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Kursad Turksen *Editor*

Cell Biology and Translational Medicine, Volume 17

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. Amongst topics explored in this volume are repair and regenerative aspects of stem cells in different disease state. 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.

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

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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Endothelialization and Inflammatory Reactions After Intracardiac Device Implantation

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Abstract

Background: Due to the advances in catheter-based interventional techniques, a wide range of heart diseases can now be treated with a purely interventional approach. Little is yet known regarding biological effects at the intracardiac implantation site or the effects on endothelialization and vascular inflammation in an in vivo environment. Detailed knowledge of ongoing vascular response, the process of endothelialization, and possible systemic inflammatory reactions after implantation is crucial for the clinical routine, since implants usually remain in the body for a lifetime.

Methods: For this narrative review, we conducted an extensive profound PubMed analysis of the current literature on the endothelialization processes of intracardially implanted devices, such as persistent foramen ovale (PFO) occluders, atrial septal defect (ASD) occluders, left atrial appendage (LAA) occluders, transcatheter aortic valve implantations (TAVIs), and leadless pacemakers.

Christoph Edlinger and Vera Paar have equally Contributed to this chapter.

The authors declare that there is no conflict of interests regarding the publication of this paper. All companies have granted image rights to depict their devices in this article.

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Additionally, the known biological activities of common metallic and synthetic components of intracardiac devices in an "in vivo" setting have been evaluated.

Results: Nitinol, an alloy of nickel and titanium, is by far the most commonly used material found in intracardiac devices. Although allergies to both components are known, implantation can be performed safely in the vast majority of patients. Depending on the device used, endothelialization can be expected within a time frame of 3–6 months. For those patients with a known allergy, gold coating may be considered as a viable alternative.

Conclusion: Based on our analysis, we conclude that the vast majority of devices are made of a material that is both safe to implant and nontoxic in long-term treatment according to the current knowledge. The literature on the respective duration of endothelialization of individual devices however is highly divergent.

Keywords

Cardiac injury · Endothelialization · Endovascular inflammation

Abbreviations

ASD	Atrial Septal Defect
ASO	Amplatzer Septal Occluder
ATP	Adenosine Triphosphate
CMs	Cardiomyocytes
DAMPs	Damage-Associated Patterns
ECs	Endothelial Cells
ECM	Extracellular Matrix
EPCs	Endothelial Progenitor Cells
GSO	Gore Septal Occluder
HMGB1	High-mobility Group B1
HSPs	Heat Shock Proteins
ICAM-1	Intercellular Adhesion Molecule 1
IFN-γ	Interferon Gamma
LAA	Left Atrial Appendage
MERTK	Myeloid–Epithelial–Reproductive Tyrosine Kinase
MMPs	Matrix Metalloproteinases
MI	Myocardial Infarction
NOAC	New/"Non-Vitamin K" – Oral Anticoagulants
PCI	Percutaneous Coronary Intervention
PDGF	Platelet-Derived Growth Factor
PET	Polyethylene Terephthalate
PFO	Persistent Foramen Ovale
PRRs	Pattern Recognition Receptors
RAGE	Receptor for Advanced Glycation End Products
ROS	Reactive Oxygen Species
SMCs	Smooth Muscle Cells
TAVI	Transcatheter Aortic Valve Implantation
TGF-β	Transforming Growth Factor Beta
TLRs	Toll-Like Receptors
Treg	Regulatory T-cells
VEGF	Vascular Endothelial Growth Factor
VSD	Ventricular Septal Defect

1 Introduction

Interventional cardiology is a rapidly evolving field in modern clinical medicine. Although the enormous therapeutic potential was not immediately recognized after Forßmann carried out the first catheterization of the right heart in 1929 in a heroic self-experiment, the idea was taken up and further developed by Cournand et al., who are today regarded as founders of interventional cardiology (Forssmann-Falck 1997; Nicholls 2020).

Grüntzig's first successful percutaneous coronary intervention (PCI) in 1977 marked the dawn of a new era in clinical cardiology as catheter-based therapy changed from a purely diagnostic tool to an interventional treatment option for acute coronary syndrome (Ar et al. 1979).

During recent years, interventional cardiology has undergone an enormous transformation as catheter technology has developed rapidly and is

used in many different cardiological diseases. Today, in addition to coronary heart disease, a wide range of valvular diseases or congenital heart defects, such as atrial septal defect (ASD) (Sievert et al. 1998) and persistent foramen ovale (PFO) can be successfully treated using a catheter-based interventional approach. Even the catheter-based implantation of cardiac pacemakers is widely used in countless clinics today.

Permanent intracardiac placement of devices represents an enormous challenge for product engineers, as all components must offer exceptional stability and durability combined with low weight and small size. In addition, any device exposed to blood flow in an "in vivo" environment must be safe in terms of hemostasiological interactions; more precisely, the device must not initiate thrombogenic or even hemolytic cascades. Furthermore, the materials components must have an extremely low allergic potential, both at the implantation site and, in case of systemic reactions, in the entire organism.

All the devices must undergo extensive testing on safety and durability for market approval in order to achieve CE certification. In addition, an optimally designed intracardiac device should also have acceptable biocompatibility, especially rapid endothelialization, to reduce the duration of anticoagulant use with the goal of reduced bleeding risk and to safely discontinue use of endocarditis prophylaxis.

The aim of this narrative review is to provide an overview of the current state of knowledge on endothelialization in common intracardiac devices as well as an overview of the known in vivo interactions, and the important components of different devices.

2 Intracardiac Devices

2.1 PFO Occluders

The patent foramen ovale (PFO) is an essential component of intrauterine circulation, allowing blood to bypass fetal lungs. Although spontaneous occlusion should occur shortly after birth, this physiological process is absent or incomplete in up to 27.3% of the population (Hagen et al. 1984).

In the vast majority of cases, a PFO does not have a clinical relevance. Nevertheless, it may play a crucial role in the genesis of migraine headaches or even cryptogenic stroke (Saver et al. 2017). In symptomatic patients, interventional treatment with an occluder can be considered to permanently close the opening. Recent studies (CLOSE, REDUCE, RESPECT LT, and DEFENSE PFO) showed that interventional PFO occlusion (Fig. 1a) was associated with a significant reduction in recurrent stroke compared to drug therapy (Mas et al. 2017; Saver et al. 2017; Søndergaard et al. 2017). Based on the long-term results of RESPECT and the results of the REDUCE study, two occluder devices, the AMPLATZER PFO closure and GSO (GORE Medical, Flagstaff, AZ, USA) received US Food and Drug Administration (FDA) approval for secondary stroke prevention in 2016 and 2018, respectively.

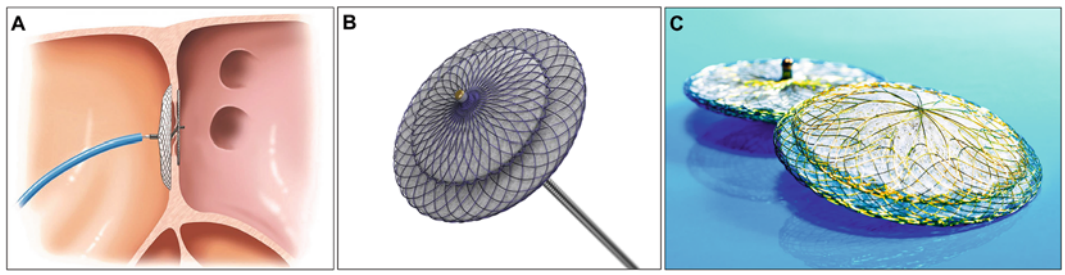

Fig. 1 PFO occluders. (**a**) Schematic representation of the regular position of a PFO occluder. (**b**) Amplatzer™ PFO Occluder. (**c**) Figulla Flex II® PFO Occluder. Pictures provided by Occlutech International AB

The later developed Flex II PFO occluder (Occlutech, Jena, Germany) received CE certification for clinical use in Europe in 2009. According to the manufacturer, more than 33,000 such devices have been delivered worldwide.

2.1.1 Amplatzer PFO Occluder

The Amplatzer occluder (Abbott Cardiovascular, North Chicago, Illinois, USA), first implanted in 1997, has been very well described elsewhere (Meier 2005; Madhkour et al. 2019). In brief, it is a double disc made of Nitinol mesh, the inside of which is made of polyester fabric.

A thin neck, which consists of the tightly woven wires of the discs, serves as a connector. The neck is rotated around its longitudinal axis so that it can be extended in principle (Fig. 1b). The two discs are sewn together with polyester fabric for better stabilization (Scalise et al. 2016).

The special feature is the so-called "shape memory," which means that the device returns to its original shape after being stretched through the guiding catheter.

The Amplatzer PFO Occluder is available in three different sizes (18 mm, 25 mm, 35 mm), whereby the size specification is based on the size of the right-sided disc. The most frequently implanted occluder is the medium size. The small version has its special value in the case of a small PFO with a largely stable septum primum, whereas the 35 mm version is used in the case of an extremely redundant septum primum and possibly in the case of an atrial septal aneurysm.

According to the current literature, the chances of success are very high, so that complete closure of the shunt can be assumed in well over 90% of cases (Bruch et al. 2002; Greutmann et al. 2009).

Residual shunts require surgical intervention only in rare cases, although the actual average duration until complete endothelialization "in vivo" is not completely clear. In a recent position paper, the German Cardiology Society recommends dual antiplatelet therapy with aspirin and clopidogrel for a period of 6 months. Should there be a concomitant indication for oral anticoagulation, this will be given as monotherapy, with NOACs being preferred.

2.1.2 Gore Septal Occluder

The Gore Septal Occluder (GSO) has been approved for the treatment of PFOs in Europe for 10 years now by means of CE certification.

In contrast to the more commonly implanted Amplatzer Occluder, it is intended to offer advantages in difficult anatomical conditions. The device consists of five nitinol wires formed into a left atrial and a right atrial disc. The outer frame is coated with polytetrafluoroethylene film. For implantation, the device comes already loaded on a 10 French introducer catheter. A special safety feature is the integrated retrieval cord, which can be used to retrieve the device if necessary. When fully deployed, two circular discs are formed, facing each other, which can be fixed in place by a locking mechanism in the center.

2.1.3 Flex II PFO Occluder

The Flex II PFO occluder (Occlutech, Jena, Germany) consists of two self-expanding woven nitinol discs (Fig. 1c). The special feature of this device is a central pin on the left atrial disc and also a ball socket joint connection. The complete closure is achieved by two biocompatible polyethylene terephthalate patches.

The superiority of this device is the flexibility and ability to angulate in order to achieve the maximal adaptation to the interatrial septum. This offers an advantage for the complex anatomical variations (Neuser et al. 2016).

2.2 ASD Occluders

Atrial septal defect (ASD) is a relatively common congenital heart defect with a birth prevalence of 1.43:1000 live births and an expected survival rate into adulthood of 97% (Anderson et al. 2002; Anderson 2016; Lee et al. 2018). ASD of the ostium secundum is the most common type, occurring in 70% of all patients with ASD, followed by ASD of the ostium primum (10%) and ASD of the sinus venosus (5–10%) (Moons et al. 2009). The "true atrial septum," that is, the tissue directly separating the atrial cavities, is

restricted to the base of the oval fossa and the surrounding inferoanterior margins. Defects of the true atrial septum are called "secundum defects." Atrial septal defects are usually well tolerated in children, but can cause significant complications in adults (Campbell 1970). Early closure is therefore recommended and can be achieved using catheter deployment in the majority of cases. A symptomatic benefit can be seen at any age (Komar et al. 2014) Since left ventricular compliance decreases with age or in the presence of conditions that can increase left atrial pressure (e.g., high blood pressure, ischemic heart disease, cardiomyopathy, aortic and mitral valve disease), the left–right shunt can increase due to ASD (Le Gloan et al. 2018; Kumar et al. 2019). However, conditions that reduce right ventricular compliance (e.g., pulmonary arterial hypertension, pulmonary stenosis, right heart disease, tricuspid valve disease) may eventually reverse the shunt and cause cyanosis (Le Gloan et al. 2018).

A left–right shunt leads to right ventricular volume overload, which in turn results in right ventricular dilatation. It is well tolerated throughout childhood, despite a pulmonary–systemic flow ratio that can exceed 3:1. The pulmonary vascular system is also able to absorb the increased blood flow at low pulmonary artery pressure for many years. A persistent large left–right shunt leads to increased right atrial and right ventricular dilatation from late childhood onwards, which in some patients leads to arrhythmia and a progressive increase in pulmonary vascular resistance (Le Gloan et al. 2018). Severe pulmonary vascular disease is rare (<5%), unless there are other associated factors (Nashat et al. 2018).

2.2.1 Amplatzer Septal Occluder

The Amplatzer® Septal Occluder (ASO) (Abbott Cardiovascular, North Chicago, Illinois, USA; former: St. Jude Medical, Inc., St. Paul, Minnesota) consists of nitinol–titanium memory wire mesh infused with polyester patches that facilitate occlusion and endothelialization (Fig. 2a). It consists of a smaller right and a larger left disc connected by a waist; the difference in size of both discs is 4 mm (Nassif et al. 2016).

ASO has been shown to be a practical, safe, and effective treatment option for ASD (Masura et al. 1997; Podnar et al. 2001; Masura et al. 2005; Cardoso et al. 2007; Knepp et al. 2010). Nevertheless, complications such as implant embolization, mispositioning, and fracture may occur in rare cases. Moreover, cases of erosion/perforation, cardiac arrhythmia, cardiac tamponades, and even infectious endocarditis have been reported (Sievert et al. 1998; Chessa et al. 2002; Fischer et al. 2003; Balasundaram et al. 2005; Sadiq et al. 2012).

2.2.2 Occlutech ASD Occluder

The Figulla Occlutech ASD closures (Occlutech, Jena, Germany) consist of individually braided, very thin (40–150 μm or 0.00157–0.00590 inches) nitinol strands. All strands end proximally

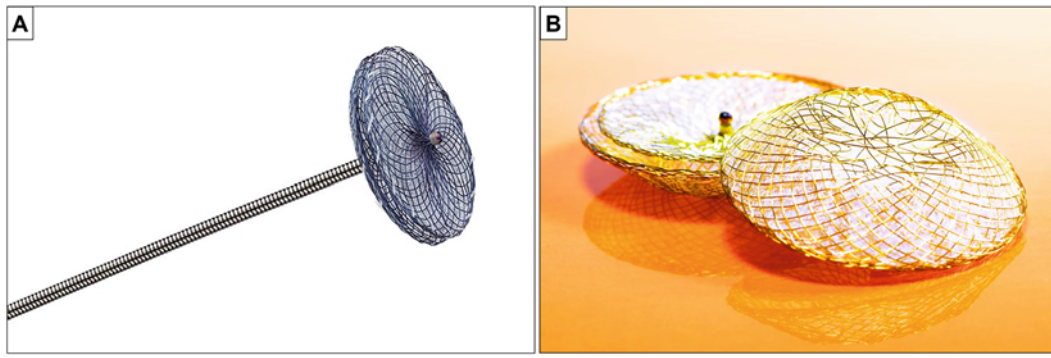

Fig. 2 ASD occluders. (**a**) Amplatzer™ Multi-Fenestrated Septal Occluder – "Cribriform". (**b**) Occlutech® Fenestrated Atrial Septal Defect Occluder. Pictures provided by Occlutech International AB

and therefore do not require clamping to the left disc (Fig. 2b). This results in a smaller amount of uncovered metallic material. The ultrathin fabrics made out of polyethylene terephthalate (PET) of the device promote the endothelial growth after implantation as well as the defect closure (Pedra et al. 2016). The design of the Flex II ASD occluder is intended to allow ideal alignment of the septum, which in turn should increase feasibility and patient safety during implantation. The device is made of Titanium oxide–covered nitinol, which should result in the lowest possible release of nickel.

2.3 Left Atrial Appendage Occluders

The term "LAA occluder" refers to devices that can be used to close the left atrial appendage (LAA). In patients with atrial fibrillation, the LAA is anatomically particularly important, as this is where the vast majority of cardio-embolic strokes originate (Alli and Holmes, 2015). In patients at high risk for bleeding complications, implantation of an occluder allows discontinuation of anticoagulants after the initial endothelialization phase. The most common product is the Watchman Device, which has been CE certified since 2005. Another product, CE certified in 2013, is the Amulet device, which is designed to provide benefits due to its wide range of available sizes, according to the manufacturer.

2.3.1 Watchman Device

The Watchman device (Boston Scientific, Marlborough, MA, USA) consists of a nitinol frame coated with a permeable 160 micron polyethylene terephthalate knit fabric on the left atrial surface (Fig. 3a) (Fountain et al. 2006). It is a parachute-shaped, self-expanding device (Kramer and Kesselheim, 2015). The PET knit fabric facilitates endothelialization over the device and serves as a filter for emboli that originate from the LAA pouch (Della Rocca et al. 2019). After femoral vein access and transseptal puncture, the Watchman device is delivered using a 12-Fr delivery catheter and is then deployed until its titanium dowel pin separates from the catheter (Fig. 3b). The Watchman device is affixed to the LAA wall by 10 fixation barbs, which are arranged around the mid-perimeter. To match different LAA orifice sizes, the device is manufactured in five sizes (21 mm, 24 mm, 27 mm, 30 mm, and 33 mm) to allow adequate placement. An adequate seal is defined as a leakage < 5 mm.

2.3.2 Amulet Occluder

The AMULET is a second-generation Amplatzer Cardiac Plug (Abbott Cardiovascular, North Chicago, Illinois, USA; former: St. Jude Medical, Inc., St. Paul, Minnesota). The self-expanding device is made of flexible, braided nitinol filled with polyester tissue. It consists of a proximal disc and a distal lobe shaped like a hockey puck

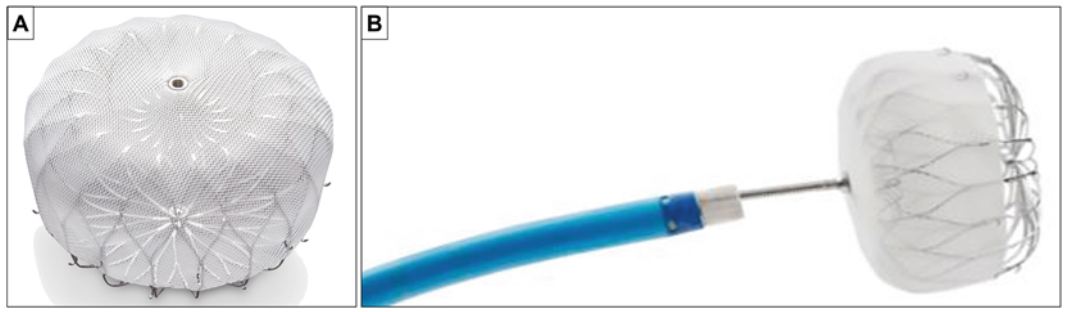

Fig. 3 Watchman Devices. (**a**) Watchman™. (**b**) Watchman™ on a 12 French delivery catheter. Pictures provided by Boston Scientific

connected by a flexible waist (Meerkin et al. 2013). The proximal disc covers the LAA orifice and the distal lobe with stabilization hooks that secure the engagement of the occluder to the LAA wall. The LAA Occluder is delivered by a 12-F or 14-F sheath into the left atrium after a transseptal puncture. It is available in eight sizes. Pre-interventional standard imaging, including transesophageal echocardiography and computerized tomography (CT) scan, is performed in order to determine the proper occluder size. The proximal disc is always slightly larger than the lobe and has a central screw.

2.4 Transcatheter Aortic Valve Implantation

Transfemoral aortic valve replacement, first performed in 2001 by Cribier et al., is beyond doubt one of the greatest achievements in interventional cardiology (Cribier et al. 2002). As alternative to conventional surgical aortic valve replacement, a minimally invasive transcatheter implantation of a valve prothesis is possible. The intervention was originally reserved for medium- to high-risk elderly patients, also due to material durability profiles. In recent years, its indication has been extended to younger patients age <75 years. Recently, transcatheter valve replacement has been approved by the FDA as the method of choice for all patients (Edlinger et al. 2020). The most important challenge is the material compatibility and the special required characteristics of the valve. Those consist of "durability, low thrombogenicity, hydrodynamics, hemocompatibility, low calcification susceptibility and crimping and deployment stability" (Rotman et al. 2018).

Various models have been developed over the years, whereby the self-expanding Medtronic valves (CoreValve, Evolut R) (Medtronic, Minneapolis, MN, USA) and the balloon-expanding Edwards valves (Sapien, Sapien XT, Sapien 3) (Edwards Lifesciences, Irvine, CA, USA) are the most widely implanted valve types (Chakos et al. 2017). The SAPIEN 3 and SAPIEN 3 Ultra are the latest generation of balloon-expanding Edwards valves. The Evolut R and Evolut PRO are the latest self-expanding valves from Medtronic (Renker and Kim, 2020). In several hospitals they consist more than 70% of the total transcatheter aortic valve implantation (TAVI) procedures.

Another important aspect concerns the valve-in-valve procedures. Here is the geometric orifice area that plays the most significant role. Therefore a valve-in-valve procedure should only be used after careful planning, because it can diminish the

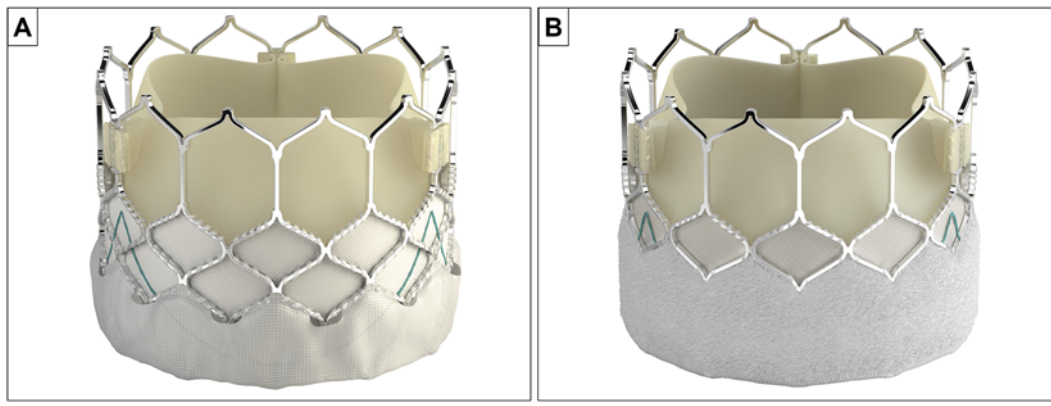

Fig. 4 Transcatheter aortic valve products of Edwards Lifesciences. (**a**) Edwards SAPIEN™; Edwards SAPIEN 3TM transcatheter heart valve. Picture provides by Edwards-Sapien. (**b**) Edwards SAPIEN™ 3 Ultra; Edwards SAPIEN 3 UltraTM transcatheter heart valve. Picture provided by Edwards-Sapien

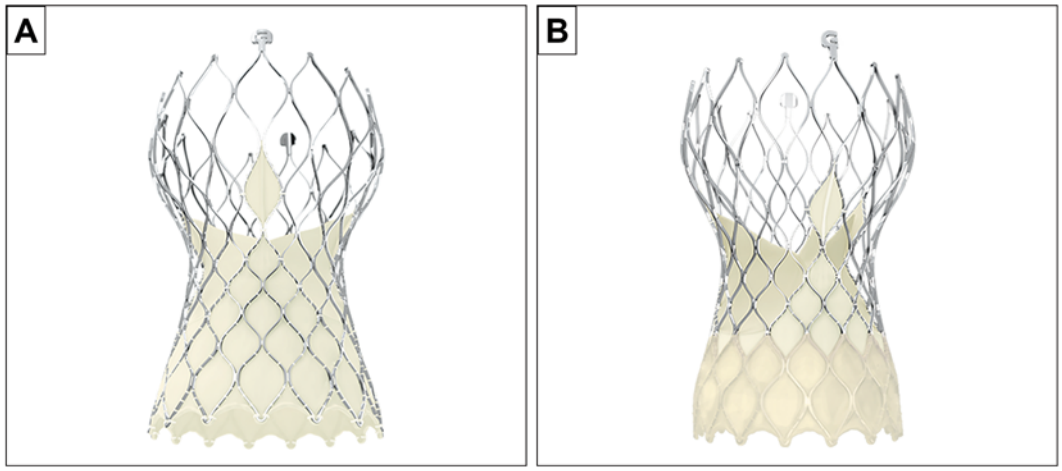

Fig. 5 Transcatheter aortic valve products of Medtronic. (**a**) CoreValve™ Evolut™ R. (**b**) CoreValve™ Evolut™ Pro. (Source: Medtronic GmbH)

hydrodynamic performance of the valve and significantly reduce the opening area (Rotman et al. 2018).

2.4.1 Edwards SAPIEN 3/SAPIEN 3 Ultra

The Edwards SAPIEN 3 (Fig. 4a) and SAPIEN 3 Ultra (Fig. 4b) consist of a cobalt–chromium stent frame, three leaflets of bovine pericardium, an inner and an outer skirt. The SAPIEN 3 has an internal skirt and an outer sealing skirt made of polyethylene terephthalate (PET) (Jose et al. 2015). In the SAPIEN 3 Ultra, the outer portion is textured PET material with a greater height in comparison to the SAPIEN 3 (Renker and Kim, 2020). The valves are manufactured in four sizes (20 mm, 23 mm, 26 mm, and 29 mm).

2.4.2 Medtronic Evolut R/Evolut PRO

Both valves consist of a self-expandable nitinol stent frame and three leaflets of porcine pericardium positioned in a supra-annular location. In contrast to the Evolut R (Fig. 5a), an external porcine pericardial sleeve has been added to the Evolut PRO (Fig. 5b) as a sealing sleeve. The Evolut R model is manufactured in four sizes: 23 mm, 26 mm, 29 mm, and 34 mm, while the Evolut PRO is only available in 23 mm, 26 mm, and 29 mm.

The valve size selection is of paramount significance for the physician and for the patient and is highly associated with the success of a TAVI procedure. In order to choose the right valve size, an MSCT Scan must be performed. Then the appropriate software should be used to quantify the aortic root.

The cut-off values of the manufacturer's sizing plan should be considered. There is often a difference between clinical valve selection and selection based on the computer software. The "device–host interaction" is very important in order to prevent complications such as obstruction of the coronary arteries, relevant aortic regurgitation after implantation as well as conduction disorders such as atrioventricular (AV) block (El Faquir et al. 2020).

A rare but sometimes life-threatening complication is the transcatheter heart valve migration into the outflow tract of the left ventricle. In this case is a balloon repositioning of the valve necessary as well as possibly a valve-in-valve procedure in order to prevent severe aortic regurgitation (Ito et al. 2017).

One of the deciding mortality as well as success parameters of a TAVI procedure – also long term – is the paravalvular regurgitation. This reinforces the importance of correct valve selection and the experience of the physician. In several publications, it is observed that in the second part of the study or cohort there are a greater number of good final results as the technique of

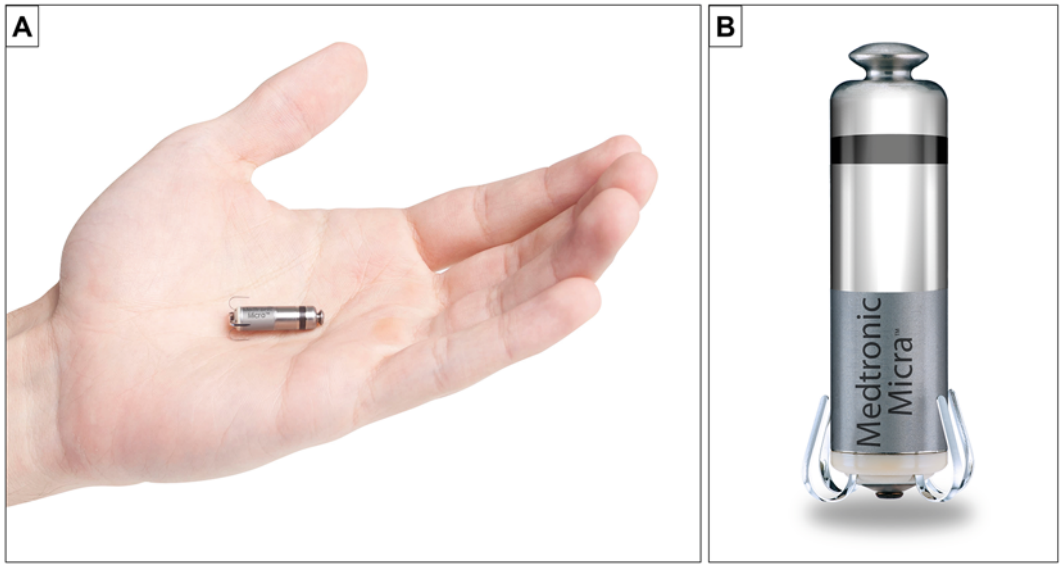

Fig. 6 Micra Pacemakers. (**a**) Illustration of Micra™ pacemaker size compared to a human hand. (**b**) Micra™. (Source: Medtronic GmbH)

the interventional cardiologist improves exponentially (Wang et al. 2021).

2.5 Leadless Pacemakers

Within the last decade, single-chamber leadless pacemaker devices have been developed, which are implanted to the inner side of the right ventricle via a steerable catheter insertion system (Reynolds et al. 2016). Currently, the most common device in clinical use is the MicraTM (Medtronic Inc., Minneapolis, MN, USA). Despite its small size of 0.8 m^3, a light capsule weight of 2.0 g, a length of 25.9 mm, and an outer diameter of 6.7 mm, it has all the features of a conventional single-chamber pacemaker system (Reddy et al. 2015). The Micra pacemaker itself consists of nitinol, gold, steel, titanium, and tungsten (Figs. 6a and 6b). The tines, with which the device is affixed to the endocardium of the right ventricle, consist entirely of nitinol.

There are now numerous clinical studies that prove both, the effectiveness and safety of the product. Medtronic postulates that the device would float in an in vivo environment at the inside of the right ventricle. According to the manufacturer, the only connection to the endothelium is through the pacemaker's anchoring system. As lead-free pacemaker technology is a relatively new topic, the effects on the intracardiac endothelium at the anchor point are not yet well understood. However, individual cases have also been published in which unexpected encapsulation was observed. . First data from autopsies show partial or even complete encapsulation (Tjong et al. 2015; Kypta et al. 2016). In the two published cases, complete endothelialization or even encapsulation is reported after 12 months and 19 months, respectively. Within one of our prior in vitro studies, we could identify a potential impact of the tungsten component in these processes of endothelialization (Edlinger et al. 2019).

3 Common Device Components

3.1 Nitinol

Nitinol, a material used in the majority of intracardiac devices, is a 55:45 nickel–titanium alloy. It is of enormous value for medical applications due to its thermal shape–memory effect and super elasticity (Ryhänen et al. 1998). Nitinol is not considered cytotoxic or thrombogenic, although its individual components, nickel and titanium,

can be detected in peripheral blood samples (Shayan and Chun 2015).

It is assumed that the immunological effects are comparable to those of stainless steel without any toxic effects (Ryhänen et al. 1998). Nitinol is widely used, for example, in many PFO and ASD occluders, as well as the tines of the Micra pacemaker are made of this material.

3.2 Titanium

Due to its good in vivo compatibility, titanium is a widely used component for implants and alloys (Ungersboeck et al. 1995). Concentrations of 50–150 μg/l can be measured in peripheral blood samples, a level considered nontoxic (Ipach et al. 2012). Data from orthopedic studies could show an increase in classical inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1beta (IL-1β) and IL-6 in patients with titanium implants. However, there is no evidence of toxicity to date (Sun et al. 2000; Östberg et al. 2015). In cardiology, for example, titanium is used in pacemaker generators with no long-term adverse effects. It is particularly important as a component of the nitinol alloy.

3.3 Tungsten

Tungsten is another component that is commonly used in medical devices. Certain publications point to toxic effects during long-term treatments (Witten et al. 2012). However, according to the current knowledge, no toxic effects are expected at normal corrosivity rates and slightly elevated serum levels are considered as normal (Peuster et al. 2003). It is considered to be a very strong and durable material as well as very resistant to corrosion. In cardiology, tungsten is widely used as a component of pacemaker probes. The Micra pacemaker also partly consists of this material.

3.4 Gold

Implants made of gold and its nanoparticles are known to be noncytotoxic and nonimmunogenic (Shukla et al. 2005). Cytokine elevations of interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), interleukin 1-beta (IL-1β), and mononuclear chemotactic protein 1 (MCP-1) are frequently found. However, these cytokine reactions are insignificant and have no influence on the cell viability (Zhang et al. 2011). For this reason, gold is of paramount value as a reserve material if the patient to be implanted suffers from a previously known allergy, for example to titanium. This is a rare allergy, which affects only the 0.6% of the total population. There are reports of patients who have been successfully implanted with a gold-plated leadless pacemaker for this indication. This was described in a 65-year-old man with a type IV allergy to titanium in the case report by Kypta et al. (Kypta et al. 2015; Goli et al. 2012).

3.5 Steel

Steel is considered to be a material of manufacturing precision, good hygiene, as well as high resistance against corrosion. It has been reported that the toxic effects of steel implants are higher than those of other components (Haynes et al. 1998). Lacey et al. observed a decrease in monocyte and macrophage survival in response to steel (Lacey et al. 2009), as well as a reduced leukocyte migration to the implant site or prosthetic implants. In addition, steel has been shown to induce abundant elevation of cytokines such as IL-1β (Haynes et al. 1998).

4 Cardiac Injury, Wound Healing, and Regeneration – A Brief Overview

Cardiac injury causes a severity-related damage of the myocardium, followed by cardiac repair or wound healing where damaged tissue is usually replaced by a fibrotic scar, as described in the literature (Deb and Ubil 2014; Talman and Ruskoaho 2016). Moreover, it has recently been reported that adult cardiomyocytes (CMs) have a slight ability to proliferate, raising the promise of promoting cardiac regeneration in humans

(Beltrami et al. 2001; Bergmann et al. 2009; Mollova et al. 2013). However, the feasibility of cardiac regeneration is largely dependent on the type and extent of immune responses, thus leading to an inflammatory response (Sattler and Rosenthal 2016; Cheng et al. 2017).

Although the implantation of an intracardiac device is necessary for the maintenance of proper cardiac pacing in various cardiac diseases, it is also accompanied by the injury of the cardiac tissue at the site of implantation. This inevitable injury initiates a complex series of tissue repair processes that comprise of the interaction and timely coordination of several cell types, cytokines, chemokines, and signaling cascades. Furthermore, there is a host reaction following the implantation of foreign biomaterial into the cardiac implantation site. This also includes blood–material interactions, inflammation, granulation, provisional matrix formation, and the fibrotic remodeling of the injured area (Gretzer et al. 2006; Luttikhuizen et al. 2006).

Generally, the immune response to cardiac injury is accomplished by the innate and the adaptive immune systems in synergy and can be divided into three phases: the pro-inflammatory phase, the proliferative phase, and the reparative phase (Lai et al. 2019).

4.1 Pro-inflammatory Phase

In the very early process after the implantation of a foreign biomaterial, a material–blood interaction occurs, whereby proteins from the blood adhere to the implant's surface. A provisional matrix based on the blood's components forms, that is, the initial thrombus or blood clot where further protein adsorption proceeds. This provisional matrix and the injured tissue are responsible for the recruitment of structural, biochemical, and cellular compartments that are essential for wound healing (Gristina 1994; Gretzer et al. 2006; Luttikhuizen et al. 2006). In this period, inflammatory cells are recruited to the site of injury to clear the damaged wound of dead cells and tissue, as well as to degrade the matrix debris. Furthermore, it initiates the processes necessary to form the reparative scar. However, it has been described that prolonged or excessive inflammation is accompanied by a poor tissue remodeling and worse outcomes in patients or animal models with myocardial infarction (MI) (Timmers et al. 2008; Arslan et al. 2010; Frangogiannis 2012).

The initial immune response is driven by molecules released from necrotic cells, the so-called damage associated patterns (DAMPs) (Arslan et al. 2010). In addition, during tissue death, dying proteases, hydrolases, and mitochondrial reactive oxygen species (ROS) are also released into the extracellular space, generating further DAMPs that trigger the inflammatory response (Kono and Rock 2008). Subsequently, these DAMP molecules bind to pattern recognition receptors (PRRs), including toll-like receptors (TLRs) and the receptor for advanced glycation end products (RAGE), that are expressed by both tissue resident cells and recruited leukocytes (Muzio et al. 2000; Chavakis et al. 2004). Among other DAMPs present in cardiac inflammation, high-mobility group B1 (HMGB1) is one of the best characterized (Andrassy et al. 2008). HMGB1 is responsible for the initiation of inflammation in myocardial infarction (MI) and cardiac ischemia by promoting the migration of immune cells through its interaction with PRRs, such as TLR2/4 (most abundant TLRs in the heart) and RAGE (Nishimura and Naito 2005; Klune et al. 2008; Sims et al. 2009). Moreover, it induces tissue healing by changing the macrophages' phenotype, favoring neoangiogenesis and promoting stem cell activation and proliferation (Bianchi et al. 2017).

Physiologically, the extracellular matrix (ECM) is responsible for the support and the maintenance of the heart's structural integrity. However, during inflammation the ECM is degraded by matrix metalloproteinases (MMPs), activated by necrotic cells, neutrophils, and macrophages. This degraded ECM can in turn act as a DAMP, driving the inflammatory pathway forward (Dobaczewski et al. 2010a). In the context of cardiac injury, a switch to a transient fibrin-based ECM is achieved (González-Rosa et al. 2011; Frangogiannis 2017), further

modulating and guiding inflammatory cells through TLRs (Corbett and Schwarzbauer 1998; Smiley et al. 2001; Flick et al. 2004) and promoting the proliferation of endothelial cells and fibroblasts (Frangogiannis 2017).

As mentioned above, dying cardiomyocytes release intracellular components, such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), as well as intracellular components like adenosine triphosphate (ATP) and heat shock proteins (HSPs), that might accelerate the ongoing immune response (Arslan et al. 2011; Kono et al. 2014). Furthermore, reactive oxygen species (ROS), which stem from mitochondria of necrotic cells or are secreted by neutrophils, constitute a key player in the promotion of immune cells to infiltrate the injured tissue. ROS contributes to the onset of the nuclear factor kappa light chain enhancer of activated B-cells (NF-κB), a main chemotactic and pro-inflammatory protein complex (Thannickal and Fanburg 2000; Gloire et al. 2006), and directly activates the so-called inflammasome, as well as cardiac resident cells, such as fibroblasts and mast cells (Gilles et al. 2003; Kawaguchi et al. 2011). The inflammasome, a multiprotein complex of receptors and cytokines, in turn promotes the immune response and triggers the expression and activation of other cytokines (Latz et al. 2013).

After the immune response has been initiated by damage-associated molecular patterns (DAMPs) and related pattern recognition receptors (PRRs), resident immune cells and non-immune cells, such as resident macrophages, endothelial cells (ECs), and fibroblasts, drive the expression of pro-inflammatory cytokines and chemokines. In cardiac device implantation, the extent of immune responses is primarily mediated by the extent of injury that happened during the implantation procedure (Zdolsek et al. 2007; Tang et al. 1998). In the presence of DAMPs, cytokines, chemokines, activated platelets, and histamine, neutrophils are the first innate immune cells that are rapidly recruited to the injured tissue (Mcdonald et al. 2010; Soehnlein and Lindbom 2010). Contemporaneously, the cardiac endothelium is activated by pro-inflammatory cytokines, such as TNF-α, IL-1β, and histamine (Duperray et al. 1995; Dewald et al. 2004; Debrunner et al. 2008). This ensemble of pro-inflammatory cytokines constitutes the inflammasome and facilitates the neutrophil transmigration between and through the endothelial wall to the site of tissue injury (Frangogiannis et al. 1998; Singh and Saini 2003). Furthermore, IL-6 seems to be a main mediator of tissue injury, since it is expressed by CMs and recruited neutrophils and macrophages (Youker et al. 1992). IL-6 in turn upregulates intercellular adhesion molecule 1 (ICAM-1) on CMs that mediates neutrophil binding and is associated with cytotoxic events (Entman et al. 1992; Youker et al. 1992).

4.2 Cell Proliferation Phase

The cellular proliferative phase is the second phase and is characterized by the expansion of neutrophils and macrophages that degrade dead cells and the matrix debris, further promoting the expression of cytokines and growth factors. Due to the pro-inflammatory and cytotoxic activity of neutrophils, excessive amounts or a prolonged presence of neutrophils have been associated with remodeling and a poor prognosis after MI (Mocatta et al. 2007; Akpek et al. 2012). On the other hand, they constitute a key factor in the resolution of inflammation and lead to a shift of the macrophages' phenotype to a reparative one (Čulić et al. 2002; Pase et al. 2012). Furthermore, they contribute to the initiation of angiogenesis during inflammation by expressing vascular endothelial growth factor (VEGF) (Gong and Koh 2010).

Generally, monocytes are a type of leukocytes that have the ability to differentiate into macrophages and dendritic cells. There are two different subpopulations of monocytes present in cardiac inflammation: the $Ly6C^{high}$ and $Ly6C^{low}$ (Hettinger et al. 2013; Yona et al. 2013). $Ly6C^{high}$ monocytes belong to the primary subset that is recruited to the injured heart, driven by MCP-1. Therefore, $Ly6C^{high}$ monocytes are commonly active in the early pro-inflammatory phase and are responsible for proteolytic and inflammatory processes. In contrast, $Ly6C^{low}$ are sometimes

known as resident monocytes due to their appearance of not being actively recruited into the injured myocardium (Geissmann et al. 2003; Nahrendorf et al. 2007). They have been shown to emerge later, in the resolution phase, demonstrating decreased inflammatory properties, as well as the expression of VEGF (Yao et al. 2012). It is not definitively clear if $Ly6C^{low}$ arise from differentiation of $Ly6C^{high}$ (Hanna et al. 2011; Yona et al. 2013), although it has been speculated that they arise from the same progenitor cells (Hettinger et al. 2013; Yona et al. 2013). In addition, two macrophage subsets (M1 and M2 macrophages) correspond with these different monocyte concentrations. M1 macrophages are present early after heart injury and are known to secrete pro-inflammatory cytokines, like IL-1β, TNF-α, IL-6, and IL-10 (Dewald et al. 2005), whereas M2 monocytes become active at the later stage of reparative heart tissue healing (Nahrendorf et al. 2010). However, the simple division of macrophages into two subsets should be considered due to the great variety of macrophage phenotypes (Martinez and Gordon 2014). The initial acute inflammatory response usually resolves within 1 week after device implantation, though it is also dependent on the extent of injury at the implant site (Gretzer et al.).

4.3 Endothelialization and Resolution of Inflammation

Finally, the conversion from inflammation to the repair phase is crucial for wound healing as a prolonged inflammatory response would lead to CM death, excessive fibrosis, cardiac remodeling, and damage. In cardiac device implantation, acute inflammation is often followed by a chronic inflammation period that is characterized by the presence of mononuclear cells, such as monocytes and lymphocytes. This chronic inflammation lasts for a short time of approximately 2 weeks and is strictly located to the site of implantation. The prolongation of the inflammation phase for greater than 3 weeks, usually a device infection is indicated (Luttikhuizen et al. 2006). Once the inflamed/injured area is cleared of apoptotic cells, the repair process is initiated and a new ECM is produced (Frangogiannis 2014). The resolution phase is mainly characterized by the recruitment of lymphocytes, the activation of fibroblasts, and the proliferation of ECs, as well as the activation of smooth muscle cells (SMCs).

Originally, it was thought that circulating endothelial progenitor cells (EPCs), as progenitors of ECs, were a source of new endothelial cells, as first described by Asahara et al. in 1997 (Asahara et al. 1997). They originate from different hematopoietic progenitor cells located in the bone marrow, such as hematopoietic stem cells, myeloid precursors, and mesenchymal stem cells (Balistreri et al. 2015). However, EPCs also stem from different nonhematopoietic tissues, such as the umbilical cord etc. (Ingram et al. 2004; Mund et al. 2012; Chan et al. 2013). In response to tissue damage, they are released into the circulation and invade the site of injury attracted to inflammatory cytokines and chemoattractant proteins. As progenitor cells, EPCs constitute a source for ECs by differentiation and further promoting the proliferation of resident ECs (Buijs et al. 2004; Li et al. 2012). Furthermore, they release several growth factors, such as VEGF and angiopoietins, and other pro-endothelial factors that promote the healing process (MCP-1), stromal cell-derived factor 1, insulin-like growth factor 1, platelet-derived growth factor (PDGF), and macrophage inflammatory protein 1a (Rehman et al. 2003; Caiado et al. 2008). In turn, these factors stimulate ECM proteins and the proliferation of SMCs. An overview of the factors released by ECs, SMCs, and inflammatory cells was already provided by Welt and Rogers (Welt and Rogers 2002).

Both lymphocytes, B- and T-cells, comprise the main cellular components of the adaptive immune system. T-cells are further divided into $CD8^+$ and $CD4^+$ subsets, whereas $CD4^+$ T cells are the main actors in the healing process. According to their secreted cytokines they are further classified into Th1 (IL-2, TNF-α, and interferon gamma (IFN-γ); Th2 (IL-4, IL-4, IL-13); Th17 (IL-17, IL-21, IL-22); and regulatory T-cells (Treg) (transforming growth factor

beta (TGF-β), IL-35) (Hofmann and Frantz 2015). More precisely, especially Tregs play a key role in the healing phase through suppressing the immune response in the damaged tissue, promoting revascularization, and initiating the shift to a reparative phenotype of the macrophages (M2 microphages; as mentioned above) (Zouggari et al. 2009; Dobaczewski et al. 2010b; Weirather et al. 2014). Particularly, TGF-β was shown to be mainly responsible for the deactivation of inflammatory macrophages in MI (Dobaczewski et al. 2011). These M2 macrophages then have the ability to express high amounts of several different MMPs and secrete anti-inflammatory cytokines, such as IL-4, IL-13, and mainly IL-10 (Frangogiannis et al. 2000). Consequently, the extent of IL-4 and IL-13 expression also determines the extent and duration of the inflammatory response. Furthermore, it was found that myeloid–epithelial–reproductive tyrosine kinase (MERTK) (Wan et al. 2013) and platelet-derived growth factor (PDGF) (Zymek et al. 2006) are crucial for the transition to a reparative status too. Furthermore, TGF-β signaling and the decline in pro-inflammatory cytokine signaling result in the activation of interstitial and perivascular fibroblasts, EC proliferation, followed by reparative myocardial fibrosis and angiogenesis (Chen and Frangogiannis 2013). Any failure of accurate regulation of Treg or TGF-β signaling may lead to excessive scar formation, an ongoing chronic inflammation (Kypta et al. 2016), such as described in the case report by Kypta et al. (Dobaczewski et al. 2011).

5 Discussion

In the last decades, an immense increase in the number of implanted intracardiac devices could be observed (Mond and Proclemer, 2011). At the same time, patients implanted with a device are getting older and therefore may live for decades with this foreign material embedded in the endocardium and exposed to blood flow (Leon et al. 2010; Proclemer et al. 2010). This is of relevance insofar as little is known about long-term toxic effects of implantable devices (Eliaz 2019; Nasakina et al. 2019). Furthermore, at present it is not known with certainty whether there is a "critical concentration" of the metallic components which, if exceeded, can be expected to cause consequential damage to health. Nitinol, by far the most commonly used alloy for intracardiac devices, appears to have a number of good properties especially during implantation and durability in long-term treatment. On the one hand, it is highly malleable, which is of enormous importance in the context of implantation (Stoeckel et al. 2004; Henderson et al. 2011; Maleckis et al. 2018); on the other hand, it is considered to be extremely durable with overall good tolerance (Eliaz 2019).

We know from numerous preliminary reports that as a result of intracardiac positioning, endothelialization on nitinol surfaces is expected to occur after only a few weeks (Zahn et al. 2001; Sigler et al. 2005a; Schwartz et al. 2010) and depends on numerous factors, such as the size of the device or, in the case of occluders, the primary interventional outcome (Granier et al. 2018). Incomplete endothelialization could lead to complications at site of the implantation, such as thrombus formation (Sigler et al. 2005b; Sellers et al. 2019). Moreover, patient-specific factors must be taken into consideration. For instance, there are known cases of patients with multiple allergies where excessive endothelialization was found in the autopsy (Kypta et al. 2016). Vice versa, it seems conceivable that endothelialization processes or the healing phase can be negatively influenced by the intake of immunosuppressive substances such as glucocorticoids (Radovsky et al. 1988) or TNF-α inhibitors (Sandberg et al. 2012). The same is conceivable for patients in whom cytotoxic substances or radiation therapies are used (Hopewell 1990). As a wide overall variation in anatomic conditions is to be expected in PFO, ASD, or LAA occlusions, positional control by transesophageal echocardiography appears essential (Krizanic et al. 2010; Saw et al. 2016).

Intracardiac pacemakers are a special case in this context, as no endothelial surface usually

forms over the implanted cardiac device due to direct contact with the blood flow (Jana 2019). According to the manufacturers, the devices should only be anchored to the endocardium at their base, while the majority of the device should remain floating in the blood flow. However, there are now several published cases reporting the contrary. Namely, a complete or at least partial endothelialization/encapsulation of the device in the right ventricular wall (Candinas et al. 1999; Esposito et al. 2002; Tjong et al. 2015; Keiler et al. 2017). Interestingly, there are also reports from autopsies where histological processing has shown a clear evidence of inflammatory processes around the encapsulated pacemaker (Dvorak et al. 2012). Exact knowledge of any expected endothelialization is of enormous clinical relevance, since an influence on the stimulation threshold is at least conceivable through the encapsulation (Stokes et al. 1991). There are also issues of what to do in the event of battery exhaustion. An extraction, as originally intended by the developers, seems unlikely in the case of complete encapsulation. It remains to be seen whether the limited space at the surface of the inner heart is sufficient to safely implant an additional device. For the Micra pacemaker, it could be shown in an animal model that up to three devices can be implanted without any problems (Omdahl et al. 2016).

Another major uncertainty is the importance of allergies in long-term use. Nitinol is an alloy which consists of 45–50% nickel (Eliaz 2019), a relevant allergen. Nickel allergies are type IV allergies, that is, a contact allergy caused by long-term exposure, usually after 24 h to a few days (Tramontana et al. 2020), and are relatively common with a prevalence of approximately 8% to 19% in adults (Diepgen et al. 2016). Whether the allergenic potential within the blood flow is particularly high, or whether a weakening occurs once endothelialization has been achieved, remains completely unclear to date. Allergies are also known to occur with exposure to titanium, which is the other component of the nitinol alloy (Fage et al. 2016). However, the incidence is significantly lower for titanium; consequently therefore the clinical relevance is probably of secondary importance (Grosse Meininghaus et al. 2020). In case of a confirmed allergy, it is possible to coat the device with a less/nonallergenic substance such as gold, which has already been done in individual cases (Kypta et al. 2015). The measurement of any metal released from cardiac devices has already been performed (Ries et al. 2003; Saylor et al. 2018). An open question for the future will be whether there are measurable parameters that can be used to estimate the degree of endothelialization. For example, it is conceivable that the metallic components could be measured as nanoparticles in peripheral blood, but their concentration would decrease during the healing phase. It may also be assumed, that with complete endothelialization achieved, the metal content might fall below the detection limit, which in turn provides important additional information for the estimated duration of the healing process.

6 Conclusion

In summary, we conclude that the vast majority of intracardiac devices meet very high safety standards from a hemostasiological point of view, and that there is currently no evidence of any therapy-limiting toxic effects in long-term treatment. Nitinol, as a component of many devices, is of particular importance in this context. However, there are currently gaps in knowledge for patients who are under immunosuppressive medication. Moreover, the impact of an optimal implantation technique on the initial healing phase and endothelialization phase has not been fully understood in many cases.

For these reasons, it seems indispensable to us that patients continue to be treated at the respective healthcare center after primary implantation and are followed up by means of imaging, so that individualized coagulation management can be determined if necessary.

7 Limitations

We could only include devices which are already in broad clinical use. Nevertheless, a number of less-established devices in the field of interventional cardiology might already have received market approval or might be in the preclinical testing phase. However, in our opinion, the current knowledge of the established devices provide a fundamental basis for the above review and the obtained conclusions.

Contributions C.E planned and coordinated the study, compiled and analyzed data, wrote the manuscript, and contributed in the final submission. V.P., E.B., S.H.K., and M.B. analyzed data, prepared figures, and contributed to manuscript preparation. F.K. analyzed data and contributed to final submission. V.D. contributed in data acquisition. K.K. carried out English-language editing of the paper. E.L., C.B., and U.C.H. revised the article critically for the content. M.L. planned the study and provided the final approval of the article.

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Articular Cartilage Regeneration in Veterinary Medicine

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Abstract

Cartilage is an avascular tissue with a limited rate of oxygen and nutrient diffusion, resulting in its inability to heal spontaneously. Articular cartilage defects eventually lead to osteoarthritis (OA), the endpoint of progressive destruction of cartilage. In companion animals, OA is the most common joint disease, and many pain management and surgical attempts have been made to find an appropriate treatment. Pain management of OA is usually the first choice of OA therapy, which is often managed with nonsteroidal anti-inflammatory drugs (NSAIDs). To avoid known negative side effects of NSAIDs, other approaches are being considered, such as the use of anti-nerve growth factor monoclonal antibodies (anti-NGF mAB), hyaluronic acid (HA), platelet-rich plasma (PRP), and mesenchymal stem cells (MSCs). The latter is increasingly being recognized as effective in reducing or even eliminating pain and lameness associated with OA. However, the in vivo mechanisms of MSC action do not relate to their differentiation potential, but rather to their immunomodulatory functions. Achieving actual regeneration of cartilage to prevent OA from developing or even revert already existing OA condition has not yet been achieved. Several techniques have been tried to overcome cartilage's inability to regenerate, from osteochondral transplantation, autologous chondrocyte implantation (ACI), and matrix-induced ACI (MACI). Combinatory use of MSCs unique features and biomaterials is also being investigated with the aim to as much as possible recapitulate the native microenvironment of the cartilage, yet so far none of the methods have produced reliable and truly effective results. Although OA, for now, remains an incurable disease, novel techniques are being developed, rendering hope for the future accomplishment of actual cartilage regeneration. The aim of this chapter is firstly to summarize known and developing pain management options for OA, secondly to present surgical attempts to regenerate articular cartilage, and finally to present the attempts to improve existing regenerative treatment options using mesenchymal stem cells, with the vision for the possible use of developing strategies in veterinary medicine.

Keywords

Articular cartilage · Biomaterials · Mesenchymal stem cells · Osteoarthritis · Regeneration · Veterinary medicine

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Abbreviations

ACI	Autologous chondrocyte implantation
Anti-NGF mAB	Anti-nerve growth factor monoclonal antibodies
BMP2	Bone morphogenetic protein-2
CD105	Cluster of differentiation 105
CD73	Cluster of differentiation 73
CD90	Cluster of differentiation 90
CD45	Cluster of differentiation 45
CD34	Cluster of differentiation 34
CD14	Cluster of differentiation 14
CD11b	Cluster of differentiation 11b
CD79a	Cluster of differentiation 79a
CD19	Cluster of differentiation 19
COMP	Cartilage oligomeric matrix protein
ECM	Extracellular matrix
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
GAGs	Glycosaminoglycans
HA	Hyaluronic acid
HIF-1α	Hypoxia-inducible factor-1alpha
HLA	Human leukocyte antigen
IGF	Insulin-like growth factor
MACI	Matrix-induced autologous chondrocyte implantation
MAT-3	Matrilin-3 protein
MMP13	Matrix metalloproteinase 13
MMP	Modified Maquet procedure
MSCs	Mesenchymal stem cells/ medicinal signaling cells
NGF	Nerve growth factor
NSAIDs	Nonsteroidal anti-inflammatory drugs
OA	Osteoarthritis
OCD	Osteochondritis dissecans
PDGF	Platelet-derived growth factor
PRP	Platelet-rich plasma
PCL	Polycaprolactone
PEG	Polyethylene glycol
PGA	Polyglycolic acid
PLA	Polylactic acid
PLGA	Polylactic-co-glycolic acid
PTHrP	Parathyroid hormone-related protein
ROS	Reactive oxygen species
RUNX2	Runt-related transcription factor 2
SOX9	SRY-box transcription factor 9
TGF-β	Transforming growth factor beta
TPLO	Tibial plateau leveling osteotomy
TSP-1	Thrombospondin-1
TTA	Tibial tuberosity advancement
VEGF	Vascular endothelial growth factor
2D	Two-dimensional
3D	Three-dimensional

1 Cartilage and Its (In)Ability to Heal

Cartilage is a connective tissue of mesodermal origin (Armiento et al. 2019). In the fetus, cartilage acts as a bone template and provides a structure for endochondral ossification (Chiara and Ranieri 2009). In the adult organism, cartilage remains in several areas in the body, such as joints, nose, ear, trachea, and intervertebral disks, playing a role of a supportive structure, shock absorber, flexibility, and movement (Hoshi et al. 2018). Four types of cartilaginous tissues are distinguished based on the cellularity, morphology, and extracellular matrix (ECM) composition: hyaline cartilage, fibrocartilage, and elastic and hypertrophic cartilage (Armiento et al. 2019). The development of certain cartilage type is dependent on the mechanical impact on the tissue. The most common is hyaline cartilage, the embryonic form of cartilage, present at the connection between the ribs and sternum, in the trachea, and on the joint surface where it resists compressive load and provides frictionless movement (Nürnberger et al. 2006). Major constituents of cartilage include a small number of cells, chondrocytes, and a large proportion of their product, ECM, embedded in an abundant interstitial fluid which represents the majority of tissue weight and is essential for joint lubrication and wear resistance (Bora Jr. and Miller 1987). In the vertebrate skeletal system, articular cartilage is highly organized (Nürnberger et al. 2006). Complex organization of articular cartilage rises from differentiation of the cartilage into four layers

(superficial, middle, deep, and calcified zone), ECM compartmentalization (collagen type I predominating in the uppermost part of the zone and collagen type II in the middle and deep zone), and orientation of collagen fibers (Nürnberger et al. 2006) (forming Benninghoff arcades, oriented mostly parallel to the articulating surface with average fibril rotating through the tissue until the orientation of collagen fibers in the middle and deep zones near the interface with bone is perpendicular to the joint surface) (Benninghoff 1925). Despite well-established cartilaginous tissue structure, there are considerable variations between the species. For example, small species such as mice have higher cellularity than larger animals (Stockwell 1971), whereas cartilage thickness is higher in smaller animals (Stockwell 1971; Frisbie et al. 2006).

2 Osteoarthritis in Companion Animals

In the adult organism, cartilage lacks blood and lymph vessels, nerves, and perichondrium. Chondrocytes are thus sustained by nutrients, gases, and cytokines delivered by the synovial fluid (Stockwell 1978). Cartilage metabolism is relatively slow. Low rate of tissue turnover, ascribed to cartilage avascularity and limited rate of oxygen and nutrient diffusion from synovial fluid, results in cartilage inability to heal spontaneously (Hayes Jr. et al. 2001). Intrinsic repair mechanisms, even in minor cartilage defects, are insufficient for the regeneration of cartilage ad integrum (Nürnberger et al. 2006). Natural repairing process of hyaline cartilage results in mechanically inferior fibrocartilage that in comparison to hyaline cartilage contains high levels of type I collagen and only a small portion of glycosaminoglycans (GAGs) and collagen type II, making it less resilient to wear, with higher-friction motion between bones (Armiento et al. 2019). Cartilage injuries may often appear asymptomatic but symptoms appear with progressive cartilage destruction (Mehana et al. 2019; Janakiramanan et al. 2006). The loss and dysfunction of articular cartilage eventually lead to osteoarthritis (OA), a clinical and pathological endpoint of progressive cartilage destruction, affecting both humans and animals worldwide. OA is a slowly progressing degenerative joint disease characterized by whole joint structural changes including varying degrees of osteophyte formation, subchondral bone change, and synovitis, leading to pain and loss of joint function (Dieppe and Lohmander 2005; Enomoto et al. 2019). OA is the most common joint disease in companion animals, especially dogs and horses (Gencoglu et al. 2020) and also geriatric cats (Clarke et al. 2005). Risk factors for OA in dogs are associated with genetics, breed and conformational predispositions, body weight, age, and neuter status (Anderson et al. 2020). In horses, changes in composition and structure properties of cartilage result from cartilage damage due to trauma, impact injuries, abnormal joint loading, excessive wear, or aging process (Gencoglu et al. 2020). In cats, idiopathic OA mediated by congenital, traumatic, infectious, nutritional, and immune-mediated causes is prevailing (Enomoto et al. 2019). The prevalence of OA is higher in older animals, but can also occur in young animals (Gencoglu et al. 2020; Anderson et al. 2020). Although the exact etiology of OA has yet to be identified, the environmental stress followed by metabolic changes in chondrocytes may play a key role in cartilage degeneration (Zheng et al. 2021): Adverse microenvironmental conditions lead to a switch in chondrocyte metabolism from a resting regulatory state in which oxidative phosphorylation is a leading metabolic process to highly metabolically active glycolysis (Zheng et al. 2021). The consequential increase in biosynthesis of inflammatory and degrative mediators and exposure of chondrocytes to proinflammatory cytokines, hypoxia, and nutrient stress are promoting signaling pathways of catabolism. Enhanced catabolism is followed by mitochondrial dysfunction, resulting in excessive production of reactive oxygen species (ROS) and oxidative damage, a hallmark of OA (Zheng et al. 2021; Mobasheri et al. 2017). The important consequence of ROS is the activation of AMP-activated protein kinase (AMPK) and consequential upregulation of the expression of

collagen type I, proinflammatory cytokines, and matrix metalloproteinases (MMP) (Zheng et al. 2021). In particular, MMP13 is known to break down collagen type II, a key structural component of cartilage ECM. Matrix degradation products further promote inflammation and prevent the cycle of degeneration to break (Bedingfield et al. 2020).

3 Pain Management of OA

3.1 Conservative Treatment

OA is currently an incurable disease (Enomoto et al. 2019) and pain management is usually the first step in cartilage therapy. In veterinary medicine, nonsteroidal anti-inflammatory drugs NSAIDs are often the first choice for the treatment of OA and can be used for long-term management of the inflammatory component of OA pain. In addition to NSAIDs, gabapentin, amantadine, and tramadol can be administered when treatment with NSAIDs is not an option. Conservative treatment of OA also relies on the use of weight management, nutritional joint support, and physical rehabilitation including laser therapy, magnetic field therapy, shock wave therapy, massage, and balneotherapy (Zylinska et al. 2018; Rychel 2010). Unfortunately, existing therapies are often associated with severe side effects, such as potential renal, gastrointestinal, or hepatic adverse reactions, and are also often not sufficiently effective (Rychel 2010).

Additional conservative treatment option for treating OA is arthrocentesis or articular puncture, performed to inject supplements such as GAGs or HA to improve the natural qualities of HA, present in the articular fluid, and to increase the mobility of the joint (Zylinska et al. 2018). IM injections of polysulfated GAGs to dogs with OA resulted in improved lameness scores in 12 out of 16 dogs. Reduced lameness was ascribed to GAGs inhibition of cartilage oligomeric matrix protein (COMP) degradation seen as a decrease in serum COMP concentration (Fujiki et al. 2007). However, these results were short-lived similar to the HA treatment. Single intraarticular injection of HA alone in dogs with naturally occurring hip OA also had only a temporary amelioration of the symptoms as measured by Canine Brief Pain Inventory. However, intraarticular injection of HA combined with corticosteroids appeared superior in positive effects compared to HA alone (Alves et al. 2020). Although intraarticular injection of HA and corticosteroids might prove useful for patients that cannot tolerate NSAIDs (Franklin and Cook 2013), based on the retrospective studies in dogs, there was weak or no evidence to support the use of HA for OA (Sanderson et al. 2009; Aragon et al. 2007). Evidence for the efficacy of HA is relatively weak due to the lack of control groups, and the limited numbers of controlled clinical studies make it difficult to suggest the superior effect of HA over the use of NSAID (Aragon et al. 2007).

3.2 Novel Pain Management Treatment Options

3.2.1 Platelet-Rich Plasma

In comparison to intraarticular injection of HA combined with corticosteroids, patient-based assessment scores in lameness and pain were better with intraarticular injection of autologous conditioned platelet-rich plasma (PRP) (Franklin and Cook 2013). PRP is an autologous product, containing an increased concentration of growth factors and bioactive proteins that may enhance the healing process on a cellular level. Besides bioactive factors such as serotonin, histamine, dopamine, calcium, and adenosine that have fundamental effects on the biological aspect of wound healing, PRP contains cytokines and growth factors, including transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) that play an important role in cell chemotaxis, proliferation, differentiation, and angiogenesis and therefore represent a potential to enhance healing of tendon, ligament, muscle, and bone (Foster et al. 2009). The advantage of PRP is primarily that it is a simple, rapid, cost-

effective, and safe way to obtain a clinical improvement of animals affected by OA (Catarino et al. 2020), although diverse methods and devices used to evaluate pain and lameness among different studies make the results difficult to compare (Vilar et al. 2018). Several studies have shown beneficial, albeit temporary, results of intraarticular injection of PRP in the treatment of canine OA. A single injection of PRP into OA joints of dogs was shown to have a positive effect estimated by the lameness grades (Catarino et al. 2020) or force platform gait analysis (Vilar et al. 2018; Venator et al. 2020), but these effects only lasted for 3 to 6 months. Prolonging management of pain was achieved by combining PRP treatment with physical therapy (Cuervo et al. 2020). In comparison to dogs, intraarticular administration of PRP in horses with naturally occurring OA indicates variable changes in kinetic gait parameters (Mirza et al. 2016). Due to differences in PRP concentrations used in different studies, optimization of number of enriched platelets, the volume applied, and concentration of growth factors used for clinical application is needed. Furthermore, characteristics of PRP products differ considerably in the amount of blood processed, method of PRP preparation, and the amount of PRP produced (Franklin et al. 2015). Despite mentioned promising results, there is a lack of data supporting the use of a particular PRP for a specific medical condition, and a consensus on the actual benefits of PRP has not yet been established.

3.2.2 Anti-nerve Growth Factor Monoclonal Antibodies Therapy

A potential alternative to pharmacological pain management in dogs and cats is analgesia using anti-nerve growth factor monoclonal antibodies (anti-NGF mAB) therapy. NGF is a soluble signaling protein, belonging to a family of neurotrophin molecules. During development, NGF has an essential role in the development of sensory and sympathetic neurons, whereas in the adult organism, NGF takes an important part in the sensitization of nociceptors after tissue injury (Mantyh et al. 2011). NGF is produced and released by peripheral tissues such as chondrocytes (Enomoto et al. 2019) and white adipose tissue depots (Ryan et al. 2008). NGF serum level was shown to be associated with stress-related conditions, for example, during transportation (Kawamoto et al. 1996) or exercise load (Matsuda et al. 1991; Ando et al. 2016), and was thus recognized as an important factor to evaluate stress status in an animal (Ando et al. 2020). Besides psychological stress, NGF was correlated also with the mechanical stress associated with OA. Isola et al. (Isola et al. 2011) reported that the concentration of NGF in synovial fluid in dogs with OA was significantly higher in comparison to healthy dogs, suggesting the involvement of NGF in OA inflammation. Similarly, as in dogs, NGF concentration in horses was also higher in synovial fluid from acutely inflamed joints and joints with chronic OA in comparison to healthy joints (Kendall et al. 2021). Some recent clinical studies used anti-NGF mAB to alleviate OA pain in animal patients and are limited to a few studies conducted on dogs and cats. For the treatment of inflammatory pain in dogs, rat anti-NGF mAB were fully caninized (Gearing et al. 2013). Canine-specific anti-NGF mAB were used intravenously in pilot, masked, placebo-controlled clinical studies to alleviate pain in dogs with degenerative joint disease (Lascelles et al. 2015). With 25 dogs included in the study, a positive analgesic effect, similar to that expected with NSAIDs, was recognized based on significantly improved patient-specific outcomes of pain and mobility and significantly increased objectively measured activity. Positive effects of the treatment were observed over 4 weeks after a single treatment with anti-NGF mAB (Lascelles et al. 2015). Similar observations were made in another study conducted by Webster et al. (Webster et al. 2014) where OA-associated pain was alleviated in dogs up to 4 weeks after IV treatment with anti-NGF mAB. Similarly, as in dogs, species-specific anti-NGF mAB were developed for pain treatment in cats (Gearing et al. 2016). In a study with 34 cats, feline-specific anti-NGF mAB were used subcutaneously to treat degenerative joint disease-associated pain. A positive analgesic effect was observed for 6 weeks

during the study with significantly increased objectively measured activity (Gruen et al. 2016). Current evidence suggests that anti-NGF mAB therapy of OA in dogs and cats and possibly in horses could be an alternative to NSAIDs and other pharmacological drugs. The efficiency of a single injection of anti-NGF mAB seems to last 4–6 weeks, but further studies are needed to better understand the level of analgesia and to determine possible adverse side effects and the long-term safety of NGF use.

3.2.3 Mesenchymal Stem Cells/Medicinal Signaling Cells

Longer-lasting pain management effects of treating OA were accomplished using adult multipotent mesenchymal stem cells (MSCs). Stem cells are undifferentiated cells with the capability of self-renewal and differentiation into different specialized cells (Morrison et al. 1997). Compared to other stem cell types such as embryonic stem cells and induced pluripotent stem cells, MSCs were recognized as the most promising type of stem cells for therapy because of the relatively simple harvest techniques, isolation, and the absence of greater ethical concerns associated with their use (Sasaki et al. 2018). For both laboratory-based scientific investigations and preclinical studies, a set of standards to define human MSCs was proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) (Dominici et al. 2006). In essence, (1) MSC must be plastic-adherent when maintained in standard culture conditions using tissue culture flasks; (2) 95% of the MSC population must express CD105, CD73, and CD90 and lack the expression of CD45, CD34, CD14, or CD11b, CD79a or CD19, and HLA class II; and (3) MSCs must be able to differentiate into osteoblasts, adipocytes, and chondroblasts under standardized *in vitro* differentiating conditions. For the identification of animal MSCs, minimal criteria are yet to be defined. MSCs are found in numerous tissues, which, when endogenously activated, act to replace dead, injured, or diseased tissue cells (Caplan 1991). MSCs of common veterinary patients, e.g., dogs, horses, and cats, have been isolated from several tissues including the bone marrow and adipose tissue (Sasaki et al. 2018; Arevalo-Turrubiarte et al. 2019; Webb et al. 2012), umbilical cord (Zhang et al. 2018; Denys et al. 2020), umbilical cord blood (Kang et al. 2012; Koch et al. 2007), muscle and periosteum (Radtke et al. 2013; Kisiel et al. 2012), gingiva and periodontal ligament (Mensing et al. 2011), peripheral blood (Sato et al. 2016; Longhini et al. 2019), endometrium (Rink et al. 2017), and placenta (Carrade et al. 2011). MSCs have also been described in several joint tissues such as synovium (Sasaki et al. 2018), synovial fluid, and synovial membranes (Arevalo-Turrubiarte et al. 2019; Prado et al. 2015) and inside the infrapatellar fat pad. One of the important aspects of the therapeutic potential of MSCs is their ability to migrate into the damaged tissue and secrete immunomodulatory and trophic bioactive factors (Caplan 2017). Therapeutic properties of MSCs, ascribed to their immunomodulatory functions, are exhibited by paracrine action, secretion of extracellular vesicles, immunomodulation mediated by apoptosis, and mitochondrial transfer (Voga et al. 2020). In veterinary medicine, the therapeutic potential of MSCs is being exploited for the treatment of various organ systems. Musculoskeletal diseases have especially been proven indicative for MSC therapy, as was shown in horses with tendon injuries (Pacini et al. 2007; Godwin et al. 2012; Dyson 2004; Smith et al. 2013; Muir et al. 2016), bone spavin (Nicpon et al. 2013), and meniscal damages (Ferris et al. 2014). Notably, as recently reviewed by our group (Voga et al. 2020), remarkable clinical outcomes of MSC treatment have also been shown in dogs (Mohoric et al. 2016; Black et al. 2007; Vilar et al. 2013; Shah et al. 2018; Harman et al. 2016; Maki 2020; Kriston-Pal et al. 2020) and horses (Magri et al. 2019; Marinas-Pardo et al. 2018) with osteoarthritic conditions, showing as significant longer-termed reduction or even elimination of pain and lameness. Based on the results of these studies, MSC treatment for OA appears safe with promising clinical outcomes, showing reduced lameness and pain associated with OA, decreasing the need for use of anti-inflammatory drugs

with their known side effects. However, in comparison to clinical evaluation, the long-term follow-ups with radiographic and CT imaging are scarce and often do not report improvements following MSC therapy, as recently reviewed by Brondeel et al. (2021). Some reduction in progression of OA, demonstrated with radiographic images, was shown in an equine model of OA in fetlock joints (Bertoni et al. 2021), but there is a need for the long-term follow-up imaging performed on actual patients where the progression of the disease is often very different from the experimentally induced pathologies. Demonstrated ability of MSCs to slow down or even stop OA progression is indicative of their well-established immunomodulatory function. One of the important features of MSCs is their tendency to home to injured or inflammation sites when administrated in vivo. However, in contrast to the initial belief that MSCs differentiate and replace damaged tissue, evidence from recent years suggest that MSCs in vivo rarely or never differentiate into the tissue at the site (Guimaraes-Camboa et al. 2017; Meirelles Lda et al. 2009) but secrete bioactive factors. The in vitro multipotency of MSCs thus cannot be directly related to their mechanisms of action in vivo. To avoid the confusion originating from the discrepancy between the name and therapeutic potential of MSCs, it was proposed by Caplan that the term "mesenchymal stem cells" should be changed into "medicinal signaling cells" (MSCs) (Caplan 2017). The actual regeneration of cartilage to prevent OA from developing or even revert already existing OA condition, therefore, remains the topic of research, which is in recent years focusing on exploiting the in vitro differentiation capabilities of MSCs as a basis for finding novel potential solutions to address this issue.

The following part of this chapter focuses on the regenerative surgical attempts to treat cartilage defects, starting with the initial MSC-free attempts to regenerate cartilage, followed by the presentation of the studies exploiting the in vitro differentiation potential of MSCs for cartilage regeneration.

4 Surgical Treatment of OA and Cartilage Defects

4.1 Conventional Treatment Options

Conventional surgical treatment of OA is indicated when conservative therapy fails or is inadequate in alleviating pain and maintaining the function of the joint (Cook and Payne 1997). In dogs, several surgical techniques for OA have been developed. Surgeries may offer treatment of the primary cause, such as cranial cruciate ligament rupture, where tibial plateau leveling osteotomy (TPLO) (Slocum and Slocum 1993), tibial tuberosity advancement (TTA) (Lafaver et al. 2007), or modified Maquet procedure (MMP) (Ness 2016) is indicated. In cases where providing pain relief and lessening the progression of future OA is needed, salvage procedures are performed, such as femoral head and neck excision (indicated in coxofemoral luxation; severe coxofemoral OA; comminuted or complicated fractures of the femoral head, neck, or acetabulum; avascular necrosis of the femoral head; or failed total hip replacement) (Harper 2017a), arthrodesis (indicated for intractable articular fractures, luxations, subluxations, or failed total joint replacement) (McCarthy et al. 2020), and total joint replacement (indicated for patients with debilitating OA secondary to trauma or joint dysplasia) (Harper 2017b).

4.2 Reparative Treatment Techniques

In contrast to salvage surgical interventions used to treat irrevocably damaged articular cartilage by removal or replacement, reparative bone marrow stimulation techniques are used to expose the subchondral bone to stimulate bone marrow and improve cartilage vascularization, enabling the diffusion of nutrients from the subchondral bone into the cartilage and stimulating bone marrow cells to reach the avascular cartilage lesion and initiate a healing response (Stupina et al. 2015). In

humans, the method of bone marrow stimulation is one of the most recommended reparative surgical techniques to treat OA (Gill and Steadman 2004). It can be achieved via drilling, chondral abrasion, or microfractures. The latter are of special interest as it can be performed arthroscopically. Light scraping, but not complete removal of calcified cartilage, is indicated to facilitate attachment of the reparative tissue to exposed calcified cartilage (Breinan et al. 2000). In veterinary medicine, objective evidence documenting the efficiency of bone marrow stimulation is not available. In a canine model of OA, chondral abrasion resulted in a fibrocartilage (Altman et al. 1992). In another study using a dog model of OA, subchondral tunneling of subchondral bone together with the injection of autologous bone marrow into the canals resulted in improved cartilage vascularization and consequently improved chondrocyte metabolism and functionality of cartilage (Stupina et al. 2012). Neither method of bone marrow stimulation resulted in hyaline cartilage formation, but rather in reparation of cartilage with the formation of fibrocartilaginous tissue. Similar was shown in horses. While microfractures increased the tissue volume in the defects (Frisbie et al. 1999) and did not cause any negative effects, this technique did not seem to have clinical effects in horses with stifle lameness diagnosed with naturally occurring OA (Cohen et al. 2009). Bone marrow stimulation results in the formation of fibrocartilage, with poor structural and mechanical properties that do not provide long-term efficacy of reparative surgical treatment techniques (Zylinska et al. 2018). Moreover, poor long-term wear characteristics of fibrocartilage do not prevent the progression of OA (Lane et al. 2004). Since the prevention of degenerative joint changes over time is one of the ultimate goals in the treatment of cartilage lesions (Burks et al. 2006), the limited intrinsic ability of cartilage to heal is proposed to alter with the regenerative treatment options that are therefore at the forefront of the cartilage treatment research.

4.3 Regenerative Treatment Options

A common feature of OA is cartilage defects that may either be associated with pain and decreased function or may appear asymptomatically (Janakiramanan et al. 2006). Either way, without treatment, cartilage defects may lead to progressive joint disease (Mehana et al. 2019; Burks et al. 2006). Treatment of cartilage defects is thus directed toward the regeneration of the defective cartilage and prevention of progression of the disease. Cartilage regeneration methods include osteochondral grafting, autologous chondrocyte implantation (ACI), matrix-induced ACI (MACI), and combinatory use of MSCs and biomaterials, aiming to replace the damaged cells and extracellular matrix while preserving the microarchitecture and biomechanical functions of the cartilage (Zylinska et al. 2018).

4.3.1 Osteochondral Transplantation

Osteochondral grafting is an attractive option for cartilage reconstruction because live homologous tissue is used. In humans, osteochondral and meniscal allograft transplantation in the knee has been performed for over 40 years (Rucinski et al. 2019; Familiari et al. 2018; De Armond et al. 2021). In animals, the majority of the studies are performed on animal models. One of the indications for using osteochondral grafts as a means for cartilage reconstruction in dogs is osteochondritis dissecans (OCD). OCD is an inflammatory condition that occurs when the diseased cartilage separates from the underlying bone. The disease can increase the risk of developing OA and it is an important cause of lameness in dogs (Schreiner et al. 2020). It was previously reported that no differences were detected between the surgical and medical treatment of OCD in 19 dogs. Medical treatment resulted in an even more rapid return to normal weight-bearing. Despite some clinical improvement, in most dogs, lameness continued and the disease progressed (Bouck et al. 1995). Albeit demonstrated to be technically feasible in canine

caudocentral humeral head, medial humeral, and medial femoral condyle, positive clinical outcomes of osteochondral autograft transfer in dogs with OCD were short-termed, with minimal donor site morbidity (Fitzpatrick et al. 2010; Fitzpatrick et al. 2009; Fitzpatrick et al. 2012). The osteochondral graft may not even render clinical changes, as was shown in a canine model of full-thickness cartilage defect, where phalangeal osteochondral graft did not result in significant functional difference compared to the nongrafted group of dogs 6, 12, or 20 weeks after surgery (Dew and Martin 1992). In comparison to OCD in dogs, osteochondral grafts in the case of subchondral bone cysts in horses that can also lead to osteochondrosis (Bodo et al. 2004) resulted in the reconstruction of the articular surface, subchondral decompression, and a renewed cartilage gliding surface. Promising clinical outcomes demand further investigation of the suitability of treatment of subchondral bone cysts with osteochondral grafts (Bodo et al. 2004).

Even though studies on animals are for the most part conducted on animal models and not the actual patients, up to 20% of procedures are unsatisfactory (Huang et al. 2004). The clinical success of the grafts is dependent on the viability of cartilage cells, the capacity of host bone to join graft cartilage, and the host's immunologic tolerance. Integration of donor allograft into recipient's bone can thus be incomplete and can cause failure (Pritzker et al. 1977). Although function and quality of life, based on owner perception, seem to improve after osteochondral grafting (Cook et al. 2008), donor site morbidity is considered a major ethical concern albeit donor sites from canine stifle are currently the only reliable available source of canine donor osteochondral autograft material (Fitzpatrick et al. 2009). Morbidity associated with autografted tissue for treating osteochondral defects could be avoided using fresh allograft tissues. In a canine model of knee cartilage defect, allografts were shown to be similar to autografts regarding bone incorporation, articular cartilage composition, and biomechanical properties (Glenn Jr et al. 2006). Despite being a promising solution for mismatch of transplanted cartilage, allografts may be immunogenic; hence the cartilage becomes vulnerable to direct injury by cytotoxic antibodies or lymphocytes or to indirect injury by inflammatory mediators and enzymes induced by the immune response. However, the literature on the immunogenicity of allografts is contradictory. In some studies, the severe immune response was demonstrated upon allograft transplantation, as shown by an induced inflammatory response, thinned, dull, and roughened cartilage of allografts, with the severely fibrotic and hyperplastic synovial membrane of the joints in dog models (Stevenson et al. 1989). In other studies, no immune response was detected (Glenn Jr et al. 2006; McCarty et al. 2016), or immune response was dependent on whether or not allografts were previously frozen or were vascularized (Stevenson et al. 1996). Freezing was reported to cause harm to the cartilage and thus lower the success rate of osteochondral transplantation (Stevenson et al. 1989). As it was demonstrated in a canine model, viable chondrocytes in osteochondral allografts at the time of transplantation are primarily responsible for the maintenance of donor articular cartilage health in the long term, confirming that not only storage but also procurement, processing, transportation, and clinical implantation are of great importance for allograft clinical use (Cook et al. 2016).

Novel systems for preserving osteochondral allografts, such as MOPS (Missouri Osteochondral Allograft Preservation System) (Cook et al. 2014), and novel methods for enhancing graft integration are being developed. A lack of osteochondral graft integration is one of the important problems in transplanting osteochondral grafts that can cause a treatment failure, especially since there is often a mismatch of transplanted cartilage regarding the contour and thickness of the injured surface (Huang et al. 2004; Hurtig et al. 2001). Also, transplantation of osteochondral grafts involves manual precise preparation of the donor graft and recipient bed. The process is user-dependent, not standardized, and subject to human error. A possible solution for bypassing the issue of

insufficient supply of available donor tissue with accurate anatomical features is a fabrication of osteochondral constructs with the use of 3D printing techniques, improving the accuracy of anatomical architecture and topology, suggesting clinical relevance for large area cartilage repair (De Armond et al. 2021; Roach et al. 2015). Additionally, enhancing graft integration was attempted by using saturating grafts with bone marrow aspirate concentrate (Schreiner et al. 2020; Stoker et al. 2018) or PRP (Stoker et al. 2018), with the assumption that growth factors, cytokines, and other proteins contained in bone marrow aspirate concentrate may enhance osteoinductive, chemotactic, and neovascular signals needed for better graft integration. For example, in an in vitro study, bone marrow aspirate concentration was shown to be superior to PRP in enhancing integration potential for canine osteochondral allografts (Stoker et al. 2018). A combination of novel graft preservation and implantation techniques may therefore result in more satisfying clinical outcomes, as was demonstrated in a study where osteochondral allograft transplantation technique using fresh unicompartmental bipolar osteochondral and meniscal osteochondral allografts and application of bone marrow aspirate concentrate were used to treat medial compartment gonarthrosis in a canine model. Clinical, radiographic, and arthroscopic assessment of the graft and joint demonstrated the maintenance of the integrity of transplants and integration into the host tissue, leading to superior outcomes without early OA progression compared to NSAID controls (Schreiner et al. 2020).

While animal models provide crucial information about disease mechanisms, the artificially induced disease cannot recreate the natural in vivo environment (Cope et al. 2019). Studies conducted on actual veterinary patients are scarce, and extensive research is still needed to prove the efficacy and usefulness of osteochondral graft transplantation on actual patients. However, advancement in allograft transplantation in animal models suggests that osteochondral grafting is worthy of further investigation also in actual veterinary patients.

4.3.2 Autologous Chondrocyte Implantation

The lack of significant cellular activity in chondral defects was indicative for the researchers that chondrocytes are needed for articular cartilage regeneration (Shortkroff et al. 1996). Autologous chondrocyte implantation (ACI) was thus developed as an alternative for treating defects of articular cartilage. In humans with full-thickness cartilage defects, the procedure was described in 1994 by Peterson et al. (Brittberg et al. 1994): Cartilage slices were obtained from an uninvolved area of the injured knee during arthroscopy. Chondrocytes were then isolated and cultured for 14 to 21 days in the laboratory and then injected into the injured area under a periosteal flap taken from the proximal medial tibia. ACI seems to be advantageous over bone marrow stimulation techniques in that the cartilage that is formed is predominantly hyaline-like, containing collagen type II (Brittberg et al. 1994; Min et al. 2007; Cherubino et al. 2003). It was demonstrated by Min et al. that cartilage regeneration after ACI is correlated with at least 4-week-long survival of transplanted chondrocytes (Min et al. 2007). Fluorescently labeled chondrocytes implanted in the goat model were shown to integrate into the surrounding tissue and become a structural part of repaired tissue, rich in collagen type II and proteoglycans (Dell'Accio et al. 2003). In the canine model, ACI was shown to be superior to bone marrow stimulation techniques based on morphology, histology, and serum marker levels, with smooth surface, less fissure, and good border integration (Nganvongpanit et al. 2009). Similar as in dogs, in three horse models of cartilage lesions of fetlock joints in the forelimb, hyaline-like cartilage was formed after ACI treatment (Barnewitz et al. 2003). In the majority of animal models, ACI is investigated in full-thickness cartilage lesions. Partial-thickness cartilage lesions represent a more hostile environment for regeneration due to avascularity, poor cellularity, and smoothness of calcified cartilage. However, in patellofemoral joints in equine models, partial-thickness defects with intact calcified cartilage were proven to be a good indication for treatment

with ACI. ACI improved cartilage healing (although less obviously as in full cartilage defects), as seen with improved histological, immunohistological, and biochemical scores, including defect filling with collagen type II and attachment to the surrounding cartilage (Nixon et al. 2011).

Although ACI has produced promising results, it was indicated in previous studies that the degree to which hyaline-like cartilage fills a defect is insufficient to integrate with surrounding tissue (Breinan et al. 1997). Significant effects after ACI treatment in dog models seem to be short-termed and degenerative changes are not prevented (Nixon et al. 2011). In attempts to enhance the filling of cartilage defects with the functional tissue, biomaterials were developed to serve as carriers of cells.

4.3.3 Matrix-Induced ACI (MACI)

In the original ACI technique, the periosteal cover was used since it was thought to have the chondrogenic potential (O'Driscoll and Fitzsimmons 2001) and stimulate subchondral bone remodeling (Russlies et al. 2005). However, with ACI, there are damage associated with periosteal harvest (Ueno et al. 2001), damage associated with the suturing of articular cartilage (Hunziker and Stahli 2008), and hypertrophy observed after periosteal grafting (Ueno et al. 2001). The downside of this method is also a non-homogenous distribution of chondrocytes due to the use of cellular suspension, together with the risk of leaking out in case of inadequate sealing (Haddo et al. 2004). These limitations were improved by using the matrix-induced ACI (MACI), where alternative covers, such as porcine-derived type I/III collagen membrane, are used. The bilayered structure of a membrane is cell occlusive at the compact side, protecting cells from diffusion and mechanical impact, and the porous side consists of collagen fibers, allowing for cell invasion and attachment (Haddo et al. 2004). Autologous chondrocytes are seeded onto the membrane, enabling the membrane to be attached to the defect with the fibrin glue eliminating periosteal harvest, and procedure is faster and with less extensive exposure, as surgical implantation could be achieved via arthroscopy or mini-arthrotomy (Cherubino et al. 2003). Besides facilitating the handling of the cells, scaffolds are also useful for immobilization and broader distribution of the cells (Nuernberger et al. 2011). The procedure is traditionally performed by arthrotomy (Cherubino et al. 2003), but arthroscopy was also shown to be possible, as was shown in some studies with equine models that underwent arthroscopic implantation of cell-polymer (Ibarra et al. 2006; Masri et al. 2007) or cell-collagen membrane constructs (Frisbie et al. 2008; Nixon et al. 2017). In several studies of equine joint defect models, treatment with MACI resulted in significantly improved cartilage compared to spontaneously healing empty controls, as shown by arthroscopy, gross healing, histology scores, and mechanical analysis (Nixon et al. 2017; Nixon et al. 2015; Griffin et al. 2015). Materials other than collagenous membranes were also used for MACI, for example, PGLA, used in eight horse models and were shown to efficiently contain a large number of chondrocytes without the risk of cell loss when implanted arthroscopically with the use of a fluid pump (Masri et al. 2007). Although ACI and MACI have produced promising results and MACI treatment indeed improved cartilage healing, characterization of MACI graft implant in animal models showed that formed tissue has inferior shear properties to native cartilage (Nixon et al. 2015; Griffin et al. 2015; Lee et al. 2003). The loss of chondrocyte capacity to produce hyaline cartilage might be associated with the cell dedifferentiation occurring during chondrocyte culturing (Rakic et al. 2017).

Although increasing the dose of articular chondrocytes was shown to improve articular cartilage repair in a sheep model (Guillen-Garcia et al. 2014), chondrocytes cultured in vitro are prone to spontaneous dedifferentiation, albeit less so when cultured in a 3D environment. It was shown by Sanz-Ramos et al. (2014) that chondrocytes cultured in a 3D collagen environment possessed a better chondrogenic capacity in vitro and in vivo than the cells expanded on a plastic surface (Sanz-Ramos et al. 2014).

Interestingly, the extent of dedifferentiation seems to vary between species. For example, sheep chondrocytes were shown to be able of spontaneous redifferentiation into hyaline-like cartilage, whereas human chondrocytes were able to redifferentiate only when stimulated by chondrogenic inducers (Giannoni et al. 2005). In the equine model, chondrocyte redifferentiation was shown to be possible under the influence of 3D collagenous microenvironment, hypoxia, and BMP2 (bone morphogenetic protein-2) and RNA interference (Rakic et al. 2017). In comparison to human and equine chondrocytes, dog chondrocytes showed no capacity to redifferentiate regardless of the inducers present (Giannoni et al. 2005). The interspecies differences in chondrocyte characteristics in culture indicate that species should be considered when extrapolating data from one species to another and that differences between species in terms of chondrocyte phenotype stability during expansion might also result in different clinical outcomes when used in ACI. In addition to interspecies differences, chondrogenic differentiation of chondrocytes was dependent also on the number of passages and aging (De Angelis et al. 2020; Acosta et al. 2006; Veilleux et al. 2004), as well as whether the cells were osteoarthritic or not (Acosta et al. 2006). While, interestingly, adult donors showed a more stable expression of some chondrogenic markers, chondrocytes from elderly animals dedifferentiated at earlier passages, associated with a reduced proliferative capacity (De Angelis et al. 2020). Chondrocyte dedifferentiation could therefore be controlled from different aspects of donor and culture factors.

Another hurdle in using ACI/MACI for the treatment of chondral defects is a need for a two-step surgery. In 2006 the evidence that ACI could be delivered without cell expansion was presented. It was proposed that mechanical fragmentation of cartilage was sufficient to mobilize embedded chondrocytes through the increased surface of tissue area. In goats, cartilage fragments were placed on resorbable scaffold hyaline-like tissue (Lu et al. 2006). The procedure was adopted also in horse models with autologous cartilage fragments on a polymer scaffold implanted in a defect within the equine femoral trochlea. Compared to two-step ACI treatment, one-step treatment with minced cartilage achieved an even higher score in arthroscopic, histologic, and immunohistochemistry evaluation and prompted a phase 1 clinical study in humans (Frisbie et al. 2009). In a study performed in dogs, it was demonstrated that 100-μm-sized cartilage particles yielded the highest number of cells and provided the most optimal cartilage regeneration, based on the autologous intrafacial implantation of the microcartilage together with the absorbable scaffold and the slow release system of the basic fibroblast growth factor (Nishiwaki et al. 2017). Another possibility to overcome the need for two-step surgery was proposed by Bekkers et al. who showed that a one-stage procedure could be achieved by combining chondrocytes or chondrons with bone marrow mononuclear cells or MSCs. In a goat model, such implantation outperformed microfracture (Bekkers et al. 2013a, b).

Despite promising results associated with ACI/MACI for treatment of chondral defects, there are still many challenges that have not yet been overcome, such as insufficient integration of implanted chondrocytes, insufficient capacity of chondrocytes to produce hyaline cartilage, dedifferentiation of cultured chondrocytes, the need for two-step surgery, and the harvesting procedure that may result in changes in the articular cartilage that potentially represent a risk of becoming clinically relevant (Lee et al. 2000). This is why in recent years other treatment options for cartilage defects are increasingly being investigated. MSCs as possible substitute cells for chondrocytes are the focus of the most recent research. MSCs seem promising candidates for replacing chondrocytes because of their immunomodulatory properties and their ability to differentiate into several specialized cells, including chondrocytes. At the same time, many novel biomaterials are at the forefront of cartilage regeneration research, aiming to (i) resemble native cartilage tissue to provide the most optimal environment for chondrogenic differentiation of MSCs and (ii) simultaneously develop clinically relevant biocompatible material for in vivo implantation.

5 Attempts to Improve Existing Regenerative Treatment Options with the Use of Mesenchymal Stem Cells

5.1 Chondrogenic Differentiation of MSCs

MSCs have in recent years received significant interest in veterinary and human medicine due to their immunomodulatory and multilineage differentiation properties. Under appropriate culture conditions, MSCs can be induced toward differentiation into different lineages such as adipocyte, osteocyte, and chondrocyte lineages (Dennis et al. 1999). Although there are some reports on spontaneous chondrogenic differentiation of MSC ascribed to either high cell density (Bosnakovski et al. 2004; Dudakovic et al. 2014), presence (Fortier et al. 1998) or absence (Cho et al. 2018) of fetal bovine serum (FBS) in cell culture media, early passages (De Bari et al. 2001), or tissue source (Naruse et al. 2004), chondrogenesis on a standard 2D polystyrene surface is commonly induced with specific culture conditions such as chondrogenic differentiation media, high cell density, and highly humid atmosphere. Chondrogenic differentiation of MSCs is commonly performed in two ways. One technique is a pellet culture – a scaffold-free three-dimensional (3D) culture with high cellular density, where cells are grown in polystyrene conical tubes to form a spherical aggregate at the bottom of a tube (Johnstone et al. 1998). Another method is a micromass culture system where cells are placed in the microwell cell culture plate as droplets of cells with high density that become coalesced to form micromasses of cartilaginous tissue (Mello and Tuan 1999). During early chondrogenesis progenitor cells condense and express collagen type I. By the 5th day, collagen type II is detected and type X collagen is detected by the 14th day. The presence of aggrecan and link protein in the cell aggregates demonstrate that aggregating proteoglycans of the cartilaginous tissue are synthesized by the newly differentiating cells (Yoo et al. 1998). Commonly recognized markers of chondrogenesis in MSCs are SOX9, collagen type II, aggrecan, GAG, and COMP (De Angelis et al. 2020). In chondrogenic differentiating media, growth factors and hormones, namely, TGF-β and dexamethasone (Li and Pei 2018; Mwale et al. 2006), are often used to induce chondrogenesis. TGF-β upregulates chondrogenesis by enhancing SOX9 expression and inhibiting osteoblast differentiation by repressing expression of RUNX2 (Pei et al. 2009), while dexamethasone potentiates the growth factor-induced chondrogenesis of MSCs in vitro, although its influence is not indispensable for chondrogenic differentiation of MSCs as it is dependable on tissue source and microenvironment of MSCs (Shintani and Hunziker 2011). Besides TGF-β, other growth factors, namely, IHH and BMP2 (Steinert et al. 2012; An et al. 2010), FGF (Handorf and Li 2011), and IGF (An et al. 2010; Patil et al. 2012), were also shown to be inducers of chondrogenesis of human MSCs. However, the molecular mechanisms of chondrogenesis are not yet fully understood.

5.2 Hypertrophy Associated with Chondrogenic Differentiation of MSCs

Due to their rapid expansion in culture, trilineage differentiation potential, and easier retrieval that is not associated with articular cartilage damage as opposed to chondrocytes, using MSCs over articular chondrocytes is thought to be advantageous, especially since chondrogenesis of MSCs can be achieved with relatively simple procedures on a standard polystyrene surface. However, the undesirable effect of differentiating MSCs toward chondrogenic lineage is the constitutive expression of hypertrophic markers in MSCs. Hypertrophic markers include collagen type X, MMP13, VEGF (Chen et al. 2019), and a novel biomarker, thrombospondin-1 (TSP-1), known by its antiangiogenic properties and recently described

as an antihypertrophic protein (Cortes et al. 2021; Gelse et al. 2011). The chondrocyte hypertrophy stage can ultimately lead to apoptosis, vascular invasion, and ossification, similarly as in the growing cartilage (Bruderer et al. 2014; Mueller and Tuan 2008). Notably, hypertrophy-related changes can also be related to pathological conditions such as OA (Tchetina et al. 2005; Walker et al. 1995; Nakase et al. 2002). Importantly, it was shown that chondrogenically differentiated MSCs with expressed hypertrophy-associated genes result in mineralization, related to endochondral ossification when transplanted to ectopic sites in severe combined immunodeficient mice (Pelttari et al. 2006). The main hesitation associated with the clinical use of MSCs is therefore their inability to recapitulate stable articular chondrocyte phenotype. Indeed, the extent of the expression of hypertrophic factors might be dependent on the protocol for induction of chondrogenesis. Micromass culture was shown to be superior to pellet culture in that induced cartilaginous tissue was larger, more homogenous, and enriched in collagen type II, while the expression of hypertrophic markers was lower than in a pellet culture (Zhang et al. 2010). Yet, MSCs cultured under either of the two chondrogenic conditions are prone to hypertrophy and matrix calcification, unlike articular chondrocytes that under the same conditions maintain a non-hypertrophy phenotype (Pelttari et al. 2006). Hypertrophy correlated with both techniques is therefore undesirable as it may cause endochondral ossification in vivo.

Reduction of chondrocyte hypertrophy is extensively being investigated by using different techniques, such as co-culturing MSCs with chondrocytes; culturing MSCs in the hypoxic atmosphere; adding hormones, proteins, or other components to the culture media; silencing hypertrophic genes; or using biomaterials to imitate the natural cell environment. Some of these techniques offer promising results, although to date none have shown clinically relevant reduction, let alone complete prevention of hypertrophic differentiation.

5.3 Attempts at Reduction of MSC Hypertrophy

5.3.1 Co-culture

Chondrogenesis of MSCs greatly depends on the microenvironment, as soluble factors from surrounding tissue/cells or direct cell-cell contact can alter gene and protein expression profiles (Grassel and Ahmed 2007). The accurate regulation of key factors involved in chondrocyte hypertrophy might enable guidance of MSCs between chondral and endochondral pathways (Dreher et al. 2020). One of the ways to reduce hypertrophic differentiation of MSCs is thus co-culturing MSCs with chondrocytes, as it was previously shown that chondrocytes provide chondrogenic signals to MSCs via paracrine secretion of soluble factors including TGF-β1, IGF-1, and BMP2 (Liu et al. 2010). Inversely, chondrocytes were also shown to be affected by paracrine secretion of MSCs, as was shown by co-culturing human adipose or bone marrow-derived MSCs, leading to reduction of hypertrophy and dedifferentiation of chondrocytes, which was partially ascribed to HGF secretion by MSCs (Maumus et al. 2013). In rats, reduced hypertrophy by MSC and chondrocyte co-culture was demonstrated by increased expression of aggrecan and collagen type II together with a reduction of collagen type X and MMP13 formation (Ahmed et al. 2014). Similarly, hypertrophy reduction was shown in 3D in vitro environment with co-cultures of bovine MSCs and ACs (Meretoja et al. 2013). Effects of hypertrophy suppression were demonstrated in several other studies where MSCs were co-cultured with chondrocytes (Fischer et al. 2010; Ramezanifard et al. 2017; Amann et al. 2017). Since there is a lack of proper chondrogenic niche, it is a great challenge to stabilize ectopic chondrogenic differentiated MSC phenotype not only in vitro but also in vivo, e.g., in subcutaneous tissue. It was previously shown that the differentiation potential of MSCs is different in vitro when compared to implantation in vivo. Yang et al. (2009) demonstrated that the proliferation rate of bone

marrow-derived rat MSCs cultured in vitro in a 3D environment was similar to self-renewal capacity during in vivo implantation (Yang et al. 2009), whereas trilineage differentiation potential was suppressed in vivo in comparison to in vitro conditions. However, it was shown by Liu et al. (2010) that chondrogenic niche within subcutaneous environment could be created by co-transplantation of MSCs and articular chondrocytes, as was shown with bone marrow-derived porcine MSCs and articular chondrocytes. Chondrogenic signals were provided by the secretion of soluble factors by chondrocytes, including TGF-β1, IGF-1, and BMP2, and not by cell-cell interactions (Liu et al. 2010). Interestingly, there are some reports about the inability of articular chondrocytes to prevent hypertrophy of MSCs in pellet cultures (Giovannini et al. 2010). Similarly, nasal chondrocytes were not able to prevent MSC hypertrophy and calcification in vivo unless parathyroid hormone-related protein (PTHrP) was added to the culture (Anderson-Baron et al. 2020).

5.3.2 PTHrP

PTHrP along with its receptors is generally accepted as an inhibitor of chondrocyte development during chondrogenesis of the growth plate (Kronenberg 2003) and is a commonly reported factor to reduce hypertrophy. Fischer et al. showed that when cultured in a chondrocyte-conditioned medium together with PTHrP, expression of collagen type X, the activity of alkaline phosphatase, and matrix calcification in human MSCs were reduced. Pulsed rather than constant application of PTHrP was shown to be even more effective in the reduction of endochondral differentiation (Fischer et al. 2014). PTHrP was shown to be effective in the reduction of endochondral ossification in several other studies investigating the effect of PTHrP on human MSCs (Mwale et al. 2010; Weiss et al. 2010; Mueller et al. 2013). However, although PTHrP was shown to reduce hypertrophy, it was also reported to simultaneously reduce GAG synthesis and thus have a negative effect on chondrogenesis in human MSCs (Browe et al. 2019). Therefore, further research is needed to better understand the role of PTHrP in the chondrogenesis of MSCs.

5.3.3 Matrilin-3

Besides PTHrP, a non-collagenous ECM protein matrilin-3 (MAT3) was reported to play a regulatory role in cartilage homeostasis. It was previously shown that mutation or deletion of human MAT3 is associated with the early onset of cartilage degenerative diseases (Stefansson et al. 2003; Borochowitz et al. 2004). Indicative chondroprotective properties of MAT3 were supported in a study conducted on human and mice chondrocytes, where it was shown that MAT3 was responsible for the upregulation of cartilage matrix components such as collagen type II and aggrecan. Moreover, it was shown to slow down cartilage degeneration by downregulation of matrix-degrading enzymes, namely, collagenase MMP13 and aggrecanase ADAMTS-4 and ADAMTS-5 (Jayasuriya et al. 2012). The role of MAT3 in slowing cartilage degeneration was shown also in vivo, where MAT3 -primed MSCs suspension slowed the progression of cartilage degeneration in the medial meniscus OA mouse model (Muttigi et al. 2020). In addition to its chondroprotective role, MAT3 was also shown to significantly reduce hypertrophy in chondrocytes and MSCs. In hypertrophic chondrocytes, MAT3 acts as a BP-2 antagonist as it was shown to inhibit BMP/SMAD 1 activity leading to downregulation of collagen X expression and thus inhibition of premature chondrocyte hypertrophy (Yang et al. 2014). In hypertrophic human adipose-derived MSCs, MAT3 significantly reduced the expression of hypertrophic markers such as collagen type X, RUNX2, and ALP (Muttigi et al. 2020). In a study conducted by Liu et al. (2018) where the chondroprotective role of MAT3 was demonstrated in vivo as well as in vitro, the role of MAT3 was ascribed to its function in promoting the expression of HIF1-α. Hypoxia-inducible factor-1alpha (HIF-1α) was shown to be a key mediator in the cellular response to hypoxia (Kanichai et al. 2008) and vital in articular cartilage homeostasis (Liu et al. 2018).

5.3.4 Hypoxia

Since the articular cartilage microenvironment is relatively low in partial oxygen pressure (~ 1–5% O_2) (Gale et al. 2019; Brighton and Heppenstall 1971), a low-oxygen environment for cell chondrogenic differentiation culture conditions was proposed as opposed to standard incubator culture conditions (~ 21% O_2). In fetal mice forelimb organ culture, HIF-1α was shown to regulate chondrocyte differentiation and function during endochondral ossification through triggering BMP2 activation and suppressing the activity of alkaline phosphatase and suppressing collagen type X expression (Hirao et al. 2006). When combined with BMP2, hypoxia and BMP2 synergistically promote the expansion of proliferating chondrocyte zone and inhibit chondrocyte hypertrophy and ossification (Zhou et al. 2015). In chondrocytes, hypoxia promoted chondrocyte rather than osteoblast commitment by suppressing collagen type X mediated by downregulation of RUNX2 activity (Hirao et al. 2006). Interestingly, in chondrocytes, hypoxic culture conditions were shown to induce the expression of PTHrP in a HIF-1alpha-dependent manner (Pelosi et al. 2013). Combining hypoxia and exogenous PTHrP may therefore result in an additive effect in maintaining high levels of GAGs while reducing ALP activity (Browe et al. 2019). Similar effects of hypoxia that were shown with chondrocytes were also shown with MSCs. Kanichai et al. demonstrated that a hypoxic cell environment together with chondrogenic culture conditions significantly enhances collagen II expression and proteoglycan deposition in rat MSCs (Kanichai et al. 2008). HIF-1α in human and murine MSCs, similarly as in chondrocytes, potentiated the expression of BMP2-induced chondrogenic markers and inhibited expression of RUNX2 and osteogenic markers in vitro (Zhou et al. 2015). As in chondrocytes, where hypoxia was shown to induce the expression of PTHrP, hypoxia was also shown to induce PTHrP and reduce MEF2C expression in human MSCs, demonstrating a pathway by which hypoxia attenuates hypertrophy (Browe et al. 2019). Based on the published results from human and murine stem cells, hypoxia seems to enhance chondrogenesis while suppressing hypertrophy. In addition, hypoxia was shown to enhance chondrogenesis also in canine and equine MSCs (Lee et al. 2016; Ranera et al. 2013). Interestingly, in another study investigating the effect of hypoxia on chondrogenesis of equine MSCs, hypoxia did not significantly increase the chondrogenesis of either synovium or bone marrow-derived MSCs, but it did downregulate the expression of hypertrophic marker collagen type X (Gale et al. 2019). Moreover, when studying hypertrophy of bovine MSCs and ACs cultured in a 3D microenvironment under different atmospheric conditions, hypertrophy was reduced in co-cultures of MSCs and ACs in both normoxic and hypoxic conditions, whereas culturing MSCs alone even increases hypertrophic differentiation in hypoxia compared to normoxic conditions (Meretoja et al. 2013). These studies indicate the possibility that there is a difference in susceptibility of MSC to hypoxic conditions between species. The effect of hypoxic culture conditions on suppressing hypertrophy in MSC chondrogenic differentiation might also be dependent on the tissue source of MSCs (Gale et al. 2019). Further studies are therefore needed to more accurately establish the role of hypoxia in MSC chondrogenesis.

Silencing genes associated with hypertrophy is another possible approach in stabilizing chondrogenic phenotype, as was demonstrated in a study conducted on equine bone marrow-derived MSCs, where it was shown that silencing the hypertrophic genes might prevent the persistence of collagen I expression and increase the collagen type II/collagen type I ratio. Introducing siRNA to cells targeting col1a1 resulted in 50% inhibition of col1 expression, suggesting the need for further exploration of the knockout strategy to limit hypertrophic differentiation of MSCs (Branly et al. 2018).

Besides abovementioned attempts to revert hypertrophy, there are also some reports of other possible ways to reduce chondrogenic differentiation-related hypertrophy. For example, it was previously shown that TGF-β and high

doses of steroid hormones together with the absence of thyroid hormones inhibit the induction of hypertrophy (Mueller and Tuan 2008; Karl et al. 2014). Pei et al. showed that TGF–βinduced chondrogenesis was enhanced when synovium-derived MSCs were transfected with histone deacetylase 4, while type X collagen expression was simultaneously reduced (Pei et al. 2009). One of the reported agents to suppress the expression of hypertrophic genes is XAT (xanthotoxin), a furanocoumarin, also named methoxsalen, otherwise used in treating various skin diseases in humans such as vitiligo and psoriasis. It was previously shown to be able to prevent bone loss in ovariectomized mice through inhibition of RANKL-induced osteoclastogenesis (Dou et al. 2016). In the following study examining the effect of XAT on chondrocyte hypertrophic differentiation, it was shown that XAT inactivates the p38-MAPK/HDAC4 signaling pathway leading to reduced degradation of HDAC4 and inhibition of RUNX2 and thus participates in maintaining chondrocyte phenotype in regenerated cartilage (Cao et al. 2017). Hypertrophy of IPSC during chondrogenesis was also reduced using lithium-containing bioceramics with bioactive ionic components (Hu et al. 2020).

Studies investigating different options to revert hypertrophy provide promising results and offer the potential for new ways of maintaining chondrogenic differentiation by suppressing endochondral ossification. However, in most of these studies, MSCs were cultured in a standard 2D environment, which is fundamentally different from their natural environment, and none of the methods described above have provided satisfactory results, preventing the application of differentiated cells in clinical use for cartilage regeneration. To further address this issue, other approaches in the induction of chondrogenic differentiation of MSCs and cartilage regeneration are being investigated, with the focus on recapitulating MSCs native environment.

5.4 Biomaterials for Mimicking Native Cartilage Tissue

5.4.1 The Influence of the 3D Structure on MSCs

The importance of mimicking cellular natural microenvironment lies in spatially and temporally complex signaling that directs the cellular phenotype. The cell, together with the ECM, growth factors, hormones, and other molecules, is connected into an entity, which guides the functioning of individual organs and the whole organism (Tibbitt and Anseth 2009). The interaction of stem cells and their niches creates a dynamic system that is being imitated by in vitro niche models to move closer to the possibility of the therapeutic use of chondrogenic differentiated MSCs. 3D cell culture mimics mechanical and biochemical properties of the natural cellular environment and consequently provides a better insight into the physiological function of MSCs (Jensen and Teng 2020), which is especially important from the therapeutic aspect of using MSCs (Egger et al. 2019). Studies investigating the influence of the 3D environment on MSCs have shown that the 3D environment provides better conditions for expressing biological mechanisms, including cell number, vitality, morphology, proliferation, differentiation, response to environmental signals, intercellular communication, migration, angiogenesis stimulation, immune system avoidance, gene expression, and protein synthesis. 3D cell environment has thus been shown to be more suitable for cell culture than 2D (Antoni et al. 2015). In 3D cultures using carriers or biomaterials, four basic groups of materials are used – polymeric, ceramic, metallic, and composite materials (Kapusetti et al. 2019) – among which the most commonly used are hydrogels, polymeric materials, hydrophilic glass fibers, and organoids (Jensen and Teng 2020).

5.4.2 Influence of Biomaterial Properties on MSCS

The mechanical, surface, and chemical properties of the biomaterial are recognized as crucial in controlling cell fate (Martino et al. 2012). Stem cells are known to be sensitive to the mechanical properties of biomaterials and can recognize a solid substrate even when they are not in direct contact with it (Schaap-Oziemlak et al. 2014). Their adhesion to the substrate depends on the elasticity of the biomaterial, suggesting that even the smallest changes in the mechanical properties of the biomaterial can affect stem cell differentiation. Thus, the different elasticities of the biomaterial have different effects on cell adhesion, proliferation, and differentiation potential. For example, higher biomaterial strength leads to greater potential for osteogenic differentiation due to increased integrin activation, and softer biomaterials increase expression of II type collagen and lipoprotein lipase, markers for adipogenic and chondrogenic differentiation, respectively (Xu et al. 2013). In addition to the mechanical properties of the biomaterial, the surface properties also play an important role in the fate of MSCs. Stem cells do not bind directly to the surface of the biomaterial. In proteinaceous solution, e.g., in cell culture medium, stem cells bind indirectly to the surface of the biomaterial by binding to pre-bound proteins because of their slower movement compared to proteins (Tamada and Ikada 1993). The binding of cells to proteins depends on the distribution and conformation of the proteins, the latter of which depends on the wettability and chemical composition of the biomaterial (Schaap-Oziemlak et al. 2014). Therefore, the manipulation of proteins bound to the surface of the biomaterial is of particular importance in controlling cell adhesion (Schaap-Oziemlak et al. 2014). The results of several studies also indicate the influence of the chemical properties of the biomaterial surface on the direction of cell differentiation (Ren et al. 2009; Curran et al. 2006; Benoit et al. 2008). The surface treatment of biomaterials with different chemical groups, e.g., methyl (-CH3), amino (-NH2), thiol (-SH), hydroxyl (-OH), or carboxyl (-COOH) groups, can have different effects on cell fate and lead MSCs to adipogenic, osteogenic, or chondrogenic differentiation (Curran et al. 2006; Benoit et al. 2008). However, the direction of cell differentiation in a 2D or 3D environment may differ with the addition of the same chemical group (Schaap-Oziemlak et al. 2014). Therefore, the 2D or 3D environment may affect the fate of MSCs differently depending on the functional chemical group.

5.4.3 General Structure of Biomaterials for Cell Encapsulation

In addition to the mechanical, surface, and chemical properties, the scaffold structure itself also importantly affects stem cells. 3D biomaterials can be microporous, nanofibrous, or composed as hydrogels. Microporous structure supports the encapsulation of cells, but due to the pore size (100 μm) being larger than the average cell diameter (10 μm), they represent a curved 2D microenvironment. Nanofibrous structures containing fibrillar ECM proteins provide a better approximation of the natural cellular environment, but their mechanical properties are too weak to handle the stress required for mechanotransduction. Hydrogels do not have these limitations, making them a suitable biomaterial for the development of an ECM-like environment. The network structure of interconnected polymer chains allows for high water content and transport of oxygen, nutrients, waste, and other soluble molecules. Hydrogels can be composed from a range of natural or synthetic materials that exhibit a wide range of different mechanical and chemical properties (Tibbitt and Anseth 2009). Compared to synthetic hydrogels, natural hydrogels not only enable but also promote their cell activities. Natural hydrogels are usually composed of ECM proteins such as collagen, fibrin, hyaluronic acid, or components from other biological sources such as chitosan (Ribeiro et al. 2017), alginate (Sun and Tan 2013), and silk (Kundu et al. 2013).

5.4.4 Natural Biomaterials to Promote MSC Chondrogenesis

For cartilage regeneration, various scaffold materials have been developed. Most commonly used biomaterials for cartilage tissue regeneration are of natural origin, which are biocompatible, contain bioactive molecules such as RGD tripeptides that enable cell adhesion, but have in most cases poor mechanical properties and high degradation rate. Natural biomaterials are composed either of polymers, for example, agarose, alginate, chitosan, and hyaluronate, or of proteins, such as collagen, gelatin, fibrin, and silk (Ge et al. 2012). On the other hand, synthetic polymers such as polyglycolic acid (PGA), polylactic acid (PLA), poly(lactic-co-glycolic acid (PLGA), or poly(ethylene glycol) (PEG) lack the binding sites for adhesion molecules and have been shown to promote the undesirable endochondral ossification (Salonius et al. 2020), but usually provide with controllable degradation rate, high reproducibility, and easy manipulation to form specific shapes (Ahmed and Hincke 2010). Due to the advantages and disadvantages of either natural or synthetic materials, hybrid materials are also thought of as promising materials for providing microenvironment resembling cartilage tissue that is suitable for induction of stem cell chondrogenesis. Below, the commonly used biomaterials for induction of chondrogenesis are described.

Collagen

One of the most extensively used biomaterials in tissue engineering is collagen as it is a key component of cartilage ECM. It is also biocompatible and easy to manipulate with. Bioactive domains in its structure allow for good adhesion of cells. Type I/III collagen membrane has been frequently used in MACI therapy (Haddo et al. 2004). However, there are several disadvantages associated with the use of collagen as a scaffold. Firstly, the use of collagen is associated with the risk of immunogenicity (Kim et al. 2020a, b). Secondly, there is also a possibility of prion transmission (Raftery et al. 2016). Thirdly, collagen does not possess suitable mechanical strength to withstand the in vivo forces (Ahmed and Hincke 2010; Raftery et al. 2016), and lastly, culturing MSCs on collagen does not prevent hypertrophic differentiation of MSCs, as shown by human bone marrow-derived MSCs cultured either on commercial type I/III membrane or collagen/polylactide composite scaffolds, both resulting in a hypertrophic state of the cells (Salonius et al. 2020).

Regarding the immunogenicity of collagen, atelocollagen – telopeptides-free collagen – provides a biomaterial with no immunogenic activity. For treatment of chondral defects in human medicine, atelocollagen combined with microdrilling is used as an enhancement of traditional microfracture technique using the off-the-shelf product (Kim et al. 2020a). Atelocollagen, obtained by salt precipitation, was also tested for chondrogenesis of MSCs. Compared to type I collagen, type I atelocollagen enhanced chondrogenic markers' expression of human adipose-derived MSCs. Moreover, reduction of chondrogenic markers' expression RUNX2, osterix, and MMP13 was observed in cells cultured on atelocollagen, indicating better suitability of atelocollagen compared to collagen for in vitro cartilage engineering applications (Kim et al. 2020b). As a less immunogenic alternative to collagen, gelatin is also used. It is produced from processed bovine or porcine bones and skin and is usually used in combination with other materials to combine positive properties of both (Ahmed and Hincke 2010). For example, the gelatin-alginate scaffold was used to demonstrate that the proliferation rate of bone marrow-derived rat MSCs cultured in vitro on the scaffold was similar to self-renewal capacity during in vivo implantation (Yang et al. 2009).

To avoid the risk of prion transmission, other sources of collagen, besides mammal, are being investigated, such as salmon skin. However, it was shown that salmon skin-derived collagen is inferior to bovine-derived collagen in several terms such as porosity, pore size, architecture, compressive modulus, capacity for water uptake, and rat MSC proliferation and differentiation (Raftery et al. 2016).

In structural and load-bearing performance, collagen plays a pivotal role, while surrounding polysaccharides are needed for internal stress management and elastic reinforcement of collagen and absorption of fluids due to their hydrophilic nature. A protein-polysaccharide scaffold was therefore thought of as a promising material for induction of stem cell chondrogenesis. When used either alone or cross-linked with dextran or chitosan, the PEG-chitosan construct was determined as the most appropriate in inducing chondrogenesis as well as in reducing hypertrophy in human bone marrow-derived MSCs (Sartore et al. 2021). To improve the mechanical strength of the scaffold, chitosan is also increasingly studied and often used in combination with collagen. The addition of chitosan to collagen not only improved the mechanical strength of collagen but also increased compressive strength and swelling ratio and prolonged the degradation rate (Raftery et al. 2016).

Hyaluronic Acid

In addition to collagen, hyaluronic acid (HA) is one of the promising biomaterials in use for chondrogenic induction of stem cells. Hyaluronic acid is a natural component of the cartilage ECM. However, HA is highly degradable in vivo and cannot bind proteins with high affinity because of the lack of negatively charged sulfate groups. Sulfated HA was therefore fabricated to encapsulate human MSCs. The sulfated HA exhibited slower degradation, improved protein sequestration, and promoted chondrogenesis. Furthermore, it suppressed hypertrophy in vitro and in vivo in the OA rat model, due to improved growth factor retention (Feng et al. 2017). When HA was added as a supplementation to a collagen hydrogel, it was shown to stimulate chondrogenic differentiation of adipose-derived human MSCs in a dose-dependent manner. Among different concentrations from 0 to 5%, 1% HA showed the best overall results in terms of SOX and Coll type II expression. Furthermore, exchanging 25% of human articular chondrocytes with 75% of adipose-derived human MSCs didn't change the chondrogenic potential of MSCs, but reduced hypertrophy and improved biomechanical properties (Amann et al. 2017).

Silk Fibroin

One of the promising biomaterials for use in tissue engineering is silk fibroin, derived from the silkworm *Bombyx mori*. It is biocompatible, has suitable mechanical properties, and is produced in bulk in the textile industry (Kundu et al. 2013). In comparison to other natural biomaterials used for tissue engineering, SF provides a remarkable combination of strength, toughness, and elasticity that are ascribed to its crystallinity, hydrogen bonding, and numerous small β-sheet crystals (Altman et al. 1992). Another advantage of SF is its ability to take the form of different shapes such as hydrogels, tubes, sponges, composites, fibers, microspheres, and films that could be used in tissue engineering (Rockwood et al. 2011). It was previously reported that silk fibroin can aid in MSC differentiation when combined with different components. It was previously shown that silk fibroin with incorporated L-ascorbic acid 2-phosphate significantly promoted collagen type I in mouse fibroblast L929 cells (Fan et al. 2012). It was shown to promote osteogenic differentiation and mineralization of human ADMSCs (Gandhimathi 2015), and in another study, it was shown that silk fibroin scaffold combined with PRP effectively induced chondrogenesis of human ADMSCs (Rosadi et al. 2019). Interestingly, it was shown by Barlian et al. that silk fibroin combined with silk spidroin promoted better chondrogenesis of human Wharton jelly's MSCs than silk fibroin alone and that cell culture medium supplemented with PRP promoted higher GAG accumulation in comparison with medium supplemented with ascorbic acid (Barlian et al. 2018). Contrary to mentioned studies where combining silk fibroin with other components was needed to induce chondrogenesis in MSCs, we have shown in our previous research that SF alone could also induce chondrogenesis in canine adipose-derived MSCs, possibly as a species-specific effect.

Decellularized Cartilage Matrix

Besides natural biomaterials such as collagen, hyaluronic acid, gelatin, chitosan, or silk fibroin, which have provided some promising results regarding chondrogenic differentiation of MSCs and reducing their hypertrophy phenotype, other ways for more accurate recapitulation of the cartilage microenvironment are being exploited. Among them, decellularized cartilage scaffolds have shown promise in providing the structural integrity of engineered tissues, better load-bearing ability, and functioning as a reservoir of signaling molecules, e.g., cytokines and growth factors, providing a specific microenvironment similar to native tissue. A hybrid natural ECM scaffold/artificial polymer polycaprolactone (PCL) was developed by combining ECM produced by bovine chondrocytes co-cultured with rabbit MSCs on electrospun microfibrous PCL. This hybrid scaffold was shown to have a positive effect on rabbit MSCs on aggrecan, collagen II, and collagen II/I expression compared to PCL controls (Levorson et al. 2014). Further, Yang et al. developed a cartilage ECM-derived acellular matrix by physically shattering human cartilage, followed by decellularization, freeze drying, and cross-linking techniques. They showed that ECM enabled attachment, proliferation, and chondrogenic differentiation of canine bone marrow-derived MSCs (Yang et al. 2008). ECM scaffold was also shown to be beneficial in reducing loss of chondrogenic phenotype as shown by using ECM scaffold derived from porcine chondrocytes seeded with rabbit MSCs in vivo compared with PGA scaffold (Choi et al. 2010).

In comparison to other mentioned biomaterials, decellularized cartilage ECM is advantageous in that it importantly recapitulates the native cartilage structure. However, achieving the complexity of articular cartilage structure regarding the mechanical stimulation to which the articular cartilage is constantly subjected and related orientation of collagen fibrils is especially challenging. The effect of mechanical loading and orientation of collagen fibrils on cartilage regeneration potential has been investigated in several studies.

5.4.5 Role of Mechanical Stimulation in Cartilage Regeneration

Since articular cartilage is subjected to constant movement and mechanical load, mechanical stimulation was proposed as a factor to affect ECM development. For example, it was shown in chicken micromass cultures that mechanical loading significantly augmented cartilage matrix production and upregulated expression of collagen type III, aggrecan, and hyaluronan synthases through enhanced expression of SOX9 and protein kinase A activity (Juhasz et al. 2014). Improvement of cartilage formation with reduction of hypertrophy was demonstrated to depend on several parameters, such as loading intensity, duration, and frequency of mechanical stimulation (Thorpe et al. 2012; Haugh et al. 2011; Zhang et al. 2015; O'Conor et al. 2013; Li et al. 2010; Bian et al. 2012). Optimal mechanical load, therefore, plays a crucial role during in vitro chondrogenesis of MSCs. Although mechanical forces importantly regulate MSC chondrogenic gene expression, sustained TGF-β exposure is usually also necessary for mechanically based chondrogenic improvement (Zhang et al. 2015; Huang et al. 2010; Goldman and Barabino 2016). Also, the dosage of growth factor was shown to importantly affect hypertrophy, in that only high levels of TGF-β stabilized chondrogenic phenotype (Zhang et al. 2015; Bian et al. 2012). There are, however, reports on mechanically induced proteoglycan synthesis in the absence of chondrogenic cytokines (Kisiday et al. 2009). In a study investigating the influence of mechanical load on porcine bone marrow-derived MSCs cultured on agarose or fibrin scaffolds, the mechanical load was even shown to override the influence of specific substrates, scaffolds, or hydrogels that have been shown to regulate MSC fate (Thorpe et al. 2012). In contrast to studies supporting the effectiveness of mechanical load in MSC chondrogenesis, it was shown that in the initiation stage of cartilage repair, the mechanical load may not necessarily positively affect the cell fate. In a study investigating the effect of chondrogenic priming of equine peripheral blood MSCs on adhesion and incorporation

into cartilage explants, it was shown that mechanical loading reduced the adhesion of cells and altered integration of MSCs into isolated cartilage explants (Spaas et al. 2015). These results are consistent with other studies investigating the effect of biomaterial properties on cell chondrogenesis, showing that mechanical properties can influence cells in terms of their spreading, migration, and differentiation (Toh et al. 2012; Vainieri et al. 2020). This indicates that adjusting biomaterial properties to match mechanical properties, alongside composition and architecture of cartilage, may prevent the incorporation of cells into the cartilage and consequently alter initiation steps of tissue repair (Vainieri et al. 2020). In support of these data, it was also previously demonstrated that mechanical load was associated with bone formation. Mechanical load led to the expression of NGF in mice osteoblasts, followed by the activation of NGF-receptor-positive sensory neurons, resulting in osteogenic cues and bone mass formation (Tomlinson et al. 2017). The data indicate that removing the mechanical load could have a positive effect on MSC in enabling them to reestablish joint homeostasis. Due to the contradictory results from different studies investigating mechanical load on MSCs, further research of the biomechanics, especially early in the disease course, will be needed to provide the data on which MSC repair strategies are needed for optimal cartilage regeneration (McGonagle et al. 2017).

5.4.6 Importance of Biomaterial Architecture

The mechanical performance of articular cartilage directly correlates with the complexity of its structure. Scaffold geometry, recapitulating native orientation of collagen fibrils forming Benninghoff arcades (Benninghoff 1925), thus also seems to play an important role in regulating the cartilage-like activity of cells. For example, bone marrow-derived porcine MSCs expressed collagen type II and synthesized GAGs to a greater extent when cultured on aligned polycaprolactone (PCL) microfibers than on randomly oriented scaffold that was more supportive of an endochondral phenotype as indicated by higher expression of bone morphogenetic protein-2 (BMP2) and type I collagen gene (Olvera et al. 2017). Similarly, mimicking aligned structures of ECM fibrils in cartilage tissue led to better chondrogenesis of human BM-MSC in a nanofibrous scaffold compared to a scaffold with randomly aligned nanofibers (Zamanlui et al. 2018). Furthermore, it was shown that chondrocytes respond differently to geometrically different scaffolds, for example, nanofibrous poly (L-lactide) scaffold more efficiently promotes the cartilage-like activity of bovine chondrocytes than microfibrous scaffolds (Li et al. 2006). A similar tendency of cells toward favoring nanoultrastructure of the scaffold was shown for MSCs. Culturing human MSCs on nanofibrous polycaprolactone resulted in an increased expression of aggrecan compared to MSCs cultured on a microfibrous scaffold (Schagemann et al. 2013). These studies indicate that nano-topographical geometry with aligned structures is favored by cell types such as chondrocytes and MSCs.

To further improve the imitation of the complex structure of cartilage tissue, Nurnberger et al. (2021) have fabricated decellularized articular cartilage scaffold treated for GAG removal and engraved with a CO_2 laser to create the well-defined structure of native cartilage. With the laser, lines and crossed lines were created allowing enough space for homogenous distribution and for the new matrix to be generated. Interestingly, it was shown that new collagen fibers perpendicularly aligned to the cartilage superficial zone, corresponding to the natural alignment of the collagen fibers, deeming superior over scaffolds that promote random matrix deposition (Nurnberger et al. 2021).

One of the novel techniques used for creating complex 3D scaffold structures is 3D bioprinting, as was shown by printing decellularized ECM cross-linked with gelatin methacrylate. Bioactive factors and cells were quantitatively and accurately placed within to form a bionic multifunctional scaffold to recognize, bind, and recruit endogenous stem cells to the site. Scaffold with implanted aptamers for specifically recognizing and recruiting adipose-derived stem cells, together with TGF-β for stem cell

chondrogenesis, resulted in a great improvement of in vivo cartilage full-thickness defects in rabbit models (Yang et al. 2021). Similarly, as in rabbit models, pig models of cartilage defects were used for testing 3D-printed hybrid scaffolds made of gelatine and hydroxyapatite. Gelatine-hydroxyapatite scaffolds, compared to gelatine scaffolds or blank controls, were shown to be the best in reducing hypertrophic markers and repairing cartilage injuries (Huang et al. 2021). 3D bioprinting allows for the fabrication of complicated yet stable structures of tissue analogs and is thus considered a very promising technology, holding considerable potential for articular cartilage repair.

The architectural complexity of cartilage tissue and its constant subjection to mechanical forces demands an understanding of complex mechanisms required for induction of stable chondrogenic phenotype with minimizing the upregulation of hypertrophic genes. Challenges faced in scaffold fabrication are achieving a layered structure mimicking highly specific hierarchical ultrastructure arrangement of ECM of cartilage, mechanical environment for cells resembling native cartilage, and providing physical and biochemical cues to control the biological environment of cells. Mimicking native mechanotransduction pathways may thus be a promising way in creating the desired environment for controlled and stable chondrogenesis. Although cartilaginous tissue structure is well established, its simulation in vitro has proven very challenging, yet novel technologies and increasing acquisition of comprehensive knowledge in regenerative medicine and tissue engineering are encouraging for future cartilage treatment options in both veterinary and human medicine.

6 Summary

Cartilage's unique characteristics encourage scientist to develop methods to overcome its inability to heal. So far, medication-mediated treatment is often the first choice of therapy; however, the therapy is focused on relieving the symptoms but cannot induce repair or regeneration and is often associated with severe side effects. Due to cartilage avascularity, bone marrow stimulation techniques were developed, which have shown some short-term beneficial effects but resulted in a formation of fibrocartilage, which is mechanically insufficient to bear loading stress. Further attempts at repairing cartilage were focused on using native tissue to produce osteochondral grafts. The main disadvantages of this method are the limited amount of donor cartilage availability, donor site morbidity, and the lack of osteochondral graft integration. To overcome the lack of significant cellular activity with osteochondral grafts, ACI was proposed. ACI seemed to be advantageous over other techniques in that the cartilage that formed was predominantly hyaline-like, containing collagen type II. However, there was an issue with the non-homogenous distribution of chondrocytes and the consequential need for periosteal coverage, resulting in damage associated with periosteal harvest. The latter was overcome with the use of MACI. Although MACI treatment improved cartilage healing, the tissue formed was still inferior to the native hyaline cartilage. Moreover, cultivating chondrocytes is associated with chondrocyte dedifferentiation and thus potentially variable treatment results. Although this was shown as possible to overcome with one-step surgery where minced cartilage instead of isolated chondrocytes were used, novel methods to substitute the use of chondrocytes are being developed. MSCs' immunomodulatory properties and multilineage differentiation ability make them attractive candidates as an alternative to chondrocytes. However, the generation of cartilage tissue from MSC is challenging as in vitro chondrogenic differentiation of MSC reflects endochondral ossification unable to maintain a stable hyaline stage. Hypertrophic development of MSCs leads to the bone formation on ectopic sites and is thus unsuitable for cartilage therapy in vivo. Other approaches in the induction of stable chondrogenic phenotype of MSCs are being investigated, with the focus on recapitulating MSCs native environment and providing MSCs the best options to express their

biological function. Many novel biomaterials are thus at the forefront of cartilage regeneration research, from standard collagen-based matrices to novel decellularized ECM cell carriers. Recapitulating the exact architecture of cartilage tissue has proven challenging yet of great importance for cartilage tissue engineering. Despite advances made in biomaterial-based stem cells therapies, each scaffold material currently used in tissue engineering approaches is still limited in possessing all the requirements needed for cartilage regeneration. Moreover, the knowledge of stem cell mechanisms of action is still elusive. A more detailed comprehensive understanding of the MSC mechanisms of action and their responses to complex structural, architectural, and geometrical properties of biomaterials is therefore needed to find the most appropriate way of delivering stable cartilage tissue formation. Combining technologies and knowledge of different scientific fields is essential for engineering a biomaterial that would fundamentally contribute to cartilage regeneration. The collaboration of scientists from interdisciplinary fields is thus of key importance for the further development of advanced cartilage therapies. Looking forward, one can be hopeful that, based on the novel cutting-edge technologies being available and progressive knowledge acquisition, we are on the verge of future developmental breakthroughs in the field of cartilage regeneration.

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Adult Stem Cell Therapy as Regenerative Medicine for End-Stage Liver Disease

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Abstract

The increased incidence of end-stage liver disease (ESLD) causes a major burden on the global health system and population health. Liver transplantation (LT) is one of the most effective treatments for ESLD patients, but its practice is extensively hampered by the scarcity of liver donors, the limited number of transplantation centers, the complexity of the procedure, and postoperative complication. In parallel, vast growing advances in cellular biology and biotechnology have opened new alternatives in clinics, including the transplantation of adult stem cells for chronic diseases such as ESLD. Numerous types of stem cells, such as mesenchymal stem cells, hematopoietic stem cells, endothelial progenitor cells, and other cells, obtained from bone marrow, umbilical cord, adipose tissue, or peripheral blood had been isolated and given to ESLD patients all over the world. Many clinical data had demonstrated promising results, indicating its potential. However, conclusive protocol and agreement on adult stem cell definition and transplantation method are still lacking, and thus further research must still be conducted.

Keywords

Adult stem cells · Cell transplantation · End-stage liver disease · Regenerative medicine

Abbreviations

BM	Bone marrow
EPC	Endothelial progenitor cells
ESC	Embryonic stem cells
ESLD	End-stage liver disease
HBV	Hepatitis B virus
HCC	Hepatitis C virus
HCC	Hepatocellular carcinoma
HLO	Human liver organoid
HSC	Hematopoietic stem cells
iPSC	Induced pluripotent stem cells
LPC	Liver progenitor cells
LT	Liver transplantation
MELD	Model for End-Stage Liver Disease
MSC	Mesenchymal stromal/stem cells
PHH	Primary human hepatocytes

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1 End-Stage Liver Disease

Liver disease is one of the major health problems in the world. It accounts for approximately two million deaths per year worldwide, one million due to complications of cirrhosis and one million to viral hepatitis (hepatitis B virus (HBV) and hepatitis C virus (HCV)) and hepatocellular carcinoma (HCC). Chronic liver disease is usually caused by prolonged excess alcohol consumption, metabolic disorders, and viral hepatitis infection (Asrani et al. 2019).

The number of end-stage liver disease (ESLD) cases is increasing resulting in a greater burden on the healthcare system (Fricker and Serper 2019). ESLD, often interchangeably called liver failure or decompensated cirrhosis, is the final stage of chronic liver disease and is associated with a high degree of mortality. The annual rates of liver disease progression to decompensated stage range from 4% for HCV to 6–10% for alcoholic cirrhosis and 10% for HBV (Asrani et al. 2019).

Liver cirrhosis is characterized by a silent, asymptomatic course that may be undetectable for years. This is usually referred to compensated cirrhosis. When the portal pressure is increased and liver function is significantly reduced, the clinical phenotype is observed. Decompensation is marked by the development of overt clinical signs, the most frequent of which are ascites, bleeding, encephalopathy, and jaundice (European Association for the Study of the Liver. 2018; Haep et al. 2021).

Liver transplantation (LT) is one (if not the only one) of the most effective treatments for any patients with ESLD. LT would extend life expectancy of the patients regardless of the natural history of underlying liver disease where LT is expected to improve the quality of life. However, in practice, LT is hampered by the shortage of donor organs, the limited number of liver transplantation facilities, and the high cost (Harries et al. 2019). Recently, the possibility of living donor liver transplantation (LDLT) can be another option. However, LDLT needs immense and complicated technical operations. And still, the donor shortage remains a concern (Au and Chan 2019; Choudhary et al. 2022).

Following LT, further, the liver recipient might suffer postoperative complications, transplant rejection, and long-term immunosuppression side effects (Feng and Bucuvalas 2017). Further, de novo malignancies are often detected in liver transplant patients undergoing daily immunosuppression regimens, one of the leading causes of late death. The incidence of de novo malignancies among transplant patients is predicted up to four times higher than in the healthy population (Herrero 2012; Manzia et al. 2019).

Since 2002, the Model for End-Stage Liver Disease (MELD) has been used to rank liver transplant candidates for ESLD (Kamath et al. 2001; Wiesner et al. 2003). It is considered an effective strategy for prioritizing candidates with a higher transplant survival benefit over those with lower survival benefit (Luo et al. 2018). This scoring system predicts liver disease severity based on serum creatinine, serum total bilirubin, and INR. It was previously shown to be useful in predicting mortality in patients with compensated and decompensated cirrhosis (Wiesner et al. 2003).

In brief, MELD score ranks patient to number 6 to >40 using the formula ($0.967*\log_e$(creatinine (mg/dL)) + $0.378 \times \log_e$(bilirubin (mg/dL)) + $1.120 \times \log_e$(INR) + 0.6431) × 10) and is suitable as a disease severity index to determine organ allocation priorities (Kamath et al. 2001). Regardless of various revisions and updates (MELD 3.0, MELD-Na, etc.) (Nagai et al. 2018; Kim et al. 2021), the change in MELD score is used as an indicator to measure the benefits of therapy following LT or other treatment regimens.

2 Liver Development and Regeneration

Liver is not only the largest internal organ in the body; it is also capable to replenish its mass by self-regeneration capacity. From a liver phenotypic point of view, it reflects the broad metabolic functions of hepatocytes as well as the liver's unique vascular anatomy, having an inflow

blood supply from both an arterial (hepatic artery) and venous (portal vein) sources (Haep et al. 2021).

Following liver injury, hepatocytes can proliferate to reinstate their morphological and physiological function. In the 1930s, liver regenerative ability in a murine model of partial hepatectomy (PH) had been evidenced. Following PH of around 70% of its total mass, the liver was recovered in about 1 week (Higgins 1931). Using thymidine tracking in the DNA, the restoration of liver mass and function was further demonstrated (Bucher and Swaffield 1964).

In the case of sustained damage such as fibrosis and impaired hepatocytes regeneration, the liver needs to activate its resident stem cells compartment. The canals of Hering and bile ductules in the human liver contain liver progenitor cells (LPC) that can differentiate toward the biliary and hepatocytic lineage (Theise et al. 1999; Libbrecht and Roskams 2002). The source of the LPC is still unclear. It has been variously demonstrated that adult mature hepatocytes can be reprogrammed into proliferative bipotent progenitor cells in response to chronic liver injury (Tarlow et al. 2014; Hu et al. 2018). A population of EpCAM+ cells has been identified within the canals of Hering and the bile ductules, serving as facultative bipotent progenitors capable of differentiating into hepatocytes and cholangiocytes (Safarikia et al. 2020).

During liver disease, the degeneration from healthy-functioning livers involves a dynamic process of hepatocyte damage leading to the reduction of hepatic function. As already known widely, the activation of stellate cells and the production of extracellular matrix (ECM) are the keystone of liver fibrosis. In the case of cirrhosis and ESLD, hepatocyte proliferation or liver regeneration is finished (Haep et al. 2021). Liver failure is also majorly influenced by the exposure to an inflammatory setting, a loss of cell-cell contact caused by cell death and ECM deposition, and changes in energy metabolism and transcriptional deprogramming of hepatocytes (e.g., HNF4α, HNF1, FOXA, HNF6, and C/EBP). Further, clinical manifestations in patients with ESLD are directly related to specific alternated metabolic pathways in failing hepatocytes (Haep et al. 2021).

ESLD is not only due to the lack of healthy hepatocytes but also to the disturbance of tissue architecture and the continuous deposition of inflammatory cells (Lorenzini et al. 2008). Thus, when ESLD occurs, it is hard for the liver to establish its capacity to regenerate.

3 Stem Cell Therapy

Cell therapy has been thought of as the source of liver regeneration (Fig. 1). For therapy applications, donor cells must act as fully functional differentiated cells, such as the expression of liver-specific markers and secretion of albumin and alpha-fetoprotein. Thus, careful protocol and cell characterization should be verified before the transplantation.

Freshly isolated primary human hepatocytes (PHH) are currently the benchmark cell type for cell therapy, but they are not readily available, dedifferentiate quickly, and rapidly die in culture (Hannoun et al. 2016). Several groups had reported methods to cryopreserve the PHH (Godoy et al. 2013; Sison-Young et al. 2017). Despite various optimization protocols, cryopreservation still has damaging effects on the viability and metabolic function (Hannoun et al. 2016). Further, a rather large number of cells (10–15% of liver mass) are needed to provide enough function (Fitzpatrick et al. 2009). So far, various studies had demonstrated the clinical application of hepatocyte transplantation in liver diseases (Lee et al. 2018). In chronic liver disease, however, there are some hassles with engraftment since the liver architecture is disrupted. It is one of the causes of the common failure in hepatocyte transplantation to date (Fitzpatrick et al. 2009).

Stem cells have the astonishing proliferative capacity, self-renewal ability, and differentiation properties. Due to their plasticity, stem cells have been proposed as a source for cell therapy. Embryonic stem cells (ESCs) are the most pluripotent cells that can become all cell types in the body. They are derived from the embryo, typically from the inner cell mass in the blastocyst.

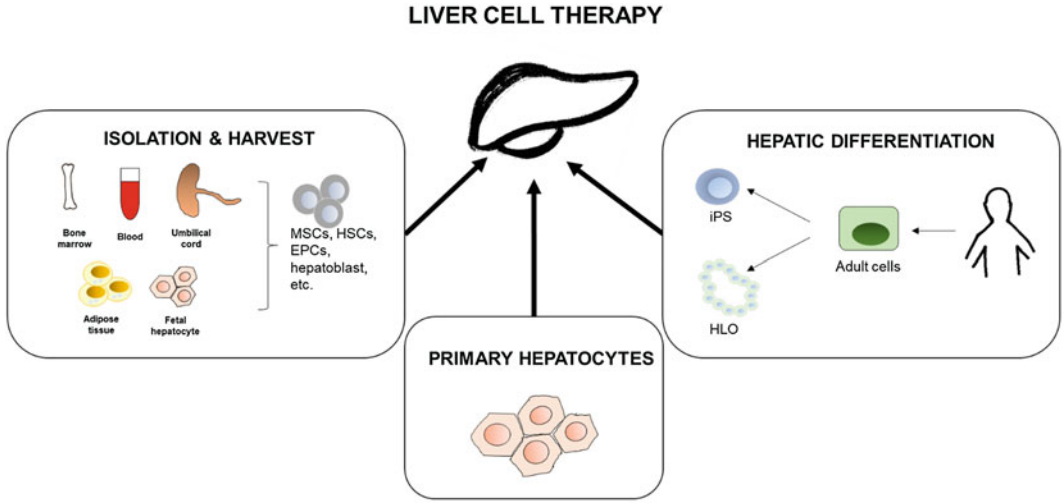

Fig. 1 Organ sources of cellular therapy for the liver

Due to its pluripotency, the human ESCs would be potent tools in regenerative medicine such as Parkinson's disease, spinal cord injury, myocardial infarction, and many more (Mountford 2008). ESCs have been demonstrated to have potential in cell therapy for liver disease. ESCs transplantation had been demonstrated to reduce liver fibrosis and to engraft the liver in rodents (Heo et al. 2006; Sharma et al. 2008; Moriya et al. 2008; Haideri et al. 2017). It is important to notice, however, that ESCs implantation may be tumorigenic where teratoma can occur (Fujikawa et al. 2005; Blum and Benvenisty 2008; Hentze et al. 2009; Stachelscheid et al. 2013). In the human study, it also has a significant ethical dilemma because it involves the destruction of an embryo to obtain the ESCs.

Adult stem cells (or somatic stem cells) can be found in a small number of undifferentiated cells in a specific area of tissue or organ in the body. Even though they are not as multipotent as the ESCs, the adult stem cells can easily be obtained and differentiated into various cells. More importantly, these cells can be ideal sources for autologous stem cell transplantation to replenish tissue damage in the same patient. The bone marrow (BM) compartment is the major source of committed progenitor (stem) cells that can develop into mesenchymal lineages and hematopoietic cells (Masson et al. 2004).

In the beginning, it was assumed that adult stem cells could differentiate only into their maturation lineages. For instance, bone marrow stem cells could only differentiate into blood cells. However, more studies demonstrated that adult stem cells are multipotent and they can differentiate into various cells. For example, bone marrow-derived stem cells could regenerate de novo myocardium (Orlic et al. 2001); skeletal (Gussoni et al. 1999), adipocytic, chondrocytic, or osteocytic lineages (Pittenger et al. 1999); microglial and perivascular cells in the brain (Corti et al. 2002; Hess et al. 2004); as well as the liver cells (Petersen et al. 1999). The injection of these cells ameliorated the outcome of diseases.

To date, there have been numerous clinical studies on adult stem cell therapy for the treatment of ESLD registered in the public database (https://clinicaltrials.gov/) even though many of these studies' results are still unavailable. As the primary outcome, usually, these studies measure the improvement of the MELD score and liver function as the success of the treatment.

3.1 Mesenchymal Stem Cells

The mesenchymal stromal/stem cells (MSC) is the most common stem cells used in clinical

therapy, in addition to being the most controversial. The term mesenchymal stem cells was firstly named in the late 1980s by Dr. Caplan for a cell type derived from bone marrow. These cells could be isolated and expanded in culture while maintaining their in vitro capacity to be induced to form a variety of mesodermal phenotypes and tissues (Caplan 1991). In regard to their multidifferentiation capacity and high self-renewal ability, MSC are a good option for promoting tissue regeneration and inhibiting fibrosis and, at the same time, lessening tissue inflammatory response (Xiang et al. 2022).

In the last three decades, however, the exponential growth of scientific articles had used this nomenclature across numerous isolated cells. In some cases, these cells are various tissue-specific cell types with the use of different cell-surface markers (Sipp et al. 2018), leading to confusion in the scientific community and clinical practice. A previous study demonstrated that "MSC" isolated from different anatomical sources (bone marrow, skeletal muscle, periosteum, and perinatal cord blood) actually differed widely in their transcriptomic signature and in vivo differentiation potential (Sacchetti et al. 2016).

Back in 2005, a working group of the International Society for Cellular Therapy (ISCT) acknowledged the MSC inconsistencies and ambiguities, and they recommended a new designation: multipotent mesenchymal stromal cells (Horwitz et al. 2005). The ISCT also proposed minimal criteria to define human MSC. First, MSC must be plastic-adherent when maintained in standard culture conditions. Second, MSC must express CD105, CD73, and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR surface molecules. Third, MSC must differentiate into osteoblasts, adipocytes, and chondroblasts in vitro (Dominici et al. 2006). Another term of medicinal signaling cells (also abbreviated as MSC) was proposed by Dr. Caplan to more accurately reflect the fact the tissue origin or disease and secrete bioactive factors that are immunomodulatory and trophic (regenerative) medicine (Caplan 2010; Caplan 2017). Regardless of the nomenclature, the capacity of the MSC in the repair of liver tissues had been widely studied with various results.

Several sources of MSC had been used in various clinical trials for ESLD, with the most common sources being umbilical cord (UC) and bone marrow (BM; autologous or allogenic). Some also take advantage of adipose tissue-derived MSC, naively or following cell differentiation (Nhung et al. 2015). As for adipose tissues, sources are broad, and cells can be collected from the subcutaneous tissue, viscera, omentum, inguinal fat pads, peritoneal fat, and other sources (Hu et al. 2019).

As expected, the results of these studies are variable. The injection of autologous BM with CD44+ phenotype had resulted in short benefit in treated patients, regardless of the delivery method (hepatic or peripheral transfusion) (Kharaziha et al. 2009; Peng et al. 2011; Amin et al. 2013; Salama et al. 2014). In these studies, MSC-injected patients had improvement in their liver function and MELD and CP scores compared to control. A meta-analysis of five studies showed that bone marrow infusion in the treatment of decompensated cirrhosis improved liver function without serious side effects at least for the first year (Pan et al. 2014). However, at least in one of the studies, the long-term outcomes were not markedly improved with no significant difference in the incidence of hepatocellular carcinoma (HCC) or mortality between the two groups (Peng et al. 2011).

A recent report from a Japanese clinical trial (UMIN Clinical Trials Registry UMIN000022601) using freshly isolated autologous adipose tissue-derived stem cells in seven patients also showed promising results. Stem cell transplantation improved serum albumin in six out of seven patients and prothrombin activity in five out of seven patients. No trial-related adverse events, which were serious or nonserious, were observed (Sakai et al. 2020; Sakai et al. 2021).

For donor transplantation, the infusion of allogenic MSC from donors was also considered a safe procedure. In patients with liver failure, donor MSC significantly increased the survival rate by improving liver function (reduction of ascites volume, increase of albumin, decrease of

bilirubin, improvement of CP and MELD score) and decreasing the incidence of severe infections (Zhang et al. 2012; Lin et al. 2017; Schacher et al. 2021). In a longer study, upon allogeneic MSC infusion (obtained from donor BM, cord blood, and umbilical cord), MELD score improved at 6 months, 1 year, and 2 years of follow-up. No serious adverse events were observed during or after infusions of MSC in patients with decompensated cirrhosis as compared to control patients (Zhang et al. 2012; Liang et al. 2017). UC-derived MSC transfusion also increased liver function and survival rate in ACLF patients, either by intravenous infusion or hepatic arterial transfusion (Shi et al. 2012; Li et al. 2016).

However, in contrast, several studies showed no benefit of MSC transplantation. A previous study indicated the unsafety of the procedure, and even mortality, following cell transplantation. In a randomized, placebo-controlled trial, from 15 autologous MSC-injected patients, there were 3 deaths registered, while the rest of the patients did not show any improvement in liver function and CP or MELD score (Mohamadnejad et al. 2013). Another study had shown that in this study, even though it was considered safe and feasible, consecutive liver biopsy examinations suggested that MSC infusion via peripheral vessel could not reach the liver in a sufficient amount; thus there were no improvements in MELD scores and serum albumin (Kantarcıoğlu et al. 2015).

3.2 Hematopoietic Stem Cells

Hematopoietic stem cells (HSC) are the most accessible source of stem cells in the body. They give rise to all lineages of blood cell differentiation. In the beginning, it was thought that CD34 (CD34+ cells) is the HSC marker in mammals; however, then it was noticed that human CD34- also had self-renewing capability and acted as primitive HSC that could give rise to CD34+ cells (Zanjani et al. 1998; Wang et al. 2003; Sumide et al. 2018).

The differentiation capacity of HSC, especially to hepatic lineage, is still limited if not controversial. Previously, it was shown that HSC could become liver cells when co-cultured with injured liver separated by a barrier (Jang et al. 2004). In mouse model studies, the transplantation of human cord blood cells CD34+ was able to repopulate the liver (even though with a very low percentage) showing the contribution of HSC (Masson et al. 2004). However, this potentiality was challenged over time. A study showed that HSC expressed mRNAs of hepatic cell markers, but could not efficiently convert into hepatocytes in vitro even in the presence of cytokines or co-cultured hepatocytes (or tissue) (Lian et al. 2006). As mentioned by Thorgeirsson and Grisham, it seemed that the hematopoietic cells are only a minor contributor to hepatocyte formation under either physiological or pathological conditions. These cells, however, may provide cytokines and growth factors that promote hepatocyte functions by paracrine mechanisms (Thorgeirsson and Grisham 2006).

In the clinical study, the application of HSC transplantation in the ESLD had been another option, even though it is not as frequent as the MSC, in line with this limitation described above. One of the first studies comprised a rather small number (phase 1); autologous CD34 was injected into five patients with liver insufficiency. Patients were previously given subcutaneously granulocyte colony-stimulating factor (G-CSF) for 5 days to increase the number of harvested CD34+ cells from the circulation. Following portal vein or hepatic artery injection of these cells, four patients showed improvement in serum albumin (Gordon et al. 2006).

In another study which used the same method, 90 ESLD patients received G-CSF followed by autologous CD34+ and CD133+ HSC infusion in the portal vein. Up to 6 months of follow-up, around 50% had near normalization of liver enzymes and improvement in synthetic function, and 14% showed stable states, compared to control group (Salama et al. 2010). From the same group in another study, stem cell transplantation was done via portal vein infusion of 50% of HSC (CD34+/CD133+), and the other 50% were differentiated to MSC and infused systemically in a peripheral vein in the presence of growth

factors. This procedure had a low incidence of complications and it improved CP and MELD score and degree of ascites of the patients. When the infusion was done in two sessions, the sustained response was continued throughout the follow-up period of 12 months (Zekri et al. 2015).

Another group had shown that the infusion of cell population with CD133+ marker (stem/progenitor cell (SPC)) in ESLD patients was feasible and safe and improved liver function transiently. The recollection of SPC after G-CSF treatment was associated with increased levels of selected cytokines potentially facilitating SPC function (Catani et al. 2017).

Hematopoietic cell isolation and injection from BM also had been performed. In this study, autologous mononuclear (CD34/CD45+) from BM was infused via the peripheral vein in nine patients. Following the procedures, no major adverse effects were noticed. Infused patients had significantly improved CP scores at 1 and 6 months together with improvement in liver biopsy (Terai et al. 2006).

3.3 Endothelial Progenitor Cells

The endothelial progenitor cells (EPC) were discovered around two decades ago (Asahara et al. 1997). These cells were purified by magnetic bead selection with the surface markers antigens CD34+ and Flk1+; in vitro, these cells differentiated into endothelial cells (Asahara et al. 1997). As in MSC the nomenclature of EPC is still under discussion, where another term "endothelial colony-forming cells (ECFC)" is also used (Prasain et al. 2012; Keighron et al. 2018). This disagreement in consensus needs a more precise characterization of these cells based on a pre-defined cellular phenotype and function (Medina et al. 2017).

By using a nonhuman primate model, the localization of injected autologous EPC/endothelial cells (EC) can be traced. At 14 days postinjection via the portal vein, these cells were found scattered in the intercellular spaces of hepatocytes at the hepatic tissues, indicating successful migration and reconstitution in the liver structure as the functional EPC/EC (Qin et al. 2018). Another study examined the benefit of BM-EPC in a rat model of liver fibrosis/cirrhosis induced by carbon tetrachloride (Sakamoto et al. 2013; Lan et al. 2018). While EPC transplantation gave a beneficial result, combined transplantation of BM-EPC and BM-derived hepatocyte stem cells exhibited maximal treatment effect (Lan et al. 2018).

The transplantation of EPC in decompensated liver cirrhosis patients had been reported. In this phase 1–2 pilot clinical trial, autologous cells were harvested from the bone marrow of patients subjected to differentiation to EPC ex vivo. Following hepatic arterial administration in 11 patients, no treatment-related severe adverse events were observed. At 90 days post-transplantation, there was a significant improvement in MELD, and five of nine patients alive showed a decreased hepatic venous pressure gradient (D'Avola et al. 2017).

3.4 Fetal Human Hepatocytes

Fetal liver is becoming an available source of cells for the treatment of liver diseases. Group of Cardinale et al. defined fetal liver as the liver developed from 10 weeks of gestation, the timing when the hematopoietic progenitor cells migrate from the aorta-mesonephros-gonad region to colonize the liver (Giancotti et al. 2022). It contains hepatic stem/progenitor cells within the ductal plates and multipotent stem/progenitor cells within large intrahepatic bile ducts and extrahepatic bile ducts (Semeraro et al. 2013).

Still, limited information is available for the clinical application of fetal liver for ESLD. An Indian clinical study of fetal liver transplantation in 25 end-stage liver cirrhosis patients showed clinical improvement observed in terms of all clinical and biochemical parameters together with a decrease of MELD in 6 months' follow-up in all patients. These cells were obtained from fetal livers of spontaneous abortions from 16 to 20 weeks of gestation and showed positivity of EpCAM+ (Khan et al. 2010). A comparable result was obtained from a study in Italy. Following fetal liver transplantation in an ESLD patient,

the MELD score decreased from 15 to 11 at 3-month and 10 at 18-month follow-up with no signs of encephalopathy. These cells expressed highly significant amounts of proliferation markers compared to adult hepatocytes (Gridelli et al. 2012).

3.5 Hepatic Lineage Differentiation

Several studies had taken another additional step for the application of the MSC. Taking advantage of the multipotency ability, MSCs obtained either from BM, UC, or adipose tissues can be subjected to a hepatic lineage differentiation in vitro before the infusion into the patient/recipient. For example, adipose-derived MSC can be differentiated into hepatocytes in 14 days' culture condition with hepatogenic medium containing dexamethasone, insulin, hepatocyte growth factor (HGF), and epidermal growth factor (EGF), followed by activation of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling pathway (Liang et al. 2009).

One of the first clinical studies using this approach in ESLD was reported in 2011. In this study, upon the isolation and the phenotyping of the autologous BM-MSC, MSC was stimulated into hepatic cells using in the presence of HGF for 7 days. The hepatic-committed lineage was then evaluated by morphological, immunophenotyping, and albumin production. Cells were then injected via the intrasplenic or intrahepatic route. The result showed that MSC-infused patients had significant improvement in ascites and serum albumin, CP, and MELD score over the control group. No difference was observed between intrahepatic and intrasplenic groups (Amer et al. 2011).

Another study used a two-step MSC differentiation into the hepatic lineage, using HGF and FGF, continued by oncostatin and dexamethasone. In this phase 2 trial, however, cells were injected intravenously. MSC-received patients showed partial improvement in liver function tests and MELD score at 3 and 6 months post-infusion. However, there was no significant difference regarding clinical and laboratory findings for MSCs transplantation of either undifferentiated or differentiated cells (El-Ansary et al. 2012).

4 Cell Reprogramming

In the last decades, advances in molecular and cellular biology technologies open exponential opportunities in the manipulation of cellular fate. One of the greatest breakthroughs of the century is the discovery that mature cells can be reprogrammed to become immature, even pluripotent cells, leading to a greatly appreciated shared Nobel Prize in Physiology or Medicine 2012 awarded to Sir John B Gurdon and Shinya Yamanaka (https://www.nobelprize.org/prizes/medicine/2012/summary/).

Back in the 1960s, John Gurdon was successful in transplanting nuclei from fully differentiated cells from the intestine of a tadpole into the cell nucleus of a frog's egg cell. The egg developed into a fully functional cloned tadpole. The transplanted nucleus promoted the formation of a differentiated intestinal cell and at the same time contained the genetic information necessary for the formation of all other types of differentiated somatic cell in a normal feeding tadpole (Gurdon 1962). This nuclear transfer technique was then widely publicized several decades later with the cloning of Dolly sheep, published in 1997 by Wilmut et al. (1997).

In 2006, by using four defined transcription factors Oct3/4, Sox2, c-Myc, and Klf4 (OSKM factors), Takahashi and Yamanaka showed that mouse fibroblasts could be reprogrammed into an embryonic stage, namely, the induced pluripotent stem cells (iPSC). These iPSC cells exhibited ESCs morphology and growth properties and ESCs marker genes. Furthermore, subcutaneous transplantation of iPSC cells into nude mice resulted in variety of tissues from all three germ layers (Takahashi and Yamanaka 2006). In the following year, this technique was then proven in a human cell. Human iPSC cells were similar to human ESC in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase

activity (Takahashi et al. 2007). Because of its ESC-like pluripotency, iPSC is a valuable tool in the basic research on the mechanisms of tissue formation, cell therapy, and patient-specific cell development.

4.1 Induced Pluripotent Stem Cells (iPSC)

First data on the iPSC differentiation to functional hepatocytes was reported in 2009 by Song et al. (Song et al. 2009). They used iPSC cell lines 3U1 and 3U2 subjected to hepatic differentiation protocol composed of four stages: endoderm induction (activin A), hepatic specification (FGF4, BMP2), hepatoblast expansion (HGF, KGF), and hepatic maturation (oncostatin M, dexamethasone, N2, B27, nonessential amino acids, and β-mercaptoethanol). The differentiated cells exhibited mature hepatocyte functions including albumin secretion, glycogen synthesis, urea production, and inducible cytochrome P450 activity (Song et al. 2009). This process takes around 21 days.

A more rapid protocol was then demonstrated. In about 12 days, iPSC could be directed into mature hepatocytes by using the protocol of endodermal induction (activin A, Wnt3a, HGF), hepatic lineage commitment (in the presence of nonessential amino acids, β-mercaptoethanol, DMSO), and hepatic (oncostatin M, dexamethasone, ITS) (Chen et al. 2012). The cells had similar gene expression profile to mature hepatocytes. Besides its functionality as mature hepatocytes including cytochrome P450 enzyme activity, secreted urea, uptake of low-density lipoprotein (LDL), and glycogen storage, these induced hepatocyte-like cells rescued lethal fulminant hepatic failure in a NOD-SCID mouse model (Chen et al. 2012).

The induction of iPSC into bipotent hepatic progenitor cells (HPC) gave rise to both mature hepatocytes and cholangiocytes (Yanagida et al. 2013). The induced-HPC from iPSC resulted in $CD13^{high}CD133+$ cells, positive markers of hepatoblast. Spheroid formation of the HPC could be induced into hepatocytes (dexamethasone, OSM) and cholangiocytes (EGF, HGF, R-spondin 1, Wnt-3a, A-83-01, and Y-27632) (Yanagida et al. 2013). The clinical application of iPSC was performed in several diseases such as degenerative and cardiovascular disease with various results (Martins et al. 2014; Bracha et al. 2017; Tsujimoto and Osafune 2021). However, for liver diseases, its application mostly is still conducted in a preclinical setting.

4.2 Human Liver Organoids (HLO)

Organoid biology is one of the fastest-growing interests in recent organ development and regeneration study. The capacity of isolated cells to self-assemble to form an entire organism was already reported in the early 1900s. When siliceous sponges are kept in confinement under proper conditions, they degenerate and gave rise to small masses of undifferentiated tissue which in turn grow and differentiate into perfect sponges (Wilson 1907).

Human liver organoids (HLO) derived from either adult stem/progenitors or pluripotent stem cells emulate the structure and cellular diversity of the human liver in vivo (Chang et al. 2021; Reza et al. 2021). Under a strict cell culture condition and the presence of correct growth factors (e.g., matrigel, TNFα), organoids can resemble a functional liver. A recent report even showed that from a single hepatocyte, organoids can be established and grown for multiple months while keeping its key morphological, functional, and gene expression features (Hu et al. 2018). However, when compared to the fetal culture, HLO derived from hepatocytes appeared to be more limited in their expansion times yet yielded organoids of very similar composition (Hu et al. 2018).

In the clinical application, HLO technology is not yet available, even though preclinical data in the animal model showed promising result. In a PH model in rat, the transplantation of HLO through portal vein is safer and more effective compared to monolayer cell transplantation, showing 70% replacement of the damaged liver (Tsuchida et al. 2019). Further, HLO in

combination with co-culture with other cell lines and advanced bioengineering tools (sheet layers, microfluidics, 3D scaffold) will increase the differentiation efficiency and enhance the functional maturity.

5 General Perspective

Stem cell therapy is a promising alternative for the treatment of ESLD, especially when the availability of donor liver for LT is scarce. Thriving development of technology in stem cell isolation and maintenance, characterization, and in vitro differentiation to hepatic cells is growing fast, thus allowing an improved method in clinical application.

In ESLD, however, at least until now, stem cell therapy application is still rather far from ideal. The biology of stem cells is still needed to be explored. Clinicians and basic scientists must know whether the transplanted cells are multipotent and self-renewable or the cells' phenotype (Fig. 2), both in donor cells and in the recipient patient, including the protocol of administration, patient's status, safety, and efficacy. Further, vast differences in the source of the cells, type of the cells, transplantation protocol, and criteria of recipients render technical hitches. The administration of stem cell injection (quantity and mode of delivery) may vary between laboratories based on each protocol and experience. Several studies were conducted to definite numbers of stem cells for the injection, while others calculate the body weight of the recipient. Similarly, several studies preferred intrahepatic administration while others via intrasplenic or peripheral vein. Therefore, so far, there is no definite indication or international consensus regarding the protocol of adult stem cells in ESLD patients.

Apart from a scientific perspective, the clinical application of cell therapy is related also to the vast speed of the internet spread. Advances in information technology significantly increase the global transfer of knowledge, including in the search for stem cell therapy in one click. As can be seen in cell therapy for regenerative medicine, the so-called stem cell tourism (Berger et al. 2016; Sipp 2017) is also a problem in hepatology and gastroenterology (Hermerén 2014). This problem requires prompt action for the regulation of cell therapy, from scientists, clinicians, professional associations, and government or authorities. Stem cell therapy for ESLD had shown some promising results, but more research and the definition of a better protocol are still significantly needed.

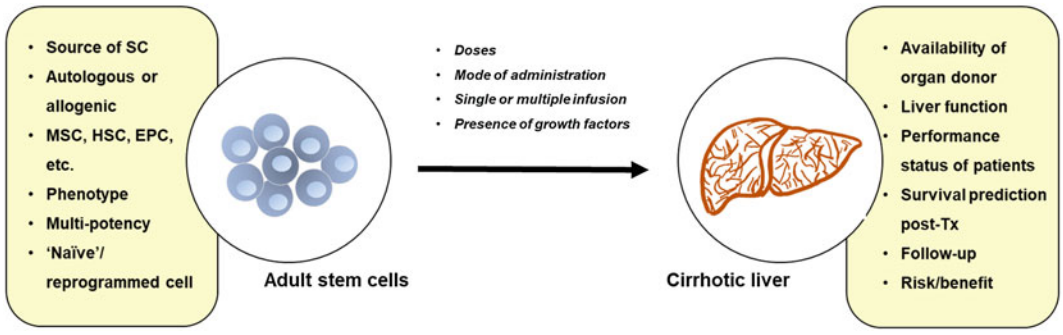

Fig. 2 Important factors for adult stem cell therapy for ESLD. Stem cell therapy would need to consider aspects both in the donor cells (source, types, phenotypes, potency) and in the recipient (liver status, patients' performance, risk/benefit), together with the mode of administration (site, presence of growth factor, doses) and correct protocol

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An Affordable Approach of Mesenchymal Stem Cell Therapy in Treating Perianal Fistula Treatment

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Abstract

The application of stem cells to treat perianal fistula due to Crohn's disease has attracted a lot of interest in recent decades. Though still a popular procedure, the existing surgical methods may be an ideal form of therapy since the recurrence rate is high, which affects the quality of life badly. Stem cell therapy offers to be a better solution in treating PF, but the utilisation is often restricted because of the manufacturing cost. Hence in this review, the selection of suitable cell sources, the use of bioreactors and preconditioning MSCs as well as modified stem cells will be discussed for a more affordable as compared with the current MSC therapy towards PF. We anticipate that exploring these approaches may give a complete picture in understanding stem cells in order to make them effective and affordable for long-term therapeutic applications.

Keywords

Manufacturing cost · Refractory Crohn's disease · Stem cells genetic manipulation · Surgery

Abbreviations

ADSCs	Adipose-derived stem cells
AGA	American Gastroenterological Association
bFGF	Basic fibroblast growth factor
BM-MSCs	Bone marrow-derived mesenchymal stem cells
CCL	C–C motif chemokine ligand
CD	Crohn's disease
CD163	Cluster of differentiation 163
CK	Casein kinase
COX2	Cyclooxygenase 2
CPF	Complex perianal fistula
CSF	Colony-stimulating factor
CXCL	Chemokine (C-X-C motif) ligand

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CXCL12	The stromal cell-derived factor-1
CXCR	Chemokine (C-X-C motif) receptor
DC	Dendritic cell
DDL4	Delta-like 4
eASCs	Expanded allogeneic adipose-derived stem cells
EGF	Epidermal growth factor
eIF2	Eukaryotic initiation factor 2
EMT	Epithelial-to-mesenchymal transition
EPG	Epidermal growth factor
EV	Extracellular vesicle
FAS	Fas cell surface death receptor
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptors
FoxP3$^+$ Treg cells	Forkhead box P3$^+$ regulatory T cells
G1 phase	Growth 1 phase
G5k3β	Glycogen synthase kinase 3 beta
GCN2	General control nonderepressible 2
GDF-15	Growth differentiation factor-15
GM	Granulocyte-macrophage
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Good Practice Manufacturing
HCAM	Homing cell adhesion molecule
HGF	Hepatocyte growth factor
HLA-G	Human leukocyte antigen G
HUMSCs	Human umbilical cord-derived mesenchymal stem cells
HUVECs	Human umbilical cord vein endothelial cells
IBD	Intestinal bowel disease
ICAM-1	Intercellular adhesion molecule 1
IDO	Indoleamine 2,3-dioxygenase
IEC	Intestinal epithelial cells
IFN-y	Interferon gamma
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor 1
IGFBP	Insulin-like growth factor-binding protein
IL	Interleukin
ILT	Immunoglobulin-like transcript
IRF-1	Interferon regulatory factor-1
ITT	Intention to treat
JAK-STAT 1	Janus kinase and signal transducer and activator of transcription 1
K	Keratin
LIFT	Ligation of the intersphincteric fistula tract
M	Matrix protein
MCP-3	Monocyte chemotactic protein 3
miRNAs	MicroRNAs
MMPs	Matrix metalloproteinases
MSCs	Mesenchymal stem cells
NF-κB	Nuclear factor kappa B
NK	Natural killer
Oct4	Octamer-binding transcription factor 4
PAMPs	Pathogen-associated molecular patterns
PDGF	Platelet-derived growth factor
PF	Perianal fistula
PGE2	Prostaglandin E_2
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
S phase	Synthesis phase
SDF-1α	Stromal cell-derived factor-1
SLUG	Snail family transcriptional repressor 2
SNAIL1	Snail family zinc finger 1
Sox2	SRY-box transcription factor 2
TC	Transitional cells
TGF-β	Transforming growth factor beta
Th	T helper
TLR4	Toll-like receptor 4
TNF-α	Tumour necrosis factor alpha
TRAEs	Treatment-related adverse events
Treg cells	Regulatory T cells
TSG6	Tumour necrosis factor-stimulated gene 6
UB-MSCs	Umbilical blood-derived mesenchymal stem cells
UC-MSCs	Umbilical cord-derived mesenchymal stem cells
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor

VLA-4	Very late antigen 4
VWBR	Vertical-Wheel™ Bioreactors
WJ	Wharton's jelly

1 Introduction

Perianal fistula (PF) is a probable consequence of Crohn's disease (CD) since as many as 26% of patients with CD eventually develop PF within 20 years after the diagnosis. This suggests that the CD cases are perhaps a good tracking parameter for PF incidences (Dudukgian and Abcarian 2011; Schwartz et al. 2019). Traditionally, the occurrence and incidence rate of PF is more common and higher in the Western world such as North America, Europe and Scandinavia as compared to the rest of the world (Ng 2014). However, there have been noticeable changes since the last decade wherein several studies on the epidemiology of CD in the Asia Pacific and developing region revealed an increasing trend, while the rate was stable or rather regressive in the Western countries (Ahuja and Tandon 2010). This shift is likely due to changes in diet, stressful lifestyles and industrialisation in developing countries (Ng 2014).

Current treatment options for PF closely follow its anatomical features. The common one is surgical intervention, namely, fistulotomy, advancement flap procedure and ligation of the intersphincteric fistula tract (LIFT) which aims for a complete healing of fistula although they are less effective in complex cases like transsphincteric fistula (Ji et al. 2021; Limura and Giordana 2015). Despite this, surgeries are complicated with a prolonged recovery period that delays patients from returning to normal life (Sanad et al. 2019). The recovery rate of surgery is less impressive, for example, the success rate for LIFT is only 65% (Lehmann and Graf 2013). Furthermore, it is not uncommon for patients to experience fistula recurrences. Emile et al. (2017) reported that 10.3% of patients relapsed and subjugated themselves to second or third surgeries. Besides healing, there is also a need to address patient satisfaction as well. Side effects like bowel incontinence affect 7% of patients after undergoing surgeries, deteriorating their quality of life by adapting to the adversaries (Dudukgian and Abcarian 2011; Panés and Rimola 2017).

Among the recent revolutionary therapeutic procedures, stem cell therapy showed significant improvement in fistula treatment. Table 1 summarises ten complete clinical trials that were being reported as of 2021, with bone marrow and adipose tissue used as the distributions for the source of mesenchymal stem cells (MSCs). MSCs injected into the tissue surrounding the fistula can restore the damaged tissues primarily through their immunomodulatory effect, stimulating a cascade of immune reactions to foster natural healing (Carvello et al. 2019; Prockop and Oh 2012). The treatment procedure is simpler and takes a shorter time of procedure and hospital stay (Park et al. 2021). Statistically, it boasts a higher success rate, lower recurrences and complete healing with higher patient satisfaction (Ciccocioppo et al. 2019; Herreros et al. 2019).

Despite the superior efficacy of stem cell therapy, its application for PF treatment is underwhelming (Gallo et al. 2020). It suffers from slow industrial growth, yet to be fully realised and made available at a wider scale. High cost of the therapy shrinks its market size, confining stem cell therapy as a last resort for very complex cases only when all other methods fail, thus making it a very niche treatment (Choi et al. 2019). Alofisel, a currently existing medicine specifically for PF based on MSCs derived from allogeneic adipose tissue, costs around $67,000 per dose which some patients need multiple doses for complete fistula closure (Scott 2018). Consequently, the unaffordable cost of treatment is deterring patients from pursuing it, relying on cheaper alternatives. The total cost of treatment is largely dependent on its production cost and numbers of in-process and final release quality assays, as well as on storage (Scott 2018).

A profitable product model is interlinked to the source, isolation and expansion techniques of MSCs. New opportunities like discovering new MSC sources can provide cheaper extraction methods. New advancements in bioreactors previously recruited for bacteria and viruses

Table 1 Clinical studies with stem cell therapy for PF from 2016 to 2021 and their current status

Author (year)/ clinical trial number (if applicable)	Study/clinical trial title	Clinical trial phases	Patient enrolment and injection dosage	Type of cell and its source	Status	Results (if applicable)
Park et al. (2016)	Allogeneic adipose-derived stem cells for the treatment of perianal fistula in Crohn's disease: a pilot clinical trial	Phase 1	Group 1, n = 3 (10×10^6 cells/mL); group 2, n = 3 (30×10^6 cells/mL)	Allogeneic ADSCs	Completed	At month 8: Complete closure was observed in group 1, 2/3 (67%); group 2, 1/3 (33%)
Panés et al. (2016)/ NCT01541579	Expanded allogeneic adipose-derived mesenchymal stem cell (Cx601) for complex perianal fistulas in Crohn's disease: A phase 3 randomized, double-blind controlled trial	Phase 3	Cx601, n = 107 (120×10^6 cells/mL); placebo, n = 105	Allogeneic ADSCs	Completed	At week 24: 57/107 (53%) Cx601 vs. 43/105 (41%) placebo achieved clinical remission
Dietz et al. (2017)/ NCT01915927	Autologous mesenchymal stem cells, applied in a bioabsorbable matrix, for treatment of perianal fistula in patients with Crohn's disease	Phase 1	12 (20×10^6 cells/mL)	Autologous ADSCs	Completed	At 6 months: 10/12 (83%) achieved complete clinical healing
Panés et al. (2018a, b)/ NCT01541579	Long-term efficacy and safety of stem cell therapy (Cx601) for complex perianal fistulas in patients with Crohn's disease	Phase 3	Cx601, n = 107 (120×10^6 cells/mL); placebo, n = 105	Allogeneic ADSCs	Completed	At week 52: 61/103 (59%) Cx601 vs. 42/101 (42%) placebo achieved clinical remission in modified ITT group
Wainstein et al. (2018)	Stem cell therapy in refractory perianal Crohn's disease: Long-term follow-up	Observational pilot study	9 (100×10^6–120×10^6 cells/mL)	Autologous ADSCs	Completed	At median follow-up of 31 months: 8/9 (89%) patients achieved complete healing
Serrero et al. (2017)/ NCT02520843	Long-term safety and efficacy of local microinjection combining autologous microfat and adipose-derived stromal vascular fraction for the treatment of refractory perianal fistula in Crohn's disease	Phase 1	10 (10.9×10^6–47.8×10^6 cells/mL)	Autologous ADSCs	Completed	At week 48: 80% of patients had clinical responses; 60% of patients had combined remission

Laureti et al. (2020)/ NCT03555773	Refractory complex Crohn's perianal fistulas: A role for autologous microfragmented adipose tissue injection	Prospective pilot study	15 (20 mL microfragmented ADSCs)	Autologous ADSCs	Completed	At 24 weeks: 10/15 (67%) patients achieved combined remission that includes clinical and radiographic assessment; 4/15 (27%) patients had improved condition
Dige et al. (2019)/ NCT03803917	Efficacy of injection of freshly collected autologous adipose tissue into perianal fistula in patients with Crohn's disease	Phase 1	21 (18 mL–104 mL)	Autologous ADSCs	Completed	At 6 months: 12/21 (57%) patients had complete fistula healing; 3/21 (14%) patients had ceased fistula secretion; 1/21 (5%) patients had reduced fistula secretion
Zhou et al. (2020)/ ChiCTR1800014599	Autologous adipose-derived stem cells for the treatment of Crohn's fistula-in-ano: An open label, controlled trial	Phase 2	ADSCs, n = 11 (5×10^6 cells/mL); placebo, n = 11	Autologous ADSCs	Completed	At 12 months: Healing rates were observed in 7/11 (64%) patients in the ADSCs group vs. 6/11 (55%) patients in the placebo group
Barnhoorn et al. (2020)/ NCT01144962	Long-term evaluation of allogeneic bone marrow-derived mesenchymal stromal cell therapy for Crohn's disease perianal fistulas	Phase 1	Cohort 1, n = 5 (10×10^6 cells/mL); cohort 2, n = 5 (30×10^6 cells/mL); cohort 3, n = 5 (90×10^6 cells/mL); placebo, n = 6	Allogeneic BM-MSCs	Completed	At 4 years: Cohort 1, 3/4 (75%); cohort 2, 4/4 (100%); cohort 3, 1/5 (20%); achieved complete clinical fistula closure vs. placebo, 0/3 (0%)
2020/NCT04519671	A phase IB/IIA study of adult allogeneic bone marrow derived mesenchymal stem cells for the treatment of perianal Fistulizing Crohn's disease	Phases 1 and 2	40 (75×10^6 cells/mL)	Allogeneic BM-MSCs	Recruiting	–
2021/NCT04791878	A phase I study of adult allogeneic bone marrow derived mesenchymal stem cells for pediatric perianal Fistulizing Crohn's disease	Phase 1	10 (75×10^6 cells/mL)	Allogeneic BM-MSCs	Recruiting	–

(continued)

Table 1 (continued)

Author (year)/ clinical trial number (if applicable)	Study/clinical trial title	Clinical trial phases	Patient enrolment and injection dosage	Type of cell and its source	Status	Results (if applicable)
2021/NCT04939337	Study to assess the safety and efficacy of allogeneic umbilical cord-derived mesenchymal stem cells (TH-SC01), for treatment of complex perianal fistulas in perianal Crohn's disease	Phase 1	24 (120×10^6 cells/mL)	Allogeneic UC-MSCs	Enrolling by invitation	–
2021/NCT05039411	A phase I study of the safety of allogeneic human umbilical cord mesenchymal stem cells (UC-MSCs) for perianal fistulas in patients with Crohn's disease	Phase 1	7 ($125–150 \times 10^6$ cells/mL)	Allogeneic UC-MSCs	Not yet recruiting	–

Numbers of clinical trials have been conducted to identify the efficiency and the efficacy of MSCs towards PF based on the injection dosage and type of cell source. There are still several clinical trials up until now to prove the safety of MSCs against PF with Crohn's disease

expansion can be modified to sustain a biological environment for the various techniques that exist for cell culture production, with limitations for MSCs (McKee and Chaudhry 2017; Damasceno et al. 2020). Hence, in this review, the market demand and the details of finding a practical and cost-effective approach to transfer MSCs from various sources for treating PF using shorter time are explored.

2 Pathophysiology of Perianal Fistula

A fistula represents a tunnel under the skin which connects two epithelial surfaces. The most prevalent among the fistulas is PF, which typically connects the rectum and drains out to the skin around the anus. Apart from CD, PF also occurs due to infection (Scharl et al. 2016). Multifactorial changes in physical behaviours and biological functions in the rectum area cause a surge in inflammation which induces an inflammatory response, such as activation of macrophages, monocytes and neutrophils (Chen et al. 2018). This leads to the secretion of pro-inflammatory cytokines, chemotactic and cell-activating peptides as well as tissue-degrading enzymes and reactive oxygen radicals, which induce local tissue injury (Scharl et al. 2016).

The release of the pro-inflammatory cytokine, tumour necrosis factor (TNF), stimulates the expression of transforming growth factor-beta (TGF-β) which leads to the production of β-integrin that act as a catalyser to the onset of epithelial-to-mesenchymal transition (EMT) (Scharl and Rogler 2014). EMT redifferentiates epithelial cells located on the inner lining of the rectum into fibroblastic-like cells with migratory capability and penetrates adjacent tissue (Panés and Rimola 2017; Scharl and Rogler 2014). TGF-β also triggers interleukin-13 (IL-13) and increases the secretion of matrix metalloproteinases (MMPs) which are associated with cell-invasive aid (Scharl and Rogler 2014). The overactivation of β-integrin and MMPs marks the birth of the fistula. Once the wastes from the body pile up, during defecation, the intraluminal pressure drives the wastes into subcutaneous tissues (Bataille et al. 2004). The accumulation of wastes leads to the formation of abscesses which are potentially the source of bacterial infection. The luminal pressure makes the tunnel become longer until an external opening is formed (de Zoeten et al. 2013). As a result of this mechanism, the deep penetrating tract develops into PF. Based on the pathophysiology and the severity of fistulas, precise classification of PF in patients has been highlighted in order to come out with the best clinical strategy (Marzo et al. 2015). There are three classifications of PF, which are Parks classification, St James University Hospital classification and American Gastroenterological Association (AGA) classification shown in Fig. 1 (Panés and Rimola 2017).

3 Mechanism of Action of MSCs

The main mechanism of action of MSC entirely is attributed to its paracrine factors such as cytokines, chemokines and growth factors (Park et al. 2018). MSCs release secretomes that help to suppress inflammation, increase cell proliferation and repair damaged tissue (Park et al. 2018). Here we briefly explain the mechanism that is likely to happen upon the injection of MSCs to the side of the fistula. The mechanism of action is also shown in Fig. 2.

3.1 Homing Ability

After injection into the fistula tissue, MSCs enter the vascular system and migrate into the injured site through a homing mechanism (Li et al. 2019; Ullah et al. 2019). Several cell signalling molecules play a crucial role for efficient homing (Spees et al. 2016). For example, the presence of pro-inflammatory cytokines like TNF-α activates endothelial cells in blood vessels to induce intercellular adhesion molecule 1 (ICAM-1), vascular

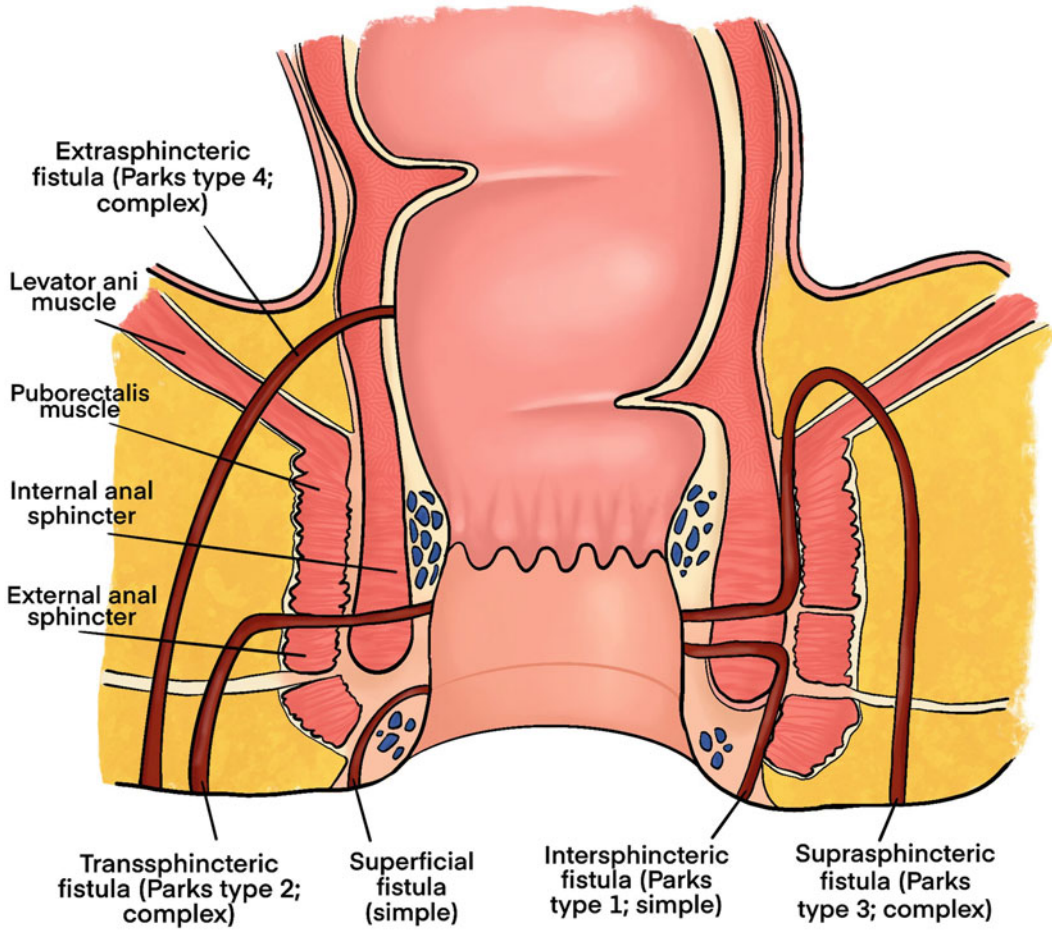

Fig. 1 Classification of PF. Superficial fistula: A fistula without interception with sphincter or muscular structure (Agha et al. 2013). Intersphincteric fistula: Located in intersphincteric plane. Fistula located in between internal anal sphincter until the external anal sphincter (Agha et al. 2013). Transsphincteric fistula: A fistula that will travel through the external sphincter (high or low) to pass some distance distally in the intersphincteric plane before going through the external sphincter (Agha et al. 2013). Suprasphincteric fistula: Fistula that perforates through the intersphincteric plane before piercing the levator ani and descending through the ischioanal fossa (Agha et al. 2013). Extrasphincteric fistula: Fistula starts from the external anal sphincter that tracks across the levator ani to the perineum (Agha et al. 2013)

cell adhesion molecule 1 (VCAM-1) and P-selectin activation for increasing adhesion of MSCs to the endothelial cells (Teo et al. 2012). The movement of MSCs on vascular cell surfaces prompts the upregulation of ligands such as cluster of differentiation 44 (CD44), homing cell adhesion molecule (HCAM) and CD49d (Andreas et al. 2014). Integrins like very late antigen 4 (VLA-4) are formed by galectin-1 to regulate the adhesion of MSCs. Platelet- and neutrophil-derived growth factors such as fibroblast growth factor (FGF) as listed in Table 2 are released by MSCs to interact with basic fibroblast growth factor (bFGF) in endothelial cells to regulate the adhesiveness of galectin-1 to P-selectin (Langer et al. 2009).

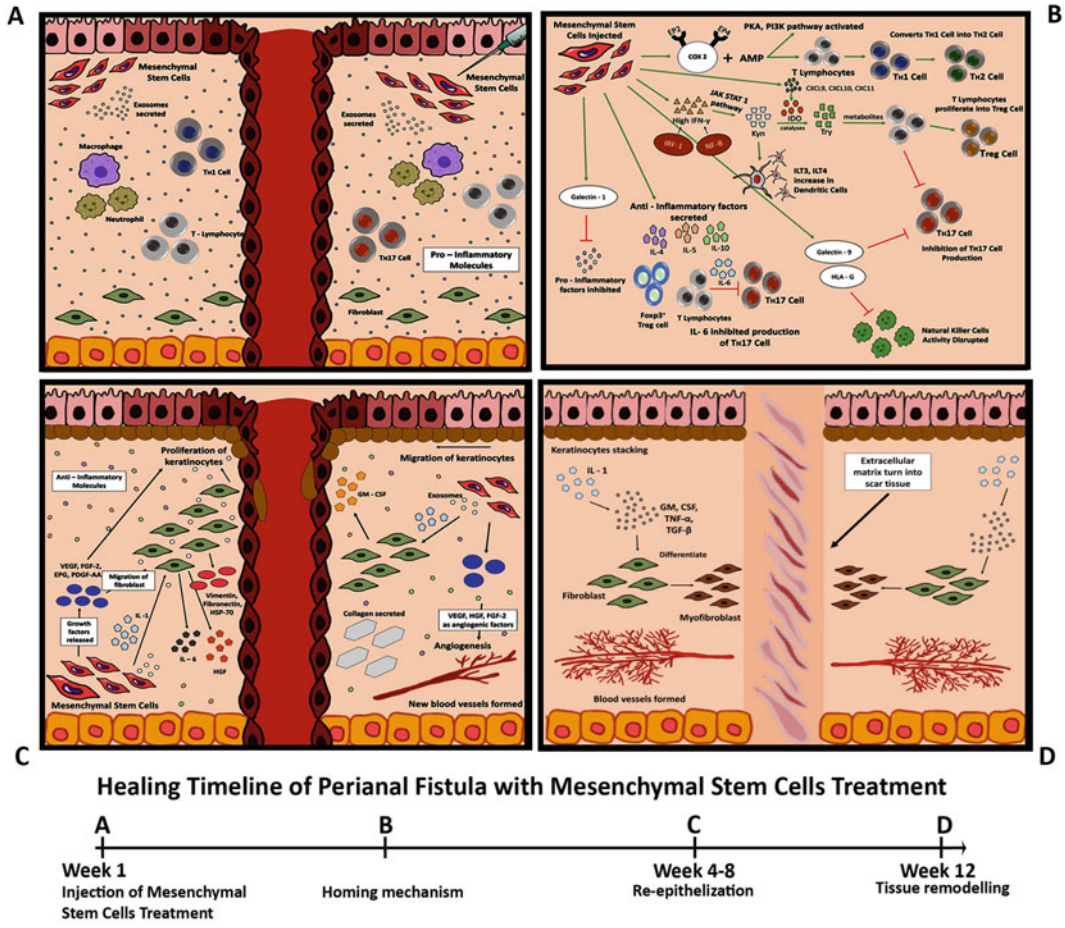

Fig. 2 Healing time of PF with MSCs treatment. (**a**) MSCs were injected into the active inflammation site. The active inflammation site contains an abnormal amount of pro-inflammatory molecules such as TNF-α, IL-1β, TGF-β and others including TH1 and TH17. (**b**) Mechanism of action of MSCs to the active inflammation site including suppressing the production of pro-inflammatory factors and anti-inflammatory factors which are secreted. The production of TH17 is inhibited by the anti-inflammatory factors and Th1 is converted into Th2. Pathway protein kinase (PKA), phosphoinositide 3-kinase (PI3K) and JAK STAT1 activated. (**c**) Re-epithelisation of tissue around the fistula. The abundance of anti-inflammatory molecules balances out the pro-inflammatory molecules. Growth factors such as VEGF, FGF-2, EPG and PDGF-AA are released to assist the migration of fibroblast and the proliferation of keratinocytes to the damaged tissue. The growth factors also contributed to the angiogenesis process, allowing the secretion of collagen. (**d**) Tissue remodelling leaving the extracellular matrix turns into scar tissue and the fistula is healed. New blood vessels completely formed

3.2 Anti-Inflammatory Mechanism

Once it has migrated to the injured site, microenvironment of the injured tissues which consist of pro-inflammatory cytokines secreted during inflammation such as interferon gamma (IFN-y), TNF, IL-1 and IL-17 activates the immunomodulation role of MSCs (Waterman et al. 2010; Sangiorgi and Panepucci 2016). Several immunoregulatory factors like IL-10, prostaglandin E_2 (PGE2), human leukocyte antigen G (HLA-G), indoleamine 2,3-deoxygenate (IDO) 1 and IDO2 and chemokines like chemokine C-X-C motif receptor (CXCL) 9, CXCL10 and CXCL11 will be secreted by MSCs as listed in Table 3 (Li et al. 2018). Once the MSC receptors bind to the

Table 2 Growth factors secreted by MSCs

Author (year)	Growth factors secreted by MSCs	Function
Joel et al. (2019)	HGF	Exert anti-inflammatory signals by causing MSCs to inhibit the proliferation and/or activities of $CD4^+$ Th1, Th17, $CD8^+$ T cell and NK cells
Wang et al. (2014)	bFGF	Proliferates and promotes differentiation of fibroblasts
Delafontaine et al. (2004)	IGF-1	Tissue growth factor with effects to influence blood glucose level
Zhao et al. (2013)	PDGF	Promotes migration of fibroblasts
Tamama et al. (2010)	EGF	Promotes cell regeneration by stimulating cell proliferation
Panek-Jeziorna and Mulak (2020)	FGF-19	Growth factor with potential anti-inflammatory properties
Langer et al. (2009)	FGFR	Interact with bFGF in endothelial cells to arbitrate the adhesiveness of galectin-1 to P-selectin
Zhao et al. (2013)	FGF-2	Attracts leukocyte recruitment to the inflammation site
		Increases the migration of fibroblasts and the functional roles of keratinocytes. Initiates vascularization in the damaged tissue
Ho et al. (2012)	GDF-15	Antiapoptotic, antihypertrophic and anti-inflammatory properties in response to oxidative stress or pro-inflammatory signalling molecules
Zhao et al. (2013)	VEGF	Angiogenesis factor which involves immunology
		Increases the migration of fibroblasts and the functional roles of keratinocytes

The growth factors secreted by MSCs and its function are described. Most of the growth factors show immunomodulatory and anti-inflammatory properties which may benefit in PF treatment

cytokines, chemokines and growth factors in the microenvironment, production of the anti-inflammatory paracrine factors is initiated (Waterman et al. 2010). The paracrine factors will tune the function of the T lymphocytes, macrophages, neutrophils, natural killer (NK) cells, dendritic cells (DC) and B lymphocytes for immunosuppression activities (Wang et al. 2014).

IDO production is activated by the Janus kinase and signal transducer and activator of transcription 1 (JAK-STAT 1) signalling pathway (Ji et al. 2017). In this pathway, the nuclear factor-beta (NF-β) and interferon regulatory factor-1 (IRF-1) bind to upstream IFN-γ-responsive elements of the IDO gene, thus promoting gene expression (Sohni and Verfaillie 2013). IDO behaves as a switch that initiates a cascade of reactions to promote immunosuppression (Luz-Crawford et al. 2013). It directs the monocyte to differentiate into anti-inflammatory and immunosuppressive type 2 macrophage (Francois et al. 2010). Moreover, CXCL12 and CXCR4 secreted by MSCs draw near the T cells to enable IDO to catabolise the tryptophan in T cells (Sohni and Verfaillie 2013). Tryptophan, which is a necessary molecule for T-cell survival, is then broken down into metabolites like kynurenine, quinolinic acid and picolinic acids (Weber et al. 2006). The deficit in tryptophan numbers retards the T-cell multiplication and growth, thus stunting their numbers (Moffett and Namboodiri 2003). This forces a change in the metabolic pathway of ATP production from glycolysis to oxidative phosphorylation and activates a stress response in the immune cells eukaryotic initiation factor 2 (eIF2) and general control nonderepressible 2 (GCN2) through the accumulation of uncharged tRNA (Zhu et al. 2011). Consequently, the arrested cell growth declines their physiological roles, mediating fas cell surface death receptor (FAS)-regulated lymphocyte apoptosis. On the contrary,

Table 3 Paracrine factors secreted by MSCs in PF treatment

Author (year)	Anti-inflammatory factors secreted by MSCs	Function
Schraufstatter et al. (2015)	Complement component C5	Anti-inflammatory cytokine, increases survival of MSCs under oxidative stress
Weiss and Dahlke (2019)	COX2	Anti-inflammatory cytokine, involves in reducing pain
Solodeev et al. (2018)	Fas ligand	Improves cell survival during inflammation tissue damage
Hartung (1998)	G-CSF	Reduces inflammatory activity by inhibiting the secretion or function of the main inflammatory mediators
Luz-Crawford et al. (2013)	IDO 1 & 2	Catabolic enzyme with immunosuppressive properties through kynurenine pathway
Miguel-Hidalgo et al. (2007)	ICAM-1/CD54	Modifies the immunosuppressive functions of MSCs by facilitating the interaction between pro-inflammatory macrophages and MSCs
Lee et al. (2018)	IL-1	Anti-inflammatory cytokine that can inhibit Th17 polarisation
Amorin et al. (2014)	IL-4	Treg cell differentiation and Th2 cells
Pripp and Stanišić (2014)	IL-5	Anti-inflammatory cytokine, increases the development of new nerve cells in the hippocampus and lowered the quantity of potentially damaging inflammation in the brain
Amorin et al. (2014)	IL-10	The most potent anti-inflammatory cytokine, high levels are predicted to involve to the ageing secretome Treg cell differentiation and Th2 cells
Nilsson et al. (2019)	IL-13	Anti-inflammatory cytokine, involves in reversing ageing
Liu et al. (2018)	MCP-3 (CCL7)	Participates in anti-inflammatory responses through binding to its receptors to facilitate the recruitment of immune cells
Park et al. (2006)	PGE2	Vasodilator, reduces inflammation
Luz-Crawford et al. (2013); Mallis et al. (2018)	HLA-G	Inhibits T-cell differentiation into Th1 and Th17. Allows apoptosis of T and B cells
		Stops cytolysis of $CD8^+$ cells and induces development of $CD4^+$, $CD25^+$, $FoxP3^+$ Treg cells
		Disrupts activities of NK cells
Sohni and Verfaillie (2013)	CXCL9, CXCL10, CXCL11, CXCL12	Immunomodulatory roles in MSCs wound healing
Sohni and Verfaillie (2013)	CXCL12, CXCR4	Draws near T cell towards MSCs
Akiyama et al. (2012)	TGF- β	Development and maturation of Treg cell differentiation
Wang et al. (2014)	TSG6	Anti-inflammatory properties
Gieseke et al. (2010)	Galectin-1	Downregulates the pro-inflammatory cytokines TNF-α, IFN-y, IL-2, IL-10
Pripp and Stanišić (2014)	Galectin-9	Anti-inflammatory cytokine, increases the development of new nerve cells in the hippocampus and lowered the quantity of potentially damaging inflammation in the brain
Whelan et al. (2020)	CCL2	Regulates macrophage recruitment, accelerates wound healing

The paracrine factors secreted by MSCs that may involve in PF treatment are stated and its role in PF treatment is also described based on several studies

kynurenine upregulates inhibitory receptors like immunoglobulin-like transcript (ILT) 3 and ILT4 in the DC, while co-stimulating cytokines are suppressed in parallel. HLA-G expressed by MSCs also induce T and B immune cells to undergo apoptosis (Mallis et al. 2018). They further end the cytolysis of antigen-activated $CD8^+$ cells and encourage the development of $CD4^+$, $CD25^+$ and forkhead box $P3^+$ regulatory T cells ($FoxP3^+$ Treg cells).

Galectin-1 secretion by MSCs downregulates the pro-inflammatory cytokines such as IFN-y, IL-2, TNF-α, IL-10 and so on (Gieseke et al. 2010). Together with galectin-9, biological activities of T cells are controlled to reduce T and B lymphocyte numbers (Mallis et al. 2018). Activated MSCs also regulate immunoglobulin (lg) E and IgG concentration by retarding the growth of B cells (Wang et al. 2014). MSCs downregulate IL-2 and IL-15 to keep the natural killer cells dormant, while $CD14^+$ is inhibited by MSCs to stop DC differentiation (Spaggiari and Moretta 2013).

3.3 Cell Proliferation Stage

Activation of GCN2 plays a role in promoting differentiation of Treg cells and downregulates IL-6. Without adequate IL-6, T helper (Th) 17 cells' activities are suppressed (Liu et al. 2020). The tryptophan metabolites, kynurenine, induce the production of tolerogenic DC (Regmi et al. 2019). All the metabolites overall exhibit toxic effects towards $CD4^+$ Th1 and $CD8^+$ T cells but are substantially harmless towards immunosuppressing cells like Th2. This impact prompts T helper cells to start developing into Th2 cells and decrease the development into Th1 cells (Weiss and Dahlke 2019). On the other hand, MSCs block TNF-α secretion by promoting IL-10, and IL-4 secretion also helps in increasing the Treg cell differentiation and Th2 cells (Amorin et al. 2014).

IDO, HLA-G, galectins and other secretomes carry out an extra role of inhibiting the differentiation and development of T cells into Th1 and Th17 (Luz-Crawford et al. 2013). This inhibition forces the macrophages to express growth factors, TGF-β which plays a vital part in the development and maturation of Treg cell differentiation (Akiyama et al. 2012). At the same time, HLA-G also disrupts the protoplasmic activities of NK cells (Mallis et al. 2018).

Furthermore, MSC exosomes can polarise macrophages from pro-inflammatory matrix protein 1 (M1) into anti-inflammatory M2 phenotypes once triggered by the availability of pro-inflammatory cytokines such as chemokines, IL-1β, IL-12 and TNF-α (Murray 2017). It is found that miR-223 in exosomes abates inflammation and helps to speed up healing through macrophage M2 polarisation, while M2 macrophage is brought about by Th2 cytokines and chemokines like TGF-β, IL-10 and M2 markers such as IL-1ra, CD163 and C-C motif chemokine 22 (Murray 2017; Zhuang et al. 2012).

3.4 Re-epithelisation and Tissue Remodelling

MSCs help to remodel the damaged tissues by accelerating wound healing (Nie et al. 2011; Whelan et al. 2020). MSCs will differentiate to fibroblasts and express vimentin, fibronectin and heat shock protein 47 once embedded into the injured site IL-1 secreted by MSCs influences gene expression for other chemokines involved in the metabolic cascade chain like granulocyte-macrophage (GM), colony-stimulating factor (CSF) and TNF-α (Hamilton 2008; Shingyochi et al. 2015). MSCs also differentiate into keratinocytes, producing keratin (K) 5 and K14, integrins, cytokeratin 5, cytokeratin 14, cytokeratin 19, desmoglein 3 and cytokeratin 6α proteins for keratinocyte assembly (Shingyochi et al. 2015). Other than that, they can also differentiate endothelial cells into blood vessel walls (Ebrahimian et al. 2009). Growth factors such as vascular endothelial growth factor (VEGF), epidermal growth factor (EPG), fibroblast growth factor 2 (FGF-2) and PDGF-AA that are secreted by MSCs increase the migration of fibroblasts and the functional roles of keratinocytes (Zhao et al. 2013). VEGF, HGF and FGF-2 also act as angiogenic factors by initiating vascularisation in the damaged tissue.

New blood vessels help in transporting nutrients and oxygen for the cell proliferation of fibroblasts and keratinocytes. At the same time, MSCs secrete IL-1 which directs the migration of fibroblast to the wound, excreting IL-6, HGF and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Zhao et al. 2013).

Lastly, during remodelling, keratinocytes stack up in alignment through epidermal

stratification (Santoro and Gaudino 2005). The mechanical tension from the tissue activates TGF-β, and splice variant fibronectin triggers proto-fibroblast differentiation into smoother myofibroblasts, increasing proliferation. When the wound gap closes, the excess capillaries slowly disappear, leaving behind a completely remodelled tissue (Sorg et al. 2017; Hinz et al. 2001).

4 A more Affordable MSC Therapy in Treating PF

Stem cell therapy was widely investigated in various kinds of diseases, including PF. As compared to the other treatments, stem cell therapy has a relatively high healing rate, which is up to 59% with a low complication rate, 13%–18.5% (Fig. 3). Hence, stem cell therapy is a good alternative for treatment that gives a long-term outcome with fewer relapses (Georgiev-Hristov et al. 2018). Table 4 shows the current conventional treatments and its respective cost. Unfortunately, the high cost appears to be a major bottleneck, causing the limited industrial growth towards a wider market base, especially in the low-income countries. The entire process from the manufacturing process until the delivery of the services requires a high amount of cost and resources, thus causing MSCs as a premium therapy that only PF patients with a higher tier of income could be able to afford. Therefore, several techniques and alternative approaches are being suggested in the following section, making MSCs a more affordable therapy.

4.1 Cell Source

MSCs can be derived from a variety of sources such as the adipose tissue, bone marrow and umbilical cord. Among these various sources, BM-MSCs and adipose tissue-derived MSCs (ADSCs) are commonly utilised in PF treatment due to their easy accessibility (Musiał-Wysocka et al. 2019). Two approaches are involved in obtaining adult MSCs, which are either autologous or allogeneic. Autologous MSCs have the advantage in preventing transplant rejection and performing better cell tolerability; however it is not readily available where it needs to be further isolated and expanded, which may not be suitable in emergency treatment (Molendijk et al. 2015). This leads to allogeneic MSCs which could be pre-cultured in advance and are ready to use anytime. Nonetheless, a healthy donor can be selected to obtain functional and normal MSCs, and these cells may be cryopreserved and readily available for future use (Molendijk et al. 2015).

Cheng et al. (2020) reported that a majority of the reported clinical trials used BM-MSCs and ADSCs in treating PF, with an authorised product available using the latter source. Although these well-studied findings reported a visible healing rate towards PF, several challenges remain unsolved. Firstly, both BM-MSCs and ADSCs recorded low proliferation rates, which were proven by Amable et al. (2014). Besides, age becomes a limiting factor in obtaining high-quality adult tissue-derived MSCs. According to Bustos et al. (2014), using aged BM-MSCs reported a decrease in immunomodulatory activity which was caused by the lowered expression level of chemokine and cytokine receptors that are involved in cell migration. Further, additional steps are needed to extract MSCs from adipose tissue and bone marrow, for example, by bone aspiration and liposuction, and these may increase the risk of contamination if mishandling of samples occurs throughout the workflow (Mazini et al. 2021). With the limitation of adult tissue-derived MSCs, the umbilical cord seems to be an ideal cell source selection in PF treatment. UC-MSCs proved to have a better proliferation rate and cellular migration ability while possessing similar immunomodulatory characteristics with adult tissue-derived MSCs (Omar et al. 2014). For example, WJ-MSCs are reported to have higher expression levels of IL-10, TGF-β and VEGF compared with adult tissue MSCs, which perform better immunosuppressive ability against diseases, and it has been proven since the last decade (Weiss et al. 2008).

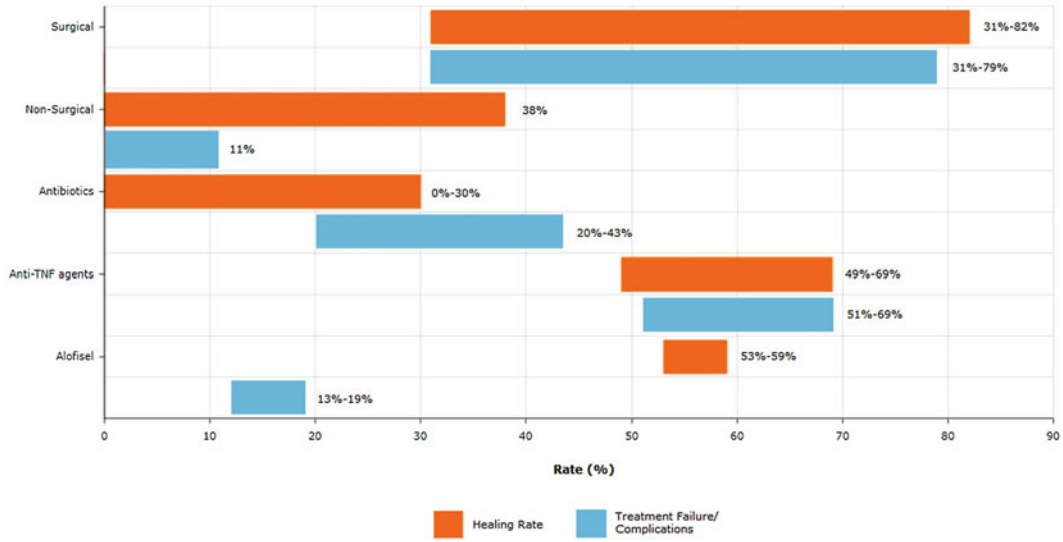

Fig. 3 Graph of treatment success and complication rate of current treatment of PF. Stem cell therapy (Alofisel) has a relatively high healing rate, which is up to 59% with a low complication rate, 13%–19%. Treatment using anti-TNF agents has both high healing and complication rate, which are up to 69%. The healing rate of treatment using antibiotics is low, 0%–30% with high complication rate, which is up to 43%. The healing rate of non-surgical treatment is up to 38% with low complication rate, 0%–11%, while surgical treatment gives highest healing rate, 31%–82% with the highest complication rate, which is up to 79%

4.2 Manufacturing Scales

To produce MSCs in a large quantity as a commercial product for treating PF, a Good Manufacturing Practice (GMP)-compliant production process using a bioreactor is crucial. Traditionally, MSCs are cultured in monolayer flasks, have a simple handling process and are generally low in cost (Rodrigues et al. 2011). However, monolayer culture technology has a higher risk of contamination due to the open system coupled with low cell yield (Mizukami and Swiech 2018). This has prompted for the use of bioreactor for cell expansion to upsize the scale of cell manufacture. Since MSCs are anchorage-dependent, the microcarriers that are present in the bioreactor are used for cell attachment and cell growth by providing higher surface area to volume, allowing a higher yield of MSCs in a shorter time, thus reducing the cost of production (Panchalingam et al. 2015). Moreover, stirred tank bioreactors with impellers achieved a homogenous culture system as it mixes the culture more regularly. As compared with traditional culture using flasks, stirred-tank bioreactors are closed systems that reduce the risk of contamination and with parameter control and monitoring systems which are able to monitor the cultured cells easier (Nienow et al. 2014). According to Mizukami et al. (2017), hollow fibre bioreactor was used in clinical trials testing for intestinal bowel disease (IBD), and successful expansion of up to 11-fold of MSCs in 5 days was reported.

4.3 Preconditioning of MSCs

Preconditioned MSCs under modified culture conditions would help in preserving their therapeutic effects, and this approach has been well studied in stem cell therapy in recent years (Ocansey et al. 2020). For instance, preconditioning of MSCs using IFN-γ seems to be a promising approach in conferring anti-inflammatory effect which was proven by Noone et al. (2013). IFN-γ will specifically target T cells or NK cells and suppress its activity by the release of prostaglandin E2. The suppression of

Table 4 Current conventional treatments (surgical, non-surgical, medical, stem cells) and their cost

Author (year)	Category		Estimated average cost (USD)	No. of patients	Healing rate (%)	Follow-up (weeks)	Complication
van Koperen et al. (2009)	Surgical	Fistulotomy	$2,855	28	82	343	Fistula recurrence (18%)
							Faecal incontinence (61%)
							Total: 79%
Galis-Rozen et al. (2010)		Seton drainage	$2,688	17	59	104	Fistula recurrence (40%)
							Faecal incontinence (6%)
							Total: 46%
Bessi et al. (2019)		Advancement flap	$3,015	34	68	58	Treatment failure (33%)
Gingold et al. (2014)		LIFT	$2,876	15	60	8	Treatment failure (40%)
Senéjoux et al. (2016)		Fistula plug	$2,965	54	31	12	Abscesses formation (7%)
							Plug avulsions (9%)
							CD flare (2%)
							Abdominal pain (2%)
							Miscellaneous (11%)
							Total: 31%
Grimaud et al. (2010)	Non-surgical	Fibrin glue	$2,627	36	38%	8	Abscess formation (11%)
	Medical treatment						
Thia et al. (2009)	Antibiotic	Metronidazole	$9.8 for ten tablets (500 mg) (8)	7	0	10	Abscess (42.9%)
Thia et al. (2009)		Ciprofloxacin	$19 for ten tablets (500 mg) (9)	10	30	10	Abscess (20%)
Bouguen et al. (2013)	Anti-TNF agents	Infliximab	$1,229 (100 mg) (11)	156	69	250	Fistula recurrence (40%)
							Abscess formation (29%)
							Total: 69%
Castaño-Milla et al. (2015)		Adalimumab	$3,120 (40 mg) (13)	46	49	52	Treatment failure (51%)
	Medical treatment						
Panés et al. (2016)	Stem cells	Alofisel (Cx601)	$67,000	107	53	24	TRAEs:
							Anal abscess (6%)
							Proctalgia (5%)
							Procedural pain (1%)
							Fistula discharge (1%)
							Total: 13%
Panés et al. (2017)				107	59	52	TRAEs:
							Anal abscess (13%)
							Proctalgia (5%)
							Procedural pain (1%)
							Total: 19%
Ramezankhani et al. (2020)		Cupistem	$5,000	43	80.8	96	–

immune cells activity will then prevent it from presenting cytotoxicity characteristics (Noone et al. 2013). Also, according to a recent study by Yu et al. (2019), preconditioning of MSCs with IFN-γ and IL-1β upregulates the production of PGE2 and IDO, thus improving the immunomodulatory property of MSCs and increasing its efficacy towards treatment.

Nicotinamide (NAM) can also be used in cell culture to act as a cell supplement. NAM belongs to the family under vitamin B3 and has been used widely for treatment of various diseases such as diabetes, Alzheimer's disease and cancer (Meng et al. 2018). However, studies have shown that using NAM for cell culturing could enhance cells' performance (Meng et al. 2018). This may be due to the role of NAM as a direct effector on ROCK signalling pathway inhibition, thus improving cell survival and inducing cell differentiation (Watanabe et al. 2007; Zhang et al. 2021). Not only that, studies have also proved the role of NAM to inhibit casein kinase 1 (CK1), which involves in CK1 signalling pathway to induce apoptosis (Janovská et al. 2020). Besides this, several studies have proposed that preconditioning of MSCs under hypoxic conditions presented an improvement in proliferation rate. For example, Haque et al. (2013) stated that the proliferation rate and the cell doubling time that cultured for BM-MSCs under hypoxic conditions reported a significant increase as compared to ambient oxygen concentration.

4.4 Genetically Modified MSCs

Genetically modified MSCs have been used widely in recent years in treating various diseases (Varkouhi et al. 2020). Although limited studies are available on using engineered MSCs against PF treatment, there are still several clinical trials reported showing better wound healing rates and improved immunomodulatory effects against certain diseases. These techniques would be useful as a reference to further examine the efficacy of PF treatment. Table 5 summarises the use of genetically modified methods and their effect on immunoregulatory function. Here we have briefly explained the therapeutic benefits of genetically modified MSCs as well as the cost that needs to be taken into consideration before introducing this approach to the clinical.

4.4.1 Improving Migration

The cellular migratory ability is crucial in MSCs treatment where the transplanted or injected MSCs would need to migrate to the injured tissues. The improvement of cell migration can be enhanced by modifying miRNAs. According to the in vitro study in a rat model, overexpression of miR-9-5p would help in upregulating the β-catenin signalling pathway, which takes part in most of the cellular regulatory processes in MSCs, such as differentiation and migration (Li et al. 2017; Pai et al. 2017). Overexpressing miR-9-5p would help to suppress the activity of glycogen synthase kinase 3 beta (G5k3β) and casein kinase 1 alpha (CK1α), which act as inhibitors towards the production of β-catenin. The inactive inhibitors will thus help in preventing the degradation of β-catenin, promote the formation of β-catenin and enhance the signalling pathway at a higher level (Pai et al. 2017). The overall process will thus be promoting sufficient MSCs to migrate towards injured tissues. Modification of miR-9-5p of MSCs in PF treatment may provide similar results with the study mentioned, where further studies need to be conducted to test its efficacy of it.

Enhancement of the cellular migratory process could also be achieved by modifying CXC chemokine receptors, in specific CXCR4 and CXCR7. Based on a study by Devetzi et al. (2018), overexpressing both genes will induce the activity of stromal cell-derived factor-1 (SDF-1) which acts as key chemokines in mediating homing of transplanted MSCs to the injured tissues. This results in promoting paracrine signalling to the nearby cells, thus maximising the efficacy of the wound healing process (Devetzi et al. 2018). According to a study by Du et al. (2013), an animal model has been used to determine the effect of CXCR4 and CXCR7 overexpression in BM-MSCS towards early liver regeneration. The results showed

Table 5 Types of genetic modification process

Author (year)	Genetic modification	Source of MSCs	Experimental model	Transfection method	Transfection efficiency	Purpose	Target mechanism
Du et al. (2013)	Overexpression of CXCR4 and CXCR7	Rat BM-MSCs	Rat with liver injury	Viral (adeno-associated virus)	Approximately 98%	Improves cell migration	Increase level of SDF-1α, VEGF and HGF
Han et al. (2014)	Overexpression of Sox2 and Oct4 gene	Human ADSCs	Human ADSCs	Non-viral (liposomal)	Approximately 98%	Reduces premature senescence of MSCs	Extending S phase in cell cycle by increasing the production of cyclin D1
Li et al. (2017)	Overexpression of miR-9-5p	Rat BM-MSCs	Artificial made wound cells	Non-viral (liposomal)	High	Improves cell migration	Induce β-catenin signalling pathway by suppressing activity of CK1α and G5k3β
Fang et al. (2016)	Exosomal miR-21, miR-23a, miR-125b and miR-145	WJ-MSCs	Mouse model with skin injury	Non-viral (microinjection)	High	Improves wound healing process	Induce the translocation of β-catenin to the injured tissue
Li et al. (2016)	Exosomal miR-181c	UC-MSCs	Mouse model with burn injury	Non-viral (microinjection)	High	Anti-inflammatory	Suppresses macrophage activity to produce IL-6
							Downregulates the secretion of inflammatory cytokines IL-1β and TNF-α
							Increase of IL-10 production
							Inhibits TLR4 expression
Liang et al. (2016)	Overexpression of exosomal miR-125a	Human ADSCs	HUVECs	Non-viral (microinjection)	Approximately 90%	Promotes re-epithelisation	Downregulating the expression of DLL4 thus promotes the formation of epithelial tip cells

Different types of genetic modification approaches of MSCs are described together with its purpose and target mechanism. This may help as a guide to involve genetic modification techniques in PF treatment, where exosomal microRNAs are mostly used

improving cellular migration into rat liver graft, promoting regeneration of hepatocytes in the rat's liver (Du et al. 2013). Although the study was only conducted in animal models, altering CXC chemokine receptors may be a promising technology in PF treatment and should be further examined.

4.4.2 Reduced Premature/Replicative Senescence

Modifying octamer-binding transcription factor 4 (Oct4) and SRY-Box transcription factor 2 (Sox2) genes would help in reducing premature senescence of MSCs. Based on a study by Han et al. (2014), the overexpression of these two genes was transfected in human ADSCs with the help of a PB-CA vector. The findings concluded that overexpressing of Sox2 and Oct4 will prolong the duration of the synthesis (S) phase in the cell cycle, where DNA replication occurs. It is important to monitor the processes in each phase of the cell cycle, as the overall events will eventually affect the cell proliferation performance (Resnitzky et al. 1994). High expression levels of Sox2 and Oct4 will trigger the production of cyclin D1 in high amount, thus accelerating the transition from non-proliferative growth 1 (G1) phase into proliferative S phase, prolonging the duration in DNA replication and resulting in extended MSCs growth and expansion (Han et al. 2014). This may be also included in PF treatment, where the yield of MSCs will be increased and may be suitable in a larger scale of production.

4.4.3 Cost

The cost of genetically modified MSCs is still unknown in disease treatment as it has only been used in smaller-scale experiments, consisting only of phase I or phase II clinical trials (Damasceno et al. 2020). Nevertheless, exosome therapies are still yet to be approved by FDA, which means that all the experimental studies reported are still not being commercialised in the market (An Introduction to Exosome Therapy and Its Costs 2020). Further studies are needed to identify its cost-effectiveness on large-scale production, and the cost should be affordable for every patient, especially in PF treatment.

4.5 Cell-Free Therapy

Cell-free therapy involves the utilisation of exosomes derived from MSCs, and this approach is gaining much interest in recent years mainly in human disease treatment. Several research have shown the potential of involving exosomes derived from MSCs in the wound healing process, which may benefit PF treatment. Facilitation of the wound healing process could be achieved by transfecting WJ-MSCs exosomes into the injured tissues. Fang et al. (2016) stated that WJ-MSCs contain exosomal miR-21, miR-23a, miR-125b and miR-145, and these miRNAs were being proved in promoting myofibroblast and scar formation, accelerating the overall wound healing process. Not only that, treating injured tissues by using WJ-MSCs exosomes improves the re-epithelialising of tissues by promoting translocation of β-catenin into the injured wound to enhance the formation of skin cells and promotes migration (Fang et al. 2016).

Exosomes released from WJ-MSCs would also contribute to the regulation of the immune system by suppressing the activity of macrophages to secrete IL-6, which reduce the inflammatory response (Song et al. 2020). Li et al. (2016) also mentioned that miR-181c-expressed exosomes secreted from WJ-MSCs can downregulate the release of inflammatory cytokines IL-1β and TNF-α while promoting IL-10 production, leading to anti-inflammation of cells. Nevertheless, exosomes derived from ADSCs were reported to have a great contribution towards the re-epithelialisation process. This is due to the presence of overexpressed miR-125a in the exosomes acting as a pro-angiogenic factor, where it encourages the development of endothelial tip cells by lowering the expression level of angiogenic inhibitor delta-like 4 (DLL4) (Liang et al. 2016).

5 Remaining Challenges and Conclusion

Several limitations and challenges remained to be addressed in MSC therapy for PF treatment. In normal circumstances, MSCs will differentiate into fibroblast and myofibroblast in the phase of wound healing, forming scar tissue on the site of injury. However, excessive proliferation of myofibroblast may occur during tissue remodelling, thus causing fibrotic disease or excessive scarring on the remodelled tissue (Darby et al. 2014). Although most of the patients may not be affected, some may feel discomfort or pain at the scarring site (Dwarkasing and Schouten 2013). Regardless of MSC source, the production of stem cells requires GMP compliance to ensure the products are safe to be used for the patients. However, maintaining a lower cost while practising GMP is a crucial, yet challenging, aspect for manufacturing companies. Besides that, implementing a bioreactor system in MSCs production remains unclear. Detailed financial planning should be performed by considering several financial aspects, such as the cost of the bioreactor as well as on the maintenance. On the other hand, the efficacy of using genetically modified MSCs in PF treatment remains uncertain due to limited studies available in the industry. Therefore, further detailed examination and clinical trials would need to be conducted if genetically modified MSCs are to be introduced in treating PF for both its safety and efficacy index. In conclusion, developing affordable PF therapy is important to satisfy the current market demand, especially targeting the Asia Pacific market with the increase of fistulising CD incidence rate in the next 20 years.

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Virus, Exosome, and MicroRNA: New Insights into Autophagy

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Abstract

Autophagy is known as a conserved self-eating mechanism that contributes to cells to degrade different intracellular components (i.e., macromolecular complexes, aggregated proteins, soluble proteins, organelles, and foreign bodies). Autophagy needs formation of a double-membrane structure, which is composed of the sequestered cytoplasmic contents, called autophagosome. There are a variety of internal and external factors involved in initiation and progression of

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autophagy process. Viruses as external factors are one of the particles that could be associated with different stages of this process. Viruses exert their functions via activation and/or inhibition of a wide range of cellular and molecular targets, which are involved in autophagy process. Besides viruses, a variety of cellular and molecular pathways that are activated and inhibited by several factors (e.g., genetics, epigenetics, and environment factors) are related to beginning and developing of autophagy mechanism. Exosomes and microRNAs have been emerged as novel and effective players anticipated in various stages of autophagy. More knowledge in these pathways and identification of accurate roles of them could help to provide better therapeutic approaches in several diseases such as cancer. We highlighted the roles of viruses, exosomes, and microRNAs in the autophagy processes.

Keywords

Autophagy · Cancer · Chemoresistance · Exosome · MicroRNA · Viral infection

1 Autophagy

Although autophagy was recognized around 50 years ago in mammalian cells, its molecular function was revealed vastly in the past decade. Autophagy usually occurs as an evolutionary conserved mechanism in all eukaryotic cells for sustaining cell homeostasis. Recent studies have revealed that autophagy is one of the vital biological mechanisms, which is related to health, longevity, differentiation, starvation, homeostasis, cell survival, adaptation, elimination of microorganisms, and cell death (Shafabakhsh et al. 2021). This process begins with the formation of double-membrane vesicles (DMVs), which is generally termed autophagosome, as well as by various processes, such as fusing with lysosomes. This event leads to degradation/recycling of components which exist in cytoplasmic lysosomes (Cuervo 2004). Critical roles of autophagy process in longevity, homeostasis, and cell death have been recently demonstrated (Mizushima 2007). In eukaryotic cells, autophagy includes microautophagy, macroautophagy, and chaperone-mediated autophagy (CMA), three key intracellular pathways. Core molecular machinery of autophagy referred to subset of autophagy-related (ATG) proteins is essential for autophagosome formation (Fig. 1) (Mizushima 2007). P53 and Bcl-2 protein\families with dual regulatory properties play significant roles in autophagy induction (Yoon et al. 2012; Singletary and Milner 2008). Autophagy is involved in various pathologies, such as neurodegenerative and age-related disorders, infections, and inflammatory/immunity diseases, and especially in invasion and cancer progression (Yang and Klionsky 2010). Increasing evidences show the importance of autophagy in cancer and support the concept when it gets disturbed, it can lead to an accelerated tumorigenesis. Also, comparative evidences have shown that degradation of autophagy or proteolysis in tumors is less than normal cells (Yang et al. 2011). Anticancer role of autophagy is due to elimination of damaged cell component and inhibition of tumor growth. However, autophagy can cause tumor cells withstand stress in undesirable conditions leading to survival. Stress-induced autophagy may result in resistance to treatment and result in the progression of tumor cells (Yang et al. 2011; Kondo et al. 2005). Additionally, the efficacy of autophagy inhibitors, along with chemotherapy, in preventing tumor growth and inducing cell death is far better than the chemotherapy alone. Recent investigations have shown that autophagy may play an important role in drug resistance. It means that autophagy may contribute to increase tumor cells resistance to chemotherapeutic

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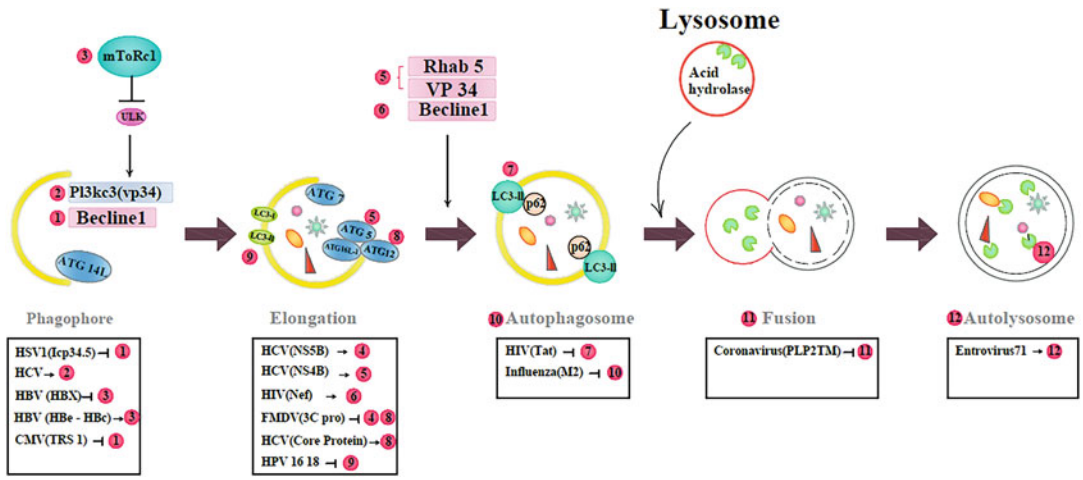

Fig. 1 A schema various stages of autophagy. Viruses, through the production of different proteins, can affect different stages of autophagy, such as the early stages of autophagy (phagophore and elongation), and the ending stages (Autophagosome and autolysosome), in order to survive in the host cell longer. Some of the viruses that can affect the autophagy process are hepatitis C and B viruses and HIV. For example, the hepatitis C virus by producing NS5B protein influences the ATG5 autophagy regulatory protein, which proceeds autophagy in the elongation phase, or, by producing the NS4B protein, affects the Rhab5 factor and drives autophagy from the elongation phase to autophagosome formation. Hepatitis B virus targets and impedes mToR protein kinase through the production of HBX protein, increases the efficiency of the ULK protein, and leads to the initial phase of autophagy, the formation of phagophore. It has also been observed that some of the proteins of the virus (HBe and HBc) inhibit the early stage of autophagy by increasing the efficiency of the mToR protein kinase. HIV produces nef and thus affects one of the major proteins in autophagy called BECN1, which causes the autophagy to progress from the elongation stage to the formation of autophagosome, or by producing TAT stops a factor necessary for the formation of autophagosome (LC3-II-PE complex), losing the autophagosome form. There are other viruses that can apply their effects on the autophagy process. For example, coronaviruses prevent lysosomal incorporation with the PLP2TM protein and impede the formation of autolysosomes. Enterovirus 71 affects and disables autolysosomes. Influenza virus via M2 protein destroys autophagosome. HSV prevents the formation of phagophore by producing Icp34.5 and inhibiting BECN1

and anticancer agents. Therefore, autophagy regulation can be considered as an appropriate therapeutic target in the therapy of cancer. Thus, various autophagy-modulating approaches may be assumed to circumvent chemoresistance (Huang et al. 2016; YiRen et al. 2017). Mounting evidences have revealed that autophagy along with chemotherapy and its association with chemoresistance can be a new therapeutic goal to succeed in cancer treatment.

2 MicroRNA and Autophagy

2.1 Regulation of Autophagy by MicroRNAs

MicroRNAs (miRNAs) are a group of noncoding small RNA molecules (~19–22 nucleotides long) which regulate protein-coding genes (Mollazadeh et al. 2019; Neshati et al. 2018; Letafati et al. 2022; Mousavi et al. 2022; Balandeh et al. 2021; Razavi et al. 2021; Mirzaei and Hamblin 2020). The main miRNA mechanisms are translational repression and mRNA degradation. In the nucleus, RNA polymerase II (RNAPII) produces long primary transcripts (pri-miRNAs), which acts as a substrate for RNase III enzymes and Drosha-DGCR8 complex (a microprocessor that is essential for miRNA maturation) to produce precursor miRNAs (pre-miRNAs). Then, pre-miRNA is exported from the nucleus into the cytoplasm by exportin-5 and Ran-GTP. In the cytoplasm, pre-miRNA is cleaved by another RNase III enzyme, Dicer, into miRNA duplexes approximately 19–22 nucleotides long. Mature miRNA is incorporated into RNA-induced

silencing complex (RISC) where it remains stable and binds to its complementary target mRNA. miRNAs are involved in many major biological functions such as intracellular signaling, cellular metabolism, differentiation, pathological processes, and regulation of gene expression (Su et al. 2015). Some miRNAs are only expressed in specific cell types. Expression patterns of miRNAs are unique to individual tissues and differ between cancer and normal tissues (Jafari et al. 2018). Aberrant expression of miRNAs is associated with multiple human diseases, such as metabolic disease, neurological disorders (Tavakolizadeh et al. 2018), cardiovascular complications, viral diseases (Keshavarz et al. 2018), immune-related diseases, and especially malignancies (Bartels and Tsongalis 2010).

Autophagy-related protein 7 (ATG7) was recently considered as a potential target of miR-96-5p. The aberrant expression of this miRNA reduces autophagy activity (Yu et al. 2018a). Based on the current data, miR-20a-5p inhibits cell proliferation and autophagy and promotes apoptosis through negative regulation of ATG7 (Yu et al. 2018b). Moreover, the overexpression of miR-140-5p/miR-149 inhibits apoptosis and promotes autophagy by downregulating fucosyltransferase1 (FUT*1*) (Wang et al. 2018a). According to the investigation conducted by Liu et al. (2017a) miR-20a negatively relates to autophagy/lysosome pathway. They reported that miR-20a inhibited autophagy and lysosomal proteolytic activity through targeting several key regulators of autophagy, including BECN1, ATG16L1, and sequestosome 1 (SQSTM1) (Liu et al. 2017a). Various molecular components involve in autophagy cascade, including Atg1/unc-51-like kinase (ULK) complex, Beclin-1/class III phosphatidylinositol 3-kinase (PI3K) complex, Atg9 and vacuole membrane protein 1(VMP1), two ubiquitin-like protein (Atg12 and Atg8/LC3) conjugation systems, and proteins which mediate fusion between autophagosomes and lysosomes (Kroemer et al. 2010). Some of these core components of autophagy pathway are direct targets of miRNAs (such as miR-30a, miR-23a, and miR-129-5p) and have key roles in the inhibition/induction of autophagy process (Fig. 2) (Xiao et al. 2015; Guo et al. 2017a; Zhu et al. 2009; Sadri Nahand et al. 2021; Pourhanifeh et al. 2020a, b; Rezaei et al. 2020; Jamali et al. 2020). In the following, the role of miRNAs in the regulation of autophagy and their potential molecular mechanisms has been reported in some disorders.

Meng and colleagues revealed the clinical significance of miR-138 in patients with malignant melanoma, which inhibits cell proliferation and induces apoptosis. Overexpression of miR-138 increases cell autophagy by LC3 protein induction as well as the suppression of PI3K/AKT/mTOR and PDK1 (Meng et al. 2017). It was exhibited that the upregulation of miR-18a-5p in melanoma cell lines and tissues had promising role in melanoma pathogenesis mediated by EPHA7 silence leading to tumor development as well as apoptosis and autophagy blockage (Guo et al. 2021).

Long et al. reviewed the association between miRNAs and autophagy in colorectal cancer (CRC) and concluded that miRNA-regulated autophagy could be up- or downregulated in various CRC conditions associated with the tumor microenvironment. In this context, it can referrer to the roles of miR-140-5p and miR-502 in inhibition of autophagy in chemotherapy of CRC stem cells; miR-214, miR-183-5p, and miR-31 in inhibition of autophagy in radiotherapy of CRC; and miR-124, miR-18a, and miR-210 in promotion of autophagy in metabolism and hypoxia of CRC. Also, blockage of autophagy in inflammatory bowel disease could be mediated via miR-142-3p, miR-143, miR-130a, etc. (Long et al. 2020).

In hepatocellular carcinoma (HCC), autophagy could be reduced via miR-490-3p/ATG7 (Ou et al. 2018) or microRNA-181a/Atg5 axis, suggestive of the profounding value of autophagy deficiency in HCC (Yang et al. 2018). Jin et al. showed that miR-513b-5p attenuated tumorigenesis of liver cancer cells in HCC via inactivation of PIK3R3-mediated autophagy (Jin et al. 2021). Zhang et al. demonstrated that downregulation of miR-638 in human liver cancer led to a noticeable reduction in malignancy of liver cancer cell accompanied by increase of autophagosomes and

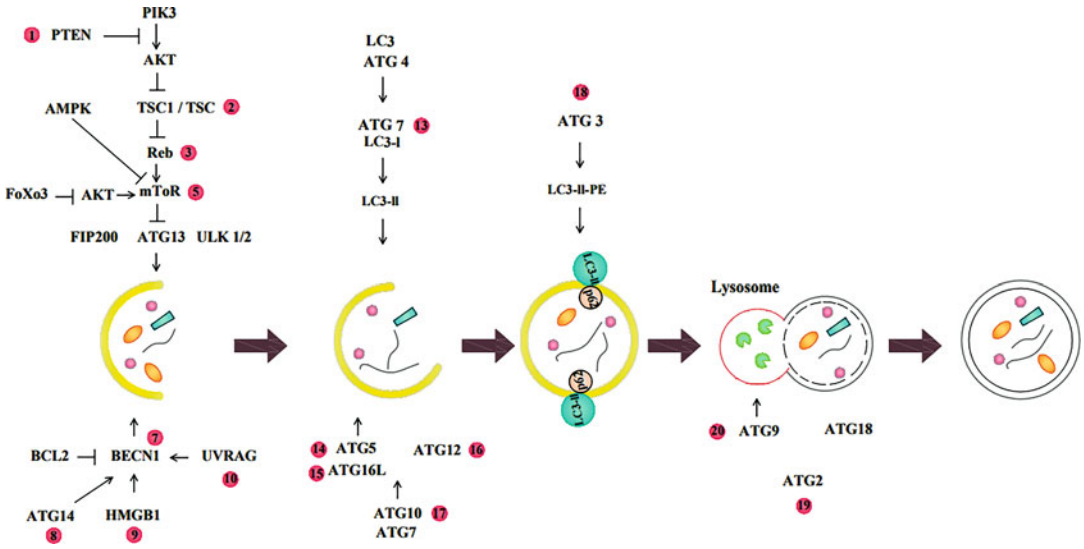

Fig. 2 Various factors involved in the formation of the autophagy mechanism, each of which is affected by different microRNAs that somehow regulate the autophagy steps. The PTEN protein by inhibiting the PIK3-akt pathway paves the way for autophagy to start, increasing the expression of mir-21 that inhibits this protein, thus activating the pik3-akt pathway, and preventing the onset of autophagy (1). Autophagy starts/miR-193b-3p declines, and this protein is most produced and autophagy occurs more (2). Reb has an incremental effect on the mTOR protein that activates this pathway and prevents the formation of the initial autophagy phase. The expression of miR-199a-5p decreases, the inhibitory effect on Reb is inactivated, and autophagy is inhibited (3). Foxo3 disables Akt and causes the MPT pathway to be deactivated/miR-27a decreases its expression, and the foxo3 protein is further produced and autophagy continues to function (4). mTOR, one of the important pathways involved in the autophagy mechanism, has an inhibitory effect on this process and does not allow autophagy to begin and applies its effect on the ULK1/2 factor/miR-7 declines, and its inhibitory effect on this pathway is removed, and the autophagy does not start (5). ULK1/2 is one of the important factors for the onset of autophagy and phagophore formation, declining miR-26b and its inhibitory effect on ULK2, and autophagy begins its pathway, but the expression of miR-290-295 cluster is increased, and the ULK1 protein level is reduced, and the phagophore is not formed, so autophagy does not occur (6). Beclin-1 is somehow one of the important proteins in the development of phagophore and the onset of autophagy. The expression of mir-20a increases, and its inhibitory effect on the gene does not allow the formation of proteins and, accordingly, autophagy does not begin, but miR-30a expression reduces, the Beclin-1 gene is more expressed, and autophagy starts (7). ATG14 has an increased effect on Beclin-1 and makes phagophore more likely to form miR-135a expression increases, thereby inhibiting ATG14 gene and autophagy formation (8). HMGB1 stimulates the Beclin-1 gene and causes the autophagy to start its first phase/miR-34a expression decreases, and its inhibitory effect on the HMGB1 gene is removed, and Beclin-1expression increases (9). UVRAG interferes somehow behind the initial pathway of autophagy and reaches the formation of autophagosome/the miR-183 which disrupts the process by targeting and inhibiting the gene (10). FIP200, present in ULK complex and is effective in the formation of phagophore/miR-224-3p expression, is increased, and an inhibitory effect on this gene is increased, and the initial phase of autophagy does not occur, but miR-20b, which declines, causes an increase in the expression level of FIP200, and phagophore is formed (11). AMK with inhibitory effect on MTOR pathway and TSC1/TSC2 stimulation inhibits autophagy. The expression of miR-185 is reduced, AMK is more expressed, and autophagy is more active (12). ATG7 is a factor accelerating the conversion of lc3-I to Lc3-II, which is an important process for the onset of autophagosome formation. miR-490-3p expression declines and further stimulates its target and ATG7, and autophagy continues (13). ATG5 is a protein that causes autophagy to evolve from the phagophore formation phase to the next formation of the process. miR-181a is increased, and most of the ATG5 gene is inhibited, and this functional trend is disrupted (14). ATG16L is a factor that accelerates the formation of autophagosomes. The expression of miR-130a is increased, the level of the ATG16L protein decreases, and the autophagy is inhibited/expression of the miR-410 decreases, and this process continues (15). The activity of ATG12 is similar to that of ATG16L. The expression of MIR-23a is reduced, its inhibitory effect on this gene is reduced, and autophagy continues its process. miR-378 inhibits the autophagy process by inhibiting the gene (16). ATG10 is a protein that stimulates the activity of ATG5, ATG16L, and ATG12 proteins and accelerates the process of autophagosome formation. miR-20 has an inhibitory effect on this protein, which can disrupt this activity (17). ATG3 is a factor to stimulate the formation

autolysosomes, suggestive of tumor-suppressive role of miR-638 via silence of EZH2 (Zhang et al. 2021a).

In osteosarcoma (OS), miR-210-5p induced epithelial-mesenchymal transition (EMT) and oncogenic autophagy via PIK3R5/AKT/mTOR axis (Liu et al. 2020a). Also, upregulation of miR-22 in OS suppressed autophagy and induced apoptosis resulted in increased sensitivity to cisplatin (Meng et al. 2020). In prostate cancer (PC) cells, overexpression of miR-381 increased cellular autophagy and apoptosis, while decreased cell proliferation mediated by reelin (RELN) suppression (Liao and Zhang 2020). Deng et al. recognized that miR-493 respectively activated cytotoxic autophagy and reduced invasion of PC cells via up-modulation of BECN1 and ATG7 (Deng et al. 2020).

In cervical cancer cells, miR-211 overexpression targeted autophagy and apoptosis through Bcl-2 regulation (Liu et al. 2020b). Besides, aberrant expression of miR-106a in cervical squamous cell carcinoma (CSCC) was related to malignancy parameters of CSCC tissues. Based, overexpression of miR-106a elevated CSCC growth and suppressed autophagy via binding to 3UTR of LKB1 in human papilloma virus (HPV) 16-positive CSCC (Cui et al. 2020). Consistently, miR-378 has a potential impact on cervical cancer progression via binding to ATG12-regulated autophagy (Tan et al. 2018). In the ovarian cancer (OC), increased expression of miR-34 activates apoptosis and autophagy followed by significant reduction in the proliferation of cancerous cells (OVACAR-3 cells) via silencing Notch 1 (Jia et al. 2019). Shao et al. identified that miR-1251-5p upregulation had oncogenic effects on human ovarian cancer via preventing TBCs (negative modulator of autophagy) (Shao et al. 2019).

In bladder cancer, reduced expression of miR-221 facilitated autophagy through increasing TP53INP1 levels, indicative of the valuable importance of miR-221 as therapeutic targets in this malignancy (Liu et al. 2020c). Dai et al. represented the *tumorigenic* capacity of miR-130 in bladder cancer cells as it was proved by autophagy induction through blocking CYLD (Dai et al. 2020). Also, Zhang et al. displayed that upregulation of miR-21 in bladder tumor cells (T24 cells) promoted T24 cells progression alongside with apoptosis and autophagy obstruction via downregulation of, Beclin-1, PTEN, caspase-3, LC3-II, and E-cadherin (Zhang et al. 2020a). Similarly, Rezaei et al. focused on the impacts of up-/downregulation of miRNAs in the different lung diseases including lung cancer either in in vitro and in vivo conditions or human. In this regard, up- and downregulation of respectively miR-210 and miR-181 inactivated autophagy, while down- and upregulation of respectively miR-3127-5p and miR-21 activated autophagy (Rezaei et al. 2020).

In esophageal squamous cell carcinoma (ESCC), autophagy is triggered by miR-503 via PKA/mTOR pathway followed by inhibition of ESCC invasiveness (Wu et al. 2018). In another study, Li et al. focused on the effect of miR-126 on apoptosis and autophagy of ESCC cells and found that miR-126 expression was increased in ESCC followed by enhancement of apoptosis and autophagy; however, miR-126 inhibition reversed current trend via suppression of STAT3 (Li et al. 2020a). Phatak et al. (2021) clarified that miR-141-3p could act as an oncogene in esophageal cancer cells via binding to TSC1 mRNA which led to tumor progression as well as autophagy reduction (Phatak et al. 2021).

In gastric cancer (GC) cells, miR-let-7a/Rictor/Akt-mTOR axis modulates autophagy activity

Fig. 2 (continued) of LC3-PE, which causes the LC3 protein binding to phosphatidylethanolamine and the formation of autophagosome and maintains its stability. The expression of miR-1 is reduced, this factor is further developed, and autophagosome is formed (18). ATG2 protein is effective in the formation of autolysosome. The expression of miR-143 is increased, the ats2 gene is suppressed, and this process is disrupted (19). ATG9 is an agent for stimulating the formation of autolysosome and accelerating the process of lysosome fusion with autophagosome/miR-29a expression which is decreased and the level of atg9 increased, and this trend continues (20). Akt is a stimulant factor for the mTOR pathway and prevents the formation of autophagy/miR-185 which targets this gene and inhibits autophagy (21)

(Fan et al. 2018). Among another regulators of autophagy in GC, it can mention miR-183 which its downregulation blocks apoptosis and autophagy via interacting with MALAT1 and SIRT1 through PI3K/AKT/mTOR pathway (Li et al. 2019a). Li et al. evidenced that miR-133a-3p could strengthen autophagy and proliferation of GC cells via downregulation of FOXP3 (Li et al. 2020b). In breast cancer cells, transfection of MCF-7 with miR-26b mimic reduced autophagy dependent to irradiation through silence of DRAM1 (Meng et al. 2018). Ai et al. (2019) clarified that overexpression of miR-107 in breast cancer cell lines (MDA-MB-231 and MDA-MB-453 cells) causes significant reduction in cellular autophagy, proliferation, and metastasis via silencing HMGB1. ULK1 and lysosomal protein transmembrane 4 beta (LAPTM4B), autophagy-related mediators, have also been identified as direct targets of miR-489 which is downregulated in the most of breast cancer cells and several drug resistant breast cancer cell lines (Soni et al. 2018a).

In the metabolic diseases such as osteoporosis, the condition can be exacerbated via miR-15 overexpression which modulates osteoblast genesis and autophagy alongside with downregulation of USP7 (Lu et al. 2021). Wang et al. provided evidences that in osteoarthritis (OA), joint disease, miR-140-5p/miR-149 could affect autophagy, apoptosis, and proliferation of chondrocytes via their potential target, FUT1 (Wang et al. 2018a). Also, miR-20 has a pivotal impact on OA evidenced by inhibition of autophagy and chondrocytes proliferation through ATG10/PI3K/AKT/mTOR axis (Vojtechova and Tachezy 2018). Besides, He et al. (2018) assigned that the inhibition of miR-20 promoted proliferation and autophagy machinery in articular chondrocytes by targeting ATG10 via PI3K/AKT/mTOR signaling pathway (He and Cheng 2018). Furthermore, pathogenesis of intervertebral disc degeneration (IDD) can be influenced by miRNA-regulated autophagy including decreased autophagy facilitated by upregulation of miR-210 and miR-202-5p via targeting ATG7 (Lan et al. 2020). Yun et al. (2020) highlighted the promising role of miR-185 in preventing IDD via improving cell survival and suppressing apoptosis and autophagy of nucleus pulposus cell via blockage of galectin-3/Wnt/β-catenin pathway (Yun et al. 2020). Similar results have been achieved by miR-142-3 overexpression in controlling and inhibiting IDD (Xue et al. 2021).

Moreover, evidences are in a favor of miR-145-3p in exerting autophagic flux in multiple myeloma (MM) via *HDAC4* inhibition (Wu et al. 2020). In the neurodegenerative disorders such as Parkinson's disease (PD) defined by dopaminergic neurons apoptosis, Wen et al. (2018a) confirmed that AMPK/mTOR-regulated autophagy and apoptosis could be a potential therapeutic platform as this axis can be inhibited by miR-185 overexpression leading to prevention of dopaminergic cells death in PD model (Wen et al. 2018a). Similarly, Li et al. (2018a) observed that autophagy in PD could be triggered by miR-181b/PTEN/Akt/mTOR axis in a way that overexpression of miR-181b is associated with increased cell viability. Also, Lu et al. (2020) conducted similar research on PD model and reached to the findings that upregulation of miR-133a in a PD cell model increased cell proliferation and inhibited autophagy and apoptosis by binding to 3 UTR of RAC1 (Lu et al. 2020). Wen and colleagues demonstrated that overexpression of miR-185 inhibited autophagy and apoptosis through regulating the AMPK/mTOR signaling pathway in PD (Wen et al. 2018b). In Alzheimer's disease (AD), the amounts of miRNA-101a was significantly decreased in patients as well as in vivo model and resulted in autophagy regulation through the MAPK pathway (see Table 1) (Li et al. 2019b). Another novel therapeutic option in AD could be proposed by upregulation of miR-16-5p or downregulation of BTG2, which inhibit neuronal damage and autophagy (Dong et al. 2021). Yang et al. (2020) pinpointed that melatonin could reduce neuronal death and autophagy in cerebral ischemia-reperfusion injury (CIRI) mechanistically through regulation of miR-26a-5p/NRSF as well as JAK2-STAT3

Table 1 microRNAs and autophagy

miRNA	Expression	Target	Inhibition/ induction	Disease	Note	Ref
miR-26b	Down	ULK2	Induction	Prostate cancer	Downregulation of mTOR	Clotaire et al. (2016)
miR-21	Up	Rab11	Inhibition	Renal ischemia-reperfusion	Reduction of Beclin-1andLC3-II expression and upregulation of p62	Liu et al. (2015a)
miR-185	Down	mTOR AMPK	Induction	Parkinson	Increase of neuronal apoptosis through elevating AMPK/ mTOR signaling pathway activity, upregulation of Beclin-1, LC3-I/ LC-II	Wen et al. (2018a)
miR-96-5p	Up	FOXO1	Inhibition	Breast cancer	Increase of migration, invasiveness, and proliferation by decreasing apoptosis	Doan et al. (2017)
miR-502	Down	Rab1B DHODH	Induction	Colon cancer	Increase of cell proliferation and metastasis	Zhai et al. (2013)
miR-100	Down	mTOR IGF-1R	Inhibition	HCC	Decrease of LC3B-II and Akt proteins enhance tumor growth	Ge et al. (2014)
miR-30a	Down	Beclin-1	Induction	Breast cancer Lung cancer Glioma	–	Zhu et al. (2009)
miR-143	Down	ATG2B HK2	Induction	Non-small-cell lung cancer (NSCLC)	Promotion of cell proliferation, metastasis and Warburg effect	Wei et al. (2015)
miR-23a	Down	ATG12	Induction	Melanoma	Increase of the expression of RUNX2 reduces miR-23a Increase of metastasis and invasion via blocking AMPK-RhoA pathway	Guo et al. (2017a)
miR-130a	Up	ATG16L	Inhibition	COPD	Enhancement of apoptosis and increase of the development of COPD	Li et al. (2016a)
miR-193b-3p	Down	TSC1	Induction	Amyotrophic lateral sclerosis (ALS)	Increase of cell survival by increase of TSC1 expression, and decrease of mTORC1 activity, apoptosis	Dhital et al. (2017)

(continued)

Table 1 (continued)

miRNA	Expression	Target	Inhibition/ induction	Disease	Note	Ref
let-7i	Up	IGF-1R	Induction	Ankylosing spondylitis (AS)	Protection of T cell from apoptosis through (PI3 K)/Akt and MAPK signaling pathways, LC3B-II increase in T cell and p62 decline, inhibition of mTOR	Hou et al. (2014a)
miR-20a-5p	Down	ATG16L1	Induction	Ischemic kidney injury	The hypoxia downregulated HIF-1α and miR-20a-5p expression, increase of LC3-II	Wang et al. (2015a)
miR-20a	Up	THBS2	Induction	Cervical cancer tissue	The miR-20a deficiency led to trigger the decrease of autophagic activity in cervical cancer cell lines	Zhao et al. (2015a)
miR-338-5p	Up	PIK3C3	Inhibition	Colorectal cancer	Promotion of metastasis and cell migration, decline of ATG14, LC3-II and Beclin-1 expression	Ju et al. (2013)
miR-301a	Up	NDRG2	Induction	Prostate cancer	Hypoxia-induced miR-301a expression, increase of cell viability and decrease of cell apoptosis, promotion of PTEN expression	Guo et al. (2016)
miR-301b	Up	NDRG2	Induction	Prostate cancer	Hypoxia-induced miR-301b expression, increase of cell viability and decrease of cell apoptosis, promotion of PTEN expression	Guo et al. (2016)
miR-290-295 cluster	UP	Atg7 ULK1	Inhibition	Melanoma	Promotion of cell Proliferation and migration Increase of melanoma cells resistance to glucose deficiency	Cheng et al. (2012)

(continued)

Table 1 (continued)

miRNA	Expression	Target	Inhibition/ induction	Disease	Note	Ref
miR-185	Down	AKT1 RICTOR RHEB	Inhibition	HCC	Increase of cell proliferation by overexpression of mTOR Decrease of apoptosis via Bcl-2, upregulation of cyclin D1	Zhou et al. (2017)
miR-96-5p	Up	ATG7	Inhibition	Liver fibrosis	TGF-β1 promotes miR-96-5p expression, inverse cell proliferation, inhibition of mRNA, and protein levels of α-SMA and Col1α1	Yu et al. (2018b)
miR-101	Down	EZH2	Induction	HCC	Increase of chemoresistance and decline of apoptosis	Xu et al. (2014)
miR-101	–	–	Inhibition	Liver ischemia/ reperfusion injury (LIRI)	miR-101 can inhibit autophagy and reduce LIRI by activating the mTOR pathway	Song et al. (2019)
miR-101a	Down	–	Inhibition	Alzheimer's disease (plasma)	miRNA-101a could regulate autophagy by targeting the MAPK pathway	Li et al. (2019b)
miR-129-5p	Up	Beclin-1	Inhibition	Prostate cancer	Increase of resistance to the Norcantharidin (NCTD)	Xiao et al. (2016)
miR-140-5p/ miR-149	Down	FUT1	Inhibition	Osteoarthritis	Decrease of chondrocyte proliferation, overexpression of IL-1β, and promotion of apoptosis	Wang et al. (2018a)
miR-124	Down	Bim	Inhibition	Parkinson	Increase of apoptosis and inhibition of autophagosome accumulation and lysosomal depletion	Wang et al. (2016)
miR-124	–	p62/p38	Induction	Parkinson	miR-124 can suppress neuroinflammation during the Parkinson's disease development via targeting autophagy, p62, and p38	Yao et al. (2019)

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Table 1 (continued)

miRNA	Expression	Target	Inhibition/ induction	Disease	Note	Ref
miR-224-3p	Up	FIP200	Inhibition	Cervical cancer	Promotion of cell proliferation	Fang et al. (2016)
miR-143	Down	GABARAPL1	Induction	Gastric cancer	Increase of resistance to the quercetin	Du et al. (2015)
miR-22	Up	PTEN	Inhibition	Diabetic nephropathy	Increase of renal tubulointerstitial fibrosis, increase of glucose inducing miR-22 and promoting AKT/mTOR pathway	Zhang et al. (2018a)
miR-130a	Down	ATG2B DICER1	Induction	Chronic lymphocytic leukemia	Promotion of cell proliferation	Kovaleva et al. (2012)
miR-181a	Up	MTMR3	Inhibition	Gastric cancer	Promotion of cell proliferation, metastasis and inhibition of apoptosis	Lin et al. (2017)
miR-21	Up	PTEN	Inhibition	HCC	Increase of resistance to sorafenib, promotion of AKT pathway	He et al. (2015a)
miR-409-3p	Down	Beclin-1	Induction	Colon cancer	Increase of resistance to oxaliplatin	Tan et al. (2016)
miR-30a	Down	Beclin-1	Induction	Renal carcinoma	Increase of resistance to sorafenib, upregulation of ATG5 and decrease of apoptosis	Zheng et al. (2015)
miR-503	Up	PRKACA	Inhibition	Esophageal carcinoma	Promotion of cell proliferation, metastasis, increase of PKA/mTOR signaling pathway activity	Wu et al. (2018)
miR-143	Up	ATG2B	Inhibition	Crohn's disease	Blockage of autophagy in intestinal epithelial cells, decline of autophagosome and autolysosome formation, downregulation of IκBα Promotion of pro-inflammatory cytokine expression: IFN-γ, TNF-α, and IL-8	Lin et al. (2018)

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Table 1 (continued)

miRNA	Expression	Target	Inhibition/ induction	Disease	Note	Ref
miR-423-3p	Up	Bim	Induction	Gastric cancer	Increase of cell proliferation, invasion and migration, upregulation of LC3, and decrease of P62 and apoptosis	Kong et al. (2017)
miR-135a	Up	Atg14	Inhibition	HCC	Factor VII-increased miR-135a, decrease of LC3A/B protein level, promotion of mTOR activation	Huang et al. (2017)
miR-34a	Down	HMGB1	Induction	Acute myeloid leukemia (AML)	Inhibition of apoptosis Increase of LC3 level, enhancement of chemoresistance	Liu et al. (2017b)
miR-34	Down	Notch 1	Induction	Ovarian cancer cell lines	miR-34 can be inhibiting ovarian cancer cells proliferation by triggering apoptosis and autophagy. It suppresses cell invasion through targeting Notch 1	Jia et al. (2019)
miR-142-3p	Down	HMGB1	Induction	Non-small-cell lung cancer (NSCLC)	Promotion of mTOR, AKT, and P13K activation, increase of chemoresistance	Chen et al. (2017a)
miR-142-3p	Down	HMGB1	Induction	Acute myelogenous leukemia (AML)	Increase of drug resistance in AML cells, inhibition of apoptosis	Zhang et al. (2017a)
miR-142-3p	Down	KLF9	Inhibition	Human ectopic endometrial tissues	Upregulation of mir-142-3p levels can restrict autophagy and induce apoptosis of CRL-7566 cells	Ma et al. (2019)
miR-30b	Down	Atg12, Atg5	Induction	Hepatic ischemia-reperfusion injury (IRI)	Upregulation of LC3-II and increase of autophagosomes	Li et al. (2016b)
miR-30b	–	–	Induction	Vascular calcification	Restoring of miR-30b expression can promote autophagy	Xu et al. (2019a)

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Table 1 (continued)

miRNA	Expression	Target	Inhibition/ induction	Disease	Note	Ref
miR-199a-5p	Down	Rheb	Inhibition	Ankylosing spondylitis (AS)	Enhancement of mTOR signaling pathway, decrease of LC3, Beclin-1, and ATG5 expression, Increase of pro-inflammatory cytokines: TNF-α, IL-17, and IL-23	Wang et al. (2017a)
miR-320	Up	HIF-1α	Induction	Retinoblastoma (RB)	Upregulation of HIF-1α and hypoxia, increase of LC3 and Beclin-1 expression, decrease of p62 and p-mTOR	Liang et al. (2017)
miR-32	Up	DAB2IP	Induction	Gastric cancer	Increase of radioresistance in GC, decline of apoptosis and mTOR	Wu et al. (2016a)
miR-224	Up	Smad4	Inhibition	Hepatitis B Virus-associated HCC	Hepatitis B reducesAtg5 and Beclin-1, increase p62, inhibits the formation of autophagosome, blocks TGF-β signaling pathway, and promotes cell proliferation and metastasis	Lan et al. (2014)
miR-181a	Down	p38 JNK	Induction	Parkinson	Enhance p38 MAPK/JNK signaling pathways and apoptosis	Liu et al. (2017c)
miR-410	Down	ATG16L1	Induction	Osteosarcoma	Increase of chemoresistance and inhibition of apoptosis	Chen et al. (2017b)
miR-449a	Down	CISD2	Inhibition	Glioma	Increase level of BCL-2 and cell proliferation, downregulate Beclin-1	Sun et al. (2017a)
miR-378	Up	ATG12	Inhibition	Cervical cancer	Increase migration, invasiveness, proliferation, and metastasis	Tan et al. (2018)
miR-33	Up	ABCA1	Inhibition	Atherosclerosis	Decrease autophagosome formation and LC3 in macrophage	Ouimet et al. (2017)

(continued)

Table 1 (continued)

miRNA	Expression	Target	Inhibition/ induction	Disease	Note	Ref
miR-32	Up	DAB2IP	Induction	Prostate cancer	Increase radioresistance in prostate cancer cells, promote autophagy through the mTOR-S6K pathway	Liao et al. (2015)
miR-7	Down	mTOR	Inhibition	HCC	Increase cell proliferation	Wang et al. (2017b)
miR-26b	Down	DRAM1	Induction	Breast cancer	Increase radioresistance in breast cancer cell	Meng et al. (2018)
miR-20a	Up	BECN1 ATG16L1 SQSTM1	Inhibition	Breast cancer	C-myce promotes miR-20a and elevates ROS level and DNA damage	Liu et al. (2017a)
miR-638	Up	TP53INP2	Inhibition	Melanoma	Inhibit apoptosis via block p53, increase methylation at CpG islands, enhance melanoma metastasis	Bhattacharya et al. (2015)
miR-212	Down	SIRT1	Induction	Prostate cancer	Increase angiogenesis and cellular senescence	Ramalinga et al. (2015)
miR224-3p	Down	ATG5 FIP200	Induction	Glioblastoma	Hypoxia inhibits miR224-3p and mTOR activity, increase levels of ATG 16,12,13 and ULK1	Guo et al. (2015)
miR-183	UP	UVRAG	Inhibition	Colorectal cancer	Decrease of apoptosis and autophagosome formation, increase of cell proliferation	Huangfu et al. (2016)
miR-183	Down	SIRT1	Induction	Gastric cancer tissue and cell lines	miR-183 can enhance gastric cancer cell viability and inhibit cell apoptosis by promoting autophagy MALAT1-miR-183-SIRT1 axis and PI3K/AKT/mTOR pathway are involve in autophagy of gastric cancer cells	Li et al. (2019a)
miR-29	Down	–	Induction	Retinal pigment epithelial cells	Overexpression of miR-29 can induce autophagy of ARPE-19 cells and primary human retinal pigment epithelial cells	Cai et al. (2019)

(continued)

Table 1 (continued)

miRNA	Expression	Target	Inhibition/ induction	Disease	Note	Ref
miR-29a	Down	ATG9A TFEB	Induction	Pancreatic cancer	Increase resistance to gemcitabine, autophagy flux, autophagosome formation and autophagosome-lysosome fusion, overexpression of LC3B and decrease p62, promote cell proliferation, migration and invasion	Kwon et al. (2016)
miR-29a	Up	PTEN	Inhibition	Pathological cardiac hypertrophy (rat model)	miR-29a can inhibit autophagy by regulating the PTEN/AKT/mTOR signaling pathway	Shi et al. (2019)
miR-29b-3p	Down	SPARC	Inhibition	Blood samples of heart failure (HF) and hypoxia-induced H9c2 cells	Hypoxia led to downregulation of miR-29b-3p level and induces autophagy and apoptosis of H9c2 cells miR-29b-3p suppresses apoptosis and autophagy by targeting SPARC in hypoxia-induced H9c2 cells	Zhou et al. (2019)
miR-30a	Down	BECN1	Induction	Diabetic cataract	Hgh glucose-promoting apoptosis	Zhang et al. (2017b)
miR-24-3p	Up	DEDD	Induction	Bladder cancer	Increase of cell proliferation, invasion, migration and LC3, decline of apoptosis and p62	Yu et al. (2017a)
miR-138	Down	Sirt1	Induction	Lung cancer	Increase cell proliferation, invasion, metastasis, EMT and AMPK signaling pathway, decrease apoptosis and mTOR activity	Ye et al. (2017)
miR-490-3p	Down	ATG7	Induction	HCC	Increase cell proliferation, decrease apoptosis	Ou et al. (2018)
miR-20a-5p	Down	ATG7	Induction	Neuroblastoma	Increase of LC3-II/ LC3-I and autophagosome formation, decline of apoptosis	Yu et al. (2018b)

(continued)

Table 1 (continued)

miRNA	Expression	Target	Inhibition/ induction	Disease	Note	Ref
miR-23a	Down	ATG12	Induction	Melanoma	Increase cell proliferation, invasion and metastasis	Guo et al. (2017b)
miR-138	Down	PDK1	Inhibition	Malignant melanoma	Promote PI3K/ AKT/mTOR signaling pathway, decrease levels of LC3, caspase-3 and Bax	Meng et al. (2017)
miR-181a	Up	Atg5	Inhibition	HCC	Decline of apoptosis	Yang et al. (2018)
miR-489	Down	ULK1 LAPTM4B	Induction	Breast cancer	Increase of chemoresistance	Chen et al. (2018)
miR-30a	Down	Beclin-1	Induction	Medulloblastoma	Increase cell proliferation and LC3B level	Singh et al. (2017)
miR-214-3p	Down	Atg12	Induction	Sporadic Alzheimer's disease	Increase levels of LC3bII and Beclin-1, and enhance number of GFP-LC3-positive autophagosome vesicles and apoptosis	Lv et al. (2016)
miR-214	–	PTEN	Induction	Ischemic heart disease (H9c2 cell line)	Oridonin can induce apoptosis and autophagy by regulating PI3K/ AKT/mTOR pathway via overexpression of miR-214	Gong et al. (2019)
miR-20a	Down	RB1CC1/ FIP200	Induction	Breast cancer	Decrease of mTOR activity	Li et al. (2016c)
miR-20b	Down	RB1CC1/ FIP200	Induction	Breast cancer	Decrease of mTOR activity	Li et al. (2016c)
miR-181b	Down	PTEN	Induction	Parkinson	Decrease of PI3 K/Akt/mTOR signaling pathway	Li et al. (2018a)
miR-181b	Up	CREBRF	Induction	Gallbladder cancer	miR-181b inhibits tumor suppression mediated with ginsenoside Rg3 of gallbladder carcinoma through inducing autophagy flux by targeting CREBRF	Wu et al. (2019)
miR-20	Up	ATG10	Inhibition	Osteoarthritis (OA)	Decrease of PI3 K/Akt/mTOR signaling pathway and enhancement of proliferation in chondrocytes	Vojtechova and Tachezy (2018)

(continued)

Table 1 (continued)

miRNA	Expression	Target	Inhibition/ induction	Disease	Note	Ref
miR-222	Up	PPP2R2A	Inhibition	Bladder cancer	Increase of Akt/mTOR signaling pathway, enhanced resistance of bladder cancer cells to cisplatin	Zeng et al. (2016)
miR-125b	Up	APC	Induction	Colorectal cancer	CXCL12/CXCR4 promotes miR-125b expression and elevates Wnt/β-catenin signaling pathway, EMT and cell invasion, enhances resistance of colorectal cancer cells to fluorouracil	Yu et al. (2017b)
miR-125a	Up	–	Inhibition	Thyroiditis (mice)	Overexpression of miR-125a can be inhibits autophagy by targeting PI3K/ Akt/mTOR signaling pathway in mouse model of thyroiditis	Chen et al. (2019a)
miR-218	Down	YEATS4	Induction	Colorectal cancer	Increase resistance to the oxaliplatin (L-OHP), inhibit of apoptosis	Fu et al. (2016)
miR-22	Down	HMGB1	Induction	Osteosarcoma	Increase drug resistance in osteosarcoma cells, promote cell proliferation, migration, and invasion	Guo et al. (2014)
miR-1	Down	ATG3	Induction	Non-small-cell lung cancer (NSCLC)	Increase drug resistance in NSCLC cells	Hua et al. (2018)
miR-27a	Down	FoxO3a	Induction	Traumatic brain injury (TBI)	Increase level of Beclin-1 and decrease p62	Sun et al. (2017b)
miR-27a	Up	SYK	Inhibition	Melanoma tissues	Depletion of miR-27a lead to induced autophagy and apoptosis of melanoma cells through the activation of the SYK-dependent mTOR signaling pathway	Tang et al. (2019)
miR-31	Down	–	Induction	Colorectal cancer-associated fibroblasts (CAFs)	Elevation of levels of Beclin-1, ATG, DRAM, and LC3, decrease of apoptosis, increase of radioresistance	Yang et al. (2016a)

(continued)

Table 1 (continued)

miRNA	Expression	Target	Inhibition/ induction	Disease	Note	Ref
miR-140-5p	Down	Smad2 ATG12	Induction	Colorectal cancer	Decrease of TGF-β signaling pathway and necrosis, increase of cell proliferation, metastasis, and invasion	Zhai et al. (2015)
miR-let-7a	Down	Rictor	Inhibition	Gastric cancer	Upregulation of Akt/mTOR signaling pathway	Fan et al. (2018)
miR-221	Up	TP53INP1	Inhibition	Colorectal cancer	Increase cell proliferation, decrease level of LC3	Liao et al. (2018)
miR-107	Down	HMGB1	Inhibition	Breast cancer tissue and cell line	miR-107 can suppress autophagy, migration and proliferation of breast cancer cells through targeting HMGB1	Ai et al. (2019)
miR-107	Down	TRAF3	Induction	Osteoarthritis chondrocytes	Upregulation of miR-107 can inhibit the activation of NF-κB and AKT/mTOR pathway by targeting TRAF3 genes. Also, miR-107 overexpression suppresses apoptosis and promotes autophagy	Zhao et al. (2019a)
miR-223	Up	Atg16l1	Inhibition	Brain microglial cells (BV2 cells) and	miR-223 can inhibit autophagy and induce CNS inflammation by targeting ATG16L1 Expression level of miR-223 was upregulated in CNS and spleen during experimental autoimmune encephalomyelitis (EAE) progression	Li et al. (2019c)

(continued)

Table 1 (continued)

miRNA	Expression	Target	Inhibition/ induction	Disease	Note	Ref
miR-365	–	ATG3	Inhibition	HCC tissue and cell line	Enforced expression of miR-365 led to significant inhibition of the ATG3 expression in hepatocellular carcinoma cells LncRNA PVT1 can promote autophagy by sponging miR-365 in HCC	Yang et al. (2019a)
miR-206	Down	STC2	Induction	Head and neck squamous cell carcinoma (HNSCC) tissue and cell line	Enforced expression of miR-206 can lead to enhanced autophagy of HNSCC cells	Xue et al. (2019)
miR-206	–	–	Inhibition	Osteoarthritis (rat model)	miR-206 can inhibit autophagy and apoptosis of osteoarthritis cells by activating the IGF-1-mediated PI3K/AKT-mTOR signaling pathway	Yu et al. (2019)
miR-93	–	BECN1, SQSTM1, ATG5, ATG4B	Inhibition	Glioblastoma cancer	miR-93 can inhibit autophagy activity by downregulation of autophagy regulatory genes level	Huang et al. (2019a)
miR-99a and miR-449a	Down	Beclin-1	Inhibition	Thrombosis (serum sample)	Upregulation of miR-99a and miR-449a can inhibit beclin-1 expression levels and autophagy	Zeng et al. (2019)
miR-216a	Down	MAP1S	Inhibition	Colorectal cancer	miR-216a can act as a tumor suppressor miRNA and inhibit autophagy through the TGF-β/MAP1S pathway	Wang et al. (2019a)
miR-18a	Up	BDNF	Inhibition	Cardiomyocytes (from an acute myocardial infarction (AMI) rat model)	miR-18a can lead to inhibiting autophagy and promoting senescence of cardiomyocytes after AMI by targeting BDNF	Lin et al. (2019)

(continued)

Table 1 (continued)

miRNA	Expression	Target	Inhibition/ induction	Disease	Note	Ref
miR-155	–	–	Inhibition	Human umbilical vein endothelial cells (HUVECs)	Downregulation of miR-155 expression can lead to decreasing oxidant-induced injury and inducing cell proliferation by upregulating autophagy	Chen et al. (2019b)
miR-506-3p	–	SPHK1	Inhibition	Osteosarcoma cancer cell	miR-506-3p can initiate epithelial-to-mesenchymal transition (EMT) and inhibit autophagy of osteosarcoma cancer cells by targeting SPHK1	Wang et al. (2019b)
miR-326	–	XBP1	Induction	Parkinson's disease (mouse model)	miR-326 can inhibit nitric oxide synthase (iNOS) expression and induce autophagy of dopaminergic neurons via targeting XBP1	Zhao et al. (2019b)
miR-1251-5p	Up	TBCC	Induction	Human ovarian cancer cell lines and tissues	miR-1251-5p can induce autophagy and act as an oncogene to suppress TBCC and α-/β-tubulin expression	Shao et al. (2019)
miR-217	–	NAT2	Induction	CCL4-induced liver injury (rat models)	miR-217 can induce apoptosis and autophagy and inhibit proliferation of hepatocytes by targeting NAT2	Yang et al. (2019b)
miR-34a-5p	Up	–	Induction	CIH-induced HCAECs	Overexpression of miR-34a-5p can contribute to chronic intermittent hypoxia (CIH)-induced human coronary artery endothelial cell (HCAEC) autophagy by Bcl-2/ Beclin-1 pathway	Lv et al. (2019)

pathway (Yang et al. 2020). It was suggested that neuronal deficit and autophagy in ischemic stroke could be abolished by miR-378 trough targeting GRB2, while lncRNA MEG3 could sponge the miR-378 and activate the expression of GRB2 (Luo et al. 2020).

Shi et al. clarified that miR-126 loss of function could activate myocardial autophagy induced by Beclin-1 and contributed in acute myocardial infarction (AMI) development (Shi et al. 2020). In contrast, miR-18a downregulation had protective effects against AMI via activation of BDNF expression and inhibition of Akt/mTOR axis (Lin et al. 2019). In the Su et al. study (Su et al. 2020), it was manifested that downregulation of miR-30e-3p lessened autophagy and activated apoptosis and injury in cardiomyocytes under ischemia/hypoxia conditions potentially through Egr-1 regulation (Su et al. 2020). MiRNA-regulated abnormal apoptosis and autophagy of cardiomyocyte have a great of importance in heart failure (HF). Alongside with reduced expression of miR-29b-3p in HF patients, the level of this miRNA was decreased in an in vitro HF model under hypoxia condition followed by elevated apoptosis and autophagy via inactivation of SPARC and regulation of TGF-β1/Smad3 cascade (see Table 1) (Zhou et al. 2019).

In the liver complications such as liver fibrosis characterized by hepatic stellate cell (HSC) activation, the regulation of HSC autophagy has attracted research interests. There is line of evidence shown that introduction of miR-96-5p into HSCs (LX-2 cells) is accompanied by repressing autophagy in the cells via ATG7 regulation (Yu et al. 2018c).

In the renal problems including renal tubulointerstitial fibrosis (TIF) as a main result of diabetic nephropathy (DN), accumulating data implicated the major role of miRNAs in the autophagy regulation. Zhang et al. findings represented that miR-22 partially targets PTEN-blocked autophagy followed by TIF development (Liu et al. 2018a). Furthermore, p53/miR-214/ULK1 axis affects autophagy dysregulation in diabetic kidney disease (DKD) (Ma et al. 2020). Moreover, Liu et al. disclosed that the expression of miR-25-3p was increased in polycystic kidney disease (PKD) model via interacting with ATG14-activated autophagy as well as promoting proliferation of renal cell (Liu et al. 2020d). Table 1 lists some miRNAs regulating autophagy in some human cancer cells.

2.2 MiRNAs Interactions in Chemo-Induced Autophagy

Increasing data have reported that autophagy, along with chemotherapy and its association with chemoresistance can be a new therapeutic platform to succeed in cancer treatment. To find the correlation between miRNAs and chemotherapy-induced autophagy, experimental investigations were reviewed. More importantly, the cross talk between miRNAs (modulators of multiple pathways) and autophagy holds promise to overcome chemoresistance in malignancies (Soni et al. 2018b).

Chen and colleagues found that *miR-519a not only plays a role in* glioma by regulating STAT3-mediated autophagy pathway but also affects autophagy in glioblastoma multiforme (GBM) cells and also temozolomide (TMZ) chemosensitivity. The results showed that miR-519a enhanced the sensitivity of GBM cells to TMZ. Also, a significant association was found between miR-519a effects and autophagy. Overall, miR-519a promoted autophagy in glioblastoma through targeting STAT3/Bcl-2 signaling pathway (Li et al. 2018b). Besides, overexpression of miR-29b in GBM cells inhibited cell survival, activated apoptosis and autophagy, and sensitized tested cells to TMZ (Xu et al. 2021). Because of TMZ importance in the treatment of glioblastomas and its ability to induce autophagy, Xu and colleagues assessed the regulatory role of miR-30a in glioblastoma cells treated with TMZ. They revealed that miR-30a increases U251 glioblastoma cells' chemosensitivity to TMZ through direct target of Beclin-1 and inhibition of autophagy (see Table 2) (Xu et al. 2018a). In an in vivo study, Chakrabarti and colleagues proved that antitumor activities of luteolin and silibinin, chemotherapeutic agents, were augmented due to the overexpression of miR-7-1-3p leading to

Table 2 Dysregulated expression of miRNAs in chemotherapy and their function in autophagy cascade

MicroRNA	Expression	Target	Drug/chemotherapy/radiotherapy	Inhibition/induction of autophagy	Type of disease	Ref
miR-519a	Up	STAT3 Bcl-2	Chemotherapy (temozolomide)	Induction	Glioblastoma	Li et al. (2018b)
miR-199a-5p	Down	Beclin-1	Chemotherapy (cisplatin [also known as diamminedichloridoplatinum (II) (DDP)]	Induction	Osteosarcoma (OS)	Li et al. (2016d)
miR-18a	Up	mTORC1	Radiotherapy	Induction	Colon cancer	Qased et al. (2013)
miR-22	Up	HMGB1	Chemotherapy	Inhibition	Osteosarcoma (OS)	Li et al. (2014a)
miR-22	–	MTDH	Chemotherapy (cisplatin)	Inhibition	Osteosarcoma cancer cells	Wang et al. (2019c)
miR-155	Up	–	Chemotherapy	Induction	Osteosarcoma (OS)	Chen et al. (2014a)
mIR-152	Up	ATG14	Chemotherapy (cisplatin)	Inhibition	Ovarian cancer	He et al. (2015b)
miR-214	Up	UCP2	Chemotherapy (tamoxifen, fulvestrant)	Inhibition	Breast cancer	Yu et al. (2015a)
miR-30a	Down	Beclin-1	Chemotherapy (temozolomide)	Induction	Glioblastoma	Xu et al. (2018a)
miR-7-1-3p	Up	XIAP	Luteolin Silibinin	Inhibition	Glioblastoma	Chakrabarti and Ray (2016)
miR-199a-5p	Down	ATG7	Chemotherapy (cisplatin)	Induction	HCC	Xu et al. (2012)
miR-199a-5p	Down	DRAM1	Chemotherapy (Adriamycin)	Inhibition	Acute myeloid leukemia (AML)	Li et al. (2019d)
miR-423-5p	Up	p-ERK 1/2	Chemotherapy (sorafenib)	Induction	HCC	Stiuso et al. (2015)
miR-125b	Up	EVA1A	Chemotherapy (oxaliplatin)	Inhibition	HCC	Ren et al. (2018)
miR-216a	Up	Beclin-1	Radiotherapy	Inhibition	Pancreatic cancer	Zhang et al. (2015a)
miR-410-3p	Up	HMGB1	Chemotherapy (gemcitabine)	Inhibition	Pancreatic ductal adenocarcinoma (PDAC)	Xiong et al. (2017)
miR-140-5p	Up	IP3k2	Chemotherapy	Induction	Osteosarcoma	Wei et al. (2016)
hsa-miR-302a-3p	Down	ULK1	Chemotherapy (5-fluorouracil)	Induction	Colon cancer	Hou et al. (2014b)
hsa-miR-548ah-5p	Down	ATG16L1	Chemotherapy (5-fluorouracil)	Induction	Colon cancer	Hou et al. (2014b)

hsa-miR-30a-5p	Up	PIK3R2	Chemotherapy (5-fluorouracil)	Induction	Colon cancer	Hou et al. (2014b)
hsa-let-7c-5p	Up	BCL2L1	Chemotherapy (5-fluorouracil)	Induction	Colon cancer	Hou et al. (2014b)
hsa-miR-99b-5p	Up	mTOR	Chemotherapy (5-fluorouracil)	Induction	Colon cancer	Hou et al. (2014b)
hsa-miR-23a-3p	Up	BCL2	Chemotherapy (5-fluorouracil)	Induction	Colon cancer	Hou et al. (2014b)
hsa-miR-195a-5p	Up	BCL2	Chemotherapy (5-fluorouracil)	Induction	Colon cancer	Hou et al. (2014b)
miR-34a	Up	FoxO3	Lipopolysaccharide (LPS)	Induction	Acute lung injury (ALI)	Song et al. (2017)
miR-15a-3p	Up	Bcl-2	Polygonatum odoratum lectin (POL)	Induction	Lung adenocarcinoma	Wu et al. (2016b)
miR-1290	Down	GSK3β	Polygonatum odoratum lectin (POL)	Induction	Lung adenocarcinoma	Wu et al. (2016b)
miR-193b	Up	–	Chemotherapy (5-fluorouracil)	Induction	Esophageal cancer	Nyhan et al. (2016)
miR-193b	Down	FEN1	Chemotherapy (epirubicin)	Induction	Osteosarcoma cells	Dong et al. (2019)
miR-384-5p	Down	Beclin-1	Streptozotocin (STZ)	Induction	Diabetic encephalopathy	Wang et al. (2018b)
miR-101	Up	STMN1 RAB5A ATG4D	Chemotherapy (cisplatin)	Inhibition	HCC	Xu et al. (2013)
miR-30a	Up	Beclin-1	Chemotherapy (doxorubicin)	Inhibition	Osteosarcoma	Xu et al. (2016)
miR-30b	–	ATG5	Chemotherapy (cisplatin)	Inhibition	Gastric cancer	Xi et al. (2019)
miR-221/222	Up	ATG12	Chemotherapy (dexamethasone)	Inhibition	Multiple myeloma	Xu et al. (2018b)
miR-142-3p	Up	ATG5 ATG16L1	Chemotherapy (sorafenib)	Inhibition	HCC	Zhang et al. (2018c)
miR-21	Down	–	Chemotherapy (etoposide, doxorubicin)	Induction	Chronic myeloid leukemia	Seca et al. (2013)
miR-137	–	ATG5	Chemotherapy (doxorubicin)	Inhibition	Pancreatic cancer cells	Wang et al. (2019d)
miR-224-3p	–	ATG5	Chemotherapy (temozolomide)	Inhibition	Glioblastoma and astrocytoma	Huang et al. (2019b)
miR-146a	–	TAF9b/P53 pathway	Doxorubicin (DOX)	Induction	Human AC16 cell line	Pan et al. (2019)

inhibition of autophagy and induction of apoptosis in glioblastoma cells (Chakrabarti and Ray 2016). In addition, miR-224-3p weakened resistance to TMZ in glioblastoma cells (LN229 cells) via abolishing autophagy under hypoxia via ATG5 downregulation (Liu et al. 2020e).

Xiao et al. (2016) investigated the role of miR-199a-5p in reducing chemoresistance to cisplatin or diamminedichloridoplatinum (II) (DDP) in OS. They showed that treatment of OS cells with DDP attenuated the expression level of miR-199a-5p; increased the level of various proteins, such as Beclin-1 and LC3; and induced autophagy machinery, which highlights the relationship between treatment cytotoxicity, autophagy inhibition, and their effects on chemoresistance (see Table 2) (Li et al. 2016d). Chen and colleagues observed that overexpression of miR-155 during chemotherapy induced autophagy leading to mediate chemoresistance in OS (Chen et al. 2014a). Wang et al. noted that upregulation of miR-22 in OS cells (MG-63) increased *sensitivity to cisplatin* mediated via negative regulation of autophagy by down-expression of MTDH (Wang et al. 2019c). Alongside, miR-193b/FEN1 axis ameliorated the epirubicin sensitivity of OS cells through autophagy induction (Dong et al. 2019). miR-375 could be another target to sensitize OS to cisplatin as its overexpression in cisplatin-resistant OS models delayed tumor progression and autophagy via targeting ATG2B (Gao et al. 2020a). Qased et al. investigated the role of miR-18a in autophagy process in HCT116 (human CRC cells). To do so, HCT116 cells were irradiated, and the expression levels of miR-18a were subsequently measured in the cells. The results showed that the radiation led to increased expression level of miR-18a and enhanced autophagy induction (Qased et al. 2013). Li et al. showed that the expression levels of miR-22 are enhanced during chemotherapy and target HMGB1, which results in inhibition of HMGB1-induced autophagy (see Table 2) (Li et al. 2014a).

He et al. reported that miR-152 plays an important role in autophagy regulation and drug resistance in ovarian cancer (OC) (He et al. 2015b). They showed that miR-152 was significantly downregulated in cisplatin-resistant cells. It has been reported that overexpression miR-152 leads to induction of apoptosis in cisplatin-resistant cancer cells as well as a decrease of cisplatin-induced autophagy. In this in vitro study, it was documented that ATG14 downregulation by EGR1-miR-152 sensitizes ovarian cancer cells to cisplatin-induced apoptosis through inhibiting cyto-protective autophagy (He et al. 2015b). Vescarelli et al. verified that miR-200c considerably sensitized chemoresistant OC cells to olaparib via regulating NRP1 (Vescarelli et al. 2020). In addition, miR-29c-3p overexpression inhibited autophagy which in turn reversed cisplatin resistance of OC by downregulation of FOXP1/ATG14 pathway (Hu et al. 2020). Esfandyari et al. (2021) demonstrated that miR-143 overexpression in cervical cancer cells (CaSki cells) could increase cisplatin sensitivity of treated cells via induction of apoptosis and autophagy (Esfandyari et al. 2021). Tamoxifen (TAM) and fulvestrant (FUL) are considered as effective drugs for patients with ER-positive breast cancer, but the rate of response to these therapies is limited because of various barriers, such as endocrine resistance. In this regard, Yu and colleagues found that miR-214 enhanced breast cancer cells sensitivity to TAM and FUL through autophagy inhibition (Yu et al. 2015a). In a comparable study on breast cancer, Soni et al. identified that miR-489 enhanced sensitivity to doxorubicin (Dox) as a result of autophagy inhibition dependent to LAPTM4B downregulation (Soni et al. 2018b).

Xu et al. reported that miR-199a-5p downregulation induced by cisplatin enhances drug resistance through activating autophagy in HCC (Xu et al. 2012). Soni et al. evaluated the role of miR-155-5p on Adriamycin (ADR)-resistant liver carcinoma cells (HepG2/ADR), and their findings indicated the effects of miR-155-5p as sensitizer of ADR, activator of apoptosis, and inhibitor of autophagy via attaching to ATG5 3UTR (Soni et al. 2018b). Also, higher expression of miR-541 inhibited the autophagy in HCC cells by targeting ATG2A and RAB1B leading to promising response to sorafenib (Xu et al. 2020a). In another

study, it was revealed that upregulated miR-142-3p increased sensitivity of HCC cells to sorafenib by targeting ATG5 and ATG16L1 as negative modulators of autophagy (Zhang et al. 2018b). Similar findings have been reported for miR-101/RAB5A/STMN1/ATG4D axis in the HCC cells (HepG2) which improved the response to cisplatin due to inhibition of autophagy mechanism (Xu et al. 2013). Consistently, Ren et al. demonstrated that miR-125b/EVA1A axis-mediated autophagy reversed resistance of HCC cells to oxaliplatin (Wei-Wei et al. 2018).

Chemoresistance of nasopharyngeal carcinoma (NPC) has been investigated by Zhao et al. (2020) study in which they verified that miR-1278 expression was decreased in NPC tissues associated with worse chemotherapy response. Nonetheless, upregulation of miR-1278 dramatically raised anticancer effects of cisplatin in NPC cells together with reduced autophagy via inhibiting ATG2B (Zhao et al. 2020).

Yang et al. (2021) showed that miR-136-5p upregulation not only had negative effects on malignant progression of laryngeal squamous cell carcinoma (LSCC) and hypopharyngeal squamous cell carcinoma (HPSCC) cells but also reversed cisplatin resistance in the tested cells via inactivation of ROCK1 Akt/mTOR axis (Yang et al. 2021).

Recently, Xi et al. explored the lncRNA MALAT1/miR-30b/ATG5 axis in cisplatin resistance of GC cells (AGS/CDDP and HGC-27/CDDP) and documented that miR-30b attenuated cisplatin resistance by reduced expression of not only MALAT1-activated autophagy but also ATG5 (see Table 2) (Xi et al. 2019). In another study, Chen et al. (2020a) identified that miR-30a could sensitize gastrointestinal stromal tumors (GISTs) cells to imatinib (IM) via silence of Beclin-1-regulated autophagy (Chen et al. 2020a). Also, He et al. (2020a) discovered that miR-153-5p upregulation in oxaliplatin (L-OHP)-resistant CRC cells could overcome L-OHP resistance via silencing Bcl-2-induced autophagy (He et al. 2020a). Furthermore, Liu et al. (2020f) indicated that lncRNA NEAT1 upregulation sponged miR-34a in CRC. Additionally, NEAT1 inhibition significantly slowed down CRC tumorigenesis and elevated sensitivity of cells to 5-fluorouracil (5-FU). miR-34a overexpression also showed comparable trends with NEAT1 inhibition via binding to autophagy components (HMGB1, ATG4B, and ATG9A) (Liu et al. 2020f). The role of miRNA in chemoresistance of pancreatic cancer (PC) cells was evaluated by miR-137 overexpression in PANC-1 cell lines. The results indicated that miR-137 chemo-sensitized the cells to Dox via ATG5-triggered autophagy (Wang et al. 2019d).

The main hurdle for the proper treatment of multiple myeloma (MM) is still chemoresistance. Of note, the cross talk between miRNA dysregulation and autophagy illustrated that miR-221/222 could suppress dexamethasone (Dex) sensitivity in MM cells via inhibition of autophagy associated with ATG12/p27-mTOR axis (Xu et al. 2019b). In various studies, drug resistance in non-small-cell lung cancer (NSCLC) has been investigated. In a research conducted by Hua et al., overexpression of miR-1 reversed cisplatin resistance in NSCLC by suppression of ATG3-regulated autophagy (Hua et al. 2018). Therefore, miRNAs have regulatory roles in chemoresistance due to their effects on autophagy induction. These mediators should be further investigated in numerous in vivo and in vitro studies to find the molecular mechanisms related to resistance. Table 2 lists the effects of autophagy-related miRNAs on some human cancer chemotherapy.

3 Exosome and Autophagy

Exosomes, membrane-coated vesicles with 30–120 nm size, are released by several cells, such as lymphocytes, platelets, epithelial cells, mast cells, dendritic cells, neurons, and endothelial cells (Théry et al. 2002; Hashemipour et al. 2021). Exosome has main roles in biological events, including inflammation, tumorigenesis, metastasis, and response to therapy (Kharaziha et al. 2012). Various researches have demonstrated that exosomes can also be considered as diagnostic means and targeted drug delivery system. It has been identified that almost all biological body fluids, including blood, serum,

saliva, milk, amniotic fluid, semen, breast milk, and urine contain exosomes (Keller et al. 2011; Lässer 2015).

Exosomes carry diverse unique molecular cargos, including lipids, proteins, and nucleic acid fragment. Some of the proteins are involved in assembly, movement, and organization of exosomes (e.g., annexins, actins, tumor susceptibility gene 101, vesicle-associated membrane protein 8, and fibronectin) and observed in the structures of exosome. Furthermore, a cluster of proteins known as exosome surface markers, such as CD9, CD63, CD81, and CD82, are useful for the detection of exosomes (Zhao et al. 2015b; Barclay et al. 2017). Mounting evidence has established that exosomes have a wide range of roles in human pathological and physiological processes. Since exosomes deliver their constituents into recipient cells, they are able to play a prominent role in cell signaling and local/distant cell-to-cell communication (Lakkaraju and Rodriguez-Boulan 2008; Van Niel et al. 2006). These data demonstrated that exosomal molecular constituents can represent disease conditions (Feng et al. 2013). The idea of the RNAs presence in exosomes has attracted great attention in the research of exosomal RNAs, especially miRNAs as potential diagnostic biomarkers (Taylor and Gercel-Taylor 2008). Recent experiments have demonstrated that exosomal miRNAs are resistant to RNase degradation and thus remain stable in circulating plasma and serum. On the other hand, they are easily evaluated, are minimally invasive, and have high sensitivity and specificity. This evidence indicates that exosomal miRNAs are ideal biomarkers for early clinical diagnostic applications (Lin et al. 2015; Li et al. 2014b).

As cited above, the autophagic process contains five key stages including initiation, nucleation, elongation and maturation, fusion, and degradation (Li et al. 2020c). mTOR acts as the regulator of the initiation stage, and its activation is associated with prohibition of autophagy, whereas its inactivation is able to induce autophagy. It has been revealed that mTOR and the ULK complex (consist of ULK1, FIP200, and autophagy-related protein 13 [Atg13]) is inactivated and activated, respectively, in stress situations. Beclin-1, an essential component for autophagosome formation, in combination with Vps34 and Atg14L produces a complex, which is necessary for induction autophagy nucleation (Liang et al. 1999; Levine et al. 2015; He and Klionsky 2009; Kihara et al. 2001). In the elongation along with maturation stage, two ubiquitin-like conjugation systems are warranted to facilitate autophagosome membrane expansion. The first system involves the microtubule-associated protein light chain 3 (LC3)-phosphatidylethanolamine (PE) complex. LC3 is cleaved by Atg4 at its C terminal to produce intracellular LC3-I, which is conjugated with PE in the ubiquitin-like reactions of Atg7 and Atg3. The lipid form of LC3 (LC3-II) is attached to the autophagosome membrane (Yu et al. 2015b). The second system involves the Atg12-Atg5-Atg16 complex, in which Atg12 is conjugated with Atg5 via ubiquitin-like reactions of Atg7 and Atg10. The Atg12-Atg5 conjugate interacts noncovalently with Atg16 to form a large complex. While lysosomes bind to autophagosomes to form autolysosomes in the fusion stage, cargo within autolysosomes will be degraded in the degradation stage. Autophagy is tightly modulated to keep homeostasis. Following autophagy initiation, lots of Atg proteins collaborate to manage the next stages of autophagy. It is yet not clear that autophagy conveys protective or detrimental effects in diseases (Saha et al. 2018; Xiong 2015). For example, lack of autophagy is associated with excess amount of tau and synuclein proteins, which induces neurodegenerative disorders. Evidences are in support of the fact that autophagy has a dual effects on cancer cells and initially acts as a tumor inhibitor; however, later it defends tumor cells against the immune system's attacks (Sharma et al. 2021; Hassanpour et al. 2020). Likely, it has been demonstrated that autophagy regulates cardiac and hepatic disorders positively and negatively, respectively. Thus, the control of autophagy via exosomes can have various positive and negative effects on a variety of diseases (Xing et al. 2021).

The role of exosomes in cellular stresses has been evidenced. However, some researches

indicate that the interaction between exosomes and autophagy machinery may preserve intracellular protein and homeostasis (Baixauli et al. 2014). In addition, autophagy induction due to nutrient deprivation leads to inhibited exosome secretion (Fader and Colombo 2009). There are some exosomal proteins markers related to autophagy mechanism. Dias et al. showed that PRNP (prion protein gene) is essential to promote the release of exosomes regulating CAV1/caveolin-1-suppressed autophagy (Dias et al. 2016). Moreover, significant levels of autophagy proteins, including WIPI2, LC3, NBR1, and p62, are present in exosomal fractions secreted by apilimod-treated cells (Hessvik et al. 2016). Importantly, different exosomal and autophagic proteins can be applied as potential biomarkers regarding the type of cancer (Salimi et al. 2020).

Also, the role of exosomal miRNAs in autophagy regulation has been demonstrated by various investigations. Yang et al. reported that high serum levels of exosomal miR-30a were observed in AMI patients. Also, they observed that inhibition of miR-30a increased the expression level of Beclin-1, Atg12, and LC3-II/LC3-I known as the regulators of core autophagy machinery and contributed to preserve the hypoxia-induced autophagy (Yang et al. 2016b). Liu and colleagues conducted a study on AMI rat model and in vitro model of hypoxic H9c2 cells to investigate the cardioprotective role of miR-93-5p-encapsulating exosomes released from adipose-derived stromal cells (ADSCs) in ischemia-induced cardiac damage. They found overexpression of inflammatory cytokines as well as miR-93-5p in both patients and rat models with AMI. In addition, the comparison of the protective effects of exosomes on infarction-induced cardiac damage revealed that exosomal treatment containing miR-93-5p derived from ADSCs caused more protection than simple exosomes (Liu et al. 2018b). Also, Li et al. highlighted the impact of bone marrow-derived mesenchymal stem cells (BMMSCs)-derived exosomes enriched in miR-29c on negative regulation of autophagy in cardiac ischemia/reperfusion (I/R) injury through PTEN/Akt/mTOR pathway (Li et al. 2020d). Similarly, human umbilical cord mesenchymal stem cells-exosome (hucMSC-ex) abolished coxsackievirus B3 (CVB3)-activated myocarditis due to upregulation of autophagy function mediated by AMPK/mTOR axis and reduction of cardiomyocyte death (Gu et al. 2020). Santoso et al. (2020) demonstrated that induced pluripotent stem cells and their differentiated cardiomyocyte-delivered exosome (iCM-Ex) treatment had cardioprotective effects against post-MI via improvement of autophagy machinery in vivo and in vitro (Santoso et al. 2020). Besides, Li and colleagues isolated exosomes released by human aortic smooth muscle cells and identified that isolated exosomes contained miR-221/222. They found that miR-221/222 could target 3′UTR of PTEN. Also, overexpression of miR-221/222 downregulated the expression of ATG5, LC3-II and Beclin-1, suggestive of the inhibitor role of exosomal miR-221/222 in autophagy process (Li et al. 2016e).

Yuwen et al. (2017) reported that the expression level of exosomal miR-146a-5p in NSCLC is correlated with chemosensitivity and chemotherapy response to cisplatin. Low levels of miR-146a-5p in serum exosomes were detected in advanced NSCLC patients. In both NSCLC cells and exosomes, the expression level of miR-146a-5p was gradually decreased due to chemoresistance to cisplatin. In addition, miR-146a-5p also inhibited the autophagy through targeting Atg12 (Yuwen et al. 2017). Wang et al. investigated the role of tumor environment such as acute shear stress (ASS) in NSCLC invasion. Their data indicated that ASS activated cell death by exerting the secretion of autophagy and exosome components via SIRT2/TFEB axis (Wang et al. 2020a). In the severe lung injury and respiratory deficit, Wei et al. illustrated that huMSC-ex-delivered miR-377-3p could improve acute lung injury (ALI) induced by lipopolysaccharide through targeting RPTOR followed by autophagy activation (Wei et al. 2020).

Exosomal miR-1910-3p derived from breast cancer cell attenuated metastasis, growth, and autophagy via MTMR3 suppression and NF-κB and wnt/β-catenin signaling induction (Wang et al. 2020b). Since exosomes loaded with

miR-1910-3p increased autophagy and breast cancer development via silencing MTMR3 and inducing NF-κB and wnt/β-catenin pathway, it could be considered as a diagnostic biomarker for breast cancer (Wang et al. 2020b). Moreover, hucMSCs-ex transferring miR-224-5p could hamper cellular apoptosis and mount proliferation and autophagy in breast cancer via silence of HOXA5 (Wang et al. 2021a). In another interesting study, Han et al. (2020), showed that exosome-shuttled miR-567 repressed autophagy and chemo-sensitized breast cancer cells to trastuzumab via interacting with ATG5 (Han et al. 2020). Additionally, the anticancer effects of gemcitabine in breast cancer (luminal-b type) could be improved using exosome-overexpressed small interfering RNA (siRNA) MTA1, which suppressed autophagy and EMT/HIF-α pathway (Li et al. 2020e).

In the field of thyroid research, papillary thyroid cancer (PTC) cell exosome-delivered SNHG9 lncRNA could prevent autophagy flux and upregulate apoptosis of human normal thyroid epithelial cell line (Nthy-ori-3 cell) mediated by YBOX3/P21 pathway (Wen et al. 2021).

In cisplatin-resistant GC, Yao et al. manifested that the levels of exosomal circ-PVT1 and miR-30a-5p were respectively upregulated and downregulated, while the silence of Circ-PVT1 reversed cisplatin resistance via reducing autophagy alongside with increasing apoptosis through miR-30a-5p/YAP1 axis (Yao et al. 2021). Comincini et al. evaluated the expression levels of exosomal miR-17 and miR-30a to diagnose celiac disease and discovered that miR-17- and miR-30a-regulated ATG7 and BECN1 known as two key executor of autophagy (Comincini et al. 2017).

Beclin-1 contains three main domains including coiled coil (CCD), evolutionarily conserved (ECD), and Bcl-2-homology-3 (BH3). Several proteins through binding to the various domains of Beclin-1 and forming different complexes regulate autophagy activity (Wirawan et al. 2012). Beclin-1 is encoded by BECN1, which is located on chromosome 17q21 and was shown to be targeted via miR-30a (Zhu et al. 2009). Exosomal miR-30a is capable of prohibiting autophagy via targeting the Beclin-1 pathway and maintains a mandatory role in liver fibrosis and MI. It was revealed by Yang et al. (2016b) that hypoxic cardiomyocytes prohibit autophagy through secreting miR-30a and, thereby, cause cardiomyocyte damage. So, it can be expected that autophagy level can be increased by targeting miR-30a, and, thereby, cardiomyocyte damage will be decreased. In contrast to findings of Yang et al., Zhang et al. found out that epigallocatechin gallate acts as a protective agent for MI through overexpression of exosomal miR-30a and, consequently, prohibiting autophagy and apoptosis (Zhang et al. 2020b). An animal study that was conducted by Xu et al. (2019c) also demonstrated that exosomal miR-30a through prohibiting autophagy decreased the level of cardiomyocyte apoptosis in rats with MI/reperfusion injury. Autophagy becomes active throughout hypoxia and displays protective effects by modifying cell survival. Nevertheless, as myocardial hypoxia continues, excessive autophagy occurs, which causes accumulation of a quite amount of toxic components and, as a consequence, cell death. In Yang et al.'s study, autophagy was inhibited by exosomal miR-30a; hence, there was a lack of protective autophagy in cardiomyocytes, which contributed to cardiomyocyte apoptosis. However, in other studies performed by Zhang and Xu, excessive autophagy was the reason behind cardiomyocytes damage. Exosomal miR-30a is able to decrease the level of cardiomyocyte apoptosis via prohibiting excessive autophagy. Moreover, it has been unveiled that excessive autophagy can induce liver fibrosis. It was shown that in a hepatic fibrosis model that was establish by Chen et al. (2017c), the expression level of exosomal miR-30a, secreting via hepatic stellate cells, was decreased. The upregulation of miR-30a may have the capacity to improve liver fibrosis through prohibiting autophagy mediated by the Beclin-1 pathway.

Li et al. (2021) revealed that osteosarcoma (OS)-secreted exosomal lncRNA OIP5-AS1 regulated autophagy and angiogenesis via reduction of miR-153 and enhancement of ATG5 expressions (Li et al. 2021). In spite of pro-tumor effects of hBMSC-derived exosomes on OS progression via autophagy elevation, knockdown of

ATG5 in OS cells attenuated oncogenic effects of hBMSC exosomes (Huang et al. 2020).

Reportedly, in osteoarthritis (OA) mice model, intra-articular administration of OA exosomes loaded with ATF4 had protective effects against chondrocyte apoptosis via activating autophagy (Wang et al. 2021b). Furthermore, in IVDD model, it was confirmed that normal cartilage end plate stem cell-derived exosomes (N-Exos) had a better therapeutic impact on stopping nucleus pulposus cell apoptosis and delay in IVDD progression in comparison with degenerated cartilage end plate stem cell-derived exosomes (D-Exos) via induction of PI3K/AKT/autophagy pathway (Luo et al. 2021). Also, the effects of human umbilical cord mesenchymal stem cell-derived exosomes (hucMSC-ex) on tissue damages make them as a promising tool in the regenerative medicine. Based, Jia et al. (2018) discovered that hucMSC-ex enriched with 14-3-3ζ reversed cisplatin-activated nephrotoxicity via interaction with ATG16L-induced autophagy (Jia et al. 2018).

It has been made clear that the levels of anti-inflammatory cytokines and miR-30d-5p are reduced following acute ischemic stroke (AIS). Jiang et al. recognized that exosomes derived from miR-30d-5p-overexpressing ADSCs could overcome autophagy-induced cerebral damage via increasing polarization of M2 microglial/macrophage (Jiang et al. 2018). Chen et al. (2020b) noticed that exosome-delivered circSHOC2 released from ischemic-preconditioned astrocyte (IPAS) potentiated neuronal protective effects against ischemic cerebral injury by affecting autophagy through the miR-7670-3p/SIRT1 axis (Chen et al. 2020b). Recently, Pei et al. verified that astrocyte-released exosomes (AS-Exo) suppressed neuronal autophagy and alleviated neuronal injury and apoptosis in an in vitro model of ischemic injury via overexpression of miR-190b and downregulation of Atg7 (Pei et al. 2020). It has been observed that hucMSC-ex could breakdown blood-brain barrier (BBB) and target substantia nigra leading to protection of dopaminergic neurons via activation of autophagy in a PD model (Chen et al. 2020c). Ma et al. (2021) analyzed the amounts of lncRNA LINC00470 in glioma-derived exosomes from patients and concluded that overexpressed LINC00470 could abrogate autophagy and raise glioma cells proliferation via binding to miR-580-3p which in turn inactivated WEE1 and induced the PI3K/AKT/mTOR pathway (Ma et al. 2021). Programmed death-ligand 1-containing exosomes (PD-L1-ex) derived from glioblastoma stem cell (GSC) enhanced autophagy and reduced apoptosis via AMPK/ULK1 pathway cascade resulted in enhanced resistance to TMZ, while knockdown of PD-L1 reversed these effects (Zheng et al. 2021). There is line of evidence shown that Schwann cells (SCs) have regenerative role following peripheral nerve injury. In this context, Yin et al. discovered that ADSC-Exos loaded by miR-26b blocked SC autophagy and improved the myelin sheath regeneration in the sciatic nerve injury model via targeting Kpna2 (Yin et al. 2021). Due to the improvement of inflammation secondary to spinal cord injury (SCI) via anti-inflammatory effects of peripheral macrophages (PMs), Zhang et al. represented that PM-derived exosomes (PM-Exos) could promote spinal cord recovery via enhancement of microglial autophagy and anti-inflammatory microglia polarization mediated through PI3K/AKT/mTOR pathway (Zhang et al. 2021b).

In type 2 diabetes mellitus (T2DM) rats, He et al. uncovered that hucMSC-ex promoted hepatic lipid and glucose metabolism potentially by enhancing the autophagosomes via AMPK pathway (He et al. 2020b). Likewise, Zhang et al. reported that liver I-/R-induced injury could be alleviated by huMSC-ex-transmitted miR-20a via regulating apoptotic and autophagic genes including caspase-3, P62, mTOR, and LC3-II (Zhang et al. 2020c). Further, Zhu et al. (2020) verified that ADSC exosome carrying mmu_circ_0000623 inhibited liver fibrosis through autophagy induction (Zhu et al. 2020). Since liver fibrosis can be driven by HSC activation, Wang et al. (Wang et al. 2020c) displayed that natural killer (NK) cell-derived exosome (NK-Exo) attenuated HSC activation via inhibiting TGF-β1 mechanistically through overexpression of miR-223 and inhibition of ATG7-induced autophagy (Wang et al. 2020c).

All in all, studies have recently demonstrated that autophagy has regulatory properties in exosomal production and its release. The link between Atg5 and V1V0-ATPase and their role in induction of exosome production has been documented by Chen et al. (2018). They found that cells with Atg5 and Atg16L1 deficiency exhibit reduced exosome production, but it's not dependent on Atg7 and canonical autophagy. It has been shown that Atg5 affects the production of exosomes by reducing the acidifying of endosomes and disrupting the acidification of V1V0-ATPase. Because of the role of autophagy and exosomes in metastasis, Atg5 is able to induce invasion and metastasis (Guo et al. 2017c). Abdulrahman et al. evaluated the role of autophagy in exosome production and processing. They found that the induction of autophagy by rapamycin, mTOR inhibitor, suppressed the release of exosomal prions; however, the inhibition of autophagy resulted in increased release of both exosomes and prions (Abdulrahman et al. 2018). Totally, further studies were collected in Table 3.

Table 3 Exosome and autophagy

Type of cargo	Exosome source	Effect on autophagy	Type of disease	Note	Ref
miR-146a-5p	Serum	Inhibition	Non-small-cell lung cancer (NSCLC)	miR-146a-5p upregulated and decrease level of Atg12	Yuwen et al. (2017)
miR-93-5p	Adipose-derived stromal cells (ADSCs)	Inhibition	Acute myocardial infarction (AMI)		Liu et al. (2018b)
miR-30d-5p	Adipose-derived stromal cells (ADSCs)	Induction	Acute ischemic stroke (AIS)	Enhancement of M2 microglial/macrophage polarization and reduce of M1 microglial/macrophage polarization. Inhibition ischemia-induced neuronal damage via decreasing of TNF-α, IL-6, and iNOS secretion from M1 microglial cells. Downregulation of Beclin-1 and Atg5. Induction of expression anti-inflammatory cytokines IL-4 and IL-10 from M2 microglial cells	Jiang et al. (2018)
miR-181-5p	Adipose-derived mesenchymal stem cells (ADSCs)	Induction	Liver fibrosis	miR181-5p-ADSC block of STAT3/Bcl-2/Beclin-1-dependent signaling pathway and decrease liver fibrosis	Qu et al. (2017)
miR-30a	Serum H9c2 cell	Inhibition	Acute myocardial infarction (AMI)	Hypoxia promotes expression of miR-30a in cardiomyocytes and increases apoptosis and elevates Atg12 and Beclin-1 protein levels	Yang et al. (2016b)
miR-17	T98G cells	Induction	Celiac disease (CD)	miR-17 downregulated and increase of expression level of ATG7	Comincini et al. (2017)
miR-30a	T98G cells	Induction	Celiac disease (CD)	miR-30a downregulated and increase of expression level of BECN1	Comincini et al. (2017)
miR-221/222	Human aortic smooth muscle cells (HAoSMCs)	Inhibition	–	miR-221/222 upregulation in HUVECs, reduction of PTEN, LC3-II, ATG5, and Beclin-1protein levels. Increase of SQSTM1/p62 level and Akt signaling pathway	Li et al. (2016e)

(continued)

Table 3 (continued)

Type of cargo	Exosome source	Effect on autophagy	Type of disease	Note	Ref
MSC exosome (miR-125b)	Neonatal mice cardiomyocytes (NMCMs) cell	Inhibition	Myocardial infarction (MI)	Decrease of p53/Bnip3 signaling pathway and save myocardial from death	Monaco et al. (2017)
HucMSC exosome (14-3-3ζ)	NRK-52E cells	Induction	Acute kidney injury (AKI)	HucMSC exosome-delivered 14-3-3ζ attached the ATG16L protein and induced autophagosome formation and as a result elevated cisplatin resistance and cell proliferation and reduced apoptosis	Jia et al. (2018)
Exosomes derived from gefitinib-treated (Exo-GF)	PC9 cells	Induction	Non-small-cell lung cancer (NSCLC)	Enhancement cisplatin resistance, overexpression of Bcl-2 and LC3-II protein levels, decrease of Bax and p62 protein levels	Li et al. (2016f)
NA	H9C2 cells	Induction	Myocardial ischemia-reperfusion injury (MIRI)	Exosomes derived from mesenchymal Stem cells enhance cardiomyocyte autophagy, inhibit cell apoptosis and ROS production through H2O2, promote AMPK pathway and decrease Akt and mTOR pathways	Liu et al. (2017d)
HucMSC exosomes	NRK-52E cells	Induction	–	HucMSC exosomes block cisplatin-induced mitochondrial apoptosis and secretion of inflammatory cytokines, decrease of mTOR and NF-KB, increase levels of ATG5 and ATG7	Wang et al. (2017c)

4 Inhibition or Stimulation of Autophagy by the Virus

Viruses are known as intracellular parasites that are highly dependent on the host for their cell cycle. Hence, after entrance, they reprogram the target host cell to meet their basic needs (Fehr and Yu 2013; Bagga and Bouchard 2014). As we cited before, autophagy has a crucial role in preserving cellular hemostasis by participating in different physiological processes, such as, but not limited to, cell differentiation and development, starvation, and degradation of abnormal products. Additionally, it has been shown that autophagy is produced in response to stress conditions such as infection with viral viruses (Senft and Ze'ev 2015; Mizushima and Levine 2010). Also, in response to viral infections, autophagy becomes active by innate immune system to degrade viruses (Deretic et al. 2013). Additionally, autophagy also takes part in activation of adaptive immune system by accelerating antigen processing (Paludan et al. 2005; Romao et al. 2013). Xenophagy is a type of selective lysosomal degradation pathway that is vital for eliminating pathogens especially bacteria and viruses (Levine 2005). Although autophagosomes potentially are detrimental for invading viruses, several viruses have shown to be able to convert the autophagosome to their home during replication. The autophagosome provides a membrane-bound, protected site to produce their progeny, where their metabolites can be utilized as source of energy for viral replication. Another unique class of autophagy, called lipophagy, targets intracellular lipid droplets, and this process can also be captured by viruses. Lipid droplets are considered as the optimal source for viral assembly since the viruses have the potential to stimulate lipophagy provide the high values of

ATP needed for viral replication (Choi et al. 2018; Heaton and Randall 2011). Taken together, according to recent findings, viruses are developing new strategies to fight or use autophagy to facilitate their replication. Herein, we sought to provide a brief review on how autophagy fights against viral viruses and, thereafter, how the viruses disrupt the autophagic pathway to escape form immune system reactions and prompt their replication.

Recently, several studies have reported that the aim of virus interference with host cell autophagy is to promote the life cycle of virus and avoid detection by the host immune system. The diverse set of viruses are able to dysregulated autophagy machinery (Glick et al. 2010; Jackson 2015). The viral proteins directly or indirectly interact with autophagy components leading to enhance or block autophagy (Mack and Munger 2012). For instance, coronavirus papain-like protease, termed PLP2, induces autophagy via interacting with Beclin-1 (Chen et al. 2014b). Although some viral proteins inhibit the autophagy via interaction with Beclin-1, HIV-Nef and HSV-1 ICP34.5 proteins are capable of inhibiting autophagy-dependent Beclin-1 (Orvedahl et al. 2007; Kyei et al. 2009a; Campbell et al. 2015a). Beclin-1 has Bcl-2 homology 3 (BH3) domain and, through this domain, interacts with anti-apoptotic Bcl-2 family members (Oberstein et al. 2007). This interaction inhibits Beclin-1 assembly to the pre-autophagosomal structure, thereby preventing autophagy (Liang et al. 1998).

The importance of apoptosis and Bcl-2 proteins in immune system regulation and responses to stresses has provided evolutionary pressures on viruses to acquire the genes encoding pro-survival Bcl-2 proteins (Neumann et al. 2015). Large DNA viruses, such as γ-herpesviruses 68 (γ-HV68), adenovirus, Epstein-Barr virus (EBV), and Kaposi's sarcoma-associated herpesvirus (KSAH), mimic the pro-survival Bcl-2 proteins leading to hijack the intrinsic pathway of apoptosis for their purposes (Kvansakul et al. 2017). Liang et al. (2008) reported that murine gamma-herpesvirus 68 (MγHV68) Bcl-2 protected virus-infected cells against apoptosis, also repressed autophagy through its direct binding to Beclin-1 (Liang et al. 2008). In addition to suppressing autophagy by the vBcl2/Beclin-1 complex, KSHV also inhibits this process by viral homolog of cellular FLICE-like inhibitor protein (v-FLIP). Both KSHV v-FLIP and cellular FLIP directly interact with the autophagy-protein ATG3 in competition with LC3 protein. It has been demonstrated that, to suppress autophagic programmed cell death, this interacting ability of KSHV v-FLIP is required (Mack and Munger 2012; Irmler et al. 1997; Thome et al. 1997; Lee et al. 2009). The biochemical evidences show interaction of different HCV and HBV proteins with autophagy machinery components. Nonstructural protein 3 (NS3) of HCV was found to co-localize and associate with the immunity-associated GTPase (IRG) family M that it known autophagy pathway regulator in response to the bacterial infection (Grégoire et al. 2011a; Singh et al. 2006). Core protein of HCV activates autophagy through EIF2AK3and ATF6 UP pathway and/or upregulating Beclin-1 expression (Wang et al. 2014a; Liu et al. 2015b). Moreover, this core protein represses apoptosis and enhances autophagy in hepatocytes through upregulating Beclin-1 (Liu et al. 2015b). Small surface proteins of HBV interact with LC3 and HBV-HBx protein interacts with VPS34 (Sir and Tian 2010; Li et al. 2011a). Sir and colleagues reported that HBx through binding to phosphatidylinositol 3-kinase class III, a critical enzyme in the initiation of autophagy, leads to enhanced activity of this enzyme and thus activates the early autophagic pathway (Sir and Tian 2010).

Espert et al. have shown that autophagy-dependent cell death is activated after binding of HIV envelope glycoprotein to CXCR4 on T cells (Espert et al. 2006; Espert et al. 2007). Bcl-2-associated athanogene 3 (BAG3) is known as a pro-autophagic and anti-apoptotic factor in many normal and neoplastic cells (Rubinstein and Kimchi 2012; Behl 2011; Rosati et al. 2011). Bruno and colleagues reported that transfection of HIV-1 trans-activator (Tat) protein into glioblastoma cells results in increasing BAG3 levels leading to stimulate the autophagic pathway, while silencing of BAG3 results in disrupted balance between autophagy and apoptosis (Bruno et al. 2014). As mentioned earlier, autophagy process involves the formation and maturation of autophagosomes.

Recent studies have showed that interferon-γ (IFN-γ) activates autophagosomes to participate in immunity defense (Deretic 2006). HIV-Tat protein suppresses the formation of autophagosome. In other words, this protein disrupts the IFN-γ signaling pathway through repression of STAT1 phosphorylation and, consequently, inhibits the IFN-γ-induced autophagy (Li et al. 2011b). Additionally, influenza matrix protein 2 and human parainfluenza virus Type 3 phosphoprotein interrupt the maturation of autophagy through blocking autophagosome degradation (Ding et al. 2014; Gannagé et al. 2009).

One of the most important regulators of autophagy is the mammalian target of rapamycin (mTOR), which moderates the balance between autophagy and growth in response to environmental stress and physiological conditions (Cuyàs et al. 2014). Kinase mTOR is the downstream target of PI3K-Akt signaling pathway, which is activated by growth factor receptors and neurotropism as well as promotes cell differentiation, growth, and survival and also reduces apoptosis (Manning and Cantley 2007; Brunet et al. 2001; Hanada et al. 2004). It has been observed that suppression and activation of PI3K/AKT/mTOR pathway lead to promote and inhibit autophagy, respectively (Heras-Sandoval et al. 2014). Surviladze et al. reported that contamination of HaCaT cells with HPV-16 pseudovirions activates thePI3K/Akt/mTOR signaling pathway leading to autophagy inhibition (Surviladze et al. 2013). KSHV-K1, a viral protein, activates thePI3K/Akt/mTOR signaling pathway in endothelial cells and B lymphocytes (Mack and Munger 2012; Tomlinson and Damania 2004; Wang and Damania 2008). Also, HBV induces autophagy in HepG2 cells transfected with HBx through regulating the PI3K/Akt/mTOR pathway (Wang et al. 2013a). It is believed that autophagy plays an important role in the regulation of cancer progression and development and in determining of tumor responses to anticancer treatments. It has been observed that oncolytic viruses (OVs) interact with autophagy in infected tumors to ensure their own survival and replication advantage (Jiang et al. 2011). While an increasing number of OVs are reported to induce autophagy in infected tumors, some OVs choose to subvert or evade it (Zhang et al. 2006; Moloughney et al. 2011). For instance, Rodriguez-Rocha et al. showed that adenoviruses induce autophagy to promote virus replication and oncolysis in lung cancer A549 and H1299 cells (Rodriguez-Rocha et al. 2011). This concept suggests an insightful indication to OV therapy to improve the quality of life and survival of patients with cancer. Therefore, viruses and viral products can effect on the stimulation or inhibition of autophagy. Searching for using these agents to control stress conditions should be more focused.

HCMV belongs to β-herpesvirus family, which has shown to be transmissible via different body fluids. HCMV is known as one of the biggest viruses since its genome contains of 236 kilobases (Plotkin and Boppana 2019). Albeit it has been demonstrated that primary infection mainly is asymptomatic, the congenital form of the virus can be accompanied by several complications including, but not limited to, disabilities and death. HCMV was shown to have the potential to favor cancer through transformation of infected cells when infecting normal tissues by regulating several signaling pathways (Herbein 2018). The virus modulates autophagy in a dual fashion (Joseph et al. 2017; Nahand et al. 2021). At early phases of infection, it contributes to autophagic vesicle formation. On the contrary, later, it inhibits autophagy via producing some proteins (Chaumorcel et al. 2012). By far, two viral proteins, namely, TRS1 and TRS2, that participate in autophagy prohibition in cooperation with Becline-1 have been explored. It has been demonstrated that simultaneous expression of TRS1 and IRS1 is necessary for prohibition of autophagy in virus infection (Mouna et al. 2016). Recently, viral components with the ability of regulating latency and lytic reactivation, especially those in the uLb' gene region, have been at the center of focus. These viral components are capable of limiting virus replication via moderating immune system response and viral latency through expressing quite a few virus proteins. For instance, a viral protein, namely, UL138, through autophagy machinery, can

modulate adaptive immunity of fibroblast when it presents to MHC-1 (Tey and Khanna 2012; Mlera et al. 2020). However, recent evidence clarified that prohibition of autophagy is associated with extreme CD8 + T-cell response because of the internalization of molecules in MHC-I (Loi et al. 2016). Expressing viral proteins derived from HMCV genes 1 and 2 (IE1 and IE2) is essential for immunomodulation and reactivation of host cell virus (Suares et al. 2021; Reddehase and Lemmermann 2019). IE2 is able to modulate gene expression by interacting with UL84 and itself along with a number of cell transcription factors. IE2 protein has a mandatory role in synthesis of viral DNA and was shown to have the potential to counteract host responses (Li et al. 2020f; Møller et al. 2018). Lately, it has been shown that upregulation of IE2 can contribute to autophagy in cells infected with the virus (Zhang et al. 2021c). Briefly, it has been found out that when a cell is infected with HMC, viral proteins result in autophagosomal vesicle formation. Later, the proteins prohibit vesicle-to-lysosome binding, which leads to loss of their degradative capability.

HTLV-1 is a complex type C virus belongs to *Retroviridae* family and contains an envelope which derived from the cell membrane of host (Martin et al. 2016). The virus first was extracted from patients who were suffering from rapidly growing T-cell lymphoma (ATLL) with cutaneous involvement (Martin et al. 2016). Additionally, it has been shown that HTLV-1 has a major role in other diseases including development of poliomyelitis, arthropathy, HTLV-1-associated myelopathy, facial nerve palsy, and infectious dermatitis (Futsch et al. 2018). It has been reported that approximately 5–20 million individuals carry the virus globally; however, a small proportion (3–5%) of them progress secondary ATLL (Gessain and Cassar 2012; Schierhout et al. 2020). Tax is known as a regulatory protein maintaining a crucial role in HTLV-1 replication and, hence, is needed for the virus propagation. It also plays a crucial role in ATLL development since it cooperates with more than 100 cellular proteins to increase cell signaling, inhibit apoptosis, contribute to cell cycle dysregulation, disrupt DNA repair, and stimulate proto-oncogenes (Mui et al. 2017). It was shown that the virus is able to prohibit the binding between autophagosomes and lysosomes through a mechanism involving tax. As a result, quite a few autophagic vesicles, which are not degraded, appear, and these vesicles are great for virus replication (Tang et al. 2013). Hence, Tax protein combines with the IKK complex to induce NF-kB and Beclin-1 activity. Cell adhesion molecule 1 (CADM1) is a glycoprotein belonging to the type 1 transmembrane cell adhesion family, which is part of immunoglobulin superfamily and is taken into account as a marker of T cells infected with HTLV-1 in (Nakahata et al. 2021; Chen et al. 2015). Tax and NF-kB stimulation and degradation of NF-kB negative regulator, namely, p47, are necessary for CADM1 expression. The main mechanism behind p47 degradation is autophagy, and autophagy can be detected in the majority of HTLV-1 infected ATLL cells (Sarkar et al. 2019). HBZ is another crucial essential viral protein for progression of ATLL (Akram et al. 2017). Recent evidence found out that HBZ can prohibit autophagy as well as apoptosis and, in contrast, stimulate brain-derived neurotrophic factor (BDNF) and its receptor expression (Baratella et al. 2017; Mukai and Ohshima 2014). HBZ can exert different effects based on its location; its expression in cell nucleus and cytoplasm is associated with tumor development and stimulation of inflammation, respectively. Its entry to cytoplasm from nucleus is associated with activation of mTOR via PPP1R15A expression, which is a regulator subunit of protein phosphatase1 (Mukai and Ohshima 2014). Same to other viruses, infection with HTLV-1 is associated with formation of autophagosomes and prohibition of binding to lysosomes so as to inhibit degradation. As a consequence, a great amount of autophagosome vesicles will appear, which provides a suitable environment for the virus formation and, moreover, a physical barrier, which limits the progression of cellular processes (Ren et al. 2015).

Since 2019, the world is witnessing a pandemic caused by a new virus called SARS-CoV-2, causing COVID-19 infection (Khatami et al. 2020). It has been reported that at least

270 million individuals infected with SARS-CoV2 and near 5.3 million people have died because of that (Worldometer 2020). Although its mortality rate is not considerably high, it is highly infectious (Sanche et al. 2020). COVID-19 infection symptoms are broad ranging from fatigue, fever, tiredness, and cough to acute respiratory distress syndrome, MI, stroke, renal injury, and death (Xu et al. 2020b). Albeit some mechanisms have been proposed for sever form of the disease, the exact mechanism behind the diseases pathology is yet not clarified and required more studies (Gorshkov et al. 2020). It has been demonstrated that for the virus replication and transcription, there is a need to DMVs to be formed, indicating the fact that the virus may hijack the autophagosomal machinery to assist DMV formation (Carmona-Gutierrez et al. 2020). Hence, autophagosomes play a crucial role in infection replication by using viral replicase proteins (Cottam et al. 2011). In support of that, also, it was found out that NSP6, a viral replicase protein, colocalized with DMVs positive for LC3, showing a probable correlation between the virus replication and autophagy (Cottam et al. 2011; Bello-Perez et al. 2020). Furthermore, Fulvio et al. designed a study to explore the mechanism that coronaviruses such as mouse hepatitis virus and SARS hijack the formation of EDEMosome, and vesicles participate in the regulation of endoplasmic reticulum degradation, in order to produce the DMVs needed for the virus replication. They declared that mouse hepatitis disrupts two endoplasmic reticulum-associated degradation (ERAD) regulatory proteins, namely, EDEM1 and OS-9, degradation via trapping them into DMVs (Reggiori et al. 2010). This represents that SARS-CoV2 is able to facilitate the virus replication within the infected individual by escaping from autophagy.

Enhanced amount of processed form of LC3B and LC3B-II and an accumulation of SQSTM1, supporting the fact that SARS-CoV2 infection contributed to decreased autophagic flux (Hayn et al. 2021). An experimental study illustrated that although stimulation of autophagy using rapamycin cannot affect the virus considerably, activation of innate immune using interferons keeps the virus sensitive. Therefore, the virus escapes from antiviral mechanism of autophagy. In order to understand the mechanism behind anti-autophagy effects of SARS-CoV2, Hayn et al. (2021) evaluated the effect of 29 of the 30 SARS-CoV-2 proteins on autophagy. They found out that while NSP15 expression is associated with reduced number of autophagosomes positive for LC3B, ORF3a, E, M, and ORF7a expression was associated with accumulation of LC3B. Moreover, the authors showed that E, M, ORF3a, and ORF7a inhibit autophagic flux. It is of importance to note that the reduction of autophagosomes for Nsp15 expression was improved following administration of rapamycin, proposing that possibly Nsp15 impacts mTOR axis. While upon E, ORF3a, and ORF7a expression, the values of processed LC3B-II enhanced, Nsp15 expression led to decrease but not substantial in LC3B-II values. In consistent with this finding, ORF3a, ORF7a, E, and Nsp15 expression is associated with higher values of SQSTM1. Noteworthy, while M expression is associated with higher values of processed LC3B, it was not able to prohibit the degradation of SQSTM1, showing that M cannot inhibit autophagy. Immunofluorescence assay demonstrate that although overexpression of ORF3a, E, and ORF7a is associated with higher numbers of LC3B-positive puncta, M expression is associated with elevated LC3B localization. Also, following Nsp15 expression, decrease in number of autophagosomes was observed. Moreover, the authors proposed that the role of SARS-CoV2 proteins including M, ORF3a, ORF7a, and Nsp15 in autophagy is virtually similar to their function in SARS-CoV-1 and bat coronavirus RaTG13 (Hayn et al. 2021; Koepke et al. 2021). A very recent study showed that ORF3a can intensely prohibit autophagic flux by preventing the fusion of autophagosomes with lysosomes (Zhang et al. 2021d). It was shown that ORF3a colocalized with lysosomes and interacted with VPS39, which is a subunit of the homotypic fusion and protein sorting (HOPS) complex. The interaction between VPS39 and ORF3a contributes to inhibition of -HOPS binding to RAB7, which inhibited the assembly of a fusion

machinery, contributing to increase levels of autophagosomes. These findings shed light on the mechanism behind the virus escape degradation, which is disrupting the fusion of autophagosomes with lysosomes (Zhang et al. 2021d). Taken together, the spread of SARS-CoV-2 virus can be limited with using approaches targeting autophagy.

Several drugs, for instance, azithromycin, chloroquine, and hydroxychloroquine, have been considered since these drugs are capable of modulating autophagy signaling pathways (Gao et al. 2020b). The fact that the mentioned medications are able to inhibit endocytic pathway and, thereby, inhibit SARS-CoV2 replication constitute a rational for considering using these drugs in patients who are infected with the virus (Gao et al. 2020b). In clinical settings, inconsistent findings regarding the benefits of these drugs in COVID-19 patients have been reached. Some studies revealed that hydroxychloroquine administration is associated with lower mortality rate in severe COVID-19 patients (Yu et al. 2020; Meo et al. 2020); however, several studies demonstrated that these medications were not able to decrease mortality from infection with SARS-CoV2 (Molina et al. 2020; Singh et al. 2020). Noteworthy, it has been found that these medications are associated with prolonged QT interval, which can lead to cardiac arrhythmia and sudden cardiac death (Chorin et al. 2020; Jankelson et al. 2020). Thus, more investigations are warranted to evaluate the advantageous and disadvantageous of autophagy modulator drugs to limit the virus infection progression. Table 4 lists the effects of viral infection on the regulation of autophagy during some viral diseases.

5 Autophagy Supporting Viral Replication

RNA viruses hijack autophagy for replication. During the autophagy process, DMVs are formed, which maintain a crucial role in poliovirus replication by creating a promising environment for poliovirus replication and keeping polioviruses RNAs away from innate immune receptors recognition and degradation.

Polioviruses, a member of picornavirus family, lack a membrane envelope. Autophagy was shown to be inducer of poliovirus replication, and its inhibition was shown to associated with reduced virus replication (Jackson et al. 2005; Dales et al. 1965). Besides, infection with poliovirus increases the level of LC3 in puncta and expresses two nonstructural poliovirus proteins 2BC and 3A, contributing to lipidation and formation of LC3 and DMVs, respectively, which makes link between the virus replication and autophagy. Similar to polioviruses, foot-and-mouth disease virus and CVB3 exploit autophagy for replication (Berryman et al. 2012; Robinson et al. 2014).

Hepatitis C virus also can trigger autophagy via increasing levels of autophagosomes and using autophagosomal membranes, which is the site for the virus replication (Shrivastava et al. 2011; Dreux and Chisari 2009; Ait-Goughoulte et al. 2008). Nonetheless, the capacity of HCV in stimulating the fusion of lysosome with autophagosomes is still the matter of debate. Several studies have claimed that the virus stimulates autophagosomes and inhibits the autophagosome and lysosome fusion to enhance viral replication and limit virus degradation (Taguwa et al. 2011; Sir et al. 2008a, b). A study stated that HCV enhances the levels of autophagosomes without any change in the levels of autophagy protein degradation, which is (Sir et al. 2008b). Dreux et al. demonstrated that although the autophagy proteins are key components in the translation process of incoming HCV genome, it is not essential for maintenance of the infection (Dreux et al. 2009). However, Ke et al. revealed that the viral replication is totally dependent on the whole autophagic process through complete autolysosome maturation (Ke and Chen 2011b). At early phase of infection with HCV, the interaction between the HCV RNA-dependent RNA polymerase NS5B and ATG5 was observed, which highlights the importance of ATG5 for infection initiation. Blocking ATG5 expression was shown to be associated with the virus replication and maintenance (Guévin et al. 2010).

Table 4 Autophagy and viruses

Virus	Inhibition/ induction	Viral product	Target	Sample	Note	Ref
B19	Induction	–	–	In vitro	B19-infected cells survive by cellular autophagy	Nakashima et al. (2006)
Adenoviruses	Induction	–	–	In vitro	Ad E1a and E1b activate LC3 conversion and Atg12-Atg5 complex formation	Rodriguez-Rocha et al. (2011)
PRRSV	Induction	–	–	In vitro	Autophagy is triggered in pulmonary alveolar macrophages by PRRSV infection	Liu et al. (2012)
HP-PRRSV	Induction	–	–	Bystander cells	Induced apoptosis and autophagy in thymi of infected piglets	Wang et al. (2015b)
Mouse norovirus (MNV)	Induction	–	–	In vitro	MNV infection triggers autophagy in host cells and appears to block the downstream degradation of autophagosomes.	O'Donnell et al. (2016)
Rotavirus	Dysregulation	RV-vsRNA1755	IGF-1R	In vitro	RV-vsRNA1755 targets IGF-1R, blockading the PI3K/Akt pathway and triggering autophagy, but it ultimately inhibits autophagy maturation	Zhou et al. (2018)
Influenza A	Inhibition	Matrix protein 2 (M2)		In vitro	M2 protein blocks autophagosome degradation	Gannagé et al. (2009)
Influenza A	Inhibition	M2	LC3		IAV utilizes a mimicry of host protein short linear motifs (SLiMs) to hijack autophagy	Beale et al. (2014)
HSV-1	Inhibition	ICP34.5	Beclin-1	In vitro	Inhibition of Beclin-1-dependent autophagy	Orvedahl et al. (2007)
Human parainfluenza virus type 3 (HPIV3)	Induction	Matrix protein (M)		In vitro	Viral M protein is sufficient to induce mitophagy by bridging autophagosomes and mitochondria	Ding et al. (2017a)
Zika virus	Induction	NS4A and NS4B		In vitro	Akt-mTOR signaling to inhibit neurogenesis and induce autophagy	Liang et al. (2016)
Coronavirus	Induction	PLP2-TM	Beclin-1	In vitro	PLP2-TM activates autophagosome formation but prevents its fusion with lysosomes	Chen et al. (2014b)

(continued)

Table 4 (continued)

Virus	Inhibition/ induction	Viral product	Target	Sample	Note	Ref
HIV	Inhibition			CD4+ T cells, U937 cells	The autophagy protein Beclin-1, LC3 II and autophagosomes were found to be markedly decreased	Zhou and Spector (2008)
HIV	Inhibition	Nef	Beclin-1	In vitro	Nef acts as an anti-autophagic maturation factor through interactions with the autophagy regulatory factor Beclin-1	Kyei et al. (2009a)
HIV	Inhibition	Nef	Beclin-1	MOLT-4 cells	During permissive infection, Nef binds BECN1 resulting in mammalian target of rapamycin (MTOR) activation, TFEB phosphorylation and cytosolic sequestration, and the inhibition of autophagy	Campbell et al. (2015a)
Dengue virus	Induction	–	–	Mice	Dengue virus induces amphisome and autophagosome formation as well as the autophagic flux in the brain of infected mice	Lee et al. (2013)
Subgroup J avian leukosis virus (ALV-J)	Inhibition	–	–	In vitro		Liu et al. (2013)
Encephalomyocarditis virus	Induction	2C 3D	–	In vitro	2C and 3D were shown to be involved in inducing autophagy by activating the ER stress pathway	Hou et al. (2014c)
HBV	Induction	HBx	Phosphatidylinositol 3-kinase class III	In vitro Mice	Interestingly, in contrast to starvation-induced autophagy, this enhancement of autophagy by HBV does not lead to an increased autophagic protein degradation rate	Sir and Tian (2010)
HBV	Induction	HBx		HepG2.2.15 cells	Novel function of HBx in increasing autophagy through the upregulation of Beclin-1 expression	Tang et al. (2009a)
HBV	Induction	HBx		HepG2 cells	HBx activates the autophagic lysosome pathway in HepG2 cells through the PI3K-Akt-mTOR pathway	Ju et al. (2013)
HBV	Induction	HBx		Chang cell	HBX induces autophagy via activating DAPK in a pathway related to Beclin-1, but not JNK	Zhang et al. (2014)

HBV		HBx		Huh7 cells	HBx-induced autophagosome formation is MTOR inhibition-independentRepressive effect of HBx on lysosomal function is responsible for the inhibition of autophagic degradation, and this may be critical to the development of HBV-associated HCC	Liu et al. (2014)
HBV	Induction	HBV small surface protein		Huh7 cells	SHBs partially co-localized and interacted with autophagy protein LC3	Li et al. (2011a)
HBV	Induction	HBx	–	Huh7 cells	HBV can be promoting autophagy by the interaction of HBx and c-myc to affect miR-192-3p-XIAP, which in turn regulates Beclin-1	Wang et al. (2019e)
Bluetongue virus (BTV)	Induction	–	–	In vitro	The BTV1-induced inhibition of the Akt-TSC2-mTOR pathway and the upregulation of the AMPK-TSC2-mTOR pathway both contributed to autophagy initiation	Utama et al. (2011)
BTV1	Induction	–	–	BSR cells	BTV1-induced inhibition of the Akt-TSC2-mTOR pathway and the upregulation of the AMPK-TSC2-mTOR pathway both contributed to autophagy initiation	To et al. (2020)
HIV	Induction	Env	CXCR4	In vitro	Autophagy is specifically triggered after Env binding to CXCR4, leading to apoptosis	Espert et al. (2006), Espert et al. (2007)
HIV	Inhibition	Vif	Human autophagy-related protein 8 family proteins	In vitro	The C-terminal part of viral infectivity factor interacts with microtubule-associated protein light chain 3	Borel et al. (2015)
HIV	Induction	Tat	BAG3	Human glial cells	Tat protein is able to stimulate autophagy through increasing BAG3 levels in human glial cells	Bruno et al. (2014)
HIV	Inhibition	Tat		Human primary blood macrophages	HIV-1 Tat protein suppressed IFN-g-induced autophagy processes, including LC3B expression HIV-1 Tat suppressed the induction of autophagy-associated genes and inhibited the formation of autophagosomes	Li et al. (2011b)

(continued)

Table 4 (continued)

Virus	Inhibition/ induction	Viral product	Target	Sample	Note	Ref
HCV	Induction		UPR	In vitro	HCV induces the unfolded protein response (UPR), which in turn activates the autophagic pathway	Ke and Chen (2011a)
HCV	Induction	–	–	Huh7	HC V inhibited the AKT-TSC -MTORC1 pathway via ER stress, and the inhibition of the AKT-TSC -MTORC1 pathway contributed to upregulating autophagy	Huang et al. (2013)
HCV	Inhibition	–	–	Monocyte	HCV-positive sera block autophagy during monocyte differentiation LC3 II level increased in monocytes cultured in the presence of HCV-positive sera	Granato et al. (2014)
HCV	–	NS5B	ATG5	Huh7 and C5B cells	HCV utilizes ATG5 as a proviral factor during the onset of viral infection	Guévin et al. (2010)
HBV	Inhibition	HBeHBc		Human ($n = 40$) In vitro	HBe and HBc proteins of HBV activate the mTOR signaling pathway to inhibit autophagy in neutrophils	Hu et al. (2018)
HBV	Induction	HBx				
West Nile virus (WNV)	Induction			In vitro	West Nile Virus-induced LC3 lipidation	Beatman et al. (2012)
Foot-and-mouth disease virus	Inhibition	$3C^{pro}$	ATG5-ATG12	PK-15 cells	FMDV suppresses NF-κB, IRF3, and autophagy by degradation of ATG5-ATG12 via 3Cpro	Fan et al. (2017)
High-risk HPV	Inhibition	–	–	Tissue sample (uterine cervical cancer, $n = 270$)	Persistent HPV infection may stabilize ATAD3A expression to inhibit cell autophagy and apoptosis as well as to increase drug resistance	Chen et al. (2011)
Influenza A virus	Induction	–	–	Cell lines (A549 cells, MDCK, 293T, WT MEFs, and autophagy-deficient MEFs)	IAV infection increased levels of the autophagosomal marker "microtubule-associated protein light chain 3-II" (LC3-II), at early stage of infection	Khalil (2012)
Human bocavirus (HBoV)	Induction	–	–	Human bronchial epithelial cells	Microtubule-associated protein 1A/1B light chain 3 (LC3) II and autophagy protein 5 were increased in HBoV-transfected HBECs, whereas, the mRNA	Deng et al. (2017)

					and protein levels of LC3-I and sequestosome 1 were decreased	
Porcine hemagglutinating encephalomyelitis virus (PHEV)	Induction	–	–	Neuro-2a cells	PHEV infection induces atypical autophagy and causes the appearance of autophagosomes but blocks the fusion with lysosomes	Ding et al. (2017b)
Bovine viral diarrhea virus (BVDV)	Induction	–	–	MDBK cell	BVDV NADL infection triggers autophagosome formation and increases autophagic activities Beclin-1 and ATG14 expression levels were increased as a result of BVDV NADL infection	Fu et al. (2014a)
BVDV	Induction	E^{rns} and E2		MDBK cell	BVDV-NADL infection induced autophagy and significantly elevated the expression levels of autophagy-related genes, Beclin-1 and ATG14, at 12 h postinfection in MDBK cells	Fu et al. (2014b)
Enterovirus 71 (EV71)	Induction	–	Beclin-1	Hep2, vero	EV71 infection resulted in the reduction of cellular miR-30a, which led to the inhibition of Beclin-1, a key autophagy-promoting gene that plays important roles at the early phase of autophagosome formation	Fu et al. (2015)
EV-71	Induction	–	–	Human rhabdomyosarcoma, neuroblastoma cells and in vivo	The specific viral proteins encoded contributed to the inhibition of the mTOR/p70S6K pathway and the induction of autophagy	Huang et al. (2009)
EBV	Induction	–	–	EBV-associated nasal NKTCL ($n = 35$)	Enhanced autophagy and reduced expression of lysosomal enzymes induced regional ACD under EBV infection in natural killer/T-cell lymphomas	Hasui et al. (2011)
Avibirnavirus	Induction	VP2	HSP90AA1	DF-1 cells, 293T cells	HSP90AA1 binding to the viral protein VP2 resulted in induction of autophagy and AKT-mTOR pathway inactivation	Hu et al. (2015)

(continued)

Table 4 (continued)

Virus	Inhibition/ induction	Viral product	Target	Sample	Note	Ref
HCV	Induction	Core protein		QSG-7701	HCV core protein can enhance hepatocytes autophagy through upregulating Beclin-1	Liu et al. (2015b)
HCV	Induction	Core protein		Huh7 hepatoma cell line	Core protein activates autophagy through EIF2AK3 and ATF6 UPR pathway-mediated MAP1LC3B and ATG12 expression	Wang et al. (2014a)
HCV	Induction	–	Class III PI3K-independent pathway	Huh7 hepatoma cell line	–	Sir et al. (2012)
HCV	Induction	NS4B		Huh7.5 cells	Rab5 and Vps34 are involved in NS4B-induced autophagy	Su et al. (2011)
HCV	Induction	–	–	Huh7.5 cells	HCV induces autophagy by upregulating Beclin-1 and activates mTOR signaling pathway	Shrivastava et al. (2012)
HPV16 and 18	Inhibition	–	–	104 cases of cervical cancer tissues	The expression levels of Beclin-1 and LC3B were significantly lower in cervical cancer cells	Wang et al. (2014b)
Flavivirus	Induction	NS4A		Epithelial cells	Expression of *Flavivirus* NS4A is sufficient to induce PI3K-dependent autophagy and to protect cells against death	McLean et al. (2011)
Simian virus 40	Induction	Small T antigen	–	–	The novel role for the SV40 ST antigen in cancers, where it functions to maintain energy homeostasis during glucose deprivation by activating AMPK, inhibiting mTOR, and inducing autophagy as an alternate energy source	Kumar and Rangarajan (2009)
Varicella-zoster virus	Induction	–	–	Human skin vesicle MRC-5 cells	–	Takahashi et al. (2009)
HHV-8	Induction	RTA	–	RTA-inducible BCBL-1cells (TRExBCBL1-RTA)	Autophagy is involved in the lytic reactivation of HHV-8	Wen et al. (2010)
EBV	Induction	LMP1		B cells		Lee and Sugden (2008)
HCMV	Inhibition	TRS1	Beclin-1	MRC5 cells	The Beclin-1-binding domain of TRS1 is essential to inhibit autophagy	Chaumorcel et al. (2012)

HCMV	Induction	–	–	THP-1 cells	HCMV could induce autophagy, and the capacity of promoting autophagy may be weakened in the latent infection	Liu et al. (2017e)
Porcine circovirus type 2 (PCV2)	Induction	–	–	PK-15 cells	PCV2 might induce autophagy via the AMPK/ERK/TSC2/mTOR signaling pathway in the host cells	Zhu et al. (2012)
HSV-1	Inhibition	Us11	PKR	HeLa cells and fibroblasts		Lussignol et al. (2013)
HPV-16	Inhibition	–	–	HaCaT and 293T cells	The HPV-host cell interaction stimulates the PI3K/Akt/mTOR pathway and inhibits autophagy	Surviladze et al. (2013)
HIV-1	Induction	ASP	LC3	U937 andCOS-7 cells		Torresilla et al. (2013)
Influenza A virus	Induction	NS1	–	CV-1 cells	NS1 stimulates autophagy indirectly by upregulating the synthesis of HA and M2	Zhirnov and Klenk (2013a)
EBV	Induction	Rta	–	293T cells	Autophagic activation is caused by the activation of extracellular signal-regulated kinase (ERK) signaling by Rta	Hung et al. (2014)
HTLV-1	Induction	Tax	–	U251 cells	Tax-triggered autophagy depends on the activation of IKKTax can be degraded via manipulation of autophagy and TRAIL-induced apoptosis	Wang et al. (2014)
HTLV-1	Induction	Tax	–	–	Tax induces Bcl-3 expression Bcl-3 acts as a negative regulator of NF-κB activation and promotes autophagy in HTLV-1-infected cells	Wang et al. (2013c)
HTLV-1	Induction	Tax	–	Human astroglioma cells	HTLV-1 Tax protein induces autophagy via IKK in human astroglioma cells: a protective mechanism against death receptor-mediated apoptosis	Zheng et al. (2014)
EBV	Induction	LMP2A	–	MCF10A cells	MP2A may inhibit anoikis and luminal clearance in acini through induction of autophagy	Fotheringham and Raab-Traub (2015)
DENV	Induction	NS1	–	HMEC-1 cells	–	Chen et al. (2016)
HIV-1	Inhibition	–	–	Monocytic cells		Van Grol et al. (2010)

(continued)

Table 4 (continued)

Virus	Inhibition/ induction	Viral product	Target	Sample	Note	Ref
					HIV-1 impairs autophagy in bystander macrophages/monocytic cells through Src-Akt signaling	
Japanese encephalitis virus	Induction	–	–	N2a cells	–	Jin et al. (2013)
Japanese encephalitis virus	Induction	C, M and NS3		BHK-21, PK-15 and N2A cells	–	Wang et al. (2015a)
Coxsackievirus A16	Induction	2C and 3C		HeLa cells	CA16 infection inhibited Akt/mTOR signaling and activated extracellular signal-regulated kinase (ERK) signaling, both of which are necessary for autophagy induction	Shi et al. (2015)
Coxsackievirus B3	Induction	–	–	HeLa cells	CVB3 might directly or indirectly induce autophagy via AMPK/MEK/ERK and Ras/Raf/MEK/ERK signaling pathways in the host cells	Xin et al. (2015)
Coxsackievirus B3	Induction	2B		HeLa cells	56 V in the loop region of 2B is critical for the induction of autophagy	Wu et al. 2016c)
γHV68	Inhibition	vBcl2	Beclin-1	NIH3T3 cells	Viral Bcl-2s displays enhanced anti-autophagic activity than cellular Bcl-2	Liang et al. (2008), Liang et al. (2006)
γHV68	Inhibition	M11	Beclin-1	COS7 cells	M11-Beclin-1 BH3 domain binding is required for autophagy inhibition by M11	Sinha et al. (2008)
Bluetongue virus	Induction			BSR cells	BTV- induced disruption of cellular energy metabolism contributes to autophagy	(Lv et al. (2016)
Influenza A virus H5N1	Induction		mTOR signaling	MEF cells	H5N1 causes autophagic cell death through suppression of mTOR signaling	Ma et al. (2011a)
Influenza A virus H5N1	Induction			Mouse lungs and human A549 cells	Autophagy induced by live H5N1 virus in human A549 cells depends on signaling through the Akt-TSC2-mTOR pathway The hemagglutinin protein of H5N1 virus may induce autophagy in A549 cells	Sun et al. (2012)

Newcastle disease virus	Induction			U251 cells		Meng et al. (2012a), Kang et al. (2017)
Newcastle disease virus	Induction	NP and P proteins		A549 cells	NDV NP and P proteins induced autophagy through activation of the ER stress-related UPR pathway	Cheng et al. (2016)
Avian reovirus	Induction	–	–	Chicken fibroblast cells and vero cells	The class I PI3K/Akt/mTOR pathway contributes to ARV-triggered autophagy	Meng et al. (2012b)
RABV GD-SH-01	Induction	–	–	Human and mouse neuroblastoma cell lines	Autophagy is induced by GD-SH-01 and can decrease apoptosis in vitro. Furthermore, the M gene of GD-SH-01 may cooperatively induce autophagy	Peng et al. (2016)
HSV-1 HSV-2	Induction	–	–	SIRC cell line		Petrovski et al. (2014)
Enterovirus 71 (EV71)	Induction	–	–	Suckling mouse model	EV71 infection can induce autophagosome, amphisome and autolysosome formation, and the structural protein VP1 and nonstructural protein 2C of EV71 were distributed around the autophagosome and amphisome	Lee et al. (2014)
Pseudorabies virus	Inhibition	–	–	PK-15 cells	Alphaherpesvirus US3 tegument protein may reduce the level of autophagy via activation of the AKT/mTOR pathways in PRV infected cells	Sun et al. (2017c)
Zika virus (ZIKV)	Induction	–	–	Human umbilical vein endothelial cells (HUVEC)	–	Peng et al. (2018)
HIV-1 HIV-2	Induction	–	–	Jurkat and CD4+ T cells	HIV is able to induce the autophagic signaling pathway in HIV-infected host cells, which may be required for HIV infection-mediated apoptotic cell death	Wang et al. (2012)
Sendai virus (HVJ-E)	Induction	–	–	NSCLC cells	HVJ-E could induce autophagy in NSCLC cells via the PI3K/Akt/mTOR/p70S6K signaling pathway	Zhang et al. (2015b)
Rotavirus (RV)	Induction	–	–	HT29 cells, MA104 cells	RV infection can be activating autophagy machinery during RV infection through upregulation and downregulation of miR-99b and let-7g expression levels, respectively	Mukhopadhyay et al. (2019)

HCV dynamically modulates autophagy by expressing ultraviolet radiation resistance-associated gene protein (UVRAG) and Rubicon to increase its replication (Wang et al. 2015c). At the early stages of viral infection, upregulation and downregulation of Rubicon and UVRAG, respectively, by the virus inhibit the autophagosomes maturation and thereby increase the levels of autophagosomes, leading to virus replication (Wang et al. 2015c). Additionally, immunity-related GTPase family M protein (IRGM), an IFN-inducer GTPase, was shown to be able to modulate autophagy process by interacting with several autophagic proteins (Grégoire et al. 2011b). Hansen et al. showed that IRGM by promoting autophagy and Golgi fragmentation induces the virus replication. IRGM stimulates Golgi fragmentation via modulation of Golgi apparatus-specific brefeldin A-resistant guanine nucleotide exchange factor 1 (GBF1) and AMPKα (Hansen et al. 2017). In summary, HCV is able to regulate autophagy process to induce the virus replication.

According to findings of related studies, it can be concluded that flaviviruses take benefits from the close connection between ER and autophagy processes. At first, it was believed that stimulation of autophagy in those infected with flaviviruses is only related to the ER stress-related UPR signaling pathway. On the other hand, it was shown that several nonstructural proteins of West Nile virus (WNV) and DENV are able to stimulate autophagy irrespective of the UPR (Blázquez et al. 2014; Miller et al. 2007). Analyses of neural progenitor cells infected with Zika virus (ZIKV) disclosed that the infection causes a huge remodeling of ER and, moreover, vesicular packet formation, which are assumed to be the spots of ZIKV replication (Offerdahl et al. 2017; Cortese et al. 2017). Infection of skin fibroblast is associated with autophagosomes formation, leading to higher levels of ZIKV replication (Hamel et al. 2015). Furthermore, enhancement in lapidated form of LC3 along with decrement in ATG16L1 expression, a vital autophagy gene, in placentae infected with ZIVK, indicates the fact that autophagy plays a crucial role in vertical transmission of ZIKV (Cao et al. 2017). Liang et al. clarified the mechanism responsible for fetal neurological defects causing by ZIKV. They found out that two proteins exist in ZIKV, namely, NS4A and NS4B, in cooperation with each other inhibit the Akt-mTOR signaling pathway, which contributes to autophagy activation and defective neurogenesis (Liang et al. 2016). Upon early phase of ZIKV and DENV infection, inhibition of FAM134B, which acts as an autophagy receptor, enhances the virus replication. The viruses use their NS3 protease to cleave FAM134B, leading to limit ER and autophagosomes formation (Khaminets et al. 2015; Lennemann and Coyne 2017).

It was shown that two HIV proteins Gag and Nef modulate the autophagy process through interacting with LC3 and Beclin-1, which, finally, causes higher viral replication. During early phase of autophagy, Gag protein interacts with C3, which leads to higher levels of Gag processing and HIV levels in macrophages (Kyei et al. 2009b). Also, during the maturation stage of autophagy, Nef protein of HIV inhibits autophagy maturation via binding to Beclin-1 and, thereby, keeps the virus safe from degradation. Thus, the interaction between the virus and autophagy increases HIV load and replication through inducing early-stage autophagy but prohibits late stages (Kyei et al. 2009b). Nevertheless, it has been detected that during permissive infection, the virus inhibits autophagy so as to prevent the degradation of proteolytic. In the normal situation, mTOR by phosphorylating transcription factor EB (TFEB) limits TFEB translocation. TFEB is able to induce autophagy and lysosomal activation when it transfers to the nucleus. In doing so, TFEB should become dephosphorylated, which is dependent upon mTOR inhibition. For stimulating autophagy within macrophages infected with HIV, the interaction between TLR8 and HIV should be occurred, which is dependent on the dephosphorylation and nuclear translocation of TFEB. The authors also observed that during permissive infection, the interplay between Nef and Beclin-1 contributed to phosphorylation of TFEB, mTOR activation, cytosolic sequestration, and, thereby, autophagy inhibition (Campbell et al. 2015b).

A number of experimental studies have declared that autophagy inhibition causes

prohibition of HBV replication, which represents the fundamental role of autophagy in HBV life cycle (Table 3). The studies have utilized cells that were infected with HBV, or transfected with HBV, or exhibiting HBV DNA replication. It was found out by Sir and his colleagues that triggering autophagy by HBV is dependent on the presence of HBx, which increases its activity through binding to PI3KC3. Therefore, autophagy along with PI3KC3 modulates the majority of HBx impacts on HBV replication (Sir et al. 2010a, b). Either inhibition of PI3KC3 or Atg7 contributes to decrease in HBV replication (Sir et al. 2010b). A study found out that autophagy inhibition decreases pgRNA packaging and HBV RNA values to some extent while inhibited HBV DNA replication remarkably (Tang et al. 2009b). Therefore, it can be concluded that this phenomenon indicates that autophagy exerts its effects on HBV replication mainly at the viral DNA replication stage of the viral life (Sir et al. 2010b). Another study similarly found positive effects of autophagy on HBV replication; however, the effects were mostly seen at the stage of envelopment (Rautou et al. 2010). Li et al. designed a study to evaluate the association between autophagy and HBV by suppressing autophagy using 3-methyladenine and siRNA duplexes that suppress fundamental genes need for autophagosome formation. The investigators explored that autophagy inhibition is able to suppress the virus replication notably and stimulating autophagy using starvation and/or rapamycin increases the virus replication (Li et al. 2011c). These inconsistent findings can be explained by using different HBV strains or sublines of Huh7 cells in the relevant studies. Also, a study unveiled ROS HBV capsid assembly in the existence of Hsp90; however, it was observed that ROS without Hsp90 decreases the virus assembly (Kim et al. 2015). Another pathway responsible for HBV-induced autophagy is ROS/JNK signaling pathway. In doing so, ROS/JNK signaling pathway modulates the interaction between Beclin-1 and Bcl-2, which is crucial for activation of autophagy (Zhong et al. 2017). Additionally, it has been shown that HBV has the potential to favor its replication by subverting autophagy Atg5-12/16L1 complex, without any need for Atg8/LC3 lipidation, which is a vital process for autophagosomes maturation (Döring et al. 2018). At the same time, several studies have claimed that autophagy triggered by HBV inhibits the virus replication. Wu et al. demonstrated that autophagy following infection with HBV is able to degrade envelope proteins (Wu et al. 2016d). For the first time, Lazar et al. demonstrated that HBV decreases the level of envelope protein through that the ERAD signaling pathway. Simultaneous expression of the virus envelope proteins and EDEM1 caused huge envelope protein degradation, which was blocked through EDEM1 inhibition (Lazar et al. 2012). Furthermore, a study revealed that AMPK activation is able to limit the virus production by inducing autophagy, suggesting the therapeutic value of targeting AMPK for HBV management (Xie et al. 2016). Collectively, it can be said that the precise relationship between the virus replication and autophagy merits extra studies.

Also, infection with influenza A virus (IAV) is able to induce enhanced levels of autophagosomes that needed for viral replication (Zhou et al. 2009). A study showed that the virus increases the levels of autophagosomes by inhibition of their fusion with lysosomes, and the presence of matrix 2 (M2) ion-channel protein for prohibition of autophagosomes degradation is pivotal (Gannagé et al. 2009). Another research displayed that M2 escapes from autophagy using its LC3-interacting region (Beale et al. 2014). M2 interacts with LC3 and induces LC3 re-localization to the plasma membrane, and disruption of this interaction downregulates virion budding and stability. The NS1 is another IVA protein that induces autophagy via overexpression of M2 and hemagglutinin (HA) (Zhirnov and Klenk 2013b). Recently, the interplay between M2 protein and MAVS signaling pathway was demonstrated, which leads to MAVS aggregation and, thereby, stimulates MAVS-mediated antiviral innate immunity. Furthermore, it was shown that M2 triggers ROS generation, which is a crucial factor for autophagy activation (Wang et al. 2019f). Additionally, H5N1, a major avian pathogen, has the potential

to induce autophagy via prohibiting mTOR (Ma et al. 2011b).

As we mentioned before, Beclin-1 is a fundamental modifier of autophagy process that forms two distinct complexes, one with Atg14 that is needed for autophagosome formation and the other with UVRAG, which is essential for autophagosome maturation (Levine et al. 2015). In a study that was conducted by Qu and his associates, it was demonstrated that infection with SARS-CoV-2 is associated with incomplete autophagy response, which was shown to be needed for effective virus replication. Moreover, the investigators disclosed that although infection with SARS-CoV-2 stimulates autophagosomes formation, the infection contributes to prohibition of autophagosome maturation and block autophagy by inhibiting fundamental genes involved in the virus replication (Qu et al. 2021). They analyzed expression of 26 proteins expressing by the virus and found out that ORF3a expression is associated with incomplete autophagy. The ORF3a interplays with UVRAG to promote and prohibit expression of PI3KC3-C1 (Beclin-1-Vps34-Atg14) and PI3KC3-C2 (Beclin-1-Vps34-UVRAG), respectively. In summary, the authors shed light on how ORF3a inhibits autophagy and, thereby, prompts SARS-CoV-2 replication, which provides a therapeutic potential of targeting autophagy for COVID-19 treatment (Qu et al. 2021).

6 Conclusion

Autophagy is known as conserved intracellular process which transfers cytoplasmic materials to lysosomes for degradation though autophagosomes. This process emerges to be relevant to the pathogenesis of various diseases, and its regulation could have therapeutic value. It has been indicated that a sequence of cellular and molecular signaling pathways by several internal and external factors is involved in initiation and progression of autophagy. Viruses are one of main factors which exert their pathogenesis effects via affecting on autophagy processes. Besides viruses, a wide range of internal factors including genetic and epigenetic factors could influence on underlying pathways involved in autophagy processes. Very recently, microRNAs and exosomes have been emerged as critical players in the autophagy processes, given that exosomes and microRNAs are able to change behavior of host cells via targeting of a large number of cellular and molecular signaling pathways. Hence, more insights into the various signaling pathways that are targeted by exosomes and microRNAs could pave the way to the finding and designing new therapeutic approaches.

Conflicts of Interest The authors have declared that no competing interest exists.

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Epitranscriptomics Changes the Play: m^{6}A RNA Modifications in Apoptosis

Azime Akçaöz and Bünyamin Akgül

Abstract

Apoptosis is a form of programmed cell death that is essential for cellular and organismal homeostasis. Any irregularities that disturb the balance between apoptosis and cell survival have severe implications, such as improper development or life-threatening diseases. Thus, it is highly critical to maintain a proper rate of apoptosis throughout development. In fact, several complex transcriptional and posttranscriptional mechanisms exist in eukaryotes to critically regulate the rate of apoptotic processes. Recent studies suggest that not only RNA sequences but also their modifications, such as m^{6}A methylation, play a fundamental role in these transcriptional and posttranscriptional processes. A specific set of proteins, called writer, eraser, and reader of m^{6}A marks, modulate the rate of apoptosis by determining the m^{6}A repertoire and the fate of certain transcripts associated with apoptosis. In this *Review*, we will cover the dynamic m^{6}A RNA modifications and their impact on modulation of apoptosis.

Keywords

Apoptosis · Epitranscriptomics · m^{6}A RNA modification

Abbreviations

ac^{4}C	N4-acetylcytidine
circRNA	Circular RNA
CP	Cisplatin
DISC	Death including signaling complex
Fas-L	Fas ligand
hm5C	Hydroxymethylcytosine
lncRNA	Long noncoding RNA
m^{1}A	N1-methyladenosine
m^{5}C	5-methylcytosine
m^{6}A	N6-Methyladenosine
m^{6}Am	N6,2′-O-dimethyladenosine
m^{7}G	7-methylguanosine
miRNA	microRNA
ncRNA	Noncoding RNA
TGCT	Testicular germ cell tumors
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Y	Pseudouridine

1 Introduction

Deoxyribonucleic acids (DNA) and ribonucleic acids (RNA) were thought to harbor solely canonical nucleotides until the detection of the first

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distinct chemical moiety, deoxy-5-methylcytosine, on DNA in 1948 (Hotchkiss 1948). Subsequently, similar modifications were also reported to exist on RNAs, such as 5-ribosyluracil (Davis and Allen 1957), pseudouridine (Y) (Cohn 1960), 2′-O-methylribose (Rabczenko and Shugar 1971), 5-methylribouridine and 5-methylribocytosine (Grosjean 2005). This novel and exciting area of RNA modification, called epitranscriptomics, refers to altered chemical structure of RNA without any change in the ribonucleotide sequence and implies extensive regulatory effects on various layers of gene expression (Saletore et al. 2012). RNA modifications were first reported to exist on ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs). Ensuing advances in detection methods have led to the discovery of other types of RNA modifications on mRNAs and noncoding RNAs (ncRNAs) (Motorin and Helm 2011).

Currently, almost 170 modifications have emerged in all RNA types (Wiener and Schwartz 2021). At least, thirteen different chemical modifications have been reported to exist on mRNAs, which include 7-methylguanosine (m^7G), 2′-O-methylated at the ribose (cOMe), N6,2′-O-dimethyladenosine (m^6Am), N6-methyladenosine (m^6A), 5-methylcytosine (m^5C), pseudouridine (Ψ), N1-methyladenosine (m^1A), N4-acetylcytidine (ac^4C), hydroxymethylcytosine (hm5C), 3-methylcytidine (m^3C), cytosine to uridine (C to U) editing, m^7G, Nm, and 7,8-dihydro-8-oxoguanosine (Nachtergaele and He 2018; Anreiter et al. 2021). The type and location of modification appear to modulate different aspects of RNA processing, such as mRNA abundance (Jia et al. 2011), splicing (Xiao et al. 2016), export (Zheng et al. 2013), stability (Huang et al. 2018), and translation (Zhou et al. 2015). Elucidation of epitranscriptomics processes should have implications in furthering our understanding of biological processes such as cell cycle, proliferation, development, and cell death (Zhu et al. 2020). In fact, the existing evidence clearly suggests that RNA modifications modulate various types of cell death, including apoptosis (Lin et al. 2019; Vu et al. 2017; Wang et al. 2020). In this *Review*, we will cover a succinct description of apoptosis followed by a detailed discussion on RNA m^6A marks and their impact on apoptotic processes.

2 Apoptosis

Apoptosis is described as a mode of programmed cell death characterized by a series of biochemical and morphological features resulting in elimination of excess cells (Elmore 2007). Initial morphological hallmarks of apoptosis include condensation of the chromatin as well as shrinkage of the cell followed by fragmentation of the condensed nucleus. The process is then followed by detachment of the cell from the surrounding environment and formation of cytoplasmic blebs (Kerr and Wyllie 1972). Throughout the apoptotic process, cellular organelles maintain their compact structures. Subsequently, the cell fragmentation results in the formation of apoptotic bodies, which are bound to the plasma membrane and include fragmented nuclear materials, packed organelles, and condensed cytoplasm (Kurosaka et al. 2003). Phagocytosis of apoptotic bodies is carried out by neighboring cells, macrophages, and parenchymal cells. The maintenance of membrane integrity ensures that all of these events are completed without causing any inflammation (Saraste and Pulkki 2000; Kurosaka et al. 2003). Characteristic biochemical features of apoptosis also include the fragmentation of DNA and proteins by caspases, cysteine proteases (Saraste and Pulkki 2000).

There are two canonical pathways of apoptosis: (1) intrinsic and (2) extrinsic (Julien and Wells 2017). The intrinsic pathway can be triggered by many extra- or intracellular stimuli such as toxins, radiation, oxidative stress, or treatment with chemotherapeutic agents (Sivamani and Kar 2015; Pistritto et al. 2016). It is mediated by mitochondria and results in activation of caspase -9, -3, -6 and -7 (Jan and Chaudhry 2019; Xu and Shi 2007). However, death receptors and their ligands are involved in activation of the extrinsic apoptotic pathway. The binding of extracellular ligands such as tumor necrosis factor (TNF) alpha, Fas ligand (Fas-L) or TNF-related apoptosis-inducing ligand (TRAIL) to their receptors

activates death including signaling complex (DISC) and activates caspase -8, -10, -3, -6 and -7 (Sivamani and Kar 2015; Carneiro and El-Deiry 2020). Both pathways terminate with the activation of effector caspases, which degrade all nuclear materials by stimulating endonucleases and proteases (Elmore 2007). Other than the intrinsic and extrinsic pathways, a perforin/granzyme pathway is triggered by cytotoxic T lymphocytes and natural killer cells to eliminate infected cells (Igney and Krammer 2002; Nirmala and Lopus 2020). Perforin released by immune cells disrupts the membrane of the target cells while granzymes facilitate DNA fragmentation in a caspase-independent manner.

Apoptosis plays a critical role in the maintenance of organismal homeostasis. Consequently, apoptosis must be tightly regulated as part of cellular hemostasis, development, and elimination of pathogens or diseases. The existing evidence clearly documents the significance of transcriptional regulatory mechanisms that target proapoptotic and antiapoptotic proteins (Budhidarmo and Day 2015; Hotchkiss et al. 2009). Different stages of apoptosis can also be regulated by posttranscriptional mechanisms (Guttman and Rinn 2012). We have reported the contribution to this process of microRNAs (miRNAs) and circular RNAs (circRNAs) (Erdoğan et al. 2018; Tuncel et al. 2021; Yaylak et al. 2019). Recent studies suggest that both transcriptional and posttranscriptional regulatory mechanisms may be modulated by RNA modifications (Zhao et al. 2016). Especially, m^{6}A modifications have been documented to regulate apoptotic mechanisms through a diverse array of regulatory factors (Huang et al. 2019; Liu et al. 2018a, b, 2019; Vu et al. 2017; Wei et al. 2019; Xu et al. 2019).

3 m^{6}A RNA Modification

m^{6}A RNA modification is a highly dynamic and reversible process that forms the basis of gene regulation by m^{6}A RNA methylation. The first example of m^{6}A RNA modification on eukaryotic mRNAs was reported in 1970s (Desrosiers et al. 1974; Perry and Kelley 1974). Of numerous RNA chemical marks, m^{6}A methylation constitutes 80% of all mRNA modifications. Adenosine residing in a highly conserved consensus sequence, called the DRACH motif ([G / A / U] [G> A] m^{6}AC [U> A> C], undergoes m^{6}A methylation upon the reception of cellular signals that activate a special set of methylation enzymes (Niu et al. 2013). m^{6}A marks may be distributed throughout the body of the transcript. The abundance of m^{6}A-methylated residues is attained by a combinatorial effect of methyltransferases (writers) and demethyltransferases (erasers) (Fig. 1). The impact of m^{6}A modification is then dictated by reader proteins that recognize the m^{6}A site and probably recruit other proteins that dictate the fate of the transcript (Huang and Yin 2018).

3.1 Deposition of m^{6}A Modification

A multiprotein writer complex carries out the m^{6}A methylation of the transcriptome in a highly specific manner. The core complex consists of METTL3, METTL14, and WTAP, and other writer proteins (Table 1) have been reported to interact with the core complex. METTL3 has a consensus methylation motif-I that contains an Adomet binding site and a consensus motif-II that harbors the catalytic domain (Bokar et al. 1996). In fact, METTL3 is the only enzyme with a catalytic site in the core complex. Although METTL14 possesses a methyltransferase domain, it contributes to m^{6}A methylation merely by enhancing the catalysis in the presence of METTL3. METTL14 also helps change the local RNA structure to increase its binding efficiency with the writer complex (Liu et al. 2014). WTAP, on the other hand, stabilizes the core complex by interacting with METTL3-METTL14 and dictates the localization of the core complex (Ping et al. 2014). RBM15, VIRMA, and HAKAI are responsible for recruiting the core writer complex to specific RNA sites for selective methylation (Bawankar et al. 2021; Liu et al. 2018a, b; Ortega et al. 2003; Patil et al. 2016; Wen et al. 2018).

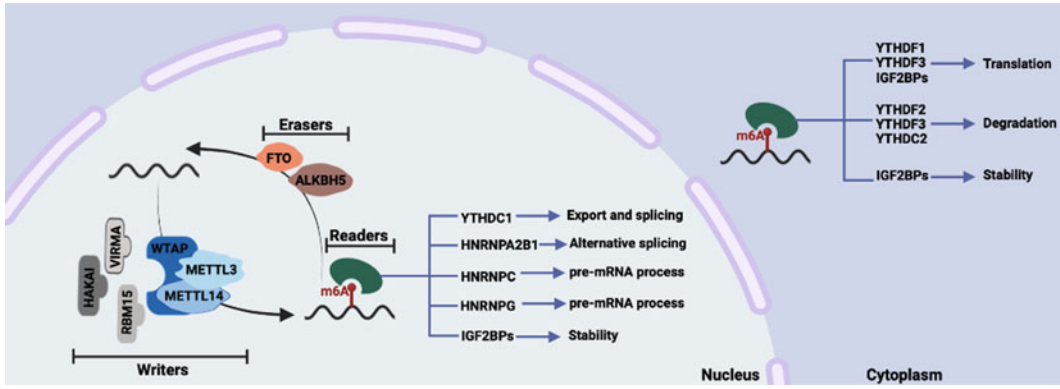

Fig. 1 Mechanism of m^{6}A modification

Table 1 The function of writers, erasers, and readers

	Name	Function	References
Writers	METTL3	Catalysis of m^{6}A methylation on adenosine	Bokar et al. (1996)
	METTL14	Boosts the catalytic activity by stabilizing the interaction between substrates and the writer complex	Liu et al. (2014)
	WTAP	Interacts with and stabilizes METTL3-METTL14	Ping et al. (2014)
	RBM15	Recruits the writer complex via its RNA-binding domains and methylates target mRNAs	Patil et al. (2016)
	VIRMA	Guides the core complex to specific RNA sites	Ortega et al. (2003)
	HAKAI	Interacts with WTAP for its target mRNAs	Bawankar et al. (2021)
Erasers	FTO	Removes m^{6}A marks in two steps via oxidation	Jia et al. (2011)
	ALKBH5	Directly removes the methyl group without oxidative demethylation	Zheng et al. (2013)
Readers	YTHDF1	Promotes translation	Wang et al. (2015)
	YTHDF2	Regulates RNA stability	Du et al. (2016)
	YTHDF3	Role in translation and mRNA decay	Shi et al. (2017)
	YTHDC1	Responsible for mRNA export and splicing	Roundtree et al. (2017)
	YTHDC2	Reduces mRNA stability	Mao et al. (2019)
	HNRNPA2B1	Modulates alternative splicing	Alarcón et al. (2015)
	HNRNPC	Role in pre-mRNA processing	Liu et al. (2015)
	HNRNPG	Role in pre-mRNA processing	Liu et al. (2015)
	IGF2BP-1/2/3	Enhances mRNA stability	Huang et al. (2018)

Erasers are RNA demethyltransferases that remove m^{6}A residues from RNAs. FTO, which is localized both in the nucleus and the cytoplasm, has been identified as the first demethylase that eliminates the methyl group in a two-step reaction that involves oxidation (Jia et al. 2011). Subsequently, ALKBH5 was reported as an eraser protein that directly discards the m^{6}A residue without oxidative demethylation (Zheng et al. 2013). Interestingly, ALKBH5 is enriched in the nuclear speckles. Although ALKBH5 plays a role in gene expression primarily by regulating splicing and the nucleocytoplasmic export of RNAs (Zheng et al. 2013), FTO appears to function in mRNA splicing, cell differentiation, and other gene regulatory processes (Jia et al. 2011).

3.2 Fates of m^{6}A Methylated RNAs

A special set of proteins, called reader proteins, dictate the fate of m^{6}A-methylated RNAs by

directly or indirectly interacting with m^{6}A marks (Fig. 1). YT521-B-homology-(YTH)-domain-containing proteins (YTHDF1, YTHDF2, YTHDF3, YTHDC1, and YTHDC2) recognize and bind preferentially to the m^{6}A residues (Wang et al. 2014). There appears to be a specificity between the type of reader protein and its effect on the mRNA fate. For example, YTHDF1 and YTHDF3 enhance the translational efficiency of m^{6}A-methylated RNAs (Li et al. 2017; Shi et al. 2017; Wang et al. 2014). On the other hand, YTHDF2 and YTHDC2 appear to modulate the stability of m^{6}A-methylated RNAs by recruiting the RNA degradation machinery (Du et al. 2016; Mao et al. 2019). YTHDF3 has a dual function in that it cooperates with YTHDF1 and YTHDF2 to modulate translation and mRNA degradation, respectively (Li et al. 2017; Shi et al. 2017). YTHDC1 is a reader protein primarily involved in the splicing and nucleocytoplasmic transport of mRNAs (Xiao et al. 2016). The reader proteins of the heterogenous nuclear ribonucleoprotein (HNRNP) family recognize m^{6}A-methylated mRNAs via alteration of the mRNA structure. HNRNPA2B1 modulates alternative splicing and processing of primary miRNAs (Alarcón et al. 2015; Wu et al. 2018). HNRNPC and HNRNPG have a role in pre-mRNA processing (Liu et al. 2015). In addition, insulin-like growth factor 2 binding proteins (IGF2BPs) strengthen the stability and enhance the translation efficiency of transcripts with m^{6}A marks (Huang et al. 2018). In brief, m^{6}A residues possess the capability of dictating multiple fates of mRNAs, such as stability, translation, alternative splicing, and degradation. By doing so, m^{6}A methylation has the potential to modulate a variety of biological processes, such as apoptosis.

4 m^{6}A Modifications in Apoptosis

Recent years have witnessed a significant progress in the regulation of apoptosis by the m^{6}A methylation machinery. Initial efforts have been geared toward understanding the relationship between apoptosis and m^{6}A modifications particularly from the perspective of cancer (An and Duan 2022). As expectedly, apoptotic stimuli could activate or inactivate methyltransferases or demethylases targeting either pro-apoptotic or anti-apoptotic transcripts, resulting in the spatial or temporal regulation of apoptotic pathways. In turn, transcript-specific recognition of m^{6}A marks by readers modulates a series of molecular effects such as enhanced stability, degradation, or translation efficiency of pro- and anti-apoptotic transcripts (Fig. 2).

The importance of m^{6}A RNA methylation was first documented in HepG2 cells, in which *METTL3* knockdown led to apoptosis (Dominissini et al. 2012). Congruently, *METTL3* overexpression was reported to reduce the rate of apoptosis through the elevated m^{6}A methylation of *PTEN*, *BCL2,* and *MYC*, whose translation efficiency is enhanced upon methylation in human hematopoietic stem/progenitor cells (HSPCs) (Vu et al. 2017). *METTL3*-mediated modulation of apoptosis was shown in testicular germ cell tumors (TGCT) in which *METTL3* upregulation decreased cisplatin (CP) sensitivity by targeting ATG5 (Chen et al. 2021). ATG5 promotes autophagy by inhibiting apoptosis, leading to chemoresistance. Similarly, *TFAP2C* m^{6}A methylation enhances the stability of the transcript, resulting in enhanced cell viability under CP treatment conditions in seminoma (Wei et al. 2020). m^{6}A modification of *BCL2* under CP and TNF-alpha treatment conditions positively regulates its mRNA stability. Increased BCL2 in turn blocks apoptosis and enhances invasion in TGCT and temporomandibular joint osteoarthritis (Peng et al. 2021; He et al. 2022). Interestingly, METTL3 and ALKBH5 inversely modulate the m^{6}A RNA methylation of the *TFEB* transcript in hypoxia/reoxygenation-treated mouse cardiomyocytes (Song et al. 2019). *TFEB* transcripts m^{6}A-methylated in their 3′ untranslated regions (UTRs) exhibit a lower stability, leading to an increase in the rate of apoptosis in cardiomyocytes. Modulation of apoptosis is not limited to the METTL3 component of the core writer complex. For example, METTL14 silencing reduces the apoptotic rate via m^{6}A RNA methylation of miR-375 that in turn inactivates the mTOR

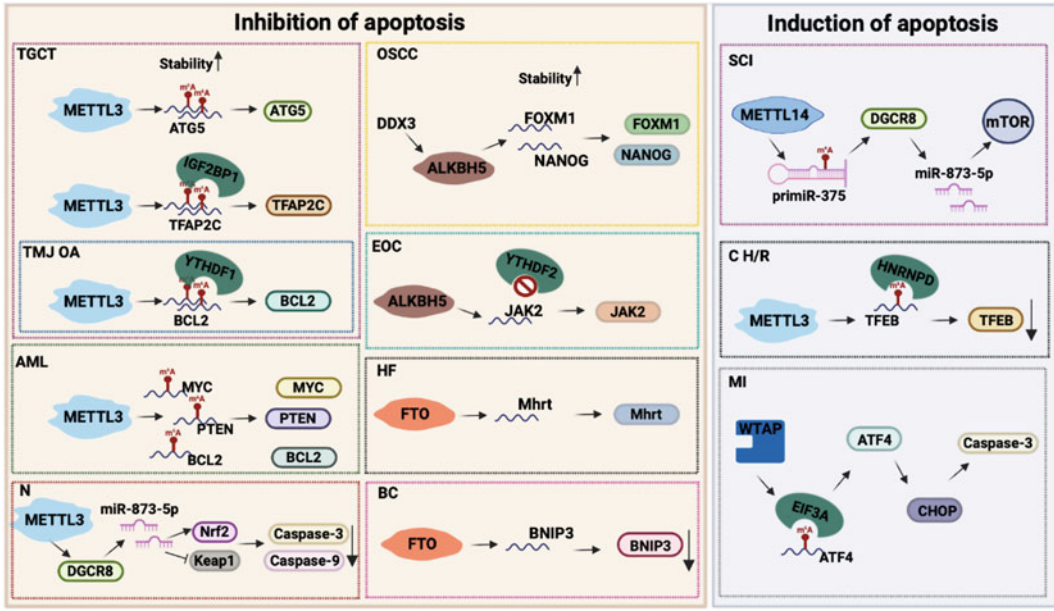

Fig. 2 m^{6}A-mediated regulatory mechanisms in apoptosis. *TGCT* testicular germ cell tumor, *TMJ OA* temporomandibular joint osteoarthritis, *AML* acute myeloid leukemia, *N* nephrotoxicity, *OSCC* oral squamous cell carcinoma, *EOC* epithelial ovarian cancer, *HF* heart failure, *BC* breast cancer, *SCI* spinal cord injury, *C H/R* cardiomyocyte treated with hypoxia/reoxygenation, *MI* myocardial infarction

pathway in the spinal cord injury (Wang et al. 2021a, b). Additionally, WTAP was reported to facilitate m^{6}A modification on *ATF4* in myocardial infarction (Wang et al. 2021a, b). Consequently, enhanced translation of the *ATF4* transcript promotes the ER stress and apoptosis in myocardial infarction. microRNAs can be targeted by the m^{6}A RNA methylation machinery as well as mRNAs. For example, METL3-mediated modification of miR-873-5p leads to downregulation of caspase -3 and -9 via Keap1 and Nrf2 in mouse renal tubular epithelial cells (Wang et al. 2019).

The dynamic nature of m^{6}A marks on pro- or antiapoptotic RNAs may be modulated by erasers through demethylation of these transcripts and thereby controlling the rate of apoptosis. For example, the DDX3-ALKBH5 axis removes the m^{6}A residues from *FOXM1* and *NANOG* and reduces the rate of apoptosis in head and oral squamous cell carcinoma by enhancing their translation rates (Shriwas et al. 2020). ALKBH5 was also reported to function in a loop with HOXA10 to facilitate chemoresistance in epithelial ovarian cancer cells (Nie et al. 2021). ALKBH5-mediated demethylation of *JAK2* stabilizes the transcript and inhibits apoptosis, leading to chemoresistance to cisplatin. FTO is another eraser whose overexpression downregulates apoptosis by inducing the m^{6}A modification of *MHRT* in myocardial cells (Shen et al. 2021). FTO also eliminates the m^{6}A marks from *BNIP3* in breast cancer cells, and downregulation of BNIP3 results in the blockage of apoptosis (Niu et al. 2019).

There exists strong evidence for the critical role of writers, erasers, and readers on regulation of apoptosis through the differential m^{6}A methylation of specific transcripts. However, the extent of m^{6}A methylation under apoptotic conditions is unknown. Recently, we employed cisplatin as a universal inducer of apoptosis to examine the scope of m^{6}A methylation through m^{6}A miCLIP-seq analysis in HeLa cells (Akcaoz et al. 2022). Our analyses revealed that a total of 972 transcripts are subjected to differentially m^{6}A methylation in HeLa cells. Interestingly, 132 mRNAs associated with apoptosis are

targeted for m^{6}A methylation under cisplatin-induced apoptosis. Further analyses have uncovered a METTL3-p53-NOXA axis that might be important in regulating the intrinsic apoptotic pathway in HeLa cells.

In conclusion, the m^{6}A methylation machinery, including writers, erasers, and readers play an essential role in orchestrating the apoptotic processes through m^{6}A methylation of various transcripts (Fig. 2). Writers and erasers determine the abundance and location of m^{6}A residues on transcripts while readers recognize these m^{6}A marks and dictate the fate of transcripts. One of the pressing questions in the field is what determines the pathway-specific induction of apoptosis and whether different mechanisms function in a pathway-specific manner. Yet another interesting question is how the m^{6}A methylation machinery communicates with the apoptotic pathways that results in a pathway- or cell-specific response under apoptotic conditions. The existing information suggests that readers might play a pivotal role in serving as a link between the m^{6}A machinery and apoptotic pathways. Uncovering these mechanisms shall certainly pave the way for discovering novel targets that can be exploited both in basic and translational research.

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

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The Fingerprints of Biomedical Science in Internal Medicine

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Abstract

With the development of numerous advances in science and technologies, medical science has also been updated. Internal medicine is one of the most valuable specialized fields of medical sciences that review a broad range of diseases. Herein, the internal medicine specialist (internist) is obliged to do diagnostic measures to evaluate disease signs and symptoms. In recent times, biomedical sciences as the new emergence science (including cellular and molecular biology, genetics, nanobiotechnology, bioinformatics, biochemistry, etc.) have been capable of providing more specific diagnostic methods together with techniques for better understanding the mechanism of the disease and the best diseases modeling and offering proper therapies. Accordingly, the authors have tried to review the link between biomedical sciences and medicine, particularly internal medicine.

Keywords

Advanced technology · Biomedical research · Internal medicine · Medical informatics · Molecular diagnostic techniques · Regenerative medicine

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Abbreviations

CAR T cell	Chimeric antigen receptor T cells
CFTR	Cystic fibrosis transmembrane conductance regulator

CNB	Core needle biopsy
CT	Computed tomography
DNA	Deoxyribonucleic acid
EBUS	Endobronchial ultrasound
ECG	Electrocardiography
ELISA	Enzyme-linked immunosorbent assay
EMBL	The European Bioinformatics Institute
EMG	Electromyography
FNA	Fine needle aspiration
GGBN	Global Genome Biodiversity Network
GI	Gastrointestinal
GWAS	Genome-wide association studies
HRT	Hormone replacement therapy
IBD	Inflammatory bowel disease
IL	Interleukin
KNB	Knowledge Network for Biocomplexity
MR	Magnetic resonance
MRA	Magnetic resonance angiography
MRI	Magnetic resonance imaging
NCBI	National Centre for Biotechnology Information
NGS	Next-generation sequencing
PCR	Polymerase chain reaction
PET	Positron-emission tomography
PTH	Parathyroid hormone
RNA	Ribonucleic acid
SCID	Severe combined immunodeficiency
SPECT	Single-photon emission computed tomography

1 Introduction

Before the advent of medical science, people believed that the cause of many diseases was supernatural and should resort to magic for treatment (Major 1954, Ackerknecht and Haushofer 2016). Gradually, the use of medical sciences with the approach of herbal medicine was formed among ancient civilizations such as Greece, Babylon, Egypt, China, and India (Organization 2002; Weatherall et al. 2006; Hajar 2012; Jamshidi-Kia et al. 2018). With the passage of time and the creation of numerous advances in science and technologies such as pharmacology and drug production, medical science has also been upgraded (Weatherall et al. 2006). In this respect, medical specialists are trained for the treatment of special disorders in various organs of the body (Weiland et al. 2015). Herein, internal medicine as one of the most efficient specialized fields of medical sciences examines and treats a wide range of diseases, disorders, and syndromes such as rheumatologic, immunologic, allergic, endocrine and metabolic, infectious, pulmonary, etc. On the other hand, during facing an unknown cause disease, an internal medicine specialist can usually give the best advice, because they are known as specialists with comprehensive information on a wide range of diseases (West and Dupras 2012). In all specialized medical disciplines, the physician is required to use methods to diagnose the signs and symptoms of the disease and ultimately prescribe appropriate treatment. In this context, choosing the proper diagnostic methods is often a challenging issue, because many of the signs and symptoms are nonspecific or common to many diseases (Crombie 1963; Organization 2018). Hereupon, in recent centuries, the emergence and development of biomedical sciences with different approaches including cellular and molecular biology, genetics, nanobiotechnology, bioinformatics, biochemistry, etc. have been able to prepare more specific diagnostic methods (e.g., molecular imaging) along with methods to study the mechanism of the disease and provide the best practices for diseases modeling. Moreover, it can also offer modern therapies (e.g., regenerative medicine). In other words, biomedical sciences have played a significant role in the advancement of medical science around the world (Cambrosio and Keating 2001; Wade and Halligan 2004; Quirke and Gaudillière 2008; Gwee et al. 2010; Blann and Ahmed 2014; Fuller 2017). Here, the authors have sought to review the connection between biomedical sciences and medicine, especially internal medicine, as one of the most general medical disciplines.

2 Internal Medicine: Background and Present Status

Internal medicine as a branch of medicine deals with the prevention, diagnosis, and medical treatment of diseases in adults through understanding the basic pathological causes of symptoms and signs of patients. Throughout the history of medicine, during the nineteenth and twentieth century, the combination of three way of medical thinking including the anatomoclinic, the physiopathologic, and the ethiopatogenic mentality led to the emergence of new conception, holistic medicine or medicine of the person, in Europe. In this regard, the "internal medicine" expression originated from a German term *Innere Medizin*. Additionally, owing to the first written book about the internal diseases and the first convened world congress of internal medicine during that period, the twentieth century has been mentioned the golden century of internal medicine (Fordtran et al. 2004; Amatriain 2007). Nowadays, internal medicine has diverse subspecialties including rheumatology, pulmonary disease, hematology, endocrinology, nephrology, gastroenterology, etc. that the internists can follow specialty training in internal medicine if they wish.

2.1 Common Diagnostic Methods

In addition to history taking and accurate physical examination, how to reach differential diagnosis and final diagnosis is an important step in managing the patients. Nowadays, novel achievements in technology and basic sciences had shed light on the future of different fields of medicine and improve the diagnosis and management of the patients. In this regard, internal medicine has also benefited greatly in clinical approaches by applying para clinical investigations including laboratory and imaging investigations. The common diagnostic methods including imaging, laboratory data, pathology, etc. have their own pros and cons. Different imaging technologies assist the internists a lot in different areas and subspecialties. Laboratory testing plays a pivotal role in screening, diagnosis, treatment planning, and follow-up not only in internal medicine but also in every field of medicine. In recent years, new immunologic-based tests such as enzyme-linked immunosorbent assay (ELISA) and Western blot analysis are routinely applied in medicine (Swanson et al. 2018). Imaging is known as one of the noninvasive diagnostic method in medicine. Different imaging techniques like X-ray, computed tomography (CT) scan, magnetic resonance imaging (MRI), ultrasound, positron-emission tomography (PET), etc. through providing images from internal tissues and organs help internists in the diagnosis and management of diseases. The major advantage of MRI and ultrasound over the previous modalities is lack of X-ray and reduced the exposure to ionizing radiation. Due to the ability of MRI in showing the soft tissues and vasculature with high resolution, it is widely applied in many fields of medicine. Different methods of MRI are available: functional MRI (brain mapping) and magnetic resonance (MR) spectroscopy (measuring the chemical components of tissues, e.g., the brain tumors). One of the best modalities with high sensitivity for diagnosing the acute ischemic stroke is diffusion-weighted magnetic resonance imaging. PET as a functional imaging technique is broadly performed to evaluate the malignancies and their spread. Fluoroscopy, angiography, magnetic resonance angiography (MRA), and single-photon emission computed tomography (SPECT) are some the other imaging techniques (Ahn et al. 2002; Hansell et al. 2009; Kang et al. 2009; Goodarzi et al. 2019d). Pathological study is another diagnostic method which needs bio-fluids and tissue samples. Various methods are applied for obtaining tissue samples including fine needle aspiration (FNA), core needle biopsy (CNB), and open incisional/excisional biopsies. Hence, pathological study unlike the other methods often is an invasive diagnostic method (Tayanloo-Beik et al. 2020). Furthermore, there are other modalities applied in subspecialties of internal medicine. Accordingly, endoscopy, colonoscopy, rectosigmoidoscopy, endobronchial

ultrasound (EBUS), electrocardiography (ECG), echocardiography, and electromyography (EMG) are some of these modalities. Although all of the above-discussed methods most of the time assist the internists, sometimes these common diagnostic techniques are not sufficient enough, and their sensitivity and specificity are different. Hence, novel tests and methods are required to progress the diagnosis and management of patients.

2.2 Common Treatment Options

Internists, unlike the surgeons, commonly deal with medical treatments. Prevention, diagnosis, and treatment are the basis of an internist career. Prevention has some levels that internal medicine can concern with various levels in different ways. Accordingly, recommendations for changing lifestyle, screening, and treatment for preventing/limiting the progress of diseases and their complications are examples of internal medicine role in prevention. Besides the wide range of treatments applied in internal medicine, the medication has an essential role among other options. Hormone therapy including hormone replacement therapy (HRT), insulin therapy, corticosteroid therapy, levothyroxine, and parathyroid hormone (PTH) replacement therapy is considered as one of the treatment bases in internal diseases (Forsblad d'Elia and Carlsten 2006; Cutolo 2010; Gluvic et al. 2015). Recently, interventional therapy by novel achievements finds a special place in various subspecialties of internal medicine. In this regard, coronary angioplasty with or without stenting in cardiology, endoscopic and colonoscopic intervention for sphincterotomy, stent placement, stone removal, polypectomy, clip application, submucosal injections in gastroenterology, radiology-guided intra-articular/periarticular/myofascial trigger point injection in rheumatology, ultrasound-guided renal biopsy, and insertion of peritoneal dialysis catheters in nephrology are some examples of interventional therapies (Khan 2005; Efstratiadis et al. 2007; Lee-Kong and Feingold 2017; Ramírez and Plasencia 2018; Tseng et al. 2019).

3 Biomedical Science: Subsets and Applications

Biomedical sciences (*biomedicine*) include a set of natural science disciplines which help advance the goals of medical science by using physiological and biological principles. The mentioned natural science disciplines include cellular and molecular biology, genetics, biochemistry, bionanotechnology, bioinformatics, bioengineering, microbiology, embryology, and physiology (Kirschner et al. 1994, Cambrosio and Keating 2001, Pal 2007, Quirke and Gaudillière 2008, Nass et al. 2009, Arjmand et al. 2020d, e).

3.1 Cellular and Molecular Biology

Cellular and molecular biology is a science which generally studies the function, evolution, and development of the cellular structures of living organisms and their molecular basis. The mentioned studies include investigating cells and molecules' interaction with each other and the environment (Weatherall 1998; Karp 2009; Wei and Huang 2013; Alberts et al. 2018). In other words, it evaluates cellular and molecular signaling and metabolic pathways as well as the cell cycle regulation. Furthermore, molecular biology strongly overlaps with some other biological sciences including genetics and biochemistry (Swanson 2018). Indeed, cellular and molecular biology studies can help physicians to understand the precise pathogenesis of the disease (Beenhouwer 2018; Williams and Silverman 2018). On the other hand, it can be important for the development of diagnostic methods. In other words, cellular and molecular diagnoses, including the analysis of different cell phenotypes and tissue derivatives along with the measurement of various macromolecules and metabolites, can indicate the presence of a disease and abnormal body function (DeBerardinis and Thompson 2012; Tan 2016; Raghavendra and Pullaiah 2018). Further, cellular- and molecular-based diagnoses can improve simulating the disease in appropriate preclinical models (including cell-

based and animal models) and lead to select the most appropriate treatment options. Additionally, investigations based on cellular and molecular sciences have opened a new window of therapeutic approaches, including cell therapy and regenerative medicine (Weatherall 1998; Wang et al. 2013).

3.1.1 Biochemistry

Biochemistry (investigation of chemical compounds and essential chemical processes in living organisms) and medicine share a relationship of mutual collaboration. In other words, biochemical experiments have shed light on multiple phases of the disease (Kogut 1977; Baynes and Dominiczak 2009). Herein, different diseases are classified as being associated with the main types of biochemical molecules (including proteins, carbohydrates, lipids, and nucleic acids) and their related signaling pathways (Stryer et al. 2002; Blanco and Blanco 2017).

3.1.2 Genetics

Genetics as a branch of biology investigates the genetic material in an organism (including deoxyribonucleic acid (DNA) or ribonucleic acid (RNA)), genes (as sequences of nucleotides in DNA or RNA), genetic variations, and heredity (Griffiths et al. 2000). Also, genetic studies (by high-throughput methods, i.e., genome-wide association studies (GWAS) and next-generation sequencing (NGS)) can play a pivotal part to develop understanding of the several disease (e.g., diabetes, obesity, arthritis rheumatoid, Alzheimer's disease, Parkinson's disease, etc.) mechanisms via evaluation the specific involved biological pathways in pathogenesis and implementing accurate diagnostic methods (Edwards 1963; Claussnitzer et al. 2020; Jackson et al. 2020) (Fig. 1). Accordingly, understanding the mechanism of disease can lead to ameliorating therapeutic tactics and finding novel biomarkers and drug targets. Moreover, advances in genetic studies have led to the emergence of a new and effective treatment called gene therapy as approach for improving mutant genes (altered genes) or site-specific modifications (Abati et al. 2019).

3.2 Bioinformatics

Bioinformatics is the use of computing, statistics, and research techniques to collect, analyze, and handle data in recent biology and medicine. In this context, physicians and biologists can detect the structure of biological molecules, e.g., nucleic acids and proteins, via accessing the Internet and bioinformatics-related websites (Table 1), together with simple bioinformatics methods (Lesk 2019; Azodi et al. 2020; Baxevanis et al. 2020). Additionally, bioinformatics has become an important component of omics (genomics, transcriptomics, proteomics, and metabolomics) (Fig. 2) investigations (Mayer 2011; Schneider and Orchard 2011; Yadav 2015). The goal of omics investigations is to identify and quantify the biological molecules on which the structure, dynamics, and function of organisms depend (Horgan and Kenny 2011; Agharezaee et al. 2018; Arjmand 2019; Gilany et al. 2019a, b c; Goodarzi et al. 2019a; Khatami et al. 2019; Larijani et al. 2019a, b; Mehrparavar et al. 2019; Mehrparvar et al. 2020; Tayanloo-Beik et al. 2020). Moreover, the broad omics information achievement can lead to biology development and contribute to the emergence of system biology (research area which focuses on the understanding of whole biological processes, i.e., metabolic pathways and gene regulation) (Chen and Snyder 2012; Yan et al. 2018). On the other hand, individual omics evaluation is expected to lead to substantial improvement in personalized medicine (Chen and Snyder 2013; Ibrahim et al. 2016).

3.3 Bioengineering

Bioengineering uses a range of sciences such as mathematics, biomechanics, tissue engineering, and polymer science to design and develop some areas (including medical devices, diagnostic instruments, biocompatible products, ecological engineering, agricultural engineering, etc.) in order to improve living a healthy lifestyle in this modern world (Valentinuzzi et al. 2017; Sharma and Khurana 2018).

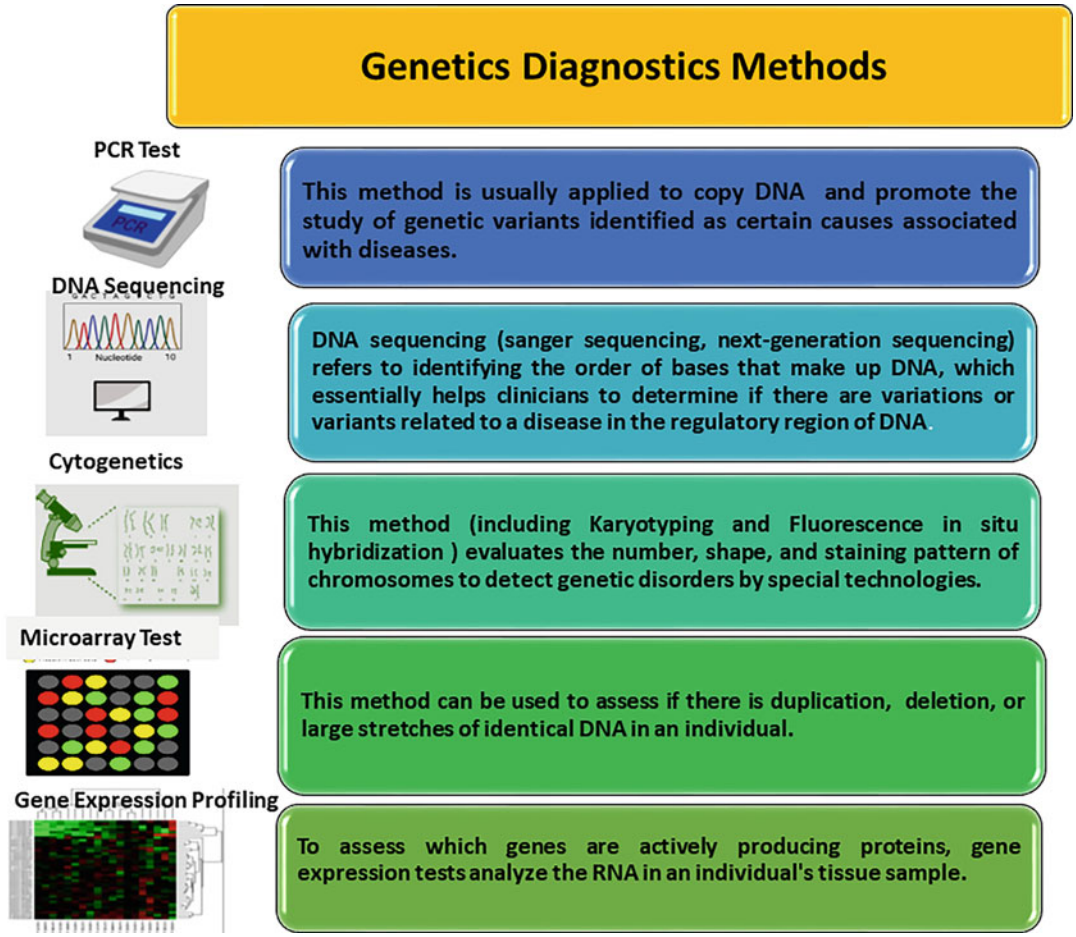

Fig. 1 Genetics diagnostic methods. Genetics diagnostic methods are including polymerase chain reaction (PCR), DNA sequencing, cytogenetics, microarray testing, and gene expression profiling which help look for disease mechanisms (Dwivedi et al. 2017)

3.4 Bionanotechnology

Bionanotechnology, which involves many scientific fields such as cellular and molecular biology, physical sciences, bioengineering, chemistry, nanotechnology, and medicine, can incorporate biological molecules into nanotechnological applications. In other words, it uses knowledge of the characteristics acquired by living organisms on the evolutionary path for technological purposes. Hereupon, the production and design of multifunctional nanoparticles focuses on improving diagnostic techniques, drug delivery system, and therapeutic approaches (Kumar et al. 2013; Ramsden 2016; Zhang et al. 2017; Rauta et al. 2019).

3.5 Microbiology

Microbiology that investigates microscopic organisms (viruses, bacteria, fungi, protozoa, and archaea) includes fundamental evaluation of microorganisms' physiology, cell biology, biochemistry, and ecology. In this respect, it offers services to help diagnose and manage infectious diseases (Glazer and Nikaido 2007; Brooks 2013; Murray et al. 2020).

Table 1 Some of the useful bioinformatics websites

Websites	Application and services
Allen brain atlas	It can provide a unique online public source of broad gene expression, connectivity, and neuroanatomical data about the brain in mice, humans, and nonhuman primates
BLAST	It can be applied to understand functional and evolutionary connections between sequences and recognize gene family members
ChemSpider	It can provide instant access to more than 67 million chemical structures from hundreds of data sources
The European bioinformatics institute (EMBL)	It provides a freely accessible and up-to-date comprehensive collection of molecular data resources
ExPASy	It can provide access to over 160 databases and software resources for the study of genomics, proteomics, structural biology, evolution and phylogeny, system biology, and medical chemistry as an extensible and integrative portal.
Global genome biodiversity network (GGBN)	It can provide a set of vocabulary designed to describe samples of tissue, DNA, or RNA linked to voucher specimens and samples of tissue
Knowledge network for biocomplexity (KNB)	It is an international repository designed to promote environmental and ecological studies around biocomplexity
National Centre for biotechnology information (NCBI)	It can provide access to biomedical and genomic data

Altschul et al. 1997; Andelman et al. 2004; Kanz et al. 2005; Dong 2008; Pence and Williams 2010; Artimo et al. 2012; Barrett et al. 2012; Droege et al. 2014

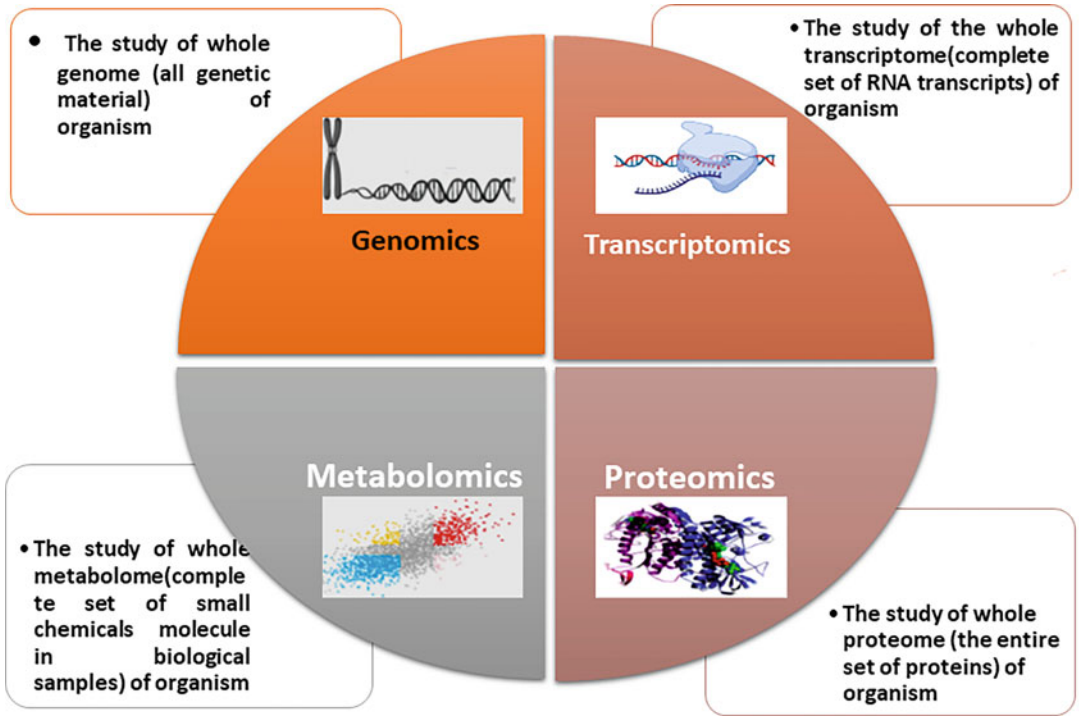

Fig. 2 Omics technologies. Omics technologies seek to the studying of whole genome (genomics), mRNAs (transcriptomics), proteome (proteomics), and metabolome (metabolomics) in specific biological sample (Arjmand 2019; b; Larijani et al. 2019a, b)

3.6 Embryology

Embryology studies the evolutionary and development procedure of various tissues of the living organism from the embryonic stage. In addition, embryological investigations can be effective in the treatment process of fertility-related disorders as well as advancing tissue engineering studies (Patten 1954; De Ferraris and Muñoz 2009; Appasani and Appasani 2010).

3.7 Physiology

Physiology is a branch of biology which concentrates on the biomolecules, cells, and organs' mechanisms of function in a living organism. Indeed, it evaluates the chemical and physical mechanisms (Withers 1992; Ganong 1995; Feder et al. 2000).

4 Molecular Diagnostics and Multi-Omics Approaches

Molecular diagnostics is a group of techniques used to examine biological markers in the genome, proteome, and metabolome. In the recent decades, molecular diagnostics has undergone a period of rapid growth and development (Chehab 1993; Buckingham 2019). Moreover, to advance the goal of achieving proper treatment, it is important to introduce new high-throughput technologies in a clinical molecular diagnostic laboratory. In this context, one of the promising technologies for accelerating the detection process is molecular detection by analytical omics along with using different nanotechnologies (application of numerous nano-devices and nano-systems) (Quezada et al. 2017; Chakraborty et al. 2018; Mukherjee et al. 2020).

4.1 Molecular Imaging

Molecular imaging as part of medical imaging techniques focuses on the use of specific imaging molecules (special probes, i.e., metal ion and radioactive isotope which is injected into a specific anatomical location of living organisms) and imaging modalities (i.e., MRI, CT scan, and PET scan) with the aim of noninvasively studying at the molecular and micromolecular level. The mentioned imaging technique is used to identify metabolic pathways and tissue structures and to evaluate small laboratory animals. Recently, it is applied specifically for infectious diseases, congenital abnormalities, and cancer subjects, from diagnosis to therapy (Aghayan et al. 2014b; Abou-Elkacem et al. 2015; Haris et al. 2015; Saadatpour et al. 2016; Saadatpour et al. 2017).

4.2 Single-Cell Multi-Omics Analysis

Recent technical advancements (including groundbreaking single-cell assays) are promising to overcome the limitation of genome-wide assays (which offers an average of a large number of cells). Accordingly, single-cell sequencing is now becoming available for genomes, transcriptomes, proteomes, and metabolomes, and it provides unprecedented insights into basic biology and biomedicine. Single-cell multi-omics profiling may fix problems that are difficult for other techniques. Hereupon, the genotypic and phenotypic heterogeneity of bulk tissue can be analyzed by single-cell sequencing technology. Indeed, it promises to extend our knowledge of the fundamental processes that control both health and disease (Bock et al. 2016; Hu et al. 2018; Packer and Trapnell 2018; Lee and Hwang 2020; Samir et al. 2020).

5 Advanced Preclinical Models

To simulate a human disease condition (e.g., psychiatric disease), using preclinical models in biomedical studies has become near-universal. Additionally, preclinical models can increase knowledge of cellular signaling pathways and recognizing possible drug targets and novel treatment options (Pan et al. 2020; Scearce-Levie et al. 2020). In this respect, since the past, the use of animal models (especially mammalians) has been popular (Goodarzi et al. 2019d; Larijani et al.

2019a, b; Arjmand et al. 2020b; Baradaran-Rafii et al. 2020). Herein, extrapolating outcomes from models to humans have become an important topic in the evaluation process of the novel treatments. Moreover, based on recent investigations, some biological conditions (such as mental development) have been described which are unique to the human and cannot be modeled in other organisms. In this context, to overcome the mentioned limitations, the advent of in vitro approaches to 3D cell culture systems (employing the genetic engineered stem cells derived from various tissues) or organoids as fast-emerging technology has drawn extensive attention. Organoids (Fig. 3) are able to regenerate, reorganize themselves, and display the function of organs (Li and Izpisua Belmonte 2019; Maximino and van der Staay 2019; Duque-Correa et al. 2020; Jimenez-Palomares et al. 2020; Kim et al. 2020).

6 The Next Generation of Treatments

New breakthroughs in science show great promise in the future of medicine through novel alternative treatments. In this respect, cell-based and gene-based therapies in recent decades greatly progress and become some light of hope for the treatment of incurable diseases. On the other hand, a modern mantra emerging in healthcare is personalized medicine which is powered by providing the clinical, genetic, and environmental knowledge of each individual.

6.1 Cell-Based Therapies

Recently, cell therapy and regenerative medicine are extensively considered in various area of

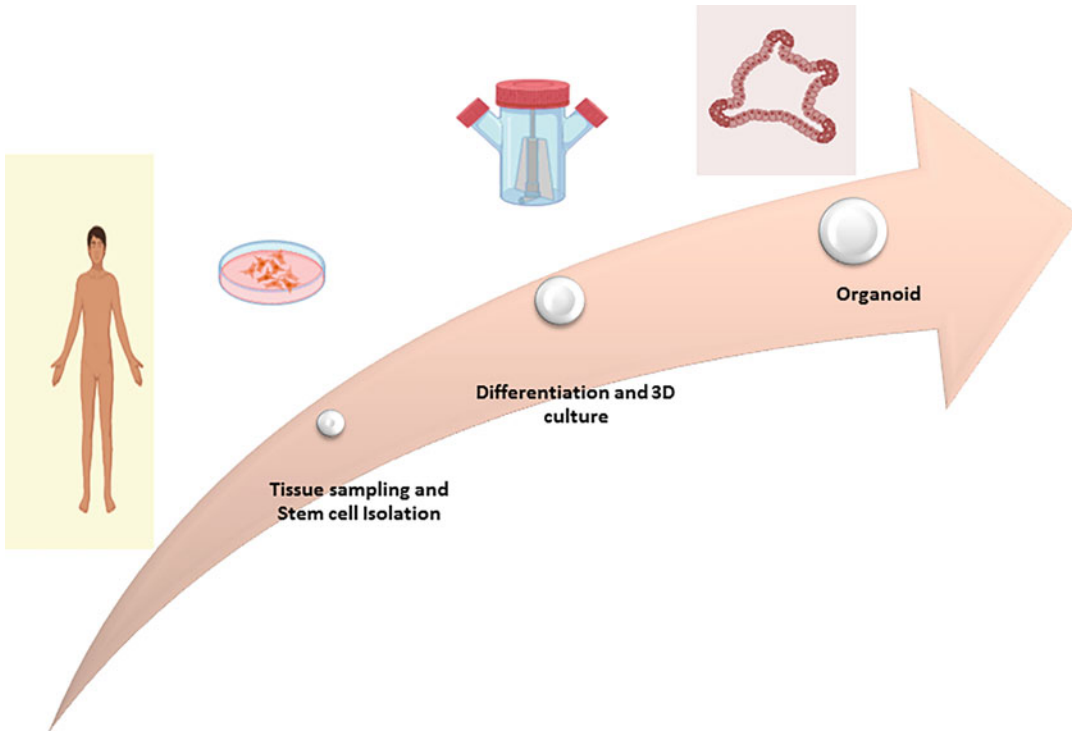

Fig. 3 Three-dimensional (3D) organoid culture. Organoids are 3D cell cultured models which include a population of self-renewing stem cells that differentiate into multiple forms of organ-specific cells and finally show a spatial organization similar to their origin organ and are capable of imitating a highly physiologically certain functions of that organ (Kaushik et al. 2018)

medicine, especially in internal medicine (Arjmand and Aghayan 2014; Arjmand et al. 2017a; Goodarzi et al. 2018b, 2019b, c; Aghayan et al. 2020; Arjmand et al. 2020c, 2021; Ebrahimi-Barough et al. 2020; Hosseini et al. 2020). Different types of stem cell are now available and can be used to generate the healthy and functioning specialized cells, which can then replace diseased or dysfunctional cells. Investigations of cell therapy for cirrhosis, perianal disease of inflammatory bowel disease (IBD), cystic fibrosis of the lung, different types of lymphoma, chronic angina, heart failure, myocardial infarction, ischemic stroke, critical limb ischemia, Parkinson' disease, Alzheimer' disease, spinal cord injuries, etc. have significant progress, but most of them are not yet ready for routine clinical application (Saberi et al. 2008; Aghayan et al. 2014a; Arjmand et al. 2014, 2019a; Goodarzi et al. 2014; Larijani et al. 2014; Derakhshanrad et al. 2015; Goodarzi et al. 2015; Larijani et al. 2015; Shirian et al. 2016; Soleimani et al. 2016; Arjmand et al. 2017c; Rahim and Arjmand 2017; Goodarzi et al. 2018a, c; Payab et al. 2018; Rahim et al. 2018a, b, c, d; Larijani et al. 2020; Payab et al. 2020; Roudsari et al. 2020a, b). Hence, although regenerative medicine and cell therapy as a new technique of treatment have received widespread attention in recent decades, there are a lot of pitfalls in this field (Cho et al. 2015; Nguyen et al. 2016; Shah et al. 2018; Yong et al. 2018; Abramson et al. 2019; Chen et al. 2019; Guo et al. 2019; Hayes Jr et al. 2019).

6.2 Gene-Based Therapies

Gene therapy assists in preventing/treating/or curing disorders through introducing genes into cells (Hashemi et al. 2016; Arjmand et al. 2019b, 2020; Hasanzad and Larijani 2019). This technique is used in treating several inherited disorders; however, it is more feasible for diseases with a single gene involved. There are two types of gene therapy: somatic gene therapy and germ cell gene therapy. Results of the somatic gene therapy unlike the germ cell gene therapy will not be continued by the patients' offspring. Somatic gene therapy is acceptable for diseases such as cystic fibrosis, muscular dystrophy, cancer, inherited blindness, Parkinson's disease, etc. (Verma and Weitzman 2005; Larijani et al. 2019a, b). The first approved gene therapy is introduced in 1990 for severe combined immunodeficiency (SCID) (Ferrua and Aiuti 2017). Thereafter, this technique is applied for other blood disorders like thalassemia, hemophilia, and sickle cell anemia. Also, investigations for gene therapy of cystic fibrosis through introducing normal cystic fibrosis transmembrane conductance regulator (CFTR) gene are ongoing (Yan et al. 2015). Application of gene therapy in the field of cancer treatment was a hot topic in recent years. Accordingly, introducing tumor suppressor genes like p53 or cytokine encoding genes (such as IL 2) and chimeric antigen receptor T cells (CAR T cells) is an example of this application (Ginn et al. 2018). However, gene therapy is not considered to be applied routinely because of some safety and ethical problems such as probability of gene therapy effects on descendants of the patient.

6.3 Regenerative Personalized Medicine

Personalized medicine in biomedical sciences is a special modern treatment method in the current century. The inherent heterogeneity of patients, which indicates different prevalence, different clinical symptoms, and different treatment responses in individuals, families, and ethnicities, is considered to be a very important element in the method described. Personalized medicine needs an examination of the genotype, physiology, and clinical and behavioral details of each individual in order to diagnose and create a personalized treatment plan rapidly. The use of bioinformatics and omics technologies is very

helpful in this context (Arjmand et al. 2017a, b, 2020b; Arjmand and Larijani 2017).

7 Conclusion and Future Perspectives

Internal medicine focuses on both acute and chronic diseases. In recent years, combination of technology with basic and medical sciences ameliorates the disease managements. Common available treatment options can cure some of the diseases. On the other hand, due to diverse reasons such as lack of knowledge about the pathophysiology of some diseases and the absence of effective therapeutic options, there are no curative treatments for some of the other diseases. In such circumstances, the goal of management is palliative care and to prevent the progression of disease and its consequences. Further, better understanding the etiology of diseases, new discoveries in basic sciences, novel advances in technology, and proper combination of these elements are required. Here through the experiments on human tissue and fluid samples carried out in high-tech laboratories, biomedical scientists have a huge effect on the development of innovative therapies for human diseases. In other words, the emphasis of biomedical research in medicine has specific advantages for the future of internal medicine and offers satisfaction with the application of fundamental science to the resolution of clinical problems. Therein, in the coming years, medical practice is expected to continue to evolve toward improved and personalized healthcare, with new techniques to be implemented in order to recognize and improve existing clinical limitations, with an increased effort to tailor medical services to each patient's unique characteristics. In order to understand and direct tailored strategies for an effective clinical context, technological advances and clinical trials can help. Hereupon, also the innovation in stem cell-based technology would enable this pathway such mentioned therapies can be readily accessible in medical procedures.

Compliance with Ethical Statements

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Mesenchymal Stem Cell-Derived Extracellular Vesicles: Progress and Remaining Hurdles in Developing Regulatory Compliant Quality Control Assays

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Abstract

Regenerative medicine is shaping into a new paradigm and could be the future medicine driven by the therapeutic capabilities shown by mesenchymal stem cell-derived extracellular vesicles (MSC-EVs). Despite the advantages and promises, the therapeutic effectiveness of MSC-EVs in some clinical applications is restricted due to inconsistent manufacturing process and the lack of stringent quality control (QC) measurement. In particular, QC assays which are crucial to confirm the safety, efficacy, and quality of MSC-EVs available for end use are poorly designed. Hence, in this review, characterization of MSC-EVs and quality control guidelines for biologics are presented, with special attention given to the description of technical know-how in developing QC assays for MSC-EVs adhering to regulatory guidelines. The remaining challenges surrounding the development of potency and stability of QC assays are also addressed.

Keywords

Bioprocessing · Conditioned media · Exosome · Microvesicles · Regenerative medicine · Secretomes

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Abbreviations

μm	Micrometers
AD	Adipose tissue
AFM	Atomic force microscopy
BM	Bone marrow
CB	Cord blood

CDSCO	Central Drugs Standard Control Organisation
DLS	Dynamic light scattering
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EMA	European Medicines Agency
EVs	Extracellular vesicles
FC	Flow cytometry
FDA	Food and Drug Administration
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ISEV	International Society for Extracellular Vesicles
MFDS	Ministry of Food and Drug Safety
MISEV	Minimal information for studies of extracellular vesicles
MSC-EVs	Mesenchymal stem cell-derived extracellular vesicles
MSC-Exo	Mesenchymal stem cell-derived exosome
MSC-MVs	Mesenchymal stem cell-derived microvesicles
MSCs	Mesenchymal stem cells
MVBs	Multivesicular bodies
MVs	Microvesicles
MWCO	Molecular weight cutoff
nm	Nanometers
NTA	Nanoparticle tracking analysis
PCR	Polymerase chain reaction
PMDA	Pharmaceuticals and Medical Devices Agency
QC	Quality control
qPCR	Quantitative polymerase chain reaction
RPS	Resistive pulse sensing
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Scanning electron microscopy
sEVs	Small extracellular vesicles
TEM	Transmission electron microscopy
TRPS	Tunable resistive pulse sensing
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling
UC	Umbilical cord
WB	Western blot

1 Introductions

Mesenchymal stem cell secretome, the extracellular vehicles (MSC-EVs), has sparked attention in the last few years because of their increasing biological relevance in normal physiology and disease states (Xunian and Kalluri 2020; Hartjes et al. 2019). They have broader therapeutic effects due to its ability to carry a considerable number of functional therapeutic molecules in the form of mRNA, micro-RNAs, long-coding RNAs, DNA, and metabolites (Maumus et al. 2020). This unique characteristic allows MSC-EVs to be utilized in many forms such as the replacement of live cells in stem cell transplantation or as a vehicle in delivering specific targeted therapeutic agents (Maumus et al. 2020).

Though up to date more than 40 MSC-EV studies have been conducted broadly in preclinical animal models, however, only a few have been approved for clinical studies in which most of them are in the not yet recruiting or available phases and are confined to wound healing process and autoimmune, neurological, and cardiovascular diseases (Witwer et al. 2019; Lee et al. 2021). Inconsistent bioprocessing of EV production especially in large scale-up and lack of standardized quality control (QC) assay are the key factors downgrading the therapeutic effects of MSC-EVs products (Witwer et al. 2019; Nguyen et al. 2020). While attention has been given in addressing the issues related to the former, researchers still have difficulty in designing a proper QC assay to align with regulatory requirements.

MSC-EVs are classified as medicinal biological products by leading regulatory agencies, which means that MSC-EV-based products need to be approved by competent authorities before administration for clinical use. For the approval of MSC-EV products to be

considered, compliance to Good Manufacturing Practices (GMP) and strict regulatory practices during the in-process QC of product, finished product QC, and stability studies are crucial to ensure the safety, efficacy, and quality of the MSC-EVs available to end use. Current MSC-EV QC assays are centralized to address the characterization as there is a certain degree of overlap in terms of sizes and markers between the various subtypes (exosomes, microvesicles, apoptotic bodies). While a certain degree of attention is given to address the QC-related assays on physical properties, safety, and purity, potency assays that overall predict the biological function of the EVs are often neglected (Ludwig et al. 2019). Further, most of these QC assays were developed without proper analytical validation resulting in inconsistency in release criteria.

Hence, this review will address the progress and challenges in designing regulatory compliant QC assays that cover major key elements such as identity, purity, safety, and potency. We anticipate that a validated QC assay will lead to the better therapeutic efficacy of MSC-EVs.

2 Biological Characterization of MSC-EVs

MSC-EVs are non-replicated lipid bilayer vesicles and generally secrete into the extracellular environment (Witwer et al. 2019). Typically, EVs are divided into three subtypes – apoptotic bodies, microvesicles (MVs), and exosomes – which are generally distinguished by their sizes (Fig. 1). Exosome being the smallest of all of them with a diameter size between 40 and 120 nanometers (nm), has the main role in regulating intercellular communication carrying specific biomolecular information such as RNAs, proteins, and lipids in their intracellular compartments and transporting them to the target cells (Andaloussi et al. 2019; Colombo et al. 2014). Exosome can be easily distinguished from other subtypes by characterising marker proteins such as tetraspanins (CD9, CD63, CD81, CD82), membrane transport proteins (annexins, Rab), heat shock proteins (Hsp60, Hsp70, Hsp90), and multivesicular bodies (MVBs) formation proteins (Alix, TSG101) (Zhang et al. 2019) that are found on its membrane surface. This is followed by MVs which are in the range of size of 50 to 1000 nm and have similar function and content as exosomes (Andaloussi et al. 2019). MVs also mediate intercellular communication via the delivery of contents to recipient cells and contain cytosolic and plasma membrane-associated proteins, mRNAs, miRNAs, nucleic acids and lipids in their intracellular compartments (Andaloussi et al. 2019; Doyle and Wang 2019). The protein markers present on the membrane of MVs are

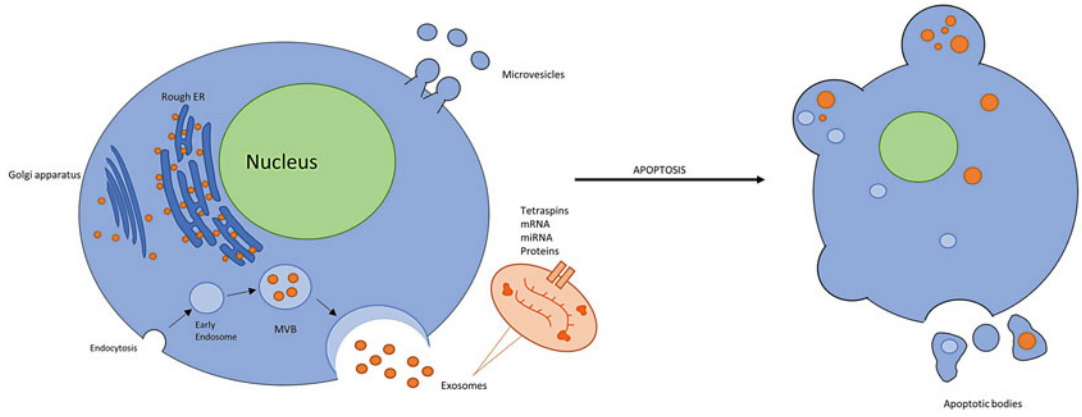

Fig. 1 The biogenesis and content of extracellular vesicles which include the subtypes of extracellular vesicles, microvesicles, and apoptotic bodies

integrins, selectins, and CD40 ligands that function as adhesion molecules (Andaloussi et al. 2019). Apoptotic bodies are larger EVs with a size between 500 and 2000 nm and formed during apoptosis. It contains a portion of a dying cell known as nuclear fractions and cell organelles and can be recognized by having large amounts of phosphatidylserine (Andaloussi et al. 2019).

Due to the overlapping of sizes, characterization markers, and functions between the subtypes of EVs, the International Society for Extracellular Vesicles (ISEV) has published minimal criteria for studies of extracellular vesicles (MISEV) in defining EVs, especially those from MSCs (Thery et al. 2018). According to guideline which is known as MISEV 2014 (later being adopted as MISEV 2018), MSC-EVs that are sized greater than 200 nanometers (nm) in diameter are termed large EVs while EVs that are smaller than 200 nm in diameter are termed small EVs (sEVs) and are named based on its contained biochemical compounds or their originating cell type (Thery et al. 2018).

ISEV further suggested that each research of EVs should be (1) outlined by quantitative measurement of EV source (e.g., total amount of conditioned medium, initial cell seeding, and final cell count); (2) characterized to the utmost practicable to determine the quantity of EVs (e.g., quantification of particles, protein, lipids, nucleic acid); (3) verified for the presence of components specific for particular EVs subtypes (e.g., characterization by protein composition or surface marker); and (4) examined for the presence of non-vesicular, co-isolated components. In other words, EVs should be characterised by the content such as protein or particle concentration, minimum three positives and one negative protein marker and two analytical measures of single extracellular vesicle. The positive protein markers of EVs include transmembrane and cytosolic protein while negative protein markers are composed of apolipoproteins A1/2 and albumin. In addition, EVs subtype can be characterized by subcellular compartments such as the nucleus, mitochondria, or extracellular protein with biological function like growth factors, cytokines or the extracellular matrix (Thery et al. 2018).

2.1 Current Scenario on MSC-EV Characterization Assays

Most of the characterization assays related to downstream activities of MSC-EVs are largely based on identifying and quantifying (Table 1). Apart from directly looking at the number of particles with the same range of sizes, the assays in this category also measure various components that are present in the EVs such as the specific protein markers and the contents of nucleic acids and proteins. Currently, nanoparticle tracking analysis (NTA) is the most commonly used method to determine the size and the number of particles followed by dynamic light scattering (DLS) and resistive pulse sensing (RPS). On the other hand, single vesicle analysis by electron microscope (EM) is more popularly used than atomic force microscopy (AFM) in most research to characterize the morphology and structure of EVs. In terms of evaluating protein marker quantification and expression, a majority of researchers used Bradford, Western blot (WB), or flow cytometry (FC) analysis. While most of the researchers are using compendial testing which is also known as pharmacopoeia standards to check on safety, only a handful of research had focused on reporting on potency testing. Further, despite many studies providing quantitative values, none of the research reported on the ratio (e.g., protein/particle, lipid/particle, or lipid/protein) to estimate EV purity which has been recommended by the ISEV.

3 Quality Control Regulation for MSC-EVs

Quality control consists of procedures done to warrant that the service or product fulfills certain requirements or achieves a certain degree of quality (Sachan et al. 2014). The Food Drug and Administration (FDA) defines biologics as products that can be used in the diagnosis,

Table 1 QC-related assays being used in MSC-EV research

Author	Cell origin	Phase	Disease	Isolation/ purification	QC assay			
					Identity/quantity	Purity	Safety	Potency
Nassar et al. (2016)	Human CB-MSC-EVs	Phase II/III	Chronic kidney disease	Differential centrifugation Density gradient ultracentrifugation	Total protein content TEM SEM FC			
Zhang et al. (2018)	Human UC-MSC-Exo	Phase I	Refractory macular holes	Differential centrifugation	Total protein content SEM WB			
Shi et al. (2021)	Human AD-MSC-EVs	Phase I	Lung injury	Differential centrifugation PEG concentration	Total protein content TEM NTA WB		Gram stain – Microscopy Sterility test – BacT/ALERT Mycoplasma – qPCR Endotoxin – Limulus assay	
Gatti et al. (2011)	Human MSC-MVs	Preclinical	Acute and chronic kidney injury	Differential centrifugation	Total protein content TEM SEM DLS		Endotoxin – Limulus assay	
Bruno et al. (2012)	MSC-MVs	Preclinical	Acute kidney injury	Differential centrifugation	Total protein content TEM SEM DLS FC		Endotoxin – Limulus assay	
Li et al. (2012)	Human UC-MSC-Exo	Preclinical	Liver fibrosis	Differential centrifugation Ultrafiltration	Total protein content TEM WB			
Arslan et al. (2013)	MSC-Exo	Preclinical	Myocardial ischemia/ reperfusion injury	HPLC Ultrafiltration Tangential flow filtration				

(continued)

Table 1 (continued)

Author	Cell origin	Phase	Disease	Isolation/ purification	QC assay			
					Identity/quantity	Purity	Safety	Potency
Bian et al. (2013)	Human BM-MSC-Evs	Preclinical	Myocardial infarction	Differential centrifugation	Total protein content TEM FC WB			Cell proliferation assay Cell migration assay Tube formation assay
Zhou et al. (2013)	Human UC-MSC-Exo	Preclinical	Cisplatin-induced nephrotoxicity	Density gradient centrifugation Ultrafiltration	TEM WB			
Zhu et al. (2013)	Human BM-MSC-MVs	Preclinical	Acute lung injury	Differential centrifugation	Total protein content SEM			
Chen et al. (2014)	Murine BM-MSC-MVs	Preclinical	Pulmonary arterial hypertension	Differential centrifugation Ultracentrifugation	Total protein content TEM NTA FC			
Zhang et al. (2014)	Human UC-MSC-MVs	Preclinical	Renal ischemia/ reperfusion injury	Differential centrifugation	Total protein content TEM FC	TUNEL assay		
Cruz et al. (2015)	Human BM-MSC-EVs	Preclinical	Allergic airway inflammation	Differential centrifugation Ultracentrifugation	Total protein content TEM NTA			
Doeppner et al. (2015)	Human BM-MSC-Exo	Preclinical	Poststroke neuroregeneration	Ultrafiltration PEG precipitation Ultracentrifugation	Total protein content NTA WB		Bacterial contamination – PCR and infectious serology	
Monsel et al. (2015)	Human BM-MSC-MVs	Preclinical	Severe pneumonia	Ultracentrifugation	Total protein content SEM WB			

Teng et al. (2015)	Murine BM-MSC-Exo	Preclinical	Myocardial infarction	Precipitation (ExoQuick-TC)	Total protein content TEM FC			Cell proliferation assay Tube formation assay
Zhang et al. (2015a)	Human UC-MSC-Exo	Preclinical	Cutaneous wound healing	Differential centrifugation Ultrafiltration	Total protein content TEM NTA WB			
Zhang et al. (2015b)	Murine BM-MSC-Exo	Preclinical	Traumatic brain injury	Precipitation (ExoQuick)	Total protein content TEM NTA WB			
Zhao et al. (2015)	Human UC-MSC-Exo	Preclinical	Acute myocardial ischemic injury	Differential centrifugation MWCO concentration Density gradient centrifugation Ultracentrifugation	Total protein content TEM NTA WB			
Lin et al. (2016)	AD-MSC-Exo	Preclinical	Renal acute ischemia/ reperfusion injury	Ultracentrifugation	EM WB			
Ophelders et al. (2016)	Human BM-MSC-EVs	Preclinical	Preterm hypoxic-ischemic brain injury	Ultrafiltration PEG precipitation Ultracentrifugation	Total protein content NTA WB		Tested for the presence of bacteria, viruses, and endotoxins	
Tamura et al. (2016)	Murine BM-MSC-Exo	Preclinical	Liver injury	Differential centrifugation Ultrafiltration Ultracentrifugation	Total protein content TEM TRPS FC			
Zhang et al. (2016)	BM-MSC-Exo	Preclinical	Myocardial repair	Differential centrifugation Precipitation (ExoQuick-TC)	Total protein content TEM FC WB			Cell proliferation assay Cell migration assay Tube formation assay

(continued)

Table 1 (continued)

Author	Cell origin	Phase	Disease	Isolation/ purification	QC assay			
					Identity/quantity	Purity	Safety	Potency
Zou et al. (2016)	Human MSC-EVs	Preclinical	Renal ischemic reperfusion injury	Ultracentrifugation	Total protein content TEM FC NTA			
Bai et al. (2017)	Human UC-MSC-Exo	Preclinical	Autoimmune uveitis	Differential centrifugation Ultrafiltration	Total protein content EM WB			
de Castro et al. (2017)	Human AD-MSC-EVs	Preclinical	Allergic asthma inflammation	Differential centrifugation Ultracentrifugation	Total protein content SEM DLS			
Drommelschmidt et al. (2017)	Human BM-MSC-EVs	Preclinical	Inflammation-induced preterm brain injury	Ultrafiltration PEG precipitation Ultracentrifugation	NTA WB		HIV, HCV, HBV – Multiplex PCR Microbiological contamination – BacTAlert bottles	
Gangadaran et al. (2017)	Murine BM-MSC-EVs	Preclinical	Hindlimb ischemia	Differential centrifugation Density gradient ultracentrifugation	NTA TEM WB			Cell migration assay Cell proliferation assay Tube formation assay
Haga et al. (2017)	BM-MSC-EVs	Preclinical	Lethal hepatic failure	Differential centrifugation Ultracentrifugation	Total protein content NTA TEM			
Mao et al. (2017)	Human UC-MSC-Exo	Preclinical	Inflammatory bowel disease	Differential centrifugation Density gradient centrifugation Ultrafiltration	Total protein content TEM NTA WB			

Song et al. (2017)	Human UC-MSC-Exo	Preclinical	Sepsis	Differential centrifugation Ultrafiltration	Total protein content TEM WB			
Stone et al. (2017)	Human UC-MSC-EVs	Preclinical	Lung ischemic/ reperfusion injury	Differential centrifugation	Total protein content NTA Imaging FC			Cell migration assay
Wang et al. (2017)	MSC-EVs	Preclinical	Myocardial infarction	Ultracentrifugation Differential centrifugation	Total protein content TEM WB			Cell proliferation assay Cell migration assay Tube formation assay
Ahn et al. (2018)	Human UC-MSC-EVs	Preclinical	Neonatal hyperoxic lung injury	Differential centrifugation Ultracentrifugation	TEM NTA			
Bandeira et al. (2018)	AD-MSC-EVs	Preclinical	Silicosis	Differential centrifugation Ultracentrifugation	Total protein content TEM/SEM NTA FC			
Cho et al. (2018)	Human AD-MSC-Exo	Preclinical	Atopic dermatitis	Differential centrifugation	TEM NTA WB FC			Cell-based assay
Jiang et al. (2018)	Human UC-MSC-Exo	Preclinical	Liver injury	Differential centrifugation Ultracentrifugation MWCO concentration Density gradient centrifugation Ultrafiltration	TEM NTA FC			

(continued)

Table 1 (continued)

Author	Cell origin	Phase	Disease	Isolation/ purification	QC assay			
					Identity/quantity	Purity	Safety	Potency
Sun et al. (2018a)	Human UC-MSC-Exo	Preclinical	Spinal cord injury	Differential centrifugation MWCO concentration Ultracentrifugation Ultrafiltration	Total protein content TEM DLS WB			
Sun et al. (2018b)	Human MSC-Exo	Preclinical	Type 2 diabetes mellitus	Differential centrifugation Ultrafiltration	Total protein content WB TEM NTA			
Wu et al. (2018)	Human UC-MSC-Exo	Preclinical	Inflammatory bowel disease	Differential centrifugation Density gradient centrifugation Ultrafiltration	Total protein content TEM NTA WB			
Hao et al. (2019)	Human BM-MSC-EVs	Preclinical	Lung injury	Differential centrifugation Ultracentrifugation	Total protein content SEM NTA FC WB			
Shi et al. (2019)	Human UC-MSC-Exo	Preclinical	Acute myocardial infarction	Differential centrifugation MWCO concentration ExoQuick-TC	Total protein content TEM NTA WB			
Shiue et al. (2019)	Human UC-MSC-Exo	Preclinical	Nerve injury-induced pain	Differential centrifugation Ultrafiltration	Total protein content TEM WB FC			
Varkouhi et al. (2019)	Human UC-MSC-EVs	Preclinical	Acute lung injury	Differential centrifugation	Total protein content TEM FC			

EM electron microscope, *TEM* transmission electron microscope, *SEM* scanning electron microscope, *NTA* nanoparticle tracking analysis, *DLS* dynamic light scattering, *TRPS* tunable resistive pulse sensing, *FC* flow cytometry, *WB* Western blot, *HPLC* high-performance liquid chromatography

prevention, and treatment of medical disorders (Code of Federal Regulations 2021). Hence, biological products, such as MSC-EVs, are subjected to several QC regulations, which cover aspects such as identity, quantity, purity, sterility, potency, and stability. Each country has its own regulatory body, governing the quality of biological products with its own set of QC guidelines (Table 2).

3.1 Establishing QC Assays for EV-Based Products

Many characterization assays are established and being used routinely in MSC-EV research work; however, the main stumbling block is that only a handful of these assays are being developed into a proper QC assay. In this regard, the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) has proposed the feasibility of developing any assays into QC assays with specific acceptance criteria to ensure the assays' reproducibility, reliability, and therapeutic value. To develop a good QC assay, ICH Q2 (R1) guideline highlights the need to conduct validation experiments based on the eight important parameters which include linearity, specificity, range, accuracy, robustness, precision, quantitation limit, and detection limit (Table 3). Here, we briefly explain

Table 2 Guideline subsections of each regulatory aspect for biological products by several regulatory bodies

	Guideline subsection by each regulatory body				
Regulatory aspects	FDA (USA) – Code of Federal Regulations Title 21, Subchapter F, Chap. 1, Part 610 (Code of Federal Regulations 2021)	EMA (Europe) – ICH Q5C – Quality of Biotechnological Products: Stability Testing of Biotechnological/ Biological Products and ICH Q6B – Specifications: Test Procedures and Acceptance Criteria for Biotechnological/ Biological Products (European Medicines Agency 1994a, 1994b)	MFDS (Korea) – Regulation on Approval and Review of Biological Products (Ministry of Food and Drug Safety 2003)	PMDA (Japan) – Guideline for the Quality, Safety, and Efficacy Assurance of Follow-On Biologics (Pharmaceuticals and Medical Devices Agency 2009)	CDSCO (India) – Biosimilar Guideline 2016 (Central Drugs Standard Control Organisation 2016)
Identity	610.14 identity	–	Article 28 (review criteria for biologics)	–	6.3.2 product characterization
Quantity	–	Q6B 2.1.5 quantity	–	6. Specifications and test procedures	6.3.1 analytical methods
Purity	610.13 purity	Q6B 2.1.4 purity, impurities, and contaminants	Article 28 (review criteria for biologics)	6. Specifications and test procedures	6.3.2 product characterization
Sterility	610.12 sterility	Q6B 2.1.4 purity, impurities, and contaminants	Article 28 (review criteria for biologics)	6. Specifications and test procedures	–
Potency	610.10 potency	Q6B 2.1.2 biological activity	Article 28 (review criteria for biologics)	6. Specifications and test procedures	6.3.2 product characterization
Stability	–	Q5C	–	4.4 stability testing	6.3.4 stability

Table 3 List of QC assays corresponding to ICHQ2 (R1) guidelines

			ICH validation criteria based on ICH Q2 (R1)								Other parameters		
Author	Assays' category	Assays	Specificity	Linearity	Range	Accuracy	Precision	Detection limit	Quantitation limit	Robustness	Acceptance range	Cost	Technicality
Adan et al. (2016); Maas et al. (2015)	Identity/ quantity	Flow cytometry (FC)	/	/	/	×	/	x	/	/	Percentage of positive or negative expression markers	↔	↓
Choudhary and Ka (2017)		Scanning electron microscopy (SEM)	/	/	/	/	/	x	/	x	The morphology and structure, either intact or not intact	↑	↑
Dragovic et al. (2011); Maas et al. (2015)		Nanoparticle tracking analysis (NTA)	/	/	/	x	x	/	/	/	The amount of particle size	↓	↓
Ghosh et al. (2014); Hartjes et al. (2019)		Western blot (WB)	/	/	/	/	x	x	/	/	Either presence or absence of the bands	↓	↓
Koritzinsky et al. (2017); Zhao et al. (2015)		Bradford assay	x	/	/	/	/	x	/	x	Detect the presence and concentration of protein in the sample of 5–50 µg/ml	↓	↓
Lu et al. (2017); Williams and Carter (1996)		Transmission electron microscopy (TEM)	/	/	/	/	/	x	x	/	The morphology and structure, either intact or not intact	↑	↑
Sharma et al. (2018)		Atomic force microscopy (AFM)	/	/	/	/	x	x	/	/	The amount of particle size	↔	↓

Doeppner et al. (2015)	Safety	Infectious serology	x	/	x	/	/	/	x	x	Positive result from this test is able to detect the presence of certain antibodies	↓	↓
Enderle et al. (2015); Garibyan and Avashia (2013); Montero-Calle et al. (2021)		Quantitative polymerase chain reaction (qPCR) for mycoplasma	/	/	/	/	/	x	/	x	Able to determine the exact CT value and also either the presence or absence of the bands for mycoplasma	↔	↓
Iwanaga (2007); Mehmood (2019)		Limulus assay	x	/	/	/	/	/	/	x	Gelation reaction once exposed to endotoxin from gram negative bacteria	↓	↓
U.S. Food and Drug Administration (2018)		BacT/ ALERT	/	/	/	/	x	/	/	x	The culture will change to yellow with the presence of microorganism contamination	↓	↓
Tripathi and Sapra (2021)		Microscopy gram stain	/	/	/	/	/	x	/	x	Detect the presence of contamination of either gram positive or gram negative microorganisms	↓	↓

Legend: ↑ = high, ↓ = low, ↔ = moderate

the role of each of these parameters and how important they are in shaping up a good QC assay.

Specificity is defined as the capacity of an assay to assess the existence of components that may be anticipated to be present within the analyte. For instance, in terms of MSC-EVs for identity or quantity purposes, specificity denotes the size of MSC-EVs and typically carries markers such as CD63, CD9, and CD81. In this regard, assays such as NTA, Bradford, WB, or FC can be used to validate the specificity of MSC-EVs. Other assays such as scanning electron microscopy (SEM), transmission electron microscopy (TEM), or AFM may be used; however, we reckon that the assays are less specific as compared to the former assays due to the limitation of the instrument itself which only visualizes the morphology and structures of MSC-EVs. In terms of safety-related assays, specificity refers to the ability to differentiate MSC-EVs from contaminants such as body fluids given that we derived the exosomes from the blood and qPCR-based assays tend to have higher specificity as compared to other assays (Ludwig et al. 2019).

This is followed by the validation of the linearity of an assay which is defined as the ability to acquire experimental results that have a directly proportional relationship to the concentration of analyte within a sample. Generally, the acceptance coefficient of determination (R-squared) is 0.99, and one who performs assays such as NTA, Bradford, total protein content, WB, and qPCR has to validate the linearity of these assays. Next, range is defined as the range between the lower and upper concentrations of analyte within a sample. With this in mind, AFM has the highest range as it can detect particle size as close as 1 nm up until 120 nm. On the other hand, accuracy is the degree of closeness between the experimental value and the value acknowledged as the conventional true value or approved reference value. For example, FC does not meet the requirement of accuracy criterion for MSC-EVs as it cannot determine the sample concentration accurately due to the swarming effect and its insensitivity toward lower size range solutes.

The precision of an assay demonstrates the proximity of agreement among a set of results obtained from several samplings of a sample under prearranged conditions. Moreover, as stated in the ICH guidelines, precision is to be tested using homogenous and authentic samples; if not possible, artificially prepared samples can be used for the investigation. To give an example for the precision criterion, Hartjes et al. and Kurian et al. stated that although AFM has a better range, it has a very low reproducibility as the technique is highly dependent on the sample size, such that it can only image a maximum height of 10–20 micrometers (μm) within a total scanning area of 150 × 150 μm (Hartjes et al. 2019; Kurian et al. 2021). The validation for precision is further divided into three subcategories, namely, repeatability, intermediate precision, and reproducibility. Precision under the identical operational conditions over a short period is expressed by repeatability and requires at least nine determinations that cover a defined range for the assay or a minimum of six determinations at 100% test concentration. Finally, intermediate precision is expressed within-laboratories variations which include different days, analysts, and equipment. Reproducibility is expressed by the prevision between laboratories which is usually as a mean for methodology standardization. In short, techniques that qualify for the categories of range, accuracy, and precision should be able to detect a range of different size EVs while maintaining optimum accuracy and precision.

Penultimately, the detection limit of a test is referred to as the least amount of analyte within a sample which could be detected. For example, techniques that qualify for this category should have the ability to detect the presence of EVs although in a low concentration. Other than that, the quantitation limit of a test is the least amount of analyte within a sample that can be precisely and accurately quantified. This parameter is mainly used for low concentrations of compounds in a sample and to determine impurities or degradation products within an analyte. For example, the low penetration of EM beam and vacuum conditions required the sample to be ultrathin

and completely dry affecting the morphology of EVs. Finally, assays that qualify for the robustness category should show reliability with a deliberate variation. For example, the low scanning speed of AFM requires a longer time to obtain an accurate image. This leads to thermal drift causing variation in image quality that can affect the analytical condition which in turn produces an invalid result. In a nutshell, to ensure the repeatability and precise result, a combination of several QC assay techniques should be implemented so that the overall result obtained will be able to fulfill the ICH validation guidelines. For example, the Bradford test, TEM, SEM, FC, and qPCR can be used in the quality control process along with NTA as they complement each other, hence producing reliable results.

4 Remaining Challenges

Hurdles persist among currently available QC assays such as challenges in developing specific potency assays and ascertaining the life span of EVs. Potency assay consists of biological (in vitro or in vivo) or nonbiological assays which test the specific biological capabilities of the product (USFDA 2011). Establishing QC-related potency assays for MSC-EVs is more challenging compared to other pharmaceutical or biological products due to several factors as listed below:

1. Differences in EV preparations may result in enrichment of different components within MSC-EVs resulting in a change of therapeutic outcomes (Gimona et al. 2021).
2. Different donors of MSC-EVs have different biological properties and components which may change the therapeutic outcome (Gimona et al. 2021).
3. The mechanism of therapeutic potential of MSC-EVs is still vague as it can be involved in more than one different pathological process making it difficult to predict the potency of MSC-EVs (Gimona et al. 2021).
4. MSC-EVs from different MSC sources can vary in therapeutic potency such that suppression of T-cell proliferation is higher in AD-MSC-EVs than BM-MSC-EVs (Adlerz et al. 2020).
5. The spatiotemporal site of action remains unknown making it challenging to determine the precise biodistribution of MSC-EVs within a cell/tissue (Gimona et al. 2021).

Once the dynamic biological activities of EVs have been identified, currently available QC assays can be customized to comply with potency assay requirements. For example, Bruno and colleagues found that combining RT-PCR, enzyme-linked immunosorbent assay (ELISA), immunosorbent assays, and antibody assays was able to measure the full potency of the MSC-EVs but only for acute kidney injury (2009). Another study on myocardial ischemia injury showed that using a combination of enzymatic assays was also able to determine the potency of the MSC-EVs (Lai et al. 2010). Within these two examples, the combination of currently available assays such as RT-PCR, ELISA, and enzymatic assays can comply with the potency assay requirements such that it is specific, accurate, precise, and quantitative. However, due to the diverse attributes of MSC-EVs, combinations of different types of potency assays can only be specific to their respective pathological processes.

Another important aspect that needs to be considered is the life span which is orchestrated by stability data collectively contributed by factors such as temperature, light, and handling procedure. Nevertheless, a poorly designed QC assay may result in contradicting outcomes. For example, a study showed that 4 days' preserved exosomes at −80 °C are not stable as compared to freshly prepared ones (Maroto et al. 2017). Surprisingly, Jeyaram and Jay reported that the optimum storage condition for exosomes was −80 °C, contradicting the former study (Jeyaram and Jay 2017). This shows the important validation of the analytical procedure for each selected QC assay to ensure consistent results.

Further, majority of the purity tests that detect only endotoxins and mycoplasma contamination often neglect potential viruses contamination. Viruses are capable of enclosing themself into

EVs due to convergence of pathways (van der Grein et al. 2018). It is also known that the size ranges of EVs and viruses are similar which could lead to EV preparation being susceptible to viral contamination (Gyorgy et al. 2011). However, implementing QC to detect the presence of viral contamination is an uphill task because most methods, such as the PCR multiplex assay, are not product-specific. To test for viral contamination, short fragments of DNA are needed to complement specific parts of transcribed viral DNA. In short, current methods are only capable of detecting specific target viruses, and development for nonspecific target assays for viral detection should be carried out.

5 Conclusion

Despite these challenges, there is still room to improve QC assays for MSC-EVs. For QC tests that involve categories such as identity, purity, and quantity, apart from complying with ICH guidelines, efforts also should be taken to establish a similar QC procedure between various laboratories around the world which leads to a standard reference. Such an initiative can also be arranged by the ISEV as part of a compliance program. The issue remains that the enrichment method for isolation of EVs still remains with challenges that are difficult to keep up with as there is a lack of consensus concerning isolation steps for EVs (Stam et al. 2021). Additionally, the preparation of MSC-EVs remains expensive and has limited scalability, and if these preparations were scaled up, QC assays for MSC-EVs would be difficult to maintain. Moreover, the key to developing a potency assay is determining and mapping the pathological processes in preclinical animal models as it will lead to a better understanding of MSC-EVs' potency. In terms of extending the life span of EVs, lyophilization of EVs can be adopted. For example, a study by Charoenviriyakul and colleagues demonstrated that EVs could be lyophilized with the use of trehalose without affecting their stability and structure which allows exosomes to be preserved at room temperature which is useful for many applications (Charoenviriyakul et al. 2018). However, immortalized MSC-derived EVs would have special considerations for QC assays as they divide infinitely and express unique gene patterns that sometimes cannot be found in regular EVs (Rohde et al. 2019). With this, it is possible to develop separate assay criteria for MSC-derived EVs and immortalized MSC-derived EVs. Furthermore, special surface markers such as tetraspanins on MSC-derived EVs should be taken into consideration when developing an identification assay as it provides specificity only to EVs. With the combination of these suggestions and improvements, precision of a standardized QC assay can be developed to not only serve as a gold standard for MSC-EVs research but to also enhance its therapeutic value.

Conflict of Interest Kong-Yong Then and Soon-Keng Cheong are directors of CryoCord Sdn Bhd and declare direct share interest in the company, whereas all other authors declare no conflict of interest.

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Pharmacological Effects of Caffeic Acid and Its Derivatives in Cancer: New Targeted Compounds for the Mitochondria

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Abstract

Cancer is a complex pathology of great heterogeneity and difficulty that makes the constant search for new therapies necessary. A major advance on the subject has been made by focusing on the development of new drugs aimed to alter the metabolism of cancer cells, by generating a disruption of mitochondrial function. For this purpose, several new compounds with specific mitochondrial action have been tested, leading successfully to cell death. Recently, attention has centered on a group of natural compounds present in plants named polyphenols, among which is caffeic acid, a polyphenol that has proven to be a powerful antitumoral agent and a prominent compound for studies focused on the development of new therapies against cancer.

In this review, we revised the antitumoral capacity and mechanisms of action of caffeic acid and its derivatives, with special emphasis in a new class of caffeic acid derivatives that target mitochondria by chemical binding to the lipophilic cation triphenylphosphonium.

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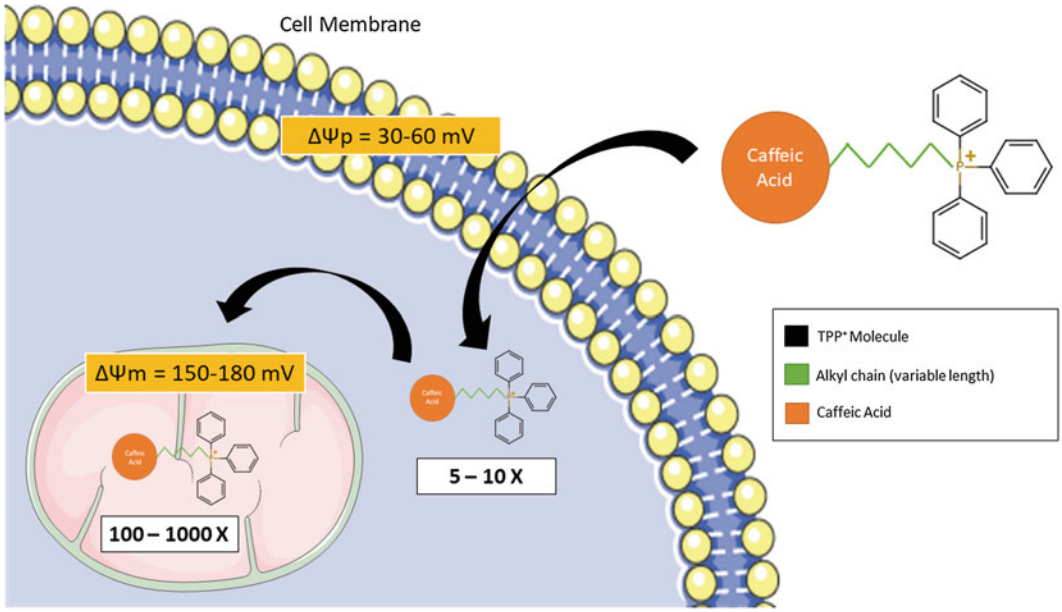
Graphical Abstract



Keywords

Caffeic acid · Cancer · Cancer therapy · Mitochondria · Triphenylphosphonium cation

Abbreviations

α-KG	Alpha-ketoglutarate
ΔΨp	Plasma membrane potential
ΔΨm	Membrane potential of the MII
2-HG	2-Hydroxyglutarate
AMPK	AMP-activated protein kinase
CA	Caffeic acid
CAPE	Caffeic acid phenethyl ester
CAPPE	Caffeic acid phenylpropyl ester
Cyt c	Cytochrome c
DR5	Death receptor 5
DRP-1	Dynamin-related protein 1
ETC	Electron transport chain
FDA	Food and Drug Administration
GSH-Px	Glutathione peroxidase
HIF	Hypoxia-induced factor
IMM	Inner mitochondrial membrane
IC_{50}	Inhibitory concentration 50
MDIVI-1	Mitochondrial division inhibitor 1
MitoCaA	Mitochondriotropic caffeic acid
MitoCA	Mitochondriotropic cinnamic acid
MitoFA	Mitochondriotropic ferulic acid
Mitop-CoA	Mitochondriotropic p-coumaric acid
mIDH	Mutant isocitrate dehydrogenase
MMP	Mitochondrial membrane potential
MMP2	Matrix metalloproteinase 2
MMP9	Matrix metalloproteinase 9
mtDNA	Mitochondrial DNA
mtROS	Mitochondrial ROS
mTOR	Mechanistic target of rapamycin
NFκB	Nuclear factor kappa B
O_2-	Superoxide anion
OH-	Hydroxyl radical
PI3-K/Akt	Phosphoinositide 3-kinase/protein kinase B
p38	Mitogen-activated protein kinase
ROS	Reactive oxygen species
SI	Selectivity indexes
SODs	Superoxide dismutases
TCA	Tricarboxylic acid
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TPP^+	Triphenylphosphonium
VEGF	Vascular endothelial growth factor

1 Background

Noncommunicable diseases represent nowadays the main cause of death in almost every country in the world. Between them, cancer emerges as one of the most significant by reporting an incidence of 24.5 million new cases and 9.6 million deaths in 2017 (Fitzmaurice et al. 2019). In Chile, studies indicate from a total of 106,388 deaths registered in 2017, 27,504 were due to cancer, therefore representing 25.9% of all deaths (Comité Nacional de Estadísticas Vitales 2017) and placing this disease among the pathologies responsible for the largest number of deaths country and worldwide.

Cancer has a great heterogeneity in its origin, course, and response to treatment, which is one of the greatest challenges to achieve, due to different patient response, time-tumor progression, type of tissue, genetic and epigenetic mutations, and metabolic alterations (Prasetyanti and Medema 2017). Due to the high degree of variability, various types of treatments have been developed, chemotherapy being one of the most used of those with clinical efficacy. For instance, main drugs used in chemotherapy are classified according to the type of molecular target and mechanism of action in alkylating agents that cause direct damage to DNA – antimetabolites which correspond to analogs of endogenous molecules that are necessary for DNA and RNA synthesis; mitosis inhibitors which act by altering the formation of the mitotic spindle; and topoisomerase inhibitors that are enzymes that regulate the DNA supercoiling. However, there have been reports of several side effects and drug resistance; therefore, the greatest issue is to improve effectiveness and selectivity against cancerous cells by providing other chemical alternatives. As a result, over the last few years, the search for new pharmacological targets has become relevant in order to develop novel drugs with specific action. Within them, mitochondria have turned out to exhibit special characteristics, allowing the design of selectively targeted drugs (Frattaruolo et al. 2020). As is widely known, this organelle plays a fundamental role in the cell, by participating in vital functions such as ATP production, cycle control, production of metabolic proteins, and cell signaling and death, in addition to being closely related with cellular metabolic stress since it is responsible for reactive oxygen species (ROS) production (Grasso et al. 2020).

2 Mitochondria in Cancer Cells

Cancer cells present various genetic, epigenetic, and metabolic alterations, which lead to the loss of normal cell functions and the acquisition of abnormal survival and proliferation capacities. In this reprogramming phenomenon, the mitochondrion plays a fundamental role by transforming its normal bioenergetic metabolism into an elevated glycolytic metabolism that triggers mitochondrial dysfunction, which is known as the Warburg effect (Grasso et al. 2020; Anderson et al. 2018a).

In cancer, the mitochondria manifest a series of alterations as mutations in mitochondrial DNA (mtDNA), affecting the synthesis of enzymes that participate in the tricarboxylic acid (TCA) cycle and the synthesis of complexes that participates in the electron transport chain (ETC). As a consequence, the production of reductor electron equivalents NADH and FADH2 is altered, resulting in the accumulation of TCA cycle intermediates depending on the affected enzyme. These intermediates can act as oncometabolites, as is the case of fumarate and succinate (Grasso et al. 2020). In addition, tumoral cells present a high rate of ROS production, which is a key feature in carcinogenesis as it correlates with the progression of malignancy, as ROS toxicity may induce the alteration of intracellular pathway signaling (Idelchik et al. 2017). The overproduction of ROS induces the accelerated metabolism and the redox imbalance that cancer cells possess, which prevents the neutralization of ROS. When cells are immersed in hypoxia conditions or a high metabolic rate, electrons can leak from complexes I and III of the ETC and conjugate with O_2 forming a superoxide anion (O_{2-}), which is the main cellular ROS. In this sense, if O_{2-}

cannot be neutralized, it will lead to the oxidation of cellular macromolecules such as DNA, lipids, and proteins (Grasso et al. 2020). As it is well known, ROS neutralization is given by cellular antioxidant mechanisms such as superoxide dismutases (SODs) – which catalyze the reaction of production of H_2O_2 from O_{2-} – and the catalase (CAT) and glutathione peroxidase (GSH-Px) enzymes that catalyze reactions where H_2O is produced from H_2O_2– (Idelchik et al. 2017).

Additionally, mitochondria in tumoral cells are characterized by presenting an abnormal inner mitochondrial membrane (IMM) potential that is higher when compared to normal cells, therefore presenting a more electronegative charge (Kalyanaraman et al. 2018; Modica-Napolitano and Weissig 2015).

3 Antineoplastic Agents Targeting Mitochondrial Function

Given the fact that mitochondria participate in multiple metabolic functions, researchers may employ different strategies to develop more effective drugs, such as targeting the TCA cycle, ETC, mitochondrial biogenesis, or the mitochondrial apoptotic pathway (Frattaruolo et al. 2020; Grasso et al. 2020; Anderson et al. 2018a). Among drugs under study or between those already approved by the US Food and Drug Administration (FDA) are those that operate on the TCA cycle such as CB-839, CPI-613, ivosidenib (AG-120), enasidenib (AG-221), and vorasidenib (AG-881) (Frattaruolo et al. 2020; Grasso et al. 2020; Anderson et al. 2018a, b; Konteatis et al. 2020) (Fig. 1).

In this sense, it has been described that CB-839 is a specific inhibitor of glutaminase. This enzyme converts glutamine into glutamate, in what is supposed to be a physiological process in cells, upregulated in cancer. Excess of glutamate has been associated with an increase in cell proliferation and malignancy (Grasso et al. 2020; Chen and Cui 2015). Additionally, CPI-613 corresponds to a lipoate analog that inhibits the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase enzymes, therefore preventing the incorporation to the TCA cycle of carbons derived from glucose or glutamine (Anderson et al. 2018a; Stuart et al. 2014). Further,

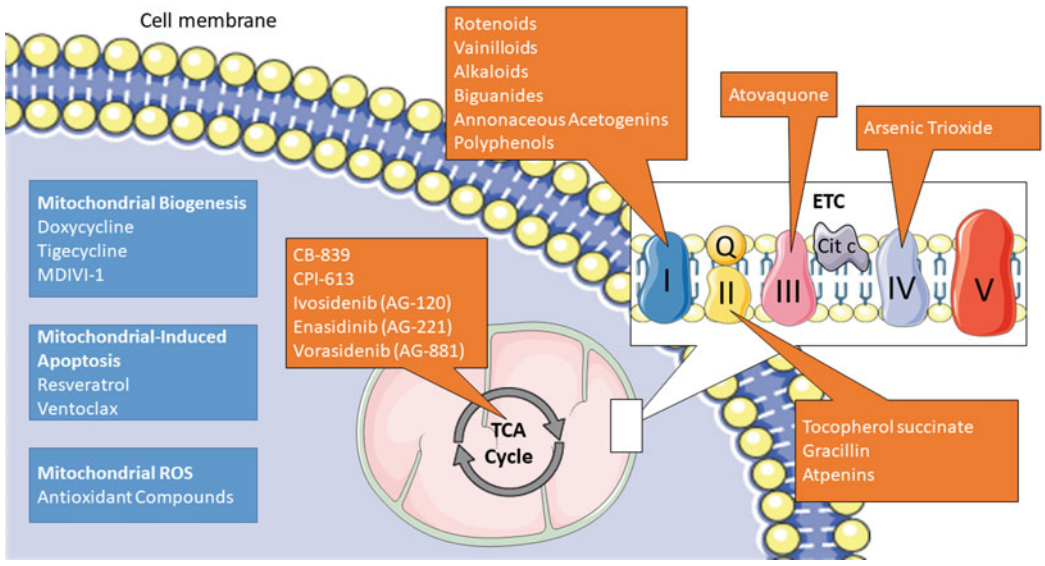


Fig. 1 Agents with antineoplastic effect that act on the mitochondria with action on biogenesis, apoptosis and mitochondrial ROS, TCA cycle, and CTE in their different complexes are described

ivosidenib, enasidenib, and vorasidenib work by inhibiting mutant isocitrate dehydrogenase (mIDH) enzymes. Under normal conditions, isocitrate dehydrogenase should catalyze the conversion of isocitrate to α-ketoglutarate (α-KG); however, when mutated it can catalyze the conversion of α-KG to the oncometabolite 2-hydroxyglutarate (2-HG) (Frattaruolo et al. 2020; Anderson et al. 2018a, b; Abou Dalle and DiNardo 2018). Particularly, ivosidenib inhibits the mutant mIDH1 enzyme, enasidenib inhibits mIDH2, and vorasidenib inhibits both mIDH1 and mIDH2, as described above (Konteatis et al. 2020; Abou Dalle and DiNardo 2018; Popovici-Muller et al. 2018). The mutual mechanism of action consists of blocking the active site that catalyzes the conversion reaction of α-KG to 2-HG (Anderson et al. 2018a, b).

Another strategy that has been developed is to target the different complexes of the ETC (Frattaruolo et al. 2020; Urra et al. 2017; Ashton et al. 2018). As a result, there are several series of drugs described as inhibitors of complex I that can be classified into rotenoids, vanilloids, alkaloids, biguanides, annonaceous acetogenins, and polyphenols (Urra et al. 2017). The latter have been recently attracted attention for their multiple beneficial effects, including antitumoral action (Zhou et al. 2016). Complex I-targeted drugs can inhibit complex I in a competitive or noncompetitive way, being common for competitive compounds to have a hydroquinone/quinone structure, while noncompetitive compounds, such as metformin and other biguanides, can bind non-competitively to different domains of complex I (Frattaruolo et al. 2020). Drugs targeting complex II include α-tocopherol succinate, gracillin, and atpenins. These agents have been described to increase ROS production, leading to apoptosis in cancer cells (Frattaruolo et al. 2020). Among the complex III inhibitor drugs is atovaquone, a ubiquinone analog that can competitively inhibit complex III of ETC as a result of its structural similarity to CoQ10 (Fiorillo et al. 2016). Finally, between drugs whose action is focused on complex IV, the more relevant is arsenic trioxide, a drug used to treat acute promyelocytic leukemia (Ashton et al. 2018).

In addition, researchers have also developed pharmacological compounds interfering with mitochondrial biogenesis, either by preventing the processes of transcription and translation of mtDNA or by altering the fission and fusion dynamics of this organelle (Frattaruolo et al. 2020; Anderson et al. 2018a). Between those that interfere with the DNA-translation process are doxycycline and tigecycline, compounds commonly used as antibiotics that have shown antitumoral effects in many cancer cell lines. As bacteriostatics, they can exert their effect by binding to the 30S ribosomal subunit of bacteria due to the structural similarities between this subunit and mitochondrial 28S ribosomal subunit. Consequently they can block the entry of aminoacyl-tRNA to the A-site of the ribosome, inhibiting the process of elongation and translation of mitochondrial proteins (Dong et al. 2019; Protasoni et al. 2018). Likewise, mitochondrial division inhibitor 1 (MDIVI-1) and indomethacin inhibit mitochondrial fission – an increased process in cancer cells – by blocking dynamin-related protein 1 (DRP-1), which affects mitochondrial dynamics (Frattaruolo et al. 2020; Anderson et al. 2018a; Mazumder et al. 2019).

Another pharmacological strategy is to restore mitochondrial-induced apoptosis, as occurs with resveratrol and venetoclax by inducing mitochondrial release of cytochrome c (Cyt c), therefore initiating the apoptotic cascade by activating the caspase pathway (Frattaruolo et al. 2020; Anderson et al. 2018a).

In recent years, drugs that can inhibit mitochondrial ROS (mtROS) production have been proposed as new strategy, especially those based on antioxidant compounds capable of selectively targeting the mitochondria (Grasso et al. 2020). The accumulation of mtROS produces several alterations in various signaling pathways, activating survival and proliferation factors such as hypoxia-induced factor 1 (HIF-1) and hypoxia-induced factor 2 (HIF-2). As a consequence, it has been described that cells present an elevated angiogenesis and glycolytic enzyme activity that allow the maintenance of ATP production for the tumor cell, despite of increased mtROS (Dickerson et al. 2017). Although excessive

mtROS production is a key process in the development of cancer since it leads to the oxidation of cellular macromolecules (Grasso et al. 2020) and malignant cell transformation, it has been largely observed that very high increases in its production cause the death of cancer cells (Idelchik et al. 2017; Dickerson et al. 2017). However, mechanisms of action of many drugs that causes mtROS accumulation have not yet been thoroughly elucidated.

4 Polyphenols as Antitumoral Agents

Nowadays, the approach of antitumoral therapy focuses on the beneficial effects of polyphenols, a group of compounds that contain two or more phenolic groups. Given their chemical structure, polyphenols have been extensively described as antioxidant, anti-inflammatory, antimicrobial, and antitumoral agents (Zhou et al. 2016). They can be classified into different categories based on their structure and number of phenolic rings, such as stilbenes, lignans, phenolic alcohols, flavonoids, and phenolic acids. This last group can be subdivided into derivatives of hydroxybenzoic acid or hydroxycinnamic acid (Quiñones et al. 2012). Within hydroxycinnamic acid derivatives is caffeic acid (CA), which exhibits powerful anti-inflammatory, antitumoral, and antioxidant effects, thus controlling oxidative stress by free radicals that constitutes a key process in cancer progression (Caffeic acid 2020). The biological effects exhibited by CA are closely related to its chemical structure. Therefore, its powerful antioxidant effects are associated with the presence of a catechol group in its structure, which is known for its great reducing capacity resulting from two hydroxyl groups (Damasceno et al. 2017).

Antioxidants can be classified as primary or secondary according to their mechanism of action. If they react directly with the radical, they are classified as primary antioxidants, while if they have an indirect effect on radicals, they are classified as secondary antioxidants (Damasceno et al. 2017). CA exhibits both mechanisms. As a primary antioxidant, CA has demonstrated direct neutralization of free radicals by donating protons from its hydroxyl groups to form stable compounds that are not able to produce oxidative damage to cell structures. In the process, CA acquires a semiquinone structure when it has one oxidized group and then an o-quinone structure when both groups are (Damasceno et al. 2017) (Fig. 2). As a secondary antioxidant, CA acts through the chelation of transition metals, which catalyze the decomposition of H_2O_2 in

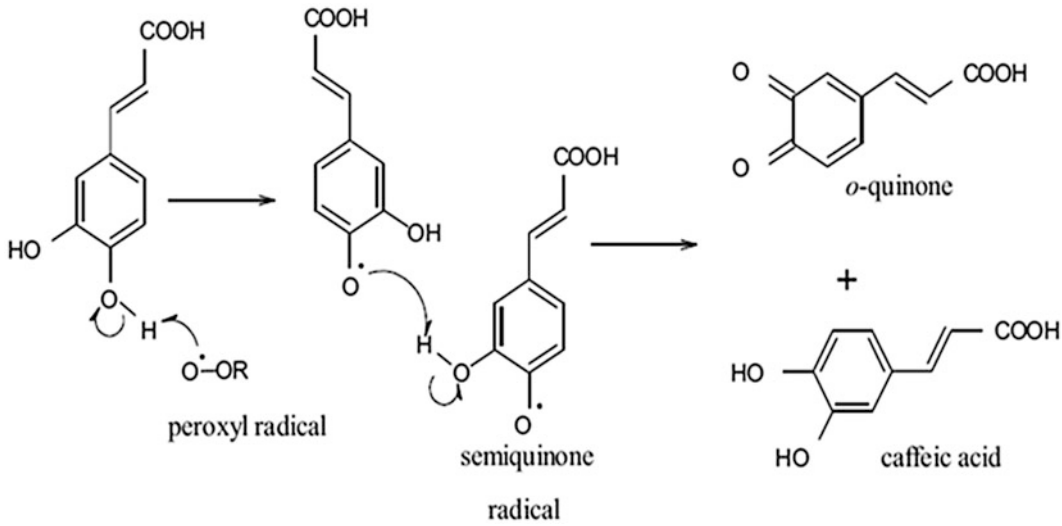


Fig. 2 Possible mechanism involved in the antioxidant activity of caffeic acid. (Damasceno et al. 2017)

hydroxyl radical (OH-). This radical has a great redox potential, which is why it produces oxidative damage in the cell by reacting with lipids, proteins, and nucleic acids (Damasceno et al. 2017). In this process, CA also undergoes structural transformation to semiquinone and o-quinone.

As mentioned above, high concentration CA can behave as prooxidants, and it is mainly to this effect that their effective antitumoral and proapoptotic capacities are associated (Damasceno et al. 2017). Therefore, the administration of high concentrations of CA to cancer cells results in the production of very high quantities of free radicals in presence of O_2 or Cu^{+2}, thus causing extensive oxidative damage and consequently triggering death in tumor cells, without significant side effects on non-cancerous cells, since they have a normal antioxidant balance (Damasceno et al. 2017).

5 Caffeic Acid and Its Derivatives with Antitumoral Action

Numerous studies have shown that CA and its derivatives have proven to be effective on different types of cancer, exhibiting antiproliferative, proapoptotic, antiangiogenic, and antimetastatic effects (Chiang et al. 2014; Monteiro Espíndola et al. 2019; Kabała-Dzik et al. 2018). As in colorectal cancer, CA derivatives such as caffeic acid phenethyl ester (CAPE) and caffeic acid phenylpropyl ester (CAPPE) (Fig. 3) have been described with antiproliferative effects by inducing cell cycle arrest as resulting from the suppression of the mechanistic target of rapamycin (mTOR) and the phosphoinositide 3-kinase/protein kinase B (PI3-K/Akt) signaling pathways (Chiang et al. 2014). Both targets induce cell proliferation and are overexpressed in cancer. The activation of the PI3-K/Akt pathway improves cell proliferation by increasing levels of cyclin D1, which is involved in the progression of the cell cycle from G1 to S phase, hence the antiproliferative effect resulting from the inhibition of this pathway induced by these derivatives.

In addition, CAPE and CAPPE produce an increased activity of the AMP-activated protein kinase (AMPK), which is defined as an energy sensor involved in the maintenance of cellular energy homeostasis. Increased AMPK activity is inversely associated with the risk of cancer by

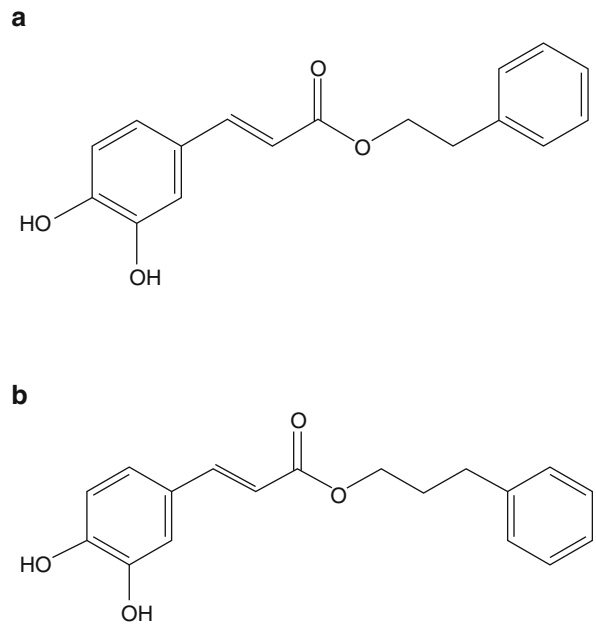


Fig. 3 Chemical structure of CAPE (**a**) and CAPPE (**b**). (Chiang et al. 2014)

suppressing mTOR activity and increasing apoptosis in cancer cells (Chiang et al. 2014).

As indicated by Monteiro et al., studies in hepatocarcinoma have demonstrated the antiangiogenic and antimetastatic capacity of CA and CAPE. The latter has shown the activation of intrinsic and extrinsic apoptotic pathways (Monteiro Espíndola et al. 2019). In addition, the antiangiogenic capacity of CAPE is given by the inhibition of HIF-1α, which has an increased expression in tumor cells due to the hypoxic environment in which they develop, thus increasing the expression of vascular endothelial growth factor (VEGF) (Monteiro Espíndola et al. 2019). Furthermore, CAPE has an antimetastatic capacity exerted through inhibiting the expression of matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9), by suppressing the expression of nuclear factor kappa B (NFκB) (Monteiro Espíndola et al. 2019). These molecules are known for their role in the degradation of the extracellular matrix, which is why they can promote metastasis as a result of their overexpression in cancer.

Additionally, CAPE successfully altered the mitochondrial membrane potential (MMP) in vitro, causing the release of Cyt c from the MMI. This event increases the activation of caspase 9, promoting apoptosis through the intrinsic pathway. Moreover, CAPE also activated the extrinsic pathway of apoptosis that is mediated by the apoptosis-inducing ligand related to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), through upregulation of the death receptor 5 (DR5) resulting from the activation of mitogen-activated protein kinase (p 38) (Monteiro Espíndola et al. 2019).

In studies with breast adenocarcinoma conducted by Kabała-Dzik et al., CA and CAPE have demonstrated a dose- and time-dependent cytotoxic effect, showing greater effects at 48 h rather than 24 h, with a concentration of 100 μM for CA compared to 10 μM for CAPE (Kabała-Dzik et al. 2018). CAPE showed a more powerful cytotoxic capacity than CA, presenting a lower IC_{50} (inhibitory concentration 50) value than the one exhibited by CA (Kabała-Dzik et al. 2018). CA and CAPE also induced the inhibition of migratory capacity in a dose-dependent manner, showing again that CAPE was more potent than CA (Kabała-Dzik et al. 2018).

6 Mitochondriotropic Derivatives of Caffeic Acid

Given the extensive evidence promoting caffeic acid and its derivatives as powerful antitumoral agents, the idea of creating mitochondria-targeted compounds based on CA emerged to maximize the arrival at their site of action (Teixeira et al. 2018). To achieve this goal, several methods have been developed including those based on compounds linked to triphenylphosphonium (TPP^+) (Zielonka et al. 2017).

TPP^+ is a lipophilic cation widely used to direct various molecules to the mitochondria since its delocalized positive charge allows selective accumulation within this organelle. This process occurs in two phases. First, the compound enters the cell guided by the electrical attraction generated by a negative plasma membrane potential ($\Delta\Psi p$) of 30–60 mV. Second, it enters the mitochondria as a result of the even more negative membrane potential of the MMI ($\Delta\Psi m$), which is between 150 and 180 mV. Therefore, TPP^+ acts as a driving force for its accumulation in the mitochondria against the concentration gradient, reaching intracellular concentrations 100–1000 times higher. This feature leads to micromolar range concentrations of these compounds, thus achieving millimolar concentrations within the mitochondria (Zielonka et al. 2017). Furthermore, the incorporation of an alkyl chain serves as a link between TPP^+ and the compound of interest, giving different degrees of lipophilicity depending on its length, being more lipophilic with a longer chain length and vice versa (Zielonka et al. 2017) (Fig. 4).

There are only a few studies of CA derivatives linked to a mitochondrial target delivery system such as TPP^+. Consequently, Teixeira et al. developed a series of mitochondriotropic antioxidants derived from CA named "AntiOxCINs." These antioxidants are constituted from a primary compound of CA linked to TPP^+ which they have

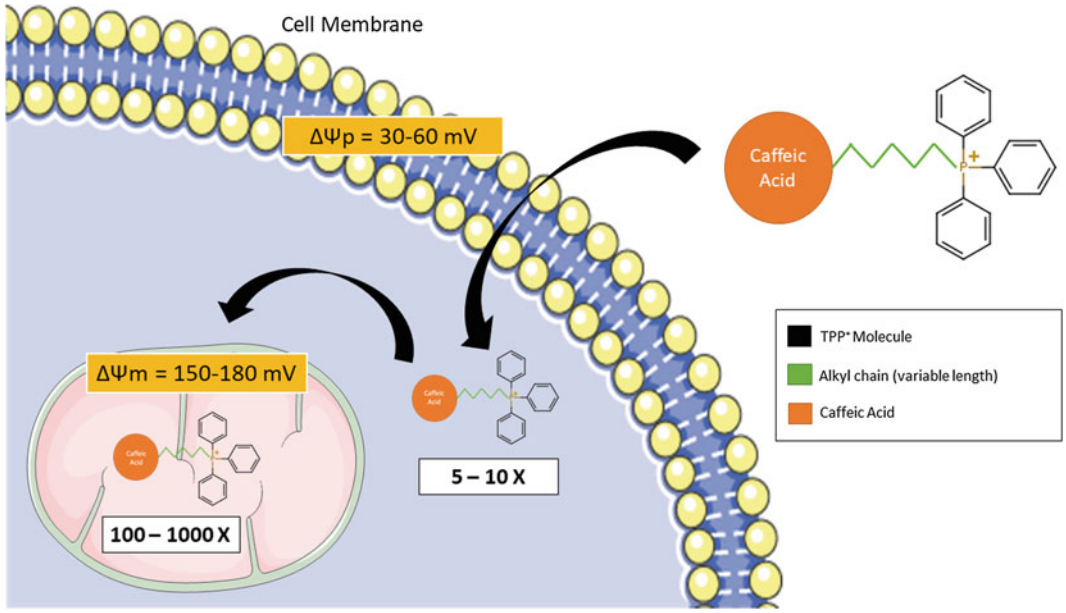


Fig. 4 General structure of TPP^+-based mitochondriotropic compounds and their intracellular and intramitochondrial accumulation driven by differences in plasma membrane and inner mitochondrial membrane potential, respectively

called "compound N° 1." Then, they synthetized novel compounds that present the catechol characteristic of CA, named as "compounds N° 22," "N° 23," and "N° 24." In addition, they synthetized another group of compounds derived from pyrogallol group named as "compounds N° 25," "N° 26," and "N° 27." Both groups of new compounds are identical, except for the length of the alkyl chain which can be of six, eight, or ten carbons, as shown in Fig. 5 (Teixeira et al. 2017). The results obtained show that AntiOxCINs present a high chelating capacity on transition metals (even similar to the activity exhibited by EDTA, a known chelating agent), and an increased mitochondrial uptake and ability to prevent mitochondrial membrane lipid peroxidation, compared to compound N° 1. In addition, the compounds were shown to induce the opening of the mitochondrial permeability transition pore (mPTP) (Teixeira et al. 2017), which has a known role in cell death. Its opening causes the mitochondrial release of Cyt c, thus initiating apoptotic signaling pathways (Grasso et al. 2020). Additional tests were performed on compounds N° 24 and N° 25, as they proved to be the most promising ones. The results illustrated their ability to prevent ROS and Fe^{+2}-related cytotoxicity in HepG2 cells at concentrations of 2.5 μM (compound N° 24) and 100 μM (compound N° 25), within incubation periods of 48 h. In addition, these derivatives did not induce proapoptotic changes related to nuclear morphology or mitochondrial depolarization in normal cells, suggesting their safe use in every type of cells (Teixeira et al. 2017).

Furthermore, in a study developed by Li et al. (2017), mitochondriotropic compounds based on hydroxycinnamic acid derivatives were synthetized, named "MitoHCAs" (Li et al. 2017). The mitochondriotropic character of these compounds was also achieved by binding to TPP^+ (Li et al. 2017). In relation to these novel compounds, four new mitochondriotropic compounds based on p-coumaric (Mitop-CoA), caffeic (MitoCaA), ferulic (MitoFA), and cinnamic (MitoCA) acids were synthesized (Fig. 6) and were subjected to several tests evaluating their antioxidant and antiproliferative capacities (Li et al. 2017). The results obtained showed that MitoCaA was the most powerful antioxidant since it inhibits lipid peroxidation

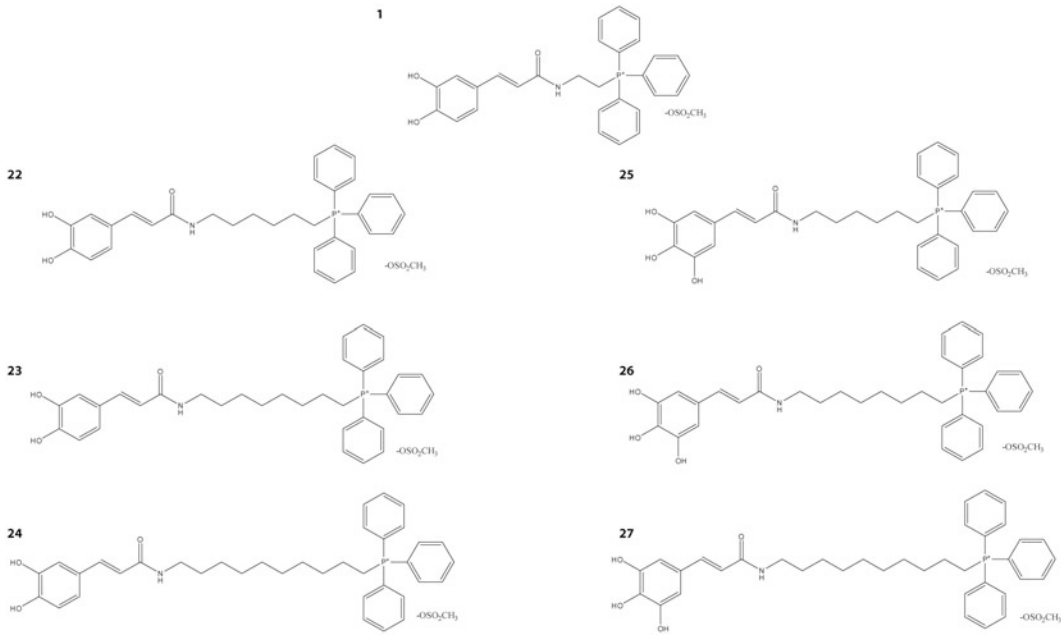


Fig. 5 Chemical structure of the caffeic acid mitochondriotropic derivatives "AntiOxCINs" developed by Teixeira et al. (2017). Compounds Nº 1, 22, 23, 24, 25, 26 and 27

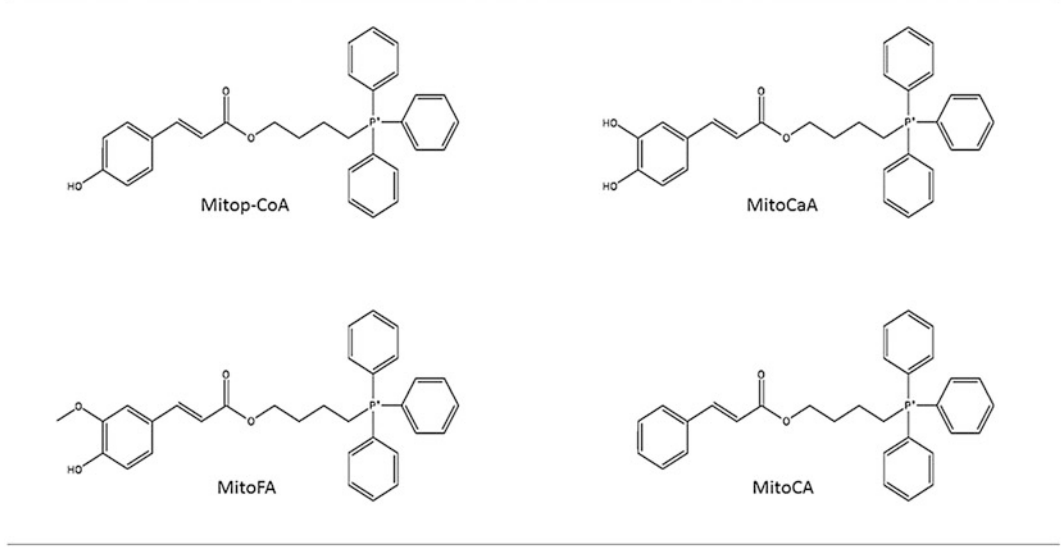


Fig. 6 Chemical structure of the hydroxycinnamic acid derivatives "MitoHCAs" developed by Li et al. (2017)

and exhibits a concentration-dependent behavior (Li et al. 2017). Subsequently, similar results were obtained when comparing the antioxidant capacity of MitoHCAs against endogenous ROS (H_2O_2) (Li et al. 2017). In addition – in order to comprehend the antioxidant mechanism by which MitoHCAs reduce H_2O_2 – results showed that MitoHCAs do not have direct radical elimination capacity and neither exert changes in the production of mitochondrial O_2^{-}. However, they do

affect the expression and activity of antioxidant enzymes: MitoCaA, MitoFA, and Mitop-CoA increased significantly the activity of the GSH-Px and CAT enzymes, suggesting this is the mechanism by which MitoHCAs decrease H_2O_2 (Li et al. 2017). Furthermore, both MitoCaA and Mitop-CoA were found to have an inhibitory effect on mitochondrial SOD, while MitoFA did not (Li et al. 2017). Additionally, antiproliferative capacity of MitoHCAs against human hepatoma HepG2 cells and normal cell lines (human liver L02 and WI38 diploid human fibroblasts) was also evaluated, resulting in a selective inhibition of cell viability over HepG2 cancer cells compared to normal cells (Li et al. 2017). MitoCA, MitoFA, and MitoCaA compounds demonstrated the most potent antiproliferative capacity against HepG2 cells at 48 h of cell treatment (Li et al. 2017). In addition, selectivity indexes (SI) for MitoHCAs were calculated, which correspond to the quotient between the antiproliferative activity of the compounds in normal cells and the same activity but in HepG2 cancer cells. The compounds with the higher SI were MitoCA, MitoFA, and MitoCaA. The latter was determined as the most selective (Li et al. 2017). Since MitoCaA was the compound with the highest antioxidant and antiproliferative capacity, additional studies were performed. The results showed that MitoCaA caused mitochondrial fragmentation in HepG2 cells in a dose-dependent manner, causing donut-shaped morphology and a discontinuous mitochondrial network at a concentration of 20 μM. Under the same experimental conditions, the compound failed to produce mitochondrial damage in normal L02 cells (Li et al. 2017), although apoptotic assays did demonstrate that MitoCaA possesses a dose-dependent apoptotic effect (Li et al. 2017). MitoCaA was able to induce the apoptosis mechanism through the opening of mPTP and its consequent release of mitochondrial Cyt c (Li et al. 2017). These results differ from the tests performed by Teixeira et al., where it was reported that CA-based mitochondriotropic derivatives would not have an effect on mPTP opening. The difference could be attributed to structural variability in molecular targets and mechanisms of action, despite they originated from the same compound, which requires further studies.

7 Conclusion

Polyphenols and especially CA have been described before as antitumoral, antimicrobial, and antioxidant agents. The novel mitochondriotropic derivatives of caffeic acid have shown to maximize their effects on tumor cells by allowing its selective accumulation within the cancer mitochondria, an organelle on which they exert their effects by altering the redox state and ultimately leading to mitochondrial-mediated apoptosis process. The effects of increased lipophilicity and the mitochondrial targeting product of the link to TPP^+ moiety are reflected in more potent compounds than those without TPP^+. The selectivity of these compounds for cancer cells is also highlighted, which could be given by the difference in MMP between normal and cancerous cells, the latter exhibiting a higher MMP that leads to the selective accumulation of mitochondrial derivatives. This would indicate a safe use of these compounds, also reducing side effects, a common problem in chemotherapy due to the low selectivity of these drugs. Therefore, in initial studies on tumor cell lines, mitochondriotropic derivatives of CA appear as promising and powerful antitumoral agents for future development of new molecules with targeted approach for cancer therapy, given their increased potency and selectivity.

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Author Contributions H.B., G.A.V., and M.C. wrote the manuscript. H.B. and G.A.V. did all the figures and table. G.A.V., G.C., J.A.J., and MC edited the manuscript.

Conflicts of Interest The authors declare no conflict of interest.

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Systematically Assessing Natural Compounds' Wound Healing Potential with Spheroid and Scratch Assays

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Abstract

Understanding cellular processes involved in wound healing is very important given that there are diseases, such as diabetes, in which wounds do not heal. To model tissue regeneration, we focus on two cellular processes: cellular proliferation, to replace cells lost to the wound, and cell motility, activated at the wound edges. We address these two processes in separate, drug responsive, in vitro models. The first model is a scaffold-free three-dimensional (3D) spheroid model, in which spheroids grow larger – to a certain extent – with increased time in culture. The second model, the scratch wound assay, is focused on cell motility. In conjunction with collagen staining, it analyzes changes to the coverage of the wound edge and wound bed. Our workflow gives insights into candidate compounds for wound healing as we show using manuka honey (MH) as an example. Spheroids are responsive to oxidative damage by hydrogen peroxide (H_2O_2) which affects viability but mostly produces disaggregation. Conversely, MH supports spheroid health, shown by size measurements and viability. In two-dimensional scratch wound assays, MH helps close wounds with relative less collagen production and increases the loose cellular coverage adjacent to and within the wound. We use these methods in the undergraduate research laboratory as teaching and standardization tools, and we hope these will be useful in similar settings.

Keywords

Collagen stain · Manuka honey · Murine stem cells · Tissue regeneration · Viability

Abbreviations

DAPI	4′,6-Diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
MRI	Montpellier Ressources Imagerie
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
PBS	Phosphate buffered saline
SDS	Sodium dodecyl sulfate

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1 Introduction

The science of wound healing has its roots in the human need for survival. Since ancient times, people from all over the world have found healing substances in their environment. In general, reports of natural compounds' healing abilities are limited to case studies, while more systematic assessment of their properties is reserved for purified fractions or individual active ingredients (Atanasov et al. 2021). Generally processes of regeneration after wound are described to include hemostasis (clot formation to limit blood loss), inflammation to remove damaged cells, proliferation with formation of immature tissue (granulation tissue) and contraction of the wound, and finally, maturation of the new tissue to restore tissue functionality (Rodrigues et al. 2019). To provide an in vitro system for assessment of such natural compounds in wound healing, we have focused on two aspects of tissue regeneration: cellular proliferation in a 3D in vitro model and cell motility measured in 2D scratch wound assays. We report here our efforts to standardize a workflow for in vitro testing of candidate natural products for wound healing. Prevalent animal models in the wound healing field can be thus displaced or substituted by mini tissue models that can be generated in vitro from relevant cells and contribute important scientific insights to tissue regeneration.

Spheroids have been chosen as the model 3D systems; as such, there are thousands of publications reporting their fabrication and attempts at standardization for application to high throughput assays (Brüningk et al. 2020; Virador et al. 2019). Our spheroids are made of NIH 3T3 cells (fibroblasts, according to ATCC information) which self-aggregate in the absence of extracellular matrix or scaffold. The term fibroblast describes a broad spectrum of cellular populations which are considered mesodermal cells, are not parenchymal, and have a prominent role in generating and maintaining the extracellular matrix; however, the distinction between fibroblasts and stromal cells is unclear and more so when their murine origin is embryonic or neonate (Robey 2017). Regardless of these debates, fibroblasts are generally agreed to be precursors of various features of the mesenchyme; they can be activated in response to various signals and participate in inflammation (Buckley et al. 2001), wound healing, or cancer, by secreting growth factors and proteolytic enzymes (Kalluri 2016). The cells we used in this work are the original Swiss albino 3T3 isolated from mouse embryos by Todaro and Green (Todaro and Green 1963). While spheroids formed from the adipogenic NIH 3T3-L1 subset have been well characterized (Graham et al. 2019), there is limited information on how spheroids of the original Swiss albino cell line are formed and sustained in the absence of scaffold. Here we offer a summary of our observations on the formation of these spheroids which appears to be chiefly dependent on the plastic surface and well size.

We established a reproducible workflow to form spheroids in a 96-well format, as well as time and end points to screen for natural compounds for wound healing. By testing cell viability as an end point, we intended to find compounds which consistently increased spheroid viability in a set time. These compounds would then be taken to the well-established scratch wound healing assay in 2D to verify their ability to increase the cell motility needed to close the wound. We added collagen staining of the 48-h wounds to our procedure to demonstrate whether the compound increases or decreases collagen production. In our analysis of scratch wounds, we looked at the traditional ratio of wound closure, but we also included the analysis of the loose space around the wound, providing insight on potential effects of the compound on cellular subpopulations affected by the wound. As an example of our workflow applied to a natural compound, we present results obtained with MH, a kind of honey native to New Zealand, produced by bees which pollinate the manuka bush flower (*Leptospermum scoparium*) and for which there are many reports showing positive effects in wounds (Tashkandi 2021).

2 Materials and Methods

2.1 Cells and Culture Conditions

The cells we used in our experiments are NIH 3T3 fibroblasts (CCL-92, ATCC, Manassas, VA). Cells were expanded and multiple vials were kept frozen under liquid nitrogen. A set of experiments was conducted from one expanded vial. Cells were used for a low number of passages (typically less than 7). Cells were monitored for mycoplasma contamination by visually assessing DAPI (4′,6-diamidino-2-phenylindole) stained samples. For regular culture maintenance and to expand cells for 3D experiments, cells were grown in T75 flasks in DMEM with high glucose, supplemented with 10% v/v bovine calf serum and 1% penicillin-streptomycin, at 37° C in a humidified atmosphere with 5% CO_2. Flat bottom 96-well plates (Corning, NY) were used for monolayer cell viability tests and flat bottom 12-well plates (Corning, NY) for scratch wound assays. For 3D spheroids, polystyrene round bottom low adhesion plates (Greiner Bio1, Cat 650,970, Monroe, NC) or non-tissue culture treated polystyrene flat well multiwell plates (VWR, 10861–556 or 10,861–558) were used, and similar sized tissue culture treated polystyrene flat well plates were used for comparison (e.g., CELLTREAT Scientific Products, Pepperell, MA).

2.2 Spheroid Formation for Compound Screening

Subconfluent cells with more than 90% viability as assessed by Trypan Blue staining were passaged with a 0.48 mM EDTA in PBS rinse (VERSENE, ThermoFisher, Waltham, MA) and exposure to 0.25% Trypsin/EDTA. Cells were centrifuged at 700 RPM for 5 min and resuspended in fresh media before seeding onto various surfaces as detailed in figure legends. In round bottom low adherence 96-well plates, 50,000 cells per well in 50 μl media were seeded. Outer wells contained only 100 μl of PBS. When spheroids had formed (typically by day 3 after seeding), 50 μl media containing the test compounds were added to each well. Cultures were monitored daily, and viability was assessed by adding MTT at the end of 1 week.

2.3 Dissolving Natural Compounds: Spheroid Treatment

MH, a product of New Zealand, was purchased from Costco (Y.S Eco Bee Farms, Sheridan, IL LOT # 9178 – the same lot was used throughout the project), and a stock solution of 1 g/ml was prepared as follows: first, 4 g of MH were weighed and dissolved in 1 ml of deionized water. The stock vial was kept at 37 °C for a few hours to overnight. When the honey stock was a fine suspension/solution, the total volume was assessed, and then the final solution was diluted with deionized water to a final concentration of 1 g/ml (w:v). From this stock solution, half-log dilution series was prepared with deionized water.

Commercially available 3% hydrogen peroxide from a freshly opened bottle was used to produce a half-log dilution series using deionized water. Care was taken to use a very small volume of each compound stock to produce final concentrations in spheroid medium.

When spheroids had formed (typically by day 3 after seeding), 50 μl media containing the test compounds were added to each well. This brought the total volume in well to 100 μl. Cultures were monitored daily.

2.4 Cell Viability and Stains

The MTT reduction assay (Berridge and Tan 1993) was used as follows: each well containing one spheroid received 10 μl MTT (Sigma-Aldrich, St. Louis, MO) dissolved in water to a final concentration of 0.5 mg/ml MTT. The spheroids were incubated at 37 °C and 5% CO_2 for 45 min, and then solubilizing reagent (10% SDS in 0.01 M HCl) was added. The plate was

maintained overnight at room temperature in the dark after which the amount of MTT formazan formed was measured at 450 nm in a Tristar2 Multimode Reader LB942 (Berthold Technologies, Oak Ridge, TN).

Mito tracker green (Molecular Probes) was dissolved in DMSO and added to the cultures at a 100 nM final concentration for 15 min in incubator prior to fixing.

Rhodamine-phalloidin (Molecular Probes) was dissolved in methanol and added to the cultures at nanomolar concentration according to manufacturer's recommendation. DAPI (Invitrogen) was dissolved in deionized water and added to the cultures according to manufacturer's recommendation.

Mason Trichrome stain to detect collagen in scratch wound assays was done as follows: Forty-eight hours post scratch, after documenting the live cell culture, cells were fixed with 4% paraformaldehyde, and then the protocol http://www.ihcworld.com/_protocols/special_stains/masson_trichrome.htm was followed.

2.5 Scratch Wounds

Subconfluent NIH 3T3 cells were seeded in 24-well plates at 10000 cells per well. Prior to seeding, a line was drawn on the underside of the wells to facilitate recording the same location of the wound over time. Two days later, medium was removed and a scratch was made by gliding a 100 µl pipette tip vertically through the center of the well, followed by a PBS rinse to eliminate floating debris and addition of media containing the test compounds. Pictures were taken immediately (time 0) at 24 and 48 h to document scratch reduction.

2.6 Imaging and Measurements

Fluorescence images were taken in an EVOS M5000 microscope with a highly sensitive 3.2 MP monochrome CMOS camera (2048 × 1536) with 3.45-µm pixel resolution (Thermo Fisher). For brightfield images, an EVOS XLCore (AMEX 1000, Invitrogen) was used. To measure spheroids, using brightfield or phase contrast, the focus was adjusted so that the images represented a circular cross section of the spheroid at its center, with the diameter of the circle matching that of the spheroid.

For scratch wounds, images were taken in the same area of each wound using the previously drawn marker and the wound as reference. The MRI Wound Healing plugin for ImageJ/Fiji® was used to analyze scratch wound images using the variance method (filter radius, 10; radius open, 4; min size, 10,000) https://github.com/MontpellierRessourcesImagerie/imagej_macros_and_scripts/wiki/Wound-Healing-Tool.

Original images, converted to 8-bit images, were used as input and processed with a fixed threshold value of 20 to find the area that contained no cells. Adjusting the selection threshold in the plugin settings up to a fixed threshold number of 100 allowed for distinction between empty space and loose cellular coverage. Ratio of closure was calculated by dividing the area given by ImageJ (in pixels) at the specific time by the area at time 0 in the same well.

To quantitate large cells, present in the wound area, ImageJ "analyze particles" plugin was used on 8-bit images with size (100-infinity) and circularity (0.50–1.00) settings. These settings were adopted after comparing the data obtained with results obtained by visual counting.

2.7 Histology

Spheroids were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 15 min, rinsed with PBS, and carefully placed between lens paper and then embedded and sectioned using routine protocols (summarized in https://www.corning.com/catalog/cls/documents/protocols/CLS-AN-431_DL.pdf). Hematoxylin- and eosin-stained sections were imaged in an EVOS XLCore microscope at 10 x magnification.

2.8 Statistics

Experiments had an N = 3 (three independent experiments) with six to eight biological replicates per condition unless indicated. Data were analyzed with Microsoft Excel and with

GraphPad Prism software (GraphPad, San Diego, USA). All the statistical analyses were performed with GraphPad Prism. Data are reported as mean ± SD *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$.

3 Results

3.1 NIH 3T3 Spheroids Self-Assemble from Cell Sheets in the Absence of Extracellular Matrix

CCL-92 is a 3T3-Swiss albino embryonic cell line from *Mus musculus*, house mouse, which grows as adherent monolayers on flat tissue culture surfaces. In 24-well non-tissue culture treated polystyrene plates, cells formed spontaneous clusters of varied shapes ranging from small colonies (between 100 and 200 μm in diameter) to larger spheroids (300 and 800 μm in diameter depending on plate and time in culture). We compared morphological stages of these spontaneous clusters and timing in various flat bottom plates. For example, at day 10 after plating, the non-tissue culture treated plate (VWR) had formed small colonies in 6 out of 24 wells, spheroids in 7 out of 24 wells, and stretched ribbons in 5 wells, while the remaining 6 wells contained small pieces of cell sheet curling at the edges. Sarstedt flat bottom tissue culture plate showed similar adhesions and pulling sheets which took longer to detach, and only fragments of cell sheets or flat sheets with ruffled edges were observed at day 10. In tissue culture treated plates with special adhesion properties for difficult to grow cells, for example, CELLTREAT, the pulling from the edges was observed in 15/24 wells, but the cell sheets never completely detached and sheet or cell clusters did not form even after 3 weeks in culture (Fig. 1a, top panels), while various shaped tissue clusters appeared in the VWR non-tissue culture plate in the same time frame (Fig. 1a, bottom panels). Most of the larger spontaneous clusters had tubular morphologies, and some resembled incomplete toroids. Detailed observation of the stages of cluster formation indicated that 2D compact cell sheets formed in various regions of the plate with one or more strong adhesions to the edges of the plate or to each other (Fig. 1b). In cases when the compact sheets had a stronger attachment to the plate, the edges curled (Fig. 1c), but in most cases complete rolling of the sheet edges occurred to form spheroids or pouches or wells filled with colonies and small spheroids (Fig. 1d). In some cases, adhesions on symmetrical ends of the cell sheet stretched and rolled over small colonies engulfing them (Fig. 1e). These observations agree with a recent report (Granato et al. 2017) of spontaneous clusters formed by human dermal myofibroblasts. Histology of typical tissue aggregates indicates that they possess a thick wall and some have a lumen (Fig. 1f–i).

Since it appeared that all different tissue aggregates formed in a similar fashion, we focused on documenting the formation of small spheroid pouches in 96-well round bottom plates. For reproducible spheroids, we found it was very important to put the plate after seeding on a flat surface at room temperature absent of any vibration, before putting it into the incubator, which is also proposed as a technique to reduce edge effect (Lundholt et al. 2003; White et al. 2019). First, we used Costar 7007 plates, made of ultralow attachment polystyrene. In these plates, the spheroids did not appear to go through the process of folding but appeared spheroidal in shape from the day of seeding. These spheroids had an average size of 0.15 mm^2 at day 12 with an increase to 0.19 mm^2 at day 15 and a progressive decrease to 0.17 mm^2 by day 23 consistent with spheroid maturation (Graham et al. 2019). Based on these observations, we speculate that spheroids made in those ultralow attachment plates may be similar to those made from cancer cells described as a "closely packed, spherical geometry of cells" (Mueller-Klieser 2000), but they do not appear to have undergone sheet retraction and folding.

We screened other 96-well polystyrene plates and found that CellStar suspension plates produced tissue structures from cell sheets. By observing the same well at days 4, 5, and 7 post seeding, we could document that spheroid formation begins by folding, rolling, and finally detaching from symmetrical stress fiber adhesions

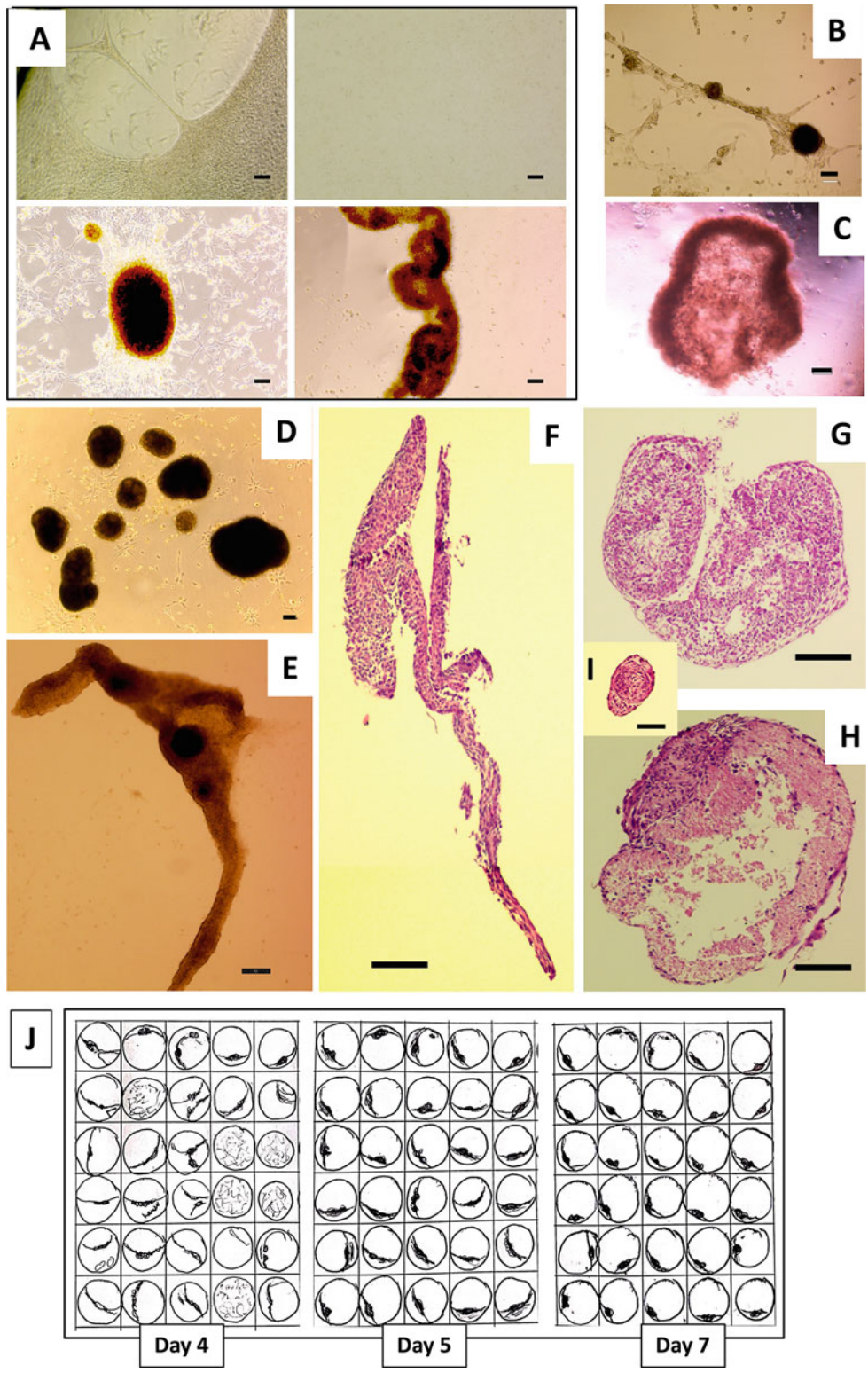

Fig. 1 Examples of tissue aggregates from cell sheets formed in low adhesion plates
(**a**) Three-week-old cultures of NIH3T3 cells (CCL92). Top panels show cells grown in CELLTREAT plates, left image shows the edge of the plate where sheets start pulling away, and right image shows tightly attached

to the concave well edges, thus undergoing similar stages to the non-tissue culture 24-well plates (Fig. 1j). Then, increased stretching was accompanied by rolling of the sheet like a candy wrapper, prior to formation of the spheroid, which resembled the closing of a pouch. In a small percentage of cases, flat sheets with ruffled edges were also observed in the 96-well plate due to imperfect pouch formation. Of note, these observations and drawings were part of a "Introduction to Scientific Research" class in a community college setting aimed at familiarizing students with cell biology and fostering scientific curiosity and systematic scientific skills.

3.2 Manuka Honey Is a Spheroid-Supporting Compound

To standardize 3D assays for multiwell plates, spheroids are preferred as they are more regular in size and reproducible. In preliminary experiments using MTT, we determined that 1-week treatment of spheroids without a medium change does not significantly alter their viability (data not shown) possibly because, once formed, spheroids have low metabolic activity compared with cells that are actively dividing (Granato et al. 2017; Fukushima et al. 2019).

Once we could reliably produce spheroids in 96-well plate and characterize their growth, we focused on standardizing methods to assess natural compounds reported as wound healing aids, many of which are part of botanical complex mixtures. To assess drug responsiveness of our model, we first chose H_2O_2, to induce oxidative stress (Sies 2017), and we compared its effect on confluent monolayers and on spheroids (Fig. 2a, b, c). As expected, the decrease of viability in response to H_2O_2 was more pronounced in monolayers. After testing a new candidate compound in monolayer, we proceeded to test spheroid drug responsiveness by assessing viability in a standardized manner in 96-well round bottom plates as shown in Fig. 2d.

Next MH with its antioxidant, antibacterial (Carter et al. 2016), and wound healing properties (Bulman et al. 2017; Frydman et al. 2020; Mokhtar et al. 2020; White 2016) was chosen to test our workflow. We found that when MH was present in the culture from the time of cell seeding (in a range of concentrations from 0.3 to 100 mg/ml), spheroids formed by day 3 in 60% of the wells compared to 20% in its absence. From this early data, we concluded that MH was a spheroid-supporting compound, whereas H_2O_2 was a spheroid-damaging compound, and this was supported by the viability data (Fig. 2e) where H_2O_2 produced a small but consistent decrease in viability while MH produced a dose-dependent small but consistent increase in viability suggesting a small increase in proliferation (Fig. 2f). Our observations indicated that spheroids responded to oxidative damage by initially disassembling, consistent with oxidative stress effects on tight junctions (Gangwar et al. 2017). Interestingly, spheroids were able to reassemble similarly to the findings of (Brüningk et al. 2020), and this reassembly was prevented by a second addition of H_2O_2 (data not shown). We speculate that this ability to reassemble may be one of the reasons for the more limited

Fig. 1 (continued) monolayer. Bottom panel, examples of cell clusters formed in wells of VWR non-tissue culture treated plates. (**b**) Colonies are formed as cell clusters pull away from each other. (**c**) An incomplete spheroid formed by rolling the edges of a flat sheet. (**d**) Small colonies and spheroids 200–500 μm in diameter. (**e**) Several colonies formed on a sheet are enveloped as the sheet rolls and gathers. (**f**) Hematoxylin-eosin stained section of a long cluster with tubular end. (**g,h**) Hematoxylin-eosin stained sections of typical 500 μm spheroids. (**i**) Hematoxylin-eosin stained section of a small 200 μm colony. (**j**) Detailed drawings of spheroid formation in 96-well low adhesion plate containing 50,000 cells per well; the same well is drawn at day 4, 5, and 7 post seeding. Scale bars, 100 μm

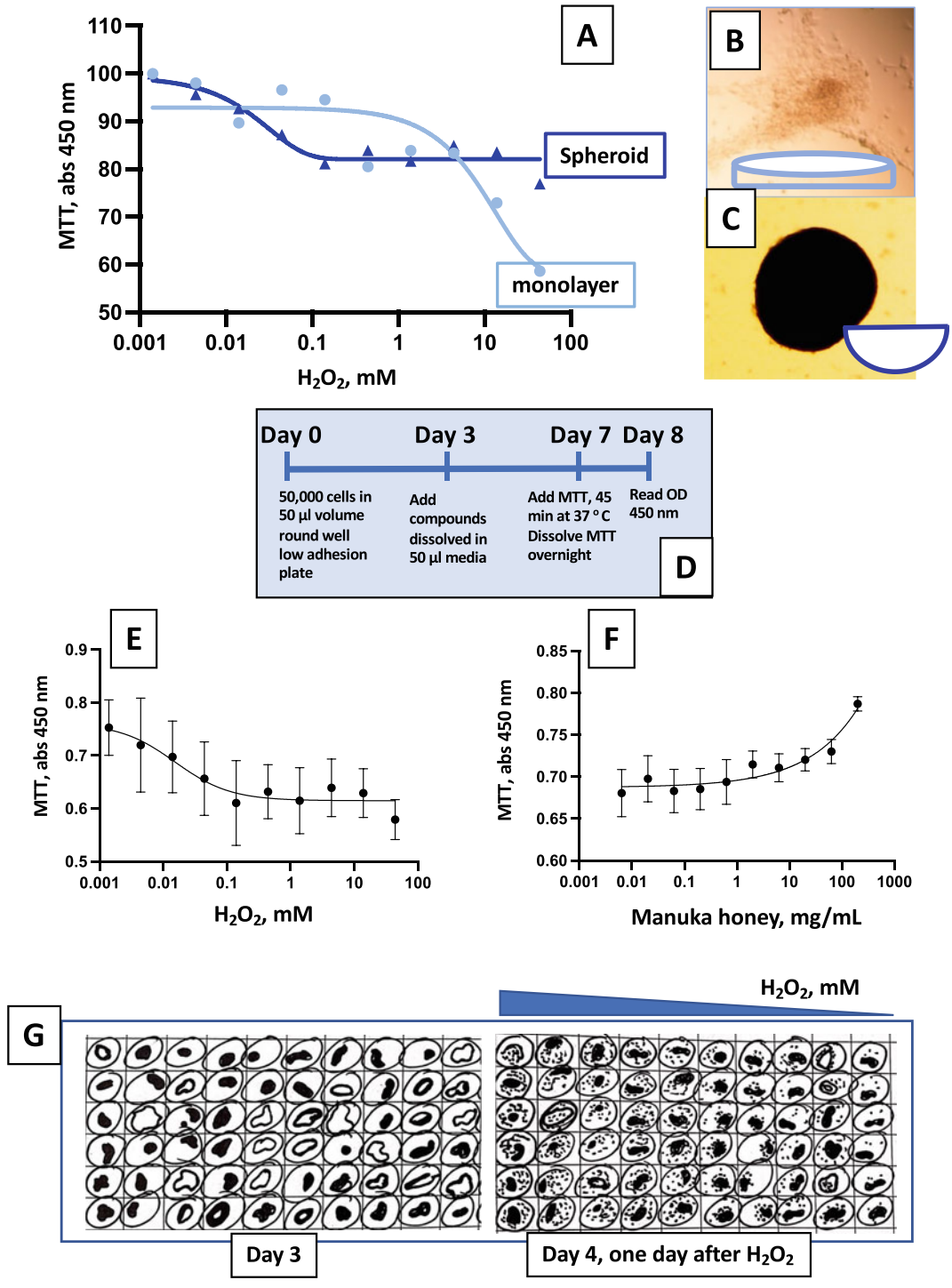

Fig. 2 Spheroids are responsive to drug treatments Response of confluent monolayer of 10,000 cells per well (light blue line and panel B) vs. spheroids (dark blue line and panel C) to increasing concentrations of H_2O_2. D. Standardized treatment scheme applied to spheroids for all compounds tested. E. Cell viability in response to H_2O_2 treatment measured by MTT absorbance (450 nm). F. Cell viability in response to MH treatment measured by MTT absorbance (450 nm). Summary data from three independent experiments with six technical replicates for each concentration. G. H_2O_2 causes disaggregation of the spheroids. Left panel, spheroid plate at day 3 prior to adding H_2O_2; right panel, spheroid plate at day 4, after 24 h of addition of H_2O_2 at the same concentrations as shown in panel E

decrease in viability observed when comparing to H_2O_2 treated monolayers.

Next, we measured spheroid surface area at several concentrations of H_2O_2 and MH. We found that at low H_2O_2 concentrations when viability is decreasing, spheroids have smaller sizes than control (Fig. 3a, b), but at higher than 0.1 mM spheroids actually became bigger, perhaps due to swelling or to different patterns of shrinkage and regrowth as seen in other studies (Brüningk et al. 2020). For MH we found a small but consistent increase in spheroid size at the concentrations we tested (Fig. 3c, d).

3.3 Manuka Honey Increases Cell Motility in Scratch Wound Assays

After MH had been established as a spheroid-supporting compound, we used the scratch wound assay to investigate the compound's effect on cell motility. Our method to produce and document the scratch wounds was adapted from (Pinto et al. 2019). Scratch wound assays are typically analyzed by segmenting the images with imaging programs to document and quantitate the amount of closure of the wound in a given amount of time, generally 24 h. For example, the MRI wound healing plugin is extensively cited in these studies (Kauanova et al. 2021). Basically, the method highlights different areas in the image by replacing each pixel with the specified neighboring area through a variance filter with a specific radius to effectively differentiate the aspect of tissue from the empty areas (also see (Suarez-Arnedo et al. 2020)). These studies generally look for disappearance of the empty space in the wound bed. Using ImageJ plugin "MRI wound healing tool" to follow the decrease in empty space in the presence of MH, we found no significant difference (Fig. 4a).

We realized that analyzing the images of the empty space did not provide sufficient

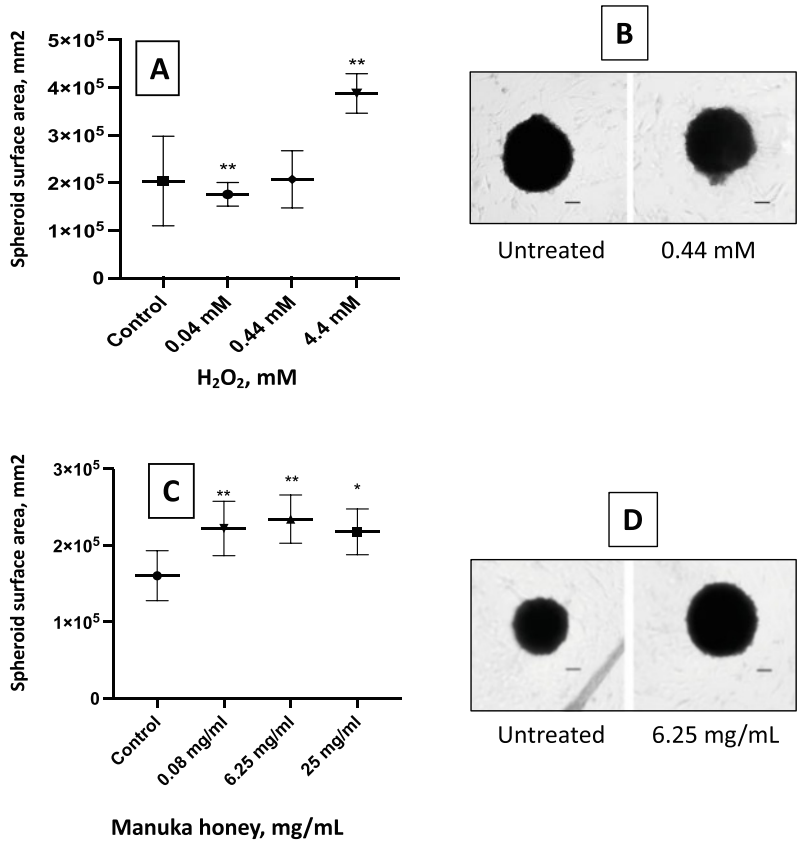

Fig. 3 MH is a spheroid-supporting compound (**a**) Size change in spheroid in response to H_2O_2. (**b**) Representative spheroid pictures taken at day 7 of the treatment protocol left spheroid control, right spheroid with 0.44 mM H_2O_2. (**c**) Size change in spheroid in response to MH. (**d**) Left spheroid control, right spheroid with 6.25 mg/ml MH. Experiments were carried out twice with six technical replicates per concentration, and one representative experiment is shown

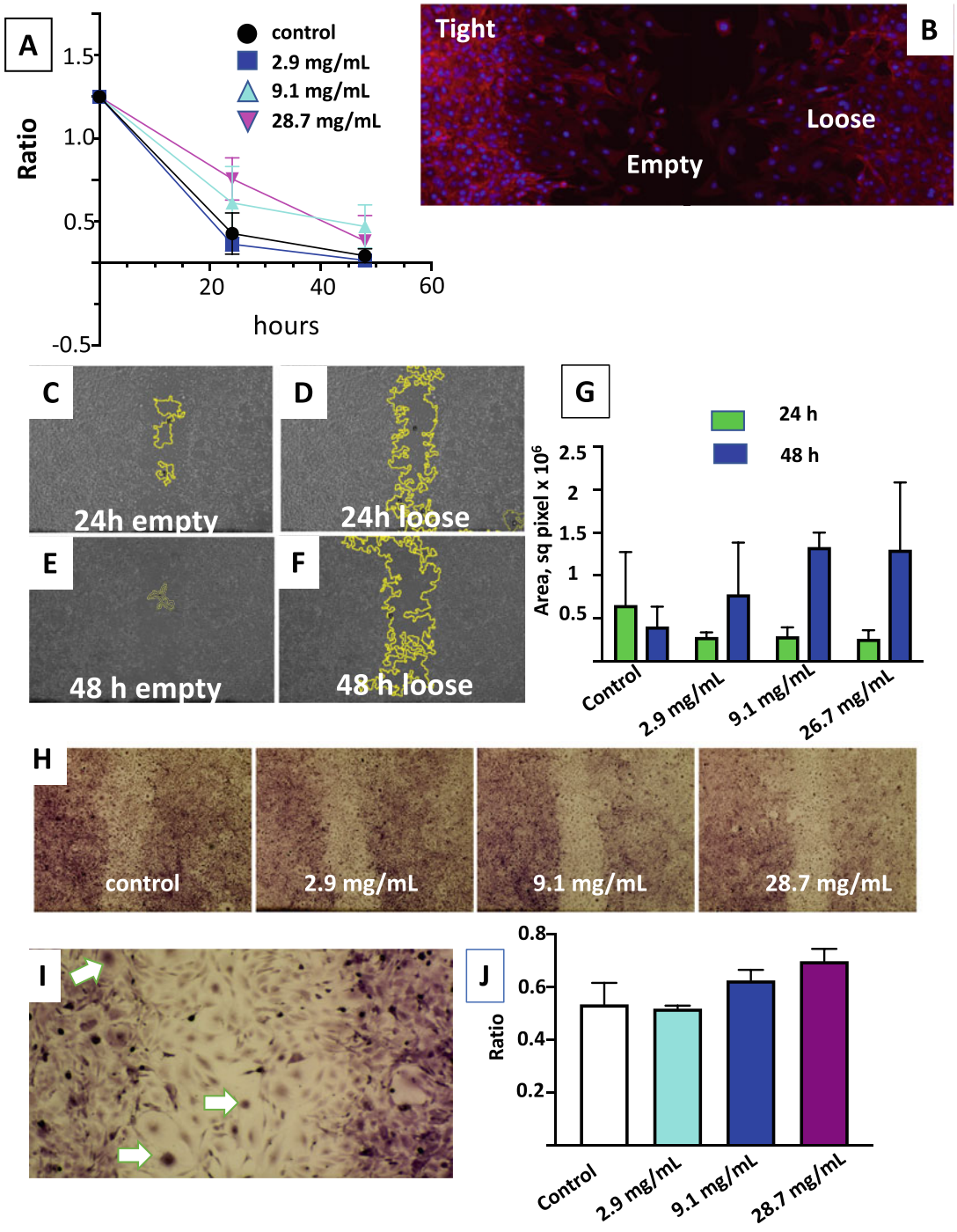

Fig. 4 Scratch wound assays treated with MH (**a**) Closure of the wound, ratio of empty space (treated vs. control) at 24 and 48 h post wound. Unpaired t test, NS. (**b**) Rhodamine-phalloidin stained wound demonstrating three kinds of cellular coverage: empty, loose, and tight. (**c**) Cellular coverage quantified with ImageJ MRI wound healing tool by assigning fixed thresholds to each image; as example, 24-h scratch treated with 2.9 mg/ml honey, threshold 20 to quantify empty space. (**d**) Twenty-four-hour scratch treated with 2.9 mg/ml honey, threshold 100 to quantify loose space. (**e**) Forty-eight-hour scratch treated with 2.9 mg/ml honey, threshold

information regarding the closure of wounds because the wound margin did not completely disappear at 24 h or even at 48 h in our experiments, and this margin was clearly noticeable when we stained for collagen; therefore, we looked for alternative ways to analyze our images which could provide insights as to what effects the compound might have on the complex processes occurring around and within the wound.

With the MRI wound healing plugin, we first tried to divide the images into three regions based on visual assessment of their morphology which correlated with the pixel density obtained from ImageJ; we could thus distinguish between empty space; loose coverage, characterized by motile cells; and the remaining tight coverage, denser cellular coverage surrounding empty and loose coverage. In other words, loose-empty space represents visual culture heterogeneity away from the homogeneous tight space (Fig. 4b). As we saw no significant change in the tight coverage at 24 h, we focused on analyzing empty and loose coverage by the ImageJ variance method after choosing two thresholds that were representative of empty space and loose space (Fig. 4c–f). Next, we followed the empty and loose space at 24 and 48 h post wound with various concentrations of MH, and we found there was a trend toward increased loose space with MH treatment at 48 h (Fig. 3g), indicating an increase in culture heterogeneity. We wondered if that correlated with an increase in fibrotic cells and stained for collagen in the 48-h wounds. Interestingly, our results suggest that MH does not increase collagen but rather decreases it (Fig. 4h–i), and this supports recent reports of scarless healing with MH (Singh et al. 2018).

We wondered what cells could contribute to the measured increase in loose space and turned our attention to flat giant cells, which appear to be interpreted by the MRI tool as empty space within covered space. These may be myofibroblast precursor cells, or perhaps fibrocytes (Reilkoff et al. 2011; Tomasek et al. 2002), mesenchymal cells with features both of fibroblasts and macrophages involved in tissue remodeling. Visual assessment and quantitation of flat giant cells in scratch wound images within and outside the wound area using ImageJ suggested that the number of such cells increases in MH treated scratch wounds, particularly in the wound area.

Taken together, our results presented here give added quantitative evidence to qualitative and anecdotal studies of MH applications in human wound healing.

4 Discussion

4.1 Scaffold-Free Spheroids from NIH 3T3 Cells Form by Substrate-Specific Collective Cellular Processes from Cell Sheets

There are different forms of 3D cellular aggregates broadly grouped under the terms "spheroids" and "organoids" (a very good current review of the nomenclature is found in (Decarli et al. 2021)). Both kinds of aggregates may form spontaneously in culture from cellular populations which contain a large proportion of stem cells or early progenitors. Organoids, characterized by their ability to organize in a manner similar to their tissue of origin, usually contain multiple cell types (Clevers 2016); they arise in tissue culture either by disaggregation of tissues and limited culturing or from coaxing stem

Fig. 4 (continued) 20 to quantify empty space. (**f**) Forty-eight-hour scratch treated with 2.9 mg/ml honey, threshold 100 to quantify loose space. (**g**) Loose minus empty space for each MH concentration. (**h**) Collagen staining 48 h post scratch in MH treated wounds. (**i**) Flat giant cells observed within and outside the margins of the wound. (**j**) Ratio of flat giant cells occupying the space within the wound to total flat giant cells in the image after 48 h. Data are representative of at least two independent experiments with three technical replicates per condition

cells into aggregation by modifications of their environment. The term spheroid is reserved for multicellular spherical shaped clusters of cells from homogeneous cell populations or cell lines of various tissue origins. Such cellular aggregates clump together and maintain their spherical shape with or without exogenous extracellular matrices. For difficult to culture cells, various scaffolds made of hydrogel materials are required (Caliari and Burdick 2016). The spheroids we describe here are very similar in their appearance to those shown by (Granato et al. 2017) formed using human dermal cells aggregated in hanging drops and collected in agarose coated plates, whereas our spheroids form and are maintained in the absence of scaffold. Similar to spheroids from cancer cell lines, commonly used in 3D screens for drug discovery, which are relatively easy to form and maintain scaffold-free, our spheroids are also good models for 3D screens because the absence of scaffold decreases complexity and variability for the screen. This study adds to the relatively scarce literature characterizing spheroid cultures with fibroblasts in the absence of hydrogel materials (Jorgenson et al. 2017; Graham et al. 2019).

It is clear that the stages of formation of spheroids differ greatly among various cell types (Smyrek et al. 2019; Livoti and Morgan 2010) and particularly if one compares cancer line derived spheroids to those originated from progenitor populations. Our work sheds light into a specific mode of formation of stem cell derived spheroids from collective rolling of cell sheets which is highly dependent on interaction with the surface. Using sarcoma cells on polyacrylamide substrates (Beaune et al. 2018) observed cell sheet collective behavior to be highly dependent on surface rigidity. We report here that, only using low adhesion plates, the stages of spheroid formation consisted of the production of a thin layer of cells spreading through the surface which pulled and broke in various places in the same time frame (roughly within 1 min) to produce a collective rolling edge of cells that quickly gathered to a pouch to close a spheroid if the surface area was small. With the same kind of adhesion and increased surface area, the stages of aggregate formation were similar but produced varied shapes and sizes.

4.2 Using Spheroids and Scratch Wound Healing Assays to Screen Natural Compounds

Botanical complex mixtures have been used since ancient times to heal minor wounds and scratches. Despite many hurdles to the research and development of natural compounds, there is renewed interest in their therapeutic potential, especially as sources for new antimicrobial treatments. Important considerations when characterizing natural compounds are their difficult solubility and availability to the cells in culture, the inherent difficulties in bioactive compound isolation and in elucidating its cellular target (Atanasov et al. 2021), and the fact that synergisms between various components with beneficial therapeutic outcomes (Schmidt et al. 2007) may obscure the contribution of a component in the mixture to the overall bioactivity of a natural compound.

We start characterization of spheroid-supporting or spheroid-disrupting compounds by measuring cell viability with the colorimetric MTT assay. The use of this method is not without controversy due to low reproducibility (Stepanenko and Dmitrenko 2015), but, in our experience, it serves didactic purposes. Our students visualize cellular activity under the microscope after a few minutes' incubation, and they also learn to collect colorimetric data and, more importantly, learn to appreciate the differences between monolayers and spheroids (Fig. 2a). Even though it is currently accepted that 3D in vitro models are superior to monolayers in drug screening, it is harder to find the most relevant end point to assess bioactivity (White et al. 2019). In our case, since we have characterized the formation of the spheroids, preventing formation via oxidative stress and supporting it with compounds such as MH, we are now in the position to set up screening for compounds that facilitate pulling and rolling of sheet edges to aid spheroid formation and support its stability.

Scratch assays are relatively simple assays that have provided important information about cell motility and have been used for drug screening. However, more information can be gained from more sophisticated image analysis as others have recently shown (Kauanova et al. 2021). Our image analysis focuses on measuring the clearly different region near and within the wound that is covered by highly motile cells. This phenomenon is useful as a unit of study because its speed of formation and cellular components can be altered by the presence of natural compounds such as MH.

There is some controversy in the literature as to whether or not spheroids may be fibrosis models. Some argue they are, because they can contain activated myofibroblasts (Kisseleva 2017), whereas others (Granato et al. 2017; Avagliano et al. 2019) suggest that spheroids are not fibrosis models because the myofibroblasts within them become deactivated and that their formation closely resembles the physiological modes of skin wound healing. Spheroids from dysregulated transcriptional coactivator with PDZ-binding motif (TAZ) grow more than normal (Jorgenson et al. 2017), and these could be considered a model of fibrosis. Our results with spheroids formed with NIH 3T3 cells support Granato's conclusions suggesting our spheroids are physiologically stable since (a) acidification of the media does not happen in spheroid containing wells while it happens in actively growing monolayers (our unpublished observations) and (b) there is no significant change in spheroid size after 2 weeks in culture. Though we have yet to ascertain the reason for some increase in size of the spheroids with MH, this change does not seem to be due to increased ECM production (collagen) because in our scratch wound assays, visually the amount of collagen accumulated in cells nearing the wound was less in MH treated wounds in accordance with a recent report of scarless wound healing with MH (Singh et al. 2018). Our analyses of the scratch wounds also uncovered the possible contribution of flat giant cells to the increased loose coverage with MH treatment. It is possible that MH increases the number and proliferation of these cells which may be proto myofibroblasts (Tomasek et al. 2002; Avagliano et al. 2019) or fibrocytes (Reilkoff et al. 2011). We still do not know the nature of these large cells, just that they are part of the loose coverage, and our future work will address this.

4.3 Usefulness of Our Proposed Workflow

With the workflow presented here, we can get insights on cellular events that may be modified by a wound healing compound. This can be useful for further characterization of the compound; similar to MH, other compounds that produce a moderate increase in viability can then be taken to the scratch assays in 2D to verify their ability to increase the cell motility needed to close the wound. In our analysis of scratch wounds, we look at the traditional ratio of wound closure, but we also include the analysis of the loose space around the wound, providing insight on potential effects of the compound on cellular subpopulations affected by wound. We added collagen staining of the 48-h wounds to our workflow to demonstrate whether the compound increases or decreases collagen production as a readout for fibrosis.

There are many case reports or anecdotal reports about natural compounds used in wound healing, many of which are based on animal testing, while our workflow uses in vitro systems that do not rely on animal testing.

Finally, we use these methods to teach cell biology and introduction to research to undergraduate students with very limited experience in the lab. The workflow was established as a collaborative effort among the students in a low budget setting. We hope these methods can be adopted and expanded in similar settings.

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Contributions G. V. performed experiments, analyzed data, and reviewed the manuscript; M. A. analyzed data and prepared figures; L. P., S. A., M. V., D. C., W. T., and N. T. performed experiments and analyzed data; and V. V. conceived the studies, performed experiments, analyzed data, and wrote the manuscript. All authors read and approved the manuscript.

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