activity of CD8+ T cells within the CNS by secreting IFN-γ and increasing the expression of major histocompatibility complex (MHC) class II in microglia and MHC class I antigens in oligodendrocytes (Septyaningtrias and Susilowati 2021). Hence, the CD8+ T cells mediate viral clearance from resident glial cells and CD4+ T cells from oligodendrocytes (Savarin and Bergmann 2018). Notwithstanding their antiviral activity, T cell responses are also associated with CNS tissue injury by inducing cytolysis and demyelination and instructing myeloid cells to initiate tissue damage (Savarin and Bergmann 2018). Compared to non-hospitalized COVID-19 patients, the hospitalized ones displayed increased blood levels of cytotoxic follicular helper cells and cytotoxic T helper cells and reduced levels of regulatory T cells (Meckiff et al. 2020). Moreover, infiltration of T cells in perivascular spaces and brain parenchyma was detected in patients with SARS-CoV-2 infection (Bryce et al. 2020; Hanley et al. 2020). However, the relevance of this identified T cell profile in patients with neuroinflammatory complications remains to be elucidated.

3.1.4 Autoimmunity

Besides the cytokine- and adaptive cell-mediated inflammatory injury to the CNS tissues, the SARS-CoV-2 infection may as well induce autoimmune responses. Currently, the underlying mechanisms of SARS-CoV-2-triggered autoimmune CNS complications are poorly understood; however, the cross-reactivity between the SARS-CoV-2 and CNS epitopes and "bystander activation" of pre-primed auto-reactive T cells might be postulated (Ehrenfeld et al. 2020). In line with molecular mimicry hypothesis, immune responses triggered by SARS-CoV-2 infection and directed to combat the virus might cross-react with the self-antigens in the cerebrovascular endothelial cells or neurons and result in an autoimmune inflammation. The inflamed CNS tissue might generate damage-associated molecular patterns (DAMPs) and autoantigens that stimulate the inflammasome platforms and predispose to autoimmunity (Talotta and Robertson 2020). The S protein was shown to play a distinct role in induction of immune-mediated neuropathology given its high immunogenic potential (Lyons-Weiler 2020). Autoimmune complications of the

CNS so far reported are: anti-N-methyl-D-aspartate receptor encephalitis (Monti et al. 2020; Panariello et al. 2020), Bickerstaff encephalitis (Llorente Ayuso et al. 2021; Ayuso et al. 2020), autoimmune meningoencephalitis, and acute disseminated encephalomyelitis (ADEM) (Paterson et al. 2020).

3.2 Compartmentalized Neuroglial Responses

The CNS-resident cells may be involved in neuroinflammation either by circulating cytokines or viral proteins leaked into the CNS through the breached BBB (Fig. 1b). In the first scenario, pro-inflammatory cytokines from the systemic circulation stimulate the microglial cells and astrocytes to produce cytokines (Wu et al. 2020a). In the second scenario, viral proteins and molecular complexes (e.g., nuclear protein high mobility group box 1) from peripheral tissue injured cells might reach the CNS and behave as DAMPs and pathogen-associated molecular patterns (PAMPs) (Iadecola et al. 2020a). Consequently, both DAMPs and PAMPs may launch an innate immune response in the cells expressing toll-like receptors (TLRs), such as CNS-resident macrophages, microglia, and astrocytes, which release pro-inflammatory cytokines (Iadecola et al. 2020b). Both scenarios result in a neuroinflammatory environment (Dogan et al. 2020), ultimately leading to CNS tissue damage.

3.2.1 Astrocyte Responses

Despite the presence of ACE-2 receptors in astrocytes (Chen et al. 2021), it is unclear whether SARS-CoV-2 can infect the astrocytes. Preliminary data from a recent histopathological study found that the affected brain tissue displayed foci of SARS-CoV-2 infection and replication, particularly in astrocytes (Tavcar et al. 2021). The susceptibility of human astrocytes to SARS-CoV-2 infection was confirmed in the same study, showing that the replication of SARS-CoV-2 occurs in neural stem cell-derived human astrocytes in vitro through a mechanism that involves a spike-NRP1 interaction (Tavcar et al. 2021). Astrocytes play important roles in orchestrating the immune responses to neuroinfection and neuroinflammation since reactive astrocytes promote immune-mediated inflammation and neuronal damage by secretion of pro-inflammatory cytokines (Tremblay et al. 2020). Astrocytes produce IL-1β, IL-6, IL-8, IL-12, IL-17, and TNF-α that exert neurotoxic effects and alter neuronal survival (Choi et al. 2014; Sofroniew 2014). Their detrimental effects are enhanced by the release of other cytotoxic factors such as nitric oxide (NO), reactive oxygen species (ROS), nitrogen oxygen species (NOS), and glutamate (Tremblay et al. 2020). Furthermore, damaged neurons release the adenosine triphosphate, which activates the purinergic receptor P2X7 (P2X7) expressed by microglia and astrocytes and results in increased Ca^{2+} influx and glutamate release (Ribeiro et al. 2021). This glutamate level increase is the main mechanism of excitotoxicity that leads to neuronal cell damage and subsequent neurodegeneration (Mahmoud et al. 2019). In addition, the astrocyte-released chemokines CXCL10, CXCL12, chemokine (C-C motif) ligand (CCL) 2, and CCL5 recruit peripheral leukocytes and resident microglia into the inflamed CNS (Choi et al. 2014; Sofroniew 2014). Increased levels of CCL2 mRNA were identified in astrocytes following human coronavirus OC43 (HCoV-OC43) infection (Aghagoli et al. 2020), while binding of MCP1 to the C-C chemokine receptor type 2 is known to alter the permeability of the BBB (Stamatovic et al. 2005). Postmortem studies in deceased COVID-19 patients are indicative of reactive astrogliosis due to increased expression of glial fibrillary acidic protein in the white matter (Reichard et al. 2020). Aside from the many inflammatory and neurodegenerative roles mentioned above, astrocytes have a protective role by secretion of anti-inflammatory cytokines, such as IL-4, IL-5, and TGF-β, and neurotrophic factors, such as the brain-derived neurotrophic factor (Sofroniew 2014).

3.2.2 Microglial Responses

Microglia are primarily scavenger cells critically involved in immune surveillance and inflammatory responses within the CNS that can acquire one of the two functional states, M1 (pro-inflammatory) or M2 (immunoregulatory) (Lloyd and Miron 2019). The M1 microglia produce pro-inflammatory cytokines promoting and maintaining neuroinflammation, while the M2 microglia mediate tissue repair and neuronal survival (Lloyd and Miron 2019). Upon viral invasion, microglia phagocytize apoptotic neuronal and glial cells and pathogens and remove cellular debris from the sites of injury (Tremblay et al. 2020). The M1 phenotype microglia secrete pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-8, and IL-12, and chemokines, such as CCL4, CCL5, CXCL2, CXCL4, CXCL9, and CXCL10, which are responsible for neuronal cell damage and demyelination (Benarroch 2013). The secretion of pro-inflammatory cytokines is mediated by the NLR family pyrin domain containing 3 (NLRP3) inflammasome that is induced by the activation of microglial P2X7 receptors (Ribeiro et al. 2021), and their cytotoxic effects are amplified by ROS and NO radicals produced by activated microglia (Benarroch 2013). Incidentally, the microglia express both ACE and ACE-2 receptors, the latter of which mediates SARS-CoV-2 infection, and the imbalance between their expression contributes to the transition from the M2 toward the M1 state (Cui et al. 2019). The SARS-CoV-2 transfected cells release a significant amount of exosomes that stimulate the activation of pro-inflammatory genes from microglia leading to severe neuroinflammation within the CNS (Mishra and Banerjea 2021). Microglia sense and strongly respond to IL-6 and IFN-α in various neuroinflammatory diseases, including viral CNS infections (West et al. 2019). As reported in previous studies, the level of coronavirus neurovirulence is related to its differential ability to induce the secretion of pro-inflammatory cytokines, including IL-6 and TNF-α, in microglial cells in the mouse brain and spinal cord compartments (Li et al. 2004). In addition, microglial cells are essential for the regulation of CNS responses to other coronavirus infection, as their depletion results in enhanced viral replication and evasiveness from the adaptive immunity (Wheeler et al. 2018), which suggests that besides the cytokine profile, microglia may also affect the ability of the virus to infect neuronal cells and the viral intra- and interneuronal transport within the CNS compartments after SARS-CoV-2 infection.

3.2.3 Neuronal Responses

As discussed previously, neuronal and glial cells might be infected by SARS-CoV-2; however they display a different susceptibility. SARS-CoV-2 can infect neurons in vitro and cause neuronal death, but data from the CSF and autopsy studies do not provide consistent evidence of direct CNS invasion (Iadecola et al. 2020b). Experimental studies on CNS cells derived from the human pluripotent stem cells particularly highlighted the increased susceptibility of dopaminergic neurons but not of cortical neurons or microglial cells (Yang et al. 2020). SARS-CoV-2 replication inside the neurons leads to cellular stress, damage, and death not only of infected neurons but also of bystander cells (Song et al. 2021). Similarly, experimental studies on coronavirus-associated neuropathogenicity evidenced that HCoV-OC43 induced cell death of mice hippocampal and cortical neurons through apoptosis of both infected and non-infected cells, with some of the mice developing chronic encephalitis with persistence of HCoV-OC43 in affected neurons for longer periods of time (Jacomy et al. 2006). Ultimately, the structural and functional integrity of the neurons might be altered by the deleterious effects of the adaptive and innate immune systems (see Sect. 3.1), on the one hand, and by viral replication inside the cells, on the other hand. However, due to the absence of MHC antigens in neurons, the elimination of viruses from the neurons depends merely on the antiviral activity of cytotoxic T cells, which can further injure CNS tissue.

3.3 Clinical Spectrum of Neuroinflammatory Syndromes

The neuroinflammatory syndromes associated with the SARS-CoV-2 infection can involve the brain (meningitis, encephalitis, and acute necrotizing encephalopathy), spinal cord (acute transverse myelitis, acute necrotizing myelitis), or both (acute disseminated encephalomyelitis) and may even occur without any preceding respiratory manifestations (Nepal et al. 2020). These CNS syndromes are considered para- and post-infectious immune-mediated complications of the SARS-CoV-2 infection.

3.3.1 Meningitis/Encephalitis

Several cases of probable meningitis/encephalitis due to SARS-CoV-2 infection have been reported, where patients have presented with fever, altered mental status, seizures, meningeal signs, and various focal symptoms, including sensory and motor deficits, hiccups, myoclonus, and opsoclonus (Paterson et al. 2020; Nersesjan et al. 2021). In most cases, lymphocytic pleocytosis and elevated protein levels in the CSF and focal brain abnormalities on brain imaging were evidenced (Paterson et al. 2020). A direct causal relation of SARS-CoV-2 and encephalitis was suggested by the presence of low levels SARS-CoV-2 in the CSF (Huang et al. 2020b) and the perivascular lymphocytic infiltrates within the temporal lobe of COVID-19 patients (Efe et al. 2020). Since SARS-CoV-2 was not detected within the brain tissue but increased levels of IL-1β and IL-6 were identified in the CSF, an exaggerated systemic inflammatory response triggered by the SARS-CoV-2 replication in other cells and tissues may be the most plausible explanation for this causal relation (Bodro et al. 2020a). In addition, autoimmune brainstem encephalitis (Paterson et al. 2020; Khoo et al. 2020a; Khoo et al. 2020b), rhombencephalitis (Wong et al. 2020), and Bickerstaff encephalitis (Llorente Ayuso et al. 2021) were also observed in SARS-CoV-2 infected patients. Treatment included steroids, plasmapheresis, antibacterial treatment, and antiseizure medication (Keyhanian et al. 2020).

3.3.2 Acute Necrotizing Encephalopathy (ANE)

Acute necrotizing encephalopathy is a rare immune-mediated disorder that follows an acute febrile usually viral illness with clinical presentation of altered mental status, focal neurological deficits, seizures, and high rates of disability (Wu et al. 2015). The neuroimaging features of ANE are multiple and symmetrically distributed lesions (sometimes with hemorrhages) within the thalamus, basal ganglia, brainstem, and subcortical white matter (Pohl et al. 2016). In SARS-CoV-2 patients, cases with widespread cerebellar lesions (Ciolac et al. 2021), with brainstem involvement and aplastic anemia (Dixon et al. 2020), and with myocarditis (Elkady and Rabinstein 2020) were described in SARS-CoV-2 patients. The CSF findings were unremarkable, most frequently without detectable SARS-CoV-2 RNA (Nersesjan et al. 2021). The cytokine storm triggered by the SARS-CoV-2 infection is thought to cause these cases of ANE (Mehta et al. 2020). Therefore, therapeutic approaches, including corticosteroids, intravenous immunoglobulin (IVIG), and plasma exchange showed varying degrees of response with outcomes ranging from death to satisfactory recovery (Nersesjan et al. 2021).

3.3.3 Acute Transverse Myelitis (ATM)

Acute transverse myelitis is an inflammatory and usually idiopathic spinal cord disease causing acute inflammation of the gray and white matter in one or more adjacent spinal cord segments. A growing number of ATM cases associated with SARS-CoV-2 infection are being reported (Mondal et al. 2020; Garg et al. 2021). Most of the cases were associated with the emergence of longitudinally extensive transverse myelitis (LETM) (Mondal et al. 2020; Garg et al. 2021), and just one case reported whole spinal cord involvement (AlKetbi et al. 2020). The neurological manifestations of COVID-19-related ATM included motor, sensory, and autonomic symptoms, and cases with available MRI were

characterized by an abnormal signal over multiple segments of the spinal cord (Garg et al. 2021). Additionally, patients presented a nonspecific CSF inflammatory profile with elevated protein levels and lymphocytic pleocytosis, while SARS-CoV-2 RNA was detectable in the CSF of a minority of patients (Garg et al. 2021). Patients received corticosteroids, IVIG, or plasma exchange with favorable outcomes in majority of patients.

3.3.4 Acute Necrotizing Myelitis (ANM)

Acute necrotizing myelitis is a rare inflammatory disorder of the spinal cord characterized by hemorrhages and cavitation on spinal MRI. Along with ATM, two cases of ANM associated with SARS-CoV-2 infection were recently described (Sotoca and Rodríguez-Álvarez 2020; Maideniuc and Memon 2020). The clinical presentation was marked by limb weakness and sensory and bladder symptoms, while the MRI findings for both cases presented as LETM, with T2-hyperintense lesions, spinal cord swelling, and areas of necrosis, and the CSF showed high protein levels and cell counts and no oligoclonal bands (Sotoca and Rodríguez-Álvarez 2020; Maideniuc and Memon 2020). Both patients responded positively to high-dose steroids and plasma exchange.

3.3.5 Acute Disseminated Encephalomyelitis (ADEM)

The ADEM is a demyelinating, usually monophasic, disorder occurring after a viral infection or vaccination and manifesting clinically with multifocal deficits and encephalopathy (Pohl et al. 2016). Neuroimaging presentation includes bilateral and asymmetric lesions within the subcortical white matter, cortical gray-white matter junction, thalamus, basal ganglia, cerebellum, and brainstem (Pohl et al. 2016). Spinal cord lesions occur in one third of the patients and involve multiple segments (Pohl et al. 2016). The classical presentation of ADEM in patients with SARS-CoV-2 infection has been reported in several case studies (Paterson et al. 2020; Parsons et al. 2020; Novi et al. 2020). The clinical manifestations ranged from headache, sensory and motor deficits to cranial nerve involvement, encephalopathy, and seizures, while the MRI showed hyperintense lesions, with some of the patients developing hemorrhagic changes and a few presenting concomitant myelitis (Paterson et al. 2020). No traces of SARS-CoV-2 RNA were detected in the CSF of patients or in the neuropathological samples of the brain tissue (Paterson et al. 2020). The applied treatment included steroids or IVIG with outcomes varying from death to partial recovery (Paterson et al. 2020; Reichard et al. 2020; Parsons et al. 2020).

3.4 Illustrative Case 1

A young male in his 40s complaining of fever, breathlessness, and cough for the last 2 days was admitted to the COVID-19 intensive care unit (ICU) with altered mental status, bulbar palsy, and bilateral limb ataxia. The naso−/oropharyngeal swabs were positive for SARS-CoV-2 infection, and the CSF analysis revealed lymphocytic pleocytosis and presence of red blood cells, without detectable SARS-CoV-2 RNA. His chest computed tomography (CT) showed bilateral pneumonia with ground glass appearance, and the brain MRI was characterized by widespread cerebellar and thalamic lesions (Fig. **2**). Other potential causes, including herpes simplex virus 1 and 2, cytomegalovirus, and Epstein-Barr virus, were excluded. The clinical, CSF and imaging findings were indicative of an ANE. During hospitalization, along with cerebellar and brainstem symptomatology, cognitive and behavioral impairment dominated the clinical presentation. The patient showed good response upon pulse steroid and sedation therapy, with a favorable outcome at discharge.

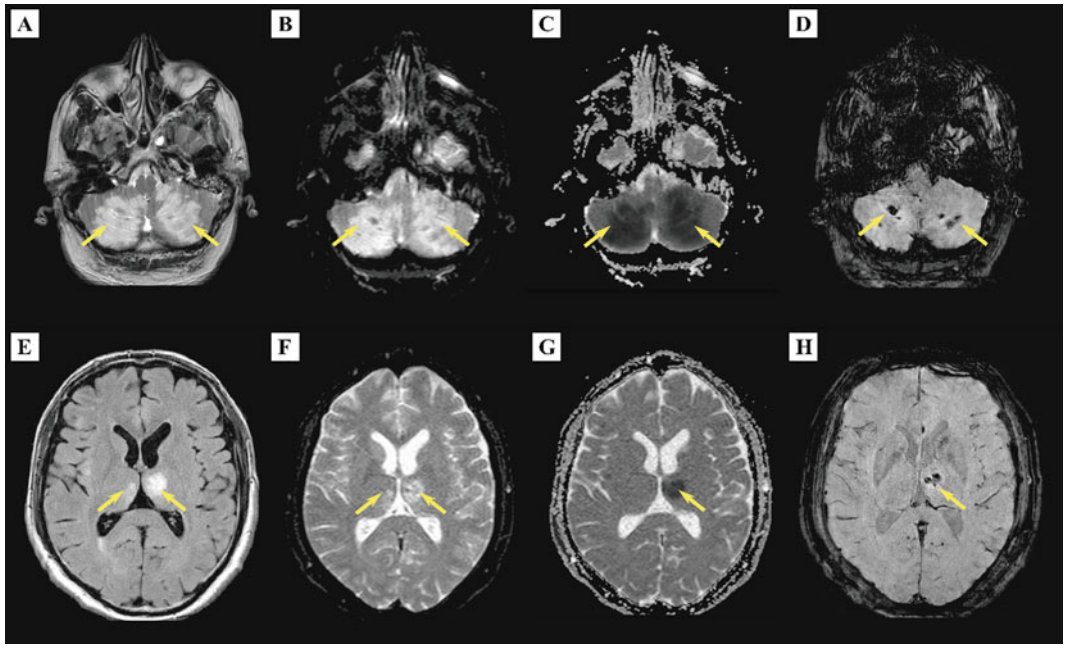

Fig. 2 Brain imaging from the illustrative case 1 (acute necrotizing encephalopathy). Bilateral cerebellar and thalamic lesions (arrows) displaying a hyperintense signal on axial fluid-attenuated inversion recovery images (**a, e**), restricted diffusion (cytotoxic edema) on diffusion-weighted images (**b, f**) and apparent diffusion coefficient maps (**c, g**), and hemorrhages on susceptibility-weighted images (**d, h**); personal courtesy of DC

4 SARS-Cov-2 Priming Effects in Neurodegeneration

4.1 Mechanisms Linking SARS-Cov-2 to Neurodegenerative Diseases

Neurotropic viruses can precipitate the neurodegenerative processes within the CNS via multiple mechanisms, among which the most extensively documented are chronic neuroinflammation and accelerated cellular senescence (Fig. 1b). These mechanisms also extrapolate to SARS-CoV-2 pathogenicity and were hypothesized to trigger the molecular cascades of neurodegeneration that translate into new-onset neurodegenerative disorders or aggravate the preexisting ones.

4.1.1 Inflammatory Mechanisms of Neurodegeneration

Involvement of the immune system pathways is a common feature of neurodegenerative and neuroinflammatory disorders. Experimental studies assessing the effects of neurotropic coronaviruses on the CNS showed the recruitment of innate and adaptive immune cells, which are responsible for neuroglial injury (Wang et al. 2006a, b). As previously mentioned in Sect. 3.1, infiltrating peripheral immune cells and CNS-resident cells release pro-inflammatory cytokines, including IL-1, IL-6, and TNF-α, which cause glutamate-mediated excitotoxicity and, eventually, neuronal and axonal damage (Ribeiro et al. 2021). Additionally, these cytokines can induce degeneration of normal neurons through the upregulation of NF-kB and mitogen-activated protein kinase (Hong et al. 2016).

Microglial cells play a critical role in the immune responses of coronavirus-infected mice developing encephalitis (Lavi and Cong 2020). In fact, moderate to intense activation of microglial cells was the most remarkable pathological feature identified on postmortem histopathological

examination of patients with SARS-CoV-2 infection (Hanley et al. 2020). Persistent microglial activation is associated with neurotoxicity, cellular senescence, and subsequent progression of neurodegenerative disorders (Hickman et al. 2018). Damage by microglia during SARS-CoV-2 infection could be mediated by activated microglial NLRP3 inflammasome, which promotes the production of pro-inflammatory cytokines and the aggregation of pathogenic peptides, as well as mitochondrial dysfunction and apoptosis, which contribute to neurodegeneration (Ising et al. 2019).

The contribution of T and B cells from the adaptive immune system to the neurodegenerative processes following the SARS-CoV-2 infection is less clear; however, perivascular and pericapillary infiltration of T cells without B cells in severe cases of COVID-19 was evidenced (Hanley et al. 2020). T cell infiltration is frequently observed in neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD) (Hong et al. 2016), and similar autoimmune mechanisms of neurodegeneration related to coronavirus infection were previously suggested by the identification of anti-CoV antibodies in the CSF of PD patients (Fazzini et al. 1992). Therefore, it is reasonable to hypothesize that SARS-CoV-2-induced autoimmune responses within the CNS tissue might promote the neurodegenerative processes as well.

Neutrophils contributed to oligodendrocyte loss and demyelination through a CXCL1-dependent mechanism, in mouse models of viral exposure (Marro et al. 2016). Such apoptosis of oligodendrocytes can occur upon the activation of mitochondrial pathways with caspase-8 as upstream signal (Liu et al. 2006). Since ACE-2 is expressed by oligodendrocyte progenitor cells (Chen et al. 2021), SARS-CoV-2 could potentially affect the differentiation of oligodendrocytes and aggravate the demyelination. Indeed, in patients with severe SARS-CoV-2 infection, the levels of neurofilament light chain, a marker of axonal injury and axonal neurodegeneration, were found to be high and increased over time (Kanberg et al. 2020). Similarly, higher levels of glial fibrillary acidic protein, a marker of astrocyte injury, were detected in moderate and severe SARS-CoV-2 infection (Kanberg et al. 2020). This data aligns with a previous report of axonal damage mediated by CD4+ and CD8+ T cells that has been observed in areas of demyelination and in adjacent areas with intact myelin after coronavirus infection (Dandekar et al. 2001).

Considering all the aforementioned, it can be postulated that the intricate interplay between the virus-induced peripheral and central immune responses mediated by the CNS-resident and adaptive immune cells and their cytokines converges on common neuroinflammatory pathways that drive the pathogenesis of neurodegeneration.

4.1.2 Accelerated Neurosenescence

Cellular senescence can contribute to neurodegenerative processes through several mechanisms, including chronic inflammation, promotion of oxidative stress and mitochondrial dysfunction, reduction of the CNS regenerative potential, and loss of neuronal function (Martínez-Cué and Rueda 2020; López-Otín et al. 2013). Viral infections, including the SARS-CoV infection, interfere with many of the pathways involved in cellular aging (López-Otín et al. 2013). Some of those pathways involve ACE-2, DPP4, and furin, the receptors used by SARS-CoV-2 to enter the host cells, which were previously found to be implicated in the acceleration of cellular senescence (Sfera et al. 2020). The SARS-CoV-2 binding to ACE-2 is likely to impair the hydrolysis of angiotensin II and further promote its deleterious effects on cellular physiology via oxidative stress and apoptosis, which in turn affects neurogenesis, neuronal differentiation, and neuronal survival (Sfera et al. 2020). Another ACE-2-mediated mechanism leading to neurodegeneration is chronic inflammation through the release of ROS and pro-inflammatory mediators from the activated glial cells (Abiodun and Ola 2020). Like ACE-2, DPP4 is also engaged in cellular aging processes. It is expressed on the surface of senescent cells and promotes their elimination

through an antibody-mediated mechanism involving the NK cells (Kim et al. 2017). Meanwhile, the furin-mediated cleavage of the SARS-CoV-2 S protein produces the S2 protein that can directly inhibit p53 and, thereby, impair the elimination of virus-infected and senescent cells (Singh and Singh 2020). Moreover, SARS-CoV-2 can directly affect the proper removal of senescent cells by interfering with the mitochondrial functions and iron metabolism of these cells (Sfera et al. 2020).

Other molecular pathways associated with aging include those associated with the ubiquitin-proteasome, tau proteins, and α-synuclein. The ubiquitin-proteasome system allows cells to degrade viral proteins after viral invasion; however, viruses can hijack this system and adopt strategies to evade it, leading to altered proteostasis and accumulation of toxic insoluble proteins (Liu et al. 2012). Therefore, the seeded protein aggregation induced by SARS-CoV-2 may contribute to the long-term post-infectious complications including neurodegeneration (Tavassoly et al. 2020). The distribution and hyperphosphorylation of tau proteins are considered the pathological hallmarks of tauopathy-related neurodegeneration; however, after neuron exposure to SARS-CoV-2, neuronal stress and death due to the abnormal intracellular distribution of tau proteins and hyperphosphorylation were observed in 3D models of human brain organoids (Ramani et al. 2020). Another feature of neurodegenerative diseases is the intracellular accumulation of misfolded α-synuclein protein, an innate neuron-specific inhibitor of viral replication that reduces neuronal injury and lysis during viral CNS infections (Beatman et al. 2016). A study performed in patients with SARS-CoV-2 infection did not find any differences in serum and CSF levels of α-synuclein between those with and without neurological symptoms (Blanco-Palmero et al. 2021). However, the serum and CSF levels of α-synuclein may not reliably mirror the true CNS levels of α-synuclein, as aggregates of α-synuclein in the neuronal cytoplasm might co-localize strictly with viral antigens (Marreiros et al. 2020), implying the necessity of immunohistochemical staining of brain samples to fully detail the effect of SARS-CoV-2 on α-synuclein.

In summary, SARS-CoV-2 invades the CNS by affecting the intracellular protein synthesis machinery, function of endoplasmic reticulum and mitochondria, and proteostasis, which promotes misfolded protein aggregation, mitochondrial oxidative stress, accelerated neurosenescence, and apoptosis that culminate in neurodegeneration.

4.2 Alteration of Clinical Trajectories in Neurodegenerative Diseases

As SARS-CoV-2 infection manifests with a prominent systemic inflammatory response, it is highly probable that it can influence the progression of neurodegenerative diseases.

Parkinson's disease alone does not increase the risk of SARS-CoV-2 infection and development of the COVID-19 disease (Fasano et al. 2020); however, after mild-to-moderate SARS-CoV-2 infection, PD patients experience a significant deterioration of, both, motor and non-motor symptoms (especially fatigue) that occur independently of the patients' age and disease duration (Cilia et al. 2020). The clinical deterioration of PD patients was associated to the systemic effects of SARS-CoV-2 infection and altered pharmacokinetics of dopaminergic therapy (Cilia et al. 2020). In addition, SARS-CoV-2 infection considerably affected the quality of life, mental health, and physical activity of PD patients (Shalash et al. 2020), with higher incidence of depression and anxiety (Salari et al. 2020). Whether the SARS-CoV-2 infection can precipitate the occurrence of PD is currently unknown. It can be hypothesized that higher susceptibility of dopaminergic neurons to SARS-CoV-2 (Yang et al. 2020), and upregulated expression of α-synuclein in response to viral infections (Tulisiak et al. 2019), might trigger the neurodegenerative processes; however, the viral infection alone is unlikely to induce neurodegeneration, unless it occurs in conjunction with a susceptible genetic background and cellular senescence (Tulisiak et al. 2019). Interestingly, a case of

probable PD after SARS-CoV-2 infection was described in a young male, who developed parkinsonian motor symptoms, including bradykinesia, tremor, and rigidity, shortly after the acute onset of respiratory manifestations (Cohen et al. 2020).

Alzheimer's Disease Dementia was found to be a risk factor for higher mortality rates related to the SARS-CoV-2 infection (Bianchetti et al. 2020). Increased levels of pro-inflammatory cytokines, including IL-1 and IL-6, in the elderly (Rea et al. 2018) may account for exaggerated immune responses that might increase the probability of poor outcomes following the SARS-CoV-2 infection. Worsening of neuropsychiatric symptoms, mainly agitation, apathy, and aberrant motor activity, was observed in AD patients with concomitant SARS-CoV-2 infection (Lara et al. 2020). As patients recovering from the SARS-CoV-2 infection display a range of cognitive symptoms (Hellmuth et al. 2021), its long-term consequences on cognitive performance might as well be expected. A synergistic effect of systemic inflammation and brain amyloid-stimulated IFN response was proposed to exacerbate the neurodegenerative processes and clinical symptomatology in AD patients (Naughton et al. 2020; Ferini-Strambi and Salsone 2020). As a part of the CNS innate immune system, amyloid is known to entrap viral particles and consequently induce microglial activation and type I IFN responses (Naughton et al. 2020). A subset of microglial cells, the "neurodegenerative phenotype," associated with the amyloid plaques is characterized by high expression levels of IFN-related genes, which further promote the pro-inflammatory responses and synapse degradation (Naughton et al. 2020).

Creutzfeldt-Jakob Disease (CJD) Systematic studies on the possible effects of SARS-CoV-2 infection on the evolution of CJD are currently lacking, yet preliminary insights were provided by several case reports (Young et al. 2020; McMurran et al. 2020). The first case described a patient with the concurrent onset of SARS-CoV-2 infection and CJD, which initially presented with fever and confusion and, later, developed a full-blown clinical profile of CJD, including impaired comprehension, myoclonic jerks, and mutism (Young et al. 2020). The second case features an elderly patient with a typical neuropsychiatric presentation of sporadic CJD (myoclonus, cognitive decline, hallucinations, and delusions), which clinically deteriorated during the hospitalization and died soon thereafter (McMurran et al. 2020). The SARS-CoV-2-induced inflammatory (IL-1, IL-6, IL-12, INF-γ, and TNF-α) and immune (activated microglia) responses were suggested to precipitate and/or accelerate the prion-driven neurodegeneration (Young et al. 2020; Mabbott et al. 2020). High levels of TNF-α and INF-γ, the cytokines found to correlate with viral loads in SARS-CoV-2 infection, stimulate reactive astrocytes with neurotoxic effects, thereby accelerating the neurodegenerative processes in prion diseases (Mabbott et al. 2020).

Future experimental and clinical studies will reveal the underlying molecular mechanisms and the long-term implications of SARS-CoV-2 in neurodegeneration.

4.3 Illustrative Case 2

A female patient in her 60s presenting with headache, fever, dry cough, and shortness of breath was admitted to the COVID-19 ICU. Her nasal and oropharyngeal swabs were positive for SARS-CoV-2 infection, and her chest CT revealed bilateral infiltrative consolidations. Blood analyses were unremarkable, except for high levels of C-reactive protein. Two months prior to the admission, she was diagnosed with sporadic CJD manifesting with ataxia, myoclonus, and cognitive impairment, yet she was able to walk and carry out some of the daily activities. The brain diffusion-weighted MRI showed extensive signal abnormality over the cerebral cortex and basal ganglia bilaterally (Fig. 3a–d). After her respiratory symptoms improved, she was transferred to the general neurology ward. However, during the hospitalization her neurological status significantly deteriorated – she was unable to walk, her cognitive and behavioral manifestations became worse, and epileptic seizures emerged.

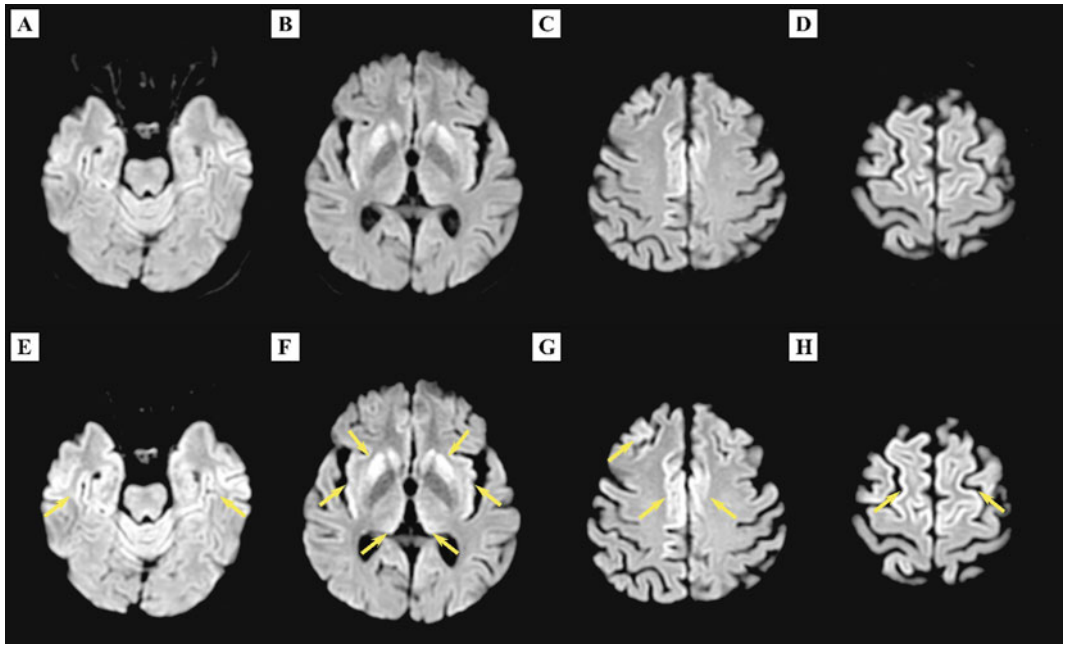

Fig. 3 Brain imaging from illustrative case 2 (Creutzfeldt-Jakob disease). Diffusion-weighted images displaying a hyperintense signal in the cortical ribbon over the frontal, parietal, insular, and cingulate cortices, as well as bilateral putamina, caudate, and thalamus pulvinar before the SARS-CoV-2 infection (**a**–**d**). Repeated MRI performed 1 month after the SARS-CoV-2 infection was marked by a more enhanced signal (arrows) over the same regions (**e**–**h**); personal courtesy of DC

On repeated MRI, a more intense signal over the cortical and subcortical structures, as compared to the previous MRI scan, was observed (Fig. 3e–h). Following a regular seizure, she had respiratory depression that required transfer to the ICU and intubation. Unfortunately, after several days of mechanical ventilation, she passed away despite the efforts to resuscitate her.

5 Therapeutic Approaches Counteracting Neuronal Damage

Several major therapeutic approaches might reduce the harmful effects of SARS-CoV-2 infection on the CNS via direct or indirect mechanisms. Vaccination is used to prevent the infection and/or reduce its severity, while antiviral therapy interferes with the cell's life cycle, and treatment that targets the immune responses (Riva et al. 2020; Vabret et al. 2020). Pharmacological therapies, commonly used for neurological complications in other viral infections, have also been applied in the treatment of SARS-CoV-2-associated neuroinflammatory manifestations, including steroids, IVIG, and plasmapheresis for ANE, ATM, ANM, ADEM, and autoimmune encephalitis (Keyhanian et al. 2020). However, several other medications aimed to modulate the immune responses to SARS-CoV-2 infection and induce viral clearance, like remdesivir, hydroxychloroquine, lopinavir/ritonavir, tocilizumab, interferons, and convalescent plasma, have shown some beneficial effects on disease activity (Chibber et al. 2020) and might be used for preventing/treatment of neuroinflammatory conditions.

Remdesivir received the FDA authorization as an emergency medication for severely ill hospitalized adult and pediatric patients with confirmed or suspected SARS-CoV-2 infection

(Lamb 2020). Remdesivir displays a broad-spectrum antiviral activity against several RNA viruses and can inhibit the replication of SARS-CoV-2, alleviate disease symptoms, fasten the recovery rate, and reduce the mortality rate (Frediansyah et al. 2020). However, it is unclear if remdesivir offers a superior benefit over dexamethasone, which is widely available and less expensive (McCreary and Angus 2020). Also, it is debatable whether remdesivir or dexamethasone have beneficial effects on the neurological manifestations of SARS-CoV-2 infection.

Tocilizumab is a monoclonal antibody that inhibits the activity of IL-6. This particular cytokine initiates the cytokine storm via the Janus kinase/signal transducer and activator of transcription or mitogen-activated kinase/NF-κB-IL-6 pathways (Saha et al. 2020). By binding to either IL-6 or its membrane-bound and soluble receptors for IL-6 (Saha et al. 2020), tocilizumab prevents signal transduction to the inflammatory mediators that recruit B and T cells, thus suppressing the cytokine storm and reducing the neuroinflammatory manifestations (Muccioli et al. 2020).

Melatonin was shown to act via an anti-inflammatory, anti-oxidative, and immune-enhancing mechanism able to restore the BBB hemostasis and prevent the entry of SARS-CoV-2 to the CNS (Romero et al. 2020). According to recent data, some of these beneficial effects were related to the upregulation of sirtuin-1, interference with TLRs, inhibition of NLRP3 inflammasome activation, and suppression of NF-κB signaling (Hardeland and Tan 2020). Melatonin also downregulates the expression of MMP9, which is involved in the immunoinflammatory responses mediated by neutrophils and alteration of BBB integrity (Martin Gimenez et al. 2020). Furthermore, melatonin can shift the cell's metabolism from glycolytic to oxidative phosphorylation by inducing the circadian brain and muscle Arnt-like protein-1 gene (Bmal1), which disinhibits the pyruvate dehydrogenase complex (PDC) and impedes viral inhibition of Bmal1/PDC (Martin Gimenez et al. 2020). Consequently, PDC drives the mitochondrial conversion of pyruvate to acetyl-coenzyme A, which is particularly relevant for immune cell activation, which exit the quiescent phenotype and enter the cell cycle (Anderson and Reiter 2020). Melatonin may also synergistically potentiate the anti-inflammatory actions of other endogenous substances, such as vitamin D, which also seems to be beneficial in the prevention and treatment of SARS-CoV-2 infection (Hardeland and Tan 2020).

Currently, several therapeutic approaches are being evaluated in clinical trials, including immunotherapy with NK cells, iron chelators, DPP4 inhibitors, and angiotensin receptor blockers (ARBs).

Immunotherapy with NK Cells This treatment modality has proved effective in amyotrophic lateral sclerosis and multiple sclerosis and is currently being studied for other neurodegenerative disorders (Maghazachi 2013; Garofalo et al. 2020). Exogenous NK cells are being evaluated in clinical trials in COVID-19 patients for the enhancement of host antiviral defenses and clearance of senescent cells (Schurink et al. 2020).

Iron Chelators As RNA viruses are more sensitive to iron chelators, they may be beneficial for the treatment of COVID-19 and are being currently assessed in clinical trials (Dalamaga et al. 2020). Iron chelators enhance the elimination of virus-infected and senescent cells by upregulation of p53, a role that has brought them into the field of neurodegenerative disorders (Ma-Lauer et al. 2016; Muñoz-Fontela et al. 2011). The SARS-CoV-2 S2 antigen appears to directly block p53, likely triggering ferroptosis, a type of cell death believed to be involved in both COVID-19 and neurodegeneration (Sfera et al. 2020). Taken together, iron chelators may inhibit SARS-CoV-2 by withholding iron from the virus and upregulating the expression of p53.

Angiotensin Receptor Blockers Preclinical studies have found that ARBs suppress the natural resistance-associated macrophage protein 1 gene, which encodes the divalent metal transporter 1 (DMT1). The DMT1 is responsible for

the absorption of non-heme iron in the duodenum, and, by suppressing its gene expression, ARBs might offer a protective effect against the SARS-CoV-2 infection at this level (Sfera et al. 2020). Consequently, ARBs could be promising therapeutics for CNS complications and are being tested in ongoing clinical trials in COVID-19 patients (Sfera et al. 2020). Since ARBs were demonstrated to also enhance the activity of p53, they might decrease the development of cellular and immune senescence, thereby reducing the risk of neurodegeneration (Sfera et al. 2020).

Gliptins or DPP4 inhibitors used to prolong the incretin half-life, potentiate meal-induced insulin secretion, and treat type 2 diabetes (Drucker 2006) have shown a variety of effects that could be beneficial to COVID-19 pathology in rodent models (Mehta et al. 2020). At least three parallel-group randomized controlled trials investigating the effects of DPP4 inhibitors on the prognosis for COVID-19 are currently ongoing. Two of them are examining the effects of linagliptin added to background insulin therapy, while the third one is the open-label "Effect of Sitagliptin Treatment in COVID-19-Positive Diabetic Patients (SIDIACO)" trial in Italy that evaluates the effects of sitagliptin as an add-on treatment to standard care with nutritional therapy with or without insulin treatment (Scheen 2021). As gliptins may lower ferroptosis, a phenomenon that plays a major role in AD and PD, these agents may also prove beneficial for neurodegenerative disorders (Guiney et al. 2017; Yan and Zhang 2020). Since DPP4 marks senescent cells, their recognition and elimination by NK cells may avert their subsequent accumulation (Kim et al. 2017).

6 Conclusion

Neurological complications of the SARS-CoV-2 infection are not uncommon and may occur with minimal or no respiratory manifestations. Evolving data suggests that the SARS-CoV-2 can invade the CNS via hematogenic, neurogenic, or enteric routes by exploiting several receptors like ACE-2, DPP4, BSG, TMPRSS2/TMPRSS4, and NRP1. The mechanisms of SARS-CoV-2 neuropathogenicity range from systemic hyperinflammation, activation of innate and adaptive immune signaling pathways, and triggering para-infectious autoimmunity to direct viral damage of the CNS tissue, accelerated neurosenescence, and neurodegeneration. Several therapeutic approaches selectively targeting these neuropathogenic mechanisms have been proposed and currently are being explored for potential clinical applications. Future studies will be essential in elucidating the molecular machinery involved in the SARS-CoV-2-induced CNS injury and will open new avenues to combat its short- and long-term consequences in the post-COVID-19 era.

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The Probable Protective Effect of Photobiomodulation on the Inflammation of the Airway and Lung in COVID-19 Treatment: A Preclinical and Clinical Meta-Analysis

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Abstract

Preliminary studies also show that many of the fatalities of COVID-19 are due to over-activity of the immune system, and photobiomodulation (PBM) therapy mainly accelerates wound healing and reduces pain and inflammation. Therefore, this systematic review and meta-analysis was conducted to evaluate the probable effect of the PBM therapy on the lung inflammation or ARDS and accelerate the regeneration of the damaged tissue. We systematically searched major indexing databases, including PubMed/Medline, ISI web of science (WOS), Scopus, Embase, and Cochrane central, using standard terms without any language, study region, or type restrictions. Of the 438 studies found through initial searches, 13 met the inclusion criteria. After applying the exclusion criteria, the main properties of 13 articles on 384 animals included in this meta-analysis with a wide range of species include rat (n = 10) and rabbit (n = 3). The analysis revealed that PBM therapy reduced *TNFα* (SMD:-3.75, 95% CI: −4.49, −3.02, $P < 0.00001$, $I^2 = 10\%$), *IL-1β* (SMD:-4.65, 95% CI: −6.15, −3.16, $P < 0.00001$, $I^2 = 62\%$), and *IL-6* (SMD:-4.20, 95% CI: −6.42, −1.97, $P = 0.0002$, $I^2 = 88\%$) significantly compared with the model controls. Hence, PBM therapy increased *IL-10* significantly compared with the model controls (SMD:-4.65, 95% CI: −6.15, −3.16, $P < 0.00001$, $I^2 = 62\%$). PBM therapy also reduced MPO activity (SMD:-2.13, 95% CI: −3.38, −0.87, $P = 0.0009$, $I^2 = 64\%$) and vascular permeability (SMD:-2.59, 95% CI:

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-4.40, -0.77, $P = 0.0052$, $I^2 = 71\%$) in the lung using the Evans blue extravasation technique significantly compared with the model controls. This systematic review and meta-analysis revealed that the PBM therapy does utilize beneficial anti-inflammatory effect, modulation of the immune system, lung permeability, or bronchoalveolar lavage on lung damage in both animal models and clinical studies. However, animal model and clinical studies appear limited considering the quality of the included evidences; therefore, large clinical trials are still required.

Keywords

Anti-inflammatory · COVID-19 · Meta-analysis · SARS-CoV-2

Abbreviations

ARDS	Acute respiratory distress syndrome
BALF	Bronchoalveolar lavage fluid
LLLT	Low-level laser therapy
MERS	Middle East respiratory syndrome
MOOSE	Meta-analyses of Observational Studies in Epidemiology
MPO	Myeloperoxidase
PBM	Photobiomodulation
PML	Pulmonary microvascular leakage
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-analyses
SARS	Severe acute respiratory syndrome
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SD	Standard deviation
SMD	Standardized mean difference
WOS	ISI web of science

1 Introduction

Coronaviruses are a large family of viruses that cause a range of respiratory infections in humans, ranging from the common cold to more severe illnesses such as Middle East respiratory syndrome (MERS) and severe acute respiratory syndrome (SARS) (Chathappady House et al. 2021). A recent coronavirus of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a new type of this family that was introduced to the world in late 2019, is the cause of COVID-19 disease (Lai et al. 2020). The body's response to COVID-19 disease depends on a number of factors, including age, sex, immune system, HLA profile, number of ACE2 receptors, environmental factors, underlying diseases, and other unknown factors (Schultze and Aschenbrenner 2021; Haybar et al. 2020). Preliminary studies also show that many of the fatalities of COVID-19 are due to over-activity of the immune system, which is known as the cytokine storm (Ragab et al. 2020).

The lethal response of the immune system to the SARS-CoV-2 is a major issue that has been discussed since the beginning of the disease pandemics and can be the cause of many COVID-19-related deaths (Costela-Ruiz et al. 2020). The risk of COVID-19-related acute respiratory distress syndrome (ARDS) in patients during hospitalization was directly related to increased neutrophil count and decreased lymphocyte count (Gibson et al. 2020). Measurements of interleukin levels indicate the extent of the cytokine storm in COVID-19 and are associated with disease severity (Leisman et al. 2020). Large amounts of cytokines can cause widespread (systemic) inflammation that can damage various organs in the body and lead to multi-organ dysfunction syndrome (Rowaiye et al. 2021).

Currently, there is no treatment protocol as the gold standard for COVID-19 disease, and only supportive protocols that can reduce the inflammation in the severe conditions are often recommended (Ramphul et al. 2021). There is substantial debate about the beneficial effects of antiviral drugs, while dexamethasone has recently been shown to be effective in reducing mortality (Ranjbar et al. 2021).

COVID-19 disease causes ARDS by inducing extensive damage to lung tissue; so far, histological results obtained from lung biopsy of patients with COVID-19 showed extensive alveolar

injury, chronic inflammatory discharge, and severe lung edema (Pannone et al. 2021; Borczuk et al. 2020). Cytokines appear to play a special role in the pathogenesis of lung disorders and ARDS associated with COVID-19 (Rabaan et al. 2021). Studies so far have shown that severe and destructive inflammation is the leading cause of death in patients with COVID-19 (Merad and Martin 2020). Dramatic increases in cytokines (TNF-α, IL-1β, IL-6, IL-8, G-CSF, and GM-CSF) and inflammatory chemokines (MCP1, IP10, and MIP1α), destructive monocyte proliferation, and inflammatory macrophages represent only one aspect of the destructive role of inflammation in COVID-19 (Burgos-Blasco et al. 2020). Although at first glance, cytokine inhibition may be considered as immunosuppression and therefore harmful to COVID-19, inhibition of these cytokines is actually an anti-inflammatory effect rather than suppression (Pinheiro et al. 2021). Thus, cytokine inhibition not only does not interfere with the clearance of the SARS-CoV-2 but also helps to cure patients with COVID-19 by reducing inflammation (Zhang et al. 2020; Cavalli and Dagna 2021).

Photobiomodulation (PBM), formerly known as low-level laser therapy (LLLT), phototherapy, biological stimulation, cold lasers, or soft lasers, gives us a completely unique feature of a non-invasive method that enhances recovery by using low-level beams (Hamblin 2016). The main use of PBM therapy is to accelerate wound healing and reducing pain and inflammation. Given the anti-inflammatory properties of PBM, it seems to be a sensible method to control the symptoms of COVID-19, especially when ARDS is present (Vetrici et al. 2021). However, the role of PBM therapy in COVID-19-induced inflammation of the airway and lung remains controversial (Nejatifard et al. 2021).

Therefore, this systematic review and meta-analysis was conducted to evaluate the probable effect of the PBM therapy on the lung inflammation or ARDS and accelerate the regeneration of the damaged tissue. Moreover, the potential effects of the PBM therapy on modulation of the immune system, lung permeability, or bronchoalveolar lavage were also investigated.

2 Laser Therapy

2.1 Overview

Laser therapy is a modern technology that is used in various fields such as treating disorders, problems, and musculoskeletal pain using a laser device. To achieve the desired therapeutic result, the patient's physical condition is assessed, which includes age, sex, skin color, type and severity and weakness of pain, etc.; according to these assessments, frequency, mode, power, and other laser parameters are adjusted. Then, by preparing the affected area, the laser is applied to the patient's body. Powerful laser waves pass through the skin, muscles, and underlying tissues of the body and penetrate deep into the affected areas. The energy emitted by the laser stimulates the damaged cells and increases the blood circulation in the damaged tissue and increases their metabolism to a considerable extent. As metabolism and blood circulation increase, the ability of cells to begin the process of regeneration and repair increases, and the healing process is formed faster in the body. In fact, the laser enters the body much sooner to repair the damaged areas. This accelerates the treatment and faster recovery of the patient (Hamblin et al. 2016).

2.2 Methods

This meat-analysis was conducted in accordance with the Meta-analyses of Observational Studies in Epidemiology (MOOSE) (Stroup et al. 2000) and Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) (Liberati et al. 2009) guidelines. We systematically searched major indexing databases, including Pubmed/Medline, ISI web of science (WOS), Scopus, Embase, and Cochrane central, using standard terms without any language, study region, or type restrictions (Table 1).

Studies on the use of PBM in lung injury modeling with samples collected from lung tissue to observe IL-1β, TNF-α, IL-10, IL-6, myeloperoxidase (MPO) activity, and pulmonary

Table 1 Search terms

Database of published studies, dissertations, and conference proceedings	Search strategy	Number of hits	
		Main term specific	Total
PubMed/MEDLINE	("Radiotherapy" [mesh] OR "low-level light therapy"[mesh] OR "Photochemotherapy"[mesh] OR "phototherapy"[mesh] OR "lasers"[mesh]) AND ("COVID-19"[mesh] OR "SARS-CoV-2"[mesh] OR "SARS-CoV-2 variants" [supplementary concept] OR "viral envelope proteins"[mesh] OR "SARS virus"[mesh] OR "respiratory distress syndrome"[mesh])	285,679 171,737	315
Embase	("radiotherapy"/exp. OR radiotherapy OR "low-level light therapy"/exp. OR "low-level light therapy" OR ("low level" AND ("light"/exp. OR light) AND ("therapy"/exp. OR therapy)) OR "photochemotherapy"/exp. OR photochemotherapy OR "phototherapy"/exp. OR phototherapy OR "lasers"/exp. OR lasers) AND ("COVID 19"/exp. OR "COVID 19" OR "sars cov 2"/exp. OR "sars cov 2" OR "viral envelope proteins"/exp. OR "viral envelope proteins" OR (viral AND envelope AND ("proteins"/exp. OR proteins)) OR "sars virus"/exp. OR "sars virus" OR (("sars"/exp. OR sars) AND ("virus"/exp. OR virus)) OR "respiratory distress syndrome"/exp. OR "respiratory distress syndrome" OR (respiratory AND ("distress"/exp. OR distress) AND ("syndrome"/exp. OR syndrome)))	26,589 53,904	14
ISI web of science	("Radiotherapy" OR "low-level light therapy" OR "Photochemotherapy" OR "phototherapy" OR "lasers") AND ("COVID-19" OR "SARS-CoV-2" OR "SARS-CoV-2 variants" OR "viral envelope proteins" OR "SARS virus" OR "respiratory distress syndrome")	7,532 4,785	38
Scopus	("radiotherapy" OR "low-level light therapy" OR "Photochemotherapy" OR "phototherapy" OR "lasers") AND ("COVID-19" OR "SARS-CoV-2" OR "SARS-CoV-2 variants" OR "viral envelope proteins" OR "SARS virus" OR "respiratory distress syndrome")	33,111 149,269	63
Cochrane central	"Radiotherapy" OR "low-level light therapy AND "COVID-19" OR "SARS-CoV-2"	262 33	8

microvascular leakage (PML) were included. Studies with no induced lung injury and cytokine measurement not in lung tissue, not using PBM therapy, were excluded. We collected information regarding all outcome measures. First outcome of interest includes assessment of IL-1β, TNF-α, IL-10, IL-6, MPO activity, and PML from lung tissue. Second outcomes were number of cells in bronchoalveolar lavage fluid (BALF), including neutrophils, macrophages, lymphocytes, and total cells. Two authors (FR and HR) independently performed the title and abstract screening. Any disagreement was resolved either by double checking the reference paper or discussion with a third author. Two authors (FR and HR) independently conducted the methodological quality assessment with especial consideration to potential sources of risk of bias. We used the Cochrane Collaboration's quality assessment tool for risk of bias assessment in RCTs (Higgins et al. 2011).

Any disagreement was resolved either by double checking the reference paper or discussion with a third author. Data extraction was performed by one reviewer (FR) and double checked by another author (BA). Authors extracted data, including author's name, publication year, country, intervention, comparators, and outcomes of interest. In case the outcomes of interest were missing, we contact the authors three times; besides, if the outcomes were only presented in figures, we used WebPlotDigitizer to extract the data (Rohatgi A). Median and range were converted to mean and standard deviation (SD) using standard formula.

2.3 Data Analysis

We used RevMan 5.3 software for data analysis, as well as used standardized mean difference as effect size. If data were present as median and range, we used Wan et al.'s methods to estimate the mean and standard deviation (Wan et al. 2014). The biochemical units, such as LDL and TC, were transformed from mg/dL to mmol/L as appropriate. Heterogeneity was described as the total variability (I^2). The significant heterogeneity was tested by χ^2 test. Low heterogeneity was indicated as $I^2 < 40\%$. In case the heterogeneity was significant ($I^2 > 75\%$), the source of heterogeneity was detected before meta-analysis. We conducted sub-group analyses based on various comparators. To assess publication bias, we used funnel plots.

3 Results

Of the 438 studies found through initial searches, 13 met the inclusion criteria (Fig. 1) (Aimbire et al. 2005, 2006; Aimbire et al. 2008; da Cunha Moraes et al. 2018; de Lima et al. 2010, de Lima et al. 2011a, b, 2013a, b, de Lima et al. 2014; Fazza et al. 2020; Miranda da Silva et al. 2015; Oliveira Jr. et al. 2014). After applying the exclusion criteria, the main properties of 13 articles on 384 animals were included in this meta-analysis with a wide range of species including rat (n = 10) and rabbit (n = 3) (Table 2).

3.1 Methodological Quality

All 13 included studies divided and assigned the animals randomly to either PBM or control. Moreover, neither the data analysis nor the animal modeling and PBM administration indicate blinding method. In addition, the data about the inflammatory factors varied significantly between included studies. The dose of PBM varied greatly among included studies, and none of the selected studies mentioned the safety issues regarding the dose of laser.

3.2 Meta-Analysis

TNFα Of the 13 included studies, 6 measured *TNFα* (Aimbire et al. 2006, da Cunha Moraes et al. 2018, de Lima et al. 2014, de Lima et al. 2010, Miranda da Silva et al. 2015, Oliveira Jr. et al. 2014), which were included in the meta-analysis. Of this six studies, two studies include two different groups of PBM and controls (de Lima et al. 2010, Oliveira Jr. et al. 2014). The analysis revealed that PBM therapy reduced *TNFα* significantly compared with the model controls (SMD:-3.75, 95% CI: −4.49, −3.02, $P < 0.00001$, $I^2 = 10\%$) (Fig. 2).

IL-1β Of the 13 included studies, 4 measured *IL-1β* (Aimbire et al. 2008, da Cunha Moraes et al. 2018, de Lima et al. 2010, Oliveira Jr. et al. 2014), which were included in the meta-analysis. PBM therapy reduced *IL-1β* significantly compared with the model controls (SMD:-4.65, 95% CI: −6.15, −3.16, $P < 0.00001$, $I^2 = 62\%$) (Fig. 2).

IL-6 Of the 13 included studies, 3 measured *IL-6* (da Cunha Moraes et al. 2018, Miranda da Silva et al. 2015, Oliveira Jr. et al. 2014), which were included in the meta-analysis. PBM therapy reduced *IL-6* significantly compared with the model controls (SMD:-4.20, 95% CI: −6.42, −1.97, $P = 0.0002$, $I^2 = 88\%$) (Fig. 2).

IL-10 Of the 13 included studies, 4 measured *IL-10* (da Cunha Moraes et al. 2018, de Lima, Moreira, et al. 2011, Miranda da Silva et al. 2015,

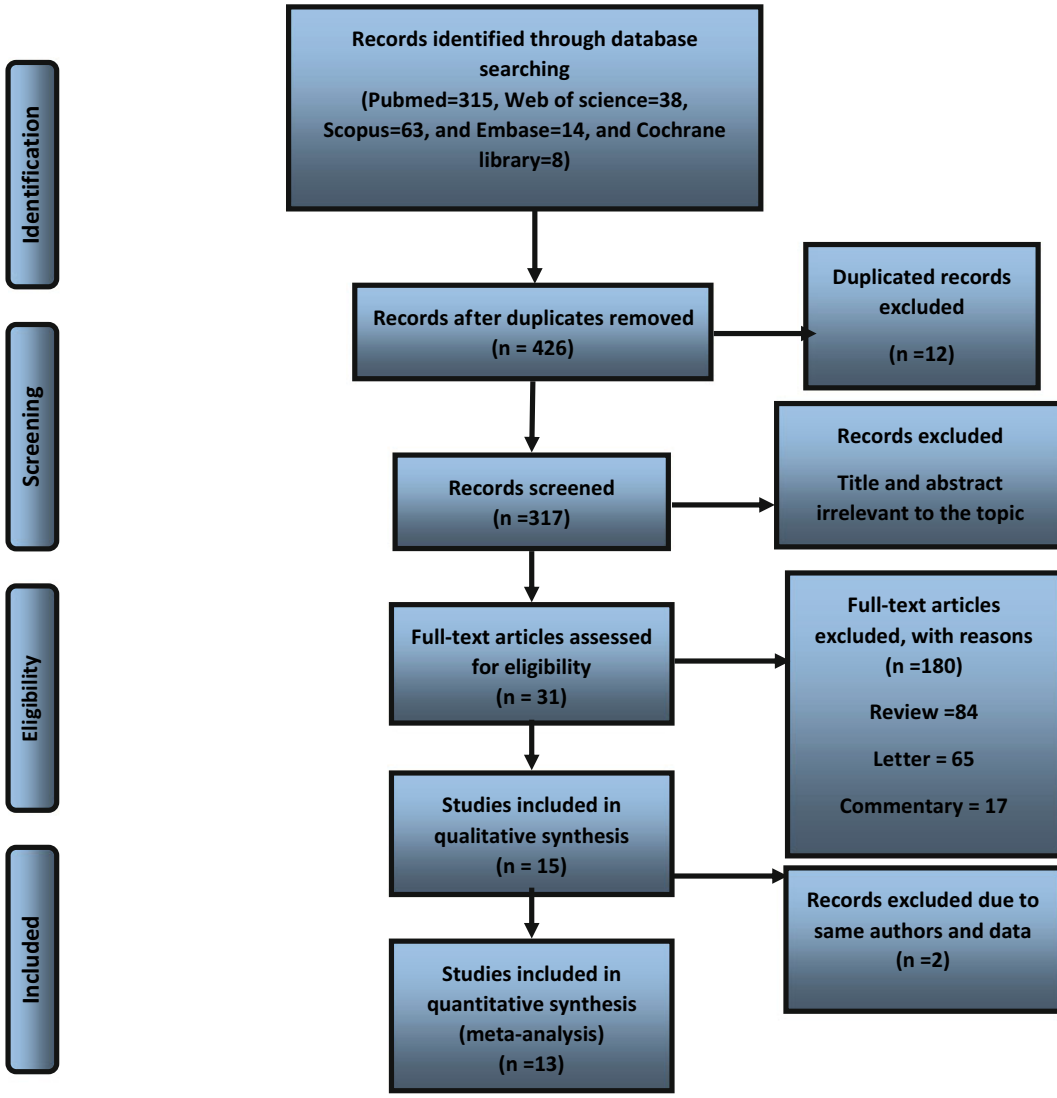

Fig. 1 Flowchart of study selection process

Oliveira Jr. et al. 2014), which were included in the meta-analysis. PBM therapy increased *IL-10* significantly compared with the model controls (SMD:-4.65, 95% CI: −6.15, −3.16, $P < 0.00001$, $I^2 = 62\%$) (Fig. 2).

Myeloperoxidase (MPO) Activity Of the 13 included studies, 4 measured MPO activity in lung tissue that was included in the meta-analysis (Aimbire et al. 2008, de Lima et al. 2014, Miranda da Silva et al. 2015, de Lima, Vitoretti, et al. 2013). PBM therapy reduced MPO activity significantly compared with the model controls (SMD:-2.13, 95% CI: −3.38, −0.87, $P = 0.0009$, $I^2 = 64\%$) (Fig. 3a).

Pulmonary Microvascular Leakage (PML) Of the 13 included studies, 3 measured (4 measurements) (Aimbire et al. 2008, de Lima et al. 2013a, Miranda da Silva et al. 2015) PML in lung tissue that were included in the meta-analysis. PBM therapy reduced vascular permeability in the lung using the Evans blue extravasation technique significantly compared with the

Table 2 Basic characteristics of the included studies

Study ID, reference	Animals	Sex	Total (no. per group)	Lung disorder model	PBM technique and dose	Outcome	Note
Aimbire et al. 2006, Brazil	Wistar rats 200–220 g	Male	35 (7)	LPS	A diode laser (Ga-AsI-Al; model Thera lase) with doses of 0.04, 0.11, and 0.22 J	TNFα	TNFα activity in bronchoalveolar lavage
Aimbire et al. 2005, 2008, Brazil	Wistar rats 230–250 g	Male	84 (7)	LPS	A diode laser with an output power of 30 mW and a wavelength of 660 nm (model, laser unit, Kondortech) with dosage of 7.5 J/cm^2	MOP, neutrophils, IL-1β, PML	Effect of PBM on lung permeability and bronchoalveolar lavage and IL-1β
de Lima et al. 2010, Brazil	Wistar rats 150–180 g	Male	35 (7)	LPS	GaAsAl diode laser (model Thera lase, Brazil) operating in the wavelength of 650 nm with a dose of 1.3 J/cm^2	TNFα, MOP, neutrophils, IL-1β	The inflammatory mediators that are driven for PBM
de Lima et al. 2011a, b, Brazil	Wistar rats 150–180 g	Male	63 (7)	i-IR	A 660 nm laser diode (MM optics, CW diode laser, São Carlos, SP) with a dose of 5.4 J	TNFα, MOP, neutrophils, IL-10, PML	TNF-a and IL-10 in reperfusion-induced
de Lima et al. 2013a, b, Brazil	Wistar rats 220–250 g	Male	35 (7)	i-IR	A 660 nm laser diode (MM optics, CW diode laser, São Carlos, SP) with a dose of 6.9 J/cm^2	TNFα, ICAM-1, GSH L, anti-inflammatory protein HSP70	PBM could modulate the acute lung inflammation by HSP70
de Lima et al. 2014, Brazil	C57/Bl6 mice 20–22 g	NA	28 (7)	i-IR	A 660 nm laser diode (MM optics, CW diode laser, São Carlos, SP) with doses of 1, 3, 5, 7.5 J/cm^2	MPO, IL-1β, IL-6, and TNFα	The effects of PBM on the lung inflammatory response in a model of ARDS
Oliveira Jr. et al. 2014, Brazil	C57/Bl6 mice 25–30 g	Male	38 (6–9)	LPS	Infrared laser administration [continuous wave, 830 nm, 3 J/cm^2	MPO, IL-1β, IL-6, TNFα, neutrophils	The effects of PBM on the lung inflammatory response in a model of ARDS
Miranda da Silva et al. 2015, Brazil	Wistar rats 220–250 g	Male	18 (6)	FAI	Infrared laser (CW diode laser- MMOptics, São Paulo, Brazil) with a dose of 12.86 J/cm^2	MPO, IL-1β, IL-6 and TNFα, neutrophils, macrophage, lymphocyte	The mechanisms of PBM with regard to lung inflammation
da Cunha Moraes et al. 2018, Brazil	C57/Bl6 mice 19–22 g	Female	24 (8)	COPD	A diode laser (power 30 mW, energy density of 3 J/cm^2 at 660 nm of wavelength)	MPO, IL-1β, IL-6, IL-10, IL-17, TNFα, neutrophils, macrophage, lymphocyte	LLLT is effective in reducing lung inflammation
Fazza et al. 2020, Brazil	Wistar rats 220–250 g	Male	24 (6)	PMV VILI	Infrared laser (photon lase III, aluminum gallium arsenide – AlGaAs)	IL1-β, IL-6 and TNF-α, CXCL2, IL-10, neutrophils, macrophage, lymphocyte	The effect of LLLT on the inflammation response of VILI

BALF bronchoalveolar lavage fluid, *PBM* photobiomodulation, *LPS* lipopolysaccharide, *MOP* myeloperoxidase activity, *PML* pulmonary microvascular leakage; intestinal ischemia and reperfusion (i-IR), *FAI* formaldehyde inhalation, *PMV* protective mechanical ventilation, *VILI* ventilator-induced lung injury

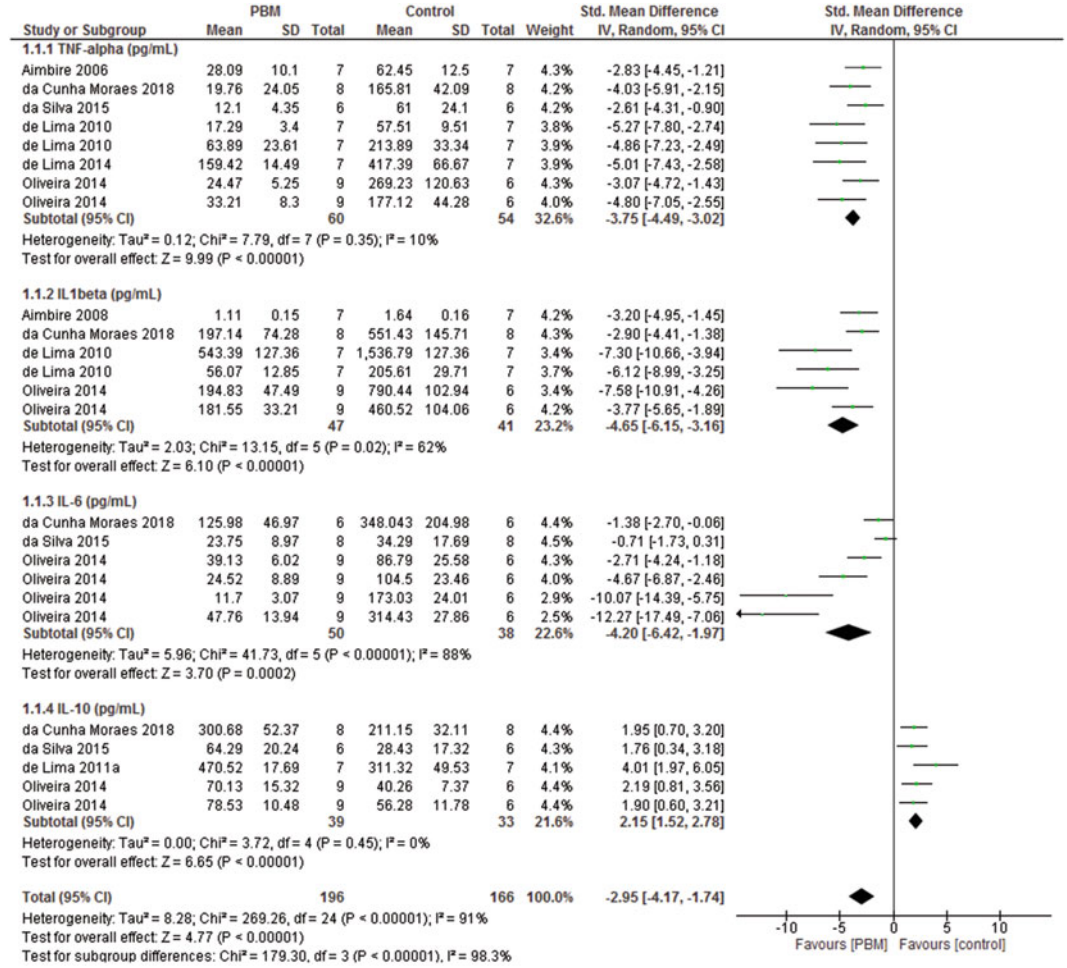

Fig. 2 Comparison of the effect on *TNF-α*, *IL-1β, IL-6, and IL-10* between PBM therapy and control in the animal lung injury models. The figure represents the SMD result of the overall experimental data, and the horizontal lines represent the 95% CIs for each study. *TNF-α*, tumor necrosis factor-α; *IL-6*, interleukin-6; *IL-1β*, interleukin-1β; *IL-10*, interleukin-10; SMD, standardized mean difference; CI, confidence interval; PBM, photobiomodulation; SD, standard deviation; IV, independent variable

model controls (SMD:-2.59, 95% CI: −4.40, −0.77, $P = 0.0052$, $I^2 = 71\%$) (Fig. 3b).

Neutrophils Of the 13 included studies, 7 measured the number of neutrophils in BALF (Aimbire et al. 2008, da Cunha Moraes et al. 2018, de Lima et al. 2011a, de Lima et al. 2011b, Fazza et al. 2020, Miranda da Silva et al. 2015, Oliveira Jr. et al. 2014), which were included in the meta-analysis. Of this seven studies, two studies include two different groups of PBM and controls (Fazza et al. 2020, Oliveira Jr. et al. 2014). The analysis revealed that PBM therapy reduced the number of neutrophils significantly compared with the model controls (SMD:-3.71, 95% CI: −5.36, −2.05, $P < 0.00001$, $I^2 = 77\%$) (Fig. 4).

Macrophages Of the 13 included studies, 3 measured the number of macrophages in BALF (da Cunha Moraes et al. 2018, Fazza et al. 2020, Miranda da Silva et al. 2015), which were included in the meta-analysis. The analysis

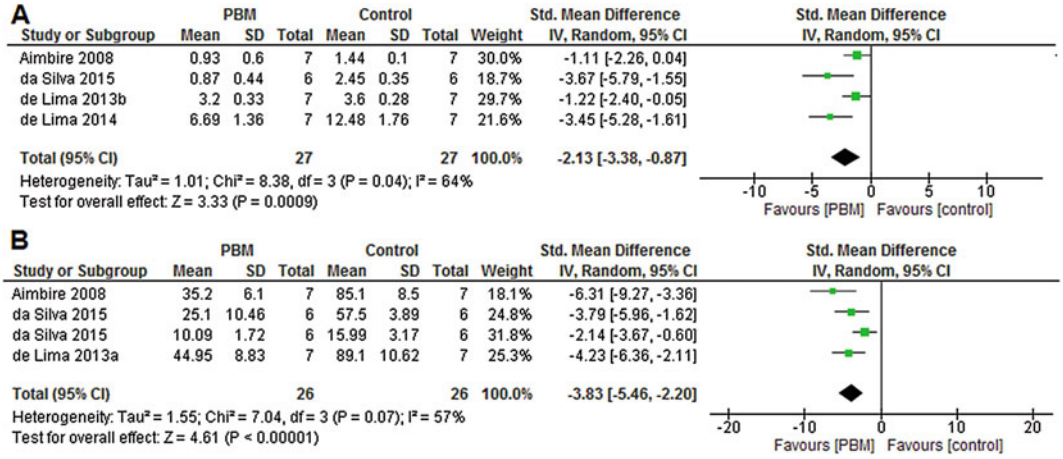

Fig. 3 Comparison of the effect on the MPO activity in lung tissue (**a**) and vascular permeability in the lung using the Evans blue extravasation technique (**b**) between PBM therapy and control in the animal lung injury models. The figure represents the SMD result of the overall experimental data, and the horizontal lines represent the 95% CIs for each study. *SMD* standardized mean difference, *CI* confidence interval, *PBM* photobiomodulation, *SD* standard deviation, *IV* independent variable

revealed that PBM therapy reduced the number of macrophages significantly compared with the model controls (SMD:-1.43, 95% CI: −2.83, −0.02, $P = 0.05$, $I^2 = 86\%$) (Fig. 4).

Lymphocytes Of the 13 included studies, 5 measured the number of lymphocytes in BALF (da Cunha Moraes et al. 2018, de Lima, Albertini, et al. 2013, Fazza et al. 2020, Miranda da Silva et al. 2015, Oliveira Jr. et al. 2014), which were included in the meta-analysis. The analysis revealed that PBM therapy reduced the number of lymphocytes significantly compared with the model controls (SMD:-4.37, 95% CI: −6.84, −1.90, $P = 0.0005$, $I^2 = 86\%$) (Fig. 4).

Total Cells Of the 13 included studies, 5 measured the number of total cells in BALF (Aimbire et al. 2008, da Cunha Moraes et al. 2018, Fazza et al. 2020, Miranda da Silva et al. 2015, Oliveira Jr. et al. 2014), which were included in the meta-analysis. The analysis revealed that PBM therapy reduced the number of total cells significantly compared with the model controls (SMD:-3.54, 95% CI: −5.30, −1.79, $P < 0.0001$, $I^2 = 84\%$) (Fig. 4).

3.3 Sensitivity Analyses

There was a substantial variation in the comparators of included studies; thus, we performed a sensitivity analysis of the effects of various comparators on study outcomes. We found no statistically significant differences between PBM therapy and control in sensitivity analysis. We observed any publication bias using funnel plots for immunologic factor measures and BALF cell outcomes**, in which a**ll plots appeared to be non-symmetrical with obvious publication bias (Fig. 5).

4 Discussion

4.1 Overview

Our meta-analysis of present evidences may shed light on the protective, anti-inflammatory, and anti-oxidative effects of PBM therapy in lung injury-induced animal model with especial focus on COVID-19. Human experience in experimental and animal models of lung disease, ARDS,

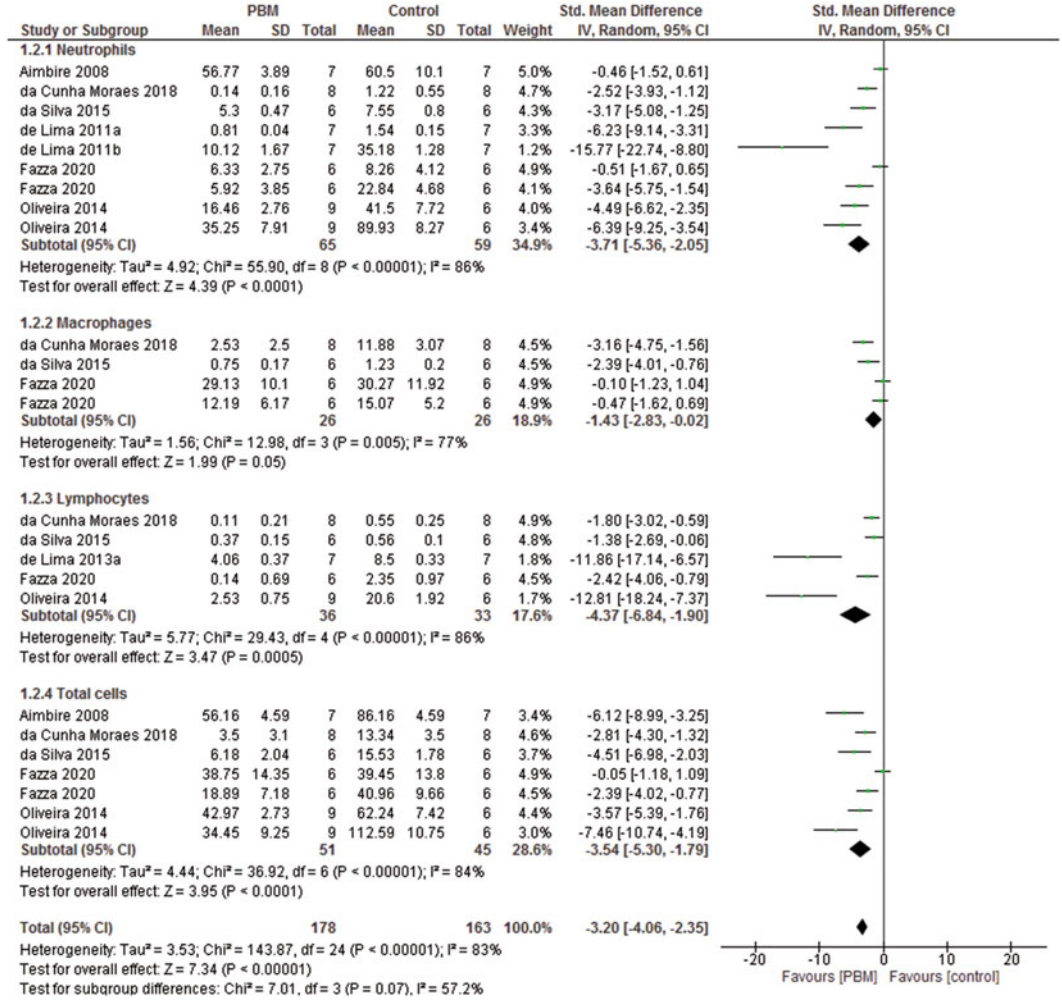

Fig. 4 Comparison of the effect on BALF cells *between* PBM therapy and control in the animal lung injury models. The figure represents the SMD result of the overall experimental data, and the horizontal lines represent the 95% CIs for each study. *SMD* standardized mean difference, *CI* confidence interval, *PBM* photobiomodulation, *SD* standard deviation, *IV* independent variable

and other infections has shown that PBMT has cellular and molecular effects at several levels against cytokine storms (Jahani Sherafat et al. 2020). PBMT reduces pro-inflammatory interleukins (IL-1β, IL-6 levels, MIP-2, mRNA expression TNF-α, etc.) (Cardoso et al. 2020). In this context our meta-analysis also showed the significant beneficial effect of PBMT in reducing IL-1β, IL-6, and TNF-α. IL-10 is an anti-inflammatory cytokine which can reduce the tissue injury with adjusting the production of other inflammatory cytokine. Studies have shown that the PBM can increase the IL-10 generation and improve the balance of inflammatory processes. Our pooled analysis also showed that PBMT re-regulates anti-inflammatory cytokines such as IL-10, which lead in an increase in IL-10 values. IL-10 is an immune-activating cytokine that plays a fundamental role in restraining host immune response against pathogen infections; thus, dysregulation of IL-10 is accompanied with greater immunopathological response to infection

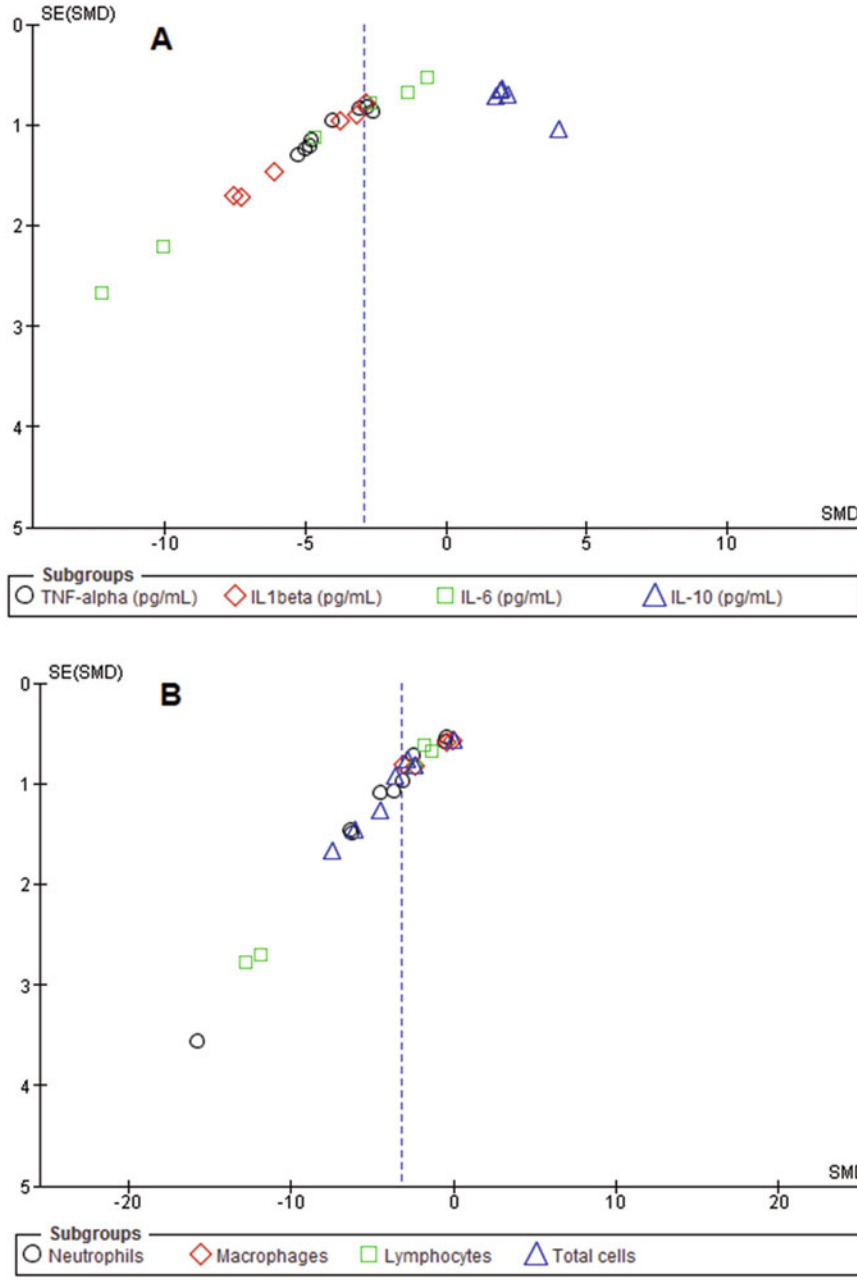

Fig. 5 Funnel plot analysis of variables of interest. The standardized mean difference is plotted against the standard error of the standardized mean difference. A, different cytokines in lung tissue; B, different BALF cells

along with more risk of developing many autoimmune diseases (Iyer and Cheng 2012) due to the fact that IL-10 levels increase in inflammatory diseases; therefore, there are many therapeutic opportunities for interventions through this cytokine pathways in patients with COVID-19 (Dhar et al. 2021).

PBMT reduces pulmonary vascular leakage and activates macrophages, T cells, and neutrophil infiltration. The P2X7 receptor (P2X7r) is

recognized as a potential new therapeutic target in the pathogenesis of COVID-19 (Pacheco and Faria 2021). P2X7r is primarily expressed in many cells and a major factor in the activation of cytokine storms and lung pathology in response to viruses. PBMT regulates P2X7r expression and reduces collagen deposition. Due to the benefits of PBMT and the lack of established treatments for COVID-19 diseases, it seems that PBMT can be useful in controlling COVID-19 as an alternative or preventative treatment, especially in severe cases. According to our pooling results, TNF-α was decreased by PBMT, in which this reduction could be due to decrease in the mRNA expression of TNF-α and its production level (Saxena et al. 2020). This change in the mRNA expression of TNF-α in viral infections has been reported previously (Fang et al. 2003). TNF-α activates neutrophil adhesion and IL-6 generation and can increase the coagulation and edema in the acute lung injury (Kany et al. 2019). Although normal levels of TNF-α are important for the regulation and persistence of immune responses to its overproduction which can lead to some inflammatory or autoimmune diseases, especially COVID-19, therefore, neutralizing TNF-α or blocking its receptors can be an effective therapeutic strategy in controlling and treating such diseases (Danlos et al. 2021, Feldmann et al. 2020). IL-6 is a pleiotropic cytokine and has a key role in ARDS pathophysiology, of which higher levels of IL-6 in the lung and plasma are related to the poor prognosis of the disease (Goldman et al. 2014). PBMT can decrease the IL-6 levels during the acute lung inflammation or ARDS, especially in patients with COVID-19 (Jahani Sherafat et al. 2020). Besides, IL-1β is the main inflammatory cytokine to the initiation of inflammation and causes poor prognosis in ARDS patients (Meduri et al. 1995). Neutrophils are the main source of this cytokine (Tecchio et al. 2014). It has been found that the PBMT can reduce the production of this cytokine.

4.2 Clinical Implications

Using search terms through selected databases, there were only five studies on using PBM therapy in patients with COVID-19 (Table 2). Of five studies, only one clinical trial included PBM therapy to treat COVID-19, and the rest four were either case report or case series (Pelletier-Aouizerate and Zivic 2021, Sigman et al. 2020a, b, Teixeira et al. 2021, Vetrici et al. 2021) (Table 3).

Table 3 Basic characteristics of the clinically included studies

Study ID	Study design (no. of cases)	Sex/age	Laser technique and dose	Outcome
Pelletier-Aouizerate and Zivic 2021	Case reports (2)	F/69 F/53	RL-PBMT, 3 times daily for 10 days, 50 J/cm2 2–3 times per week RL-PBMT, 50 J/cm2	PBMT could prevent more severe respiratory distress
Sigman et al. 2020a	Case reports (1)	M/57	Once daily for 4 days, 808 nm (GaAlAs) diode, 7.2 J/cm2	PBMT is a safe and effective potential treatment and improves clinical status in COVID-19 pneumonia
Sigman et al. 2020b	Case reports (1)	F/32	Once daily for 4 days, 808 nm (GaAlAs) diode, 7.2 J/cm2	PBMT can be safely combined with conventional treatment in patients with severe COVID-19
Teixeira et al. 2021	Case series (3)	M/57 F/84 F/70	Daily for 4 days, 808 nm (GaAlAs) diode, 7.2 J/cm2	PBMT seemed to be effective in the management of COVID-19
Vetrici et al. 2021	RCT (10)	53.4 ± 17.7	28 min of PBMT with a dosage of 7.18 J/cm2 and a total energy of 3,590 J	PBMT is a safe and effective potential treatment and improves clinical status in COVID-19 pneumonia

5 Concluding Remarks

This systematic review and meta-analysis revealed that the PBM therapy does utilize beneficial anti-inflammatory effect, modulation of the immune system, lung permeability, or bronchoalveolar lavage on lung damage in both animal models and clinical studies. However, animal model and clinical studies appear limited considering the quality of the included evidences; therefore, large clinical trials are still required.

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Ethical Consideration This research was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences.

Conflict of Interest None.

Type Systematic review and meta-analysis.

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Metabolomics Signatures of SARS-CoV-2 Infection

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Abstract

For a very long time, viral infections have been considered as one of the most important causes of death and disability around the world. Through the viral infection, viruses as small pathogens enter the host cells and use hosts' biosynthesis machinery to replicate and collect infectious lineages. Moreover, they can modify hosts' metabolic pathways in order to their own purposes. Nowadays (in 2019–2020), the most famous type of viral infection which was caused by a novel type of coronavirus is called COVID-19 disease. It has claimed the lives of many people around the world and is a very serious threat to health. Since investigations of the effects of viruses on host metabolism using metabolomics tools may have given focuses

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on novel appropriate treatments, in the current review the authors highlighted the virus-host metabolic interactions and metabolomics perspective in COVID-19.

Keywords

Coronavirus · COVID-19 · Innate immune system · Metabolism · Metabolomics · SARS-CoV-2 · Viral infections

Abbreviations

COVID	Coronavirus disease
2019-nCoV	Novel 2019 coronavirus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus-2
WHO	World Health Organization
RNA	Ribonucleic acid
ACE2	Angiotensin-converting enzyme 2
ATP	Adenosine triphosphate
TNF-β	Tumor necrosis factor-beta
IL	Interleukin
IFN-I	Type I interferon
TMPRSS2	Transmembrane protease serine 2
TNF-α	Tumor necrosis factor-α
SAP	Serum amyloid P-component
CRP	C-reactive protein
SA	Serum amyloid
RAS	Renin-angiotensin system
AT1R	Angiotensin-1 receptor
T2DM	Type 2 diabetes mellitus
TLR4	Toll-like receptor 4
MyD88	Myeloid differentiation primary response 88
CD14	Cluster of differentiation 14
ROS	Reactive oxygen species
HIF	Hypoxia-inducible factor
DAMP	Damage-associated molecular pattern
TCA	Tricarboxylic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
GSH	Glutathione
LDH	Lactate dehydrogenase
mTOR	Mammalian target of rapamycin
VRCs	Viral replication compartments
HDL	High-density lipoprotein
APO	Apolipoprotein
SARS	Severe acute respiratory syndrome
MERS	Middle East respiratory syndrome
NSP	Non-structural protein
RA	Receptor antagonist
FGF	Fibroblast growth factor
GCSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IFN γ	Interferon gamma
PDGF	Platelet-derived growth factor
VEGF	Vascular endothelial growth factor
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
SAM	S-Adenosyl methionine
LH	Luteinizing hormone (LH)
GLP-1	Glucagon-like peptide-1
ACEIs	Angiotensin-converting enzyme inhibitors
ARBs	Angiotensin II receptor blockers
HO-1	Heme oxygenase-1
MCFA	Medium-chain fatty acid
NAD+	Nicotinamide adenine dinucleotide

1 Introduction

Viral infections remain to pose a significant challenge to public health and the economic constancy of societies around the world. In other words, they have for a very long time been among the premier causes behind death and failing and conveyed expanding troubles to well-being insurance and human turn of events (Rouse and Lukacher 2010; Howard and Fletcher 2012; Wang 2020a, b). Indeed, through the viral infection, the virus enters the host cell as a small pathogen (including a piece of nucleic acid in the capsid protein shell) and disrupts its normal function. Viruses needed host cell biosynthesis machinery to replicate and gather infectious

lineage. In this context, they have developed to modify many pathways in host cells (Gelderblom 1996; Chiu et al. 1997; Lodish et al. 2000). Herein, in recent years, omics investigations have shown that viruses can lead to large-scale modifications in the host cell's metabolic pathways in accordance with their own particular needs. Moreover, studies have shown that different species of viruses may alter specific metabolic pathways in the host cell (Sanchez and Lagunoff 2015a, b; Thaker et al. 2019; Martín-Vicente et al. 2020). Hereupon, understanding the metabolic pathways involved in the viral infection process can lead to the emergence of innovative therapeutic strategies through targeted repression of special metabolic pathways (Mayer et al. 2019; Purdy 2019; Keshavarz et al. 2020). Currently, the most recent type of viral infection that has begun in Wuhan, China, in late December of 2019 and involved innumerable people around the world is coronavirus disease (COVID-19 disease), which was caused by a new type of coronavirus called the novel 2019 coronavirus (2019-nCoV) or severe acute respiratory syndrome (SARS) coronavirus 2 (SARS-CoV-2) (Alavi-Moghaddam 2020; Arjmand et al. 2020; Azodi et al. 2020; Roudsari et al. 2020). Accordingly, the authors of the present review have attempted to discuss the metabolomics perspective in COVID-19.

2 Novel Corona Viral Infection

SARS-CoV-2 (a member of the *Coronaviridae* family within the *Nidovirales* order) as a highly infectious and pathogenic virus has triggered a pandemic acute respiratory disease, and according to World Health Organization (WHO) declaration, it threatens public health and safety (Sharma et al. 2020; Weill et al. 2020). The structure of the SARS-CoV-2 with crown-like appearance contains a single-stranded, positive-sense ribonucleic acid (RNA) genome which is surrounded by a lipid bilayer membrane. Each particle of the virus is approximately 50–200 nm in diameter. In addition, it has four structural proteins called spike glycoprotein (S), nucleocapsid protein (N), envelope glycoprotein (E), and membrane glycoprotein (M) (Schoeman and Fielding 2019; Li et al. 2020a, b, c). The first step in the pathogenicity of SARS-CoV-2 is the binding of coronavirus proteins to the host-cell special surface receptor named angiotensin-converting enzyme 2 (ACE2), which leads to the membrane fusion and paves the way for the virus to enter the host cell (Ni et al. 2020a, b; Shang et al. 2020; Yesudhas et al. 2020; Zhang et al. 2020a, b). In this respect, the expression and distribution of entrance receptors subsequently impact viral tropism and pathogenicity (Mansfield 2007; Maginnis 2018; Harrison et al. 2020). In general, the life cycle of the virus after entering the host cell includes escaping the immune response of the host cell and replicating the virus by regulating the machinery of the host cell for genome replication and protein synthesis (Lucas et al. 2001; Rampersad and Tennant 2018; Zhang et al. 2020a, b). Accordingly, viral and host metabolic processes are closely related, and changes in host metabolism occur during infection at all levels – cellular, tissue, organ, and physiological (Arnold et al. 2013; Ayres 2020a, b; Troha and Ayres 2020). SARS-CoV-2 infection as a multisystem condition can lead to mild to severe illness with different symptoms. The majority with the infection undergoes a mild form of the disease, while a subset of individuals reveals a severe or critical form (Funk and Ardakani 2020; Organization, W. H 2020; Roudsari et al. 2020). Herein, the host's reaction to the infection determines the type of involvement, which in severe and critical cases, especially in subjects with comorbidities such as severe obesity, diabetes, chronic obstructive pulmonary disease, hypertension, cardiovascular disease, etc., may lead to dysfunction and damaging of several systems (Basu et al. 2020; Sanyaolu et al. 2020). Furthermore, many of the pathogeneses found in COVID-19 individuals can be outcomes of various infectious condition collections that developed to critical grades, and they are not necessarily among novel infectious conditions (Cevik et al. 2020; of the International, C. S. G 2020; Shahriarirad et al. 2020).

Immune System Activation in Response to Novel Coronavirus 2019 The immune system of

the hosts normally can support the body's natural ability to protect against viruses and diseases along with the maintaining tissue homeostasis. Moreover, it can deliver antibodies to eliminate pathogens (Rouse and Sehrawat 2010; Parham 2014; Thakur et al. 2019). In this context, investigations have reported that the metabolic pathways can amplify the effector capacities of immune cells by providing sufficient energy (adenosine triphosphate (ATP)) and metabolic intermediates. Further, metabolic reprogramming can be effective to determine immune replies (Pearce and Pearce 2013; Ganeshan and Chawla 2014; Domblides et al. 2018). In the case of SARS-CoV-2 infection, it can be mediated by T lymphocyte activation and production of inflammatory mediators (e.g., tumor necrosis factor-beta (TNF-β), interleukin (IL)-6, IL-1, type I interferon (IFN-I), and CCL2) as well as generation of perforin and granzyme B (Guidotti and Chisari 2000; Chen et al. 2010; Chowdhury et al. 2020; Oliveira et al. 2020; Lewis-Wade 2020).

3 Metabolic Pathways in Immune System Activation

There are several metabolic pathways that have roles in peripheral blood and bronchioalveolar lavage fluid of COVID-19 patients (Ayres 2020a, b; Gardinassi et al. 2020). Indeed, as mentioned, SARS-CoV-2 enters the host cell using ACE2 as the receptor in different organs and tissues. Viral spike glycoprotein activation and cleaving the C-terminal part of ACE2 (via transmembrane protease serine 2 (TMPRSS2) and FURIN proteases) make this entry more facile (Fig. 1) (Ni et al. 2020a, b). Herein, it can be stated that SARS-CoV-2 infection leads to the higher secretion of cytokines including IL-6 and tumor necrosis factor-α (TNF-α) through the effects on ACE2 receptor. It can subsequently cause the higher levels of acute-phase proteins such as serum amyloid P-component (SAP), C-reactive protein (CRP), serum amyloid (SA)A1, SAA2, and C6 which seem to be considerably elevated in the severe group of patients (Shen et al. 2020). Besides the inflammatory factors and immune activation due to direct viral effects, downregulation of ACE2 after COVID-19 infection can cause that imbalance between renin-angiotensin system (RAS) and ACE2/angiotensin-(1–7)/MAS axis leading to lung injuries and other organ's damages (Ni et al. 2020a, b; Pinto et al. 2020; Rivellese and Prediletto 2020), because inhibited ACE2 and activated ACE1 make angiotensin II act through angiotensin-1 receptor (AT1R) or AT2R and provide pro-inflammatory responses in addition to stimulated aldosterone secretion. These processes can lead to hypokalemia and higher blood pressure. The risk of breathing disorder also increases due to the higher vascular permeability locally (Fig. 1) (Bornstein et al. 2020). Herein, it has been also confirmed that angiotensin 1–7 acting through Mas receptor has anti-inflammatory effects and anti-fibrotic responses along with its inhibitory influences on vascular and cell growth mechanisms (Simões e Silva et al. 2013). Hereupon, the imbalance between these pathways leading to increased activation of AT1R and also AT2R can be the case in hypertension. Besides the existing coordination between SARS-CoV-2 and hypertension, there is a probable link to type 2 diabetes mellitus (T2DM) and metabolic syndrome, because of the existence of metabolic inflammation which predisposes coronavirus-infected patients to a higher release of cytokines. Thus, the coordination of T2DM and coronavirus can lead to more serious and long-lasting lung pathology because of dysregulated immune responses. Also, the coronavirus can bind to its receptor, ACE2, in the pancreas and results in β-cell dysfunction, damaged islets, and reduced insulin release. This process can lead to hyperglycemia and transient T2DM (Bornstein et al. 2020). On the other hand, it has been explained that increased expression of genes that have roles in metabolic pathways such as heme biosynthesis, oxidative phosphorylation, and tryptophan metabolism may be linked to COVID-19 infection (Gardinassi et al. 2020). Hereupon, higher expression of genes linked to heme-hemoglobin metabolism pathway has been found that might affect sepsis secondary to pneumonia. It has been found

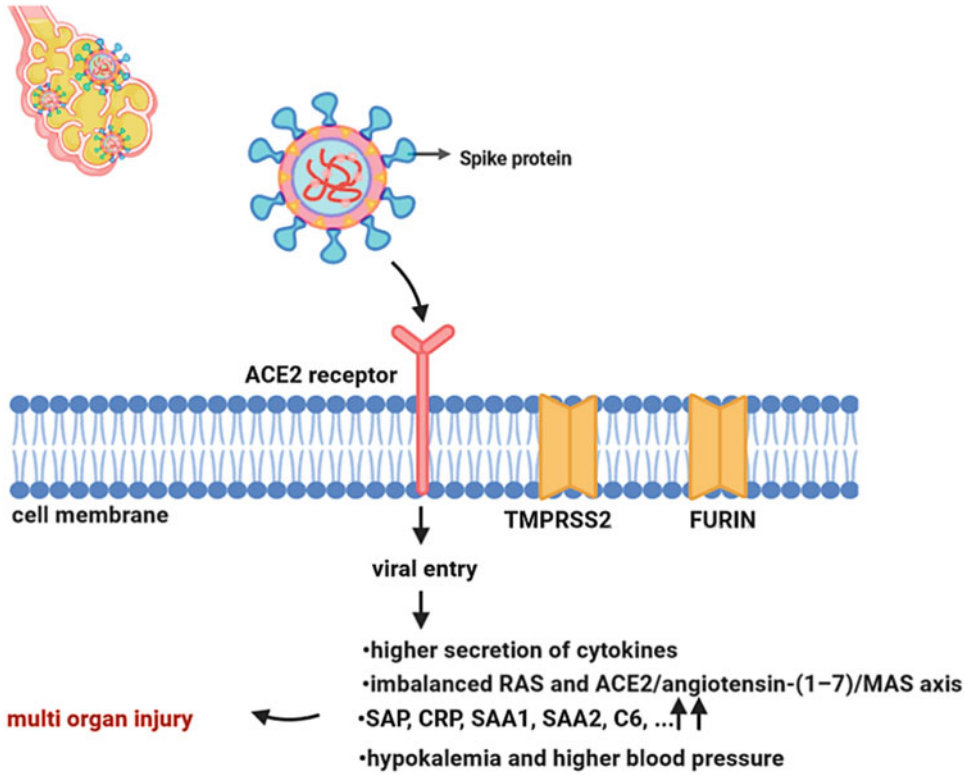

Fig. 1 A view of ACE2 receptor regulation and effects in COVID-19 infection. Spike glycoprotein activation of the virus along with the effects of TMPRSS2 and FURIN proteases on ACE2 makes viral entry more facile (Ni et al. 2020a, b). The higher secretion of cytokines; increased levels of acute-phase proteins such as SAP, CRP, SAA1, SAA2, and C6 (Shen et al. 2020); imbalanced renin-angiotensin system (RAS) and ACE2/angiotensin-(1–7)/MAS axis (Ni et al. 2020a, b; Pinto et al. 2020; Rivellese and Prediletto 2020); hypokalemia; and higher blood pressure are some of the probable effects of this viral entry (Bornstein et al. 2020). ACE2, angiotensin-converting enzyme 2; TMPRSS2, transmembrane protease serine 2; SAP, serum amyloid P-component; CRP, C-reactive protein; SA, serum amyloid; RAS, renin-angiotensin system

that heme as a part of extracellular hemoglobin has roles in sepsis and infections especially as a pro-inflammatory signaling molecule and binds specifically to Toll-like receptor 4 (TLR4) (Leite et al. 2019). Higher heme level can be mentioned as a potential inducer of the inflammatory responses in disorders with higher hemolysis or a large amount of cellular damage. It has been stated that heme can induce the secretion of TNF-α by macrophages which depends on myeloid differentiation primary response (88MyD88) and cluster of differentiation 14 (CD14) besides TLR4 (Figueiredo et al. 2007). Furthermore, hypoxia also affects the expression of genes' roles in heme biosynthesis which can also explain the contribution of hypoxia to increased mortality associated with COVID-19 sepsis. Taken together, it could be hypothesized that more heme accumulation can induce the production of cytokines and also cause intravascular coagulation which can amplify COVID-19 pathology (Gardinassi et al. 2020). Disrupted oxidative phosphorylation is also another possible constituent of metabolic pathways which is linked to higher reactive oxygen species (ROS) levels leading to sepsis via hypoxia-inducible factor (HIF)-α/sirtuin pathway. On the other hand, the existing interaction between virus and mitochondria is necessary for replication and higher viral load. For instance, mitochondria in aged cells are

unable to fulfill the hypermetabolic demands of COVID-19 sepsis; thereafter mitochondrial proteins can be presented as damage-associated molecular pattern (DAMP) that activates innate immunity. It can reveal the negative effect of aging on sepsis, too (Shenoy 2020). The modulation of tricarboxylic acid (TCA) cycle, mannose, fructose, carbon, lipid, nucleotide, protein, and galactose metabolism in addition to glycolysis and gluconeogenesis has been also reported in mass spectrometry measurements and can underlie the metabolic pathways of the disease (Gardinassi et al. 2020; Shen et al. 2020). The effects of novel coronavirus 2019 on switching host metabolism of carbon, lipids, nucleic acid, and proteins are going to be explained in more details in the next parts.

4 Switching Host Metabolism by Novel Coronavirus 2019

Some molecular changes have been found in the sera of patients with COVID-19 such as dysregulation of macrophage, degranulation of platelets, complement system involvement, and massive suppression of metabolic condition that prove the considerable alteration of protein and metabolite status in patients (Sanchez and Lagunoff 2015a, b; Shen et al. 2020). COVID-19 patient subjects have also shown an unusual rise in some biochemical parameters such as serum ferritin, erythrocyte sedimentation rate, CRP, albumin, and also lactate dehydrogenase that may underlie the ability of SARS-CoV-2 to change the host's metabolism (Singh et al. 2020). Viral genome replication through altering host nucleotide metabolism and virion assembly by assessing host amino acids are some of the benefits of their ability for changing host metabolism. They also assess carbohydrates to provide their energy source and fatty acids to form viral envelope in addition to glycoprotein involvement. In this regard, it can be understood that the host's metabolic status, medical/environmental conditions, age, and sex have important influences on the prognosis of the SARS-CoV-2 infection (Gasmi et al. 2020; Soliman et al. 2020). In the next parts, more detailed effects of the virus on host metabolism will be stated.

4.1 Carbon Metabolism

It has been clearly known that in order to access substantial levels of ATP for biological pathways, glucose utilizes aerobic metabolism. In aerobic conditions, it can also go through pentose phosphate pathway and generates nicotinamide adenine dinucleotide phosphate (NADPH). As the result, the appropriate ratio of oxidized glutathione (GSH) to glutathione is provided that take parts in antioxidant defense system and helps in destroying pathogenic microorganisms beside immune system (Nelson and Cox 2017). Inflammatory conditions make immune cells switch their mitochondrial oxidative phosphorylation to cytosolic aerobic glycolysis according to the Warburg effect. Some factors and proteins such as HIF-1α, mammalian target of rapamycin (mTOR), and serine/threonine kinase have roles in this alteration that make immune cells more phagocytic. This switch also accelerates ATP production and provides a massive amount of metabolic precursors for higher proliferation and more cytokine release (Reiter et al. 2020a, b). Therefore, aerobic glycolysis may lead to cytokine storm that in association with increased oxidative stress can result in pneumonia in COVID-19 subjects (Reiter et al. 2020a, b). It was also found that pyruvate cannot convert into acetyl-coenzyme A, when mitochondria are adopted to aerobic glycolysis. This results in a lack of available mitochondrial melatonin which has a significant antioxidant and a potent anti-inflammatory activity (Reiter et al. 2020a, b). On the other hand, under anaerobic conditions (in COVID-19 infected subjects), glycolysis pathway makes the pyruvate be generated from glucose. Then pyruvate is fermented to lactate that provides an insufficient amount of ATP (Nelson and Cox 2017; Li et al. 2020a, b, c). SARS-CoV-2 utilizes massive ATP levels and induces the anaerobic glycolysis process which forms increased levels of lactate. Continuously, hypoxia and insufficient ATP production can lead to higher amounts of blood

lactate and lactate dehydrogenase (LDH) because of unsuccessful lactate metabolism for gluconeogenesis or oxidization via citric acid cycle. ATP depletion condition can affect all of the pathways that utilize ATP, for instance, glucose uptake and pentose phosphate pathway in addition to oxidative decarboxylation of pyruvate and citric acid cycle. Insufficient ATP production also induces the production of glucose through the decomposition of hepatic glycogen. Persistent hypoxia can result in hyperglycemia condition, because this glucose has not the ability to convert into fatty acids or amino acids. On the other hand, blocked pentose phosphate pathway results in lower glutathione levels causing impaired balance between the oxidative and antioxidative status of the body. Thus, it leads to the lower ability for attenuating oxidative impairments that are accelerated in COVID-19 subjects (Li et al. 2020a, b, c). Moreover, in the metabolomics researches, higher levels of glucose and glucuronate have been also found that along with elevated bilirubin degradation product, four bile acid derivatives can show the declined detoxification activity of the liver too (Shen et al. 2020).

4.2 Lipid Metabolism

Viruses can attack synthesis and signaling of the lipids and alter the host cells in order to provide lipids for their envelopes, because lipids have a great involvement in envelopment in addition to membrane fusion and transformation that are important for viral replication (Abu-Farha et al. 2020). Thus, lipids have crucial roles in diverse stages of the virus life cycle and lipid metabolism pathway and can affect viral propagation. Herein, lipids play their functions as direct receptors for viruses in addition to their effects on forming the viral replication complex. Lipids can also provide the energy needed for viral replication (Yan et al. 2019). Positive-strand RNA viruses have the ability for remodeling cellular membrane in order to generate viral replication compartments (VRCs) that are the sites for the replication of viral RNA. It reveals that viruses have great effects on lipid metabolism along with diverse interactions with host lipid profile. Additionally, they can specifically accumulate different types of lipids such as glycerophospholipids, sphingolipids, and sterols in VRCs (Strating and van Kuppeveld 2017). In several studies, the association of COVID-19 infection with dyslipidemia has been reported; for instance, reduced serum high-density lipoprotein (HDL) cholesterol level in early stages of the disease can be seen especially in severely infected patients. Thus, declined HDL cholesterol concentration might have an association with the severity of COVID-19 (Hu et al. 2020; Li et al. 2020a, b, c). Metabolomics profiling of COVID-19 sera of patients has shown totally downregulation of over 100 lipids (sphingolipids, glycerophospholipid, and fatty acids). Herein, dysregulation of several apolipoproteins (APO) such as APOA1, APOA2, APOH, APOL1, APOD, and APOM has been also found that mostly was in association with macrophage activities and were downregulated. In addition, metabolites which have roles in lipid metabolism were dysregulated, too. Moreover, steroid hormones have been accumulated in COVID-19 infected subjects that may be linked to increased macrophage function. Decreased sphingolipid levels have been also seen in non-severe and severe patients, and it has been known that biomembranes consist of sphingolipids and glycerophospholipids that are important for the activation of immune processes. Sphingolipids also have roles in different cellular and inflammatory processes. It has been found that sphingosine-1-phosphate can promote macrophage activation and induce their migration to inflammatory sites (Shen et al. 2020). On the other hand, it should be mentioned that switching host metabolism to fasting state can result in higher levels of ketones, higher mitochondrial stress resistance, increased antioxidant defenses, and increased autophagy. It can also lead to more DNA repair and decreased protein synthesis. Thus, changes in the lipid metabolism of host are important to note and should be considered as therapeutic targets (Soliman et al. 2020). About the effects of COVID-19 infection on the host's lipid metabolism, it could be also stated that recovered patients may experience higher fat

composition and sustained loss of adipose tissue as the long-lasting effects of COVID-19 infection. Besides, acute inflammatory responses linked to COVID-19 can induce the inflammatory function of atherosclerotic plaques along with endothelial dysfunction. It can cause atherothrombotic complications that can further result in exacerbation of cardiac ischemia and injury (Radenkovic et al. 2020).

4.3 Nucleic Acid Metabolism

According to the analyses done by translatome and proteome proteomic, it has been found that SARS-CoV-2 can alter central cellular pathways including translation, splicing, and nucleic acid metabolism in addition to their effects on changing carbon and protein metabolism (Bojkova et al. 2020). It is important to be informed about the interactions between viral proteins and human cell components because of their effects on human mRNAs and non-coding RNAs (for inducing their propagation) (Sola et al. 2011). There are several pathways stated for severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS)-causing viruses that indicate how they could evade the immune system. Degrading host's mRNA, repressing host's mRNA transcription in the nucleus, and inhibiting the host's translation are mentioned as the strategies. But, the execution of SARS-CoV-2 at the molecular level has not been clearly found. In the study, it has been found that binding non-structural protein 16 (NSP16) of the virus to mRNA recognition domains (of U1 and U2 snRNAs) leads to the disruption of mRNA splicing in the host cells (infected with SARS-CoV-2-). Binding some other non-structural proteins to mRNA entry channel of the ribosome or signal recognition particle may also cause disruptions in the protein production. These may eventually lead to the suppression of interferon (IFN) responses (Banerjee et al. 2020) that are mentioned as the main clinical determinant for the severity of COVID-19 (Chen et al. 2020).

4.4 Protein Metabolism

In the laboratory tests of COVID-19 patients, the elevated levels of different products with protein structures could be seen; for instance, increased hypersensitive troponin I (hs-cTnI), IL-1B, IL-1 receptor antagonist (RA), IL-2, IL-7, IL-8, IL-9, IL-10, and D-dimer in addition to the higher levels of basic fibroblast growth factor (FGF), granulocyte colony-stimulating factor (GCSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IFNγ, platelet-derived growth factor (PDGF), TNFα, and vascular endothelial growth factor (VEGF) have been found in the patients infected with COVID-19. Also, higher levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) can be found that might be because of the abnormalities in their liver function. Some of the patients may have abnormal myocardial zymograms, with elevated levels of creatine kinase and lactate dehydrogenase. Serum creatinine may also be elevated in some of them because of their renal function damage (Banerjee et al. 2020; Chang et al. 2020; Yu et al. 2020). Impaired gonadal function with higher serum luteinizing hormone (LH) levels has been also mentioned in some reproductive-aged men infected with COVID-19 (Ma et al. 2020). The cytokine storm caused by SARS-CoV-2 can lead to a series of immune responses that eventually may cause multiple organ failure. These data noted the importance of early identification along with timely treatment of the patients (Banerjee et al. 2020). Increased levels of CRP are also another characteristic of the disease that could be mentioned as the possible biomarker for COVID-19, and it has been found to be positively linked to the appearance of lung lesions and the disease severity in the early stages of the disease (Wang 2020a, b). On the other hand, about the interactions between the virus and human proteins, 332 high-confidence protein-protein correlations can be noted that have been seen between human proteins and SARS-CoV-2 (through affinity purification mass spectrometry), and among them, 66 druggable human proteins or host factors have been detected

(Gordon et al. 2020). It has been found that amino acid residues 111–158 (related to S protein beta coronavirus) have interactions with sialic acids (on the gangliosides of host cells) (Thomas et al. 2020). In addition to the massive interaction found between coronavirus and human proteins, a couple of small protein complexes have been also mentioned that were specifically relevant to coronavirus infection; EIF4E2-GIGYF2 dimer, MAT2A-MAT2B complex, DNA-PK kinase, and the mitochondrial proteins (including PHB, PHB2, and STOML2) are the noted complexes which act on the repression of protein translation, controlling S-adenosyl methionine (SAM) synthesis, contributing to interferon induction, and regulating mitophagy in order (Perrin-Cocon et al. 2020).

5 The Importance of Metabolomics in Investigating Viral-Host Interactions

Metabolomics (as an analytical profiling technique and "omics" technologies) can quantify and compare large numbers of metabolites present in biological samples and provides a window on metabolic pathways using high-throughput analytical chemistry and analysis of multivariate results (Agharezaee et al. 2018; Rahim et al. 2018; Arjmand 2019a, b; Gilany et al. 2019; Goodarzi et al. 2019; Larijani et al. 2019; Mehrparavar et al. 2019; Tayanloo-Beik et al. 2020). Moreover, an idea of the disease severity along with the predictive outcome of the viral infection can be given by metabolite profiling or metabolomics assays. In other words, metabolomics is applied to review the impress of host-virus interaction at the metabolic level or the impacts of viruses on host cell metabolism (Voge et al. 2016; Blasco et al. 2020; Roberts et al. 2020). Indeed, metabolomics can help to provide appropriate treatment approaches via identifying unique metabolic perturbations created by viruses (Zhang et al. 2015; Byers et al. 2019).

6 Therapeutic Suggestions Targeting Metabolic Pathways

Metabolic processes of COVID-19 infection are tightly linked to the disease pathogenesis; thus, targeting metabolic pathways at different levels can result in improved defense ability against COVID-19 at its different stages (Ayres 2020a, b) (Fig. 2). Therefore, although COVID-19 is not a metabolic disease in the first place, the metabolic control of the disease is an essential therapeutic approach for it. Herein, metabolic control of T2DM and coordinated parameters such as glucose, blood pressure, and lipid levels is important in order to reduce severe complications and also to help for treating patients (Bornstein et al. 2020). Glucagon-like peptide-1 (GLP-1) agonists as a class of antidiabetic medications have positive effects on glucose metabolism and also blood pressure. They also prevent coronavirus from entering the host cell. Their influences on better metabolic pathways and also induction on the activity of protective ACE2/Mas receptor activity may lead to improved pulmonary function (Simões e Silva et al. 2013). Moreover, in order to induce and reactivate the ACE2 system in COVID-19 infected patients, angiotensin-converting enzyme inhibitors (ACEIs), angiotensin II receptor blockers (ARBs), and recombinant ACE2 may have prophylactics and therapeutic benefits (Chatterjee and Thakur 2020; Lei et al. 2020). Also, polymorphisms in ACE2 or TMPRSS2 DNA have shown a considerable association of genetic susceptibility of the disease that guides effective and personalized treatments including hydroxychloroquine and camostat. This light up the benefit of host genetic initiative for COVID-19 (Hou et al. 2020). Moreover, because of the important effects of host cell proteases to activate envelope glycoproteins and thus, viral entry, they can be also beneficial targets for antiviral intervention (Arakelyan 2020). Homocysteine also is able to activate angiotensin II (type I) receptor; thus, homocysteine-mediated transsulfuration system, vitamin B6, vitamin B12, and folic acid should be considered in treatment approaches

Fig. 2 Therapeutic suggestions targeting metabolic pathways. Some of the promising therapeutic approaches for COVID-19 targeting metabolic pathways are concluded in this figure including antidiabetic medications (Simões e Silva et al. 2013), ACEIs, ARBs, recombinant ACE2 (Chatterjee and Thakur 2020, Lei et al. 2020), homocysteine-mediated transsulfuration system-targeted drugs (Singh et al. 2020), Hb-HO-1 system-targeted drugs (Maiti 2020), oxygen therapy, regular blood glucose controlling (Li et al. 2020a, b, c), melatonin therapy (Reiter et al. 2020a, b), statins, fibrate, AM580, arbidol, LJ-001 hydroxychloroquine, methyl-β-cyclodextrin (Abu-Farha et al. 2020), and modulating sirtuin activity (Shenoy 2020). ACEIs, Angiotensin-converting enzyme inhibitors; ARBs, angiotensin II receptor blockers

(Singh et al. 2020). On the other hand, as hemoglobin dysfunction may lead to decreased oxygenation, higher free iron level, and downregulated heme oxygenase-1 (HO-1), the Hb-HO-1 system is mentioned as a suggested therapeutic target for COVID-19 (Maiti 2020). It is stated that CO (a metabolite of HO-1) levels besides endogenous HO-1 are in correlation with the severity of COVID-19 (Donnay 2020). Moreover, CO has anti-inflammatory and anti-apoptotic effects that in addition to iron chelation could be another promising target for the disease (Maiti 2020). According to negative effects of hypoxia, benefits of oxygen intake (Handy et al. 2005), and what was mentioned about unbalanced glucose aerobic metabolism during COVID-19, it is also important to correct this condition using oxygen therapy in addition to regular blood glucose controlling (with test strips) (Li et al. 2020a, b, c). The use of melatonin with its anti-

inflammatory, antioxidant, and anti-apoptotic functions (Biancatelli et al. 2020) is the other possible treatment strategy. Melatonin has been established to prevent COVID-19-induced inflammatory responses through reversing aerobic glycolysis (Reiter et al. 2020a, b). Switching the host's lipid metabolism is another suggested therapeutic approach to be used as a prophylactic or adjuvant supplement therapy. It has been proposed that coconut-rich medium-chain fatty acid (MCFA) with olive oil as a ketogenic breakfast, 8–12 h intermittent fasting, and eventually fruits and vegetables as dinner regimen may lead to the altered host's metabolism required for viral assembly and replication (Soliman et al. 2020). Moreover, because of the important role of lipid rafts and cholesterol in viral entry to host cell (by harboring ACE receptors), statins as SARS-CoV-2 protease inhibitors are mentioned to have useful therapeutic effects either to reduce the risk of cardiovascular complications or because of their antiviral and anti-inflammatory influences. This reveals the importance of targeting lipid metabolism in order to alter viral cycle (Sahebkar et al. 2016; Abu-Farha et al. 2020; Radenkovic et al. 2020). Fibrate and AM580 (as other inhibitors of cholesterol and fatty acid synthesis) besides arbidol, LJ-001, hydroxychloroquine, and methyl-β-cyclodextrin (which prevent viral entry, endocytosis, and membrane fusion) may be other lipid modifier drugs with antiviral functions (Abu-Farha et al. 2020). Tocilizumab therapy is another promising suggested treatment since it focuses on tryptophan metabolism and generating tryptophan-derived catabolites by blocking IL-6 signaling (Belladonna and Orabona 2020). On the other hand, there are some under-activated investigations that aim to clear the relevance of mitochondrial dysfunction in the presence of the organ damages linked to COVID-19 sepsis. In this regard modulating the levels of sirtuin activity and mitochondrial bioenergetics by utilizing activating and inhibiting agents is a promising therapeutic approach in experimental models proposed for sepsis. Hereupon, drugs with effects on increasing nicotinamide adenine dinucleotide (NAD+) levels followed by elevated sirtuin levels may be helpful to attenuate cytokine storm (Shenoy 2020).

7 Conclusion and Looking Forward

COVID-19, a contagious viral infection, has overwhelmed enormous populaces around the world, while vaccine and medications are as yet being created. In viral infections, the viruses attack the hosts and employs their metabolic machine to generate large numbers of progeny. In this respect, since different viruses can change the metabolic pathways of the host in different and unique approaches, evaluation of the mentioned virus-specific steps in the infectious procedure can be helpful to plan purposes of assault for broad-spectrum antiviral and vaccine approaches (Manchester and Anand 2017). In other words, investigations of the impacts of viruses on metabolism within the replication in vitro and in vivo infection using metabolomics assays have given novel experiences and gave new focuses to treatment and biomarker advancement. On the other hand, the capacity of the virus for modifying its host's metabolism to generate novel viral products has many similarities with metabolic engineering (aims to recognize and design metabolic networks for the creation of particular chemicals, medicines, and foods) (Maynard et al. 2010). Hereupon, there is a belief that it might be beneficial to take a metabolic engineering framework for combating viral infection. In this interface, considerable effort may also lead to ways of diverting current from critical paths to establish extremely robust hosts. Herein, to prevent viral products from developing, the same approaches which currently enable metabolic engineers to generate the desired products could be adapted. Nevertheless, these methods will not apply uniformly to all viral infections and also to all viral infection levels. However, in cases where viral infection is heavily dependent on metabolic factors, the approach to virology in metabolic engineering may result in drastic new knowledge about human health and biotechnology developments.

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Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Autophagic Mediators in Bone Marrow Niche Homeostasis

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Abstract

The bone marrow serves as a reservoir for a multifunctional assortment of stem, progenitor, and mature cells, located in functional anatomical micro-areas termed niches. Within the niche, hematopoietic and mesenchymal progenies establish a symbiotic relationship characterized by interdependency and interconnectedness. The fine-tuned physical and molecular interactions that occur in the niches guarantee physiological bone turnover, blood cell maturation and egression, and moderation of inflammatory and oxidative intramural stressful conditions. The disruption of bone marrow niche integrity causes severe local and systemic pathological settings, and thus bone marrow inhabitants have been the object of extensive study. In this context, research has revealed the importance of the autophagic apparatus for niche homeostatic maintenance. Archetypal autophagic players such as the p62 and the Atg family proteins have been found to exert a variety of actions, some autophagy-related and others not; they moderate the essential features of mesenchymal and hematopoietic stem cells and switch their operational schedules. This chapter focuses on our current understanding of bone marrow functionality and the role of the executive autophagic apparatus in the niche framework. Autophagic mediators such as p62 and Atg7 are currently considered the most important orchestrators of stem and mature cell dynamics in the bone marrow.

Keywords

Autophagy · Atg7 · p62 · Bone marrow · Hematopoietic stem cells · Mesenchymal stem cells

Abbreviations

Ang-1	Angiopoietin-1
Atg	Autophagy-related gene
BMPs	Bone morphogenetic proteins
CAR	Cxcl2-abundant reticular
CFU-F	Colony-forming unit fibroblast
Csf-1	Colony-stimulating factor-1
Cxcl12	C–X–C motif chemokine ligand 12
FGF2	Fibroblast growth factor 2
HSCs	Hematopoietic stem cells
IFN-γ	Interferon-γ
IL	Interleukin
LepR	Leptin receptor
LSK	$Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$
LT	Long term
MSCs	Mesenchymal/stromal stem cells
OBs	Osteoblasts

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OPG	Osteoprotegerin
p-FAK	Phospho-focal adhesion kinase
PGF2α	Prostaglandin F2α
PTH	Parathyroid hormone
RANKL	Receptor activator of NF-κB ligand
ROS	Reactive oxygen species
SCF	Stem cell factor
SLAM	Signaling lymphocyte activation molecule
ST	Short term
TGF	Transforming growth factor
VCAM1	Vascular cell adhesion molecule 1
Wnt	Wingless-related integration site

1 Introduction

The bone marrow is an intricate reservoir of interdependent and interconnected stem, progenitor, and mature cells. Schofield (1978) first proposed the concept of bone marrow niches as a cellular and chemical environment where hematopoietic stem cells (HSCs), mesenchymal/stromal stem cells (MSCs), blood, and bone progenitors/mature cells share marrow space with endothelial, reticular adventitial, neuronal, and muscle cells, establishing selective affinities for homing, maintenance, migration, and egression (Agas et al. 2015; Agas and Sabbieti 2021). Following a well-defined differentiating schedule, the HSCs give origin to adult blood cells and the MSCs to osteoblasts, chondrocytes, and fat cells (Agas et al. 2015). The physical interactions of the heterogeneous inhabitants and their release of soluble molecules guarantee distinct homeostatic profiles and topographical assemblies within the bone marrow. It is thought that there may be as many as 32 clusters of cell assemblies (Baccin et al. 2002) located next to endosteal bone or surrounding the sinusoidal and arteriolar blood vessels, giving rise to operational areas, termed endosteal and vascular niches (Agas et al. 2015; Baccin et al. 2002) able to support clonal expansion or maintain cell quiescence (Fig. 1).

HSCs are identified by the SLAM family receptors, including CD150, CD244, and CD48, which specifically recognize distinct marrow stem/progenitor cells (Kiel et al. 2005). $CD150^{+}CD244^{-}CD48^{-}$ cells are considered SLAM HSCs, while the $CD244^{+}CD150^{-}CD48^{-}$ cells are considered multipotent progenitors, and $CD48^{+}CD244^{+}CD150^{-}$ marks a restricted progenitor population (Kiel et al. 2005). HSCs are also described as a heterogeneous Lin^{-}, $Sca1^{+}$, and $C\text{-}kit^{+}$ (LSK) cell population consisting of short-term (ST) and long-term (LT) subsets and multipotent progenitors, which differentiate into lymphoid and myeloid progenitors (Agas et al. 2015; Passegue et al. 2003). LT-HSCs are mainly located in the endosteal niche (Lo Celso et al. 2009) in direct contact with osteoblasts (OBs), the most frequent component of this anatomical compartment. OBs producing angiopoietin-1, thrombopoietin, annexin-2, and osteopontin create the appropriate environment for HSC homing and maintenance through stem cell quiescence (Agas et al. 2015; Pinho and Frenette 2019; Galan-Diez and Kousteni 2017). OB ablation within the bone marrow alters the engraftment and self-renewal agenda of LT-HSCs (Bowers et al. 2015), while the age-related decline of osteopontin directly indicates HSC aging (Guidi et al. 2017). In addition, LT-HSC lodging and retention remain contingent on other cytokines, chemokines, and growth factors, such as CXCL12, stem cell factor, pleiotrophin, and vascular endothelial growth factor 2 (Agas et al. 2015; Morrison and Scadden 2014), secreted mostly by MSCs, OBs, and osteoclasts but also by fibroblasts, macrophages, and adipocytes within the endosteal area (Tamma and Ribatti 2017; Cordeiro-Spinetti et al. 2015). The osteolineage components of the endosteal area assemble significant levels of embigin and angiogenin proteins, both indispensable factors for HSC quiescence (Silberstein et al. 2016). Additionally, MSCs set up a dense extracellular matrix complex involving collagens, proteoglycans, glycosaminoglycans, fibronectin, laminin, and thrombospondin proteins. This extracellular branched framework provides surface adhesion molecules for MSC and HSC homing (Domingues et al. 2017).

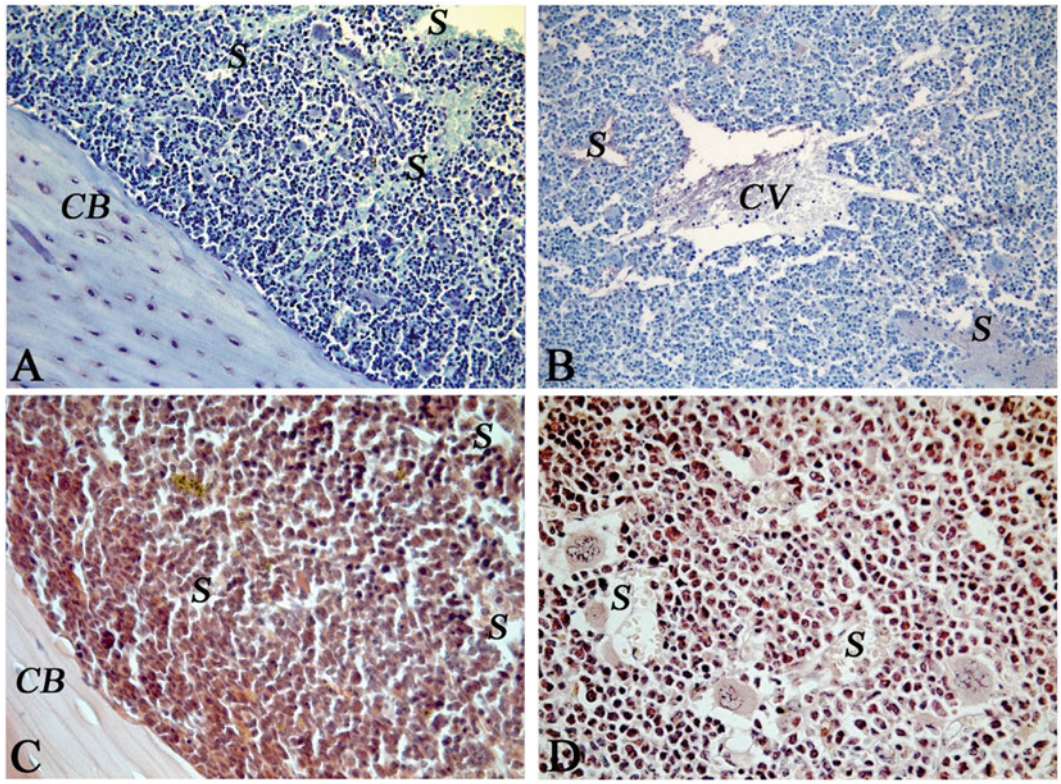

Fig. 1 Representative images of sub-metaphyseal and epiphyseal bone marrow areas from mice femur. (**a**, **b**) Histological section of the bone marrow stained with toluidine blue. (**a**) Note the close proximity of bone marrow elements near to endosteum (endosteal niche). (**b**) Central diaphyseal bone marrow region occupied by arterioles and sinusoids, which remain surrounded by stem, progenitor, and mature cells (perivascular niche). Magnification 20x. (**c**, **d**) Bone marrow endosteal (**c**) and perivascular (**d**) region stained with hematoxylin/eosin stain. Magnification 40x. Slides were imaged using a Zeiss Axioplan fluorescence microscope. *CB* cortical bone, *S* sinusoids, *CV* central vein

In sum, current research is achieving a better understanding of the bone marrow by identifying and characterizing spatiotemporal stem and mature cell affinities and further defining niche topographical features.

2 The Bone Marrow Niche Topography

Although the endosteum can be as far as five cells away from the bone marrow vasculature (Kiel et al. 2007), about 15% of the $CD150^{+}CD48^{-}$ HSCs fraction resides within the endosteal compartment, while about 60% of this subset is found next to the perivascular area (Ellis et al. 2011). The vascular niche encompasses the sinusoidal and arteriolar vessels, bordered by MSCs, HSCs, lineage-committed progenitors, and mature cells. The sinusoids *are lined with* endothelial cells in the inner part and by adventitial reticular cells in the outer part, providing a proper perivascular space for HSC adhesion and retention. Indeed, sinusoidal endothelial cells facilitate HSC homing through the release of type IV collagen, integrins, selectins, and cytokines, while adventitial reticular cells further contribute to HSC retention within the bone marrow via the release of CXCL12 and c-kit ligand (stem cell factor) (Lai et al. 2014; Czechowicz et al. 2007). In

counterpart, higher levels of reactive oxygen species (ROS) near sinusoidal endothelial cells can enhance stem cell differentiation and mobilization, while at the level of arterial endothelial cells, the formation of a low-ROS (ROS^{low}) microenvironment improves HSC quiescence and self-renewal (Ito et al. 2006). Blood vessel permeability is directly related to increased blood plasma ROS levels, which facilitate HSC migration capacity while reducing their long-term repopulation and quiescent potential (Itkin et al. 2016). HSCs surrounding sinusoidal endothelial cells (marked as Sca-1^{-}) and located more than 20 μm from bone marrow arterial endothelial cells (marked as Sca-1^{+}) can deal with either low or high ROS levels, in contrast to arterial HSCs, which experience a constant ROS^{low} background. Specifically, HSC, located within defined ROS^{low} areas distant from arteries, cohabitates next to megakaryocytes, establishing a peculiar quiescent megakaryocytic stem cell niche (Itkin et al. 2016). Indeed, 20.8% of $(Lin)^{-}CD41^{-}CD48^{-}CD150^{+}$ cells have been found in direct contact with bone marrow megakaryocytes and 48.6% of the same HSC lineage at a distance of two cells from them (Zhao et al. 2014). The spatial vicinity suggests an operational relationship between megakaryocytes and HSCs and thus is thought to indicate megakaryocyte-mediated maintenance of HSC quiescence. Megakaryocyte ablation triggers quiescent HSC activation with concurrent HSC expansion (Zhao et al. 2014).

Niche topographical features have always presented a difficult knot to untangle, and researchers have sometimes obtained differing results depending on the explicit stem cell sub-population and its specific spatiotemporal commitment and needs. A deep-imaging study of the distribution of α-catulin-GFP^{+}c-kit^{+} cells indicated that HSCs populate predominantly the diaphyseal central bone marrow area, next to leptin $receptor^{+}$ and $Cxcl12^{high}$ niche cells, rather than the bone endosteal surface. Interestingly, in $catulin^{GFP/+}$ mice, 85% of α-catulin-GFP^{+}c-kit^{+} HSCs reside in a radius of 10 μm around the perisinusoidal area but remain apart from arterioles and transition zone capillaries (Acar et al. 2015). Fully 81% of $Ki67^{-}$α-catulin-GFP^{+}c-kit^{+} non-dividing HSCs were mainly localized near sinusoids, while $Ki67^{+}$α-catulin-GFP^{+}c-kit^{+} dividing HSCs were located near the bone surface (Acar et al. 2015). Another report indicated that arterioles covered exclusively by rare $NG2^{+}$ pericytes create an optimal microenvironment for HSC quiescence; the dormant HSCs were detected near the Sca-1^{+} arterioles and arteriolar $NG2^{+}$ pericytes, in contrast to the cycling $Ki67^{+}$ HSC subset, which was located next to sinusoid-associated leptin receptor ($LepR^{+)}$ cells (Kunisaki et al. 2013). Furthermore, $NG2^{+}$ depletion destabilizes arteriolar HSC niches and drives HSCs to a non-quiescent status (Kunisaki et al. 2013).

The widespread presence of the adherence and tight junction mediators VE-cadherin and ZO-1 mainly on Sca-1^{+} arterial bone marrow endothelial cells (compared to sinusoids) further supports the effectiveness of this ROS^{low} compartment for HSC maintenance (Itkin et al. 2016). At inter- and outer-sinusoidal level, the extracellular matrix formed by a specialized laminin reticular fiber network (with laminin 421 as the main component of murine marrow) is thought to enhance HSC and progenitor cell cycling. Laminin α4 ablation blocks Lin^{-}c-$kit^{+}Sca^{+}CD48^{-}$ LT- and ST-HSC cycling at the G0 phase and puts HSPCs in a quiescent cell cycle. Moreover, in $Lama4^{-/-}$ bone marrow, researchers observed reduced HSC homing and impaired HSC recirculation between the blood and bone marrow (Susek et al. 2018).

In line with these observations, a study on Hoxb5-mCherry mice detected 94% of $Hoxb5^{+}$ HSC homing next to endothelial cells, where they establish a peculiar niche for LT-HSC lodging (Chen et al. 2016). In addition, the H-type endothelium, a specific section of arteries and capillary endothelial cells, is able to release critical HSC maintenance factors within the bone marrow reservoir (Kusumbe et al. 2016). Current findings based on $Mds1^{GFP/+}Flt3^{Cre}$ mice unearthed a novel LT-HSC population (termed MFG cells) that accounts for 12% of the total LT-HSCs and represents a quiescent bone marrow HSC fraction with potent repopulation potential. Both MDS1-GFP HSPC and MFG-HSC subsets were found

located within a range of 10 μm from the closest vessel (Christodoulou et al. 2020). The most intriguing aspect of these findings is that while MFG-HSCs are found exclusively perisinusoidal rather than peri-arteriolar, native MFG-cells are also found close to the endosteum, highlighting the spatial/operational idiosyncrasy of these cells within a cohesive endosteal-vascular niche scenario (Christodoulou et al. 2020). A further point to be noted is that hypoxia has been long considered the main niche prerequisite for stem cell quiescence (Takubo and Suda 2012). Although HSPCs and MFG-HSCs may be located far from the highly hypoxic bone marrow micro-areas, they manage to maintain their dormant capacity even in higher pO_2 settings (pO_2 > 10 mmHg) (Christodoulou et al. 2020).

Bone marrow niche homeostasis is strictly related to the behavior of MSCs, since they carry out critical functional tasks for HSC regulation and bone dynamics. Actually, given the capacity of MSCs for multilineage differentiation and self-renewal, it is thought that these fibroblast-like bone marrow inhabitants have stem cell properties, though there has long been debate on their "stemness" potency. Therefore, the scientific community has adopted a precise line to distinguish the mesenchymal stem cells from the mesenchymal stromal cells within the bone marrow: "mesenchymal stem cell" indicates a stem cell population with multilineage differentiation and self-renewal ability, whereas "mesenchymal stromal cell" comprises the heterogeneous plastic-adherent population applied mainly in tissue regenerating and immunomodulatory studies and in clinical trials (Viswanathan et al. 2019; Nolta et al. 2020). Thus, the MSC characterization used in this review corresponds to the mesenchymal/stromal "trilineage" (osteoblasts, chondrocytes, and adipocytes) of pluripotent bone marrow components.

MSCs have been depicted as nonhematopoietic ($CD45^-$) and, based on their spatial distribution, nonvascular $CD146^+$ or nonvascular $Tie2^-$ (Abarrategi et al. 2017). MSCs establish a fine-tuned regulatory network for HSC maintenance within the niche, mainly but not exclusively via the release of cytokines/chemokines and physical cell-cell interactions (Agas et al. 2015; Pinho and Frenette 2019; Crippa et al. 2019). Studies have reported dissimilar MSC sub-populations with distinct operational commitments and distribution within the bone marrow. It is worth mentioning that the $Nestin^+$ MSC subset provides angiopoietin-1 (Ang-1), interleukin-7 (IL-7), vascular cell adhesion molecule 1 (VCAM1), stem cell factor (SCF), and C–X–C motif chemokine ligand 12 (Cxcl12) for HSC maintenance (Mendez-Ferrer et al. 2010). Of note, Cxcl12 depletion from early $Nestin^-LepR^-$ mesenchymal progenitors significantly reduced HSC pool, LT-HSC repopulating activity, and HSC quiescence (Greenbaum et al. 2013). Additional findings revealed that perisinusoidal Nestin-GFP^{low} $LepR^+$ cells can supply sinusoidal milieus with elevated levels of Cxcl12 and SCF, whereas periarteriolar Nestin-GFP^{high} $NG2^+$ cells expressed predominantly high Cxcl12 levels (Kunisaki et al. 2013; Asada et al. 2017). In this context, Cxcl12 deletion in $LepR^+$ cells had no significant effect on HSC dynamics; in contrast, deletion of Cxcl12 in $NG2^+$ cells led to HSC quiescence distress and HSC mobilization out of the arteriolar niche confines (Asada et al. 2017). In addition, these findings indicate the essential role played by arteriole-associated MSCs in Cxcl12 synthesis for HSC maintenance and, as a counterpart, that of $LepR^+$ perisinusoidal cells as the foremost provider of SCF, indispensable for HSC upkeep in-house (Asada et al. 2017). Perivascular $LepR^+$ mesenchymal and endothelial cells have also been the subject of scrutiny, and one study suggested that perivascular endothelium and Lepr-expressing cells constitute the major SCF source for HSC maintenance within the vascular niche (Ding et al. 2012).

Research on MSC subsets led to the identification of CXC chemokine ligand (Cxcl)12-abundant reticular (CAR) cells. CAR cells are considered adipo-osteogenic progenitors and can release high amounts of Cxcl12 and SCF for the sustenance of HSCs and blood lineage progenitors. Specifically, CAR cells are essential for blood cell development, and they provide

support for HSC maintenance and for B cell and erythroid progenitor expansion (Omatsu et al. 2010). Perivascular CAR cells overlap with perisinusoidal LepR$^+$ cells for Cxcl12 and SCF assembly (Zhou et al. 2014), and thus both are considered to be essential sources of HSC niche factors. Recent work identified CAR/LepR$^+$ cells, a distinct MSC subset lodging near sinusoids and endosteum that, through ablation of their adipo−/osteogenic differentiation agenda by the transcription factor *Ebf1/3,* specializes for Cxcl12 and SCF release and consequently for HSC maintenance and retention within bone marrow (Seike et al. 2018). Doubtless, niche scenery reveals a well-structured cell assembly with established commitments, which reflects the singular needs of stem cells and progenies for homing, survival, expansion, differentiation, and egression.

3 Niche Maintenance and Functionality: A Matter of Intramural Mediators and Autophagic Surveillance

The operational bone marrow micro-areas or, put more simply, niches must meet the requirements of bone tissue, MSCs, HSCs, and progeny cells in terms of preservation, repair, maturation, and peculiar skills acquisition. Soluble molecules and adhesive interactions between marrow inhabitants assemble a functional architectural complex with lineage and space-related commitments. For instance, three different MSC populations have been identified as endochondral bone-forming progenitors, among them the primitive CD45$^-$Ter119$^-$Tie2$^-$AlphaV$^+$Thy$^-$6C3$^-$CD105$^-$CD200$^+$ but also the two multipotent CD45$^-$Ter119$^-$Tie2$^-$AlphaV$^+$Thy$^-$6C3$^-$CD105$^-$CD200$^-$ and CD45$^-$Ter119$^-$Tie2$^-$AlphaV$^+$Thy$^-$6C3$^-$CD105$^+$ progenitors (Chan et al. 2015). These distinctive mouse skeletal stem cell progenies differentially express receptors involved in osteogenesis, such as transforming growth factor (TGF)-, bone morphogenetic protein (BMP)-, and Wnt signaling-related receptors, which are activated by specific soluble factors within the niche (Chan et al. 2015). Intramural and paracrine signaling molecules regulate expansion of stem cells and their differentiation fate. Notably, a vast number of osteogenic factors, including BMP2, BMP7, and Wnt3a, are also derived from the niche HSCs and progenitor inhabitants (Chan et al. 2015).

In addition, most colony-forming unit fibroblast (CFU-F) activity has been attributed to a perivascular PDGFRα$^+$Sca-1$^+$CD45$^-$Ter119$^-$ (called PαS) trilineage subset, although the authors of a more recent study have argued that PDGFRα$^+$Sca-1$^-$ cells provide broader support for CFU-F-formation and release a higher number of niche mediators (like Cxcl12) than PαS cells (Zhou et al. 2014).

Ortinau et al. (Ortinau et al. 2019) classified a distinct periosteal stem cell population characterized by long-term repopulation capability and marked by Mx1 and αSMA, which is responsible for cortical bone regeneration and repair. Notably, the Mx1$^+$αSMA$^+$ periosteal stem cells constitute a bipolar, osteochondrogenic stem cell population. The authors deemed that this population has no involvement in HSC niche dynamics, in contrast to Mx1$^+$, Nestin-GFP$^+$, and LepR$^+$ bone marrow subsets. It suggested that the functional schedule of the Mx1$^+$αSMA$^+$ cells is controlled by specific molecular signatures and the release of mediators such as the CCL5 chemokine rather than the key HSC maintenance factor CXCL12 (Ortinau et al. 2019).

In turn, Nestin$^+$ and LepR$^+$ release Cxcl12 within the niche, which facilitates MSC homing throughout Cxcl12-Cxcr4 interaction and HSC retention (Lai et al. 2014; Asada et al. 2017; Bobis-Wozowicz et al. 2011). Additional evidence for the involvement of soluble factors can be seen in the Nestin$^+$Lepr$^+$Sca-1$^+$CD146$^+$ MSCs, which sustain LT-HSC self-renewal through the release of SCF and IL-11 (He et al. 2017).

The highly dynamic bone marrow landscape is governed by trophic molecules secreted by HSCs and MSCs, but also by mature cell types including osteoblasts and hematopoietic stem cells progenies. Parathyroid hormone (PTH), interferon-γ (IFN-γ), prostaglandin F2α, bone

morphogenetic proteins (BMPs), fibroblast growth factor 2 (FGF2) (Agas et al. 2017; Agas et al. 2013; Marchetti et al. 2006; Sabbieti et al. 2008; Sabbieti et al. 2017), receptor activator of NF-κB ligand (RANKL), and osteoprotegerin (OPG) (Sharaf-Eldin et al. 2016) are some examples of soluble factors involved in bone marrow and skeletal tissue homeostasis. The secretory activity of specialized clusters prompts signaling cascades and interactions indispensable for bone marrow niche functionality. In this context, adrenergic signals play a crucial role in delivering exceptional stimuli for HSC egression, the regulation of myelopoiesis and lymphopoiesis, and relief from stress signals (Maestroni 2019).

In the last decade, study of bone marrow physiology has defined new standards and roles for secretomes and cellular organelles and provided increasing evidence about the importance of autophagy in marrow dynamics. Through its peculiar task as a recycling center, the autophagic apparatus keeps under control main cellular metabolic features such as senescence, self-renewal, apoptosis, and differentiation (Chen et al. 2018). Indeed, the autophagic machinery can control the homeostatic fate of stem cells, playing crucial roles in MSC and HSC expansion and their lineage maturation agenda (Chen et al. 2018). As a cell defense tool, autophagy responds to such stressful stimuli as inflammation, hypoxia, and oxidative stress, providing a stress adaption platform for stem cell survival. Since autophagy has been correlated with HSC self-renewal and MSC proliferation and differentiation, it is currently under evaluation as a stratagem for stem cell manipulation and related clinical applications (Chen et al. 2018; Ceccariglia et al. 2020).

4 The Autophagic Apparatus and Bone Marrow Elements: Elective Affinities for Niche Dynamics

Autophagy is the cell rejuvenation process by which unnecessary material worn out organelles and pathogens are removed from the cytoplasm and degraded into lysosomes. The autophagic apparatus acts as a kind of a cell homeostasis watchdog. Indeed, the various structural gears of this "self-cannibalism" machinery can respond to starvation and other stressful conditions to guarantee cell survival, but also to provide energy and raw materials for cellular turnover (Chen et al. 2018; Ceccariglia et al. 2020). Autophagy operates in three different modes, based on the intramural executive mechanisms. In microautophagy, discarded and unwanted cell products are invoked and trapped by lysosome membrane invaginations (Gomes and Scorrano 2013). In chaperone-mediated autophagy, cytosolic chaperones recognize and trap a specific pentapeptide sequence on proteins, which they translocate and degrade into the lysosomes (Bejarano and Cuervo 2010). Finally, in macroautophagy, discarded cell components are segregated into double-membrane vesicles to form autophagosomes. These composite aggregates fuse with the lysosomal membrane to give rise to the recycling core center, the autolysosome (Agas and Sabbieti 2021; Ceccariglia et al. 2020; Klionsky and Emr 2000). Autophagosome formation is a multistage process that requires recall of exclusive factors. The assembly progression begins with the recruitment of the class III phosphatidylinositol 3-kinase (PtdIns3K) complex (involving beclin 1, Vps15, Vps34, Ambra1, and UVRAG) and the ULK1 complex (composed of ULK1, ATG13, ATG101, and FIP200). Ambra1 and TRAF6 participation further contributes to the membrane maturation process (Zhang and Baehrecke 2015). The consecutive elongation of the phagophore membrane requires the involvement of two ubiquitin-like systems. The autophagy-related gene (Atg) family proteins contribute, except other, to the realization of the ATG12–ATG5-ATG16L1 system, which interacts with MAP 1LC3/LC3 (micro-tubule associated protein 1 light chain 3). Specifically, after LC3 cleavage by ATG4 into cytosol, LC3 is converted into LC3-I and binds with phosphatidylethanolamine (PE) through an ATG7/ATG3 supportive mechanism. The LC3-I/PE interaction with the ATG12–ATG5-ATG16L1 complex enables the newly formed LC3-II to complete

the autophagosome membrane nucleation. A number of autophagosome surface proteins such as NBR1, optineurin, NDP52, Alfy, and p62 (sequestosome 1/SQSTM1) round off the autophagic apparatus and act as molecular guards for recognition and detention of cell waste products (Ceccariglia et al. 2020; Zhang and Baehrecke 2015). Macroautophagy (referred to hereafter as autophagy) culminates in the coordinated delivery of the unused or aged material to the lysosomes, where they are degraded. This process guarantees an optimal protein and organelle turnover and thus cell rejuvenation and tissue homeostasis. The macroautophagic complex and the single gears of this "self-eating" machinery regulate cell physiological patterns and meet the needs of nutrient distribution, providing the energy required for tissue maintenance and renewal.

As part of the autophagic apparatus or within a distinct autonomous operative mode, various molecules orchestrate the fate of bone marrow cells. For instance, p62 exerts a fundamental role in repossessing ubiquitinated proteins, and its action is thought to be related with osteogenesis through the attenuation of Wnt signaling (Yin et al. 2019). P62 is also considered an important molecular hub for MSC differentiation toward bone cells in adult and aged mice (Agas and Sabbieti 2021; Agas et al. 2020; Lacava et al. 2019).

In line with this observation, the osteoinductive BMP-2 cascade employs Atg7, which in turn activates Wnt16 and metalloproteinase-13 for osteoblast maturation (Ozeki et al. 2016). HSC maintenance and lineage maturation were found concomitant with the action of Atg7 (Mortensen et al. 2011a), while the absence of Atg7 leads to HSC dysfunction, impaired myeloid expansion, erythroid cell failure, and consequently severe progressive anemia (Mortensen et al. 2011b). As the spectrum of action attributed to p62 and Atg7 continues to expand, and with new understanding of their commitments in MSC and HSC operations, it would be important to pursue in-depth study of these molecules and their involvement in autophagy.

5 p62 (Sequestosome 1/SQSTM1) as Bone Marrow Homeostatic Moderator

P62 has been defined as a multitasking protein because it is involved in many facets of the cell homeostatic milieu. In fact, this molecular mediator serves as a crucial functional gear not merely in autophagy but also in cell survival and expansion, in the moderation of inflammation and oxidative stress (Jiang et al. 2015; Diaz-Meco and Moscat 2012) and in the stage management of bone marrow stem and adult cells (Agas and Sabbieti 2021; Agas et al. 2020; Lacava et al. 2019). Although p62 overexpression has been related with tumorigenesis, specifically in lung cancer (Inoue et al. 2012), breast cancer (Luo et al. 2013), and osteosarcoma (Lu et al. 2018), its normal functioning is indispensable for the steady-state cell operative agenda.

Concerning the participation of p62 in bone and bone marrow dynamics, it has been found that p62 deficiency prompts the egression into circulation of myeloid progenitors and ST-HSCs. The impaired retention of specific blood cell precursors was attributed to the compromised cross-talk of osteoblasts and macrophages due to the lack of p62 in non-hematopoietic cells within the niche. Specifically, p62−/− mice showed enfeebled osteoblast differentiation and diminished production of the chemoattractive factor Ccl4, which in turn drives Cxcl12 dysregulation and HSC egression (Chang et al. 2014). In addition, p62 was found to be involved in osteoblast maturation through attenuation of NF-κB, phospho-focal adhesion kinase (p-FAK), and p-IκBα signaling (Chang et al. 2014). Recent studies revealed that the absence of p62 triggers diseases related to low bone turnover and that exogenous administration of p62 in p62−/− mouse osteoblasts can invert osteopenia. Interestingly, p62−/− osteoblasts transfected with p62DNA showed high levels of bone-forming markers such as alkaline phosphatase, Runx2, and osterix, compared with the p62 knockout counterparts. Of note, p62 was able to enhance PTH bone-forming effects, whereas PTH

anabolic action appears to be disrupted in p62−/− adult mice (Agas et al. 2020). These findings indicate that p62 participates as a moderator of osteoblast survival and maturation. Bearing in mind also that p62 was primarily linked with osteoclastogenesis via Nfatc1 and NF-κB signaling regulation (Durán et al. 2004) and controls the monocyte differentiation agenda (Zach et al. 2018), it is reasonable to deduce that p62 contributes directly to physiological bone turnover. In fact, intramuscular administration of p62 DNA in ovariectomized adult (5 m) mice was able to invert the osteoporotic/inflammatory bone profile. This was due to augmented expression of osteo-inductive markers with simultaneous anti-inflammatory action. Exogenous delivery of p62 was able to calm pro-inflammatory cytokines and chemokines such as TNF-α, IL-6, IL-1β, and IL-17, and in parallel it moderated osteoclastogenic promoters such RANKL and NF-/κB within the bone marrow (Sabbieti et al. 2015).

There are conflicting views of the role of anabolic p62 on monocyte/osteoclast maturation. Some studies report that p62 mutations improve osteoclast nucleation and increase their size with concomitant reduction of bone volume (Daroszewska et al. 2011; Hiruma et al. 2008; Kurihara et al. 2007). Along this line, the absence of p62 has been related with mature osteoclast expansion in vitro. A solidly grounded interpretation of these results suggests that p62 can exert a regulatory function on naïve monocytes, directing them toward osteoclast lineage commitment (Zach et al. 2018).

Data obtained from p62−/− aged mice (1y and 2y old) showed augmented bone marrow adipogenesis with concurrent reduction of MSC and pre−/mature osteoblast populations. It is well established that increased bone marrow fat can lead to HSC dysregulation and MSC regression (Lacava et al. 2019; Adler et al. 2014). In this context, enhanced marrow adiposity observed in the absence of p62 causes the accumulation of reactive oxygen species (ROS) and hemosiderin and consequently the formation of a toxic habitat for the homing of MSCs and HSCs (Lacava et al. 2019). The decline of the mesenchymal Nestin$^+$, Osx$^+$, and OCN$^+$ subgroups and the increased amounts of fat cells in p62−/− marrow further highlight the key role of p62 in the lineage fate of MSCs and progenitors. This molecular hub is indispensable for safeguarding niche operations in terms of spatial and functional cohesion among the MSC inhabitants (Lacava et al. 2019). Moreover, loss of p62 increases oncogenic stress in HSCs due to inefficient elimination of damaged mitochondria and thus mitochondrial superoxide accumulation within the niche (Nguyen et al. 2019).

In sum, our information on the autophagy-related and unrelated features of p62 and their influence on bone marrow dynamics shows that this protein serves as an indispensable mechanistic gear for the operational activities of MSCs and HSCs (Fig. 2). Since p62 controls the differentiation fate and sub-population expansion of MSCs as well as the homing, maintenance, and myeloid lineage maturation of HSCs (monocytes and osteoclasts), it is seen as a jack of all trades not only in steady state but also in pathologies. This receptor carries out a wide range of functional duties affecting bone marrow cell behavior as well as inflammatory and oxidative tissue defense, the regulation of survival intramural signals and the accomplishment of autophagy.

6 The Role of Atg7 (Autophagy-Related Protein 7) in Bone Marrow Dynamics

Macroautophagic apparatus assembly is a complex, highly coordinated process in which two main mechanistic units organize a series of functional gears. The over 30 Atg proteins identified to date contribute to these two ubiquitin-like conjugation systems. Atg1, Atg9, phosphatidylinositol 3-kinase complex, and microtubule-associated Protein 1 Light Chain 3 congregate in the LC3 system, while Atg5, Atg10, Atg12, and Atg16 give rise to the Atg12 system (Feng et al. 2015). Atg7 carries out a special enzymatic function in both systems, indispensable for the congregation and expansion of autophagosomal membranes (Feng et al. 2015; Xiong 2015).

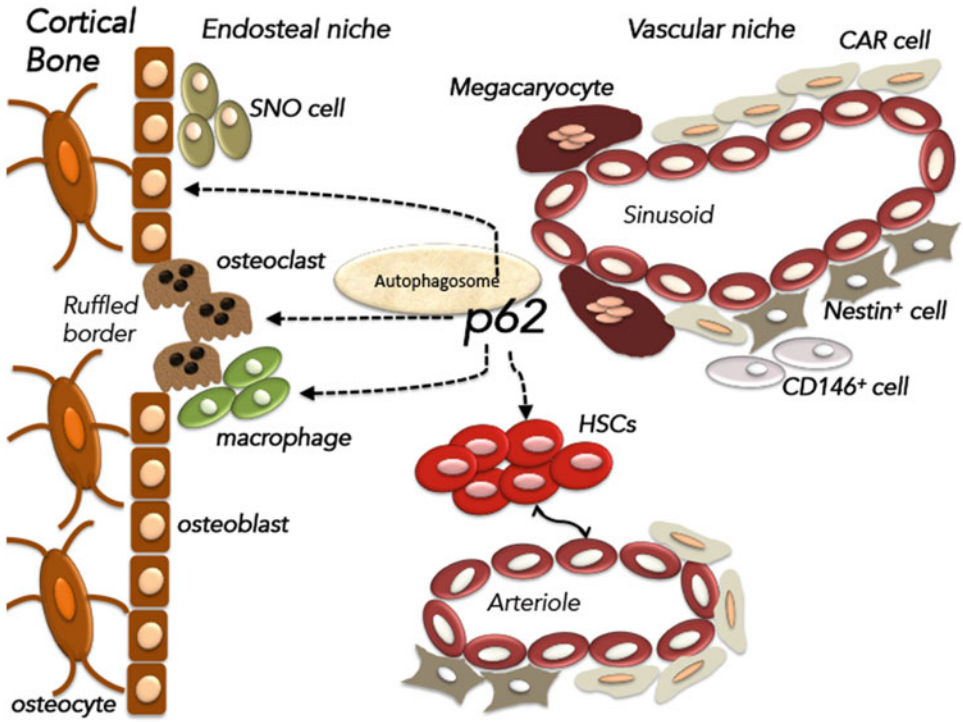

Fig. 2 p62 within an autophagy direct or indirect action orchestrates a wide range of bone marrow homeostatic features such as osteoblastogenesis, osteoclast maturation, and HSC regulation

The results of recent research have added more evidence linking Atg7 with the dynamics of bone marrow stem and mature cells. In fact, Atg7 has been correlated with HSC maintenance and multilineage maturation, as well as MSC survival. In addition, Atg7 is thought to have differentiated commitments in bone cells. Mice with Atg7 conditional deletion in HSCs and hematopoietic progenitors (Vav-Atg7$^{-/-}$ mice) revealed impaired HSC activity and maintenance, with consequent restricted myeloid and erythroid progenitor pool. The absence of Atg7 within the Lin^{-}Sca-1^{+}c-Kit^{+} (LSK) cell subset incites the intracellular amassing of high membrane potential mitochondria, augments mitochondrial superoxide release, and increases DNA damage (Mortensen et al. 2011a). Impaired autophagy in HSCs/progenitors induced by Atg7 loss leads to drastic reduction of the LSK pool and potential development of malignant myeloproliferative disorder in mice. The adverse effects observed in Vav-Atg7$^{-/-}$ mice culminated with HSC multilineage cytopenia and gradual bone marrow failure (Mortensen et al. 2011a). Recent findings support the view that Atg7 in an autophagy-related and autophagy-unrelated fashion is able to oppose hematopoietic aging. Specifically, Atg7−/− mice showed disrupted autophagy, which leads to hematopoietic growth retardation and reduced life cycle (Fang et al. 2019). In addition, vav-Atg7$^{-/-}$ mice displayed impaired monocyte differentiation into macrophages, an indication that Atg7-related autophagy supports the morphological and operational maturation of macrophages induced by colony-stimulating factor-1(Csf-1) (Jacquel et al. 2012).

In an autophagy-unrelated fashion, Atg7 exerts a crucial role in nucleosome/chromatin assembly, a process directly correlated with the aging process of the myeloid CD11b^{+}Ly6G^{-} bone marrow population. Atg7 deletion significantly reduces the number of CD11b^{+} myeloid

cells and confers an aging phenotype on this blood lineage subgroup (Fang et al. 2020).

Since niche cohesion requires physiological cross-talk among inhabitants, it is understandable that a pathological hematopoietic phenotype is seen in diminished MSC pool and bone mineral density with concurrent increase of oxidative stress and accelerated cell aging. The importance of autophagy in MSC and progenitor descendants has been studied extensively, albeit with contradictory results in some cases. Atg7 abrogation by short hairpin RNA in irradiated human MSCs pretreated with starvation caused increased ROS levels and DNA damage. These outcomes underline the key role of Atg7-related autophagy in safeguarding MSC integrity and stemness (Hou et al. 2013).

In a different view, Atg7 knockdown in MSCs exposed to critical conditions (such as hypoxia, serum deprivation, and oxidative stress) safeguards the cells against stressful microenvironmental stimuli. These outcomes indicate that as a functional autophagic gear, Atg7 plays a cytoprotective role in a physiological or a mild pathological milieu, while in severe MSC stress settings, autophagy leads to cell death (Molaei et al. 2015).

Autophagy is a well-documented moderator for MSC differentiation toward osteoblasts as well as chondrocytes. In a recent work reporting the contribution of Atg7-related autophagy to chondrogenesis, the authors argued that Atg7 is an indispensable ring for the transition of mesenchymal stem cells and pre-chondrocytes to mature cells and that its absence causes endoplasmic reticulum stress-mediated chondrocyte death (Kang et al. 2017). Moreover, in $Atg7^{flox/flox}$ mice, the impaired autophagy in chondrocytes triggered severe growth retardation of the tibia and femur, followed by mechanistic disorders in chondrocytes (Horigome et al. 2020). Parallel research revealed that conditional ablation of Atg7 or Atg5 in murine chondrocytes aggravated axial and appendicular skeletal growth due to caspase-dependent apoptosis of these cells (Vuppalapati et al. 2015). Likewise, suppression of Atg7-dependent autophagy in murine osteocytes significantly changes bone tissue homeostasis. In fact, Dmp1-Cre; Atg7-f/f mice displayed disrupted bone turnover with altered osteoclast and osteoblast population, which in turn resulted in inflammaging-like osteopenia (Onal et al. 2013).

To summarize, the vast majority of Atg7 loss-of-function studies underlined the crucial tasks of this archetypal autophagic mediator in MSC and HSC maintenance and lineage commitment and in bone tissue rejuvenation (Fig. 3). Undoubtedly, Atg7 within an autophagy-related mode, but also as distinct molecular hub, plays a remarkable role in bone and bone marrow dynamics. Further studies are required to better elucidate the autophagy-unrelated accomplishment of Atg7 in bone marrow architecture.

7 Conclusions

The bone marrow is the extraordinary, highly compartmentalized and tremendously interconnected multicellular core of the bones. Its operational micro-areas ensure MSC and HSC homing, expansion, and lineage commitment, as well as the egression and in-house allocation of progenitor and mature cells through the release and physical interaction of cytokines, chemokines, growth factors, and other molecules. For clinical applications such as bone marrow transplants, treatment of hematopoietic diseases, and the use of MSCs for their anti-inflammatory and regenerative characteristics, it is important to understand the behavior of the pluripotent stem cells and the differentiated bone marrow cell inhabitants. In the last two decades, research efforts have revealed the essential role of autophagy in the maintenance, self-renewal, and maturation of MSCs and HSCs. They have also blazed new paths for bone and bone marrow investigation by clarifying the importance of the main autophagic mediators, such as p62 and the members of the Atg family, and reporting on related gain- and loss-of-function outcomes. These archetypal autophagic gears carry out a variety of tasks, some strictly related with the autophagy complex and others unrelated, as individual mediators partaking in homeostatic signaling cascades. For instance, the presence of p62 in

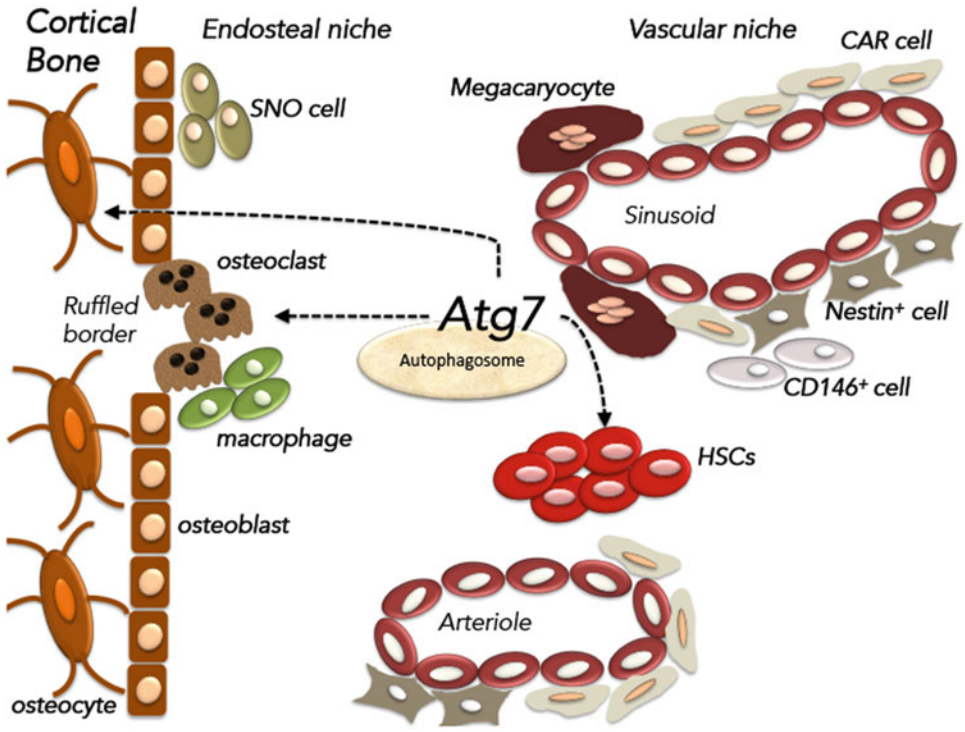

Fig. 3 Atg7 autophagy-related and autophagy-unrelated mode of action stimulates bone turnover and regulates MSC and HSC homeostatic features

MSCs has been linked to osteoblastogenesis; similarly, the absence of Atg7 has been associated with deleterious effects on bone and bone marrow homeostasis. Ongoing studies seek to deepen our knowledge of the actions of autophagic markers within the labyrinthine bone marrow interface.

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Pluripotency Stemness and Cancer: More Questions than Answers

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Abstract

Embryonic stem cells and induced pluripotent stem cells provided us with fascinating new knowledge in recent years. Mechanistic insight into intricate regulatory circuitry governing pluripotency stemness and disclosing parallels between pluripotency stemness and cancer instigated numerous studies focusing on roles of pluripotency transcription factors, including Oct4, Sox2, Klf4, Nanog, Sall4 and Tfcp2L1, in cancer. Although generally well substantiated as tumour-promoting factors, oncogenic roles of pluripotency transcription factors and their clinical impacts are revealing themselves as increasingly complex. In certain tumours, both Oct4 and Sox2 behave as genuine oncogenes, and reporter genes driven by composite regulatory elements jointly recognized by both the factors can identify stem-like cells in a proportion of tumours. On the other hand, cancer stem cells seem to be biologically very heterogeneous both among different tumour types and among and even within individual tumours. Pluripotency transcription factors are certainly implicated in cancer stemness, but do not seem to encompass its entire spectrum. Certain cancer stem cells maintain their stemness by biological mechanisms completely different from pluripotency stemness, sometimes even by engaging signalling pathways that promote differentiation of pluripotent stem cells. Moreover, while these signalling pathways may well be antithetical to stemness in pluripotent stem cells, they may cooperate with pluripotency factors in cancer stem cells – a paradigmatic example is provided by the MAPK-AP-1 pathway. Unexpectedly, forced expression of pluripotency transcription factors in cancer cells frequently results in loss of their tumour-initiating ability, their phenotypic reversion and partial epigenetic normalization. Besides the very different signalling contexts operating in pluripotent and cancer stem cells, respectively, the pronounced dose dependency of reprogramming pluripotency factors may also contribute to the frequent loss of tumorigenicity observed in induced pluripotent cancer cells. Finally, contradictory cell-autonomous and non-cell-autonomous effects of various signalling molecules operate during pluripotency (cancer) reprogramming. The effects of pluripotency transcription factors in cancer are thus best explained within the concept of cancer stem cell heterogeneity.

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Keywords

AP-1 · Cancer stem cells · Embryonic stem cells · Induced pluripotent cancer cells · Induced pluripotent stem cells · Pluripotency reprogramming · Pluripotency transcription factors · Sarcoma

Abbreviations

ABCB1	ATP-binding cassette (ABC) gene B1
ABCG2	ATP-binding cassette (ABC) gene G2
ALDH1A1	Aldehyde dehydrogenase 1 family, member A1
ALDH1A3	Aldehyde dehydrogenase 1 family, member A3
ALK	Activin receptor-like kinase
AP-1	Activator protein 1
BMP4	Bone morphogenetic protein 4
BSA	Bovine serum albumin
CD	Cluster of differentiation
CSCs	Cancer stem cells
CTGF	Connective tissue growth factor
Dppa3	Developmental pluripotency-associated 3
EMT	Epithelial-mesenchymal transition
ESCs	Embryonic stem cells
Esrrb	Oestrogen-related receptor beta
EWSR1	Ewing sarcoma breakpoint region 1
FGF-4	Fibroblast growth factor 4
GSK-3β	Glycogen synthase kinase-3β
HNSCC	Head and neck squamous cell carcinoma
HSCs	Haematopoietic stem cells
iCSCs	Induced cancer stem cells
Id-1	Inhibitor of differentiation/DNA binding 1
IGF-1 (2)	Insulin-like growth factor 1 (2)
iPCCs	Induced pluripotent cancer cells
iPSCs	Induced pluripotent stem cells
ISCs	Intestinal stem cells
JAK	Janus kinase
Klf4 (5)	Krüppel-like factor 4 (5)
LIF	Leukaemia inhibitory factor
LIN28	Protein lin-28 homolog A
MAPK	Mitogen-activated protein kinase
MEFs	Mouse embryonic fibroblasts
MEK	Mitogen-activated protein kinase kinase
MET	Mesenchymal-epithelial transition
miRNA	MicroRNA
MSCs	Mesenchymal stem cells
NSCs	Neural stem cells
Oct4	Octamer-binding transcription factor 4, also known as POU5F1 (POU domain, class 5, transcription factor 1)
PBS	Phosphate-buffered saline
RTL-1	Retrotransposon-like 1, also known as PEG11 (paternally expressed gene 11)
Sall4	Sal-like protein 4
SEM	Standard error of measurement
shRNA	Short hairpin RNA
SORE	Sox2/Oct4 response element
Sox2 (3)	SRY (sex-determining region Y)-box 2 (3)
SP	Side population
SRR2	SOX2 regulatory region 2
STAT	Signal transducer and activator of transcription
Tcf	T-cell factor
TGCT	Testicular germ cell tumours
TGFβ	Transforming growth factor β
Tfcp2L1	Transcription factor CP2-like protein 1
TRITC	Tetramethylrhodamine-isothiocyanate
Wnt	Wingless/Int-1
Yap	Yes-associated protein 1

1 Developmental Potency and Stemness

Pluripotency is the term to describe an essentially unlimited developmental potential of stem and precursor cells in terms of the range of differentiated cell types that can develop from them, and a paradigmatic cell type complying with these criteria are embryonic stem cells,

especially murine blastocyst inner cell mass-derived ESCs. The situation is somewhat more complicated with human ESCs, as on one hand, they correspond to a later developmental stage, being epiblast-derived stem cells, and, on the other hand, in vivo testing of their unlimited developmental potential including germ line is, understandably, hardly thinkable; the terms naive and primed ESCs have been coined to differentiate between ESCs of declining developmental potential, and while blastocyst inner cell mass-derived ESCs are believed to be the closest available equivalent to the in vivo ground-state pluripotency, the epiblast-derived ESCs are more remote from this ground state and are regarded as primed pluripotent ESCs. Interestingly, stem cells derived from murine primordial germ cells called embryonic germ cells are quite close to naive murine ESCs (Hackett and Surani 2014). In contrast, most adult stem cells have a very limited developmental potency, at least under the normal physiological conditions, being responsible for replenishment of only a particular spectrum of cell types in a tissue in question, their developmental potency thus ranging from totipotent (haematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), intestinal stem cells (ISCs), neural stem cells (NSCs)) to unipotent (keratinocyte stem cell, urothelial stem cell, satellite muscle cells). The developmental potency is thus to be clearly distinguished from stemness, which describes the complex phenotypic state shared by stem cells irrespective of their developmental potency and characterized by self-renewal, ability to differentiate (disregarding the developmental potency) and existence of complex self-protective mechanisms. Along the same lines, immediate developmental descendants of stem cells, precursor cells, may share a great part of their respective developmental potency, but they lose their self-renewal capacity. In this notion, stemness is believed to be manifested also by certain cancer cell, termed appropriately cancer stem cells (Hatina et al. 2013).

2 Pluripotency Stemness Regulatory Network

The pluripotency stemness is maintained by an unusually complex regulatory network of signals, signalling pathways and gene regulatory factors, with copious feedforward and feedback loops. The best way to approach this regulatory network is to dissect the cell culture requirements of embryonic stem cells that are crucial to preserve their pluripotency stemness. There are two standard culture protocols. The traditional way was the culture combining foetal bovine serum, leukaemia inhibitory factor (LIF) and a feeder layer of cells (most often mitotically inactivated mouse embryonic fibroblasts) providing additional soluble and contact signals. LIF belongs to the interleukin-6 family of cytokines signalling via gp130-containing receptor complexes using JAK-STAT (STAT-3 preferentially) pathway, with Krüppel-like factor 4 (*Klf4*) and *Tfcp2l1* as major downstream genes (Hall et al. 2009; Martello et al. 2013). The crucial factor provided by feeder cells, uncovered by expression profiling of several feeder cell lines displaying variable levels of support (Qi et al. 2004), is the bone morphogenic protein 4 (BMP4). Recognizing serine-threonine kinase receptor complexes of potentially variable composition (canonical BMPRI and BMPRII, but can also be activin receptor-like kinase – ALK-1, ALK-2, ALK-3 and ALK-6 – and activin type II receptor and activin type IIB receptor, ActR-II and ActR-IIB, respectively, Miyazono et al. 2010), the BMP4 signal activates the Smad 1/5/8-Smad 4 signalling pathway, with the *Id-1* as a prominent downstream gene (Ying et al. 2003).

It has been found that the ESCs cultured in serum/LIF exist in a semistable equilibrium between stemness and differentiation, and this ESC-provided differentiation-inducing activity has been identified as fibroblast growth factor 4 (FGF-4). Upon binding on cognate single-pass transmembrane tyrosine kinase receptor, FGF-4 engages several signalling pathways, with the

mitogen-activated protein kinase (MAPK) cascade turning out as critical for ESC differentiation (Hackett and Surani 2014). In a direct extension to this observation, culture of ESCs in presence of the specific MEK inhibitor PD0325901 significantly improves the maintenance of pluripotency stemness (Chen et al. 2020; Ying et al. 2008). The second signalling inhibitor of a similar biological impact is CHIR99021. It targets the glycogen synthase kinase-3β (GSK-3β), an essential component of the β-catenin cytoplasmic destruction complex, thereby identifying Wnt-β-catenin pathway as another pluripotency stemness signalling pathway (Chen et al. 2020; Ying et al. 2008). Its particular form is in molecular details somewhat different from the canonical Wnt-β-catenin signalling, as we know it, e.g. from the regulation of intestinal and colon stem cells and their derivative colorectal carcinoma stem cells. In the latter, stabilized β-catenin acts as a direct or indirect coactivator of the transcription factors Tcf1 and Tcf4, promoting transcriptional activation of genes under their control. In ESCs, this response is dominated by another member of the Tcf family, Tcf3, which, unlike Tcf1 and Tcf4, is a constitutive transcriptional repressor, and stabilized β-catenin abrogates this transcriptional repression (Watanabe and Dai 2011; Sineva and Pospelov 2014). The orphan nuclear receptor *Esrrb* has been identified as a crucial downstream gene target for this derepression (Martello et al. 2012).

In the centre of the pluripotency stemness regulation, a triad of transcription factors Oct4 (Pou5F1), Sox2 and Nanog lies. They are remarkable by their pronounced cooperativity; hundreds of genomic loci occupied by Oct4 are co-occupied by Sox2 and the most part of them by all the three factors (Boyer et al. 2005). Among the genes coregulated, we can find also genes for each individual factor, providing thereby a potent feedforward pluripotency stemness regulatory loop. On the other hand, a composite Oct4-Sox2 regulatory element is found in the *FGF-4* promoter as well, and this represents an opposite example of feedback regulation (Ambrosetti et al. 1997). Altogether, the pluripotency stemness machinery can be regarded as a very complex regulatory circuit (Papatsenko et al. 2018).

3 Core Pluripotency Transcription Factors in Cancer

Two of the core pluripotency transcription factors discussed above behave as genuine oncogenes, i.e. they are mutationally activated in cancer. For the Oct-4 (Pou5F1), reciprocal translocation t(6;22)(p21;q12) leading to a fusion oncoprotein between the Ewing sarcoma protein EWSR1 and POU5F1 has been identified in a range of rather rare tumours, including osteosarcoma and other bone tumours (Yamaguchi et al. 2005), soft tissue sarcoma (Deng et al. 2011), myoepithelial tumours (Antonescu et al. 2010) and salivary gland carcinoma (Möller et al. 2008). From the mechanistic point of view, the fusion oncoprotein follows the general scenario of Ewing sarcoma family oncogenic transformation – the N-terminal part of the EWSR1 gene (up to the exon 6), which encodes a very strong cryptic transcription activation domain, is in-frame fused with the almost complete *POU5F1* coding sequence. What results is a particularly strong transcription activator with the DNA-binding specificity indistinguishable from the intact Oct4 (Lee et al. 2007). *Oct4* also behaves as an oncogene in transgenic mouse system relying on its inducible tissue-specific overexpression in adult mouse tissues (Hochedlinger et al. 2005). With regard to the *Sox2*, the major oncogenic mutational mechanism is gene amplification resulting in overexpression, as evidenced in oesophageal and lung squamous cell carcinoma and glioblastoma (Bass et al. 2009; Annovazzi et al. 2011; Justilien et al. 2014).

Whereas tumours discussed above typically activate a single pluripotency factor, testicular germ cell tumours (TGCT) feature activation of an essential part of the pluripotency regulatory network, mainly thanks to the quasi-obligatory amplification of the short arm of chromosome 12 encompassing several pluripotency genes (*Gdf3*, *Dppa3* – *Stella* – and one of the *Oct4* pseudogenes) (Atkin and Baker 1982; Blanco and Tirado 2018). Oct4 itself and Nanog and LIN28 are also highly expressed in TGCT (Nettersheim et al. 2016). Interestingly, as for the Sox factors, there is a remarkable dichotomy in the particular factor overexpressed in the two major types of testicular cancer – seminoma

($Sox17^+$ $Sox2^-$) and embryonal carcinoma ($Sox17^-$ $Sox2^+$). Strikingly, both factors complex with Oct4 to cooperatively bind composite regulatory elements mentioned above, with a subtle preference for one or another particular version of them. The Sox17-containing complexes prefer binding to a particular form called compressed composite element, lacking a single G between the respective Sox- and Oct4-binding motifs, whereas Sox2 complexes prefer canonical composite elements involving the spacer guanosine residue. Consequently, downstream genes activated by either Sox-Oct4 complexes differ in part, with seminoma specific expression of oncogenes *IGF-1* and *c-myc* and a broader spectrum of pluripotency network genes in embryonal carcinoma (Jostes et al. 2020).

There is a plethora of studies available in the scientific literature exploring expression of pluripotency transcription factors (both individually and in combinations) in a wide spectrum of cancer types. It should be noticed, nevertheless, that such findings can be confounded by the existence of multiple splice variants and pseudogenes, which have been identified for Oct4, Nanog and Sall4 (Bernhardt et al. 2012). As for the former, only one isoform, Oct4A, participates in the pluripotency regulatory network. Two other splice variants, known as Oct4B and Oct4B1, may be overexpressed in cancer, with functional implications quite different from conferring pluripotency stemness. In contrast, Sall4 is expressed in two splice variants, Sall4A and Sall4B, with the B isoforms being significantly more proficient in promoting both pluripotency (Rao et al. 2010) and cancer (Ma et al. 2006). In addition, pluripotency transcription factors feature numerous pseudogenes (6–8 for Oct4, 10 for Nanog and up to 16 for Stella) (Bernhardt et al. 2012; Pain et al. 2005), and some of them might be expressed, again with a very specific function distinct from their role in pluripotent stem cells. As for the Sox2, it should not be forgotten that it is a member of a particularly complex gene and protein family counting at least 20 members (Li et al. 2016), with closely related DNA-binding specificities, and the biological relationships between individual members markedly impact the final regulatory outcome, as exemplified in TGCT above. A similar complexity may be pursued within the Klf family (Kim et al. 2017a). If not carefully checked for these complex expression patterns, the interpretation of a mere finding of overexpression of pluripotency factor(s) in cancer might be rather difficult (see Table 1).

3.1 Pluripotency Transcription Factors in Cancer Stemness

There is ample evidence that at least a good part of tumours keeps intrinsic hierarchy analogical to that found in continuously renewing tissues or even at the beginning of development. Cancer stem cells (CSCs) have been operationally defined as a cancer cell population endowed with long-term proliferative capacity due to their self-renewal and self-protection, thus driving continuous tumour growth in primary outgrowth or at recurrence or on metastatic spread. The self-protection mechanisms can be exploited to approach these cells experimentally, with side population and Aldefluor assays developing into almost universal methods of their purification, reflecting their high xenobiotic efflux pump activity (especially ABCB1 and ABCG2) and xenobiotic detoxification activity (especially aldehyde dehydrogenases ALDH1A1 and ALDH1A3), respectively. Alternatively, a series of cell surface markers, sometimes reminiscent of niche organization of the respective tissue of origin, can be used as well, including CD133, CD44, CD49f, CD90 and a few more in specific cancer types (e.g. haematopoietic stem cell markers in leukaemias). While the self-protection-based approaches reflect an intrinsic biological property of cancer stemness, the cell surface markers may frequently lack any functional link. They are often combined, both with each other and with the self-protection-based assays. Functionally, cancer stem cells feature a series of traits, like high clonogenic activity, especially in anchorage-independent settings (either in semisolid media like agar, agarose or methylcellulose, or in suspension as cancer spheres), and,

Table 1 Expression of pluripotency transcription factors in cancer

SOX2				
Cancer type	**Increased expression**	**Prognosis/stage/clinical outcome**	**Decreased expression/no expression/loss of expression**	**Prognosis/stage/clinical outcome**
Breast	Ben-Porath et al. (2008); Chen et al. (2008)	Poor prognosis (Chen et al. 2008)		
Colorectal	Saiki et al. (2009)	Lymph node metastasis (Saiki et al. 2009)		
Oesophageal squamous	Wang et al. (2009)	Poor survival (Wang et al. 2009)		
Gastric	Tian et al. (2012); Chen et al. (2016)	Enhanced tumorigenicity (Tian et al. 2012) Better treatment outcome (Chen et al. 2016)	Otsubo et al. (2008); Wang et al. (2015)	Poor prognosis (Otsubo et al. 2008) Worse clinical outcome (Wang et al. 2015)
Glioma	Guo et al. (2011)	High tumour stage (Guo et al. 2011)		
Head and neck squamous cell	Lee et al. (2014)	Tumour recurrence and poor prognosis (Lee et al. 2014)	Bayo et al. (2015)	Worse survival (Bayo et al. 2015)
Melanoma	Hadjimichael et al. (2015)			
Lung	Li et al. (2013a) Chen et al. (2012a, p. 2)	Enhanced tumorigenicity (Chen et al. 2012a)		
	Wilbertz et al. (2011); Toschi et al. (2014)	Better prognosis in early stage (NSCLC) Better prognosis (Wilbertz et al. 2011)		
Ovarian	Pham et al. (2013); Robinson et al. (2021)	A role in tumour relapse (Robinson et al. 2021) High-grade tumour (Pham et al. 2013) Better overall survival (Belotte et al. 2015)		
Oral squamous	Fu et al. (2016); Ghazi et al. (2020)	Earlier stage (Fu et al. 2016) High grade (Ghazi et al. 2020)		
Nanog				
Cancer type	**Increased expression**	**Prognosis/stage/clinical outcome**	**Decreased expression/no expression/loss of expression**	**Prognosis/stage/clinical outcome**
Breast	Ben-Porath et al. (2008); Lu et al. (2014)	Advanced stage (Lu et al. 2014)		
Colorectal	Saiki et al. (2009) Meng et al. (2010)	No clinical significance (Saiki et al. 2009) Poor prognosis and lymph metastasis (Meng et al. 2010)		
Gastric	Lin et al. (2012)	Advanced stage (Lin et al. 2012)		
Gliomas	Guo et al. (2011)	High tumour stage (Guo et al. 2011)		

Lung	Chiou et al. (2010); Li et al. (2013b); Sławek et al. (2016)	High grade/poor prognosis (Sławek et al. 2016)		
Oral squamous	Chiou et al. (2008); Belotte et al. (2015); Fu et al. (2016); Vijayakumar et al. (2020)	Advanced stage (Fu et al. 2016; Vijayakumar et al. 2020)		
Ovarian	Lee et al. (2012); Amsterdam et al. (2013); Siu et al. (2013)	Worse prognosis (Siu et al. 2013)		
Prostate	Jeter et al. (2011)	Worse prognosis (Jeter et al. 2011)		
Oct4				
Cancer type	**Increased expression**	**Prognosis/stage/clinical outcome**	**Decreased expression/no expression/loss of expression**	**Prognosis/stage/ clinical outcome**
Bladder	Xu et al. (2007); Hatefi et al. (2012) Uhlén et al. (2015)	Poor prognosis (Xu et al. 2007; Hatefi et al. 2012) Favourable prognostic factor (Uhlén et al. 2015)		
Breast	Ben-Porath et al. (2008); Shen et al. (2014); Soheili et al. (2017)	Supressed the metastatic potential (Shen et al. 2014)		
Colorectal	Saiki et al. (2009)	No clinical significance (Saiki et al. 2009)		
Oesophageal squamous	Wang et al. (2009)	Poor survival (Wang et al. 2009)		
Gastric	Matsuoka et al. (2012)	Good prognosis (Matsuoka et al. 2012)		
Gliomas	Guo et al. (2011)	High tumour stage (Guo et al. 2011)		
Head and neck squamous carcinoma	Koo et al. (2015)	Advanced stage (Koo et al. 2015)		
Intrahepatic cholangiocarcinoma	Zhang et al. (2019)	Aggressive tumour and poor prognosis (Zhang et al. 2019)		
Lung	Chiou et al. (2010); Chen et al. (2012b); Li et al. (2013b)	High grade/poor prognosis (Li et al., 2012)	Moreira et al. (2010)	
Medulloblastoma	Rodini et al. (2012)	Poor survival (Rodini et al. 2012)		
Oral squamous	Chiou et al. (2008); Fu et al. (2016); Vijayakumar et al. (2020)	Advanced stage (Ghazi et al. 2020) Non-significant role in regulation of tumour behaviour (Vijayakumar et al. 2020)		
Prostate	Matsuoka et al. (2012)	Good prognosis (Matsuoka et al. 2012)		

(continued)

Table 1 (continued)

c-Myc				
Cancer type	**Increased expression**	**Prognosis/stage/clinical outcome**	**Decreased expression/no expression/loss of expression**	**Prognosis/stage/clinical outcome**
Breast	Ben-Porath et al. (2008)			
Colorectal	Saiki et al. (2009)	No clinical significance (Saiki et al. 2009)		
Glioblastoma	Zheng et al. (2008)	Advanced stage		
Glioma	Wang et al. (2008)			
Hepatocellular	Wang et al. (2002b)	Poor prognosis (Wang et al. 2002b)		
Lung	Sławek et al. (2016)	Worse survival (Johnson et al. 1996)		
Ovarian	Uhlén et al. (2015)	Unfavourable prognostic factor (Uhlén et al. 2015)		
Renal	Uhlén et al. (2015)	Unfavourable prognostic factor (Uhlén et al. 2015)		
Urothelial	Uhlén et al. (2015)	Unfavourable prognostic factor (Uhlén et al. 2015)		
Uterine cervix	Riou et al. (1987)	Worse relapse-free survival rate (Riou et al. 1987)		
Klf4				
Cancer type	**Increased expression**	**Prognosis/stage/clinical outcome**	**Decreased expression/no expression/loss of expression**	**Prognosis/stage/clinical outcome**
Breast	Yu et al. (2011); Okuda et al. (2013) Pandya et al. (2004)	Advanced tumour		
Colon			Patel et al. (2010)	Better prognosis (Patel et al. 2010)
Head and neck squamous cell	Tai et al. (2011)	Poor prognosis	Wang et al. (2002a)	
Lung			Hu et al. (2009); Zhou et al. (2010, p. 4); Gómez et al. (2014)	Aggressive tumour (Gómez et al. 2014)
Renal	Uhlén et al. (2015)	Favourable prognostic marker (Uhlén et al. 2015)		
Thyroid			Wang et al. (2019)	

Sall4				
Cancer type	**Increased expression**	**Prognosis/stage/clinical outcome**	**Decreased expression/no expression/loss of expression**	**Prognosis/stage/ clinical outcome**
Cholangiocarcinoma	Deng et al. (2015)	Worse prognosis (Deng et al. 2015)		
Oesophageal squamous	Forghanifard et al. (2014), He et al. (2016)	Tumour invasion and metastasis (Forghanifard et al. 2014)		
Endometrial	Li et al. (2015)	Worse survival (Li et al. 2015)		
Glioma	Zhang et al. (2015)	Poor prognosis (Zhang et al. 2015)		
Leukaemia	Yang (2018)	Disease progression (Yang 2018)		
Liver	Oikawa et al. (2013); Yong et al. (2013)	Tumour growth and resistance to 5-FU (Oikawa et al. 2013) Aggressive phenotype (Yong et al. 2013)		
Hepatoblastoma	Zhou et al. (2016)	Worse overall survival (Zhou et al. 2016)		
Hepatocellular	Yong et al. (2013); Liu et al. (2014, p. 4); Jung et al. (2016)	Poor prognosis (Liu et al. 2014; Jung et al. 2016) Aggressive phenotype (Yong et al. 2013)		
Myelodysplastic syndromes	Wang et al. (2013)	Worse survival rate (Wang et al. 2013)		
Ovarian	Yang et al. (2016)	Advanced stage (Yang et al. 2016)		
Renal	Che et al. (2020)	Worse survival (Che et al. 2020)		
Thyroid	Nicolè et al. (2017)	Worse survival (Nicolè et al. 2017)		
Tfcp2l1				
Cancer type	**Increased expression**	**Prognosis/stage/clinical outcome**	**Decreased expression/no expression/loss of expression**	**Prognosis/stage/ clinical outcome**
Bladder	Heo et al. (2020)	Tumour progression (Heo et al. 2020)		
Liver	Uhlén et al. (2015)	Unfavourable prognostic marker (Uhlén et al. 2015)		
Melanoma	Uhlén et al. (2015)	Unfavourable prognostic marker (Uhlén et al. 2015)		

where appropriate, high tumorigenic activity after (xeno)transplantation into a suitable host. Inherent to the existence of self-protection mechanisms is their intrinsic therapeutic resistance, both as an experimental approach to characterize them and within clinical context, thus underlying clinical disease therapy resistance and relapse (Hatina et al. 2013).

The manifestation of pluripotency stemness, especially expression of the core pluripotency transcription factors, has been invoked as another unifying characteristic of cancer stemness. In many cases, nevertheless, this is based more on an a priori assumption rather than a hypothesis-free evidence, by just demonstrating that cancer cell populations enriched in cancer stem cells by any method discussed above express higher level of pluripotency transcription factors or their mRNAs than the corresponding non-stem fractions, and it is plagued by many of the intrinsic weaknesses discussed above, including the existence of pseudogenes, isoforms and closely related family members, which are rarely taken into account. On the other hand, it now seems to be really experimentally substantiated that a part of cancer cases (either clinical tumours or cancer cell lines) use core pluripotency transcription factors to foster their stem-like populations. The most revealing evidence has been achieved by using reporter constructs driven by the multimerized Sox-Oct4 composite regulatory elements, either originating from the *Sox2* gene itself (SRR2 enhancer – Iglesias et al. 2014; Wu et al. 2012) or from the *Nanog* gene (SORE6 enhancer – Keysar et al. 2017; Menendez et al. 2020; Pádua et al. 2020; Tang et al. 2015), which turned out to be very useful across different cancer types, but usually just in a fraction of tumour samples. This implies that pluripotency stemness might be just one of multiple possible stemness mechanisms, complementing other mechanisms within or among individual tumours, within the context of the concept of cancer stem cell heterogeneity, reviewed extensively in this series (Birbrair 2019a, b). This situation is best illustrated in TGCT that can be taken as a prototypic tumour type driven by the pluripotency stemness circuitry. Indeed, specific clinical characteristics of this tumour, like extreme cisplatin chemosensitivity, might directly derive from the biological activity of pluripotency transcription factors, especially Oct4 (Gutekunst et al. 2013). Importantly, during the development of the chemoresistant disease, tumour cells eliminate expression of both Oct4 and Nanog (Taylor-Weiner et al. 2016) and highly probably switch their stemness mechanism to another biological basis.

In this way, the pluripotency stemness (especially Oct4-dominated) might be rather antithetical to cancer stem cell self-protection. Indeed, when analysed in detail, Oct4 seems not to be a crucial factor responsible for the cancer stemness (Menendez et al. 2020; Robinson et al. 2021), and within the context of the pluripotency network, Sox2 might dominate instead these cancer stem cell populations. It may be interesting to note in this context that Sox2 can participate in additional signalling pathways, for example, Hippo-Yap (Basu-Roy et al. 2015) or Hedgehog (Justilien et al. 2014). On the other hand, the impact of Sox2 is highly context-dependent. While characterized as an amplified oncogene in oesophageal and lung squamous cell carcinoma, a transgenic model revealed its tumour-suppressive role within the context of gastric carcinogenesis, principally due to its interference with the Wnt-β-catenin signalling (Sarkar et al. 2016), and even within the context of the former group of tumours, its high expression is associated with a favourable prognosis (Wilbertz et al. 2011). A similar tumour type- and context-specific oncogenic or tumour-suppressive role has been evidenced for the Klf4 as well (Ray 2016). Plausibly, this context dependence might be at the onset of discrepancies with the earlier report showing that activation of pluripotency stemness circuit is associated with high-grade poor prognosis tumours (Ben-Porath et al. 2008).

4 Induced Pluripotent Stem Cells and Induced Pluripotent Cancer Cells

Perhaps the most conclusive evidence for a causal role of the pluripotency transcription factors in pluripotency stemness lies in their ability to

convert, in a concerted action, differentiated cells into ESC-like cells, by and large equivalent to genuine ESCs, i.e. where appropriate (in murine system) able to contribute to all the foetal and adult tissues in chimeric mice, including germ line. First established from mouse embryonic fibroblasts (MEFs) and later extended for adult fibroblasts, carcinoma-associated fibroblasts, MSCs, neuronal stem cells, melanocytes, keratinocytes and various other epithelial cells, these induced pluripotent stem cells (iPSCs) became a superstar in the current scientific literature. The most extensively used combinations include Oct4, Sox2, Klf4 and c-myc (the original combination, called in honour to its discoverer Yamanaka factors) and Oct4, Sox2, Nanog and LIN28 (later established especially for human cells), but other protocols are also available, including miRNAs, shRNAs and small molecular inhibitors (Takahashi and Yamanaka 2016). At the cell biological level, there are at least two striking parallels between the process of iPSC reprogramming and cancer progression. First, there is a remarkable similarity as for the epithelial-mesenchymal plasticity. Stepwise reprogramming, achieved by sequential transduction of Yamanaka factors, revealed that MEFs first undergo epithelial-mesenchymal transition (EMT) and become "super mesenchymal", followed by mesenchymal-epithelial transition (MET) when they approached pluripotency (both ESCs and iPSCs have a pronounced epithelial character) (Liu et al. 2013). The parallel to metastatic progression of cancer is undisputable. Transforming growth factor β (TGFβ), a notorious EMT inductor, thus can promote reprogramming if applied in the initial phase, while it had a devastating impact if applied in the second half or continuously (Liu et al. 2013). Along the same lines, the pharmacological inhibitor of the TGFβ receptor I, RepSox, is most active in a short window around a half of the reprogramming sequence (Ichida et al. 2009). Second, both during the pluripotency reprogramming and cancer, the developmental process known as cell competition is operating (Shakiba et al. 2019).

The process of pluripotency reprogramming is also subject to similar intrinsic barriers as tumorigenic transformation, including senescence, and immortalization, as well as subversion of p53- and p16-dominated checkpoints, can significantly increase reprogramming efficiency, accompanied at the same time with an increased risk of transformation (Utikal et al. 2009). Interestingly with regard to p53, there was a graded increase in both possible outcomes upon mere knockdown of p53 via shRNA and introduction of tumour-derived gain-of-function p53 mutant (Sarig et al. 2010). Indeed, iPSC reprogramming bears an inherent risk of tumorigenic transformation, representing one of the most serious clinical concerns for possible applications, e.g. in regenerative medicine (Ben-David and Benvenisty 2011; Lee et al. 2013; Wuputra et al. 2020). Interestingly, the most obvious tumorigenic effect was associated with an incomplete reprogramming, achieved by interrupting after about 2 weeks (instead leaving the process to its completion in 28 days) (Ohnishi et al. 2014).

With this in mind, it was very unexpected to find that various cancer cells could be successfully reprogrammed as well into cells that often closely approached (though rarely really achieved) iPSCs. Most notably, these induced pluripotent cancer cells (iPCCs) lose expression of typical tumour markers, and they lose (or markedly reduce) tumorigenicity, perhaps the most important expression of their transformed character, as well as the most convincing characteristic of cancer stemness (Bernhardt et al. 2012; Câmara et al. 2016; Iglesias et al. 2017).

Put simply, this constitutes a difficult dilemma – pluripotency transcription factors are copiously reported to be overexpressed in cancer, use of reporter constructs driven by pluripotency transcription factors provides access to cancer stem cells in multiple tumour types, yet forced expression of pluripotency transcription factors in cancer cells leads to their phenotypic reversion. And such reports are today rather numerous and include melanoma cells (Bernhardt et al. 2017), various gastrointestinal carcinoma cells (Miyoshi

et al. 2010), lung carcinoma cells (Mahalingam et al. 2012) as well as a spectrum of various sarcomas (Zhang et al. 2013). This way of cancer reprogramming has even been suggested as a possible way to a future therapeutic strategy (Gong et al. 2019; Miyoshi et al. 2021; Yilmazer et al. 2015).

4.1 Why?

In order to tackle this paradox, we have to concentrate on biological differences between pluripotency reprogramming (or pluripotency stemness) and tumorigenic transformation. Indeed, besides the parallels between the two processes, there are signalling pathways that are frankly antithetical, and perhaps the most important among them is the MAPK pathway. We have already mentioned that inclusion of the specific MEK inhibitor PD0325901 was an important step in formulating improved ESC culture media to prevent the FGF-4-driven autodifferentiation activity. The downstream transcription factors activated by the MAPK pathway, especially the transcription factors of the AP-1 family, consisting of various Jun and Fos dimers, have been convincingly characterized as one of the most pronounced inhibitors of both pluripotency stemness and pluripotency reprogramming (Liu et al. 2015). Along the same line, pharmacologic inhibition of activated B-Raf was reported to increase the pluripotency reprogramming of melanoma cells (Castro-Pérez et al. 2019), and activated H-ras was shown to markedly decrease iPSC reprogramming efficiency. This last example revealed a hitherto unprecedented cellular complexity of the reprogramming process, nevertheless. The pluripotency reprogramming process is inherently very inefficient, with success rate of the original Yamanaka protocol far below 1%, and there are complex signalling interactions between the successfully reprogrammed cells and the vast majority of cells that fail to achieve pluripotency stemness – the latter can be seen as a sort of niche cells promoting the acquisition of stemness by the minority of "winners". If activated *H-ras* was transduced together with Yamanaka factors into the bulk culture, it promoted reprogramming. This promoting effect was strictly non-cell-autonomous, i.e. based on a paracrine activity of "loser" cells towards the successfully reprogrammed cells. If tested for cell-autonomous effect, i.e. in individual cells that received both activated *H-ras* and the polycistronic vector coding for Yamanaka factors, then activated H-ras behaved dramatically inhibitory (Ferreirós et al. 2019). Absolutely the same has been described for another crucial cancer oncogene, Yap, the downstream effector of the Hippo pathway (Hartman et al. 2020). Of note, such a distinction between cell-autonomous and non-cell-autonomous effects is difficult to get unless deliberately pursued, thus explaining a lot of seemingly contradictory reports (Chung et al. 2016; Qin et al. 2012; Tamm et al. 2011).

Importantly, both MAPK–AP-1 and Hippo-Yap signalling pathways have dramatically different impacts in both normal adult stem cells and cancer stem cells. Jun combines with Wnt-β-catenin signalling in an intricate regulatory circuit governing ISCs and colorectal cancer stem cells and operating both feedforward (Nateri et al. 2005; Sancho et al. 2009) and feedback (Kabiri et al. 2018; Harmston et al. 2021) pathways. Side population cells of various sarcomas, neuroblastoma and urothelial carcinoma are dependent on active MAPK signalling; strikingly, in these cancers active MAPK signalling is mechanistically linked to the expression of pluripotency transcription factors (Tsuchida et al. 2008; Hepburn et al. 2012). In addition, Jun-Fos constitutes a central regulatory hub of Ewing sarcoma side population cells (Hotfilder et al. 2018), and Jun is the major effector of the stemness-promoting effect of the connective tissue growth factor (CTGF) in head and neck squamous cell carcinoma (HNSCC) (Chang et al. 2013). Intriguingly, in ESCs and iPSCs, Jun-imposed transcriptome included downregulated pluripotency stemness genes including *Nanog*, *Sall4*, *Oct4*, *Sox2* and *Esrrb*, and it was almost completely opposite to the Oct4-induced transcriptome (and, along the same lines, transcriptomes imposed by Oct4 and endogenous Jun inhibitors, either a Jun-dominant negative mutant or Jun dimerization protein 2,

were practically identical (Liu et al. 2015)). In contrast, MEK inhibitor-treated sarcoma and urothelial carcinoma cells significantly reduced their SP fraction and tumorigenicity (Tsuchida et al. 2008; Hepburn et al. 2012) instead of activating stemness as seen in ESCs and iPSCs. In CTGF-stimulated HNSCC cells, Jun is rapidly activated and subsequently directly activates pluripotency core factors Oct4, Sox2 and Nanog. Even this last aspect turned out to be more complicated if followed at single-cell resolution level. Based on the results described above, we applied CTGF to aggressive sarcoma cells, and we indeed observed a dramatic effect on anchorage-independent clonogenicity. Immunofluorescence staining failed to evidence any Oct4 induction, and staining for Sox2 and Jun revealed heterogeneous and only partially overlapping pattern (Fig. 1). Plausibly, cancer stem cells should be understood more as a collective term to describe cells bearing special functional characteristics and involving several molecularly discernible subpopulations (Hatina et al. 2019).

The existence of cancer (stemness)-promoting signalling pathways that at the same time strongly inhibit, in cell-autonomous manner, the pluripotency reprogramming could provide a straightforward explanation for the loss of tumorigenicity observed in iPCCs. In addition, it has been repeatedly reported that efficiency of pluripotency reprogramming is inversely correlated to genomic complexity of cancer cells. For example, the reprogramming efficiency gradually decreased along the melanoma progression (Castro-Pérez et al. 2019), and genomically simpler tumours like hepatoblastoma were much easier to reprogram than the corresponding complex cancer like hepatocellular carcinoma (Kuo et al. 2016). High mutational load of tumour cells and their high heterogeneity even within individual tumours or individual cancer cell lines,

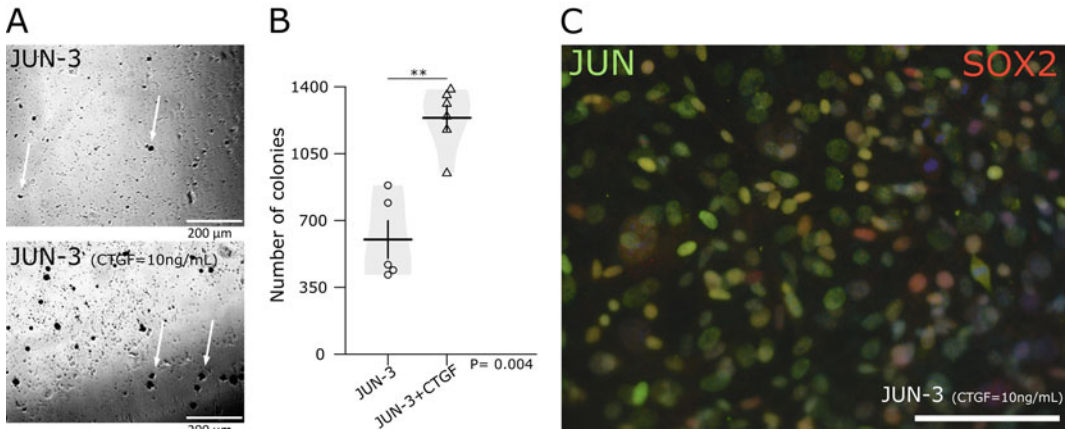

Fig. 1 The murine fibrosarcoma cell line JUN3 (Hatina et al. 2003) was routinely cultured in high glucose (4500 mg/l) Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, penicillin (final concentration 100 U/ml) and streptomycin (final concentration 100 µg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were treated with the CTGF (10 ng/ml) after 24 h serum starvation for 5 days and processed for anchorage-independent clonogenicity assay by suspending 10,000 cells in 15% methylcellulose [109] or fixed in 4% formaldehyde in PBS for 30 min, blocked in 1% BSA for 1 h and processed for immunofluorescence with overnight incubation at 4 °C with primary antibodies (anti-c-JUN mouse monoclonal antibody at 1:400 and anti-SOX2 rabbit polyclonal antibody at 1:200 dilution) followed by extensive washing and 2 h incubation with secondary antibodies (rabbit anti-mouse Atto 488; green fluorescence and goat anti-rabbit – TRITC; red fluorescence, 1:400 dilution, room temperature), extensively washed and mounted. (A) Colonies in methylcellulose were counted after 10 days; pictures were taken by the Olympus IX 81 inverted microscope equipped with the Hamamatsu Orca-ER camera. Bar: 200 µm. (B) The statistical significance is based on permutational t-test ($p = 0.004$). Each point represents an individual well. (C) Immunofluorescence pictures were taken by the Olympus IX 81 inverted microscope equipped with the Hamamatsu Orca-ER camera. Bar: 100 µm

combined with intrinsic inefficiency of pluripotency reprogramming (even distinctly lower for cancer cells than for normal differentiated cells), may inevitably select for those cancer cells with low mutational burden to be successfully reprogrammed (Lai et al. 2013), and they can't be expected to manifest advanced transformation characteristics. This relative reversion of the respective transformation status can lead to very valuable models. For example, cell lines representative of early-stage pancreatic carcinoma are unavailable, and pluripotency reprogramming seems to have helped in filling this gap (Kim et al. 2013). Specific secretome derived from reprogrammed advanced-stage pancreatic cancer cells showed potential of identifying early-stage pancreatic cancer tumour markers, with tremendous possible clinical utility for this very serious cancer diagnosis (Berger et al. 2019; Kim et al. 2017b).

4.2 Epigenetic Parallels Between iPSCs and iPCCs

Embryonic stem cells feature a very specific epigenetic landscape, with a low level of DNA methylation and bivalent chromatin modification. This is characterized by simultaneous presence of both activating (H3K4triMe) and repressive (H3K27triMe) chromatin marks, generating a poised state that can rapidly adjust gene activities to differentiation signals (Voigt et al. 2013). In contrast, differentiated cells and cancer cells feature a more polarized epigenetic regulation, with DNA methylation as a preferred mechanism of permanent gene silencing. During reprogramming, this differentiated epigenome reorganizes towards an ESC-like. This can be best documented by demethylation and activation of genes of the pluripotency network, and this switch from dependency on exogenously encoded pluripotency transcription factors, such as Yamanaka factors, whose ectopic expression starts the reprogramming process, to derepression of respective endogenous pluripotency genes, accompanied by silencing of the introduced factors, is a hallmark of successful reprogramming (Takahashi and Yamanaka 2016). Importantly, however, this genome-wide demethylation does not achieve the extent seen in ESCs. In other words, a fraction of genes still depended on DNA methylation for their silencing, and they failed to be reactivated. In addition, hundreds of genes seem to be de novo silenced during the reprogramming process (and, in fact, probably as a consequence of it) (Ohm et al. 2010). On the other hand, for imprinted genes, occasional loss of imprinting is seen in different iPSC clones (but genomic imprinting is mostly faithfully preserved in ESCs); a part of these imprinting defects may be attributable to the source differentiated cells undergoing reprogramming, but most of them occur early during the reprogramming itself (Bar et al. 2017). Among the aberrantly methylated genes, there are several proven or suspected tumour suppressors, and for the two imprinted genes standing out for showing quite frequent loss of imprinting accompanied by biallelic (and markedly increased) expression, *IGF-2* and *RTL-1*, a tumour-promoting role has been documented (Fan et al. 2017; Kessler et al. 2016; Riordan et al. 2013). In conclusion, iPSCs, as for their epigenetic landscape, seem to be somewhere in the middle between normal stem cells (ESCs and MSCs) and cancer cells (Ohm et al. 2010).

Interestingly, the opposite tendency has been documented for at least one iPCC model, obtained by reprogramming of two non-small cell lung cancer cell lines. A part of genes (over a half) on the list of aberrantly methylated genes in lung cancer, with some well-characterized tumour suppressors, were demethylated in the respective iPCC lines, and some half of them were really derepressed. On the other hand, from the list of putative lung cancer oncogenes, about a half were de novo methylated and downregulated in the wake of the reprogramming process (Mahalingam et al. 2012). We thus see a partial normalization of the cancer epigenome in the resulting iPCCs. Although no side-by-side comparison of epigenetic profiles of iPSCs and iPCCs has been, to our knowledge, published by now, they might be quite close to each other. Inasmuch as this is the result of the reprogramming process itself, or just a reflection of the specific epigenome of those rare cells that are able to

undergo reprogramming and achieve pluripotency, is not yet clear.

Parallels and differences in epigenetic processes taking place during iPSC and iPCC reprogramming can have two additional consequences. First, quite analogical to the EMT-MET plasticity observed during normal reprogramming, premature termination in cancer cell reprogramming may accentuate the invasiveness of cancer cells, which can be of benefit for identification of novel invasion-associated cancer genes (Knappe et al. 2016). Second, unlike normal pluripotency reprogramming, the EMT-MET succession may not be finished during the cancer pluripotency reprogramming, yielding a metastable EMT-MET transition state (Hiew et al. 2018), which in fact may be quite typical for metastasis-competent cancer cells (Celià-Terrassa and Kang 2016) and hence may provide a valuable insight into the biology of cancer metastasis.

5 Dose Dependency of Pluripotency Factors

Another point deserving a brief discussion is that the pluripotency factors feature a pronounced dose dependency of action. In particular, they act as stemness factors only within quite a narrow range, and underexpression, but also overexpression trigger differentiation. This has been reported for both Oct4 (Niwa et al. 2000) and Sox2 (Kopp et al. 2008); interestingly with regard to the latter, the impact on transcription of downstream target pluripotency genes dramatically changes along with elevating the Sox2 level, switching from activator as expected for the functionality of the pluripotency network to transcriptional repressor (Boer et al. 2007). The same dose dependency is true for the BMP4 as a crucial stemness signalling molecule as well. It was consistently observed that low to very low dosed BMP4 (10 ng/ml for ESCs, 0.01–0.1 ng/ml for MSCs) acts as a powerful stemness factor (Vicente López et al. 2011), whereas doses of 50 ng/ml to 100 ng/ml are consistently used to induce differentiation (Cordonnier et al. 2011). Moreover, this dose-dependent effect seems to be well conserved in cancer – autocrine BMP4 (secreted in doses of about 500 pg/ml detected in conditioned media) is protumorigenic within the context of colorectal carcinoma, including the promotion of anchorage-independent clonogenicity (one of the typical stem cell characteristics) (Yokoyama et al. 2017), whereas high-dosed BMP4 (100 ng/ml) has been repeatedly identified as a CSC differentiation factor, both for colorectal carcinoma (Lombardo et al. 2011) and glioblastoma, being even considered as a promising therapy for the latter (Nayak et al. 2020). Moreover, the iPSC reprogramming efficiency, as well as the quality (i.e. the ability to develop into an intact mouse organism), also crucially depends on the respective ratio of reprogramming factors, especially Oct4 and Sox2, with Oct4 (high) and Sox2 (low) combination being one to two orders of magnitude more efficient than other combinations (Nagamatsu et al. 2012), and this combination was also characteristic of the reprogramming mixture yielding iPSCs that could develop into "all-iPSCs" embryos (i.e. derived entirely from iPSCs) (Carey et al. 2011). Importantly most tumours, unlike normal differentiated cells, express pluripotency factors (especially Sox2 – see above), which adds to the transfected reprogramming factors and in the end possibly profoundly influences the cancer reprogramming, both regarding the reprogramming efficiency and the phenotype of the resulting iPCCs. Adding to the complexity, there are "close relatives" of both Sox2 and Klf4 within the respective protein families, which can partially substitute for them during the pluripotency reprogramming (Jiang et al. 2008), and for which a protumorigenic role and corresponding overexpression have been described in cancer – this is, for example, the case of Sox3 (Guo et al. 2018; Marjanovic Vicentic et al. 2019) or Klf5 (Liu et al. 2020; Siraj et al. 2020).

6 Conclusion

The pluripotency reprogramming has been mostly conceived within the context of regenerative medicine, to provide in theory unlimited individual patient-matched source of stem cells to be differentiated in tailored fashion and

transplanted back in order to replace disease-damaged cells. Soon it was realized that in addition to this possible direct clinical application, iPSCs offer tremendous possibilities for experimental modelling of numerous human diseases, including various neurodegenerative diseases like Huntington, Alzheimer and Parkinson diseases, amyotrophic lateral sclerosis as well as cardiomyopathy, liver metabolic disorders and urinary tract and prostate disorders (Argentati et al. 2020). Cancer couldn't be expected to stand aside, but it increasingly turns out that modelling cancer with the help of iPSC methodology will be rather a daunting task. Cancer is not a single disease, but a group of many hundreds of individual diagnoses, with unifying basic biology but each with its own genetic and epigenetic landscape. While modelling some genetically simpler cancer phenotypes like certain leukaemias (Turhan et al. 2019) or very initial stages of hereditary (Zhu et al. 2018) and in near perspective hopefully also sporadic cancers might be feasible, using iPSCs for modelling full-blown common cancers is presently beyond our reach; in fact, we do not understand the biology of such tumours in such a detail that would allow us to reproduce them in form of gene-modified iPSCs derived from normal cells of the respective patients. Although we have catalogued thousands of cancer-specific mutations and we have tools on how to introduce them with great precision into the genome of iPSCs, we are still dramatically remote from being able to reproduce cancer genome and epigenome and complex biological behaviour. Not only that a lot of oncogenic mutations, well characterized in isolation, act with pronounced context dependency, but perhaps the major hurdle is the cellular complexity of advanced tumours, with complex clonal heterogeneity and functional hierarchy. Moreover, this complexity is not static but constantly changes due to both genome instability of cancer cells and incessant reciprocal interactions with tumour microenvironment. In this view, the endeavour to generate, starting from a patient-derived iPSCs, a "cancer-on-a-dish" and use it as a screening platform within the context of personalized medicine (Papapetrou 2016) seems to be a very remote vision at best. Of course, a seemingly more straightforward solution appeared to be to start the reprogramming process with very tumour cells, which would fix all the complex (and in part still elusive) cancer genetic abnormalities in a series of tumour-derived iPSCs (or better in current nomenclature iPCCs). In our view, elaborated above, this strategy is very problematic as well. Experience shows that genetic complexity is antithetical to reprogramming capacity and a lot of signalling pathways have contradictory effects in cancer cells and in pluripotent cells. Pluripotency reprogramming in highly complex advanced tumours would thus catch at best a fraction of cancer cells, and the most aggressive cells would be probably missed. Another theoretical concept might be that tumour-derived iPSCs would be enriched in cancer stem cells, the most therapy-resistant functional cancer cell subset; even the term induced cancer stem cells (iCSCs) has been proposed in this regard (Czerwińska et al. 2018). But even this notion might be plagued with a not negligible naivety – we have seen that although pluripotency transcription factors do participate in cancer stemness, this is just one piece in the complex mosaic of biological mechanisms underlying cancer stem cells. We are far from denying the potential of pluripotency reprogramming for cancer modelling, but in our view, this would provide just one of many experimental models, and only their combined use hopefully provides us with crucial new knowledge in order to combat this devastating disease.

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Conflict of Interest None

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

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In Vitro Culturing of Adult Stem Cells: The Importance of Serum and Atmospheric Oxygen

Maša Čater and Gregor Majdič

Abstract

Adult stem cells are undifferentiated cells found in many different tissues in the adult human and animal body and are thought to be important for replacing damaged and dead cells during life. Due to their differentiation abilities, they have significant potential for regeneration and consequently therapeutic potential in various medical conditions. Studies on in vitro cultivation of different types of adult stem cells have shown that they have specific requirements for optimal proliferation and stemness maintenance as well as induced differentiation. The main factors affecting the success of stem cell cultivation are the composition of the growth medium, including the presence of serum, temperature, humidity, and contact with other cells and the composition of the atmosphere in which the cells grow. In this chapter, we review the literature and describe our own experience regarding the influence of the presence of fetal bovine serum in the medium and the oxygen concentration in the atmosphere on the stemness maintenance and survival of adult stem cells from various tissue sources such as adipose tissue, muscle, brain, and testicular tissue.

Keywords

Adipose tissue · Atmospheric oxygen · Brain · Culturing · Differentiation · Muscle · Serum · Stem cells · Stemness · Testes

Abbreviations

ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
DMEM	Dulbecco's modified Eagle's medium
EGF	epidermal growth factor
FBS	fetal bovine serum
FGF	fibroblast growth factor
GDNF	glial cell line-derived neurotrophic factor
NGF	nerve growth factor

1 Introduction

Stem cells have enormous potential in health and medical research due to their regenerative potential. Different types of adult stem cells can be

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isolated from different tissues. These cells are multipotent, meaning that they are capable of differentiating into more than one cell type, but not all cell types (Fortier 2005). Mesenchymal stem cells, a special type of adult stem cells, have become increasingly important in the field of regenerative medicine in recent years for treating certain human diseases (Giordano et al. 2007; Trounson et al. 2011; Jossen et al. 2014). They are being investigated for their therapeutic potential in inflammatory, autoimmune, and degenerative conditions in preclinical and clinical studies (Inamadar and Inamadar 2013; Ratcliffe et al. 2013).

Species and strain variations in the properties of adult stem cells from different tissues and their requirements for optimal growth have been reported in numerous publications although in general, stem cells are difficult to isolate and maintain in vitro (Baddoo et al. 2003; Peister et al. 2004; Sung et al. 2008). In addition to growth medium composition and incubation temperature, partial oxygen pressure, extracellular matrix proteins, and contacts with other cells are known factors that affect stem cell viability, proliferation, function, and differentiation (Yoshida et al. 2009). In this review, we describe the importance of serum in the culture medium and the role of atmospheric oxygen in the growth and differentiation of adult stem cells from various sources. A broad literature review is accompanied by our own studies.

2 Sources of Adult Stem Cells

Mesenchymal stem cells, also called multipotent mesenchymal stromal cells, are found in adipose tissue, bone marrow, umbilical cord, and dental pulp (Klingemann et al. 2008). Muscles contain skeletal muscle stem cells and hematopoietic stem cells (Kawada 2001; Chen and Goldhamer 2003). Spermatogonial stem cells can be isolated from testes (Goossens and Tournaye 2006; Guan et al. 2006). Adult stem cells in the mammalian brain (neural stem cells) were discovered much later than in other tissues (Altman and Das 1965; Altman 1969), and it has been shown that these neural stem cells can differentiate into neurons and glia cells in vitro (Reynolds and Weiss 1992; Ma et al. 2009). Neural stem cells, mesenchymal stem cells, muscle stem cells, epidermal stem cells, and some others meet the basic criteria for stem cells (Prockop 1997; Gage 2000; Watt 2001) as they can proliferate and differentiate into various tissues in vitro, whereas corneal stem cells and endothelial stem cells are only capable of differentiating into a single type of differentiated cell (Daniels et al. 2001; Verfaillie 2002). The most studied adult stem cells are the hematopoietic stem cells, while other adult stem cells were defined much later and are therefore less studied (Verfaillie 2002). In this chapter, we focus on adipose-derived, muscle, neural, and spermatogonial stem cells.

2.1 Adipose Tissue-Derived Stem Cells

Adipose tissue is a rich source of mesenchymal adipose tissue-derived stem cells (Zuk et al. 2010), which can differentiate into mesodermal cells such as osteoblasts, chondrocytes, adipocytes, and muscle cells (Zuk et al. 2002; Lee et al. 2004; Guilak et al. 2004; Lin et al. 2008). Therefore, these cells have an important potential for cell therapy and are used for immunomodulation in pathologies such as Crohn's disease, regenerative medicine, and aesthetic medicine (Le Blanc and Ringden 2007; Ringden et al. 2007; Abdi et al. 2008; Mirotsou et al. 2011). Adipose tissue-derived stem cells offer several advantages over other cell types. Adipose tissue is easily accessible, requiring minimally invasive surgery for its harvesting. In addition, adipose tissue contains many more progenitor cells in comparison to other tissues (e.g., bone marrow) (Strem et al. 2005). Adipose-derived stem cells also have great expansion potential (Lee et al. 2004; Kern et al. 2006) which is important for cell therapies.

2.2 Muscle Stem Cells

Muscle stem cells belong to the satellite cell population and are responsible for skeletal muscle

growth and repair. They reside between muscle fibers within the basal lamina but outside the muscle fiber (Montano 2014). Studies of muscle stem cells play an important role in the development of novel treatments for muscular disorders (Pomerantz and Blau 2008; Wang et al. 2014; Relaix et al. 2021). However, these cells are difficult to isolate and purify, so alternative myogenic stem cells, including adipose-, bone marrow-, and umbilical cord-derived mesenchymal stem cells, as well as perivascular stem cells, are being investigated for their potential as possible cell sources for treating muscle disorders (Pantelic and Larkin 2018).

2.3 Testicular Stem Cells

Spermatogonial stem cells reside inside the seminiferous tubules within the testes. They are capable of self-renewal and producing daughter cells to give rise to terminally differentiated cells, the spermatozoa. These cells are therefore responsible for the lifelong maintenance of spermatogenesis (Nagano 2003; Kubota and Brinster 2018). Since the number of spermatogonial stem cells decreases with aging, aging of their niche is a critical factor for the maintenance of these cells. Dysfunction of the niche leads to a decreased number of spermatogonial stem cells in older men (Zhang et al. 2006). Spermatogenesis can be impaired due to various congenital disorders, resulting in male infertility (Matzuk and Lamb 2008). Spermatogonial transplantation can be used to restore fertility in infertile men and to elucidate the mechanism of genetic defects in spermatogenesis (Shinohara et al. 2000).

2.4 Neural Stem Cells

The adult mammalian brain has a low regenerative capacity and is limited in its ability to replace neurons that become dysfunctional or atrophic due to acute or chronic injury. The discovery of neural stem cells opened the possibility of harnessing them for endogenous brain repair (Gage 2019). Neural stem cells are not distributed throughout the brain but are found in specific locations, especially in the ventricular-subventricular zone along the walls of the lateral ventricles and in the subgranular zone of the dentate gyrus of the hippocampus (Alvarez-Buylla and Lim 2004; Ma et al. 2005; Obernier and Alvarez-Buylla 2019). Lower number of neural stem cells is also found in the striatum, septum, and spinal cord (Palmer et al. 1995; Weiss et al. 1996b). Furthermore, the subcallosal zone, located between the white matter and the hippocampus, has also been reported to contain adult neural stem cells (Kim et al. 2016). The generation of neurons for cell therapy is promising for the treatment of neurodegenerative diseases and brain injuries.

3 The Effect of Serum on Stem Cell Growth

For the successful proliferation of adult stem cells in vitro and the maintenance of their stemness, the balance of nutrients and microenvironmental conditions is of paramount importance. The culture medium is one of the most important single factors in cell culture as it provides all essential nutrients (Butler and Jenkins 1989; Brunner et al. 2010). Serum is the most commonly used supplement in cell culture. Fetal bovine serum (FBS) is a common choice because it contains high concentrations of growth factors and other important signaling molecules such as adhesion proteins, nutrients, carrier proteins, cytokines, and hormones that, along with its buffering capacities, are required for cell survival, proliferation, and/or differentiation. FBS can be added to the medium in varying amounts from 1% to 20% to promote cell attachment and provide growth factors and vital nutrients (Harmouch et al. 2013; Forcales 2015). However, the use of FBS in cell culture can be problematic as FBS contains xenogeneic proteins and potentially pathogenic microorganisms, which presents a risk for the induction of an immunological response and the transmission of pathogens. FBS also poses a problem for optimizing cell culture conditions because its composition is not defined and

exhibits batch-to-batch variability (Heiskanen et al. 2007; Sundin et al. 2007). On the other hand, defined serum-free media have an accurate composition but are often less successful in supporting cell growth than media containing FBS (Jossen et al. 2014). This was also confirmed in our study as various adult stem cells from BALB/c mice grew significantly better in the FBS-containing media. Since serum deprivation can slow or even stop stem cell proliferation and increase cell death (Hasan et al. 1999; Cooper 2003; Shin et al. 2008), growth media should be supplemented with FBS for at least a defined period of time until stem cells reach confluence (Nonnis et al. 2016). In most cases, stem cells have been successfully cultured in FBS with no serious side effects reported (Le 2003; Berger et al. 2006; Mannello and Tonti 2007). Alternatives to FBS, including autologous or allogeneic serum, platelet lysate, and thrombin-activated platelet-rich plasma, are also in use in clinical treatments to avoid the aforementioned drawbacks of FBS (Duggal and Brinchmann 2011). In our laboratory, we performed a direct comparison between two media, commercial MesenCult medium and newly developed A20 medium, based on DMEM and containing 20% FBS.

3.1 Muscle Stem Cells

Several studies confirmed that muscle stem cells require high concentrations of FBS for their optimal growth. Most often, they are grown in medium containing 20% FBS and a high glucose content (Motohashi et al. 2014; Syverud et al. 2015; Mozzetta 2016; Čamernik et al. 2019; Boscolo Sesillo et al. 2020). Also in our laboratory, adult stem cells isolated from the muscle of a BALB/c mouse and cultured in a newly developed A20 medium containing 20% FBS grew significantly better than in a commercial serum-free MesenCult medium. In A20, cell cultures reached 80% confluence in 9 days, whereas cells in a serum-free medium did not proliferate. The cells in A20 were transplanted in several additional passages where they successfully proliferated.

3.2 Adipose Tissue-Derived Stem Cells

Like adult muscle stem cells, adipose tissue-derived mesenchymal stem cells grow best in medium containing FBS. Mouse, rat, pig, and human mesenchymal stem cells from adipose tissue are most commonly cultured in medium containing 5–15% FBS (Yamamoto et al. 2007; Arana et al. 2013; Alstrup et al. 2018). The importance of FBS in the cultivation of adipose-derived stem cells was confirmed in our laboratory. We tested the growth of adipose-derived stem cells from the BALB/c mouse in MesenCult medium without FBS and found a very slow growth and early loss of stemness. Interestingly, adipose tissue-derived stem cells also proliferated very slowly in A20 medium supplemented with FBS until hypoxic atmospheric conditions were applied. Our results point to an important fact that not only serum and media content but also atmospheric conditions play an important role in the cultivation of adult stem cells, which will be discussed in further sections.

3.3 Testicular Stem Cells

Long-term cultivation of murine spermatogonial stem cells in vitro is difficult due to low survival rate. Typically, only 10–20% of cells survive after 1 week in a culture. Medium supplements such as cytokines and growth factors, as well as serum (1–10%) are used to achieve greater proliferation over longer periods of time (Nagano et al. 1998; Kanatsu-Shinohara et al. 2003). We compared the efficiency of culturing spermatogonial stem cells from the BALB/c mouse in serum-free medium MesenCult and in A20 medium with FBS. The importance of FBS was confirmed as proliferation of cells in serum-free medium was slow and unsuccessful (Fig. 1). On the other hand, spermatogonial stem cells were efficiently cultured in A20 medium with 20% FBS, reaching 80% confluence around day nine after isolation. The cells were transplanted and grew successfully in the first passage as well.

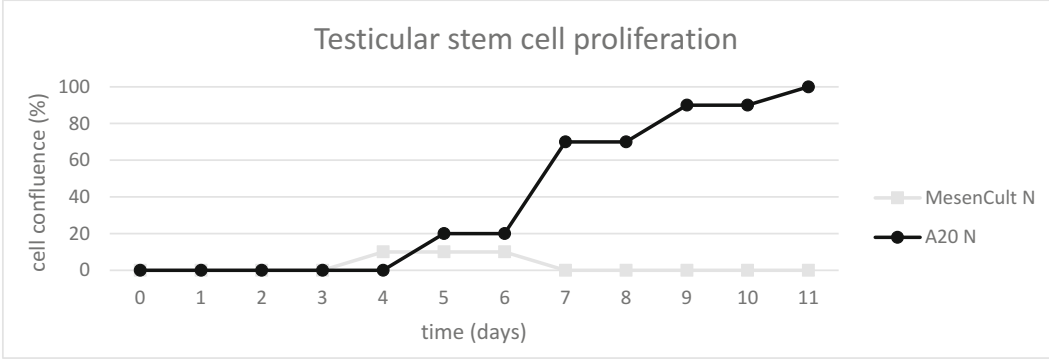

Fig. 1 Testicular stem cell growth in initial passage using serum-free MesenCult medium and A20 medium with 20% FBS. Legend: N – normoxia

3.4 Neural Stem Cells

Standard methods for culturing neural stem cells were developed in the early 1990s and include the neurosphere method and adherent monolayer culture (Reynolds and Weiss 1992; Palmer et al. 1995; Ray et al. 1995). Protocols for isolation and in vitro cultivation of neural stem cells from adult mouse whole brain in the form of neurospheres or monolayers use a serum-free medium supplemented with growth factors like epidermal growth factor and fibroblast growth factor, which are needed for the survival of cells (Walker and Kempermann 2014; Deshpande et al. 2019). However, in our study, very few neural stem cells were viable when cultured in a serum-free MesenCult medium, and they did not proliferate. Greater cultivation potential was achieved when FBS was available to the cells. Neural stem cells from all three brain regions (anterior, medial, and posterior) proliferated well in A20 medium (Fig. 2). However, we observed a much slower cell proliferation in comparison to other stem cell types. It took 2 weeks for the neural stem cells to become about 80% confluent. They were then transplanted into the next passage, where they continued to proliferate successfully.

4 The Effect of Hypoxia on Stem Cell Growth

Standard cell culture systems typically use environmental oxygen levels (20%) although the actual oxygen content in tissues is much lower. Interestingly, improved stem cell survival and reduced apoptosis have been reported when using low-oxygen partial pressure (Morrison et al. 2000; Studer et al. 2000). Several previous studies have shown improvement of stem cell culture when cells were grown in oxygen concentrations below 10% (Guyton and Hall 1996; Carreau et al. 2011). This is believed to better simulate in vivo conditions as oxygen concentrations in the brain have been reported to be around 0.5% in the midbrain, 2–5% in the cortex, and up to 8% in the pia mater (Mannello et al. 2011), about 3.8% in muscle (Carreau et al. 2011), and about 3% in the testes (Klotz et al. 1996). Interestingly, the oxygen concentration in adipose tissue varies from 4.5% to 5% in lean mice to about 1–2% in obese mice (Ye et al. 2007; Rausch et al. 2008; Netzer et al. 2015). It has been suggested that local oxygen concentration may directly affect stem cell proliferation, self-renewal, and differentiation. Stem cells seem to benefit from residing in hypoxic niches where oxidative DNA damage can be reduced (Keith and Simon 2007). When stem cells are cultured at oxygen concentrations that do not match those inside the niche microenvironment, cells undergo a number of changes such as metabolic turnover, oxidative stress, impaired motility, altered differentiation potential, and loss of stemness potential (Mas-Bargues et al. 2019).

When cells are cultured at low oxygen concentration, any available oxygen diffuses into the mitochondria, creating a hypoxic environment in

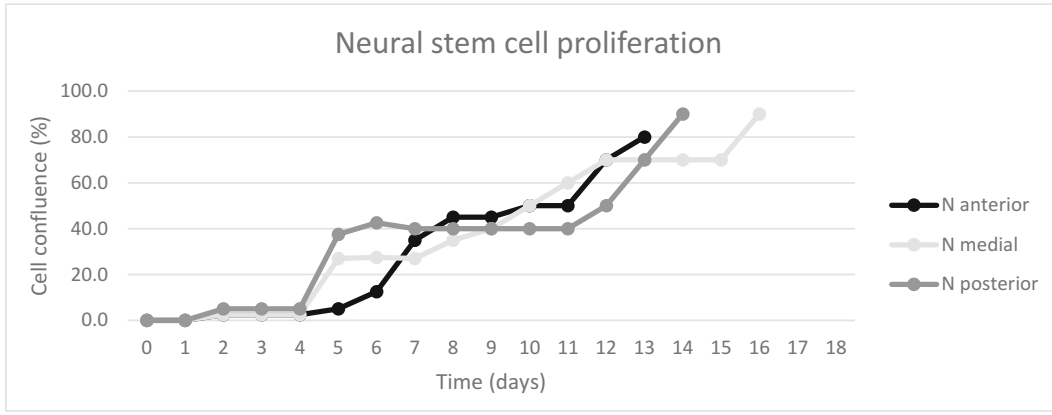

Fig. 2 Proliferation of neural stem cells from different parts using A20 medium with 20% FBS. Legend: N – normoxia

the cytosol. Hypoxia inhibits the activity of prolyl hydroxylases that regulate the activation of hypoxia-inducible factors. The original role of hypoxia-inducible factors, angiogenesis, has recently been expanded by new studies showing that they are also involved in self-renewal, stemness, and differentiation of stem cells. Under hypoxic conditions, hypoxia-induced factors are not hydroxylated and are thus stabilized to initiate their transcriptional activity (Bell and Chandel 2007). Hypoxia-induced factors are also important for the regulation of stem cell metabolism. Cells from hypoxic niches rely on anaerobic glycolysis to support ATP production. When exposed to atmospheric oxygen levels, cells are forced to decrease glycolysis and increase oxygen consumption through mitochondrial oxidative phosphorylation. This metabolic switch affects cellular function as it promotes senescence, genomic instability, and shortening life span (Estrada et al. 2012).

In our laboratory we compared the growth of adult stem cells from the BALB/c mouse in a serum-free and a serum-supplemented medium in normoxic (5% carbon dioxide, 20% oxygen, 75% nitrogen) and hypoxic atmospheres (5% carbon dioxide, 2% oxygen, 93% nitrogen). Cells from testis and muscle proliferated slightly better under hypoxic conditions when grown in MesenCult medium, but cells from adipose tissue grew only under normoxic conditions with this medium, yet not as successfully as in A20 medium with FBS. Cells from all tissues (adipose tissue, muscle, testes, and brain) grew much better in A20 medium than in MesenCult medium regardless of atmospheric conditions. However, for cells grown in A20 medium, atmospheric conditions affected only proliferation of cells from adipose tissue and brain, while cells from testis and muscle tissue proliferated at similar rates under both atmospheric conditions. Interestingly, the effect of hypoxia was the opposite for neural- and adipose tissue-derived cells. Cells from adipose tissue grew better under hypoxic conditions, while neural cells grew better under normoxic conditions.

4.1 Adipose Tissue-Derived Stem Cells

Many studies have observed low proliferation rate at the environmental oxygen concentration in adipose-derived stem cells (Efimenko et al. 2011; Kim et al. 2012, Mas-Bargues et al. 2019). Our studies correspond to these findings as the isolation of adult stem cells from the adipose tissue of the BALB/c mouse using A20 medium containing FBS was successful only in the hypoxic atmosphere, with cells visible as early as 24 h after tissue plating. Cell culture reached 80–90% confluence by day 12 (Fig. 3). The cells were then transplanted and proliferated faster in the first passage than in the passage zero,

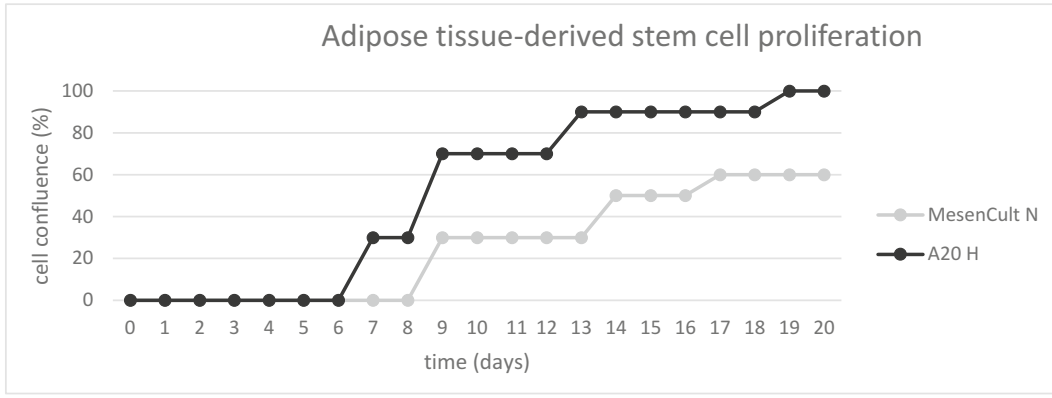

Fig. 3 Adipose tissue-derived stem cell growth curves in zero passage using MesenCult and A20 mediums. Legend: N – normoxia, H – hypoxia. Cells did not grow in A20 media in normoxic conditions and in MesenCult media in hypoxic conditions

reaching 80% confluence in 4 days. The cells were also transplanted in the second passage and reached 80% confluence in 3 days. Similar results for adipose tissue-derived stem cells have been reported previously. Hypoxia has been shown to promote stemness, proliferative capacity, and viability of adipose tissue-derived stem cells and to prevent adipogenic differentiation through negative gene regulation (Lin et al. 2006; Ye et al. 2007; Weijers et al. 2011; Yamamoto et al. 2013; Choi et al. 2014; Kakudo et al. 2015). This likely reflects the adaptation of cells to low oxygen concentrations in adipose tissue in vivo.

4.2 Muscle Stem Cells

Unsuccessful proliferation of muscle stem cells under normoxic conditions has been reported in some studies (Csete et al. 2001; Lees et al. 2008). Ambient oxygen concentration affects cell cycle regulation as p53 phosphorylation increases in cultures grown at 20% oxygen, resulting in cell cycle arrest (Chen et al. 2007). A similar effect of atmospheric conditions was observed in our laboratory. Muscle stem cell isolation and proliferation in a serum-free MesenCult medium was successful only under hypoxia (Fig. 4), with cells initially proliferating rapidly. However, their proliferation slowed considerably after reaching 50% confluence, and they never reached 80% confluence. Spontaneous differentiation occurred. On the other hand, oxygen concentration did not affect the proliferation of cells from muscles cultured in a serum-supplemented A20 medium as the cells grew at a similar rate under both normoxic and hypoxic conditions. Proliferation was successful, and cell cultures reached about 80% confluence in 9–11 days (Fig. 4). Cells were transplanted in several more passages where they proliferated successfully under both atmospheric conditions. This suggests that various factors, including growth media, affect the proliferation and self-renewal of stem cells from different tissues, and cells from different tissues show different sensitivity to oxygen concentrations. Although oxygen concentration affects proliferation of some cell types in vitro, other factors, such as media and unknown tissue-specific factors, seem to modulate the sensitivity of cells to atmospheric conditions. Since we still do not know all the factors that influence adult stem cells viability and stemness, future studies need to focus on identifying factors that influence stem cells in culture in order to develop optimal growth media and optimal atmospheric and other conditions for culturing adult stem cells from different tissues.

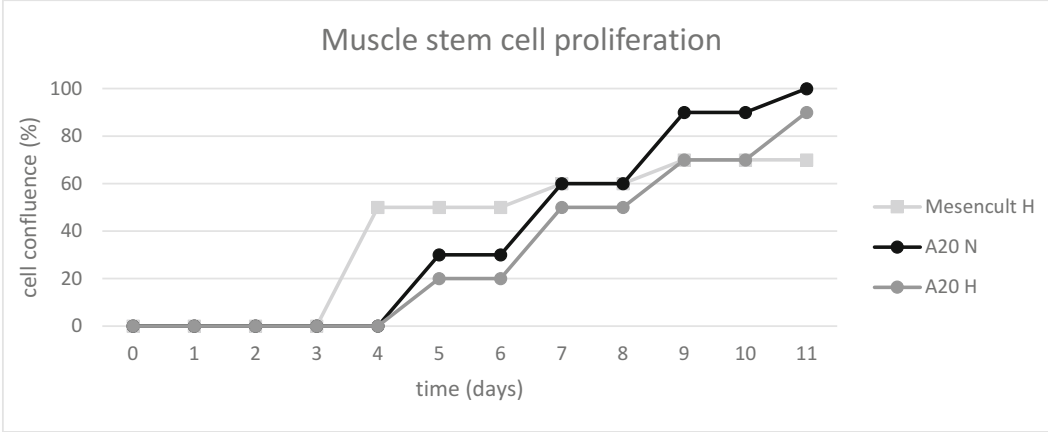

Fig. 4 Muscle stem cell growth in initial passage in both atmospheres using MesenCult and A20 mediums. Legend: N – normoxia, H – hypoxia. Cell did not grow in MesenCult media in normoxic conditions

4.3 Testicular Stem Cells

Previous studies have shown that culturing spermatogonial stem cells can be improved by reducing the atmosphere oxygen concentration. Kubota et al. (2009) and Helsel et al. (2017) used 10% of oxygen and achieved successful long-term culturing. Interestingly, however, no difference was observed in the proliferation rate under both atmospheric conditions in our laboratory. We compared the growth of adult murine spermatogonial stem cells in a serum-free and a serum-supplemented media in normoxic and hypoxic atmospheres containing 20% and 2% oxygen, respectively, and the results were similar with both media. Cells in serum-free media grew very poorly under normoxic and hypoxic conditions, whereas cells in A20 medium grew successfully regardless of atmospheric conditions (Fig. 5).

4.4 Neural Stem Cells

The effect of oxygen concentration in the atmosphere on culturing neural stem cells remains unclear as there are many controversial studies published. Some studies of culturing neural stem cells report that ambient oxygen concentration decreases their proliferation and promotes differentiation, while some report the opposite (Vieira et al. 2011; Mas-Bargues et al. 2019). We found that neural stem cells grew faster in serum-supplemented A20 medium at 20% oxygen in comparison to conditions with 2% oxygen. With A20 medium, we obtained viable cells from all brain regions studied (anterior, medial, and posterior parts of the brain). However, overall, cell proliferation of neural cells was slower in comparison to other tissues. Cells grew in both atmospheres, but proliferation was much faster under normoxic conditions (Fig. 6). Under normoxic conditions, cells were about 80% confluent in 2 weeks, whereas under hypoxic conditions, cells reached only about 40% confluence in the same time when isolated from the medial and posterior parts of the brain. This may reflect the higher requirement of neuronal cells for continuous oxygen supply. It is well known that neurons require a constant supply of oxygen and are the first cells in the body to die in hypoxia. Therefore, it would be intuitive to expect neuronal cells to grow better in an atmosphere with higher oxygen concentration.

Indeed, this has already been shown as some studies reported that optimal growth of neural stem cells occurs under normoxic conditions (Kilty et al. 1999; Kang et al. 2010). In addition to the medial part of the brain, which contains the main regions known to harbor stem cells, we have

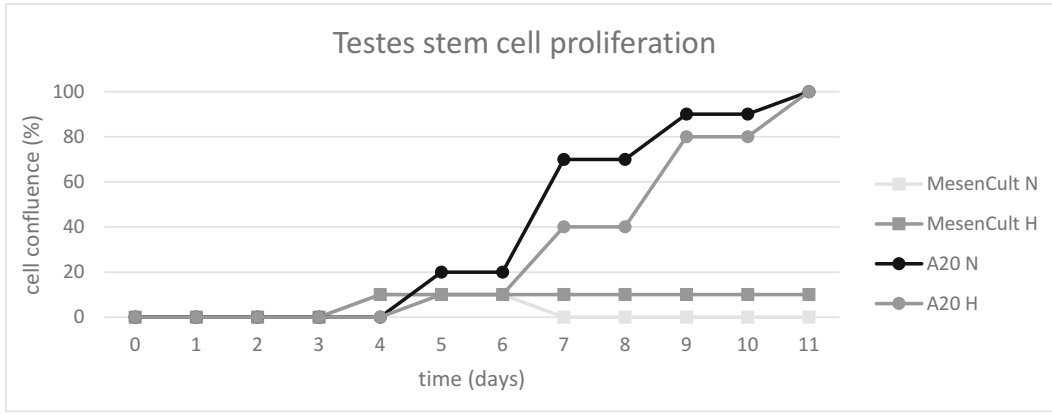

Fig. 5 Testicular stem cell growth in initial passage in both atmospheres using MesenCult and A20 mediums. Legend: N – normoxia, H – hypoxia

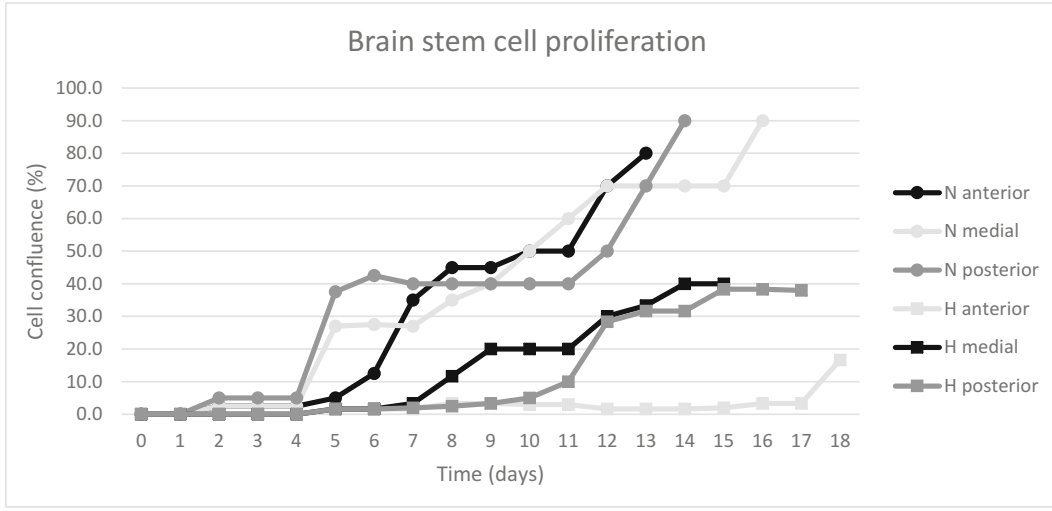

Fig. 6 Proliferation of brain stem cells from different parts in both atmospheres using A20 medium. Legend: N – normoxia, H – hypoxia

successfully isolated stem cells from the anterior and posterior parts of the brain as well. Interestingly, all three brain regions appear to be an equally good source of neural stem cells when cultured with A20 medium in normoxia. The stem cells from the anterior part of the brain most likely originated from the cortex and part of the optic nerve as these areas have been previously shown to contain stem cells (Palmer et al. 1999). Recently, it has been reported that neural stem cells are also located in the inferior colliculus, auditory cortex, and dorsal vagal complex (Li et al. 2003; Bauer et al. 2005; Volkenstein et al. 2013; Völker et al. 2019), which likely explains our positive results in obtaining stem cells from the posterior parts of the brain.

Although there are some reports suggesting that neural stem cells grow well also under hypoxic conditions, this could be a response to pathological conditions. Previous studies have shown that neural stem cell proliferation in the brain is stimulated in vivo by hypoxia as a consequence of stroke, asphyxiation, or other trauma as a

homeostatic mechanism that attempts neuroregeneration (Mannello et al. 2011; Wagenaar et al. 2018).

5 The Effect of Serum in Culture Medium and Atmospheric Oxygen Concentration on Stem Cell Differentiation

Some previous reports suggest that FBS may contain factors that could induce sensitive cells to lose their stemness and differentiate spontaneously (Brunner et al. 2010; Meenakshi 2013). However, in our study, we never observed spontaneous differentiation in the FBS-containing media even when cells grew in several passages. In contrast, cells from adipose tissue, muscle, and testis spontaneously differentiated when grown in serum-free MesenCult medium. These cells proliferated very slowly in MesenCult and appeared to have lost their stemness already during the first passage and showed signs of spontaneous differentiation into different cell types.

Apart from serum in the medium, ambient oxygen concentration in the atmosphere has been shown to have certain negative impacts on culturing adult stem cells as it can promote spontaneous differentiation of stem cells toward specialized cell lineages (Chen et al. 2007; Mas-Bargues et al. 2019). The important role of oxygen concentration in maintaining stemness was also observed in our laboratory. Interestingly, cells from different tissues often spontaneously differentiated when growing in the serum-free media but followed different differentiation pathways. In some cases, as with cells from the testes, oxygen concentration even affected the differentiation pathway of the cells.

Adult stem cells are sometimes induced to differentiate into specialized cells with the aim to use them for specific cell therapy. Usually, the addition of growth factors and neurotrophic factors into the medium is needed to direct the differentiation process into the desired cell lineage. The presence of serum also plays an important role in induced differentiation. To achieve differentiation into a particular cell lineage, some adult stem cells require FBS in the medium, together with additional inducing factors, while in some cases, a serum-free medium is required. A well-known example of heterogenous-induced differentiation demands are adult stem cells derived from adipose tissue, which can readily differentiate into osteocytes, chondrocytes, and adipocytes, depending on the presence of certain growth factors in the medium.

5.1 Adipose Tissue-Derived Stem Cells

The absence of serum in the growth medium may provoke early loss of stemness and spontaneous differentiation of isolated adipose tissue-derived stem cells. In our laboratory, morphological changes were observed in adipose tissue-derived stem cells cultured in serum-free medium within the first few days after plating when hypoxic conditions were used. Even after 3 weeks of cultivation, cells from adipose tissue grown in hypoxia with serum-free MesenCult medium remained in passage 0 because proliferation was very slow. From day nine, some spheroids with high confluency appeared. We used differential staining to investigate and confirm what type of cell differentiation occurred under the hypoxic conditions. Cells were stained with Alcian Blue and Alizarine Red S and were positive for both, indicating that spontaneous differentiation into chondrospheroids and osteocytes occurred in the cell culture under these conditions (Fig. 7).

Interestingly, proliferation of adipose tissue-derived stem cells grown in a serum-free medium at atmospheric oxygen concentration was slow and unsuccessful, but no spontaneous differentiation was observed. Similarly, no spontaneous differentiation was observed when cells were cultured in FBS-supplemented A20 medium. These results suggest that a combined effect of serum in the medium and hypoxic atmosphere is required for the best long-term proliferation of adipose-derived stem cells. The absence of serum in the medium immediately reduces the proliferation rate and triggers spontaneous differentiation, while a low oxygen concentration in the atmosphere promotes

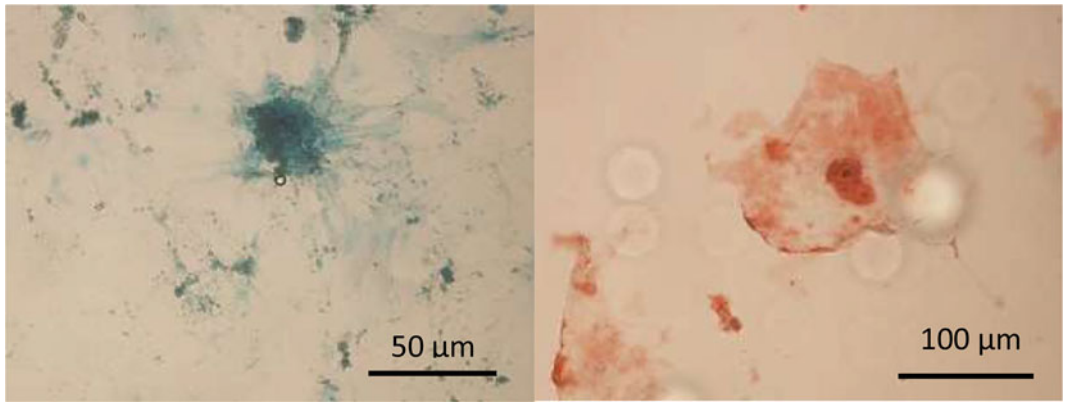

Fig. 7 Cells from adipose tissue, cultured for 3 weeks (hypoxia, MesenCult), spontaneously differentiated into chondrocyte-like cells (left) and osteocyte-like cells (right)

survival of the cells, resulting in increased spontaneous chondrogenesis and osteogenesis.

Adipose tissue-derived stem cells can be induced to differentiate into several different specialized cell types by altering the formulation of the growth medium (Zuk et al. 2002; Guilak et al. 2004). Apart from additional induction molecules, osteogenic and neural differentiation of adipose-derived stem cells require a medium without FBS, whereas adipogenic and chondrogenic differentiation media are supplemented with FBS (20% and 1–10%, respectively) (Bunnell et al. 2008). Adipocyte differentiation in vitro is induced with serum-supplemented medium and induction cocktails containing insulin, methylisobutylxanthine, hydrocortisone or dexamethasone, and indomethacin or thiazolidinedione (Halvorsen et al. 2001; Bunnell et al. 2008). In addition, stem cells from adipose tissue can be induced to differentiate into chondrocytes or the osteogenic lineage. Chondrogenesis can be promoted by adding transforming growth factor, ascorbate, and dexamethasone to a serum-supplemented medium and maintaining the cells in a three-dimensional, rounded morphology in a micromass pellet culture or within a hydrogel (Erickson et al. 2002; Awad et al. 2003). Differentiation into osteoblast-like cells is induced by the absence of FBS in the medium and by the addition of ascorbate, β-glycerophosphate, and dexamethasone (Heng et al. 2004; Bunnell et al. 2008). Adipose tissue-derived stem cells can also serve as a source of stem cells that can undergo neural differentiation. Neurospheres form when adipose-derived stem cells are cultured at high density using a neurogenic differentiation medium, which is usually a serum-free medium with the addition of antioxidants, indomethacin, insulin, and isobutylmethylxanthine (Safford et al. 2002, 2004).

5.2 Muscle Stem Cells

A high concentration of FBS in the medium is required for the maintenance of undifferentiated adult muscle stem cells in vitro, with some studies also recommending the use of hypoxic conditions (Lees et al. 2008). This was confirmed in our study as cell proliferation was arrested when cells were cultured in serum-free medium in an ambient oxygen atmosphere. Moreover, muscle stem cells cultured in serum-free MesenCult medium under hypoxia showed morphological changes after 9 days of cultivation with lipid vacuoles appearing inside the cells. Staining with Oil Red O was performed and confirmed that the cells accumulated lipid deposits and presumably spontaneously differentiated into adipose cells (Fig. 8). No such spontaneous differentiation was observed in cells grown in A20 medium containing 20% FBS under normoxic or hypoxic conditions.

For myogenic induction, cocultivation of muscle stem cells with primary myoblasts and

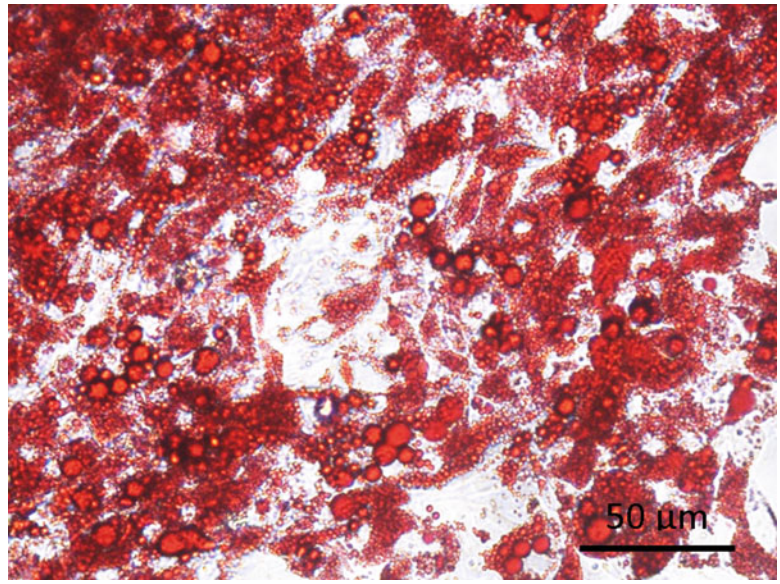

Fig. 8 Muscle-derived cells, cultured for 9 days (hypoxia, MesenCult), spontaneously differentiated into adipose-like cells with rich lipid deposits (stained with Oil Red O)

additional induction with dexamethasone and FGF is usually used (Eberli et al. 2009; Bitto et al. 2013). Stem cells isolated from adult muscle can also be a good source of autologous neural cells, useful for cell replacement in neurodegenerative and demyelinating diseases. When grown in a serum-free medium under nonadherent conditions, muscle stem cells can be induced to differentiate into neurospheres. Further cultivation under adherent conditions provokes differentiation into neurons and oligodendrocytes (Romero-Ramos et al. 2002).

5.3 Testicular Stem Cells

Serum in the growth medium is of high importance for promoting the proliferation of undifferentiated spermatogonial stem cells. In our laboratory, cells cultured in the absence of FBS spontaneously differentiated. Cells isolated from testes and grown in serum-free MesenCult medium under both normoxic and hypoxic conditions changed their morphology after only 4 days of cell culture. Interestingly, atmospheric conditions seem to influence the direction of the spontaneous differentiation. Cells grown under normoxic conditions at 20% oxygen in MesenCult medium accumulated cellular lipid inclusions, like differentiated cells from muscle tissue. Differentiation into adipocytes was confirmed by Oil Red O staining (Fig. 9, left). However, under hypoxic conditions with 2% oxygen and when grown in the same serum-free medium, cells appeared to spontaneously differentiate into chondrospheroids, which was confirmed by positive staining with Alcian Blue (Fig. 9, right). This was only observed in cells isolated from testicular tissue, and we do not know at the moment what causes these differences in spontaneous differentiation. The testis is composed of different cell types, and in vivo, these cells have different access to both oxygen and nutrients from the blood due to the composition of the testis and the testis-blood barrier. It is therefore possible that different cell types grow better under normoxic and hypoxic conditions and that these cells have different differentiation capacities. Alternatively, the same cells could respond differently to different atmospheric conditions, but this will have to be investigated in future studies.

Induced in vitro differentiation of spermatogenic cells seems to be a possible method for the treatment of male infertility. Studies of in vitro spermatogenesis have shown that FBS plays an important role and is essential to allow

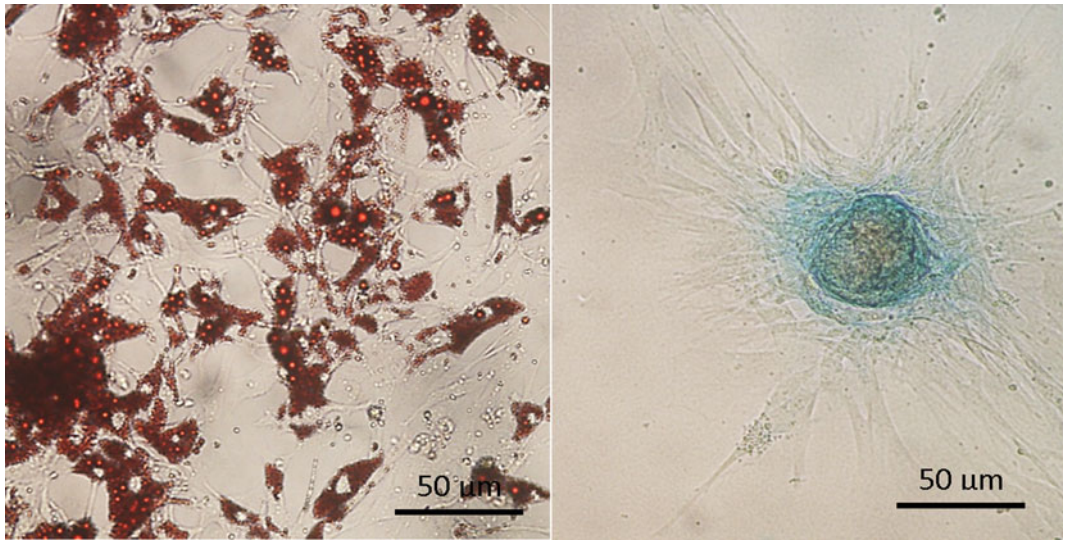

Fig. 9 Using MesenCult, testicular cells spontaneously differentiated in adipose (4 days, hypoxia, left) or chondrocyte-like cells (9 days, normoxia, right)

the progress of spermatogenesis, together with additional molecules like growth factors and hormones (Lee et al. 2006; Sato et al. 2011; Zhao et al. 2018).

5.4 Neural Stem Cells

A standard serum-free culture system for neural stem cells, also known as the neurosphere assay, allows selective growth of stem cells isolated from the adult brain. Undifferentiated neural stem cells survive and proliferate, while most other differentiated cell types die (Reynolds and Weiss 1992.) The use of certain growth factors such as EGF and FGF as mitogens can induce a consistent, renewable source of undifferentiated neural stem cells, which could be expanded into defined proportions of neurons, astrocytes, and oligodendrocytes (Gritti et al. 1995, 1996, 1999: Reynolds and Weiss 1996; Weiss et al. 1996b, 1996a). Removal of growth factors, present in stemness-promoting medium, and addition of FBS into the growth medium cause neurosphere-derived cells to differentiate (Rietze and Reynolds 2006; Liu et al. 2018). In general, undifferentiated neural stem cells express glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF). Neurotrophic factors and the presence of serum in the media are needed to induce differentiation. The regulatory factors in the serum can strongly influence the expression of neurotrophic factors. 1% FBS in the media reduces the expression of GDNF in differentiating neural stem cells, while 10% suppresses it completely (Niles et al. 2004).

A negative impact of ambient oxygen concentration on cell growth was shown also in the cultivation of neural stem cells. Cultivation under normoxic conditions can lead to spontaneous differentiation of neural stem cells toward the glial lineage (Chen et al. 2007). However, some studies report that normoxic conditions decrease proliferation and promote differentiation of neural stem cells, while some reports suggest that low oxygen concentration increases the differentiation potential of such cells (Vieira et al. 2011; Mas-Bargues et al. 2019). Controversial results have also been observed in some other types of adult stem cells and can be explained partially by the concentration of oxygen in the atmosphere and the duration of exposure used in the studies. Some of the studies used short-term hypoxia (less than 72 h), while others maintained the cells in

hypoxia permanently. Furthermore, the oxygen concentration in various studies varied between 0.1% and 5%, meaning some cells were exposed to a more anoxic environment than the others and therefore making such studies difficult to compare directly.

6 Conclusions

Many studies have shown that adult stem cells isolated from different tissues respond differently to different environmental conditions such as the content of growth media, in particular the presence or absence of FBS, and atmospheric composition. In general, serum in the media seems to have a positive effect on both cell growth and the prevention of spontaneous differentiation of stem cells. Interestingly, cells from different tissues often spontaneously differentiate when grown in serum-free media but follow different differentiation pathways. In some cases, such as cells from the testes, even atmospheric oxygen concentration can affect the differentiation pathway of cells. Apart from serum, the oxygen concentration in the atmosphere of the culture has a major impact on the stemness maintenance and survival of stem cells, so this is an essential factor to consider when culturing adult stem cells for tissue engineering and regenerative medicine.

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Mouse Models of Asthma: Characteristics, Limitations and Future Perspectives on Clinical Translation

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Abstract

Asthma is a complex and heterogeneous inflammatory airway disease primarily characterized by airway obstruction, which affects up to 15% of the population in Westernized countries with an increasing prevalence. Descriptive laboratory and clinical studies reveal that allergic asthma is due to an immunological inflammatory response and is significantly influenced by an individual's genetic background and environmental factors. Due to the limitations associated with human experiments and tissue isolation, direct mouse models of asthma provide important insights into the disease pathogenesis and in the discovery of novel therapeutics. A wide range of asthma models are currently available, and the correct model system for a given experimental question needs to be carefully chosen. Despite recent advances in the complexity of murine asthma models, for example humanized murine models and the use of clinically relevant allergens, the limitations of the murine system should always be acknowledged, and it remains to be seen if any single murine model can accurately replicate all the clinical features associated with human asthmatic disease.

Keywords

Allergy · Asthma · Mouse models

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Abbreviations

OVA	Ovalbumin
Der p	Dermatophagoides pteronyssinus
Der f	Dermatophagoides farinae
HDM	House dust mite
SPF	Specific pathogen free
ASM	airway smooth muscle
PBMCs	peripheral blood mononuclear cells

1 Introduction

Asthma is one of the most important chronic airway inflammatory diseases that affects almost 300 million people worldwide. The components of the disease are characterized by airway inflammation, variable and recurring symptoms, airway hyper-responsiveness, and chronic airflow limitations that associate with structural changes (Dharmage et al. 2019). Asthma is referred to as a complex disease where one's genetics and environmental influences play a critical role. Although evidence from twin and family studies point to a strong correlation with genetic background, the interaction between genetic and environmental factors is still under investigation (Pivniouk et al. 2020). From a clinical perspective, asthma shows differences in severity, natural history comorbidities, and medication/treatment response. Although it is difficult to strictly categorize into defined groups, the Tucson study proposed three classical phenotypes of childhood wheezing: transient early wheezers, nonatopic wheezers, and persistent atopic wheezers (Taussig et al. 1989). The term "endotype" has been proposed to define an asthma subtype characterized by a distinct pathophysiological mechanism (Akar-Ghibril et al. 2020). Recent studies revealed that parameters such as clinical characteristics, biomarkers, lung physiology, genetics, histopathology, epidemiology, and treatment response are useful in the determination of asthma sendotypes (Crescitelli et al. 2021). Currently proposed asthma endotypes include eosinophilic asthma, exacerbation-prone asthma, exercise-induced asthma, adult-onset asthma, fixed airflow limitation, and poorly steroid-responsive asthma. Among these subphenotypes, the current murine models only represent early onset Th2-type allergic asthma and eosinophilic asthma (Periz et al. 2020).

From the immunological perspective, allergic asthma is defined as a Th2-type disease state. A number of studies have demonstrated increased eosinophils and Th2-derived cytokines in the airways of children with severe asthma (Caspard et al. 2020; Cevhertas et al. 2020), while others have noted noneosinophilic patterns of airway inflammation with increased neutrophils and biomarkers of neutrophil activation (Hauk et al. 2008; Lex et al. 2005; Moeller et al. 2015). Thus, severe asthma can be associated with different endotypes that do not represent a Th1 or Th2 cytokine pattern (Fitzpatrick et al. 2010; Steinke et al. 2021).

The correct animal model should reflect the disease pathophysiology as closely as possible and is essential for the development of new therapies. Because of ethical concerns, clinical studies with allergic individuals are limited in scope and experimental murine models have become more important. Although, today there are several therapeutic and preventive animal models for allergic asthma, there remain important components to be elucidated. Data from clinical specimens suggest that some features of human asthma can be replicated in animal models, thereby providing data on disease characteristics (Stevenson and Birrell 2010). Several studies carried out in animal models have given important clues that explain the pathophysiological conditions related to the disease status. For instance, the role of Th2-type cytokines in the pathogenesis of asthma have been particularly well-studied in animal models of allergic lung inflammation.

Mouse models have been developed for almost all types of allergic disease, such as asthma (Nials and Uddin 2008; Aun et al. 2017), allergic rhinitis (Wagner and Harkema 2007), food allergy and anaphylaxis (Dearman and Kimber 2007), atopic dermatitis (Jin et al. 2009), and allergic conjunctivitis (Niederkorn 2008). These models are important to examine the mechanism of the disease and the activity of a variety of genes and cellular pathways, predict the safety of new drugs or chemicals before being used in clinical studies (Karol 1994), define the pathogenic pathways, and suggest new therapeutic options (Bice et al. 2000). Although murine models of asthma provide important insights into the disease mechanisms, there are some limitations that should be considered. Beside the genetic and physiological differences between humans and

mice, there are also limitations due to complexity of this disease. In other words, mice do not develop asthma. One can replicate important components of the disease, but no single model accurately models all the features of asthma (Wenzel and Holgate 2006; Kumar and Foster 2002; Ray et al. 2015). This is very important to take into account when choosing the correct model to address the specific experimental question. For example, chronic exposure models are required to examine many of the structural changes associated with inflammatory disease within the airways. This review focuses on the different mouse models of asthma that are currently in use and discusses the limitations of each model.

1.1 Immune Response to Allergens

If immune tolerance is overcome to certain allergens, such as aeorallergens, food allergens, and insect venom, type I and type IV hypersensitivity reactions ensue. Several factors, including genetic susceptibility, the nature of antigen that initiates the disease (antigen dose, time of exposure, route of exposure, and structural characteristics) and exposure to infections and bacteria (Burks et al. 2008), influence the type of immune response.

Specialized antigen-presenting cells, such as dendritic cells, have a crucial role in the presentation of antigenic compounds to T lymphocytes and lead to differentiation of naive T cells into Th1, Th2, Th9, Th17, and Th22 effector T-cell subsets (Goldberg and Rock 1992; Akdis and Akdis 2009). In murine models, the initial event responsible for the development of allergic disease is the generation of allergen-specific $CD4^{+}$Th2 cells (Jutel and Akdis 2011). The current view is that IL-4 stimulation promotes naive T cell differentiation into Th2 cells (Romagnani 1994; Mosmann and Sad 1996; Abdelaziz et al. 2020). The allergic immune response occurs in two main phases: first sensitization and development of memory, and later followed by the effector phase and tissue injury. In the sensitization phase, $CD4^{+}$Th2 cells secrete IL-4, IL-5, and IL-13 and mediate several functions. These cytokines induce class switching of antibody isotypes to the ε heavy chain for IgE antibody production by B cells, development and recruitment of eosinophils, production of mucus, and reduction the threshold for contraction of smooth muscle cells (Mosmann and Sad 1996; Romagnani 1994; Corry 1999). Later, this allergen-specific IgE binds to high-affinity IgE receptors (FcεRI receptors) on the surface of mast cells and basophils. This series of activation steps leads to the sensitization of patients to a specific allergen. In the final phase, reexposure to the sensitized allergens leads to activation of effector cells and tissue injury occurs. The degranulation of basophils and mast cells by IgE-mediated cross-linking of receptors is crucial in the type-I hypersensitivity reaction, which may lead to development of type-IV hypersensitivity chronic allergic inflammation. All these events require allergen-specific T cell activation in allergic individuals, while for healthy individuals peripheral, T cell tolerance prevents formation of allergic immunopathology (Hirose et al. 2017).

1.2 Mouse Models of Asthma

Mice are the most common species studied for animal models of asthma (Sagar et al. 2015). In particular, acute or chronic allergic lung inflammation models utilizing ovalbumin (OVA) or house dust mite represent severe, persistent asthma models that have been successfully established in mice. Usually, mice are systemically sensitized to allergen with alum as an adjuvant via intraperitoneal injection and allergen challenged via the airways. The number of sensitizations and challenges is decisive for the development of acute or chronic forms of this model. The nature of the lung inflammation is directly influenced by the genetic background of the mice, the allergen, type of the sensitization and challenge protocol, and contamination of the allergen with substances (e.g. LPS), which stimulate the innate immune response (Zosky and Sly 2007) (Table 1).

Table 1 Mouse models of acute and chronic asthma

Mouse strain	Sensitization	Challenge	Type of Asthma model	References
BALB/c	i.p. OVA / alum	OVA (aerosol)	Acute model	Lloyd (2007), Tomkinson et al. (2001), Hamelmann et al. (1999)
A/J	i.p. Bla g 2 and Derf 1	Allergen (oro-tracheal)	Acute model	Sarpong et al. (2003)
C57BL/6	i.p. Der p 1 / alum	HDM (aerosol)	Acute model	Tournoy et al. (2000)
BALB/c	i.p. Bet v 1 / alum	Bet v 1 (aerosol)	Acute model	Wiedermann et al. (1999a), Winkler et al. (2002)
BALB/c	i.p. OVA / alum	OVA (aerosol, 6–8 weeks)	Chronic model	Temelkovski et al. (1998)
BALB/c	i.p. OVA / alum	OVA (i.n., 10–12 weeks)	Chronic model	Henderson et al. (2002), Leigh et al. (2002)
BALB/c	-	HDM (i.n., 5 weeks)	Chronic model	Johnson et al. (2004), Ulrich et al. (2008)
BALB/c	i.p. OVA / alum	OVA (i.t.)	Chronic model	Akkoc et al. (2001)
C.B17 SCID	i.p. PBMCs	Dpt (aerosol)	Acute model	Duez et al. (1996), Duez et al. (2000)
NOD/SCID	i.p. PBMCs	HDM (aerosol)	Acute model	Perros et al. (2009)
SCID	i.p. PBMCs, Dpt/alum	Dpt (aerosol)	Acute model	Sonar et al. (2010)
C.B17 SCID	i.p. PBMCs, i.p. Der p1	Der p1 (aerosol)	Acute model	Herz et al. (2004a)
BALB/c, C57BL/6	s.c. OVA	OVA (aerosol)	Acute model	Conrad et al. (2009)
BALB/c	i.n./i.p. OVA	OVA (i.n.)	Acute model	Nelde et al. (2001)
BALB/c	Pulsed DCs i.t.	OVA (aerosol)	Acute model	Hammad et al. (2004)
BALB/c	OVA (aerosol)	OVA (aerosol)	Acute model	Renz et al. (1992)
BALB/c	i.t. OVA	OVA (aerosol)	Acute model	Blyth et al. (1996)
BALB/c	i.n. OVA	OVA (aersol)	Acute model	Williams and Galli (2000)
BALB/c	i.p. OVA	OVA (aerosol)	Acute model	Hellings and Ceuppens (2004), Mojtabavi et al. (2002)

1.2.1 Genetic Background of Mice

Various inbred mouse strains are available in laboratories (Song and Hwang 2017). However, it is not possible to develop an asthma model with all of these mouse strains. Based on the level of allergen-specific IgE and IgG1 production and the degree of airway inflammation following repeated allergen challenges, high- and low-responder mouse strains have been identified. Each different mouse strain shows a different pattern of response following immunization to allergens. There are substantial differences in the ability to induce allergic inflammation and AHR within these mouse strains. A/J and AKR/J mice display high levels of allergen-induced AHR and reactivity to methacholine (Ewart et al. 2000), while C3H/HeJ and DBA/2 mice are resistant to the development of allergen-induced AHR (McIntire et al. 2001).

In many studies, either BALB/c or C57BL/6 mice were used. BALB/c mice are known as IgE-high responders to many allergens (e.g. OVA, Bet v 1) and goat anti-mouse IgD-stimulation, whereas C57BL/6 mice are characterized by low-IgE response (Herz et al. 2004b; Bousquet et al. 2000; McMillan and Lloyd 2004; Van Hove et al. 2009; Birrell et al. 2003). In contrast, C57BL/6 mice exhibit relatively more Th1-dominant immune responses compared to BALB/c mice and are utilized more often as colitis models (Van Hove et al. 2009) (Melgar et al. 2005).

1.2.2 Allergens

In mouse models of asthma, different types of allergens have been used. Among them, OVA derived from chicken egg is a frequently used allergen that induces robust, allergic pulmonary inflammation in mice (Kumar et al. 2008). There are some advantages to using OVA in models, such as the low cost of materials, high purity, the immunodominant epitopes have been well characterized and synthetic peptides have been generated (Fuchs and Braun 2008). In addition, OVA-specific transgenic mice allow for the detailed characterization of T cell-dependent immune processes that lead to allergic sensitization and inflammation (Akar-Ghibril et al. 2020).

The method of allergen administration is important for the induction of allergic asthma, while circumventing development of tolerance. For example, repeated inhalation or oral administration of OVA induces tolerance instead of sensitization (Burks et al. 2008). For the induction of allergen sensitization, the allergen is traditionally combined with an adjuvant and administered via intraperitoneal injection. However, sensitization to OVA can also be achieved without adjuvant if injected subcutaneously (Conrad et al. 2009). Following sensitization, a series of inhaled or intranasal challenges is administered to elicit an allergic inflammatory response. OVA-induced allergic airway models may not represent the same conditions experienced by asthmatic humans, who may have more frequent and much longer periods of allergen exposure (Kumar et al. 2008).

In addition, OVA is not a relevant allergen associated with human asthma. During the last five years, a great deal of work has been performed, which has led to the development of atopy models using allergens other than OVA. For this purpose, alternative allergens that have clinical relevance, such as house dust mite (HDM) allergens (i.e. Dermatophagoides pteronyssinus (Der p) and Dermatophagoides farinae (Der f), have been used (Cates et al. 2007; Johnson et al. 2004; Sarpong et al. 2003). Compared to the OVA model, the HDM allergy model has a number of advantages. The most important advantage is that many asthmatic subjects have increased levels of HDM-specific IgE, and these people display exacerbated allergic events with HDM allergen (Maunsell et al. 1968; McAllen et al. 1970). In addition, experimental data revealed that HDM can sensitize animals via the respiratory mucosa and does not require adjuvant co-administration (Jarnicki et al. 2001). Finally, long-term application of HDM leads to chronic lung inflammation and associated airway remodeling, which resembles some additional features of human asthma (Johnson et al. 2004; Ulrich et al. 2008).

Furthermore, there are other disease-relevant aeroallergens, such as fungal allergens (e.g. Aspergillus fumigatus), ragweed, and grass pollen spores, which are being used for murine atopy models (Fuchs and Braun 2008; Wiedermann et al. 1999b; Winkler et al. 2002; Hirahara et al. 1998).

1.2.3 Sensitization and Challenge Protocols

Specific pathogen free (SPF) mice do not spontaneously develop AHR or allergic airway inflammation. Many different sensitization and challenge protocols have been compared (McMillan et al. 2002; Hessel et al. 1995; Tournoy et al. 2000; Tomkinson et al. 2001). Acute or chronic asthma models have been developed which are dependent on dose and timing of sensitization and challenge. Acute sensitization protocols usually require multiple systemic administrations of allergen, and most protocols use a combination of allergen with an adjuvant. Aluminium hydroxide ($AlOH_3$, Alum) is one of the preferred adjuvants that promotes the development of the allergen-specific Th2 response (Flach et al. 2011; Jensen-Jarolim et al. 2021). Recent studies suggest that alum induces uric acid secretion, which is required for the alum-associated Th2 adjuvant effects. However, alum also activates the inflammasome-IL-1receptor pathway, and the role of this pathway in alum-induced asthma is controversial (Kool et al. 2011). As discussed above, adjuvant-free protocols are also available, which usually require

a higher number of exposures through different routes to achieve suitable sensitization (Blyth et al. 1996). Both OVA and HDM can be used as antigen to induce pulmonary inflammation. Inhaled HDM is more successful in developing an respiratory allergy model because of the intrinsic enzymatic activity associated with the contents of this allergen (Bush et al. 2011). Extracts or purified major allergens of potent human allergens, including cockroach, ragweed, or fungi, have been increasingly used as allergens in mice and other species (Barrett et al. 2003; Kurup et al. 1997; Chapoval et al. 2002; Matsuda et al. 2021). After a sensitization period (usually 14–21 days), allergen challenge via airways is carried out for several days. The administration of allergen through airways can be applied by nebulization, intratracheal (i.t), or intranasal (i.n.) instillation. With these sensitization and challenge protocols, mice develop certain features of clinical asthma, which can be defined as increased levels of allergen-specific and total IgE, eosinophil-dominant inflammatory cell infiltration to the peri-bronchial area, goblet cell hyperplasia, basement membrane thickening, and smooth muscle cell hypertrophy and AHR to specific allergens or methacholine (Takeda and Gelfand 2009).

Although an acute allergen challenge model displays many key aspects of human asthma, there are obviously limitations when one compares these models with asthmatic patients. To overcome these limitations, several research groups have developed chronic allergen challenge models in order to reproduce more of the features associated with asthma, such as goblet cell metaplasia, epithelial hypertrophy, subepithelial fibrosis, and smooth muscle hypertrophy, which are altogether referred to as airway remodeling (Akkoc et al. 2001; Blyth et al. 1996). In order to develop a chronic asthma model, low levels of allergen should be repeatedly administered to the airways for periods up to 12 weeks. In most of the experimental models, OVA has been used as an allergen (Fernandez-Rodriguez et al. 2008; Temelkovski et al. 1998; Wegmann 2008; Akkoc and Genc 2020), and in some experimental models, environmentally relevant antigens such as HDM extract or grass pollen have been used to develop a chronic asthma model (Fuchs and Braun 2008; Maunsell et al. 1968).

1.3 Similarities and Differences Between Murine Models and Human Asthma

Mice do not exhibit asthma-like lung inflammation, which is point-by-point comparable to human asthma. There are important limitations that should be considered. Mice develop an allergen-specific Th2-type immune response using a highly artificial peritoneal allergen sensitization procedure in the presence of an adjuvant, such as alum (Debeuf et al. 2016). They do not develop spontaneous immediate and late phase airway obstruction as is seen in human asthma (Kumar and Foster 2002).

There are significant differences in the developmental features of human and mouse lungs at birth. While the human lung continues to develop from months to years after birth, mouse lungs are already mature at birth (Lodrup Carlsen and Carlsen 2001). In humans, the immature lung is exposed to environmental antigens or agents, and the immune response within the immature human lung is different compared to that of a mouse lung (Greenough et al. 2004). From the histopathological view, there are also differences in the lungs of human and mice, and this further affects the histopathological changes of chronic asthma models (Persson et al. 1997; Kumar and Foster 2002). Although submucosal glands are abundant throughout the medium and large airways of the human lung, they are only present in the trachea of mice. In the large airways of the mouse, the bronchial epithelium is not as fully stratified as in humans. This results in differences in the immune response to inhaled antigens (Lilly et al. 2005). Human asthma is restricted to conducting airways, while in mice, parenchymal and vascular components of the airways are more affected (Kumar and Foster 2002)

The role of airway smooth muscle (ASM) and myofibroblasts in the bronchi in bronchial

hyperreactivity is still under exploration (Hallstrand et al. 2011; Collins et al. 2003). In asthma, increased ASM mass is the result of hyperplasia, hypertrophy, and migration of ASM and forms one of the most important components of airway remodeling (Hirst et al. 2004; Broide 2008). ASM also contributes to the inflammatory process by producing active mediators, modifying the extracellular matrix composition, and interacting with inflammatory cells (Camoretti-Mercado 2009).

Reversible and variable airflow limitations are also key features of asthma. The most characteristic feature of asthma is the variability or periodicity of lung dysfunction, which distinguishes asthma from chronic obstructive pulmonary disease (Irvin 1987). Airway obstruction is the key feature of the disease, which is an important diagnostic criterion in the clinic. Spirometry is the easiest and most clinically useful method for measuring airflow limitation in most clinical situations (Burrows 1980). Preclinical studies for measuring respiratory function in vivo include noninvasive and invasive technologies. Both techniques have their advantages and disadvantages, and it depends on the aim of the study as to which method should be chosen (Table 2). There are two different measurements; tidal midexpiratory flow (EF_{50}) provides data on the midpoint (50%) of expiratory tidal volume in mL/s, while enhanced pause (Penh) is widely used in order to demonstrate airway obstruction in mouse models. However, strictly speaking, Penh is a better measure of distress rather than airway obstruction (Petak et al. 2001; Bates et al. 2004; Adler et al. 2004).

Critical histopathological changes lead to increased bronchial hyperreactivity. In humans, chronic airway changes, which occur over a long period of time, lead to the severe asthma phenotype, which is observed in the clinic. In contrast, due to the shorter-term experimental period and the nature of the animal model, mice do not exhibit many of the lesions that typify chronic human asthma. In particular, there is minimal chronic inflammatory changes of the airway wall and airway remodeling, such as epithelial fibrosis, epithelial proliferation, and large increase in airway smooth muscle cells in mouse (Holgate et al. 2000). However, murine models are very useful in examining the early molecular events, which may contribute to airway remodeling, such as increased Claudin-1 expression by ASM (Fujita et al. 2011).

1.4 Cytokines: From Mouse Models to Human Asthma

The entrance of an allergen into lymphatic organs initiates a series of immunologic cascades from presentation to $CD4^+$T cells by dendritic cells and

Table 2 Invasive and noninvasive techniques to measure lung function in mice

	Pros and Cons	Methods	References
Noninvasive techniques	Conscious mice Repeatable screening of respiratory function Large numbers of animals	Head out plethysmography (Tidal midexpiratory flow-EF_{50})	Vijayaraghavan et al. (1993), Glaab et al. (2002)
	Natural breathing pattern Simple handling Stress for animals Only volume and flow measurable; inhalation exposure includes nasal and gastrointestinal uptake	Barometric plethysmography (enhanced pause-Penh) (whole body plethysmography)	Hamelmann et al. (1997), Finkelman (2008)
Invasive techniques	Unstrained mice Anesthetized animal Repetitive/nonrepetitive No stres for animals	Orotracheally intubated animals (repetitive) (EF_{50})	Glaab et al. (2004), Ruckert et al. (2005)
	Gold standard parameters Inhalation exposure focus to the lungs Not very easy handling	Tracheostomized animals (nonrepetitive) (EF_{50})	Palecek et al. (1967)

induction of sensitized Th2 cells, which further releases IL-4, IL-5, IL-9, and IL-13 (Hamid and Tulic 2009). These cytokines play a role in airway eosinophilia, pulmonary lymphocytosis, and mastocytosis; goblet cell hyperplasia with epithelial cell proliferation; smooth muscle hyperplasia and increased thickness; subepithelial fibrosis; isotype switching of IgE and release from B cells; increased production of chemokines, which attracts T cells, eosinophils, neutrophils, and mast cells; and narrowing of the airways because of smooth muscle contraction (Hamid and Tulic 2009; Holgate 2008; Akdis et al. 2011).

Although allergy is correlated with Th2-type responses, some studies revealed that there is also a Th1 immune response in the lung of children with asthma (Brown et al. 2003). It is not unusual to see increased IFN-γ and IL-12 levels in human asthma (Kenyon et al. 2000). Similarly, diseases which have been characterized as Th1-dominant disorders can also display features normally associated with Th2-type immune responses. For instance, although Sarcoidosis is a Th1 disease, increased levels of IL-13 are also seen, which suggests a Th2 component to the disease (Hauber et al. 2003; Akdis et al. 2011; Meyer et al. 2010). One possibility is that Th2 responses are required at the initiation phase of asthma, while the inflammatory response, which occurs during the exacerbation phase, is not limited to Th2 responses but also requires Th1 and perhaps Th17 responses within the lung.

IL-4 and IL-13 are prototypical Th2-type cytokines, and several studies have revealed that they play a crucial role in the induction of murine asthma. Induction of IL-4 and IL-13 via both the intranasal and intratracheal routes enhances allergic airway disease (Venkayya et al. 2002). Enhanced transgenic expression of IL-4 in the airway epithelial Clara cells elicits an inflammatory response characterized by epithelial cell hypertrophy, with the accumulation of macrophages, lymphocytes, eosinophils, and neutrophils without any airway hyperreactivity or goblet cell hyperplasia (Rankin et al. 1996; Zhu et al. 1999). Selective pulmonary expression of IL-13 promotes a eosinophilic inflammatory response, mucus cell metaplasia, airway obstruction, and nonspecific airway hyperreactivity (Zhu et al. 1999). In mice, IL-13 binds to its receptor, IL-13Rα2, either in soluble form in serum or membrane-bound receptor, which is expressed by smooth muscle, while human IL-13 binds only to the membrane isoform (Chen et al. 2009). Thus, murine models have been very useful in the examination of the roles played by specific cytokines in the initiation of allergic-type pulmonary inflammation due to the availability of knock-out and transgenic models. However, murine studies should be complemented by findings in humans, which confirm the relevance of the cytokine data.

1.5 Humanized Animal Models of Asthma

A humanized mouse refers to a mouse which contains functional human genes, cells, tissues, and/or organs. For this purpose, immunodeficient mice are used to receive human cells, because they can easily accept heterologous cells due to deficiencies in the host immune system. Severe combined immunodeficient (SCID) mice were demonstrated to be a useful model to analyze human pathogenic mechanisms (Alessandrini et al. 2020). Due to an immune defect, these mice are unable to reject allogenic or xenogeneic transplants (Mosier et al. 1988; McCune 1991) and show an absence of mature and functional T and B lymphocytes (Bosma et al. 1983). It has been shown that SCID mice reconstituted with human peripheral blood mononuclear cells (PBMCs) from asthmatic patients developed a specific human IgE response, pulmonary inflammatory-type infiltrate, human Th2 cytokine production, lung inflammation, and AHR when exposed to specific allergens by inhalation (Duez et al. 1996; Pestel et al. 1994; Chiang et al. 1995; Gagnon et al. 1995; Steinsvik et al. 1999). Thus, it is possible to reconstitute components of the human immune system in mice by transferring PBMCs resulting in a human–mouse chimera with a functional human antigen-reactive immune system (Herz et al. 1998; Duez et al. 2000). The newer generation of humanized mouse models

use SCID mice crossed to IL-2Receptor gamma Knockout mice, so-called NOD or NSG mice (NOD.Cg-PrkdcSCIDIL2rgtm1WjI/SzJ), which are the more efficient immunocompromised strain (Shultz et al. 2007). These mice lack mature T, B, and NK cells. In addition, they are deficient in multiple cytokine signaling pathways as well as displaying many defects in the innate immune system (Shultz et al. 1995, 2005). Since 2000, the newest generation of immunodeficient mice is available, called NOG mice (NOD/Shi-scid/IL-2Rγnull). These mice accept heterologous cells more easily than NOD/scid mice. Thus, they represent a good humanized model as they are the most efficient recipients of human cells, resulting in successful engraftment, proliferation, and differentiation (Ito et al. 2002). The use of these animals in asthma research and preclinical drug development is an exciting development and should improve the identification of novel therapeutic compounds which will be useful in the clinic.

2 Conclusion

Recent studies clearly show that asthma is a complex syndrome, likely made up of a number of disease endotypes, each with distinct pathophysiology and possibly different underlying immunological mechanisms. Due to the diverse nature and complexity of asthma, it is difficult to represent all the features of asthma endotypes in murine models. Within those endotypes, the current murine models may only represent early onset Th2 and eosinophilic asthma.

Additional limitations of murine models, due to the anatomical and immunological differences between humans and mice, suggest that investigators should be careful in directly extrapolating data from murine studies to human disease. However, murine and human studies both support the importance of the initial Th2 response in the promotion of airway disease and the subsequent mixed Th1/Th2 response in sustaining inflammatory responses within the lung. Human clinical studies remain the gold standard for direct evidence for the clinical efficacy of new therapeutic approaches. Murine models will continue to provide important mechanistic clues, while improved mouse models may be useful for extending our understanding of the basic mechanisms underpinning asthma and for examining new therapeutic options.

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From Cells to Organs: The Present and Future of Regenerative Medicine

Yichen Wang and Yoon-Young Jang

Abstract

Regenerative medicine promises a bright future where damaged body parts can be restored, rejuvenated, and replaced. The application of regenerative medicine is interdisciplinary and covers nearly all fields of medical sciences and molecular engineering. This review provides a road map on how regenerative medicine is applied on the levels of cell, tissue, and organ and summarizes the advantages and limitation of human pluripotent stem cells in disease modeling and regenerative application.

Keywords

Cartilage damage · Hepatobiliary disease modeling · Kidney dysfunction · Organoids · Pluripotent stem cells · Regenerative medicine · Sickle cell disease

Abbreviations

ACI	autologous chondrocyte implantation
CLC	cholangiocyte-like cell
ESC	embryonic stem cell
GVHD	graft-vs-host disease
HbF	fetal hemoglobin
HbS	hemoglobin S
hESC	human embryonic stem cell
hiPSC	human-induced pluripotent stem cell
HLC	hepatocyte-like cell
hPSC	human pluripotent stem cell
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplant
HU	hydroxyurea
iPSC	induced pluripotent stem cell
OCT	osteochondral transplantation
PACI	articulated articular cartilage implantation
SCD	sickle cell disease

1 Introduction

Regenerative medicine aims to repair, rejuvenate, and replace damaged body parts that lead to disease. The idea of replacing defective and lost body parts is made possible largely by the development of stem cell technologies and tissue engineering. The field of regenerative medicine is interdisciplinary by its nature. Its applications combine the knowledge of life science, material

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science, applied mathematics, and various forms of engineering. From cell therapy to organ regeneration, regenerative medicine is bringing new hopes to various untreatable diseases of the past. Aided by innovations in gene-editing techniques, a variety of regenerative interventions are being developed to treat difficult diseases of various cellular and organ bases. Additionally, stem cells have been successful in modeling a variety of diseases. These disease models provide excellent testing ground for drug design and developmental research. Human stem cell-derived tissues are also being transplanted to animal models to create chimeras for more integrated studies. We are witnessing the advent of the new era, the era of regenerative medicine. In this review, we highlight the application of regenerative medicine in treating three different diseases of cellular, tissue, and organ basis: sickle cell disease, articular cartilage damage, and kidney dysfunction. Additionally, we highlight the groundbreaking advancements in human pluripotent stem cell disease modeling and discuss its current challenge and limitation.

2 Cells: Regenerative Medicine for Sickle Cell Disease

The etiology of sickle cell disease (SCD) is well understood for more than decades. Though it is the most common inherited hemoglobinopathy disease, an effective cure is still lacking. SCD is a monogenic disorder caused by a substitution mutation in the beta-globin gene (HBB) of chromosome 11. The mutation (A > T) at the sixth codon of HBB gives rise to the sickling hemoglobin, HbS. Structurally, the hydrophilic glutamic acid is substituted with valine that forms a hydrophobic association with alanine, phenylalanine, and leucine of the adjacent hemoglobin. Comparing to normal hemoglobin, HbS polymerizes rapidly and significantly reduced the flow rate and life span of red blood cells (Vekilov 2007).

The most common form of sickle cell disease, HbSS, constitutes the homozygous mutation of rs334. HbSS patients express no normal hemoglobin and show severe signs of anemia along with other complications. It is estimated that more than 300,000 children are born with sickle cell anemia every year globally (Piel et al. 2013b). The direct pathophysiological consequence of HbS polymerization is vaso-occlusion and hemolysis. Clinically, the disease manifests itself in various forms of acute and chronic injuries. Some of the most common symptoms include swelling and painful episodes. These symptoms are often associated with other complications such as acute chest syndrome, osteonecrosis, priapism, kidney injury, and stroke (Williams and Thein 2018).

Despite the genetic simplicity of sickle cell anemia, treating this disease had been challenging. Currently, there two FDA-approved treatments for SCD: hydroxyurea (HU) and L-glutamine. Doctors are often reluctant to prescribe HU due to its associated misinformation and poor adherence (Demirci et al. 2019). On the other hand, L-glutamine treatments are extremely expensive and usually not covered by insurance. Though these treatments can improve the patient's quality of life, neither of them completely cures the disease. Nonetheless, with the advancement in newborn screening and vaccination technology, more than 90% of SCD patients are expected to make it to their adulthood. It is expected that by 2050, there will be over 400,000 severe SCD patients (Piel et al. 2013a).

2.1 Hematopoietic Stem Cell Transplant

In 1984, a breakthrough was made in treating sickle cell disease using the concept of regenerative medicine. It was the first time that hematopoietic stem cell transplant (HSCT) was performed on an HbSS patient. The patient was an 8-year-old girl who suffered acute myeloid leukemia at the same time (Johnson et al. 1984). The source of transplant came from her HLA-matching sister. At the time, the procedure was initially aimed to treat leukemia. However, it also improved her condition of sickle cell anemia. It was not until then stem cell transplant has

become a therapeutic option for SCD (Salinas Cisneros and Thein 2020). Currently, as of early 2021, 35 clinical trials are listed on clinicaltrials.gov investigating allogeneic stem cell transplant as a treatment for sickle cell anemia. Among these 35 trials, 12 are already completed. Meanwhile, four additional trials aim to use autologous stem cell transplant with gene correcting interventions to treat sickle cell anemia. Though each of these clinical trials features a different perspective and strategy, all of them follow the same rule of regenerative medicine: repair and replace the disease-origin cells. To date, HSCT is the only therapy that cures sickle cell disease.

Hematopoietic stem cell transplantation provides long-term benefits for SCD patients. However, the applicability is yet hindered by several obstacles. HSCT is limited by the availability of HLA-matching donors. Only 15% of patients have such matches; on top of that, only 10% undergo HSCT due to the associated risks (Chakrabarti and Bareford 2007; Walters et al. 2001). It is possible to receive transplants for an unrelated HLA-matching donor. However, it dramatically increases the risk of complications such as rejection and graft-vs-host disease (GVHD).

2.2 Gene Therapy

Gene therapy that uses autologous hematopoietic stem cells provides the unprecedented potential to permanently cure SCD without the burden of donor availability and GVHD. There are three main approaches to using gene therapy for SCD. These three approaches are parallel to each other and can be summarized by these keywords: addition, induction, and correction (Fig. 1). The addition approach adds an antisickling copy of the beta-globin gene to the patient's hematopoietic stem cell (HSC) (Dever et al. 2016). It is usually done by in vitro viral transduction. The modified HSCs are then reinfused back to the patient. Another approach is to induce the expression of fetal hemoglobin (HbF) in patients (Paikari and Sheehan 2018). It has been observed that SCD does not affect newborn infants due to their high blood HbF levels (Watson 1948). The protective property of HbF was also confirmed in asymptomatic SCD patients with hereditary HbF persistence (Forget 1998). Thus, SCD can be rescued by procedurally elevating HbF levels. It can be done in two ways: enhancing the expression of HbF by stimulating HbF upregulators and knocking out HbF suppressor. A clinical trial was launched in 2018 using the later approach. Lentiviral vectors carrying shRNA-inhibiting BCL11A, a HbF silencer, were infused into severe SCD patients with the hope of boosting their HbF level (Demirci et al. 2019). The third approach is to correct the pathologic mutation that gives rise to SCD. It is the most straightforward yet most challenging approach. However, high hopes are given to the advancing CRISPR/Cas technology that can induce a double-strand break at the sickling mutation and initiate repair according to the provided template. Ideally, the patient's cells would end up with homologous copies of the normal beta-globin gene. Researchers are working hard to improve the specificity and efficiency of this technique.

2.3 The Challenge of Gene Therapy for SCD

Four significant challenges need to be addressed to improve the quality of gene therapy to treat SCDs. These challenges are efficiency, immunogenicity, specificity, and delivery vehicle. Traditional genome editing techniques are generally labor-intensive, time-consuming, and costly. More recent techniques such as CRISPR/Cas9 are relatively easier and a lot cheaper. However, it has been difficult to achieve a satisfactory delivery rate to a high number of mature cells with CRISPR/Cas9. The method is not always efficient in cutting and editing in some cases. Additionally, CRISPR/Cas9 editing may often lead to off-target effects and can result in severe consequences.

Future studies will be focused on improving the specificity, reducing the off-target effect, and the delivery method of the CRISPR/Cas9. Improvements in vector technology are also in need to widen the application of gene therapy in treating sickle cell diseases. Solving the above

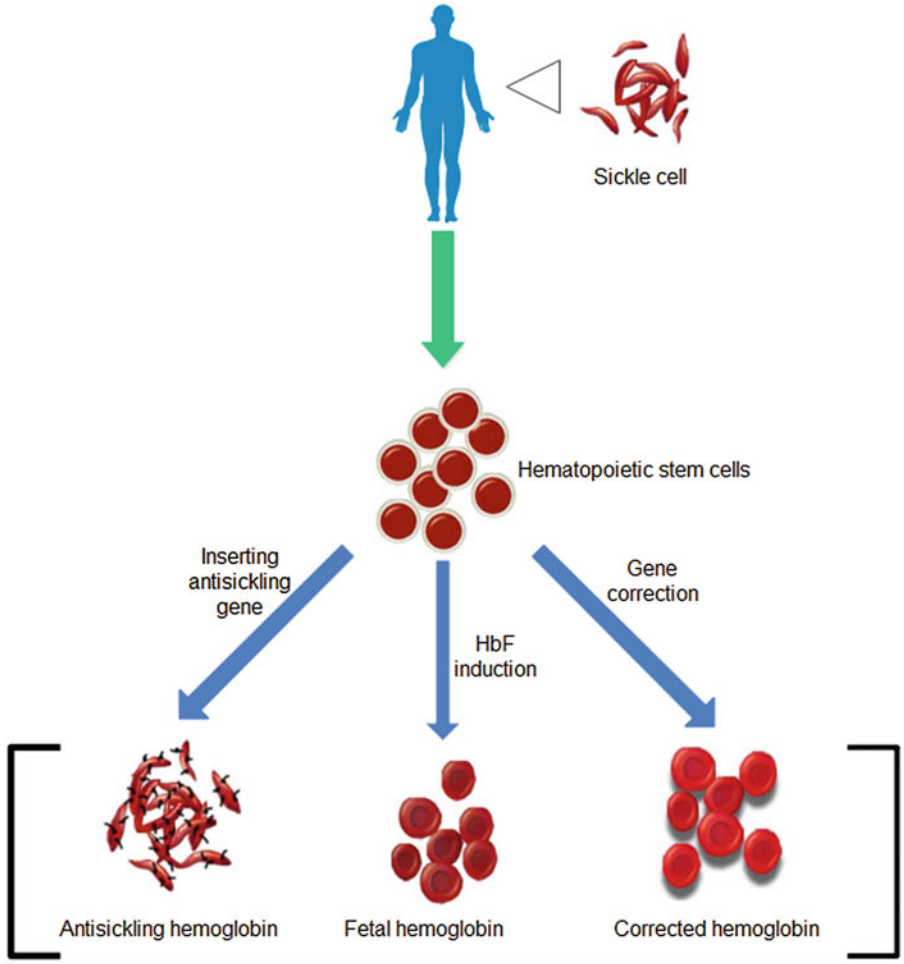

Fig. 1 The regenerative treatments of sickle cell disease The hematopoietic stem cells derived from sickle cell patients can be differentiated into improved hemoglobin types and retransfused into the patient to replace the sickle cell

challenges will also imply the future of germline gene therapy, potentially curing the patient's descendants. There will be consequential social, legal, and ethical issues based on gene editing.

3 Tissue: Repairing Articular Cartilage Injuries

The concept of regenerative medicine has achieved great success in treating various diseases on a cellular basis. In recent years, much attention in regenerative medicine has been drawn to develop advanced tissue engineering technologies. These developments have brought transformational changes to the treatments of articular cartilage damage.

Articular cartilage injury can occur due to normal wear and tear or trauma. It is particularly common in athletes and senior citizens. Patients who undergo arthroscopy are also likely to develop cartilage injuries (Kalson et al. 2010). The healing capability of cartilage injury is limited due to the lack of nerves and blood vessels in the cartilage. Left untreated, articular cartilage injury could lead to osteoarthritis and cause

various symptoms, including swelling, pain, and compromised joint movements. Nearly half of the older citizens in the United States suffer from osteoarthritis in various degrees (Jiang et al. 2020). Economically, it has become a huge burden in medical expenses. The current treatments for articular cartilage injury are obstructed by the difficulty in binding the regenerated tissue to its surrounding environments (Muhammad et al. 2019). However, the new tissue-engineered cartilage brings unprecedented advantages to articular cartilage repair.

3.1 The Current Treatments and Limitations

The current treatment strategy for articular cartilage injury is mostly based on surgical regeneration techniques (Fig. 2). Microfracture is a common bone marrow stimulation approach for cartilage repair. First developed in the early 80s, microfracture differentiates mesenchymal stem cells from fibrous cartilage (Steadman et al. 2010). However, such technique is rather unsatisfactory in repairing larger cartilage damages that are bigger than 2.5 cm^2 (Jones and Peterson 2006). Additionally, elderly patients tend to heal slowly to these procedures. Another method often used to treat cartilage injury is osteochondral transplantation (OCT) (Yamashita et al. 1985). OCT can use either autologous or allogeneic sources of osteochondral columns to fill in the defect sites of the cartilage. In the autologous case, the osteochondral columns are usually removed from the non-weight-bearing sites and transplanted into the injured cartilage. This technique is often limited by the amount of transplantable osteochondral

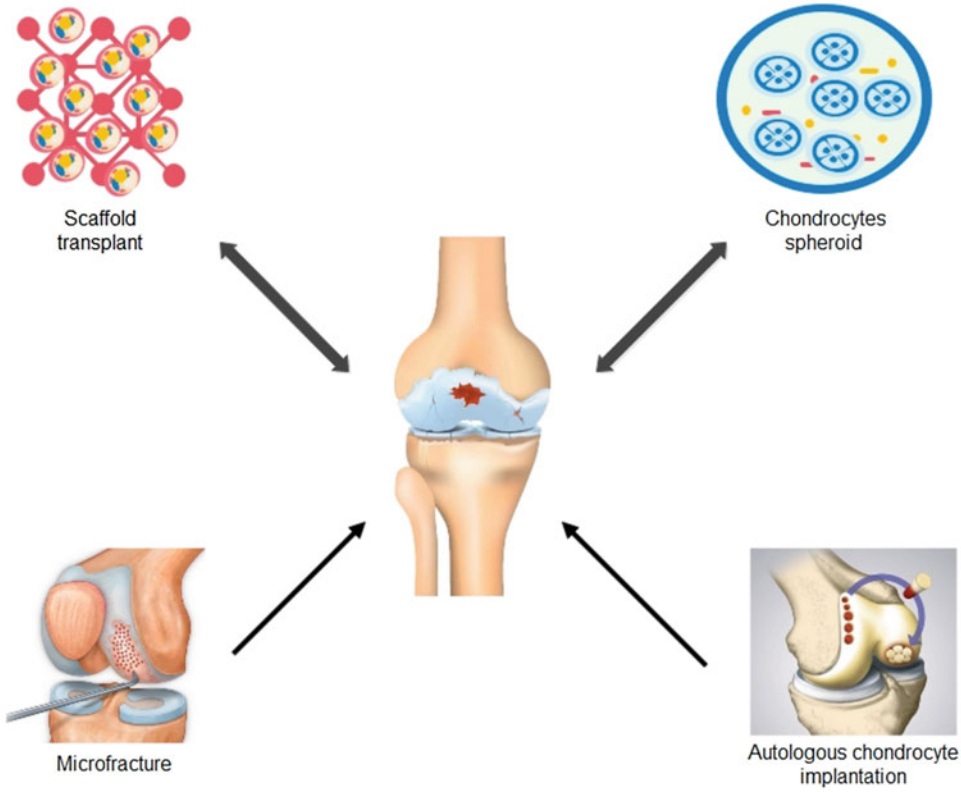

Fig. 2 The regenerative approaches to repair cartilage injury
Various regenerative approaches have been developed to elaborately repair the articular cartilage injury

columns (Andrade et al. 2016). Thus, it is only suitable for small defects. On the other hand, allogeneic osteochondral tissue transplantation is more available but risks disease transmission and is very expensive. Autologous chondrocyte implantation (ACI) is an alternative technique that harvests chondrocytes from the non-weight-bearing sites of the articular surface and transplants them into the damaged sites after in vitro expansion (Brittberg et al. 1994). However, this technique is still hindered by several limitations, such as the invasive surgical procedure and the limited number of available cells. Additionally, the in vitro expansion of the harvested chondrocytes is prone to dedifferentiation. Articulated articular cartilage implantation (PACI) is repairing the cartilage defect with the crushed allogeneic or autologous cartilage particles (Lu et al. 2006). Comparing to OCT, this technique requires less donor cartilage. However, like the previously described techniques, PACI is only suitable for small cartilage defects no larger than 3.5 cm^2 (Jiang et al. 2020).

3.2 Tissue Engineering for Cartilage Injuries

Cartilage tissue engineering combines advancements in material science, biomechanics, biochemistry, and cell biology. It has shown great promise in regenerating hyaline cartilage and repair the entire cartilage defect. Engineered tissue based on mesenchymal stem cells displays excellent proliferation potential, differentiation ability, and low immunogenicity (Harrell et al. 2019). Three main strategies are currently in use to generate tissue-engineered cartilage (Fig. 2). The first strategy is by establishing a cell-scaffold construct. The scaffold is often made of biocompatible materials such as collagen matrix and hydrogel. Chondrocytes and various types of stem cells are planted into these scaffolds before transplanting into the patients. The scaffolds offer support for the seed cells to differentiate and expand. Once completely integrated with the patient, these scaffolds slowly degrade. Another approach to generating tissue-engineered cartilage is the cell-free strategy. It is important to note that the cell-free strategy is, in fact, not free of cells. It indirectly uses stem cells, such as mesenchymal stem cells, to regenerate cartilage without directly transplanting these mesenchymal stem cells into the patients. Two subcategories fall under this technique. The first induces cartilage regeneration by stimulating bone marrow stem cells in situ combined with a transplanted biocompatible scaffold. The second approach is to integrate mesenchymal stem cell derivatives, including cytokines, various RNAs, etc., to the transplanted scaffold to stimulate the regeneration of cartilage. These "cell-free" strategies are still in the animal testing stage (Jiang et al. 2020). The last strategy commonly used to generate tissue-engineered cartilage is the scaffold-free strategy. This strategy uses chondrocytes spheroids for cartilage transplantation. It is similar to ACI. However, the difference is that ACI uses cell suspensions. The advantage of the scaffold-free approach is that it avoids the problem of developing complex scaffolds that are often unavailable. It has been challenging to develop ideal scaffolds that meet the various criteria of transplantation. These criteria include but not limited to promoting cell growth, biodegradable at an appropriate rate, and adhesive. Chondrosphere® is an approved scaffold-free product that uses chondrocytes spheroid to repair cartilage damage. The phase III clinical trial has demonstrated that Chondrosphere® is at least as effective as microfracture in patients with small cartilage effects (Armoiry et al. 2019). In patients with cartilage defects bigger than cm^2, Chondrosphere® is proven to be a more effective treatment.

To make tissue-engineered cartilages more available and practical, we must overcome several obstacles. These obstacles include producing an ideal scaffold that promotes adhesion and growth of seed cells, optimizing the differentiation and expansion of cartilage-related cells, and minimizing the tumorigenicity and heterogenicity of stem cells used. Also, the possibility of disease transmission and immune rejection of transplanted allogeneic cell sources must be addressed and prevented. Fundamentally, we need to grab a more detailed understanding of

how cartilage developments are regulated in vivo. Many interactions of immune responses between secreted factors, synovial fluids, and exosomes are yet to be determined. These interactions are the key factors to consider when optimizing the physical property of tissue scaffold and maintaining a local environment ideal for recovery.

4 Organ: Kidney Regeneration

Whole organ regeneration remains challenging at the current stage of regenerative medicine. However, significant improvements toward kidney generation have been made in recent years. The need for modern organ regeneration and transplantation is on the global rise. Kidney is the most frequently transplanted organ in the United States (Alachkar et al. 2011). Around 69,000 kidneys are transplanted every year globally according to WTO (Organization 2008). In 2014, United States alone had nearly 16,000 kidney transplants (Wragg et al. 2019). These large numbers do not nearly meet the needs of organ shortage. The average waiting time for an available kidney in the United States is almost two and a half years (Wragg et al. 2019). This wait time is much longer in other places of the world (Lee et al. 2019). More than 16% of patients die or become too sick to receive the kidney transplant while they are waiting (Cassuto et al. 2010). The extreme scarcity of kidneys has led to problems beyond the field of medicine, such as trafficking, compensated donation, and the expansion of black markets. Researchers around the world are seeking ways to grow transplantable kidneys and devices that can mimic the function of kidneys.

4.1 Organoids

Kidney organoids derived from human pluripotent stem cells (hPSCs) are given hopes to bring the future of renal replacement therapy in regenerative medicine. Numerous protocols are established to differentiate hPSCs into kidney organoids (Freedman et al. 2015; Morizane et al. 2015; Taguchi et al. 2014; Taguchi and Nishinakamura 2017; Takasato et al. 2015). These protocols use a combination of small molecules and growth factors to direct a stage-specific differentiation similar to the development of embryonic kidneys. Renal structures including nephron, glomeruli, interstitium, and collecting ducts are self-organized in these organoids. However, the functional capability of these cultures is often no match to their natural counterparts. Advanced understanding of how kidney develops in vivo are in need to optimize these differentiation protocols. Additionally, three major obstacles block the reality of renal organoid replacement therapy. These obstacles are off-target cells, vascularization, and reproducibility (Geuens et al. 2020). Kidney organoids derived from established protocol may contain up to 20% of the nonrenal cell population (Combes et al. 2019; Wu et al. 2018). These off-target cells increase in prevalence as the organoid gets larger and eventually disrupts the integrity (Geuens et al. 2020). It remains unclear how these off-target cells come to place. The cells in this off-target population resemble many cell types, including neuronal, muscle cells, cartilage, and anywhere in between (Bantounas et al. 2018; Morizane et al. 2015). The lack of vascular structure is another major difficulty in renal organoids. To date, there is no kidney organoid that shows patent vasculature. These organoids simply do not have enough endothelial cells nor the proper guiding cues from the vasculature. As organoids grow larger, cell death in the center mass becomes inevitable due to the lack of nutrients and waste exchange. The third major problem is scaling. Current kidney organoids are roughly 1/10,000 of a single human kidney by nephron counts (Geuens et al. 2020). Automated systems for generating these organoids are being constructed and improved to scale up the liver organoids production (Czerniecki et al. 2018). However, without vasculature, these organoids cannot be assembled into a functional liver.

4.2 Wearable Artificial Kidney

As the regeneration of a whole kidney remains a tremendous challenge, dialysis is the mainstream

of current kidney replacement therapy. Though dialysis provides the means to survive kidney failure, it requires significant changes in the patient's daily routine. Beyond its physical inconvenience, long sessions of dialysis are becoming a burden that affects the mental health of patients (Pereira et al. 2017). In recent years, efforts are made into the development of wearable artificial kidneys. These wearable kidney devices are currently under clinical trials (Davenport et al. 2007; Gura et al. 2005; Lee and Roberts 2008). The portability of these devices allows the patient to continue to work and travel. Typically, these devices are a few kilograms in weight and operated by battery (Salani et al. 2018). Inside the wearable artificial kidney, blood is anticoagulated and pumped through a polysulfide hollow-fiber dialyzer (Gura et al. 2009). The dialysates are pushed in a rhythm that compensates for the peak and trough of the blood flow as the dialyzer transmembrane oscillates. A blood flow of 100 mL/min is achieved with this system. To avoid the risk of accidental disconnection, wearable artificial kidneys are advised to use catheters instead of needles in a fistula. However, the consequences of continuous catheter use remain unclear (Salani et al. 2018). Some technological improvements are still needed before the general application of wearable artificial kidneys.

5 Disease Modeling with Human Pluripotent Stem Cells

Advancements in regenerative medicine bring new hope to treating diseases of various mechanisms. However, further innovations in regenerative medicine still largely rely on the development of accurate disease models. The human pluripotent stem cell (hPSC) is an excellent tool in disease modeling. The advantages of pluripotent stem cells include availability, flexibility, and genomic integrity. Once the pluripotent stem cell population is established, these cells can expand indefinitely, providing large numbers of cells for differentiation and testing. Additionally, induced pluripotent stem cells (iPSCs) can be generated from various adult cells and tissues. The abundance of iPSC and its easy maintenance offers an unparalleled advantage for developing disease models. At the same time, hPSCs are extremely flexible as they can theoretically differentiate into any cell type of the human body. The differentiation protocol of hPSCs to many cell types are well established and publicly available. The most common cell types differentiated from iPSCs include dopaminergic neurons, motor neurons, astrocytes, oligodendrocytes, cardiomyocytes, hepatocytes, pancreatic β cell, and lung epithelial cells (Fig. 3) (Abo et al. 2020; Bianchi et al. 2018; Corbett and Duncan 2019; Ehrlich et al. 2017; Hallett et al. 2015; Karakikes et al. 2015; Ma et al. 2018; Soubannier et al. 2020). Patient-derived iPSCs are often developed into the diseased cell types to identify the linkages between the patient genotype and disease phenotype as the iPSC reprogramming preserves the genomic integrity of the patient. In the past decade, iPSCs have been successful in modeling countless diseases of genetic defects. However, it remains a challenge to use iPSCs to accurately model more complex diseases, such as cancer. Nevertheless, human iPSC offers an unprecedented alternative to disease modeling. Most importantly, these cell and organoid-based disease models are compatible with high-throughput screening, providing a faster and more efficient solution to drug discovery and pathology research.

5.1 iPSC-Based Modeling of Hepatobiliary Diseases

Hepatobiliary organs are targets of detrimental diseases due to their complexity and vital functions. Despite the recent developments, it remains challenging to treat many complex hepatobiliary diseases. Some of these diseases include biliary atresia, primary sclerosing cholangitis, biliary fibrosis, liver cirrhosis, and hepatobiliary cancers. There is a desperate need for further understanding of the molecular and pathodevelopmental mechanisms of liver diseases. For the past decades, many advancements in the understanding of such

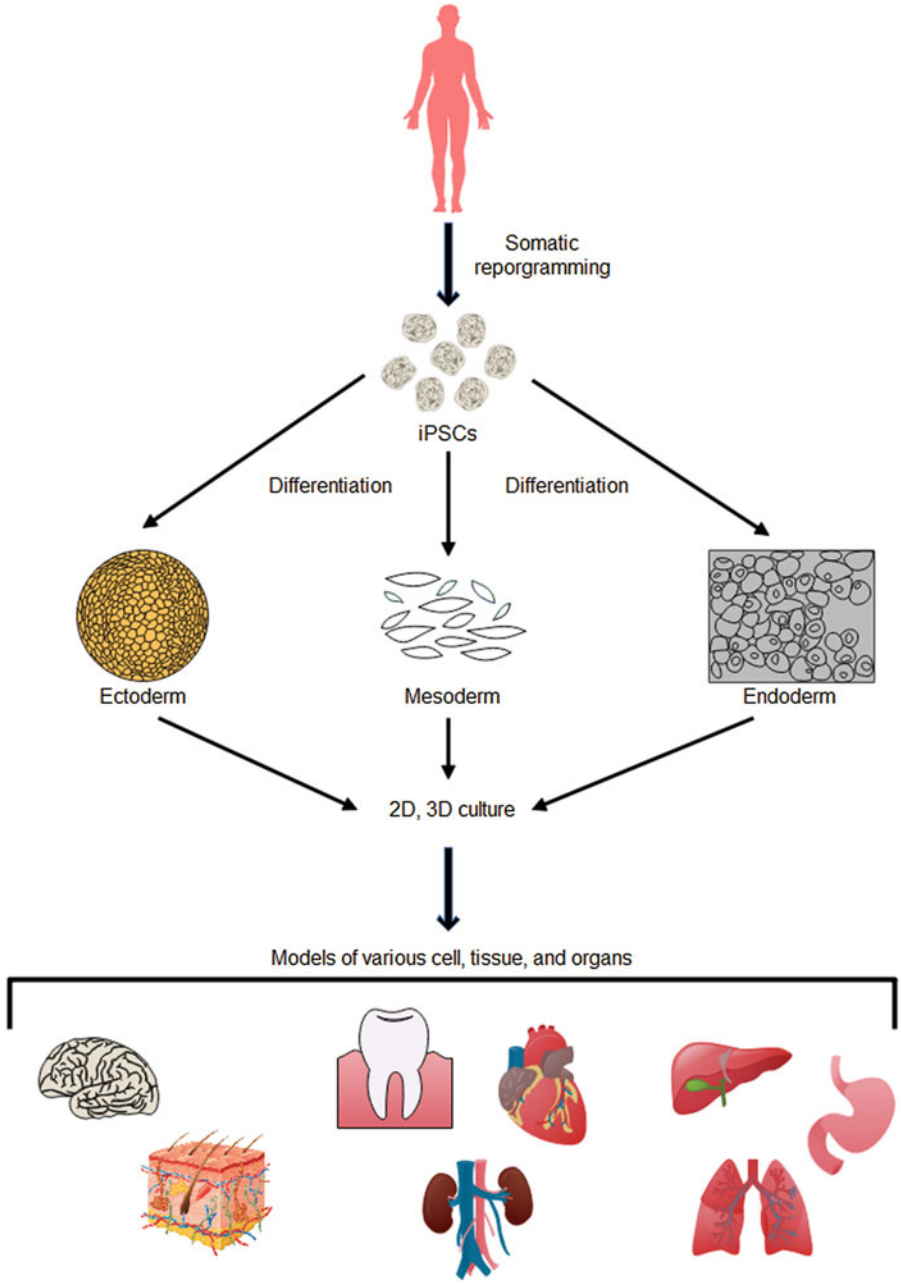

Fig. 3 Human iPSC disease models of various organ and tissue types
Patient-specific cells can be derived into iPSCs that are capable of differentiating into all three germ layers. These iPSCs preserve the genome of the patient and can be further developed into 2D and 3D disease models of various origins

diseases are supported by primary cells, tissues, as well as various animal models. However, major drawbacks of primary culture and animal models include high cost and low availability. Alternatively, immortalized disease lines are often too altered to accurately reflect their supposed physiology. Though useful in many ways, animal models of hepatology are neither close to recapitulating hepatobiliary responses in humans.

In recent years, much focus has been dedicated to creating hepatobiliary "disease in a dish." In a nutshell, this concept is enabled by the in vitro differentiation of patient-derived stem cells. Normal stem cells can also be genetically altered to express enhanced disease phenotypes, depending on the configurable culture conditions. The major advantage of this approach is that these in vitro disease models can be abundantly available and high-throughput compatible. It holds the potential to dramatically reduce the time span of fundamental research and drug discovery processes.

The differentiation protocols of human iPSC to hepatobiliary cell types, including hepatocyte-like cells (HLC) and cholangiocyte-like cells (CLC), have been reported from various sources (Corbett and Duncan 2019; Liu et al. 2011; Sampaziotis et al. 2017; Tian et al. 2016b). These protocols follow a multistep and stage-specific procedure involving a symphony of key cytokines and growth factors. When iPSCs are derived from patients of genetic diseases, the genetic linkages of disease progressions are retained in the differentiation of these iPSCs, allowing in vitro observation and drug testing. Additionally, by altering the chemical composition of iPSC culture media, researchers are also able to recapitulate the development of hepatobiliary conditions caused by environmental factors, for example, drug-induced hepatotoxicity and alcohol liver disease (Sirenko and Cromwell 2018; Tian et al. 2016a, c). Altogether genetic and environmental, iPSC models are an excellent tool for studying hepatobiliary diseases.

In the past decades, a list of hepatobiliary diseases was successfully modeled by iPSC technology, many of which have led to the discovery of promising therapy candidates. A list of these diseases and their phenotypes is included in Table 1.

5.2 The Current Limitation of iPSCs in Regenerative Applications

Three major challenges hinder the downstream applications of human iPSCs. These challenges include potential tumorigenicity, immunogenicity, and heterogenicity (Sharkis et al. 2012; Yamanaka 2020). The tumorigenicity of iPSC is mainly caused by three different reasons: incorrect patterning, reprogramming factors, and genetic abnormalities. The fate of stem cells is strongly influenced by their patterning along with other cell types. In the occurrence of incorrect or incomplete patterning, niche-specific stem cells within the transplant often end up forming tumors. One example is the emergence of neural rosettes, which will maintain normal development when it is patterned toward the cortex. However, simple in vivo injection of the same cells leads to cancerous growth (Malchenko et al. 2014). The tumorigenicity of iPSCs can also derive from the intrinsic property of reprogramming factors. The common factors used to generate iPSCs, including ct3/4, Sox2, Klf4, c-Myc, all have reported roles in cancer development. C-Myc is one of the most frequently discussed proto-oncogenes. iPSC-associated chimeric mice often develop tumors due to the reactivation of c-Myc and other reprogramming factors (Yamanaka 2020).

Another challenge iPSCs are facing is immune rejection. It has been controversial whether autologous iPSCs are immunogenic. Zhao et al. has suggested that the immunogenicity of autologous iPSC is caused by its abnormal gene expression (Zhao et al. 2011). Their research provides an example of immune rejection of autologous iPSC transplants. The teratomas formed in the iPSC-transplanted mice showed obvious signs of rejection, such as T-cell infiltration. A more recent study suggested that neoepitopes of autologous iPSCs can also originate from de novo mutations in the mitochondria (Deuse et al. 2019). However, in most cases, autologous iPSCs grafts do not trigger an immunogenic response. On the other hand, allografts of iPSCs are much more immunogenic. Nonetheless, allogeneic approaches are much preferred due to their low cost and high

Table 1 iPSC-based hepatobiliary disease models and their phenotypes

Type	iPSC-derived disease models	Model phenotypes	References
Hepatocytic	Alpha-1-antitrypsin deficiency	ZAAT polymer accumulation	Choi et al. (2013), Kaserman and Wilson (2018), and Tafaleng et al. (2015)
	Alpers syndrome	Reduced optic atrophy 1 protein	Li et al. (2015)
	Citrin deficiency	Impaired ureagenesis	Kim et al. (2016)
	Hemophilia A	FVIII deficiency	Jia et al. (2014) and Olgasi et al. (2018)
	Infantile-onset Pompe disease	Lysosomal glycogen accumulation	Yoshida et al. (2019)
	Liver fibrosis (iPSC-HSC)	Retinyl esters accumulation	Coll et al. (2018)
	Niemann-pick disease type C	Cholesterol accumulation	Soga et al. (2015)
	Tangier disease	Impaired cholesterol efflux	Bi et al. (2017)
	Wilson's disease	Rapid ATP7B degradation	Parisi et al. (2018) and Yi et al. (2012)
Biliary	Alagille syndrome	Organoid malformation	Sampaziotis et al. (2015)
	Biliary atresia	Increased fibrosis Reduced biliary differentiation	Chaudhari et al. (2019) and Tian et al. (2019)
	Cystic fibrosis	Impaired cl^- channel activity	Simsek et al. (2016)
	Polycystic liver disease	Cholangiocytic cysts	Kamiya et al. (2018)

production. Immunogenicity caused by allogeneic transplants is often mitigated by immunosuppressants. One limitation of immunosuppressants is that it is often a lifelong treatment, especially in organ transplants. However, it is possible to avoid excessive immunosuppressing medication when the graft sites are immune-privileged, for example, the central nervous system, the spinal cord, and the eye.

Heterogenicity originates from the intrinsic differences between the iPSC lines. These differences include their morphology, growth curve, gene expression, and propensity to differentiate into various cell lineages. The downstream applications such as modeling, drug testing, and therapies are hugely hurdled by heterogenicity. This problem was first addressed in mouse ESCs. It was later revealed that heterogenic mouse ESCs could be converted into a neutral "ground" state by two defined kinases: MEK and GSK3 inhibitors (Ying et al. 2008). The "ground state" of these lines characterize an undifferentiated morphology, lower DNA methylation content, and greater potential to produce chimeric mice and germline-competent ESCs. Heterogenicity is not unique to mice PSCs. Human ESC and iPSCs also have troubles with heterogenicity. The example of heterogenicity in humans can be demonstrated by the comparison of hESC and hiPSC lines. Such comparisons revealed significant differences in their gene expression, epigenetic status, and differentiation potentials (Yamanaka 2012). On the other hand, when 20 or more hESC and hiPSC lines are compared, it was shown that overlapping variations do exist. In the attempts to overcome heterogeneity, researchers attempted to convert the "primed" state of hPSCs into a "naïve" state. Multiple approaches have been taken to induce the naïve or ground state pluripotency in hPSCs (Yamanaka 2020).

6 Concluding Remarks

Regenerative medicine is a translational field that requires the constant exchange of knowledge between many related fields. Regenerative medicine is also a powerful concept that can potentially cure nearly all diseases. Instead of treating the symptoms of a disease, regenerative medicine is designed to repair the cause of disease and to replace the defect with functional tissues. The application of regenerative medicine goes way beyond what this review can capture. The purpose of this review is to demonstrate how the concept of regenerative medicine is being applied to different magnitudes, from cells to tissues to organs. The advantages and challenges of regenerative medicine mentioned above are often similar in other disease settings. The future of regenerative medicine relies on the advancements of associated fields, including iPSC differentiation technology, disease modeling, genetic engineering, material science, and, most importantly, fundamental science.

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Conflict of Interest The authors declare no conflicts of interest that pertain to this work.

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Tissue-Restricted Stem Cells as Starting Cell Source for Efficient Generation of Pluripotent Stem Cells: An Overview

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Abstract

Induced pluripotent stem cells (iPSCs) have vast biomedical potential concerning disease modeling, drug screening and discovery, cell therapy, tissue engineering, and understanding organismal development. In the year 2006, a groundbreaking study reported the generation of iPSCs from mouse embryonic fibroblasts by viral transduction of four transcription factors, namely, Oct4, Sox2, Klf4, and c-Myc. Subsequently, human iPSCs were generated by reprogramming fibroblasts as a starting cell source using two reprogramming factor cocktails [(i) OCT4, SOX2, KLF4, and c-MYC, and (ii) OCT4, SOX2, NANOG, and LIN28]. The wide range of applications of these human iPSCs in research, therapeutics, and personalized medicine has driven the scientific community to optimize and understand this reprogramming process to achieve quality iPSCs with higher efficiency and faster kinetics. One of the essential criteria to address this is by identifying an ideal cell source in which pluripotency can be induced efficiently to give

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rise to high-quality iPSCs. Therefore, various cell types have been studied for their ability to generate iPSCs efficiently. Cell sources that can be easily reverted to a pluripotent state are tissue-restricted stem cells present in the fetus and adult tissues. Tissue-restricted stem cells can be isolated from fetal, cord blood, bone marrow, and other adult tissues or can be obtained by differentiation of embryonic stem cells or trans-differentiation of other tissue-restricted stem cells. Since these cells are undifferentiated cells with self-renewal potential, they are much easier to reprogram due to the inherent characteristic of having an endogenous expression of few pluripotency-inducing factors. This review presents an overview of promising tissue-restricted stem cells that can be isolated from different sources, namely, neural stem cells, hematopoietic stem cells, mesenchymal stem cells, limbal epithelial stem cells, and spermatogonial stem cells, and their reprogramming efficacy. This insight will pave the way for developing safe and efficient reprogramming strategies and generating patient-specific iPSCs from tissue-restricted stem cells derived from various fetal and adult tissues.

Keywords

Cell reprogramming · Hematopoietic stem cells · Induced pluripotent stem cells · Limbal epithelial stem cells · Mesenchymal stem cells · Multipotent stem cells · Neural stem cells · Pluripotent stem cells · Spermatogonial stem cells · Tissue-restricted stem cells · Unipotent stem cells

Abbreviations

ADSCs	Adipose-derived stem cells
AFCs	Amniotic fluid cells
AFSCs	Amniotic fluid-derived stem cells
ASCs	Adult stem cells
DPSCs	Dental pulp stem cells
ESCs	Embryonic stem cells
FSCs	Fetal stem cells
gPSCs	Germline pluripotent stem cells
GSCs	Germline stem cells
HSCs	Hematopoietic stem cells
iPSCs	Induced pluripotent stem cells
K	KLF4
LESCs	Limbal epithelial stem cells
LT-HSCs	Long-term hematopoietic stem cells
M	c-MYC
MEFs	Mouse embryonic fibroblasts
MSCs	Mesenchymal stem cells
NSCs	Neural stem cells
O	OCT4
OK	OCT4, KLF4
OSKM	OCT4, SOX2, KLF4, c-MYC
S	SOX2
SSCs	Spermatogonial stem cells

1 Introduction

Stem cells are self-renewing cells present at the apex of the lineage hierarchy and hence serve as the founder cells during organismal development. Broadly, they are categorized into two types: embryonic stem cells (ESCs) and adult stem cells (ASCs). However, a developing fetus also possesses stem cells termed as fetal stem cells (FSCs). While ESCs are isolated from the inner cell mass of the blastocyst stage during early embryonic development, FSCs and ASCs are present as a discrete population in various tissues and organs in fetus and postnatal mammals (Van Der Kooy and Weiss 2000). ESCs are pluripotent cells that can differentiate into all the cell types belonging to three germ layers (ectoderm, mesoderm, and endoderm). In contrast, ASCs are multi-, oligo-, or unipotent cells with proliferative and self-renewing abilities, giving rise to progenitor cells, which eventually differentiate into specific terminally differentiated mature cells. Although the differentiation capability of ASCs is limited, they assist in maintaining and replenishing specific cells and tissues in an adult human body (Young and Black 2004). Because of their ability to differentiate into different types of mature cells, stem cells are considered for cell-

based therapy in various diseased conditions and disorders. Even though ESCs can be differentiated into different cell types belonging to three germ layers (Lerou and Daley 2005), they are not considered ideal for therapy because of ethical issues and their inability to be used in autologous therapy. On the other hand, there are certain obstacles to using ASCs for cell therapy, namely, age-dependent abundance, accessibility, difficulties in the isolation procedures, and limited differentiation potential (Bang et al. 2016; Saha et al. 2018a).

Circumventing all these limitations, a groundbreaking study was published in 2006, in which the researchers induced pluripotency in terminally differentiated cells (fibroblasts) to achieve a pluripotent-like state by introducing a combination of transcription factors, Oct4, Sox2, Klf-4, and c-Myc (OSKM; popularly called Yamanaka factors), using a retrovirus gene delivery approach (Takahashi and Yamanaka 2006). These cells are called induced pluripotent stem cells (iPSCs) and can be differentiated into cells of all three germ layers. Subsequently, the first human iPSCs were reported that utilized fibroblasts as a starting cell source using two reprogramming factor combinations [(i) OCT4, SOX2, KLF4, and c-MYC (OSKM; Yamanaka factors) and (ii) OCT4, SOX2, NANOG, and LIN28 (Thomson factors)] (Takahashi et al. 2007; Yu et al. 2007). Thereafter, studies reported that any somatic cell isolated from a healthy subject or diseased patient could be reprogrammed into iPSCs using integration-based and integration-free approaches (Singh et al. 2015; Menon et al. 2016; Dey et al. 2017, 2021; Borgohain et al. 2019; Haridhasapavalan et al. 2019; Ray et al. 2021), opening the prospects for biobanking and generating patient-specific cells that can be used for personalized therapy, bypassing the concern of immune rejection (Okita and Yamanaka 2011). Although iPSCs have vast potential in personalized medicine, certain challenges have to be addressed to efficiently generate quality iPSCs, like choosing an ideal starting cell source, reprogramming method, reprogramming factors, culture conditions, overcoming reprogramming barriers, and so forth (Okita and Yamanaka 2011; Brouwer et al. 2016; Omole and Fakoya 2018; Saha et al. 2018b; Haridhasapavalan et al. 2020).

One of the primary factors influencing reprogramming efficiency is the starting cell source (Okita and Yamanaka 2011). The type of cell used for reprogramming greatly influences the molecular and functional properties of the generated iPSCs (Polo et al. 2010). Fibroblasts are the most commonly used somatic cell source for the derivation of iPSCs because of their availability and well-established cell culture protocols (Raab et al. 2014; Khazaei et al. 2017). But there are few inherent limitations associated with it to be used for efficient reprogramming. Firstly, fibroblasts are of mesenchymal origin and iPSCs are of epithelial origin. Hence, the former has to undergo a mesenchymal-to-epithelial transition to be reprogrammed successfully, during which the mesenchymal-associated genes are repressed and epithelial-associated genes are expressed (Raab et al. 2014). Secondly, the reprogramming efficiency is dependent on the age of the donor from whom the cells are isolated and the passage number of these isolated and expanded cells in culture (Streckfuss-Bömeke et al. 2013; Rohani et al. 2014). Thirdly, constant skin exposure to mutagenic agents like UV rays creates some alterations in the genome, affecting the quality and clinical applicability of the reprogrammed iPSCs (Gore et al. 2011; Young et al. 2012). Due to these limitations, reprogramming efficiency and the quality of iPSCs generated is very low compared to other cell types (Kim et al. 2008; Eminli et al. 2009; Sun et al. 2009; Yan et al. 2010). Therefore, different research groups explored alternate cell types (hepatocytes, β-cells, melanocytes, muse cells, myoblasts, amniotic fluid cells, urine-derived epithelial cells, keratinocytes, dental pulp cells, blood cells, FSCs, ASCs, and so forth) for reprogramming to overcome the limitations mentioned above and generate quality iPSCs (Iida et al. 2013; Yoo et al. 2013; Raab et al. 2014; Singh et al. 2015; Menon et al. 2016; Saha et al. 2018a; Chahine 2021; Disler et al. 2021; Rogers et al. 2021; Winder and Trokovic 2021; Jamal et al. 2021; Li et al. 2021; Liu et al. 2021; Pellicano et al. 2021; Petzendorfer and Guillot 2021; Ray et al. 2021).

Recent advances in iPSCs research have led to the usage of tissue-restricted stem cells derived from adult (called ASCs) and fetal (called FSCs) tissues as promising cell sources for the generation of iPSCs. Both ASCs and FSCs have unique characteristics, notably limited self-renewal, and multi- or unipotent differentiation potential that render them easier to reprogram than terminally differentiated cells. Secondly, these cells exhibit endogenous expression of one or more Yamanaka/Thomson factors and lack the same for lineage-specific genes, thereby making them more amenable to reprogramming. Third, the tissue-restricted stem cells have been proven to have epigenetic profiles similar to ESCs than mature cells (Hochedlinger and Plath 2009). Therefore, these cells may require minimal epigenetic reprogramming than mature cells. Fourth, these tissue-restricted stem cells become independent of the expression of transgenes sooner than terminally differentiated cells (Eminli et al. 2009). Lastly, these cells may have accumulated only a few genomic aberrations compared to matured cells and are therefore genetically stable (Villa et al. 2004; Bernardo et al. 2007; De Filippis et al. 2007; Zhang et al. 2007; Meza-Zepeda et al. 2008; Giorgetti et al. 2009; Wang et al. 2019). All these reasons indicate that these tissue-restricted stem cells can be an ideal source for efficient iPSCs generation. Due to these advantages, numerous studies have observed faster reprogramming kinetics and increased reprogramming efficiencies using tissue-restricted stem cells as starting cell types for reprogramming (Kim et al. 2008; Sun et al. 2009; Ge et al. 2012; Wang et al. 2019).

In this chapter, we exclusively and comprehensively discuss the most commonly used tissue-restricted stem cells, namely, neural stem cells (NSCs), hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and limbal epithelial stem cells (LESCs), used in the generation of iPSCs along with spermatogonial stem cells (SSCs) possessing the inherent pluripotent trait. We provide a detailed overview of tissue-restricted stem cells (isolated from adult and fetal tissues; this chapter covers some of the stated stem cells common to both adult and fetal tissues) utilized as a starting cell source for cellular reprogramming, their advantages over other cell types, and critical barriers associated with their usage and applications.

2 NSCs

NSCs are one of the tissue-restricted stem cells having multipotent characteristics considered for the generation of iPSCs. Isolating and obtaining NSCs is a difficult and highly invasive procedure. NSCs are primarily localized in the ependymal lining of the subventricular zone and subgranular zone of the hippocampal dentate gyrus as a discrete population in the adult brain (Ma et al. 2009; Zhao and Moore 2018). They have been isolated from the whole brain of adult or fetal mice. Importantly, human iPSCs were also generated by reprogramming NSCs derived from the fetus (Hester et al. 2009; Kim et al. 2009a) and adult (Xie et al. 2013). The brain tissues are minced or trypsinized in NSC media and cultured until neurospheres are formed. These neurospheres are replated and grown until adherent NSCs appear (Fig. 1) (Kim et al. 2009c; Tat et al. 2010). Even though isolation of NSCs is a highly invasive procedure, they have been an efficient cell source for reprogramming to iPSCs. NSCs derived from adult mice were reprogrammed into iPSCs by retroviral transduction of four Yamanaka factors (OSKM) with high efficiency of 3.6% (Kim et al. 2008). These cells can generate iPSCs 50 times more efficiently than fibroblasts (Kim et al. 2008). iPSCs were also generated from the NSCs derived from the hippocampus tissue of epilepsy patients with approximately 0.9% reprogramming efficiency using Yamanaka factors (Xie et al. 2013).

Numerous studies have reported that NSCs endogenously express pluripotency-associated genes, like high levels of SOX2 and c-MYC (Kim et al. 2009a) or high expression of SOX2 and modest expression levels of KLF4 and c-MYC (Hester et al. 2009). Apart from this, SOX2 is the core transcription factor regulating the functional properties of NSCs (Shimozaki

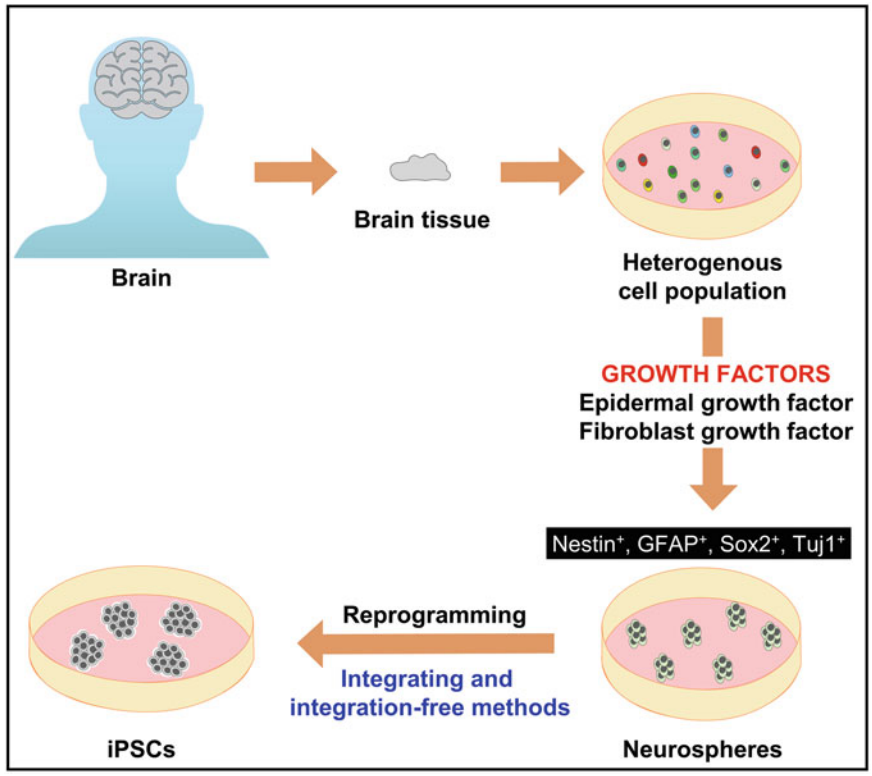

Fig. 1 Human brain tissue can be digested and cultured to derive a heterogenous cell population. From this population, NSCs can be isolated using NSC-specific markers. These stem cells form neurospheres, which can be subsequently reprogrammed to generate iPSCs using integration-based and integration-free methods

2014). Notably, adult mouse NSCs express higher endogenous levels of SOX2 and c-MYC than ESCs (Episkopou 2005; Kim et al. 2008). Therefore, by taking advantage of this endogenous expression, efforts have been made to reprogram NSCs using fewer Yamanaka factors. NSCs were successfully reprogrammed using three-factor combinations (OKM, OSK, OSM) (Kim et al. 2008), or two-factor combinations [OK alone (Kim et al. 2008; Silva et al. 2008; Hester et al. 2009), or OM alone (Kim et al. 2008)], or just using O alone (Kim et al. 2009b, a). Apart from NSCs, the neural progenitor cells, which are the intermittent stage between multipotent NSCs and mature differentiated cells, are also reported to be reprogrammed to iPSCs (Eminli et al. 2008). These neural progenitor cells have inherent SOX2 expression and, therefore, can be reprogrammed successfully using just three factors (OKM) (Eminli et al. 2008).

The expression of OK in NSCs yielded iPSCs with a reprogramming efficiency of 0.11% (Kim et al. 2008; Silva et al. 2008), whereas the same with four factors (Yamanaka factors) introduction was 3.6% (Kim et al. 2008). Later, iPSCs were also generated using the same Yamanaka factors but with a lower reprogramming efficiency of ~0.13% (Tat et al. 2010). The variation in reprogramming efficiency does reflect the region-specific cell isolation and its influence on reprogramming efficiency. While the former study (Kim et al. 2008) used cells from the mouse brain per se, in the latter case, the cells were taken from the subventricular zone of the mouse brain (Tat et al. 2010). However, the reprogramming efficiency with single factor O alone is very low (0.014% in mice and 0.004%

in humans) (Kim et al. 2009b, a). Notably, the single factor reprogramming eliminates oncogenic factors like KLF4 (Ghaleb and Yang 2017) and c-MYC (Okita et al. 2007). Hence, it can circumvent the problem of tumorigenicity when these iPSCs are employed for cell therapy.

Notably, NSCs exhibit faster reprogramming kinetics. The time taken for reprogramming mouse NSCs to iPSCs varies from one to two weeks using Yamanaka factors (Kim et al. 2008) or around 5 days using Yamanaka factors in the presence of two inhibitors (2i) and leukemia inhibitory factor (Silva et al. 2008). In contrast, mouse embryonic fibroblasts (MEFs) take ~3 weeks to be reprogrammed (Silva et al. 2008), indicating MEFs are much more challenging to reprogram than NSCs. A decrease in reprogramming efficiency and slower reprogramming kinetics was observed when a lesser number of factors were used to reprogram for both human and mouse NSCs (Table 1). Despite these limitations, using fewer factors is advantageous since the delivery of few factors in mammalian cells is easier than delivering all four factors in separate vectors. Furthermore, it will obviate the need for oncogenic factors KLF4 and c-MYC in the reprogramming cocktail and may also reduce the chance of insertional mutagenesis. Collectively, the endogenous expression of pluripotency-associated genes in NSCs makes them ideal for reprogramming with a reduced number of reprogramming factors and better reprogramming efficiencies along with faster kinetics.

Furthermore, a study reported that NSCs can attain and maintain pluripotency under feeder-free conditions and can proliferate for at least 20 passages (Choi et al. 2011). In this study, the reprogramming efficiency induced on the feeder layer was three times higher than that without the feeder layer (Choi et al. 2011). However, the presence of feeders may increase the risk of non-human pathogen transmission and immune rejection due to contamination from nonhuman antigens (Martin et al. 2005). The presence of unidentified pathogens, such as prions and viruses in these nonautologous feeder cells, could pose an increased risk of transferring pathogenic infections and a potential immune reaction. Thus, the derivation of iPSCs under animal-derived feeder-free conditions is crucial to generate clinical-grade iPSCs for various biomedical applications. Alternatively, NSCs can also be induced chemically to generate iPSCs using a cocktail of small molecules (Ye et al. 2016). Although the generation of iPSCs using this reprogramming technique is slow, there is no risk of integrating the transgene(s) in the genome (Ye et al. 2016). Hence, further optimizations are required to be worked out in employing a combinatorial approach in using small molecules with or without fewer factors *sans* oncogenes to obtain iPSCs with higher efficiency.

3 HSCs

HSCs are another source of tissue-restricted stem cells having multipotent characteristics, which are also well studied for reprogramming. HSCs can differentiate into lymphoid and myeloid progenitors, which eventually give rise to mature blood cells (Eminli et al. 2009). HSCs can be isolated from various sources, namely, cord blood, bone marrow, or peripheral blood, based on the expression of cell-specific surface markers (Fig. 2). Like NSCs, isolating HSCs from bone marrow is also an invasive procedure, but they are more efficiently reprogrammed to iPSCs than differentiated mature blood cells (Eminli et al. 2009; González et al. 2011). HSCs derived from mice were successfully reprogrammed with high efficiency using a genetically homogeneous "secondary system" expressing the Yamanaka factors (Eminli et al. 2009). These HSCs gave rise to iPSCs up to 300 times more efficiently than mature T and B lymphocytes (Eminli et al. 2009). This study showed that the differentiation stage of the starting cell has a strong influence on the kinetics and efficiency of reprogramming. Moreover, cell proliferation is presumed to be crucial for efficient reprogramming (Jaenisch and Young 2008). However, no apparent differences in reprogramming efficiency were observed between low proliferating HSCs and highly proliferating hematopoietic progenitors in

Table 1 Various studies that have reported the generation of iPSCs from NSCs

Somatic cell source(s)	Reprogramming factors	Reprogramming technique	Reprogramming efficiency (%)	Reprogramming kinetics	In vitro characterization	In vivo characterization	Reference(s)
Adult mouse NSCs	OSKM	Retroviral transduction	3.6 ± 0.5	1–2 weeks	Yes	Yes	Kim et al. (2008)
	OK		0.11 ± 0.02	2–3 weeks			
Mouse NSCs	OSKM (+2i + LIF)	Retroviral transduction	0.11	~5 days	Yes	Yes	Silva et al. (2008)
	OK (+2i + LIF)		~0.0125	2–3 weeks			
Adult mouse NSCs	O	Retroviral transduction	0.014	4–5 weeks	Yes	Yes	Kim et al. (2009b)
Human fetal NSCs	OK	Retroviral transduction	0.006	7–8 weeks	Yes	Yes	Kim et al. (2009a)
	O		0.004	10–11 weeks			
Human fetal NSCs	OK	Retroviral transduction	0.01	3 weeks	Yes	Yes	Hester et al. (2009)
Adult mouse NSCs	OSKM	Retroviral transduction	0.13 ± 0.06	ND	Yes	Yes	Tat et al. (2010)
Mouse NSCs	OSKM	Retroviral transduction	0.02	~7 days	Yes	Yes	Choi et al. (2011)
Adult human NSCs	OSKM	Retroviral transduction	~0.2	18 days	Yes	Yes	Xie et al. (2013)
Mouse NSCs	VPA, CHIR99021, 616,452, Tranylcypromine, Forskolin, Ch 55, EPZ, DZNep	Small molecules	ND	7–8 weeks	Yes	Yes	Ye et al. (2016)

O OCT4, *S* SOX2, *K* KLF4, *M* c-MYC, *NSCs* Neural stem cells, *2i* Two inhibitors, *LIF* Leukemia Inhibitory Factor, *VPA* Valproic acid, *DZNep* 3-Deazaneplanocin A, *ND* Not Determined

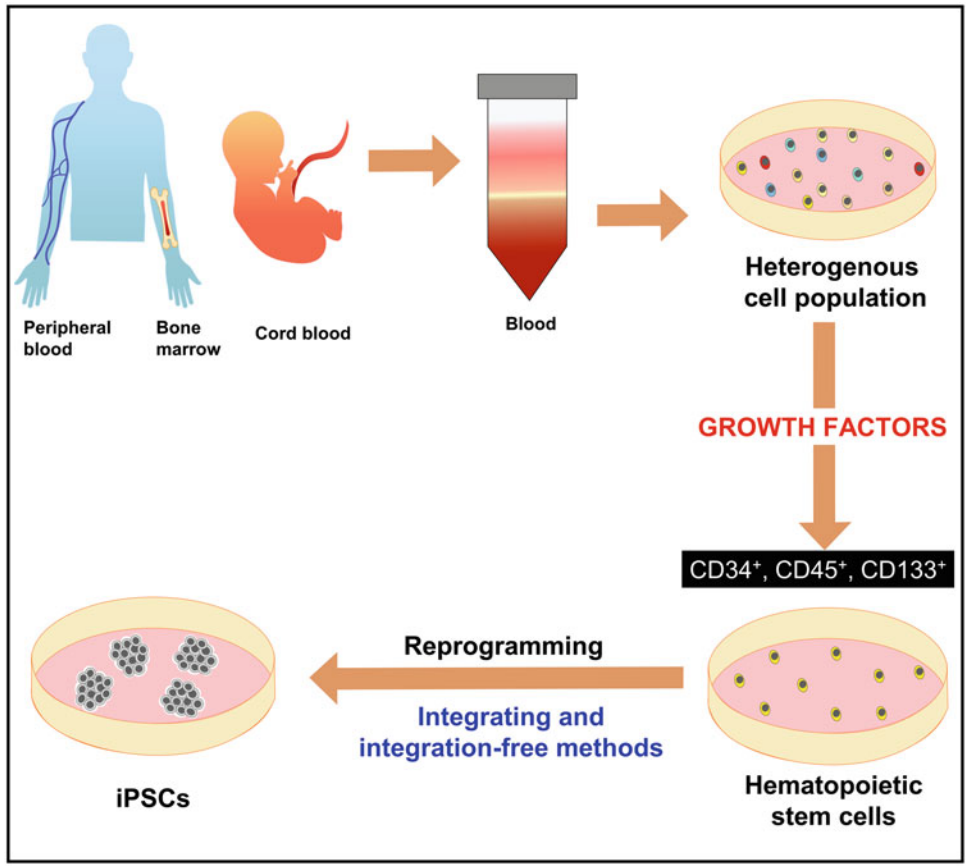

Fig. 2 Blood from human peripheral blood, bone marrow, or cord blood contains a heterogenous cell population. From this population, HSCs can be isolated using HSC-specific markers. These stem cells can be subsequently reprogrammed to generate iPSCs using integration-based and integration-free methods

this study (Eminli et al. 2009). These results implied that reprogramming efficiency is independent of the proliferation rate of cells, and it is the differentiation state, rather than the proliferation rate, which influences iPSCs formation.

Human HSCs isolated from the cord blood or bone marrow were also used as a starting cell source for iPSCs generation (Giorgetti et al. 2009; Chou et al. 2011; Kambal et al. 2011). These cells were reprogrammed by using either five (OSKM+LIN28) or three (OSK) or just two (OS) factors with comparable efficiencies (Giorgetti et al. 2009). The $CD133^+$ HSCs have a high endogenous expression of KLF4 and c-MYC than keratinocytes and fibroblasts (Giorgetti et al. 2009). In contrast, these reprogramming factors are not expressed in $CD34^+$ HSCs (Kambal et al. 2011). Furthermore, long-term HSCs (LT-HSCs) are a scarce population of cells within HSCs isolated from bone marrow and peripheral blood. HSCs isolated from cord blood will have one in 10 LT-HSCs and are characterized by the expression of 15 unique markers, CD49f being the most prominent one (Wang et al. 2019). CD49f has been reported earlier as a specific LT-HSC marker as HSCs positive for this biomarker were highly capable of producing long-term multilineage grafts (Notta et al. 2011). The $CD49f^+$ LT-HSCs are rare in peripheral blood compared to bone marrow (Wang et al. 2019). These $CD49f^+$ LT-HSCs from human bone marrow and

peripheral blood are reprogrammed within two to three weeks of infection with significantly higher efficiency, close to 50%, which is the highest ever reported using these cells (Wang et al. 2019). The $CD49f^+$ LT-HSCs were 10-fold and 100-fold more amenable to reprogramming than bulk peripheral blood $CD34^+$ cells and skin fibroblasts, respectively (Wang et al. 2019). Besides high reprogramming efficiency, the incidence of somatic single nucleotide variations and indels were low in $CD49f^+$ LT-HSCs compared to skin fibroblasts (Wang et al. 2019). A summary of various studies that have reported the generation of iPSCs from HSCs is listed in Table 2.

Apart from the HSCs population ($Lin^-/CD34^+/CD38^-/c\text{-}Kit^{-/low}$) isolated through conventional mode, several research groups have used $CD34^+$ cells isolated from cord blood and bone marrow as a cell source for the generation of iPSCs using different reprogramming methods. This cell population is heterogeneous as CD34 is a standard cell surface marker for cells belonging to hematopoietic lineage, including HSCs, hematopoietic progenitor cells, and some mature hematopoietic cells (Majeti et al. 2007). They serve as the most commonly used cell source for reprogramming besides fibroblasts because they are readily available and can be isolated using simple procedures (Haase et al. 2009; Takenaka et al. 2010; Ban et al. 2011; Chou et al. 2011; Yu et al. 2011; Nishimura et al. 2011; Ohmine et al. 2011; Meng et al. 2012; Okita et al. 2013; Su et al. 2013a; Ye et al. 2013). Although they are a heterogeneous population containing mature cells, iPSCs generated using these cells did not possess V(D)J rearrangements, unlike that seen in the case of mature lymphocytes for reprogramming (Chou et al. 2011). Notably, iPSCs can be generated from these $CD34^+$ cells using merely two factors (OS) (Meng et al. 2012). Moreover, reprogramming using these cells results in fewer single nucleotide variations than fibroblasts (Su et al. 2013b). Furthermore, it has been shown that these $CD34^+$ cells are functional and can be successfully reprogrammed into iPSCs even after storage for up to 23 years (Ye et al. 2009; Broxmeyer et al. 2011). Besides, they can also be used to generate iPSCs from patients with disorders, which can be used as a model to study and understand the mechanism of those disorders (Ye et al. 2009; Lin et al. 2015; Okumura et al. 2019). In addition, immortalized lymphocytes (lymphoblastoids) are also used as an effective somatic cell source for reprogramming (Chahine 2021). In summary, hematopoietic cells like HSCs, hematopoietic progenitor cells, and lymphoblastoids are considered potential cell sources for reprogramming. Although obtaining HSCs is difficult and LT-HSCs are a rarer population, these cells have been an excellent choice for reprogramming to iPSCs with high efficiency and fast kinetics.

4 MSCs

MSCs are multipotent stem cells of stromal origin and can be isolated from both fetal and adult sources. They have been successfully isolated from adipose tissue, hair follicles, dental pulp, bone marrow, amniotic fluid and tissues, Wharton's jelly from the umbilical cord, and so forth (Fig. 3) (Phinney and Prockop 2007). These cells can differentiate into various cell types, namely, adipocytes, osteoblasts, chondroblasts, myocytes, hepatocytes, and neuroectodermal cells. MSCs are an attractive source of cells for biomedical applications due to their multipotency, immuno-modulatory activity, and ability to provide trophic support to HSCs (Phinney and Prockop 2007). These cells are also commonly used for reprogramming to derive iPSCs, apart from the tissue-restricted stem cells discussed above. The efficiency and kinetics of reprogramming using MSCs or MSC-like cells isolated from different sources are listed in Tables 3 and 4.

4.1 Adipose-Derived Stem Cells (ADSCs)

As the name suggests, ADSCs are stem cells isolated from adipose tissue. An ADSC is defined as an MSC within adipose tissue with multipotent differentiation and self-renewal capacity. These

Table 2 Various studies that have reported the generation of iPSCs from HSCs

Somatic cell sources(s)	Reprogramming factors	Reprogramming technique	Reprogramming efficiency (%)	Reprogramming kinetics	In vitro characterization	In vivo characterization	Reference(s)
Mouse HSCs	OSKM	Lentiviral transduction	15.8	ND	Yes	Yes	Eminli et al. (2009)
CB HSCs	OSKM	Retroviral transduction	ND	12–15 days	Yes	Yes	Giorgetti et al. (2009)
	OSK		0.45 ± 0.27	12–15 days			
	OS		ND	12–15 days			
CB HSCs	OSKM	Lentiviral transduction	ND	9 days	Yes	Yes	Kambal et al. (2011)
LT-HSCs from PB and BM	OSKM	Sendai viral vector	44.5 ± 4.1	2–3 weeks	Yes	Yes	Wang et al. (2019)

O OCT4, *S* SOX2, *K* KLF4, *M* c-MYC, *HSCs* Hematopoietic stem cells, *CB* Cord blood, *LT-HSCs* Long-term hematopoietic stem cells, *PB* Peripheral blood, *BM* Bone marrow, *ND* Not Determined

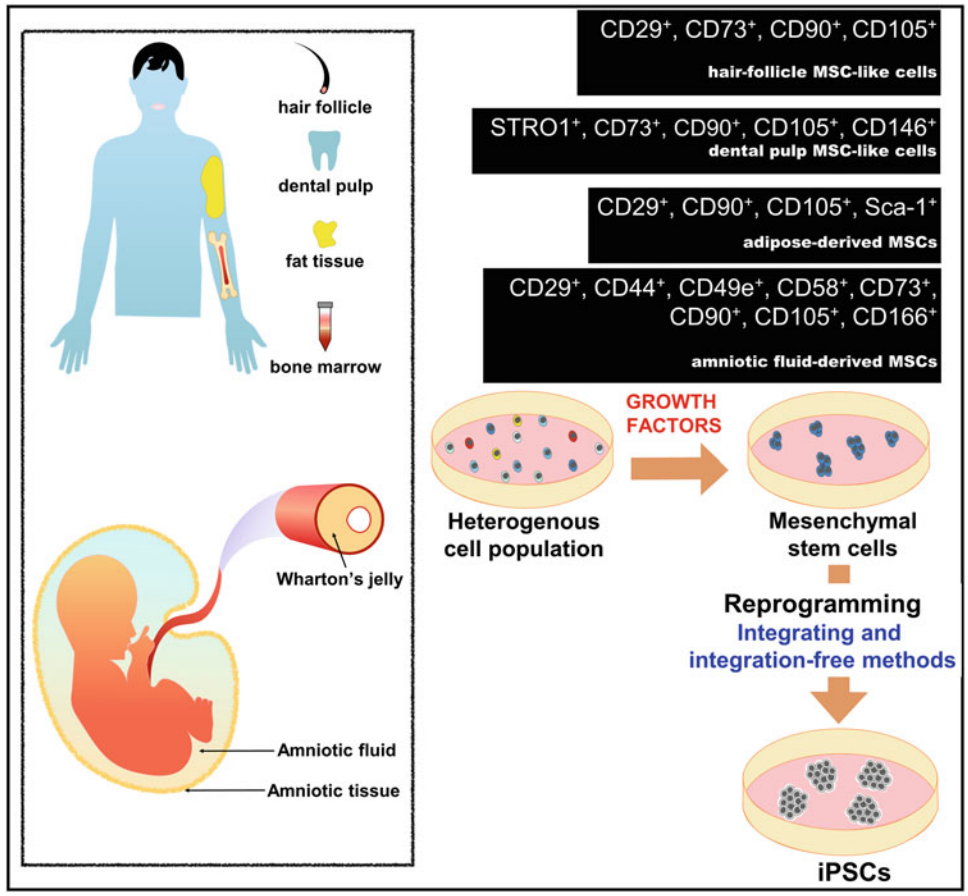

Fig. 3 Human hair follicle, dental pulp, fat, bone marrow, Wharton's jelly, amniotic fluid, or amniotic tissue can be processed to derive a heterogenous cell population. From this population, MSCs were isolated using MSC-specific markers. These stem cells can be subsequently reprogrammed to generate iPSCs using integration-based and integration-free methods

cells are isolated from patients undergoing bariatric surgery through lipoaspiration and liposuction, which are considered relatively less invasive procedures (Zuk et al. 2002; Guilak et al. 2006; Sugii et al. 2011). The derivation of ADSCs is rapid and can be achieved in less than a week (Sugii et al. 2011). The yield of ADSCs after the expansion is relatively high and is approximately 0.4×10^6 cells/ml of processed lipoaspirate isolated from the adipose tissue (Guilak et al. 2006). This vital alternative source of somatic cells can differentiate into adipogenic, osteogenic, chondrogenic, and myogenic lineages (Bunnell et al. 2008; Gao et al. 2021). Since ADSCs are multipotent, abundant, accessible, comparatively easier to derive, and proliferate more rapidly (Bunnell et al. 2008; Lee et al. 2014), these cells are also considered valuable cell sources for the generation of iPSCs (Sun et al. 2009). Human ADSCs display high endogenous expression of factors such as basic fibroblast growth factor, transforming growth factor β1, Activin A, vitronectin, and fibronectin and can serve as a feeder layer for pluripotent cells (Sugii et al. 2010, 2011). Moreover, these cells express high alkaline phosphatase levels, KLF4, and MSCs marker CD44 (Qu et al. 2012). They also express low levels of OCT4, NANOG, and REX1, enabling efficient reprogramming (Tat et al. 2010). Moreover, ADSCs possess an

Table 3 Various studies that have reported the generation of iPSCs from ADSCs

Somatic cell source(s)	Reprogramming factors	Reprogramming technique	Reprogramming efficiency (%)	Reprogramming kinetics	In vitro characterization	In vivo characterization	Reference(s)
Human ADSCs	OSKM (with feeder)	Lentiviral transduction	~0.2	15–16 days	Yes	Yes	Sun et al. (2009)
	OSKM (feeder-free)		~0.01–0.03	18–20 days			
Mouse ADSCs	OSKM (with feeder)	Retroviral transduction	0.25 ± 0.11	7–10 days	Yes	Yes	Sugii et al. (2010)
	OSKM (feeder-free)		0.4 2 ± 0.17	7–10 days			
Human ADSCs	OSKM (with feeder)	Retroviral transduction	0.74 ± 0.12	24 days	Yes	Yes	Sugii et al. (2010)
	OSKM (feeder-free)		0.008	ND			
Mouse ADSCs	OSKM	Retroviral transduction	1.14 ± 0.77	12 days	Yes	Yes	Tat et al. (2010)
Porcine ADSCs	OSKM (with feeder)	Lentiviral transduction	0.0376 ± 0.000814	7 days	Yes	Yes	Zhang et al. (2014)
	OSKM (feeder-free)		0.0153 ± 0.00106	8 days			
Buffalo ADSCs	OSKM	Retroviral transduction	ND	12 days	Yes	Yes	Deng et al. (2019)
Human ADSCs	OSKM	Retroviral transduction	ND	ND	Yes	Yes	Mao et al. (2019)
Human ADSCs	OSKM	Sendai viral vector	ND	21 days	Yes	Yes	Zhou et al. (2020)

O OCT4, *S* SOX2, *K* KLF4, *M* c-MYC, *ADSCs* Adipose-derived stem cells, *ND* Not Determined

Table 4 Various studies that have reported the generation of iPSCs from MSCs derived from different sources

Somatic cell source (s)	Reprogramming factors	Reprogramming technique	Reprogramming efficiency (%)	Reprogramming kinetics	In vitro characterization	In vivo characterization	Reference(s)
Human hair follicle MSCs	OSKM	Lentiviral transduction	~0.001	25–30 days	Yes	Yes	Wang et al. (2013a)
Human DPSCs	OSKM (double infection)	Retroviral transduction	0.1	2–3 weeks	Yes	Yes	Yan et al. (2010)
Stem cells from apical papilla	OSNL	Lentiviral transduction	0.07	2–3 weeks	Yes	Yes	Yan et al. (2010)
Human DPSCs	OSNL	Lentiviral transduction	0.03	2–3 weeks	Yes	Yes	Yan et al. (2010)
	OSNL (double infection)		0.1	2–3 weeks			
Stem cells from exfoliated deciduous teeth	OSNL	Lentiviral transduction	0.02	2–3 weeks	Yes	Yes	Yan et al. (2010)
	OSNL (double infection)		0.08	2–3 weeks			
Human DPSCs	OSKM	Retroviral transduction	ND	2–8 weeks	Yes	Yes	Beltrão-Braga et al. (2011)
Human DPSCs	OSKM	Sendai viral transduction	1.3	13–18 days	Yes	No	Pisal et al. (2018)
Human DPSCs	OSKL^{m}L + shp53 + inhibitors for TGF- β, MEK, GSK3, ROCK + sodium butyrate	Episomal vectors and small molecules	0.19	13–18 days	Yes	Yes	Chandrabose et al. (2018)
Human bone marrow MSCs	OSKM	Lentiviral transduction	ND	29–35 days	Yes	Yes	Streckfuss-Bömeke et al. (2013)
Human amniotic tissue MSCs	OSKM	Lentiviral transduction	0.4	14–20 days	Yes	Yes	Ge et al. (2012)
Human Wharton's jelly MSCs	OSKM	Retroviral transduction	~0.4	23–28 days	Yes	Yes	Cai et al. (2010)
Human Wharton's jelly MSCs	OSKM	Sendai viral transduction	ND	21–28 days	Yes	No	Miere et al. (2014)
Human Wharton's jelly MSCs	OSKM	Sendai viral transduction	ND	ND	Yes	No	Ababneh et al. (2020)
Human first trimester AFSCs	Valproic acid	NA	ND	5 days	Yes	Yes	Moschidou et al. (2012)
Human AFSCs	OSKM		≥ 0.1	1–2 weeks	Yes	Yes	

(continued)

Table 4 (continued)

Somatic cell source (s)	Reprogramming factors	Reprogramming technique	Reprogramming efficiency (%)	Reprogramming kinetics	In vitro characterization	In vivo characterization	Reference(s)
		Lentiviral transduction					Pipino et al. (2014)
Human AFSCs	O	Lentiviral transduction	0.0066	ND	Yes	Yes	Qin et al. (2016)
Human AFSCs	OS	Lentiviral transduction	0.03	ND	Yes	Yes	Qin et al. (2016)
Human AFSCs	OSKMNL + SV40LT	Episomal vectors	ND	14–30 days	Yes	Yes	Slamecka et al. (2016)
Human AFSCs	OSKM	Sendai viral transduction	0.15–0.25	3–4 weeks	Yes	Yes	Sung et al. (2020)

O OCT4, *S* SOX2, *K* KLF4, *M* c-MYC, *N* NANOG, *L* LIN28, L^m L-MYC, *shp53* short hairpin RNA for p53, *SV40LT* Simian virus 40 large T antigen, *TGF-β* Transforming growth factor- β, *MEK* MAPK/ERK kinase, *GSK3* Glycogen synthase kinase 3, *ROCK* Rho associated protein kinase, *MSCs* Mesenchymal stem cells, *DPSCs* Dental pulp stem cells, *AFSCs* Amniotic fluid stem cells, *ND* Not Determined, *NA* Not Applicable

epigenetic and genetic landscape that is more amenable for reprogramming than terminally differentiated fibroblast cells (Sun et al. 2009). Also, reprogramming ADSCs to iPSCs can deliver higher efficiency than NSCs (Tat et al. 2010), urine cells (Lee et al. 2014), keratinocytes (Sugii et al. 2010) and is far more efficient than fibroblasts (Sun et al. 2009; Sugii et al. 2010; Tat et al. 2010).

Besides humans, ADSCs have also been isolated from different species, namely, mouse, buffalo, porcine, and so forth, and efficiently reprogrammed into iPSCs using viral transduction of Yamanaka factors with reasonable efficiencies (Sugii et al. 2010; Tat et al. 2010; Zhang et al. 2014; Deng et al. 2019). The average reprogramming time to give rise to human and mouse iPSCs is 2.5 and 1.5 weeks, respectively (Sugii et al. 2011). Notably, reprogramming human and mouse ADSCs to iPSCs is 100-fold and 5-fold more efficient than human and mouse fibroblasts, respectively (Sugii et al. 2010). Furthermore, mouse ADSCs were most amenable to iPSC formation with an 8- and 38-fold improved reprogramming efficiency than NSCs and MEFs, respectively (Tat et al. 2010). A similar observation was made where porcine ADSCs were more efficiently reprogrammed than fibroblasts under feeder-free and serum-free conditions (Zhang et al. 2014). The inclusion of Leukemia inhibitory factor, CHIR99021, and PD0325901 in the medium resulted in the generation of naïve-like porcine iPSCs (Zhang et al. 2014). Moreover, another finding reported that buffalo ADSCs were reprogrammed more efficiently into naïve iPSCs under hypoxic conditions (5% of oxygen), very likely through activated hypoxia-inducible factor-1α, since they were adapted physiologically to a microenvironment where the oxygen level was 1–5% (Deng et al. 2019). Moreover, iPSCs derived by reprogramming human ADSCs have better osteogenic differentiation ability and are considered excellent for bone tissue engineering (Mao et al. 2019).

Human ADSCs were efficiently reprogrammed by viral transduction of Yamanaka factors under both feeder-dependent (Sun et al. 2009; Sugii et al. 2010; Mao et al. 2019; Zhou et al. 2020) and feeder-independent conditions (Sun et al. 2009; Sugii et al. 2010, 2011) with comparable efficiencies and reprogramming kinetics (Table 3). Sun and colleagues reported that the generation of iPSCs from human ADSCs was two times faster and 20 times more efficient than fibroblasts with efficiencies of 0.01% on feeder-free Matrigel substrate to 0.2% on MEFs as feeders (Sun et al. 2009). Although reprogramming under feeder-independent conditions takes more time (18–20 days) compared to using MEFs as a feeder layer (15–16 days), it eliminates potential variability caused by using feeder cells and negates the problem of contamination of cells from the feeder layer (Sun et al. 2009). Furthermore, using nonintegrative approaches like episomal plasmids under feeder-free conditions can yield iPSCs at higher efficiency than viral-based approaches (Lee et al. 2014). This eliminates any animal-derived pathogen transmission risk and establishes a Good Manufacturing Practices (GMP)-compliant system to produce iPSCs for therapeutic applications. A summary of various studies that have demonstrated the formation of iPSCs from ADSCs is mentioned in Table 3. All these studies suggest that ADSCs are ideal for reprogramming, especially under feeder-independent conditions.

4.2 Dental Pulp–Derived MSCs

Dental pulp serves as another exciting source of MSC-like cells. Isolation of MSC-like cells from dental pulp is accessible and less invasive (Beltrão-Braga et al. 2011). These MSC-like cells are capable of adipogenic and osteogenic differentiation (Huang et al. 2008). Additionally, these MSC-like cells are found to endogenously express some of the pluripotent stem cell markers like OCT4, NANOG, and REX1 (Huang et al. 2008). Therefore, these MSC-like cells are considered closer to pluripotent stem cells than other MSCs and differentiated mature cells (Huang et al. 2008; Beltrão-Braga et al. 2011). Moreover, MSC-like cells from dental pulp are immune privileged, and therefore, iPSCs generated from them will not be rejected by the host immune response (Gomes et al. 2010; Beltrão-Braga

et al. 2011). Hence, these cells are considered an ideal candidate for the generation of iPSCs. In fact, MSC-like cells isolated from dental pulp have been successfully reprogrammed to iPSCs using Yamanaka factors under feeder-free (Beltrão-Braga et al. 2011; Hamada et al. 2020) and xeno-free (Thekkeparambil Chandrabose et al. 2018) conditions. Efficient reprogramming was also achieved when MSC-like cells were isolated from various dental sources, namely, the dental pulp, apical papilla, and exfoliated deciduous teeth, and were reprogrammed using either Yamanaka or Thomson factors. The reprogramming efficiency increased approximately 5-fold when the cells were transduced twice with an interval of 24 h compared to single transduction (Yan et al. 2010). The iPSCs generated from reprogramming of Dental pulp stem cells (DPSCs) extracted from the natal tooth using Sendai viral mode of transduction have shown reprogramming efficiency higher than human fibroblasts (Pisal et al. 2018). The same was also true in the case of retroviral and lentiviral modes of transduction, as mentioned in Table 4. More interestingly, a subpopulation of dental pulp cells exist which expresses pluripotency-associated genes and surface markers (SSEA4, OCT3/4, SOX2, NANOG, LIN28, CD13, CD29, CD90, CD105) when cultured under specific culture conditions, termed as dental pulp pluripotent-like stem cells (Atari et al. 2012). These cells were also capable of embryoid body and teratoma formation, validating their pluripotency (Atari et al. 2012). The wide range of applications for iPSCs derived from DPSCs is reviewed in detail elsewhere (Jamal et al. 2021).

4.3 Bone Marrow–Derived MSCs

Bone marrow is a major source of MSCs, and these cells have been reported to show basal expression of OCT4 and SOX2 genes. Streckfuss-Bomeke et al. have demonstrated that the bone marrow–derived MSCs exhibit higher reprogramming efficiency (~0.12%) than fibroblasts (~0.06%) and keratinocytes (~0.03%) by using the STEMCCA system (Streckfuss-Bömeke et al. 2013). Interestingly, iPSCs derived from these cells also displayed a significantly higher differentiation potential to spontaneously beating cardiomyocytes (Streckfuss-Bömeke et al. 2013).

4.4 Hair Follicle–Derived MSCs

Hair follicles are the most accessible tissue to obtain MSCs from the human body without any invasive procedure. MSCs isolated from these human hair follicles are capable of osteogenic and adipogenic differentiation (Wang et al. 2013a). Moreover, they were negative for the major histocompatibility complex, which means that iPSCs derived from these MSCs can bypass immune rejection and may serve as an elegant source for cell therapy applications (Wang et al. 2013a).

4.5 MSCs of Fetal Origin

4.5.1 Amniotic Fluid–Derived MSCs

Amniotic fluid is found inside the amniotic sac, protecting and facilitating nutrient and gas exchange between the fetus and the mother (Fauza 2004; Roubelakis et al. 2012). This amniotic fluid is collected by a minimally invasive procedure (using a syringe) called amniocentesis (Li et al. 2009; Roubelakis et al. 2012). Amniocentesis is routinely performed as part of prenatal diagnosis (Fauza 2004; Roubelakis et al. 2012). Amniotic fluid cells (AFCs) or amniocytes represent a mixed population of cells of fetal origin (Polgár et al. 1989; Turner and Fauza 2009). This heterogeneous population of cells contains a subpopulation of self-renewing, multipotent stem cells, known as amniotic fluid–derived stem cells (AFSCs), contributing to ~1% of AFCs (Fauza 2004; De Coppi et al. 2007). These AFSCs express certain MSC-specific surface markers, namely CD29, CD44, CD49e, CD58, CD73, CD90, CD105, and CD166 (Roubelakis et al. 2012; Qin et al. 2016; Slamecka et al. 2016) and hence can be easily isolated and purified

using the same (Fig. 3) (Pipino et al. 2014; Qin et al. 2016; Slamecka et al. 2016; Sung et al. 2020). Moreover, these AFSCs express pluripotency-related genes and markers such as SSEA-4, $CD117^{+}$ (c-kit), NANOG, OCT4, c-MYC, REX1, and SOX2 (Li et al. 2009; Qin et al. 2016). Expression of all these genes and markers makes them a more desirable target for reprogramming.

Certain advantages in using AFSCs as a starting cell source are that they have the least genetic aberration induced by the environment and also have a distinct epigenetic and genetic aspect that aids in the acquisition of complete reprogramming (Fan et al. 2012; Li et al. 2013; Yan and Li 2017). More importantly, since these cells are not prone to any mutations because of external factors, patient-specific iPSCs can be generated from them and used for autologous gene therapy (Li et al. 2009; Drozd et al. 2015; Xing et al. 2018; Wang et al. 2020). A detailed perspective regarding the usage of AFSCs for reprogramming has been reported earlier (Guillot 2016; Petzendorfer and Guillot 2021). Table 4 enlists the approaches used by various groups for reprogramming AFSCs to iPSCs (Pipino et al. 2014; Qin et al. 2016; Slamecka et al. 2016; Sung et al. 2020). These cells were reprogrammed with efficiencies comparable to fibroblasts but with shorter reprogramming kinetics (Pipino et al. 2014; Sung et al. 2020). Since they already express certain pluripotency-associated markers, reprogramming was carried out using lentiviral transduction with fewer factors like OS or O alone (Qin et al. 2016). Interestingly, human AFSCs isolated during the first trimester of pregnancy could form embryoid bodies within two to three weeks upon culturing in vitro, but not teratomas in vivo (Moschidou et al. 2012). Therefore, to induce pluripotency in these cells, histone deacetylase inhibitor (valproic acid) was used in the culture media, which made them pluripotent, as confirmed by teratoma formation (Moschidou et al. 2012, 2013). Not only AFSCs but heterogenous AFCs are also widely used as a starting cell source for the generation of iPSCs. Numerous studies have used these AFCs for iPSC generation with better reprogramming efficiencies and kinetics (Li et al. 2009, 2013; Wolfrum et al. 2010; Anchan et al. 2011; Fan et al. 2012; Drozd et al. 2015; Bertin et al. 2015; Drews et al. 2015; Yan and Li 2017; Xing et al. 2018; Park and Mostoslavsky 2018; Wang et al. 2020; Yi et al. 2021).

4.5.2 Amniotic Tissue–Derived MSCs

MSCs derived from amniotic tissues express pluripotency-associated genes, such as *OCT4* and *NANOG* (Miki et al. 2005; Ge et al. 2012). Importantly, these cells do not undergo aging and are functional even after propagation for >2 years in culture (Walther et al. 2009). In addition, these cells possess immunomodulatory properties, where they can block the immune response in the host. Therefore, iPSCs generated from these MSCs also render immunomodulatory properties and can be used for therapeutic applications (Ge et al. 2012).

4.5.3 Wharton's Jelly–Derived MSCs

Wharton's jelly is a gelatinous tissue found inside the umbilical cord containing connective tissues (Stefańska et al. 2020). These tissues are usually discarded after childbirth; therefore, their usage does not pose any ethical concern (Miere et al. 2014). Wharton's jelly contains MSCs capable of adipogenic, chondrogenic, and osteogenic differentiation and display immunomodulatory properties (Stefańska et al. 2020). Hence, iPSCs-derived from these MSCs from Wharton's jelly may also bypass the host's immune system (Miere et al. 2014). Moreover, MSCs from Wharton's jelly are easily attainable and in more significant numbers than MSCs from cord blood (Fu et al. 2006; Cai et al. 2010). Because of easier availability and immunomodulatory properties, MSCs from Wharton's jelly are considered as one of the potential sources for the generation of iPSCs. Successful reprogramming of these MSCs to iPSCs has been reported using viral transduction of Yamanaka factors (Cai et al. 2010; Miere et al. 2014; Ababneh et al. 2020). Thus, MSC-like cells derived from various sources can be utilized to derive iPSCs efficiently. A summary of iPSCs derived from different types of MSCs is listed in Table 4.

5 LESCs

LESCs are unipotent stem cells located in the limbal palisades of Vogt and the inter palisade rete ridges of the corneoscleral limbus (Fig. 4) (Dua and Azuara-Blanco 2000), which are involved in the constant renewal of corneal epithelium (Ren and Wilson 1996; Ahmad et al. 2010; Rama et al. 2010; Ordonez and Di Girolamo 2012). The deficiency of LESCs causes eye complications, leading to corneal blinding (Biber et al. 2010; Pellegrini et al. 2014). Limbal stem cell transplant therapy has been employed in patients with Limbal stem cell deficiency as a treatment, but with limited success (Samson et al. 2002). The limitations of this therapy concerning culture and expansion of LESCs have prompted the exploration of the possible use of LESCs differentiated from autologous iPSCs.

In the pursuit of generating iPSCs, the primary culture of human limbal epithelial cells containing LESCs has been reprogrammed using the lentiviral mode of transduction of Yamanaka factors (Hayashi et al. 2012). The generated iPSCs have been differentiated to corneal epithelial cells, which can be used for treatment in patients with corneal damage (Hayashi et al. 2012). Isolated LESCs were reprogrammed successfully to iPSCs using a nonintegrative

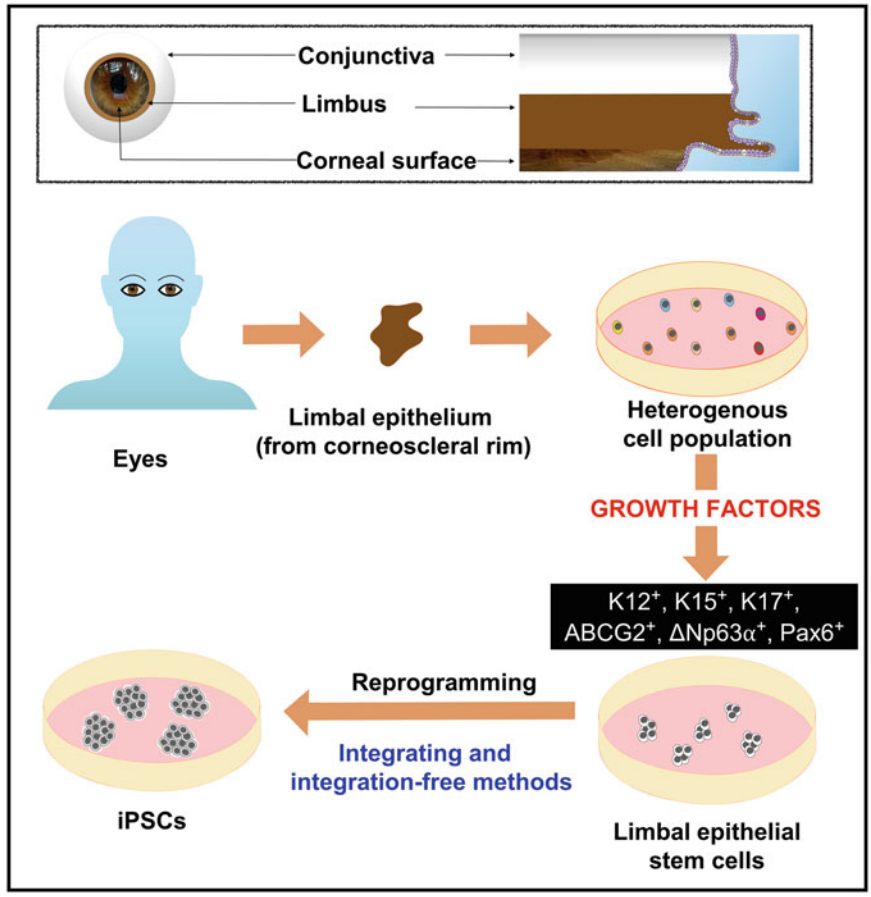

Fig. 4 Limbal epithelial tissue can be isolated from the human corneoscleral rim of an eye and cultured to derive a heterogenous cell population. From this population, LESCs can be isolated using LESC-specific markers and reprogrammed to generate iPSCs using integration-based and integration-free methods

episomal plasmid approach and under feeder-free conditions (Sareen et al. 2014). Interestingly, lower reprogramming efficiency using the lentiviral mode of transduction (~0.0005%) (Hayashi et al. 2012) has been observed compared to the episomal method of transfection (~0.005%) (Sareen et al. 2014). Although the lentiviral method of transduction is considered more efficient than the episomal approach, the difference in reprogramming efficiency might be because the former study (Hayashi et al. 2012) used limbal epithelial cells containing LESCs, while the latter (Sareen et al. 2014) used limbal epithelial cells which were enriched with LESC population to derive iPSCs. However, the latter study did not specify the exact method of LESC enrichment. LESC-derived iPSCs reported better persistence of epigenetic memory than iPSCs generated from fibroblasts (Sareen et al. 2014). The phenomenon of retaining certain epigenomic characteristics from the starting cell source makes the differentiation ability of iPSCs biased towards the starting cell source, known as epigenetic memory. Earlier studies have reported that this phenomenon is a common characteristic of iPSCs generated from other cell sources (Polo et al. 2010; Hu et al. 2010; Ohi et al. 2011; Vaskova et al. 2013). Comparatively, LESCs are easier to obtain than other tissue-restricted stem cells. To date, only two studies have been reported to use LESCs as a starting cell source for the generation of iPSCs (Hayashi et al. 2012; Sareen et al. 2014). More research is required to explore the applicability of LESCs in reprogramming.

6 SSCs

SSCs are germline stem cells (GSCs) in males, which can maintain their population by self-renewal and also differentiate into sperm during the adult male reproductive life by a process called spermatogenesis (Tagelenbosch and de Rooij 1993; de Rooij and Grootegored 1998; Ko et al. 2009; Kanatsu-Shinohara and Shinohara 2013). They belong to a distinct cell subpopulation within type A spermatogonia, which resides on the basement membrane of the seminiferous tubules (De Rooij 1998; de Rooij and Grootegored 1998). SSCs contribute to about 0.02–0.03% of the cells of testis (Tagelenbosch and de Rooij 1993). Primarily, the biological role of SSCs is the sustenance of male fertility by being the only type of ASCs that can pass on the genetic information to offspring (de Rooij and Grootegored 1998; Kanatsu-Shinohara and Shinohara 2013).

Notably, unlike somatic cells, SSCs possess inherent pluripotent characteristics similar to that of ESCs (Kanatsu-Shinohara et al. 2008; Huang et al. 2009) and do not need the introduction of any exogenous factors for their reprogramming (Lee et al. 2018). As early as 1992, pluripotent cell lines have been established from primordial germ cells under specific growth conditions (Matsui et al. 1992). Almost a decade later, testes cells from neonatal mice and SSCs from p53 knock-out neonatal mice were converted into pluripotent cells under a defined microenvironment (Kanatsu-Shinohara et al. 2004). Subsequently, extensive research was carried out to generate pluripotent cells from GSCs (Ko et al. 2009, 2012; Kossack et al. 2009; Lee et al. 2018). Moreover, pluripotent cells expressing pluripotency markers like SSEA4, TRA-1-81, OCT4, SOX2, and early hESC markers like TNAP have also been reported from a testis biopsy sample. These cells formed embryoid bodies and maintained high telomerase activity (Kossack et al. 2009). SSCs derived from both mouse and human testis have been successfully reprogrammed into pluripotent cell lines as confirmed by in vitro and in vivo assays of pluripotency (Ko et al. 2009, 2012; Kossack et al. 2009). In fact, SSCs are one of the few ASCs that display OCT4 expression (Kanatsu-Shinohara et al. 2004; Ko et al. 2009), with OCT4 required for their maintenance and colonization ability after transplantation (Kehler et al. 2004; Dann et al. 2008).

Numerous studies have shown the conversion of these SSCs to pluripotent ESC-like cells. Firstly, the testis cells are cultured under GSCs culture conditions, containing a cocktail of growth factors resulting in the formation of GSCs colonies on top of the monolayer of

testicular cells (Kossack et al. 2009). Then, these colonies are manually passaged and grown under ESC culture conditions with a feeder layer until they give rise to ESC-like colonies. These colonies are called germline pluripotent cells (gPSCs) (Fig. 5) (Kanatsu-Shinohara et al. 2004; Ko et al. 2009, 2012; Lee et al. 2018). To generate high-quality, clinical-grade pluripotent cell lines, feeder-free approaches for expanding and producing gPSCs have also been developed (Choi et al. 2014; Lee et al. 2018). The efficiency of converting SSCs into gPSCs varies depending on cell plating density with and without feeder cells (Ko et al. 2009; Lee et al. 2018). Interestingly, these mouse SSCs can attain pluripotency only till a certain age of the mouse. A study demonstrated that SSCs derived from adolescent (about 7 weeks) mice or older are almost impossible to reprogram (Azizi et al. 2016). This might be due to decreased expression of pluripotency-associated genes like OCT4, SOX2, and NANOG, and increased expression of differentiation genes with age (Azizi et al. 2016). Moreover, this might also account for varying efficiencies of SSCs derived from different aged mice to convert to gPSCs (Table 5).

Isolation and culture of SSCs is a labor-intensive process and requires expertise to identify the appropriate colonies of GSCs (Ko et al. 2010). Unlike other cell sources, the clinical application of SSCs can transcend issues of ethical concerns, immune rejection, risk of tumor formation, among others (Chen et al. 2020). Most cancer patients have to undergo chemotherapy, radiation, or both, which may result in infertility in patients. In such cases, cryopreservation of SSCs followed by their autologous transplantation into the individuals concerned will assist in

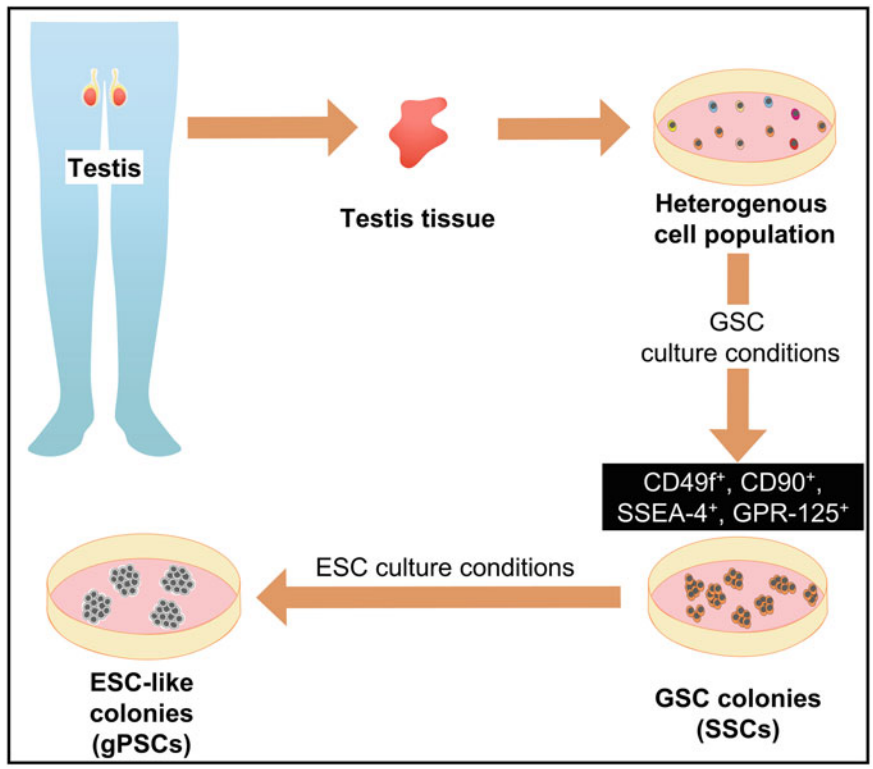

Fig. 5 Human testis tissue can be digested and cultured to derive a heterogenous cell population. From this population, GSC colonies (containing SSCs) can be isolated. These stem cells can be subsequently reprogrammed in the presence of growth factors and culture conditions to generate ESC-like gPSCs

Table 5 Various studies that have reported the generation of pluripotent cell lines from cells of testicular origin

Somatic cell source (s)	Reprogramming factors	Reprogramming technique	Reprogramming efficiency (%)	Reprogramming kinetics	In vitro characterization	In vivo characterization	Reference(s)
Neonatal mouse testis cells	None	NA	ND	4–7 weeks	Yes	Yes	Kanatsu-Shinohara et al.(2004)
Adult p53-KO mouse SSCs	None	NA	ND	~4 weeks	Yes	Yes	Kanatsu-Shinohara et al. (2004)
Adult mouse SSCs	None	NA	0.01	2–4 weeks	Yes	Yes	Ko et al. (2009)
Human SSCs	None	NA	ND	ND	Yes	Yes	Kossack et al. (2009)
Adult mouse SSCs	None	NA	ND	2–4 weeks	Yes	Yes	Ko et al. (2012)
Human testis (SSCs)	None	NA	ND	2–4 weeks	Yes	Yes	Lim et al. (2013)
Bovine testis cells	O	Electroporation	0.3	15–21 days	Yes	Yes	Wang et al. (2013b)
Neonatal mouse SSCs	OSKM	Induction of transgenes	ND	10–12 days	Yes	Yes	Bermejo-Álvarez et al. (2015)
Mouse SSCs	None	NA	ND	46–143 days	Yes	Yes	Azizi et al. (2016)
Adult mouse SSCs	None	NA	0.000369	3–5 weeks	Yes	Yes	Lee et al. (2018)

O OCT4, *S* SOX2, *K* KLF4, *M* c-MYC, *SSCs* Spermatogonial stem cells, *KO* Knock-out, *NA* Not applicable, *ND* Not determined

restoring fertility through cell therapy (Kubota and Brinster 2006; Chen et al. 2020). To date, SSCs are the only cells in an organism after birth possessing inherent pluripotency that can be transformed into pluripotent stem cells only by using specific microenvironment or culture conditions without any reprogramming factors or techniques (Kanatsu-Shinohara et al. 2004; Ko et al. 2009; Lee et al. 2018). This microenvironment-mediated generation of desired cell types from SSCs is not only limited to pluripotent cells; instead, it can be extended to specific somatic cell types like neurons (Yang et al. 2019). The neurons derived from SSCs, when transplanted in mouse models of Parkinson's disease, have been shown to restore significant sensorimotor functions (Yang et al. 2019). Moreover, SSCs have also been converted into certain other cell types, such as cardiomyocytes and hepatocytes (Guan et al. 2007; Baba et al. 2007; Fagoonee et al. 2015; Chen et al. 2016). This transdifferentiation capability of SSCs into different cell types for cell therapies is another avenue, which needs further research and exploration. A detailed review of SSCs and their biomedical applications are discussed elsewhere (Fagoonee et al. 2011; Pellicano et al. 2021).

Most studies on SSCs have been performed in animal models; the extension of the obtained results to humans will require further research. However, the undoubted potential of SSCs for the generation of pluripotent cell lines or other therapeutic usage is beyond question. Furthermore, tissues obtained from the biopsy of the testis are enough to successfully generate pluripotent cells (Kossack et al. 2009; Ko et al. 2012; Chen et al. 2016). Such unique capabilities of SSCs, beginning from microenvironment mediated conversion to pluripotent cells without any exogenous factors to transdifferentiating into different adult cell types, entails future research into it as a source for the generation of autologous cells for therapeutic purposes. The female counterpart of GSCs (from ovarian tissue) is also utilized as a source for the generation of pluripotent stem cells, which is reviewed in detail elsewhere (Disler et al. 2021).

7 Conclusion

This review has discussed the potential of tissue-restricted stem cells (derived from adult and fetal tissues) having multipotent or unipotent characteristics as a starting cell source in the generation of iPSCs and their prospective biomedical applications (Fig. 6). Being bestowed with endogenous expression of certain ESCs-specific genes, ASCs and FSCs require fewer reprogramming factors to generate iPSCs with comparable reprogramming efficiencies. For instance, fewer reprogramming factors are employed in NSCs because of the endogenous expression of pluripotency-associated genes. Significantly higher efficiencies were achieved when HSCs were used for reprogramming. LT-HSCs, although they are a rarer population of cells and difficult to isolate thus far, they have been proven to be an excellent source for reprogramming to iPSCs with the highest ever efficiency to date. In contrast, MSCs comparatively have lower reprogramming efficiencies, but they are abundantly available and easily attainable. MSCs from specific tissue sources, namely, hair follicles and dental pulp, are easier to isolate, and iPSCs reprogrammed from them can bypass immune rejection. Notably, ADSCs can be reprogrammed under feeder-free conditions efficiently. Similarly, LESCs are easier to attain, and due to their epithelial origin, a mesenchymal-to-epithelial transition does not occur during the course of reprogramming. LESCs appear to be an attractive cell source, but more research is required to realize their full potential. SSCs are considered to be much closer to pluripotent cells, and the inherent pluripotency can be unleashed solely by manipulating the culture conditions.

Tissue-restricted stem cells, thus fulfill most of the characteristics for a cell to be an ideal source for reprogramming, namely, (i) reproducibly derived and cultured from healthy and diseased human subjects, (ii) stably expandable at cell culture conditions and suitable for biobanking for long-term storage, (iii) having a "normal," unaltered genome, (iv) absence of somatic mutations and DNA rearrangements that are

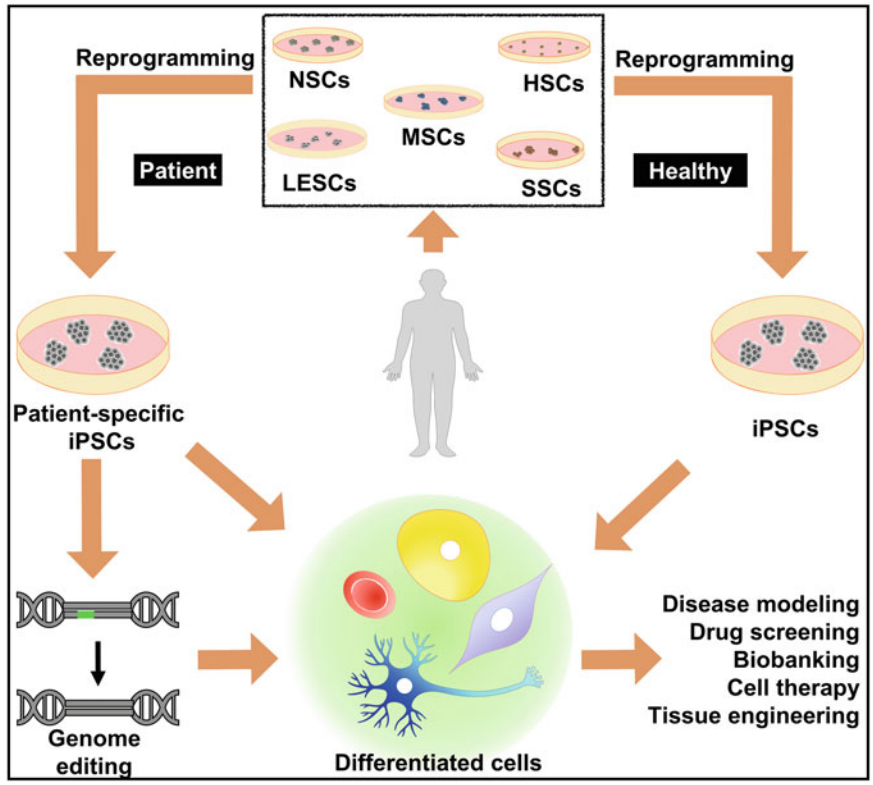

Fig. 6 Various tissue-restricted stem cells can be isolated from patients and/or healthy subjects and can be reprogrammed to generate iPSCs. These iPSCs can be differentiated into desired cell type(s) and used for various biomedical applications

usually found in terminally differentiated cells, and (v) capable of efficient reprogramming due to endogenous expression of stem cell-specific genes. To summarize, stem cells from various fetal and adult sources have been proven to be efficient somatic cell sources for the generation of iPSCs. However, there are certain hurdles in using them as a starting cell source, like availability, highly invasive collection procedures, and isolation; therefore, more research is required to make ASCs and FSCs an ideal cell source for the derivation of iPSCs and employing the reprogrammed iPSCs for various therapeutic and biomedical applications.

Additionally, the critical requirement is to create a GMP-compliant system for the generation of clinical-grade iPSCs. A rapid, reproducible, robust, and facile iPSC derivation protocol from an ideal cell source under serum-free, feeder-free, and xeno-free conditions using a non-genetic, non-viral method is highly required. More extensive research to identify ideal cell sources that will generate iPSCs retaining genomic stability with increased efficiency and kinetics is vital for biobanking and numerous clinical applications. This will help translate this promising technology to derive patient-specific iPSCs for biomedical applications.

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Declarations

Conflict of Interest The authors declare that they have no potential conflict of interest.

Ethics Approval Not applicable

Informed Consent Not applicable

Research Involving Human Participants and/or Animals None

Availability of Data and Material Not applicable

Author Contribution All authors contributed to the conception and design of this manuscript, performed data collection and interpretation, commented on the previous versions of the manuscript, read and approved the final draft of the manuscript.

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Molecular Mechanisms behind Persistent Presence of Parvovirus B19 in Human Dilated Myocardium

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Abstract

The role of parvovirus B19 (PVB19) in the pathogenesis of idiopathic dilated cardiomyopathy (DCM) remains poorly understood. Therefore, we have measured the levels of inflammation, fibrosis, apoptosis, and necrosis in endomyocardial biopsies (EMBs) and sera of nonischemic PVB19-positive (n = 14) and PVB19-negative (n = 18) DCM patients. Chronic persistence of PVB19 in myocardium did not induce significant infiltration of T cells (CD3 and CD45Ro) and macrophages (CD68), and did not secrete TNFα, IL-6, and CRB. The fibrosis in PVB19-positive EMBs was also lower compared to the virus-negative ones, while ECM degrading matrix metalloproteinase MMP1 and gelatinase MMP2 were significantly (by twofold) upregulated. In addition,

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there was no activation of neither apoptotic nor necrotic pathways. However, levels of antiapoptotic mitochondrial Bcl-2 and heat shock protein 60 (Hsp60) in PVB19-positive biopsies were almost threefold lower than in PVB19-negative ones revealing impairment of mitochondria. Altogether, data indicate that persistence of PVB19 in myocardiums of nonischemic DCM patients can cause myocardial ECM remodeling through the MMPs, such as MMP1 and MMP2, and mitochondrial impairment. The correlative analysis of measured biomarkers suggested likely further activation of apoptotic cell death pathways rather than fibrosis. Data also suggest that antiviral therapy could be beneficial for PVB19-positive DCM patients by managing further pathological myocardial remodeling.

Keywords

Apoptosis · Dilated cardiomyopathy · Fibrosis · Inflammation · MMP/TIMP · PVB19

1 Background

Viral infection is one of the leading causes of acute myocarditis with ensuing dilated cardiomyopathy and heart failure that often requires heart transplantation (Mason 2003; Kawai 1999). The chronic presence of viruses in myocardium is less harmful but is also less investigated (Kühl et al. 2005a; Tátrai et al. 2011). Currently, the most accepted models of acute viral myocarditis in human and animal models is divided into three phases: the virus entry into the myocardium, particularly endothelium cells (phase I), followed by an immune system dysregulation, increased infiltration of immune cells, and secretion of inflammatory cytokines (phase II), which leads to cardiomyocyte destruction, cardiac remodeling, and dilated cardiomyopathy (phase III) (Maekawa et al. 2007; Verdonschot et al. 2016). During the first acute phase of viral myocarditis, the direct cardiomyocyte lysis can occur further facilitating virus entrance, myocardium impairment, and cardiac dilation (Maekawa et al. 2007). The acute immune system activation sometimes can give a better further myocardial outcome or initiate worse pathological heart remodeling leading to the development of DCM (McCarthy 3rd et al. 2000), while subacute myocarditis is the most common cause of DCM (Magnani and Dec 2006).

The human myocardium can be infected by a wide variety of viruses such as adenovirus, enterovirus, parvovirus B19, hepatitis C virus, human herpesvirus type 6, Epstein–Barr virus, and others (Bowles et al. 2003; Kühl et al. 2005b; Matsumori et al. 1999; Leveque et al. 2011; Mutlu et al. 2011). However, in recent decades, a significant shift from adenoviruses or enteroviruses to parvovirus B19 (PVB19), as a most frequent cardiotropic virus in endomyocardial biopsies (EMBs) of DCM patients, has been observed (Kühl et al. 2005b; Pankuweit et al. 2000). It was shown that PVB19 may persist in 30 to 60% of healthy human hearts and be of no importance in the pathogenesis of idiopathic DCM (Lotze et al. 2010; Moimas et al. 2012). The PVB19 in DCM myocardium was confirmed to be dominating, but its relevance to future prognosis has been doubted (Kuethe et al. 2007; Zimmermann et al. 2010). However, our previously published study confirmed the direct involvement of PVB19 in the initiation of DCM in mouse model (Bogomolovas et al. 2016). In addition, other studies also suggest PVB19 being a frequent cause of patients' DCM damaging myocardium (Leveque et al. 2011). Recently, the association between PVB19 and DCM in children has been shown: the presence of PVB19 in the cardiac allograft was associated with higher adverse post-hypertensive events (Das et al. 2020). The inconsistent findings may be related to the different stages of investigated viral DCM – different intensities and/or durations of intramyocardial viral infection might have different outcomes.

Parvovirus B19 is a single-stranded DNA virus and member of the *Parvoviridae* family, which is mainly residing and replicating in erythroid progenitor cells (Verdonschot et al. 2016; Gallinella 2013). PVB19 is known as the main cause of erythema infectiosum in children, arthropathy, transient aplastic crisis, or aplasia of red blood cells (Verdonschot et al. 2016;

Anderson et al. 1984). In myocardium, PVB19 can enter endothelial cells (EC), cause vasocontraction, and damage cardiac tissue or activate an inflammation response of the immune system (Yilmaz et al. 2008). The VP1 unique region (VP1u) of the PVB19 capsid has been reported to act as a major determinant of viral tropism for erythroid precursor cells (Mrzljak et al. 2010). Our recent publication demonstrated that exposure of either human- or rat-derived ECs to recombinant VP1u was not acutely cytotoxic but led to the upregulation of cellular stress signaling-related pathways (Rinkūnaitė et al. 2021). Data also suggest that high levels of circulating PVB19 during acute infection can cause endothelial damage, even without active replication or direct internalization of virus into the cardiac cells. Beside the endothelium, the PVB19-positive myocarditis can also increase the levels of leukotrienes and prostaglandins and impair intramyocardial Ca^{2+} circulation and left ventricle functioning (Duechting et al. 2008; Bock et al. 2005; Lupescu et al. 2006; Tschöpe et al. 2005).

In addition, the inflammatory events in the viral myocarditis were shown to be responsible for the changed heart energetical potential (Wei et al. 2014), impaired balance between ECM regulating matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMP) (Pauschinger and Schultheiss 2004), initiation of cell-mediated autoimmune responses (Caforio et al. 2007), and activation of myocardial cell apoptosis or necrosis ultimately affecting the whole myocardium (DeBiasi et al. 2010). Since the main ECM component in the cardiac tissue is collagen type I (up to 85%) (Jugdutt 2003), impairment and/or degradation of its synthesis can negatively affect heart cell communication, initiate cardiomyocyte anoikis, and impinge contraction and proper heart tissue functioning (Frangogiannis 2020; Valiente-alandi et al. 2016; Johnston and Gillis 2017; Michel 2003). However, what pathophysiological processes are ongoing during persistence of PVB19 in DCM myocardium is still unclear and little investigated.

The main objective of this study was to investigate the inflammation, apoptosis, necrosis, and fibrosis in human PVB19-positive and PVB19-negative DCM myocardial tissues and sera with the purpose of evaluating which of these processes could have the highest impact on the chronic remodeling of DCM myocardium. The inflammation level has been confirmed by the intramyocardial infiltration of T cells (CD3, CD45Ro) and macrophages (CD68) and secretion of TNF-α, IL-6, and CRB. The intrinsic apoptotic pathway and mitochondria impairment-related markers (Bcl-2, Bax, caspase-9, Hsp60), extrinsic apoptotic pathway-related markers (APO1/Fas/CD95, FasL, caspase-8), and executing caspase-3 as well as collagen I synthesis−/degradation-related markers (PICP, ICTP, TGF-β1, TIMP1) were investigated both in PVB19-positive and PVB19-negative DCM myocardium tissues and sera. In addition, the levels of MMP1, MMP2, MMP9, and MMP13, markers of ECM degradation in EMBs, were evaluated immunocytochemically, while fibrosis and myocardial necrosis were investigated histochemically. Necrosis was also evaluated by the secretion of high-sensitivity troponin T (hsTnT). We hypothesize that persistent presence of PVB19 in DCM patients' myocardiums is not a benign phenomenon but negatively affects heart extracellular matrix and energetic supply-related processes that can worsen functioning of PVB19-positive DCM myocardiums compared to the PVB19-negative ones.

2 Methods

2.1 Selection of the Patients

The study group consisted of 32 consecutive patients (mean age 43.14 ± 11.86 years) with clinically suspected nonischemic DCM that were admitted to a tertiary referral center. All patients signed informed consent to include their data in the study, and all investigations conformed to the principles outlined in the Declaration of Helsinki. All patients were mainly of NYHA III groups (93–94%). All patients showed enlarged left ventricle (LV) associated with the significantly impaired systolic function (left ventricular ejection fraction (LVEF) was less than

45%) on echocardiography in association with long duration of heart failure symptoms (average 34 months).

Exclusion criteria: (1) Known causes of heart failure (hypertension, significant coronary artery and valvular heart diseases, endocrine disease, significant renal insufficiency, and abuse of drugs or alcohol; (2) acute myocarditis or a history of myocardial infarction; (3) significant coronary artery disease, defined as a proximal stenosis (50% or greater) in one or more main coronary arteries; and (4) patients were excluded if they did not give written informed consent.

2.2 Baseline Characteristics of Patients

All patients underwent a careful anamnestic and physical examination, and other routine laboratory measurements including complete blood count, high-sensitivity CRP, creatinine, and others have been done. In addition, each patient underwent standard transthoracic echocardiographic evaluation on the same day or the day before cardiac catheterization. Transthoracic echocardiography was carried out with GE Vivid 7 or 9 ultrasound system. We have used a routine protocol for conventional echocardiographic measurements. Right heart catheterization was performed to assess hemodynamic parameters: mean right atrial pressure (RAP), mean pulmonary artery pressure (PAP), pulmonary capillary wedge pressure (PCWP), right arterial pressure (RAP), and cardiac index (CI). Baseline characteristics of all patients are presented in Table 1. Both patient groups were treated according to the guidelines (McMurray et al. 2012). No significant difference in consumption of prescribed medications was observed between the groups.

Laboratory measurement of adiponectin (ANP) was done by the Millipore Adiponectin kit according to the manufacturers' recommendations (Millipore, USA). High-sensitivity troponin T (hsTnT) was measured using an Elecsys 2010

Table 1 Baseline characteristics of patients

	Virus-negative group		*Virus-positive group*		
Variable	Total no. of pts.	Value	Total no. of pts.	Value	p value
Sex (male/female)	18	16 (89%)	14	10 (71%)	0.36
Age (years)	18	45.8 ± 14.4	14	42.4 ± 9.4	0.43
NYHA					
III–IV	18	17 (94%)	14	13 (93%)	1
Cardiac parameters					
LBBB (%)	18	6 (33%)	14	1 (7%)	0.1
Permanent AF (%)	18	3 (17%)	14	1 (7%)	0.61
LVEF (%)	18	22 (20, 30)	14	24 (20, 27)	0.54
LVEDD (cm)	18	7.5 (6.6, 7.5)	14	6.8 (6.5, 7.2)	0.35
LVEDDI (cm/m2)	18	3.6 (3.5, 4.0)	14	3.6 (3.4, 3.8)	0.56
Mean Ao (mmHg)	15	95 (92, 100)	12	85 (81, 89)	0.03*
Mean RAP (mmHg)	15	13 (11, 25)	13	7 (4, 11)	0.002*
Mean PCWP (mmHg)	16	27 (21, 34)	13	15 (13, 21)	0.002*
Mean PAP (mmHg)	16	40 (29, 48)	13	27 (18, 29)	0.003*
CI (L/min/m2)	16	1.8 (1.4, 2.4)	13	2.3 (2.1, 2.8)	0.12
PVR, WU	15	3.4 (2.4, 4.7)	13	1.8 (1, 2.5)	0.02*

*Data are presented as the mean ± SD, median (25th percentile, 75th percentile), or n (%). *Significant at $p \leq 0.05$ level
Abbreviations: *NYHA* New York Heart Association functional class, *LBBB* left bundle branch block, *AF* atrial fibrillation, *LVEF* left ventricular ejection fraction, *LVEDD* left ventricular end-diastolic diameter, *LVEDDI* left ventricular end-diastolic diameter index, *Ao* aortic, *RAP* right atrial pressure, *PCWP* pulmonary capillary wedge pressure, *PAP* pulmonary artery pressure, *CI* cardiac index, *PVR* pulmonary vascular resistance

analyzer (Roche Diagnostics, Indianapolis, Indiana). Brain natriuretic protein (BNP) was measured by two-step immunoassay in human plasma using chemiluminescent microparticle immunoassay (CMIA) technology referred as Chemiflex according to the manufacturers' recommendations. Serum sample and anti-BNP-coated paramagnetic particles were combined. After incubation, samples were washed and combined with an anti-BNP acridinium-labeled conjugate. Samples were incubated and washed again and the chemoluminescence initiating mixture was added. Resulting chemoluminescence reaction has been measured by a chemoluminometer and expressed as relative light units (RLU).

2.3 Preparation of Endomyocardial Biopsies (EMBs) and Blood Samples

Right ventricular EMBs were obtained using a flexible bioptome via the right femoral vein (Cooper et al. 2007). Biopsies were taken from the right interventricular septum of the patients with confirmed absence of coronary artery disease. Collected heart tissue biopsies were immediately inserted into clean cryovials, carefully labeled, and registered. The EMBs were stored at −70 °C as retained biosamples. All biopsy samples were carefully labeled and registered before cryopreservation. EMB specimens were thawed on ice before analyzing, appropriately prepared for the assays, and immediately investigated. EMBs were subjected to conventional histochemical and immunohistochemical evaluation, ELISA assay, and DNA and RNA extraction for the amplification of viral genomes.

In parallel to the EMBs, three serum-separating (SST II) 8.5 ml tubes (BD Vacutainer®) were collected for serum sampling from each patient. Collected blood samples were kept at room temperature for 30–45 min (no longer than 60 min) to allow clotting. Samples were centrifuged for 15 min at the manufacturer's recommended speed (1,000–2000 RCF). The upper layer was carefully aspired, checked for turbidity, aliquoted into cryovials, labeled, and stored at −70 °C. Before ELISA measurements, all serum samples were thawed on ice, centrifuged at 12,000 g for 5 min, and, if necessary, appropriately diluted.

2.4 Separation of EMBs According to the Expression of PVB19

The presence of various types of viruses (adenovirus, herpes simplex viruses 1 and 2, varicella zoster virus, Epstein-Barr virus, cytomegalic virus, parvovirus B19, hepatitis C virus, entero virus, rubella virus and human herpes virus 6) was negligible and samples were eliminated. The dominating virus expression in human DCM myocardiums determined by nested PCR was PVB19 (86%); therefore, all patients' EMB samples were subdivided into two groups: PVB19-positive (n = 14) and PVB19-negative (n = 18).

Nested PCR primers were chosen for the detection of viruses including adenovirus as in (Allard et al. 2001), herpes simplex viruses 1 and 2, varicella zoster virus, Epstein–Barr virus, cytomegalic virus, parvovirus B19, hepatitis C virus, enterovirus, rubella virus as in (McIver et al. 2005), human herpesvirus 6 (HHV-6A and HHV-6B, GenBank accession nos. NC001664.2 and NC000898.1, respectively), Kirsten rat sarcoma viral oncogene homolog (KRAS, GenBank accession no. NM033360), and ubiquitin C (UBC, GenBank accession no. NM021009). Forward and reverse primers for the nested PCR detection of HHV-6, UBC, and KRAS were synthesized by Metabion Company (Martinsried, Germany). Used primers are shown in Table 2. Forward primers for the second round of PCR were labeled with 6-carboxyfluorescein (FAM) at the 5′ end.

Genomic DNA and total RNA were extracted simultaneously using ZR-Duet™ DNA/RNA Miniprep kit (Zymo Research, Irvine, CA, USA). RNA (1 μg) was reversely transcribed in 20 μl reaction volumes using random hexamers and First Strand cDNA Synthesis Kit (Thermo Fisher, Vilnius, Lithuania) according to the vendor's recommendations and diluted to 100 μl with deionized water after the reaction. All PCRs

Table 2 Primers for the detection of human herpesvirus 6 (HHV-6), Kirsten rat sarcoma viral oncogene homolog (KRAS), and ubiquitin C (UBC)

Primer	Sequence (5′ – 3′)
HHV6-N1 forward	ACCCGAGAGATGATTTTGCGTG
HHV6-N1 reverse	GCAGAAGACAGCAGCGAGATAG
HHV6-N2 forward	CATAGCAACCTTTTCTAGCTTTGAC
HHV6-N2 reverse	TCTATAACATAAATGACCCCTGGGA
UBC-N1 forward	TTCTTTCCAGAGAGCCGAAC
UBC-N1 reverse	CCCATCTTCCAGCTGTTTTC
UBC-N2 forward	TGGGTCGCAGTTCTTGTTTG
UBC-N2 reverse	CCTTCCTTATCTTGGATCTTTGCC
KRAS-N1 forward	CTTTGGAGCAGGAACAATGTCT
KRAS-N2 forward	AATCCAGACTGTGTTTCTCCCT
KRAS-N1/N2 reverse	TACACAAAGAAAGCCCTCCCC

were run on a Professional Standard thermocycler (Biometra, Göttingen, Germany) as described in (Allard et al. 2001). KRAS and UBC detection was used to validate extraction of nucleic acids and was performed in parallel according to the conditions for viral DNA and RNR, respectively. Final PCR products were tenfold diluted and analyzed by capillary electrophoresis on a Genetic Analyzer 3130*xl*, using GeneScan™ 600 LIZ™ Size Standard and Gene Mapper Software v4.1 (Applied Biosystems, Foster City, CA, USA) for sizing PCR fragments. In the case of positive result, the genomic DNA or RNR specimens of blood samples were also tested and excluded from the EMBs.

2.5 Histochemical and Immunohistochemical Assays of EMBs

The EMBs were fixed in 10% neutral buffered formalin and paraffin-embedded in a tissue processor. To estimate the extent of fibrosis, the EMB specimens were stained with connective tissue stain Masson's trichrome according to the standard protocol. The total cardiac fibrosis (including interstitial and perivascular) was assessed: keratin and muscle fibers were stained red, whereas collagen was stained blue.

Immunohistochemical staining was performed using antibodies: anti-CD3 (DAKO Hamburg, Germany), anti-CD45Ro (DAKO Hamburg, Germany) and anti-CD68 (DAKO Hamburg, Germany), anti-MMP1 (Spring Bioscience Corp., USA), anti-MMP2 (Leica Biosystems Newcastle Ltd., UK), anti-MMP9 (Leica Biosystems Newcastle Ltd., UK), and anti-MMP13 (Novus Biologicals Europe, Cambridge, UK).

The intramyocardial inflammation was diagnosed according to the criterion established by the European Society of Cardiology Working Group on Myocardial and Pericardial Diseases (an expert consensus group): immunohistochemical detection of significant focal or diffuse cellular infiltration in the EMB ($\geq$14 leucocytes/mm^2 including up to 4 monocytes/mm^2 with the presence of CD3-positive T lymphocytes $\geq$7 cells/mm^2) (Caforio et al. 2007).

2.6 Digital Evaluation of Histochemical and Immunohistochemical EMB Staining

Inflammatory infiltrates in the biopsies were immunohistochemically classified on tissue sections, according to the expression of CD3 (T lymphocytes), CD45Ro (active-memory T lymphocytes), and CD68 (macrophages). The number of positively stained cells in each biopsy sample was scored by a pathologist and expressed as number of positive cells/mm^2.

Digital images from the experimental glass slides were obtained using ScanScope Digital

Slide Scanner (Aperio, Vista, CA) at x20 magnification and archived on a devoted Spectrum Server 11.1.0.751 (Aperio). Quality control of the scanned images and all further analysis were performed using ImageScope V11.1.2.760 (Aperio) and WebScope V11.1.0.756 (Aperio).

A Genie algorithm was used to measure the extent of fibrosis, to calculate immunoassayed cells, and to evaluate the area of immunohistochemically stained elements in the myocardium. Genie (GENetic Imagery Exploration) is a pattern recognition algorithm that distinguishes spatial and morphological features based on structures (classes) provided by the user. The algorithm was run for the whole slides, ignoring the number of overlapped tissue sections on it – making the process fully automated. For more details on the Genie methodology to evaluate fibrosis, see our previous publication (Daunoravicius et al. 2014).

For this study, specific Genie classifiers were developed: (A) Basic tissue recognition Genie classifier v1 algorithm was used to identify myocardial fibrosis. We have used only spatial recognition, disabling the detection of morphological features. Total percentage of cardiac fibrosis was adjusted to a total tissue area ignoring the glass; (B) Genie pixel counting algorithm has been used to measure MMP1, MMP2, MMP9, MMP13, and PICP in myocardial biopsies. There were no suitable antibodies for the histochemical ICTP investigation. The results were shown as sum of weak/moderate/strong positive and negative pixels. In parallel, the positive pixels were counted empirically by the pathologist.

2.7 ELISA Assays in EMBs and Serums

Collected heart tissue EMBs were lysed in 100 μl of RIPA lysis buffer (Thermo Scientific Inc., USA) supplemented with protease and phosphatase mini inhibitor tablets, 1 mM PMSF, 1 mM Na_2VO_4, and 25 mM NaF according to the manufacturer's suggestion (Thermo Scientific Inc., USA). EMB samples were sonicated at 10 mV for 2 × 5 s on ice using a Bandelin Sonopuls sonicator, kept 30 min on ice, centrifuged at 12,000 g for 15 min, aliquoted, and stored at −70 °C. Protein level of serum and EMB samples was measured using a modified Lowry Protein Assay kit according to the manufacturer's recommendations (Thermo Scientific Inc., USA). Absorbance was measured with a spectrophotometer (Asys UVM 340 Microplate Reader UK – Biochrom Ltd.) set at 750 nm. A bovine serum albumin (BSA) standard curve was made to determine the protein concentration of each unknown sample. Protein concentration was expressed as mg/ml. The final concentration of estimated biomarkers by ELISA was expressed as ng/mg of protein.

Levels of apoptotic pathway-related proteins in EMBs and serum samples were measured using specific biomarkers: to identify the intrinsic/mitochondrial apoptotic pathway, the antiapoptotic Bcl-2, the proapoptotic Bax, the intrinsic apoptotic pathway initiating caspase-9 were estimated (Elabscience Biotechnology Co., Ltd., China). In addition, the mitochondrial functioning was measured by the release of myocardial intramitochondrial heat shock protein 60, (HSP60) (Assaypro, Saint Charles, Missouri, USA). The executing apoptotic caspase-3 was measured by Novus Biologicals Europe kits (Cambridge, UK). An extrinsic apoptotic pathway biomarkers APO1/Fas/CD95 (Fas receptor), FasL (Fas ligand), and extrinsic pathway initiating caspase-8 were assayed using Novus Biologicals Europe kits (Cambridge, UK).

The marker of collagen synthesis procollagen I C-terminal propeptide (PICP) was measured using Bio-Medical ELISA kit (Bio-Medical Assay Co., Ltd., China), while the marker of collagen degradation C-terminal telopeptide of type I collagen (ICTP) was measured by the Biotech kit (Shanghai BlueGene Biotech Co., Ltd., China). The fibrosis stimulating transforming growth factor beta 1 (TGF-β1) was estimated by the Invitrogen ELISA kit (Invitrogen, Paisley, UK) according to the manufacturers' recommendations.

The pro-inflammatory cytokines TNF-α, IL-6, and IL-1β were assayed in serums by solid-phase, chemiluminescent immunometric assays using

IMMULITE/Immulite 1,000 systems (Immulite, Siemens) according to the manufactures' instructions: TNF-α (Catalog No: LKNFZ (50 tests), LKNF1 (100 tests); IL-6 (Catalog No: LK6PZ (50tests), and IL-1β (Catalog No: LKL1Z (50 tests). Galectin-3 in serum samples was also estimated using ELISA assay according to the manufacturers' instructions (BG Medicine, Inc.).

2.8 Statistical Analyses

All statistical analyses were performed using the R studio package (4.0.3 version), SPSS package (version 19.0 for Windows; SPSS Inc., Chicago, IL, USA) at 5% significance level. The normality of the data distribution was tested by the Shapiro–Wilk test. Normally distributed continuous variables were expressed as the mean ± SD. Other continuous variables were expressed as the median (25th percentile, 75th percentile), and categorical data as counts and percentages. Differences in parameters of virus-positive and virus-negative patient groups were tested by Student's independent *t* test for normally distributed variables or the Wilcoxon–Mann–Whitney test for non-normally distributed variables. Comparisons of categorical variables between the groups were made using the chi-square test or Fisher's exact test if expected values were < 5. Spearman correlation coefficient was used to assess the association between variables.

2.9 Ethical Approval

The study has been approved by the Vilnius Region Biomedical Research Ethics Committee (license No.158200-09-382-103). All patients gave written informed consent. The investigation conforms to the principles outlined in the Declaration of Helsinki. The article does not contain any studies with animals performed by any of the authors.

3 Results

3.1 Summary of Chosen Biomarkers in EMBs and Serums

In this study, the intensity of inflammation, apoptosis, necrosis, fibrosis, and synthesis/degradation of the main heart ECM component collagen I ongoing in PVB19-positive and PVB19-negative EMBs and sera has been investigated (Tables 3, 4, and 5).

Summarized investigated histochemical and immunohistochemical biomarkers in both types of the EMBs are shown in Table 3. The inflammation level has been evaluated immunohistochemically by measuring the myocardial infiltrates: T cells (CD3), naive/memory T cells (CD45Ro), and macrophages (CD68) (Table 3) in EMBs. The total level of inflammation was slightly increased in PVB19-positive myocardiums compared to the PVB19-negative ones. However, the total level of fibrosis was significantly lower in PVB19-positive EMBs compared to the PVB19-negative ones that could be due to the significantly increased levels of ECM degrading MMP1 and MMP2 (Table 3).

Since inflammation can also initiate apoptosis or necrosis, appropriate biomarkers were investigated by ELISA (Table 4). The levels of intrinsic/mitochondrial apoptotic pathway regulating proteins were measured: an antiapoptotic Bcl-2 (B-cell lymphoma 2), proapoptotic Bax protein, the apoptotic cascade initiating caspase-9; apoptosis executing caspase-3, as well as mitochondria protecting heat shock protein 60 (Hsp60) have been also investigated. Although no active apoptosis was found, the negative impact of PVB19 on myocardial mitochondria (~threefold lower levels of mitochondria protecting proteins Bcl-2 and Hsp60) was observed compared to the myocardiums without PVB19 (Table 4 and Fig. 3b).

The biomarkers indicating extrinsic apoptotic pathway such as CD95 (APO1/Fas), a prototype death receptor characterized by the presence of an 80-amino acid death domain in its cytoplasmic tail, FasL (the Fas receptor ligand), and extrinsic apoptotic cascade initiating caspase-8, have been

Table 3 Intramyocardial biomarkers assessed immunohistochemically and histochemically

Variable	*Virus-negative group*		*Virus-positive group*		
	Total no. of pt.	Value	Total no. of pt.	Value	p value
Immunohistochemically assessed inflammatory infiltrates					
CD3+, cells/mm^2	17	10 (6–16)	14	11 (8.3–14.3)	0.37
CD45Ro+, cells/mm^2	17	5 (4–10)	14	6.5 (5–11.5)	0.36
CD68+, cells/mm^2	17	5 (3–7)	14	3.5 (3–5)	0.22
Inflammation, n (%)	17	9 (53)	14	9 (64)	0.52
Immunohistochemically assessed fibrotic biomarkers					
MMP1 (%)	15	6.67 ± 1.52	14	14.65 ± 2.99	0.022*
MMP2 (%)	15	2.89 ± 0.34	14	7.035 ± 1.07	0.029*
MMP9 (%)	15	10.97 ± 4.21	14	9.68 ± 1.57	0.783
MMP13 (%)	15	12.28 ± 2.53	14	12.66 ± 2.67	0.919
Histochemically assessed fibrosis					
Cardiac fibrosis (%)	18	17.97 ± 2.20	14	10.48 ± 1.68	0.012*

Data are presented as the mean ± SD or median (25th percentile, 75th percentile). *Significant at $p \leq 0.05$ level
Abbreviations: *MMP* matrix metalloproteinases, *PICP* type I procollagen carboxy-terminal propeptide, *ICTP* type I collagen carboxy-terminal telopeptide, *TGF-β1* transforming growth factor β1, *Hsp60* heat shock protein 60, *Bcl-2* B-cell lymphoma 2 apoptotic regulator, *Bax* Bcl-2-like protein 4, *Fas* Fas receptor, *BNP* brain natriuretic protein, *hsTnT* high-sensitivity troponin T

Table 4 Intramyocardial biomarkers assessed by ELISA

Variable	*Virus-negative group*		*Virus-positive group*		
	Total no. of pt.	Value	Total no. of pt.	Value	p Value
Intrinsic apoptotic pathway-related					
Bcl2 (ng/mg protein)	18	58.3 (6.8–122.1)	13	20.7 (0–107.9)	0.47
Bax (ng/mg protein)	18	3.6 (0–11.7)	13	Not detected	–
Caspase-9 (ng/mg protein)	18	41.9 (6.0–102.6)	13	20 (0–36.3)	0.12
Caspase-3 (ng/mg protein)	18	0.25 (0.01–0.58)	12	0.17 (0.08–0.3)	0.35
HSP60 (ng/mg protein)	17	22.2 (0–36.1)	13	7.6 (0–26.1)	0.88
Extrinsic apoptotic pathway-related					
APO1/Fas (ng/mg protein)	18	4.3 (2.9–6.9)	13	3.3 (2.5–4.5)	0.23
Caspase-8 (ng/mg protein)	18	1.2 (0.65–1.51)	13	1.05 (0.49–1.28)	0.39
Caspase-3 (ng/mg protein)	18	0.25 (0.01–0.58)	12	0.17 (0.08–0.3)	0.35
Fibrosis-related					
PICP (ng/mg protein)	17	0 (0–1.94)	13	Not detected	–
ICTP (ng/mg protein)	17	Not detected	13	0.03 (0–0.25)	–
TIMP1 (ng/mg protein)	18	9.6116 ± 1.4578	13	5.8772 ± 1.6243	0.266
TGF-β1 (pg/mg protein)	17	0 (0–24.2)	13	0 (0–5.34)	0.45

Data are presented as the mean ± SD or median (25th percentile, 75th percentile). *Significant at $p \leq 0.05$ level
Abbreviations: *MMP* matrix metalloproteinases, *PICP* type I procollagen carboxy-terminal propeptide, *ICTP* type I collagen carboxy-terminal telopeptide, *TGF-β1* transforming growth factor β1, *Hsp60* heat shock protein 60, *Bcl-2* B-cell lymphoma 2 apoptotic regulator, *Bax* Bcl-2-like protein 4, *Fas* Fas receptor, *BNP* brain natriuretic protein, *hsTnT* high-sensitivity troponin T

Table 5 Serum biomarkers assessed by ELISA

	Virus-negative group		*Virus-positive group*		
Variable	Total no. of pt.	Value	Total no. of pt.	Value	p value
Inflammatory cytokines in serums					
TNF-α, pg/mL	18	8.6 (7.5–9.7)	13	7.4 (5.9–9.9)	0.84
IL-6, pg/mL	18	6.01 (2.48–10.26)	13	2.01 (2.0–5.88)	0.07
CRB, mg/L	17	6.9 (4.1–15.3)	12	4.6 (1.9–17)	0.46
Intrinsic apoptotic pathway-related					
Bcl2 (ng/mg protein)	17	Not detected	14	Not detected	–
Bax (ng/mg protein)	18	2.2 (2.0–2.7)	14	2 (1.9–2.1)	0.19
Caspase-9 (ng/mg protein)	18	0 (0–0.4)	14	0.04 (0–0.9)	0.24
Caspase-3 (ng/mg protein)	18	0 (0–0.01)	13	0.006 (0–0.2)	0.47
HSP60 (ng/mg protein)	17	0 (0–0.12)	14	0 (0–0.22)	0.9
Extrinsic apoptotic pathway-related					
APO1/Fas (ng/mg protein)	17	Not detected	14	Not detected	–
Caspase-8 (ng/mg protein)	18	0 (0–0.01)	14	0.0007 (0–0.004)	0.93
Caspase-3 (ng/mg protein)	18	0 (0–0.01)	13	0.006 (0–0.2)	0.47
Fibrosis-related					
PICP (ng/mg protein)	17	0.13 (0.07–0.27)	14	0.09 (0.02–0.13)	0.13
ICTP (ng/mg protein)	17	0.013 (0.01–0.02)	14	0.009 (0.006–0.019)	0.23
PICP/ICTP	17	10 (6.18–18.24)	14	10 (0.61–14.44)	0.24
TGF-β1 (pg/mg protein)	17	12.1 (10.42–18.23)	14	14.05 (11.54–18.41)	0.32
TIMP1 (ng/mg protein)	18	6.1611 ± 0.2028	14	5.7538 ± 0.2945	0.266
Inflammatory cytokines in serums					
CRP, mg/L	17	6.9 (4.1–15.3)	12	4.6 (1.9–17)	
IL-6, pg/mL	18	6.01 (2.48–10.26)	13	2.01 (2.0–5.88)	
TNF-α, pg/mL	18	8.6 (7.5–9.7)	13	7.4 (5.9–9.9)	
Other biomarkers in serums					
Adiponectin (μg/mL)	18	26.5 (14.05–34.73)	13	14.8 (10.4–20.19)	0.06
BNP (ng/l)	18	1,165 (690–3,171)	14	715 (104–1,350)	0.08
hsTnT (pg/mL)	18	35.9 (25.5–59.7)	13	20.8 (17.6–29.9)	0.02*
Galectin-3 (ng/ml)	17	11.5 (9.74–15.11)	13	9.7 (8.45–13.9)	0.34

Data are presented as the mean ± SD or median (25th percentile, 75th percentile). *Significant at $p \leq 0.05$ level
Abbreviations: *MMP* matrix metalloproteinases, *PICP* type I procollagen carboxy-terminal propeptide, *ICTP* type I collagen carboxy-terminal telopeptide, *TGF-β1* transforming growth factor β1, *Hsp60* heat shock protein 60, *Bcl-2* B-cell lymphoma 2 apoptotic regulator, *Bax* Bcl-2-like protein 4, *Fas* Fas receptor, *BNP* brain natriuretic protein, *hsTnT* high-sensitivity troponin T

also investigated by ELISA (Table 4). Data revealed the absence of activation of extrinsic apoptotic pathway.

The investigation of inflammation-, apoptosis-, necrosis-, and fibrosis-related biomarkers in PVB19-positive versus PVB19-negative serums by ELISA (Table 5) assay showed significant absence of necrosis (~twofold lower level of secreted hsTnT). Secreted apoptotic biomarkers, similar to the EMBs, did not show activation of

apoptosis in PVB19-positive myocardiums. The levels of other inflammation-related biomarkers such as adiponectin (adipokine, which decrease is related to the obese-induced cardiovascular diseases, inflammation, and resistance to insulin), brain natriuretic protein (BNP), and C-reactive protein (CRP) also were almost twice lower in PVB19-positive serums compared to the PVB19-negative ones.

In order to better understand inflammation, fibrosis, and cell death processes ongoing during persistent presence of PVB19 in DCM myocardiums, we will evaluate the dependencies and intercorrelations of measured biomarkers showing further pathophysiological tendencies in the PVB19-positive DCM myocardiums.

3.2 The Inflammation in PVB19-Positive and PVB19-Negative DCM Myocardiums

The acute intramyocardial viral infection is usually accompanied by the intensive inflammation leading to the myocardial fibrosis or even cardiomyocyte death. However, the chronic intramyocardial PVB19 infection did not significantly increase the level of intramyocardial inflammatory infiltrates such as CD3 (T cells) and CD45Ro ("memory" T-cell subsets) compared to the PVB19-negative EMBs, whereas the level of macrophages (CD68) was even decreased (Fig. 1a). A similar tendency was observed measuring the secreted inflammatory markers TNF-alpha, IL-6, and CRB that were decreased in PVB19-positive serums compared to the PVB19-negative ones (Fig. 1b).

3.3 The Fibrosis in PVB19-Positive and PVB19-Negative DCM Myocardiums

Many studies have shown that fibrosis is strongly related to the inflammation (Thomas and Grisanti 2020). However, data of this study showed very mildly increased total level of inflammation in PVB19-positive EMBs compared to the PVB19-negative EMBs (Table 3). However, the level of fibrosis in PVB19-positive EMBs, evaluated by Masson's trichrome staining, was lower ($10.4818 \pm 1.6854\%$) compared to the PVB19-negative EMBs ($17.9724 \pm 2.2076\%$) (Fig. 2a, b). The intensity of fibrosis was quantified by Genie algorithm as written in the methods section (Fig. 2c). In addition, the cardiac fibrosis in PVB19-positive myocardiums inversely correlated with MMP1 level, which suggest the negative impact of MMPs on fibrosis (Fig. 2d). In addition, the downregulation by twofold levels of TIMP1 in myocardiums also supports the negative role of MMPs on the heart fibrosis (Table 3).

3.4 The Apoptosis in PVB19-Positive and PVB19-Negative DCM Myocardiums

Seeing that there was no intensive inflammation and fibrosis in PVB19-positive samples vs PVB19-negative samples, we have investigated the levels of apoptosis and necrosis. The chronic presence of PVB19 did not induce apoptosis in DCM myocardium: the levels of caspase-8- (external apoptotic pathway), caspase-9 (intrinsic/mitochondrial apoptotic pathway), and caspase-3 (an apoptosis executing caspase) were lower in PVB19-positive EMBs compared to the PVB19-negative ones (Fig. 3a).

However, the negative impact of PVB19 persistence on DCM myocardium was observed: the levels of Bcl-2 (an antiapoptotic protein, protecting inner mitochondrial membrane) and heat shock protein 60 (Hsp60, a mitochondria protecting chaperone) were threefold lower compared to the PVB19-negative ones (Fig. 3b). Data suggest that chronic presence of PVB19 in DCM myocardium may negatively affect energy supply in DCM myocardium and deepen pathological processes. The impaired energetic supply was shown to be typical for chronic myocardial degeneration processes (Sag et al. 2013).

In addition, the levels of secreted caspase-8, caspase-9, and caspase-3 were slightly higher in the PVB19-positive DCM patients' serums

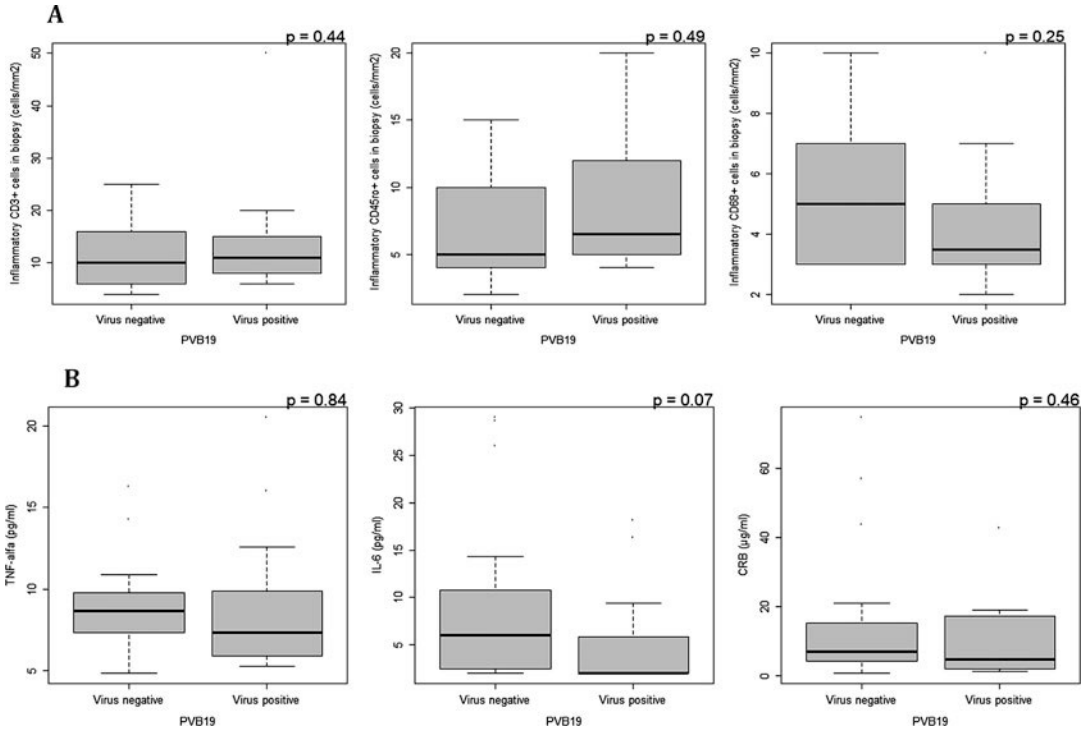

Fig. 1 Evaluation of inflammation biomarkers in PVB19-negative and -positive EMBs and serums. (**a**) Inflammatory infiltrates in EMBs. (**b**) Secreted inflammatory cytokines. The digital quantification of inflammatory infiltrates was done as described in Method part. Data are shown as means ± SD and are significant at $p \leq 0.05$

compared to the PVB19-negative ones (Fig. 3c), which might be related to the absence of apoptosis in EMBs: the more intramyocardial components are secreted, the less remains in the myocardium.

3.5 The Necrosis in PVB19-Negative and PVB19-Positive Myocardiums

The level of necrosis in PVB19-positive and PVB19-negative EMB samples was investigated histochemically by hematoxylin and eosin (H&E) staining: the necrotic cells should have bigger and swollen nuclei compared to the healthy ones. Histological staining showed only few bigger cell nuclei in PVB19-positive EMB compared to the virus-negative samples and confirmed the absence of intensive necrosis in PVB19-positive DCM biopsies (Fig. 4a). The decreased release of high-sensitivity troponin T (hsTnT), a serum marker of cardiomyocyte necrosis, in PVB19-positive patients' serums compared to the PVB19-negative samples confirmed the absence of necrosis in virus-positive myocardium (Fig. 4b).

3.6 The Correlation Analysis of Apoptotic and Fibrotic Biomarkers in PVB19-Positive Myocardiums

Since many measured biomarkers did not show the significant changes in PVB19-positive EMBs compared to the PVB19-negative ones, we have investigated their correlation tendencies in order to evaluate what processes could go on in the viral

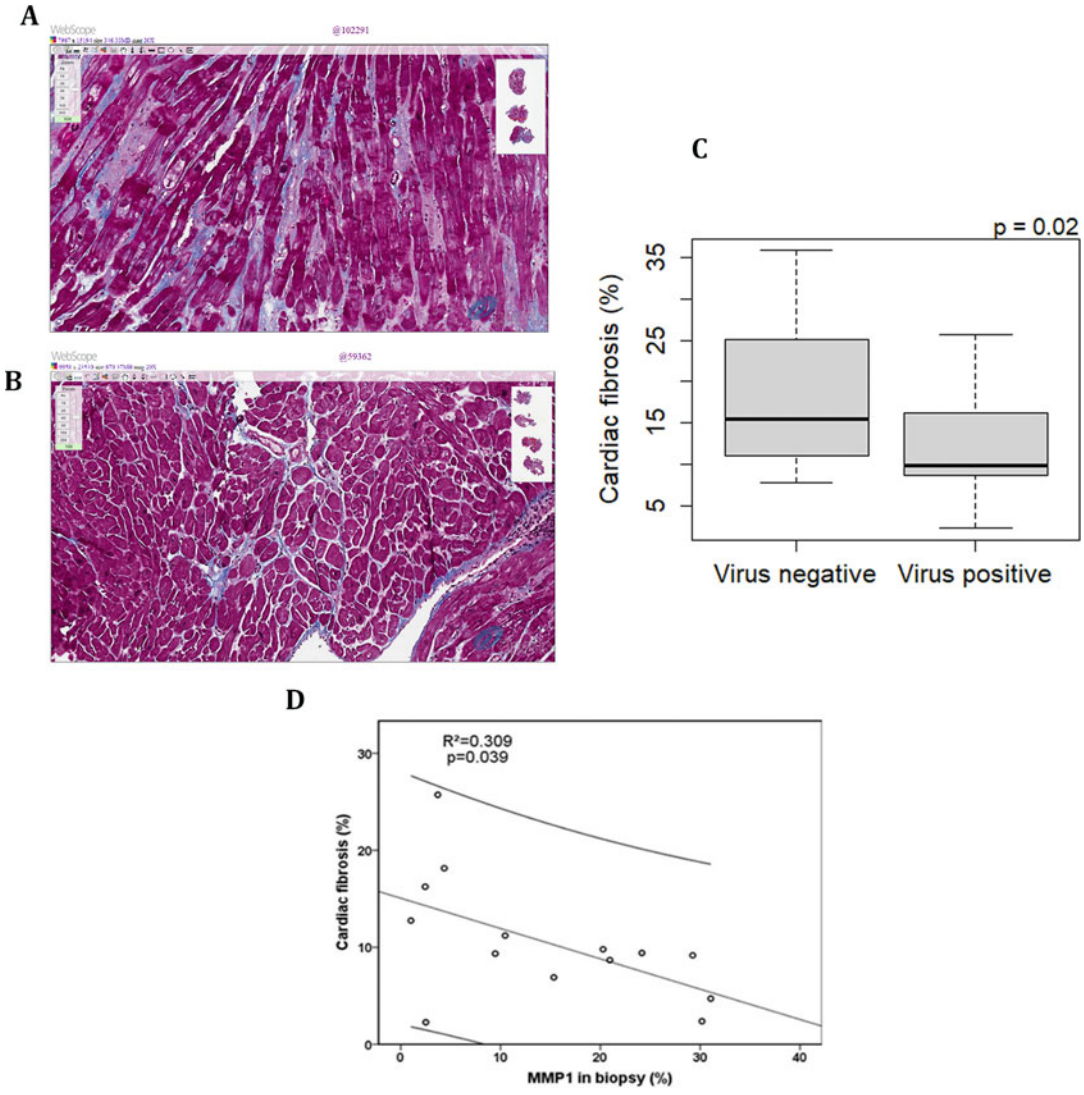

Fig. 2 The histochemical evaluation of fibrosis in virus-negative and -positive EMB samples. The microscopical images of Masson's trichrome stained PVB19-negative (**a**) and PVB19- positive EMBs (**b**) representative histological micrographs of each group are shown. (**c**) Digital quantification of fibrosis in PVB19-negative and PVB19-positive myocardium as described in method part. Data are shown as median and interquartile range and are significant at $p \leq 0.05$. (**d**) Correlation analysis of cardiac fibrosis and MMP1 in PVB19-positive EMBs

myocardium if no antiviral therapy would be applied.

The more intensive or long-lasting PVB19 infection in the EMBs might activate apoptotic cell death: the extrinsic apoptotic pathway Fas receptor significantly correlates with an executing apoptotic protease caspase-3 (Fig. 5a); both intrinsic and extrinsic apoptotic pathways can be activated in viral myocardium (caspase-9 significantly correlates with caspase-8) (Fig. 5b).

Finally, the inverse correlation between apoptotic DNA fragmentation and fibrosis in PVB19-positive EMBs (Fig. 5d) suggests that persistent presence of PVB19 in the myocardium can

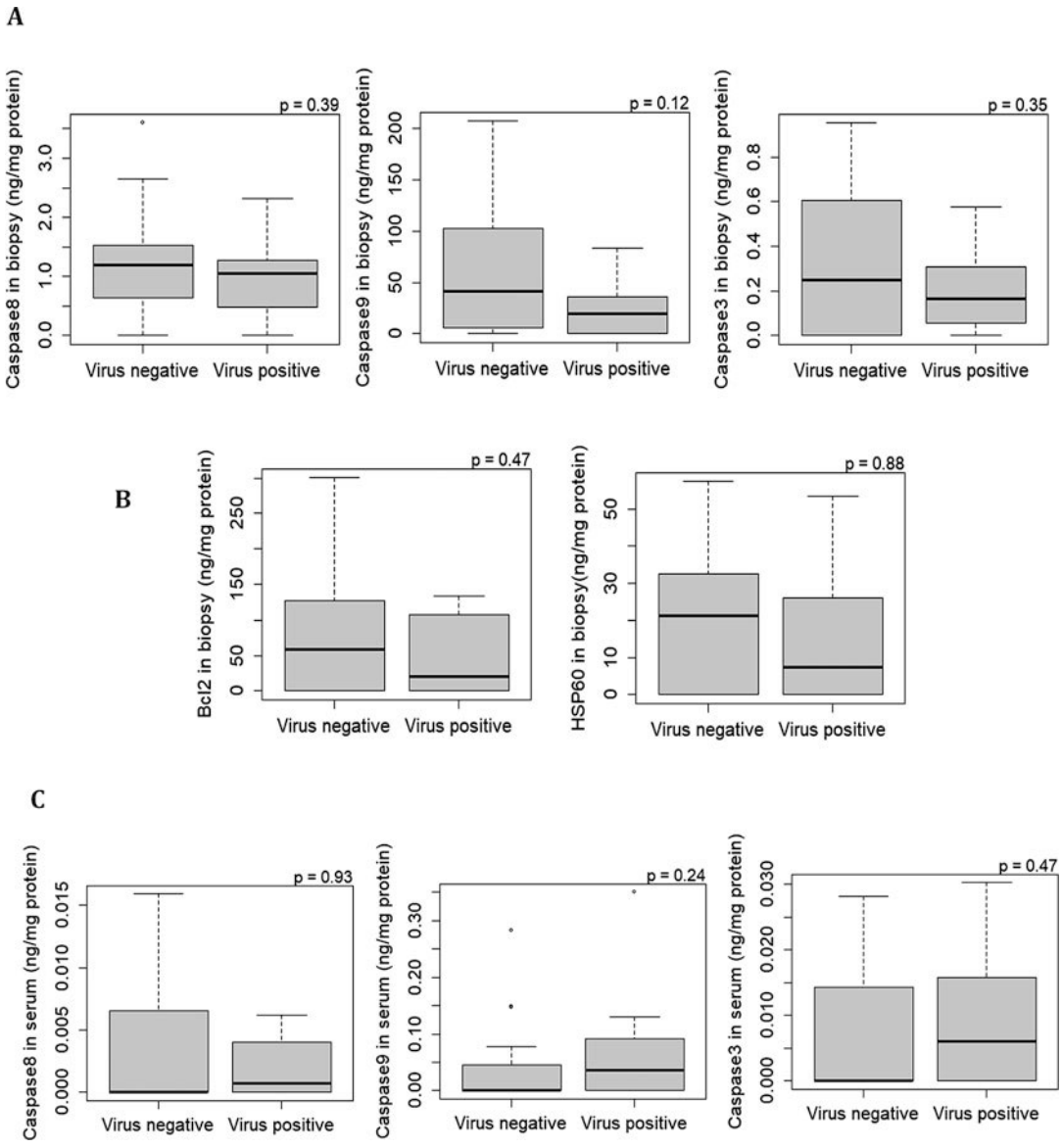

Fig. 3 The evaluation of apoptosis by ELISA in PVB19-negativeand -positive biopsies and serums. (**a**) The decreased levels of caspase 8, 9, 3 in viral EMBs. (**b**) The decrease of mitochondria protecting proteins in viral EMBs. (**c**) The increased levels of caspase 8, 9 and 3 in the PVB19-positive serums. Data are shown as median and interquartile range. Data are significant at $p \leq 0.05$

activate cell death (DNA fragmentation) rather than fibrosis (Fig. 5c) with a primary role of extrinsic apoptotic pathway in it (Fig. 5d). There is also a possibility that further presence of PVB19 in myocardium could sensitize it for further toxic exposures.

3.7 The Correlation Analysis of Secreted Biomarkers in PVB-Positive Patients' Serums

The correlation analysis of secreted biomarkers also confirmed the decrease of fibrosis in

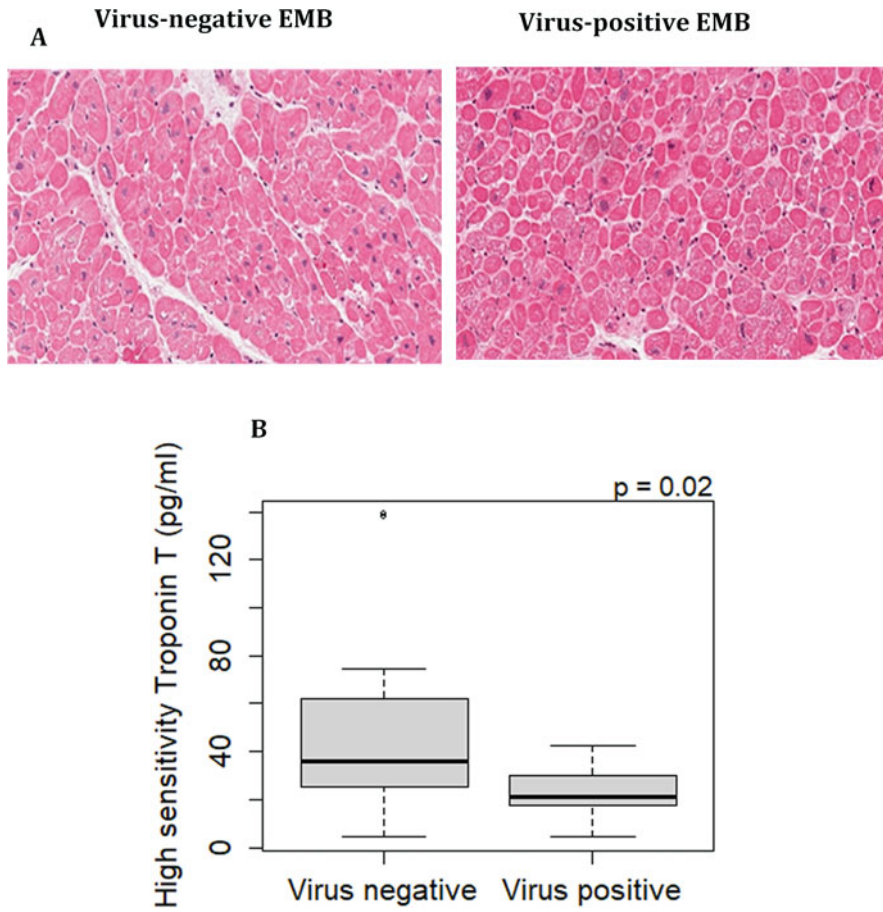

Fig. 4 The level of necrosis in PVB19-negative and -positive EMBs and serums. (**a**) The intensity of necrosis in PVB19-negative and -positive EMBs estimated histochemically (hematoxylin and eosin staining) and evaluated according to the swollen nuclei (blue staining). Representative histochemical images of each group are shown. Magnification: x10. (**b**) Secretion of high sensitivity troponin T (hsTnT) in PVB19-negative and -positive serums. Data are shown as median and interquartile range. Data are significant at $p \leq 0.05$

PVB19-positive patients' serums compared to the negative ones: secreted tissue metalloproteinase inhibitor TIMP1 significantly directly correlated with the secreted MMP9 (Fig. 6a), and inversely with collagen I synthesis biomarker PICP (Fig. 6b). So, the correlations analysis of measured both EMBs and secreted biomarkers can give an additional information about the further processes which can take place in PVB19-positive DCM myocardiums.

4 Discussion

Myocarditis was shown to be an inflammation-related disease strongly impairing myocardium, while DCM is less harmful and heterogenic and has various etiologies (Mason 2003; Merlo et al. 2019). However, more than 30% of myocarditis can give rise to DCM with progression to heart failure (Feldman and McNamara 2000). In about

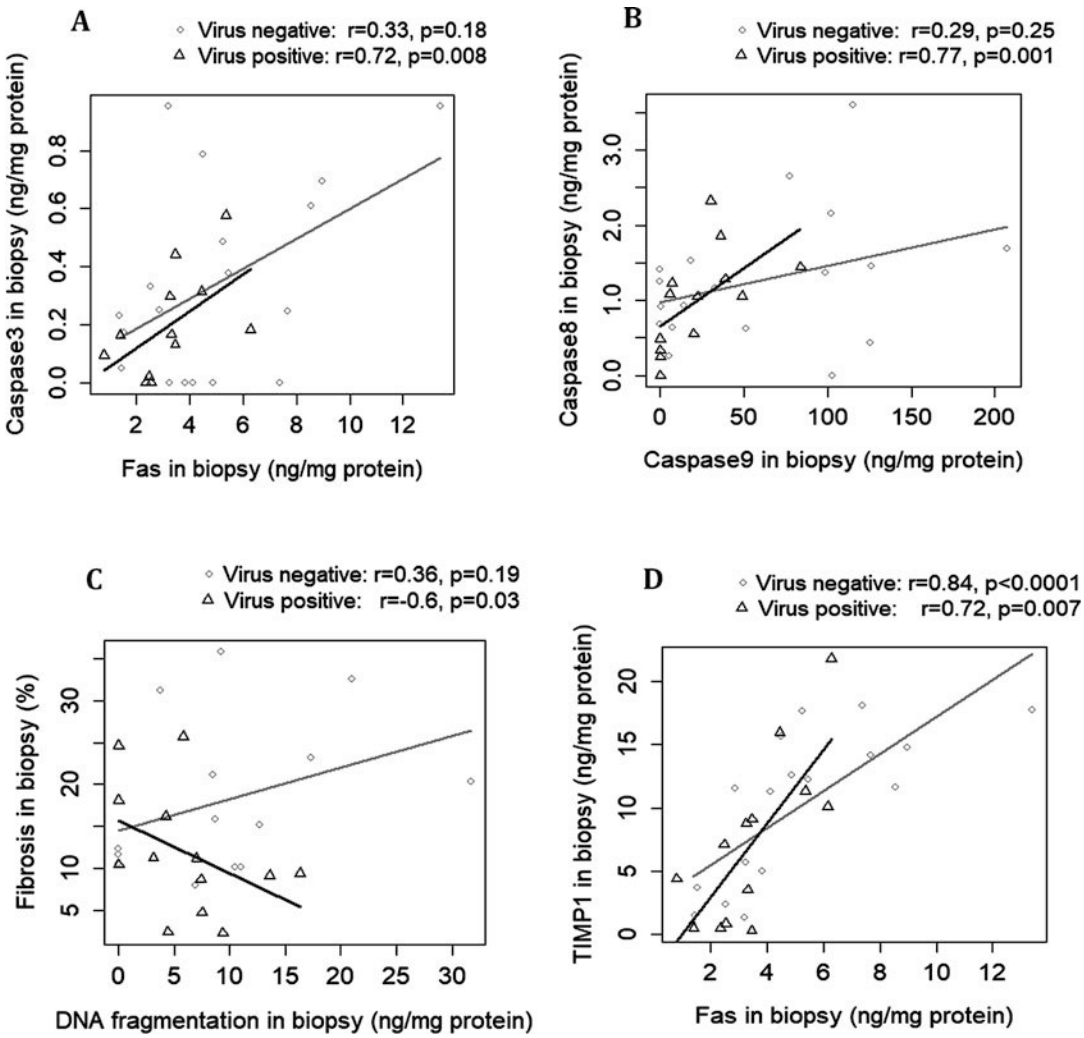

Fig. 5 The correlation analysis of apoptosis and fibrosis processes in PVB19- positive vs -negative myocardium. (**a**) Significant correlation of extrinsic apoptotic pathway activator Fas receptor with executing caspase 3 in viral EMBs. (**b**) Significant correlations of extrinsic (caspase 8) and intrinsic (caspase 9) apoptotic pathways in viral EMBs. (**c**) Significant inverse correlations between fibrosis and apoptosis-initiated DNA fragmentation. (**d**) Significant correlation between MMP1 inhibitor TIMP1 and Fas receptor in EMBs. (**e**) Data are significant at $p \leq 0.05$

50% of all idiopathic DCM cases, chronic virus persistence was observed, which was associated with a worse heart functioning compared to the virus-negative ones (Pankuweit et al. 2000; Tschöpe et al. 2005). It has long been thought that the adeno- and enteroviruses are the most cardiotropic viruses, but later on PVB19 was found in up to 50% of all DCM cases and associated with worse outcome (Zimmermann et al. 2010; Pankuweit et al. 2003; Pankuweit et al. 2005). The initial myocardial injury or acute viral myocarditis usually lasts for a very short period subsequently activating infiltration of inflammatory cells and autoimmune response, while DCM phase is most hardly diagnosed and least studied and is the most difficult to treat (Mason 2003; Zimmermann et al. 2010). It explains why many authors choose to explore

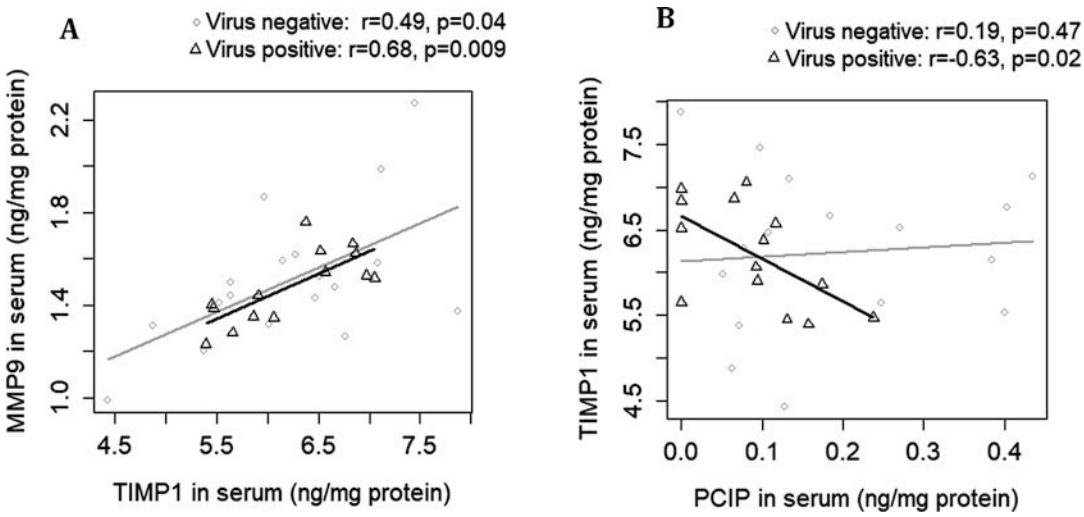

Fig. 6 The correlation analysis of secreted biomarkers in PVB19- negative and -positive serums. (**a**) The significant correlation between TIMP1 and MMP9 in viral serums. (**b**) The inverse correlation between TIMP1 and collagen synthesis markers PICP in viral serums. Data are significant at $p \leq 0.05$

the more pronounced myocarditis than DCM. In addition, the timely identification of viral myocardial infection could significantly improve therapeutic efficiency of viral DCM by preventing further myocardial remodeling. Therefore, in this study, we have investigated the ongoing inflammation, fibrosis, apoptosis, and necrosis during chronic (around 3 years from the DCM diagnosis) presence of PVB19 in idiopathic DCM myocardiums and serums. The expression of PVB19 in DCM myocardiums has been analyzed by qPCR, and all samples were divided into PVB19-negative and PVB19-positive groups for the further investigation of most typical DCM pathophysiological biomarkers and their intercorrelations.

It has been shown that the acute viral myocarditis associates with a high level of inflammation caused by a nonstructural protein (NS1) of PVB19 (Duechting et al. 2008), while other studies showed that the presence of PVB19 in myocardium is irrelevant to the acute myocarditis or DCM (Schenk et al. 2009). It is agreed that the intramyocardial inflammation needs $\geq$14 of inflammatory infiltrates ($\geq$14 leucocytes/mm^2 including up to 4 monocytes/mm^2 with the presence of CD3-positive T lymphocytes $\geq$7 cells/mm^2) (Cerqueira et al. 2002). In this study, the levels of inflammatory infiltrates in PVB19-positive and PVB19-negative myocardiums were similar: 10 and 11 of CD3-positive cells (T cells/mm^2) and 5 and 3,5 CD68-positive cells (macrophages/mm^2) in PVB19-positive and PVB19-negative EMBs, respectively. The infiltration of T cells and/or macrophages usually results in acute or chronic inflammatory processes that impair cardiac functioning by direct cytotoxic effects or by the activation of other inflammatory cells (Blanton et al. 2019; O'Rourke et al. 2019; Lafuse et al. 2020). There is also a possibility that more intensive infiltration of macrophages in viral myocardium compared to the nonviral needs more intensive stimulus than PVB19.

Similar to the EMBs, PVB19 did not stimulate secretion of IL-1, TNF-α, and IL-6. The decreased secretion of all tested pro-inflammatory cytokines might be also related to the low infiltration of macrophages, a known inducer of inflammation, in PVB19 myocardium (Lafuse et al. 2020; Martinez et al. 2008). On the other hand, significantly decreased level of IL-6 (a known myokine with antiapoptotic and heart protecting effects) (Terrell et al. 2006; Fontes et al. 2015) in PVB19-positive samples could have a negative

impact on the myocardium. On the other hand, it was shown that only chronic, not short-time, IL-6 upregulation is associated with the worse heart outcome (Terrell et al. 2006).

Many heart failure models showed fibrosis to be inflammation-related process (Bacmeister et al. 2019). The main regulator of ECM accumulation and/or degradation is matrix metalloproteinases (MMPs) family composed of more than 20 types of zinc-dependent proteases actively participating in the heart tissue functioning (Lee 2001). It has been shown that interstitial collagenases, such as MMP1 and MMP13, and gelatinases MMP2 and MMP9 are significantly expressed in mammalian myocardium and participate in the heart ECM degradation (Spinale 2002). Since the myocardium is mainly composed of collagen type I (50–85%) and III (10–45%), its degradation due to persistent presence of PBV19 can also have a crucial negative effect on the myocardium (Weber et al. 1993; Collier et al. 2012). On the other hand, the excess of collagen I synthesis can initiate myocardial stiffness in hypertensive heart disease, aortic stenosis, or late stage DCM (Díez et al. 2002; Okada et al. 1996). In congestive heart failure mice model, the activation of MMP1 was shown to be important for the left ventricle (LV) remodeling and heart dysfunction (Kim et al. 2000). The MMP1 was also confirmed to cleave mainly collagen I, while MMP2 in addition cleaves a collagen III, another important heart structural protein (Steffensen et al. 1995; Patterson et al. 2001). Moreover, the MMP1 can split the collagen I into two fragments that are further broken down into smaller fragments by MMP2 and MMP9 (Funck et al. 1997). Both MMP1 and MMP2 also can directly remodel collagen I through binding to its ά-2 chain (Gioia et al. 2010). In this study, the levels of MMP1 and MMP2 have been significantly (by 2,2- and 2,4 fold) upregulated in PVB19-positive myocardiums compared to the virus-negative EMBs that could remodel DCM myocardium. The significant inverse correlation between MMP1 and fibrosis also confirmed MMP1 participating in myocardial ECM degradation rather than in fibrosis.

The levels of other MMPs, such as MMP9 and MMP13, in the PVB19-positive vs nonviral DCM myocardiums did not differ significantly. It was shown that MMP9 can be located in mitochondria and macrophages and is released during the acute damage of myocardial cells (Moshal et al. 2008; Newby 2016). The MMP9 intramyocardial activation might also require stronger stimulus than chronic presence of PVB19 in DCM myocardium (Lin et al. 2017). Although the role of TIMP-1 in myocardial fibrosis is not well defined, the proteolytic activity of MMPs should be kept in check by TIMPs, the predominant inhibitors of MMPs in myocardium (Moore et al. 2012; Yang et al. 2019). Data of this study show that the level of TIMP1 in PVB19-positive myocardiums was almost twice lower than in PVB19-negative ones, and TIMP1 secretion strongly correlated with the secretion of MMP9 and inversely with collagen I synthesis biomarker PICP. It suggests that released TIMP1 is no longer inhibiting intramyocardial MMP9 that could degrade intramyocardial collagens or even initiate apoptosis.

The absence of intensive inflammation in PVB19-positive myocardiums also did not induce neither apoptosis nor necrosis. Despite that, the persistent presence of PVB19 in human EMBs can impair functioning of mitochondria by strong declining (~threefold) of mitochondria membrane protecting protein Bcl2 and intramitochondrial Hsp60 compared to the nonviral samples. The activation of intrinsic/mitochondrial apoptotic pathways is mainly regulated by the balance of antiapoptotic Bcl-2 and proapoptotic Bax family members (Elmore 2007) and needs harsh oxidative stress or inflammation-related stimuli (Wang et al. 2013). However, the correlation analysis of measured apoptotic biomarkers revealed a possible further activation of not only extrinsic but also an intrinsic apoptotic pathway by PVB19 infection. The presence of Hsp60 in primary cardiomyocytes was shown to protect mitochondria as well by upregulating mitochondria protecting antiapoptotic Bcl-xl and Bcl-2, and reducing the level of proapoptotic Bax (Shan et al. 2003).

It is well-known that cardiac tissue requires a high level of energy in order to properly pump

blood, while mitochondria are one of the key players in mammalian heart energy supply (Ramaccini et al. 2021) – mitochondrial dysfunction was shown to be associated with the heart diseases including DCM (Bonora et al. 2019). Moreover, a number of other mitochondrial dysfunction such as oxidative phosphorylation (OXPHOS), metabolic, fatty acid, or cardiolipin metabolism (Barth syndrome) correlate with the incidents of DCM (Marin-Garcia et al. 2000; Gebert et al. 2009). The chronic presence of PVB19 in DCM myocardium can also sensitize it to the other toxic viral infections such as coronavirus (Naneishvili et al. 2020). So, the data of this study suggest that chronic presence of PVB19 in myocardium is not an innocent phenomenon, but can slowly degrade myocardial ECM through the activation of MMPs and impair mitochondrial functioning, leading to the worse heart energy supply and tissue contraction. The antiviral therapy can be useful to prevent viral DCM and further pathophysiologic heart tissue remodeling.

5 Conclusions

Altogether, the chronic presence of PVB19 in human myocardium is not initiating myocarditis-related processes such as intensive inflammation, fibrosis, apoptosis, or necrosis compared to the nonviral myocardiums. However, the chronic presence of PVB19 in myocardiums significantly (by ~twofold) upregulated the levels of MMP1 and MMP2 that can degrade myocardial ECM, particularly collagens I and III. The decreased level of MMPs inhibitor TIMP1 (by twofold) in PVB19-positive myocardium compared to the nonviral supports participation of MMPs in ECM degradation.

In addition, the negative effects of PVB19 on mitochondria were also observed, i.e., the levels of mitochondrial membrane protecting proteins Bcl-2 and Hsp-60 in PVB19-positive biopsies were to be around threefold lower compared to the negative ones. Moreover, the correlation analysis of measured biomarkers showed possible further apoptosis activation if PVB19 persists in myocardiums. It may be that PVB19 can sensitize myocardiums to other toxic and/or viral infections.

Finally, data show that chronic presence of a PVB19 in human DCM myocardium is not a benign phenomenon: persistence of PVB19 in DCM myocardium negatively affects ECM integrity and energetic status of DCM myocardium. Data suggest that antiviral therapy for PVB19-positive DCM patients may be applicable in order to prevent further myocardial degradation.

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Limitations The main limitation of this study, due to the ethical problems, is the absence of healthy patients' samples. Despite this, the main goal of this study was to investigate the main pathophysiological processes ongoing during chronic presence of PVB19 in DCM myocardium and its impact to the further myocardium remodeling. The data suggest that antiviral therapy can be recommended to treat viral DCM.

Competing Interests The authors declare that they have no competing interest.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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