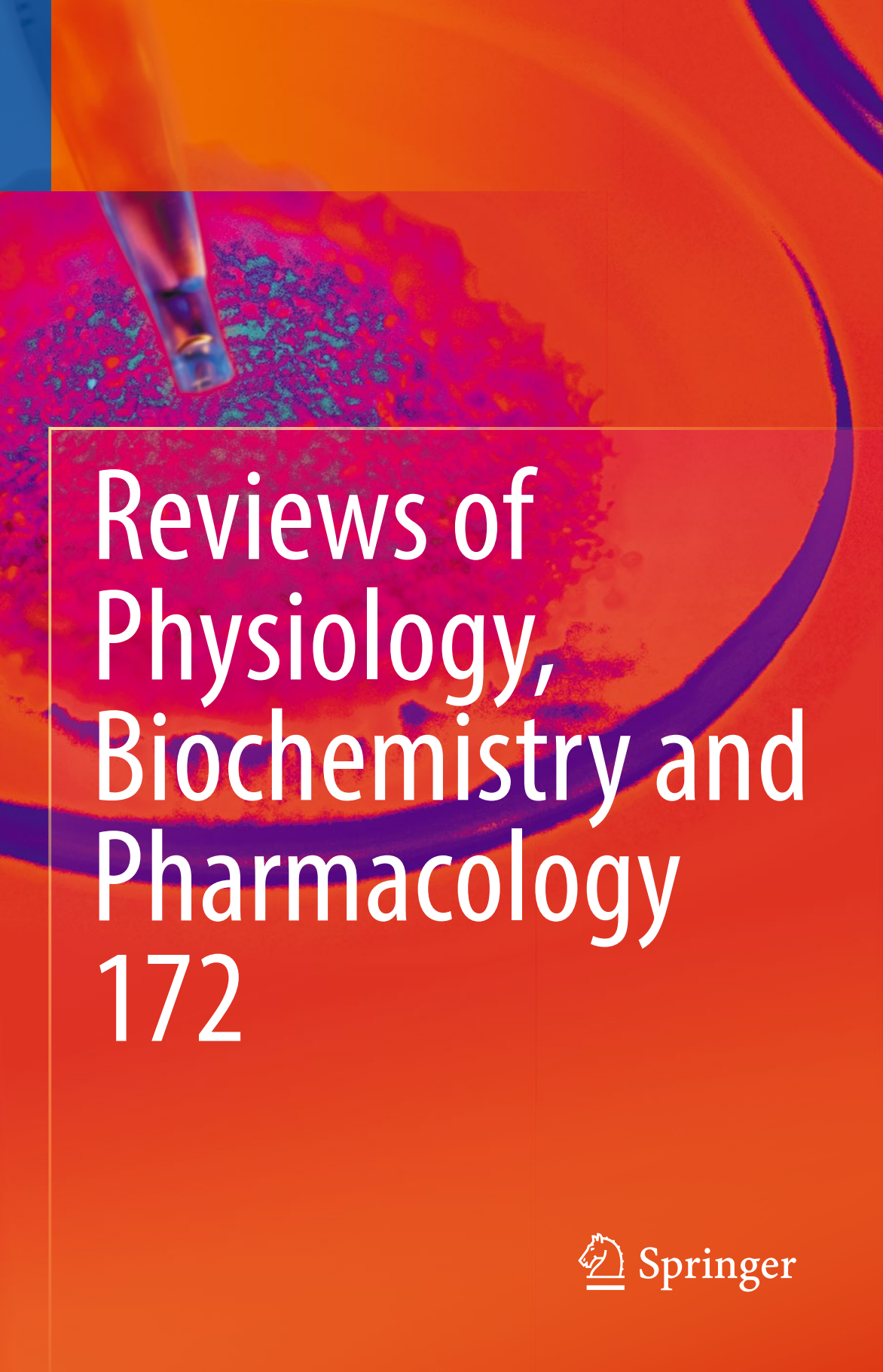
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Hepatic Stellate Cells in Liver Fibrosis and siRNA-Based Therapy

Refaat Omar, Jiaqi Yang, Haoyuan Liu, Neal M. Davies, and Yuewen Gong

Abstract Hepatic fibrosis is a reversible wound-healing response to either acute or chronic liver injury caused by hepatitis B or C, alcohol, and toxic agents. Hepatic fibrosis is characterized by excessive accumulation and reduced degradation of extracellular matrix (ECM). Excessive accumulation of ECM alters the hepatic architecture leading to liver fibrosis and cirrhosis. Cirrhosis results in failure of common functions of the liver. Hepatic stellate cells (HSC) play a major role in the development of liver fibrosis as HSC are the main source of the excessive production of ECM in an injured liver. RNA interference (RNAi) is a recently discovered therapeutic tool that may provide a solution to manage multiple diseases including liver fibrosis through silencing of specific gene expression in diseased cells. However, gene silencing using small interfering RNA (siRNA) is encountering many challenges in the body after systemic administration. Efficient and stable siRNA delivery to the target cells is a key issue for the development of siRNA therapeutic. For that reason, various viral and non-viral carriers for liver-targeted siRNA delivery have been developed. This review will cover the current strategies for the treatment of liver fibrosis as well as discussing non-viral approaches such as cationic polymers and lipid-based nanoparticles for targeted delivery of siRNA to the liver.

Keywords BMPs • Liver fibrosis • siRNA • Stellate cells • Targeted delivery

R. Omar, J. Yang, H. Liu, and Y. Gong (✉)
College of Pharmacy, Faculty of Health Sciences, University of Manitoba, 750 McDermot Avenue, Winnipeg, MB, Canada R3E 0T5
e-mail: Yuewen.Gong@ad.umanitoba.ca

N.M. Davies
College of Pharmacy, Faculty of Health Sciences, University of Manitoba, 750 McDermot Avenue, Winnipeg, MB, Canada R3E 0T5

Faculty of Pharmacy & Pharmaceutical Sciences, University of Alberta, 8613-114 Street, Edmonton, AB, Canada T6G 2H1

Contents

1 Introduction

Fibrosis of the liver is a reversible response following liver injury (Lee and Friedman 2011). Although fibrosis is an attempt to minimize the liver injury, the liver function is significantly impaired. The major causes of liver injury include chronic viral infection by hepatitis B and C; excessive alcohol consumption; nonalcoholic steatohepatitis (NASH); iron overload; autoimmune disorders, such as primary biliary cirrhosis and autoimmune hepatitis; drug-related toxicity; cholestasis; and inherited metabolic diseases such as hemochromatosis as well as Wilson's disease and Alfa 1-antitrypsin deficiency (Li et al. 2008; Adrian et al. 2007; Lee and Friedman 2011).

Hepatic fibrosis is characterized by excessive accumulation and reduced degradation of extracellular matrix (ECM). Accumulation of ECM is due to increased production and decreased degradation of ECM (Arthur 2000; Bataller and Brenner 2005). Decreased degradation of ECM is due to imbalance between matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinase (TIMPs) that regulate the ECM degradation processes (Knittel et al. 1999b; Tacke and Weiskirchen 2012). After liver injury, TIMP-1 is overexpressed resulting in decreased removal of ECM (Bataller and Brenner 2005).

Accumulation of ECM alters the hepatic architecture by forming a fibrous scar and development of nodules leading to cirrhosis (Friedman 2003). Cirrhosis increases the intrahepatic resistance to blood flow, which results in hepatic insufficiency and portal hypertension (Fig. 1). This effect leads to failure of common functions of the liver including metabolism of proteins, carbohydrates, and lipids; protein synthesis; and detoxification of chemicals, drugs, and other xenobiotic

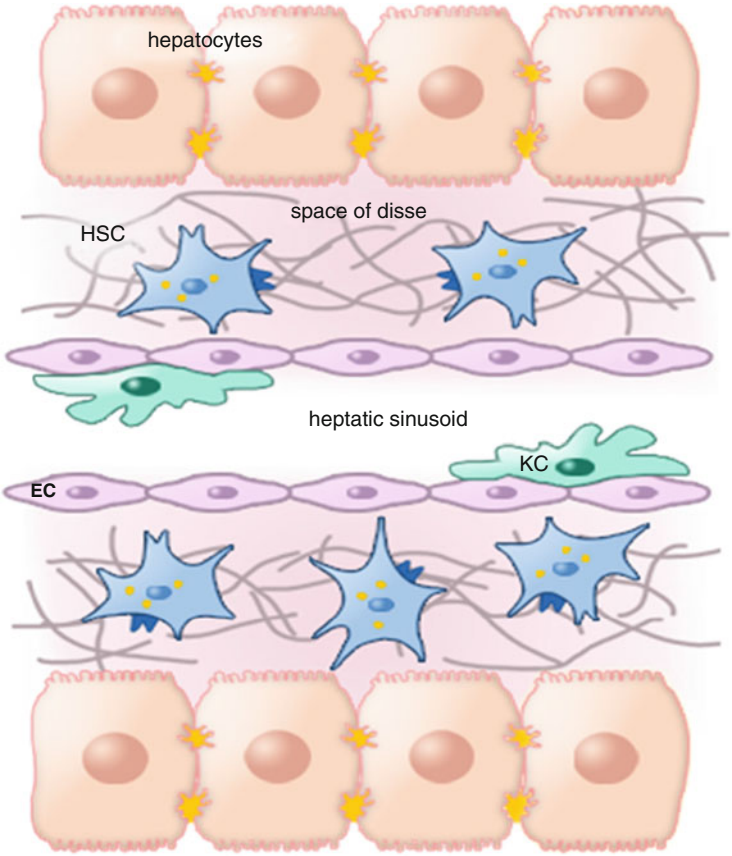

Fig. 1 Schematic representation of the liver. Kupffer cells (KC) are found in sinusoids. Hepatic stellate cells (HSC) are located in the space of Disse, between endothelial cells (ECs) and hepatocytes. ECs feature the walls of sinusoids and possess fenestration that provide a selective barrier between the blood stream and the space of Disse

compounds and their clearance from the body (Hui and Friedman 2003). There are various cells that are involved in the development of liver fibrosis such as hepatocytes, Kupffer cells (KCs), endothelial cells (ECs), and hepatic stellate cells (HSC). However, HSC are still the major player in the development of liver fibrosis as HSC are the main source of the excessive production of ECM in an injured liver (Maher and McGuire 1990; Wu and Zern 2000).

2 Hepatic Stellate Cells

Hepatic stellate cells (HSC) are also referred to as vitamin A-storing cells, fat-storing cells, lipocytes, and Ito cells. HSC are non-parenchymal cells of the liver representing 1.4% of total liver volume and 5–8% of the total liver cells. HSC are located in the perisinusoidal space which is known as the space of Disse between hepatocytes and sinusoidal endothelial cells. This anatomical position of HSC provides physical contact to the sinusoidal endothelial cells and to the hepatocytes (Hui and Friedman 2003). HSC are present in two different phenotypes that exhibit various structures and behaviors: the quiescent phenotype in the normal liver and the activated myofibroblast-like phenotype in the injured liver

2.1 Functions of HSC in the Normal and in the Injured Liver

2.1.1 Storage of Vitamin A

The primary function of quiescent HSC in the healthy liver is the storage of retinoid (vitamin A). The liver stores around 70% of the total retinoid found in the body. HSC store about 90–95% of hepatic retinoid in their lipid droplets as retinyl esters with the help of retinol-binding protein (RBP). Therefore, HSC constitute the largest reservoir of vitamin A in the body (Blaner et al. 2009).

2.1.2 Extracellular Matrix (ECM) Synthesis and Degradation

HSC are mainly responsible for the production of ECM in the liver and for the synthesis of enzymes that regulate ECM degradation (Maher and McGuire 1990); consequently, HSC are the major players in the development of liver fibrosis. Generally, the quiescent HSC in the normal liver secrete adequate amount of ECM proteins such as collagen type III, collagen type IV, and laminin. In addition, HSC secrete several degrading enzymes called matrix metalloproteinases (MMPs), such as MMP-1, MMP-8, and MMP-13 which promote ECM degradation. HSC also produce inhibitors called tissue inhibitors of matrix metalloproteinase (TIMPs), such as TIMP-1 and TIMP-2. The balance between MMPs and TIMPs regulate the ECM degradation processes (Tacke and Weiskirchen 2012; Knittel et al. 1999b).

2.1.3 Liver Development and Regeneration

HSC play an important role in liver growth and regeneration due to their anatomical position. HSC surround sinusoids in a cylindrical manner that enables them to

control the blood flow through those sinusoids and regulate the sinusoidal tone. In addition, they secrete vasoactive proteins, such as substance P, neuropeptide Y, and somatostatin (Geerts 2001). HSC have been detected within the progenitor cell zone of liver near the canal of Hering (Friedman 2008b). It has been found that HSC promote maturation of hepatic progenitor cells through cell–cell contact in a culture of mouse fetal liver-derived Thy1 cells (Baba et al. 2004; Friedman 2008b).

Furthermore, HSC express epimorphin, which is a mesenchymal morphogenetic protein that regulates liver regeneration especially after partial hepatectomy. In addition HSC produce morphogenetic proteins such as hepatocyte growth factor (HGF) (Ramadori et al. 1992) and pleiotrophin (Asahina et al. 2002) that stimulate hepatocyte proliferation and regeneration, respectively. HSC also express vascular endothelial growth factor (VEGF) that stimulates the growth of both sinusoidal and vascular endothelial cells (Ishikawa et al. 1999). Moreover, HSC secrete various molecules that regulate homeostasis process within the liver, such as endothelin-1 (Shao et al. 1999), transforming growth factor-β (TGF-β) (De Bleser et al. 1997), and erythropoietin (Maxwell et al. 1994).

2.1.4 Immunoregulation Function

When liver injury takes place, various cell types including HSC release inflammatory cytokines such as platelet-derived growth factor (PDGF), which is the most potent mitogen and chemotactic mediator (Pinzani et al. 1989; Friedman 2008b). Transforming growth factor (TGF-β1) is also released, which is the most powerful fibrogenic cytokine to HSC and plays a significant role in liver fibrosis development (Friedman 2008b).

In addition, HSC are able to interact with lipopolysaccharides (LPS) of Gram-negative bacteria (Brun et al. 2005) through the production of various chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory proteins (MIPs), as well as regulated and normal T cell expressed and secreted (RANTES). In addition, they express toll-like receptors (TLRs) and chemokine receptors including CCR5 and CXCR7 (Friedman 2008b; Marra and Pinzani 2002; Schwabe et al. 2003; Paik et al. 2003; Yi and Jeong 2013; Marra et al. 1993).

The involvement of HSC in developing inflammatory response to alcoholic liver disease is attributed to their capacity to produce complement protein 4 and neutrophil chemoattractants, such as MCP-1and macrophage colony-stimulating factor (M-CSF) (Pinzani et al. 1992; Czaja et al. 1994; Marra et al. 1993; Friedman 2008b; Maher et al. 1998; Fimmel et al. 1996).

The contribution of HSC in developing inflammatory response against viral infection has been also evaluated (Muhanna et al. 2008). HSC can act as antigen-presenting cells and modulate lymphocyte behavior (Winau et al. 2007; Vinas et al. 2003). The interaction between HSC and lymphocytes has been established (Yi and Jeong 2013; Muhanna et al. 2008). Studies showed that there is not only direct adhesion between lymphocytes and HSC (Roger et al. 2005), but also the diseased lymphocytes are ingested by HSC in hepatic fibrosis resulting in activation

of HSC (Muhanna et al. 2008). In addition, the capacity of HSC to present antigen leads to stimulation of lymphocyte proliferation (Vinas et al. 2003).

2.2 HSC and Liver Fibrosis

When the liver is injured, HSC receive signals from immune cells and from damaged hepatocytes resulting in HSC activation. The activation of HSC is a complex process that involves the interaction of various cells and inflammatory mediators. HSC activation can be divided into two major phases: initiation and perpetuation. Initiation and perpetuation may be followed by a resolution phase when liver injury resolves (Friedman 2004).

The initiation stage or the pre-inflammatory stage refers to the early changes in gene expression, which subsequently render the cells responsive to certain cytokines and other stimuli shortly after the occurrence of liver injury. The perpetuation is the stage at which the effect of these stimuli on maintaining an activated phenotype and the generation of fibrosis take place (Friedman 2008a; Lee and Friedman 2011). The resolution of fibrosis refers to the stage when apoptosis to the fibrotic HSC or reversion of these activated HSC to the normal quiescent cell phenotype occurs (Friedman 2008a).

The initial paracrine stimulation that leads to HSC activation and changes in ECM includes signals received from immune cells, damaged hepatocytes, KCs, and ECs; it involves exposure to lipid peroxides (Lee and Friedman 2011; Friedman 2008b).

The activation of HSC involves migration, proliferation, contraction, and morphological transformation from quiescent (retinoid-rich phenotype) to myofibroblast-like phenotype (Alcolado et al. 1997). The activated HSC express α-SMA protein and produce larger amounts of ECM proteins, such as collagen type I and III and laminin as compared to quiescent HSC (Bataller and Brenner 2005). In addition, the activated HSC is characterized by loss of retinoid droplets (Friedman 2008b).

Despite the contribution of other liver cells including KCs and ECs in the production of TGF-β, HSC remain the main source of production of this growth factor in the fibrotic liver (De Bleser et al. 1997). TGF-β is a very important cytokine in the development of liver fibrosis. TGF-β1 is a key factor in the regulation of HSC proliferation (Shen et al. 2003).

Another important cytokine is platelet-derived growth factor (PDGF), which is also overexpressed in liver injury. PDGF is mainly produced by HSC, KCs, and ECs (Pinzani and Marra 2001). PDGF is considered as the most potent stimulus for HSC activation (Pinzani et al. 1992; Pinzani et al. 1995; Pinzani et al. 1991), in particular for HSC proliferation (Pinzani et al. 1989). In an injured liver, HSC cells display elevated levels of PDGF and upregulation of its receptor (Pinzani et al. 1994; Wong et al. 1994). The role of PDGF in the development of liver fibrosis has been examined using a mouse with transgenic expression of PDGF-C.

This study showed that PDGF induces fibrosis development, as well as result in the development of hepatocellular carcinoma (HCC) (Czochra et al. 2006; Friedman 2008b).

BMP7 may also play an important role in liver fibrinogenesis. Although, BMP7 has a protective role in renal fibrosis by inhibiting TGF-β effect (Zeisberg et al. 2003; Wang and Hirschberg 2003), recent studies indicated that BMP7 is overexpressed in fibrotic and cirrhotic liver (Tacke et al. 2007). The profibrogenic role of BMP7 results from its activity on HSC transdifferentiation (Herrera et al. 2012). In addition, BMP7 stimulates HSC to produce collagen and fibronectin (Tacke et al. 2007).

However, a different study reported that treatment with rhBMP7 has anti-fibrotic effect in liver in agreement with its anti-fibrotic protective role in renal fibrosis (Zhong et al. 2013). This anti-fibrotic effect is attributed to inhibiting TGF-β1-induced EMT (epithelial to mesenchymal transition) (Zeisberg et al. 2003; Wang and Hirschberg 2003). Treatment with rhBMP7 was also associated with reduced expression of collagen type I and III by HSC (Zhong et al. 2013).

BMP2 and BMP4 have been also linked to liver fibrosis. A previous study reported that BMP2 and BMP4 can increase the expression of smooth muscle alpha actin (α-SMA) in cultured HSC (Shen et al. 2003; Fan et al. 2006). The expression of α-SMA is a marker of HSC transdifferentiation and transformation into their myofibroblast-like phenotype (Lee and Friedman 2011). In addition, it has been shown that BMPs have a potent effect in the regulation of transdifferentiation of HSC (Shen et al. 2003). Moreover, BMP4 itself is overexpressed in other liver diseases, e.g., bile duct-ligated liver (Fan et al. 2006) and in liver cancer tissue (Chiu et al. 2012; Maegdefrau et al. 2009).

The activated HSC play a significant role in the development of inflammatory response during liver injury as they tend to migrate and accumulate around damaged areas in response to chemotactic factors, such as MCP-1 (Czaja et al. 1994; Marra et al. 1993) and M-CSF (Pinzani et al. 1992). MCP-1 attracts activated HSC and promotes recruitment of monocytes and leukocytes (Marra et al. 1999), while M-CSF regulates macrophage accumulation and growth (Pinzani et al. 1992). Other cytokines released by HSC include PDGF, which stimulate M-CSF synthesis (Pinzani et al. 1992). PDGF is also identified as the most potent chemotactic mediators that induce migration for the activated HSC but not for the quiescent ones (Kinnman et al. 2000; Ikeda et al. 1999). Moreover HSC release cytokine-induced neutrophil chemoattractant (CINC)/IL-8 (Maher et al. 1998) and adhesion molecules, such as ICAM-1 and VCAM-1 (Knittel et al. 1999a; Lee and Friedman 2011).

Contractility of the activated HSC is a common behavior following liver injury. HSC contraction has been identified as a major cause of increased intrahepatic resistance to blood flow and portal hypertension through constricting individual sinusoids and contracting the whole cirrhotic liver (Thimgan and Yee 1999; Reynaert et al. 2002; Friedman 2008b). HSC contraction is mainly regulated by two major compounds produced by HSC; which are endothelin-1 (ET-1) and nitric oxide (NO) (Rockey 2001). ET-1 is referred to as the contraction stimulus to HSC

(Shao and Rockey 2002; Rockey and Weisiger 1996), while NO is the antagonist to ET-1(Rockey and Chung 1995). The activated HSC also produce excessive contractile filament such as α-SMA (Rockey et al. 1992; Rombouts et al. 2002).

3 Resolution of Fibrosis

Due to the significant involvement of HSC in the development of fibrotic liver, it is clear that the resolution of fibrosis is related to the reduction of HSC activation. There are two major pathways for the resolution of HSC activation: either the reversion of the activated HSC to the normal quiescent cells phenotype or their clearance through driving the stellate cell to apoptosis. The reversion of the activated HSC to the normal quiescent cells has been verified in cultured cells (Gaca et al. 2003); however, in vivo studies did not show the evidence that support this possibility. On the other hand, both in vivo and in vitro studies confirmed the incidence of apoptosis to the activated HSC during the resolution of fibrosis (Issa et al. 2001). Both CD95 (Fas), its ligand CD95L (Fas-ligand), and nerve growth factor receptor (NGFR) are expressed by the activated HSC, which promote HSC apoptosis (Shen et al. 2007). Natural killer cells (NK) can also induce HSC apoptosis by TRAIL-mediated pathway (Radaeva et al. 2006). The activated HSC also secrete soluble survival factors like insulin-like growth factor 1 (IGF-1) and tumor necrosis factor alpha (TNF-α). The excessive secretion of IGF-I and TNF-α during liver injury promotes the survival of activated HSC (Friedman 2008b; Shen et al. 2007). In addition, TGF-β and PDGF also promote HSC survival and have little antiapoptotic activity (Saile et al. 1999; Issa et al. 2001). TIMP-1 reduces apoptosis generated by serum deprivation, NGF stimulation, and cycloheximide exposure (Murphy et al. 2002). The antiapoptotic effect of TIMP-1 is based on its effect on inhibiting of ECM degradation through inhibition of MMP (Iredale 2001). Inhibition of ECM degradation suppresses apoptotic cytokines release from HSC (Murphy et al. 2002), while the pro-survival receptor such as IGF-1 is stimulated (Madge et al. 1999).

3.1 Anti-fibrotic Therapeutic Approaches

For many years, it has been believed that fibrosis is an irreversible process; however, recent clinical studies have indicated that fibrosis even cirrhosis can be reversed (Mallet et al. 2008; Friedman and Bansal 2006; Hernandez-Gea and Friedman 2011). Many animal studies reported that the removal of the primary cause is the most effective strategy for fibrosis regression especially in early stages (Ramachandran and Iredale 2009; Henderson and Iredale 2007). For example, removing of excessive iron in hemochromatosis leads to reversion of fibrosis (Powell and Kerr 1970; Blumberg et al. 1988). Using anthelmintic drugs for

treating schistosomiasis is associated with fibrosis regression (Rahoud et al. 2010). In addition, inhibition of hepatitis B virus (Lai et al. 1998; Hadziyannis et al. 2003) or hepatitis C (Poynard et al. 2002) showed significant improvement in liver function and regression of fibrosis. Furthermore, fibrosis regression has also been observed after relief of bile duct obstruction (Hammel et al. 2001). Other effective anti-fibrotic therapeutic approaches include inhibition of HSC activation, reduction of inflammation, antioxidant, stimulation of activated HSC apoptosis, inhibition of collagen synthesis, and promotion of ECM degradation (Friedman 2008a; Li et al. 2008; Rockey 2005).

3.2 Gene Therapy

Gene therapy is a new therapeutic tool that may provide a solution to manage fibrosis. Antisense oligonucleotides or functional therapeutic genes located in plasmid DNA can be used to manage fibrosis through modulation of gene expression in diseased cells (Sandig and Strauss 1996; Beljaars et al. 2002). There are various methods used for gene transfer to a fibrotic liver; however, viral vectors showed the highest efficiency as a mean for gene transfection into the target cells. Retroviruses and adenoviruses are the most common viral vectors for gene delivery both in vitro and in vivo (Grimm et al. 2008; Yi et al. 2011). Recombinant adeno-associated viruses (rAAV) have superior properties over adenoviruses, such as high cellular tropism, more stable transgenic expression for long term, and less stimulation of immune response (Di Campli et al. 1999).

TGF-β promotes HSC proliferation as well as increases the production of collagen type I and III proteins by the activated HSC (Shen et al. 2003). Various studies reported that inhibition of TGF-β release causes reduction of liver fibrosis. For example, the use of adenovirus carrying gene expressing a truncated type II TGF-beta receptor (AdTbeta-TR) to a dimethylnitrosamine (DMN)-treated rats inhibited TGF-β binding to its receptor, reduced deposition of ECM, and suppressed liver fibrogenesis (Nakamura et al. 2000). The adenoviral transfection of the gene encoding for Smad 7 that is known for inhibition of TGF-β biological activity (Wells 2000) led to a reduction of HSC activation and prevented fibrogenesis progression (Dooley et al. 2003). The delivery of TGFβRII-specific short-hairpin RNA (shRNA) into rat-activated HSC was accompanied by the knockdown of TGFβRII and reduced levels of α-SMA; collagen types I, III, and IV; and hyaluronic acid (HA) expression (Fu et al. 2011). Also, TGF-β1-siRNA has been used to knock down TGF-β1 expression in CCl4-treated mice. Another important cytokine is TNF-α, which is mainly produced by KCs. TNF-α is not only involved in activation of HSC and liver damage but also plays an important role in induction of apoptosis in HSC (Saile et al. 1999). Targeting KCs with TNF-α antisense oligonucleotides utilizing liposomes as a carrier system resulted in reduction of liver fibrosis that was induced by ethanol (Ponnappa and Israel 2002; Ye et al. 2007).

The augmentation of liver regeneration (ALR)-cloned gene plays a critical role in the regulation of liver regeneration and exerts potent anti-hepatitis effects. Therefore, the administration of ALR recombinant plasmid to a rat with hepatic fibrosis reduced the liver fibrosis, ALT, AST, and TIMP-1 expression. The administration of ALR also inhibited the expression of collagen types I and III as compared to colchicine that has been used as anti-fibrotic positive control (Li et al. 2005b).

Hepatocyte growth factor (HGF) is a major regulator of hepatocyte regeneration, and it exhibits potent antiapoptotic activity as well as has anti-fibrotic effect against hepatic fibrosis through inhibiting TGF-β1 gene and collagen type III expression levels (Inoue et al. 2003; Jiang et al. 2008; Kanemura et al. 2008).

It has been reported that the delivery of MMPs can be a promising tool for resolving liver fibrosis and advanced cirrhosis through their role in digestion of the fibrillar collagen of ECM. MMP-1, MMP-8, and MMP-13 are considered the most potent MMPs (Hemmann et al. 2007; Siller-Lopez et al. 2004). In 2011, Kim et al. utilized polyethylenimine polymer coupled to hyaluronic acid (HA) as specific ligand for the targeted delivery of plasmid DNA encoding MMP13 gene to the liver. The expression of MMP13 gene resulted in the reduction of aspartate transaminase enzyme level and also reduced the induction of liver fibrosis caused by CCl4 administration (Kim et al. 2011).The delivery of human pro-MMP-1 encoded on complementary DNA using adenoviral vector to a rat model of liver fibrosis showed significant attenuation of liver fibrosis (Iimuro et al. 2003). Similarly, the adenoviral transfection of MMP-8 gene to rat model of liver cirrhosis mediated by CCL4 administration was accompanied by significant resolution of liver damage (Siller-Lopez et al. 2004).

In advanced stages of liver damage, TIMP-1 is overexpressed, leading to more fibrosis through disposition of ECM. This is because TIMP-1 binds the degrading enzymes MMPs and inhibits their activity (Arthur et al. 1998). In 2006, Roderfeld et al. reported that the adenoviral delivery of the proteolytic inactive MMP-9 to mice of CCl4-mediated hepatic fibrosis reduced liver damage accompanied by a decrease in collagen type I expression (Roderfeld et al. 2006).

rAAV has been utilized for the delivery of IFN-gamma gene to HSC (rAAV-IFN-gamma). The anti-fibrotic effect of rAAV-IFN-gamma has been investigated both in vitro and in vivo rat model of CCl4-mediated hepatic fibrosis. The in vitro study revealed that rAAV-IFN-gamma inhibits HSC activation and decreases the expression of α-SMA, TIMP-1, and TGF-β, while the in vivo study reported that rAAV-INF-gamma could suppress the progression of the hepatic fibrosis. Furthermore, the hydroxyproline content, serum AST, and ALT level and TIMP-1 mRNA expression were significantly decreased; however, no significant changes were observed in TGF-β and MMP-13 (Chen et al. 2005).

3.3 RNA Interference

RNA interference (RNAi) is a recently discovered mechanism by which small double-stranded RNAs (dsRNAs) can regulate specific gene expression; this process can be induced either endogenously through microRNAs (miRNAs) or exogenously introduced through small interfering RNAs (siRNAs) (Kim and Rossi 2009; de Fougerolles et al. 2007). RNAi was first observed in *Caenorhabditis elegans* worm by Fire and Mello when they discovered the ability of dsRNA to knock down specific gene expression (Fire et al. 1998; Lee and Friedman 2011). In 2001, another study showed that siRNA could inhibit gene expression in mammalian cells (Elbashir et al. 2001). The first successful trial of gene silencing based on siRNA was achieved in mice against hepatitis C virus (McCaffrey et al. 2002). Recently, RNAi has become a novel therapeutic approach for the treatment of many human diseases (Okumura et al. 2008; Kim and Rossi 2008).

RNAi mechanism is activated when long pieces of dsRNA are cleaved into smaller fragments known as siRNA (21–23 nucleotides long) by the dicer enzyme (Bernstein et al. 2001). However, siRNA can be chemically synthesized outside the body and directly introduced into the cell avoiding dicer enzyme cleavage and off-target nonspecific gene silencing as well as minimizing the immune response that may result from the interaction of long pieces of dsRNA that are larger than 30 nucleotides with RNA receptor. When siRNA is formed within the cell by dicer, it binds to a protein complex called the RNA-induced silencing complex (RISC) (Buckley et al. 2004). RISC contains Argonaute-2 protein, which separates the siRNA into two single-strand RNAs followed by the cleavage of the sense strand (Matranga et al. 2005). The single antisense strand of the siRNA guides the activated RISC and selectively binds to mRNA that has a complementary sequence to the antisense strand resulting in the degradation of mRNA (Ameres et al. 2007). The cleavage of mRNA occurs by the action of endonuclease Argonaute-2 protein of RISC complex at the position between nucleotides 10 and 11 on the complementary antisense strand from the 5′-end (Rand et al. 2005). The activated RISC complex is then recycled and gets involved in multiple catalytic processes (Hutvagner and Zamore 2002). This effect lasts up to one week in rapidly dividing cells and for several weeks in nondividing cells (Bartlett and Davis 2006; Leng et al. 2009). The frequent administration of siRNA is required to maintain its inhibitory effect (Hammond et al. 2000).

3.3.1 Barriers of siRNA Delivery and Therapy

Theoretically, RNAi strategy can be used to silence almost any gene in the body, making it a novel therapeutic potential than traditional drugs. However, there are many challenges in clinical practice including safety, stability, and effective delivery of siRNA (Whitehead et al. 2009). The first obstacle is siRNA degradation by plasma RNAses as well as its rapid elimination by the renal and reticuloendothelial

system especially for naked unmodified siRNA (Peer and Lieberman 2011). Moreover siRNAs is capable of stimulating the innate immune system, which recognizes siRNAs as a foreign body and this may lead to off-target immune-mediated side effects (Burnett and Rossi 2012). In order to achieve gene silencing, siRNA must enter the cytoplasm of the target cell. However this delivery is limited by negative charge and the high molecular weight of siRNA which makes it difficult to penetrate the cell membrane by passive diffusion or without the aid of a delivery system (Bumcrot et al. 2006; Qi et al. 2014). Another barrier is the intracellular endosomal trapping effect that will guide siRNA to degradation before it reaches the target mRNA (White 2008). Moreover, siRNA application can cause off-target effect, in which siRNA may interact and induce nonspecific silencing to genes whose expression should not be targeted, as these genes may share partial sequence similarity to the target gene (Kesharwani et al. 2012; Jackson et al. 2006). The off-target gene silencing can be a significant problem and may lead to toxicity, mutation, and unexpected changes in cell behavior (Gavrilov and Saltzman 2012; Oh and Park 2009). However, these challenges could be overcome through the appropriate design of siRNA as well as the development of nanocarriers that will ensure that siRNA is delivered in an intact form to the target cells without inducing any toxic or immunological side effects. For that reason, additional optimization in siRNA biostability and cellular delivery is highly required (Elmen et al. 2005). In 2005, Jackson et al. reported that the off-target gene silencing can be minimized through substitution of 2′-*O*-methyl group on the ribose ring of the guide strand (Jackson et al. 2006). In order to perform siRNA design, many parameters should be considered, such as GC content, minimal repeated sequences that are responsible for the off-target effect, and appropriate choice of siRNA length (19–22 bps). In practice, several companies provide an effective siRNA design online with the desired properties to reduce off-target effects (Kim and Rossi 2007; Oh and Park 2009). In order to facilitate siRNA passage through biological membranes, it must be complexed with cationic polymers or cationic lipids forming positively charged or neutral polyplexes or lipoplexes, respectively, that can cross the cell membrane by fluid-phase pinocytosis. In addition, these polyplexes or lipoplexes facilitate endosomal escape (Tiram et al. 2014). There are several nanocarriers (1–1,000 nm in size) that can be utilized. However, the nanoparticles within the range between 50 and 200 nm are easily taken up by target cells via passive delivery and avoid rapid renal clearance. In addition, they can provide a large surface area that can improve drug release and extend bioavailability, bio-distribution, and efficacy of drugs (Zhang et al. 2008; Petros and DeSimone 2010; Tiram et al. 2014). Moreover, these nanocarriers can be modified with targeting moieties to achieve active targeted delivery, which can be internalized via receptor-mediated endocytosis (Fig. 2). These targeted nanoparticles can avoid or minimize off-target side effects as well as achieve the desired therapeutic effect with minimum required dose (Yang et al. 2013). Among the nanocarriers that have been successfully formulated to target liver fibrosis are cationic polymers, cationic liposomes, stable nucleic acid–lipid particle (SNALP), and polylipid nanoparticle (Fig. 3).

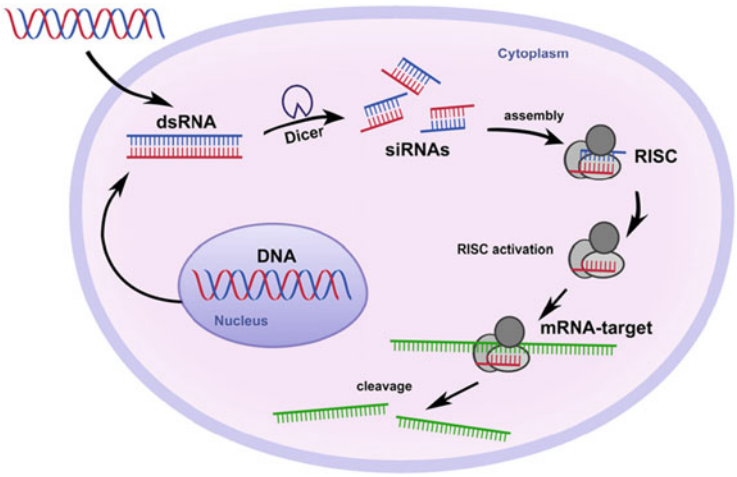

Fig. 2 Schematic graph showing mechanism of RNAi

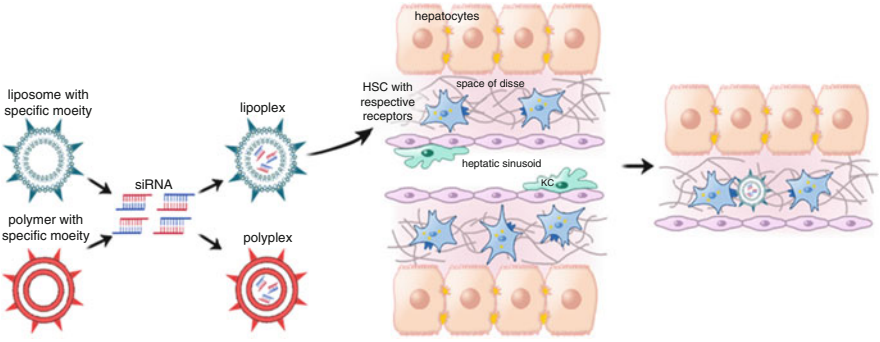

Fig. 3 Schematic overview of targeted delivery of siRNA to HSC via receptor-mediated endocytosis. siRNA interacts with liposomes or polymers that are modified with targeting moieties forming lipoplex or polyplex, respectively. Lipoplexes or polyplexes with specific ligand-modified surfaces are delivered through the circulation to activated HSC where they bind with the respective receptor

4 Lipid-Based siRNA Delivery

4.1 Cationic Liposomes

Over the last 25 years, liposomes have been extensively utilized for drug and gene delivery (Fenske and Cullis 2008). This is due to their safety, biocompatibility, and comparative ease of preparation. In addition, they demonstrate diverse range of morphologies, compositions, sizes, tissue targeting, and controlled release characteristics. Furthermore, they are able to deliver and protect various types of

therapeutic molecules of either hydrophilic or hydrophobic properties (Balazs and Godbey 2011; Montier et al. 2008; Tros de Ilarduya et al. 2010). To date the FDA has approved seven liposomal drugs for treatment of different diseases such as cancer and infectious diseases (Ozpolat et al. 2014; Petros and DeSimone 2010). Liposomes are spherical vesicles entrapping aqueous core surrounded by one "unilamellar" or more "multilamellar" phospholipid bilayer of polar head group and hydrophobic tail formed by self-assembly. In general, most liposomal preparations used for siRNA and gene delivery are mainly made of neutral and cationic lipids.

Cationic liposomes, such as DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) and DOTMA (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammonium methyl sulfate), are mainly used for siRNA delivery to the liver (de Fougerolles 2008). This is attributed to their ability to form lipoplexes with negatively charged siRNA via electrostatic interaction, as well as facilitate interaction with negatively charged cell membrane components resulting in their uptake (Verma et al. 2003; Kesharwani et al. 2012; Gomes-da-Silva et al. 2012). Moreover, cationic liposome provides protection for siRNA from enzymatic degradation and reduces renal clearance of siRNA as well (Oh and Park 2009). Cationic liposomes also provide high transfection efficiency of siRNA and exhibit relatively low cytotoxicity and immunogenicity (Kesharwani et al. 2012).

Although, cationic liposomes offer high encapsulation efficiency to siRNA, their preclinical application in vivo encountered some challenges including systemic administration-associated toxicity, poor stability in plasma, and immune response activation (Harmon et al. 2011; Knudsen et al. 2014; Ozpolat et al. 2014). However, the major concern about utilization of the cationic liposomes is their systemic administration-associated toxicity. The cationic liposome-related toxicity results from the excessive charge on the lipid surface (Simoes et al. 2005; Knudsen et al. 2014). Dokka et al. (2000) reported that liposomes' toxic effect is due to the activation and release of reactive oxygen species (ROS) as well as increased intracellular calcium levels induced by cationic liposomes. Another challenge is that cationic liposomes possess poor stability in plasma in which they tend to aggregate. This aggregation is attributed to their interaction with negatively charged serum proteins (Harmon et al. 2011). The interaction between the cationic liposomes and serum components may result in immune response stimulation, which leads to their rapid clearance from the blood by macrophages of the reticuloendothelial system (RES), and failure of siRNA release to the targeted cells. Reports have showed that the multivalent cationic liposomes like Lipofectamine® are more toxic to macrophages and other immune cells than monovalent cationic lipids, like DOTAP (Ozpolat et al. 2014; Filion and Phillips 1997; Spagnou et al. 2004). In order to overcome these challenges and to achieve successful delivery of siRNA by liposomal delivery system, important measures should be taken, such as optimization of lipid content ratio, lipid-to-siRNA ratio, size of particles zeta potential, and encapsulation efficiency. Another possible optimization could be through incorporation of specific ligand to liposome surface in order to enhance their selective interaction to the target cells (Ozpolat et al. 2014).

The development of targeted delivery system for anti-fibrotic therapeutic is a preferred approach as most of these drugs lack of in vivo cellular specificity to HSC, leading to off-target side effects. The selective targeted delivery system could increase the drug bioavailability at the target site and enhance their therapeutic efficiency with the minimum required therapeutic dose and consequently eliminate the side effects that result from off-targeting delivery (Adrian et al. 2007; Beljaars et al. 2002; Narmada et al. 2013). Ligands, such as galactosylated cholesterol, glycolipids, or galactosylated polymers, have been conjugated to liposomes and achieved significant targeting efficacy to the liver (Wu and Zern 1996; Fan and Wu 2013; Wu et al. 2002). Adrian et al. developed targeted liposomes for drug delivery to HSC composed of dilinoleoylphosphatidylcholine (DLPC), utilizing human serum albumin and mannose-6-phosphate (M6P-HAS) as a selective targeting group to HSC. M6P-HAS-coupled liposomes showed high specificity and enhanced uptake by HSC mediated by M6P/IGF II and scavenger receptors. In vitro investigation demonstrated that M6P-HAS liposomes exhibit anti-fibrotic activity on cultured HSC where the expression levels of TGF-β, collagen-I, and α-SMA in HSC were decreased (Adrian et al. 2007).

In 2008, Sato et al. developed vitamin A-coupled liposomes for selective targeted delivery of collagen-specific chaperone (gp 46) siRNA to HSC. These cationic liposomes are composed of cationic lipid (DC-6-14), cholesterol and DOPE. Vitamin A has been utilized as a selective ligand based on the function of HSC in the storage of vitamin A, as they are the only liver cells with high retinol-storing capability and high expression levels of RBP. The anti-fibrotic effect of vitamin A-coupled liposomes-gp46 siRNA has been investigated both in vitro and in three rat models of DMN, CCl4, and bile duct ligation (BDL)-mediated hepatic fibrosis. VA-lip-gp46 siRNA could silence gp46 gene expression both in vitro and in vivo as well as reversed the hepatic fibrosis in the three cirrhotic model-treated rats at a low dose of 0.75 mg/kg of VA-lip-gp46 siRNA. In addition to decreased production of collagen, hydroxyproline accumulation, bilirubin, and hyaluronate levels have been normalized in all treated rat models. It has been also revealed that treatment with VA-lip-gp46 siRNA did not stimulate the immune response where TNF-α remained normal in contrast to Lipofectamine 2000® treatment that showed significant elevation of TNF-α levels. All these findings indicate that the specific selective action of VA-lip-gp46 siRNA complex on HSC without off-targeting effect (Sato et al. 2008). Similarly, Narmada et al. have cloned HGF gene to pDsRed2 plasmid DNA vector and transfected to HSC utilizing vitamin A-coupled liposomes as a delivery system to rat model of DMN-induced liver fibrosis. Treatment with VA-lip-HGF leads to increase HGF gene expression and resolution of the existing liver damage induced by DMN administration. In addition, TGF-β1, α-SMA, and collagen-I mRNA levels decreased (Narmada et al. 2013).

Moreover, polyethylene glycol (PEG) has been utilized to modify liposomes surface forming protective barrier around liposomes surface. This protective coat prevents macrophage uptake and reduces interaction with serum components. Therefore, PEGylated liposomes, which are also referred as stealth liposomes, are

minimizing RES clearance, activating immune response, and improving liposomes stability through reducing the cationic liposomes aggregation in serum. In addition, PEG enhances the transfection efficiencies of lipoplexes in the presence of blood (Miller et al. 1998; Jokerst et al. 2011; Spagnou et al. 2004; Ozpolat et al. 2014; Balazs and Godbey 2011). Despite these advantages, PEGylated liposomes encountered some problems, such as lack of specificity for targeting cells and decreased cellular uptake of lipoplexes through inhibition of endocytosis in such way that it is dependent on the amount of PEG found on liposomes (Shi et al. 2002; Balazs and Godbey 2011). Moreover, PEGylation stabilizes DNA encapsulation into liposomes; this effect may decrease DNA–liposome complex dissociation and consequently failure to release of DNA to cytoplasm (Balazs and Godbey 2011). Although PEGylated liposomes lack specificity for targeting cells, the PEGylation provides a good surface and facilitate the conjugation of the target-specific ligand to liposomes surface like antibody, peptides, and other compounds (Ozpolat et al. 2014). In 2012, Gao et al. developed PEGylated immunoliposomes composed of DOTAP and cholesterol conjugated with anti-EGFR antibody (TLPD) for the targeted delivery siRNA to hepatocellular carcinoma (Gao et al. 2012).

4.2 Neutral Lipids

As mentioned previously, cationic liposome-related toxicity results from the excessive charge on the lipid surface; therefore, neutral lipids are commonly used in gene delivery in combination with cationic lipids to minimize the toxicity associated with the cationic liposomes; in addition, neutral lipids do not stimulate the immune response. Moreover, several in vivo studies have reported that utilization of neutral lipids with cationic ones in siRNA delivery achieved higher transfection efficiency than did cationic liposomes alone (Gavrilov and Saltzman 2012; Ozpolat et al. 2014; Halder et al. 2006; Landen et al. 2005). The most commonly used neutral lipids are 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). It is believed that DOPE leads to higher transfection efficiency than DOPC (Farhood et al. 1994; Simoes et al. 1998), as DOPE yields hexagonal tubular structure which allows DOPE to bind immediately with DNA via electrostatic interaction and stabilize DNA inside its tubules. Furthermore, reports showed that DOPE-containing cationic liposomes could destabilize the endosomal vesicles membrane, leading to endosomal escape of the lipoplex (Fig. 4). In contrast, DOPC forms lamellar layers of DNA and lipids, and it has no effect on endosomes (Koltover et al. 1998; Zuhorn et al. 2005; Balazs and Godbey 2011).

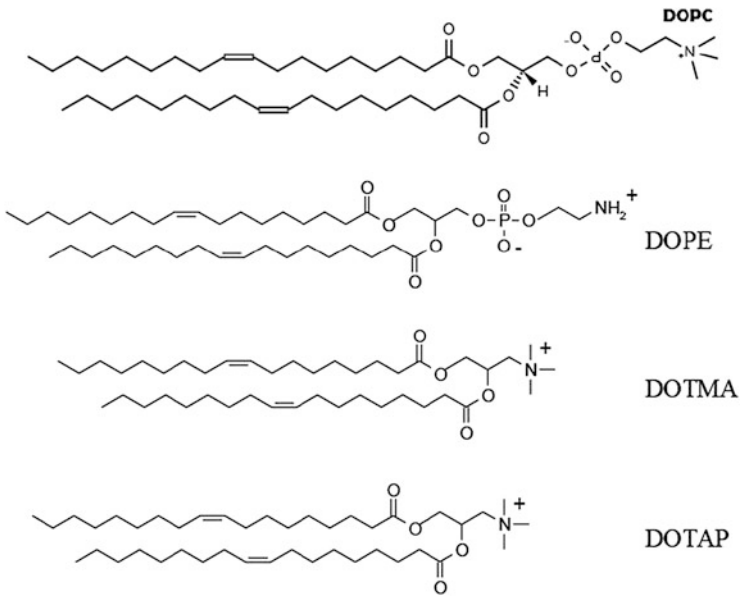

Fig. 4 Chemical structures of commonly used synthetic lipids: neutral lipids include 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) and dioleoylphophatidylethanolamine (DOPE), while cationic lipids include *N*-[1-(2,3-dioleyloxy) propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate (DOTMA) and 1,2-bis(oleoyloxy)-3-(trimethylammonio) propane (DOTAP)

4.3 Stable Nucleic Acid–Lipid Particles (SNALPs)

Solid lipid-based systems including stable nucleic acid–lipid particle (SNALP) and cationic solid lipid nanoparticle (SLN) are positively charged nanocarriers that have been recently used and have demonstrated efficacy in siRNA delivery (Kim et al. 2008).

SNALP is a lipid bilayer composed of mixture of ionizable cationic lipid (1,2-dilinoleyloxy-3-dimethylaminopropane); a neutral helper lipid, including cholesterol and fusogenic lipids; and diffusible PEG lipid. During SNALP formulation, siRNA is efficiently encapsulated within the ionizable cationic lipid bilayer (100–150 nm in size). The lipid bilayer of the cationic, neutral, and fusogenic lipid facilitates cellular uptake of siRNA due to the interaction between the negatively charged components of cell membrane and the cationic lipids. The ionizable lipid at low PH of the endosomes is cationic, which fuse with endosomal membrane allowing escape of siRNA from endosomal degradation and release to cytoplasm (de Fougerolles et al. 2007). The introduction of PEG molecule provides a neutral and hydrophilic layer that reduces particle aggregation during formulation as well as prevents particle interaction with plasma protein. PEG conjugate also protects siRNA from degradation mediated by nucleases, resulting in a higher stability of SNALP-siRNA complex (Barros and Gollob 2012; Lin et al. 2014). SNALP

nanoparticles have been utilized to deliver siRNA against HBV through intravenous injection of 30 mg/kg three times per day for up to 7 days in mice. SNALP delivery resulted in a significant inhibition of HBV DNA by about tenfold and prolonged the circulation time of siRNA (Morrissey et al. 2005). In 2006, Zimmerman et al. demonstrated that the single intravenous administration of SNALP-siRNA targeting apolipoprotein B (ApoB) at dose of 1–2.5 mg/kg cause silencing of 90% ApoB mRNA in the liver of cynomolgus monkeys. Significant reduction in serum ApoB protein, cholesterol, and low-density lipoprotein (LDL) levels has been also observed (Zimmermann et al. 2006).

Recently, two clinical trials have been launched using SNALP-siRNA formulations targeting liver diseases. In 2010, Tekmira Pharmaceuticals has completed phase I single-dose study of SNALP encapsulating ApoB siRNA for treatment of patients with elevated low-density lipoprotein (LDL) cholesterol. The preliminary results showed no evidence of liver toxicity (Barros and Gollob 2012). Another clinical trial initiated by Alnylam Pharmaceuticals was using SNALP-siRNA (ALN-TTR01) targeting hepatocyte production of both mutant and wild-type TTR to treat transthyretin-related hereditary (TTR) amyloidosis and also senile systemic amyloidosis (SSA) caused by wild-type TTR. IV infusion of ALN-TTR01 at low dose of 0.01–1 mg/kg could lower serum TTR. Treatment showed no significant increase in liver function tests (Barros and Gollob 2012).

5 Polymeric Nanoparticles

Polymeric nanoparticles are solid, biodegradable, colloidal systems that can serve as efficient nanocarriers for the delivery of nucleic acid (Egusquiaguirre et al. 2012). The delivery of siRNA using polymeric carriers is based on their ability to bind and condense siRNA into stabilized nanoparticles either via electrostatic interaction between the cationic polymer and the negatively charged nucleic acid forming lipoplexes or by chemical interaction (Tiram et al. 2014). Polymeric nanoparticles are available as natural polymers and synthetic polymers. Natural polymers for siRNA delivery include cyclodextrin, chitosan, and atelocollagen (Wang et al. 2010). While the synthetic polymers are polyethyleneimine (PEI), poly(dl-lactide-co-glycolide) (PLGA), and dendrimers (Yuan et al. 2011).

5.1 Polyethyleneimine

Polyethyleneimine (PEI) is a synthetic cationic polymer that has been widely utilized for gene delivery and recently for siRNA delivery (Wu et al. 2012). PEI is an effective transfection agent for siRNA delivery due to its high positive charge resulted from protonable amino groups. The cationic amino groups of PEI are spontaneously combined with the negatively charged phosphate group of siRNA

and form polyplexes through electrostatic interaction and a simple polycation process (Wang et al. 2010). In addition, PEI has proton sponge effect that provides strong endosomal buffering capacity and leads to endosomal escape and consequently provides higher stability and prolonged half-life for siRNA (Boussif et al. 1995). Moreover, the complete encapsulation of siRNA within PEI minimizes the immune activation (Merkel et al. 2011), and the outer surface of PEI can be modified with various targeting ligands to achieve specific gene delivery to different cell types (Kichler 2004) such as mannose for targeted DNA delivery into dendritic cells (Diebold et al. 1999), transferrin for targeting K562 cells (leukemia cells) (Kircheis et al. 1997), epidermal growth factor for targeting tumor cells (Blessing et al. 2001), antibodies for targeting hematopoietic cells (Wojda and Miller 2000), galactose for targeted gene delivery to hepatocyte (Zanta et al. 1997), and hyaluronic acid for specific delivery of siRNA to HSC (Park et al. 2011).

The weak electrostatic interactions between positively charged PEI and negatively charged siRNA facilitate the dissociation of PEI-siRNA complexes and the release of siRNA (Bolcato-Bellemin et al. 2007).

Despite these advantages, reports have showed that PEI complexes might be associated with significant cytotoxicity especially at higher molecular masses and high doses due to their highly cationic nature and lack of biodegradability (Kichler 2004; Tiram et al. 2014). The high molecular weight (HMW) PEI-related toxicity is due to its lack of stability in plasma and interaction with negatively charged serum proteins forming large aggregate which might block capillaries and activate RES (Kircheis et al. 2001; Tiram et al. 2014). Although low molecular weight (LMW) PEI forms smaller aggregate and has negligible cytotoxicity compared to the HMW PEI, the LMW PEI displays lower transfection efficiency than the HMW PEI. Several strategies have been developed to improve the transfection efficiency of the LMW PEI while maintaining its reduced cytotoxicity. One approach involves the introduction of LMW PEI into other polymeric complexes such as PEG, polyglycerol, chitosan, and PLGA (Patil et al. 2010; Singha et al. 2011; Tiram et al. 2014; Mao et al. 2006; Zhang et al. 2010). For example, in 2002, Petersen et al. synthesized polymer complex of LMW PEI and oligo(L-lactic acid-co-succinic acid), and the resulted copolymer showed a significant higher stability and transfection efficiency compared to the LMW PEI. However, the biodegradability was a concern (Petersen et al. 2002). Another study cross-linked PEG into LMW PEI via ester linkage. The PEGylated PEI displayed three times higher transfection efficiency with respect to the starting LMW PEI and the cell viability was maintained over 80% (Kircheis et al. 2001).

A third approach to reduce PEI-associated toxicity is via modifying the structure of LMW PEI. This can be done through incorporation of reversible linkage such as disulfide bridges to PEI utilizing dithiodipropionic acid as a cross-linking agent resulting in a branched biodegradable PEI with minimized toxicity on cultured cells and improved siRNA release (Gosselin et al. 2001; Luten et al. 2008; Breunig et al. 2008). Studies have also showed that the introduction of alkyl carboxyl group into the branched PEI results in reduction of the positive charge, cytotoxicity as

well as improves the stability of PEI-siRNA complex (Oskuee et al. 2010; Philipp et al. 2009).

For the targeted delivery of the liver, Zanta's group used PEI grafted with galactose which could facilitate the targeted DNA delivery to hepatocyte in the presence of 10% serum. This targeted delivery is mediated by asialoglycoprotein receptor. The target specificity of this carrier system was confirmed using galactose-free PEI which showed complete loss of transfection (Zanta et al. 1997).

In 2010, Kang's group used PEI polymer conjugated to pullulan polysaccharide to achieve specific delivery of siRNA to liver. Pullulan is used for targeting liver because it has high affinity to asialoglycoprotein receptor (Mehvar 2003). The use of PEI-pullulan/siRNA complex not only improved the liver targeting efficiency, but also it minimized in vivo toxicity and mortality rate in rats as compared to the use of pullulan-free PEI (Kang et al. 2010). Recently, another working group has also used PEI polymer conjugated to *N*-acetylglucosamine (GlcNAc) and indocyanine green (ICG) for the delivery of TGF-β1 siRNA to activated cultured HSC and to CCl4-fibrotic mice. The transfection of TGFβ1-siRNA using PEI-D-GlcNAc-ICG showed higher accumulation of PEI-D-GlcNAc-ICG/TGFβ1siRNA complex on the fibrotic mice liver than the healthy liver. The anti-fibrotic effect of PEI-D-GlcNAc-ICG/TGFβ1siRNA complex has been reflected on the levels of TGF-β1 and α-SMA which have been significantly reduced (Kim et al. 2013).

5.2 Poly(Lactic-co-Glycolic Acid) (PLGA)

PLGA is copolymer of lactic acid and glycolic acid which are linked together via ester bond. PLGA is approved by FDA as a drug carrier due to its biodegradability and biocompatibility (Zhao et al. 2012). Moreover, PLGA has recently been used for plasmid DNA and siRNA delivery. The biodegradability and compatibility of PLGA are due to hydrolysis of its ester linkages into lactic acid and glycolic acid metabolite monomers, in which both are easily metabolized by the body; therefore, a minimal systemic toxicity is associated with the use of PLGA for drug delivery. The blank unmodified PLGA nanoparticles are not efficient for siRNA delivery because they are negatively charged which means that they have low binding and encapsulation efficiency to siRNA. The negatively charged feature of PLGA also limits their endosomal escape (Singha et al. 2011; Lee et al. 2013). However, this challenge has been solved through coating of PLGA surface with cationic nanoparticles such as DOTAP and PEI which increase the electrostatic interaction between PLGA and siRNA and promote endosomal escape (Tiram et al. 2014; Danhier et al. 2012). In addition to biodegradability and biocompatibility, PLGA nanoparticles provide high stability for siRNA and they facilitate cellular uptake by endocytosis. Moreover, PLGA surface can be modified with specific ligand to target-specific tissue. Furthermore, PLGA can be incorporated with other polymeric nanoparticles to provide sustained release of siRNA (Singha et al. 2011; Lee

et al. 2013). Due to these reasons, PLGA nanoparticles are used as drug delivery systems.

5.3 Chitosan

Chitosan is a natural cationic copolymer of *N*-acetyl-D-glucosamine and D-glucosamine which are linked together through glycosidic linkage. Chitosan nanoparticles have been extensively utilized for plasmid DNA delivery and recently they have been widely investigated for siRNA delivery (Lee et al. 2013; Tiram et al. 2014). The advantages of using chitosan nanoparticles for siRNA delivery are biocompatibility and low immunogenicity. In addition, they demonstrate mucoadhesive properties and nuclease resistance as well as low cost of production (Ragelle et al. 2013; Howard et al. 2006; Saranya et al. 2011). However, high molecular weight chitosans show high cytotoxicity and lack of endosomal escape (Alameh et al. 2012). Due to reasons, several attributes must be considered to optimize the transfection efficiency and to minimize the toxicity of chitosan such as molecular weight, the degree of deacetylation (DDA) of primary amines along the chitosan chain, methods of association of siRNA with chitosan nanoparticle, as well as chitosan/siRNA ratio and the pH of media (Tiram et al. 2014; Saranya et al. 2011; Ragelle et al. 2013).

There have been several attempts to optimize siRNA delivery using chitosan. For example, in 2011, Huh's group developed new siRNA nanocarrier composed of glycol chitosan (GC) and PEI for siRNA delivery targeting red fluorescent protein (RFP). The incorporation of the cationic PEI polymer increased the stability of the formed GC-siRNA complex. In addition, in vitro finding indicated increased cellular uptake and silencing activity of siRNA (Huh et al. 2010). Another study utilized chitosan as coating surface of PLGA nanoparticle for delivery of siRNA targeting antiapoptotic gene Bcl-2. It has been found that chitosan-coated PLGA carrier system showed higher transfection and silencing efficiency of Bcl-2 siRNA compared to chitosan-free PLGA nanoparticles (Jagani et al. 2013).

5.4 Cyclodextrin

Cyclodextrins (CDs) are naturally occurring oligosaccharides composed of glucose units linked together through α-1, 4-linkages. CDs are obtained from bacteria during enzymatic digestion of cellulose. CDs have been extensively utilized for drug delivery especially for lipophilic drugs because they form funnel-like structure with hydrophilic outer surface and hydrophobic inner core that enable them to form inclusion with these insoluble drug molecules (Tiram et al. 2014; Davis and Brewster 2004). CDs are also favorable for siRNA delivery because they are nontoxic, non-immunogenic, and stable in physiological fluids and cause minimal

aggregation of erythrocytes. Also, CDs can provide protection to the payloads against enzymatic degradation. However, CD-based preparations have limited ability to escape from endosomal pathway (Kulkarni et al. 2005; Gonzalez et al. 1999). For this reason, CDs surface have been modified with imidazole group which could buffer the endosomal PH and provide protection against endosomal degradation (Mishra et al. 2006). This modification improved intracellular delivery of siRNA as well (Bartlett and Davis 2007). In 2005, Hu-Lieskovan et al. have developed new formulation based on CDs for the targeted delivery of siRNA against the EWS-FLI1 gene in tumor cells. Such delivery system is composed of β-CDs, PEG, and adamantine molecules as stabilizing agents. In addition, transferrin has been incorporated to the system as targeting moiety through binding with transferrin receptor on cancer cell surface. Using this formulation showed a successful delivery of siRNA to tumor in a murine model of metastatic Ewing's sarcoma (Hu-Lieskovan et al. 2005). This latter formulation has been modified by Calando Pharmaceuticals and developed as a new siRNA therapy called CALLA-01. CALLA-01 is the first siRNA anticancer therapeutic that has entered phase I clinical trial. It is designed to inhibit the expression of the M2 subunit of ribonucleotide reductase (R2) using cyclodextrin-based polymer (Lee et al. 2013).

6 SiRNA-Nanotherapeutics and Liver Fibrosis

Currently there have been significant developments for using siRNA-based therapy in the clinic. However, the most common application of siRNA-based therapeutics is based on the localized delivery form, e.g., age-related macular degeneration (AMD) which causes vision loss (Burnett and Rossi 2012). This study was carried out by Acuity Pharmaceuticals in 2004, which results in improved vision in some of the patients (Petros and DeSimone 2010).

In addition, there are several clinical trials based on systemic delivery of siRNA therapies that are currently being evaluated in the preclinical or clinical trials, e.g., hepatitis B virus and acute renal failure. In 2008, the first clinical trial started for siRNA therapy against a human solid tumor (melanoma) (Davis et al. 2010). Currently there are eight clinical studies for the treatment of cancer using nanoparticle-based siRNA delivery (Ozpolat et al. 2014). Calando Pharmaceuticals launched CALLA-01, which is the first siRNA anticancer therapeutic that entered phase I clinical trial. CALLA-01 is designed to inhibit the expression of the M2 subunit of ribonucleotide reductase (R2) using cyclodextrin-based polymer; after that, Tekmira, Alnylam, Silence Therapeutics, Marina, and other pharmaceutical companies have introduced siRNA nanoparticle products in either the preclinical or clinical phases (Lee et al. 2013).

The application of siRNA-based therapies in the treatment and prevention of liver diseases has been reported to be effective such as in hepatitis B virus (HBV) (Konishi et al. 2003; Morrissey et al. 2005). The prospects for siRNA-based therapeutics against hepatitis C virus have also been discussed by Chang Ho Lee

(Lee et al. 2013). The intravenous administration of HCV-specific siRNA with liposomes and purified recombinant human apolipoprotein A-I (rhapo A-I) significantly reduced HCV protein expression (Lee et al. 2009).

Another study showed that VEGF-siRNA is able to silence VEGF expression. VEGF silencing is followed by reduction in growth of hepatocellular carcinoma both in vitro and in vivo (Raskopf et al. 2008).

Adenovirus was applied to deliver siRNA to inhibit the oncogene p28GANK in hepatocellular carcinoma (HCC) cell lines and in nude mice. Results showed that siRNA for p28GANK significantly could inhibit the tumor growth both in vitro and in vivo by inducing apoptosis of HCC cells (Li et al. 2005a).

In term of liver fibrosis, TGF-β1-siRNA has been used to knock down TGF-β1 expression in CCl4-treated mice. TGF-β1 gene inhibition resulted in reduced level type I collagen and α-SMA. In addition, ALT and AST levels also decreased and consequently reduced the progression of liver fibrosis (Kim et al. 2006). Similarly, plasmid vector containing TGF-β1-siRNA has utilized to target TGF-β 1 in a rat of CCL4- and high-fat diet-mediated fibrosis. TGF-β1 siRNA successfully silenced the expression of TGF-β1 leading to inhibition of HSC activation and reduced the production of type I and type III collagen (Lang et al. 2011).

In 2006, Li et al. evaluated the anti-fibrotic activity of connective tissue growth factor (CTGF)-siRNA in a rat model of CCl4-mediated hepatic fibrosis. They observed reduction in type I and III collagen and TGF-β1 levels and inhibited HSC activation (Li et al. 2006). Two different working groups have also used CTGF-siRNA to treat the progression of hepatic fibrosis; one study was conducted in hepatic fibrosis induced by N-nitrosodimethylamine (NDMA) (George and Tsutsumi 2007) and the other recent study was against liver fibrosis in CCL4-treated rat (Li et al. 2010). Both studies demonstrated that CTGF knockdown has led to reduced HSC activation and resolution of liver fibrosis. In addition, downregulation of TGF-β1 level and reduced accumulation of connective tissue proteins in the liver have been reported in the first study (George and Tsutsumi 2007), while the latter showed decrease in α-SMA level (Li et al. 2010). In 2008, Chen et al. investigated the influence of PDGFR β-siRNA on cultured activated HSC and in rat model of liver fibrosis induced by DMN and bile duct ligation. PDGFR β-siRNA delivery was carried out utilizing hydrodynamic-based transfection method. They found that downregulation of PDGFR-β could inhibit HSC activation and proliferation and block mitogen-activated protein kinase (MAPK) pathway in vitro. Fibrotic rats treated with PDGFR β-siRNA showed significant improvement in their liver function as well as suppressed of fibrosis progression (Chen et al. 2008).

In 2009, Cheng et al. constructed and evaluated different sequences of TGF-β1 siRNA and TGF-β1 shRNA on cultured HSC-T6. Lipofectamine 2000® was used to deliver TGF-β1 siRNAs, while pyridinium liposomes were used to transfect TGF-β1 shRNA to the activated HSC-T6. There was significant decrease in TGF-β1 and TIMP-1 after transfection of HSC-T6 with both TGF-β1 siRNA and TGF-β1 shRNA, which showed synergetic effect when they are used together.

Furthermore, TGF-β1 gene silencing was accompanied with decrease in both TNF-α and IL-1β (Cheng et al. 2009).

The development of liver-targeted specific delivery system of siRNA therapeutics is an important approach to minimize the toxicity and to improve the transfection efficiency (Park et al. 2011; Kang et al. 2010). In 2010, Kang et al. used polyethyleneimine (PEI) cationic polymer utilizing pullulan polysaccharide as targeted conjugate that achieved specific delivery of PEI-siRNA complexes to liver. The use of PEI-pullulan/siRNA complex not only improved the liver targeting efficiency, but also it minimized in vivo toxicity and mortality rate in rats as compared to the use of PEI-siRNA complex (Kang et al. 2010). Another study carried out by Park et al. developed siRNA delivery system targeting HSC in the liver. They have used reducible PEI conjugated to hyaluronic acid as targeting ligand to deliver TGF-β1 for treatment of liver fibrosis. The anti-fibrotic effect of (PEI-SS)-g-HA/siRNA has been evaluated on cultured HSC-T6 and in vivo in cirrhotic mice treated with CCl4. The (PEI-SS)-g-HA/siRNA demonstrated a low toxicity and high transfection efficiency both in vitro and in vivo. Moreover, TGF-β1 siRNA/(PEI-SS)-g-HA delivery showed significant protective effect against liver cirrhosis, whereas there was significant reduction in nodule formation, as well as collagen production, α-SMA expression, and HSC activation (Park et al. 2011).

7 Conclusion

siRNA is a novel technology for knocking down gene expression that may provide a solution to manage multiple diseases including liver fibrosis.

Despite a number of challenges for siRNA application in clinical practice including safety, stability, and effective delivery of siRNA, several studies have showed that the application of siRNA-based therapies in the treatment and prevention of liver diseases are effective. Various viral and non-viral carrier systems have been developed for the targeted delivery of siRNA. Utilizing lipid and polymeric nanoparticles has made significant improvement in terms of specific site delivery, significant inhibition to the target mRNA, and improved stability of siRNA. Other undesirable effects resulting from siRNA application, such as off-target effect and stimulation of the innate immune system, must be considered and overcome. Strategies like chemical modification to siRNA and developing novel nanocarriers with specific modified surface properties showed significant improvement in crossing these barriers. Other parameters including safety and transfection efficiency of the carrier systems must be evaluated for successful translation of siRNA therapy in clinical application.

Nonetheless, there are about 25 siRNA-based drugs in clinical trials for the treatment of various diseases including viruses, cancer, and liver diseases. As a result, siRNA-based therapy has become a promising potential for the treatment of a wide range of diseases.

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Exosomes: From Functions in Host-Pathogen Interactions and Immunity to Diagnostic and Therapeutic Opportunities

Jessica Carrière, Nicolas Barnich, and Hang Thi Thu Nguyen

Abstract Since their first description in the 1980s, exosomes, small endosomal-derived extracellular vesicles, have been involved in innate and adaptive immunity through modulating immune responses and mediating antigen presentation. Increasing evidence has reported the role of exosomes in host-pathogen interactions and particularly in the activation of antimicrobial immune responses. The growing interest concerning exosomes in infectious diseases, their accessibility in various body fluids, and their capacity to convey a rich content (e.g., proteins, lipids, and nucleic acids) to distant recipient cells led the scientific community to consider the use of exosomes as potential new diagnostic and therapeutic tools. In this review, we summarize current understandings of exosome biogenesis and their composition and highlight the function of exosomes as immunomodulators in pathological states such as in infectious disorders. The potential of using exosomes as diagnostic and therapeutic tools is also discussed.

Keywords Exosomes • Host-pathogen interactions • Immune responses • Infectious diseases

Contents

J. Carrière, N. Barnich, and H.T.T. Nguyen (✉)
University of Clermont Auvergne, M2iSH, UMR 1071 INSERM/University of Auvergne, Clermont-Ferrand 63001, France

INRA USC 2018, Clermont-Ferrand 63001, France
e-mail: hang.nguyen@udamail.fr

Abbreviations

Ag	Antigen
AIEC	Adherent-invasive *Escherichia coli*
APC	Antigen-presenting cells
APOBEC3G	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
BAT3	HLA-B-associated transcript 3
BCR	B-cell receptor
CD	Crohn's disease
CMV	Cytomegalovirus
CXCL11	C-X-C motif chemokine 11
DC	Dendritic cell
DT	Diphtheria toxoid
EBV	Epstein-Barr virus
EF1α1	Elongation factor 1-alpha 1
EM	Electron microscopy
ESCRT	Endosomal sorting complexes required for transport
EV	Extracellular vesicle
FACS	Fluorescence activated cell sorting
FasL	Fas ligand
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPC1	Glypican-1
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSC	Heat-shock cognate
HSP	Heat-shock protein
HTLV-1	Human T-cell leukemia virus type 1
i.v.	Intravenous
ICAM-1	Intercellular adhesion molecule 1
IEC	Intestinal epithelial cell

IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILV	Intraluminal vesicles
LAM	Lipoarabinomannan
LF	Lethal factor
LMP1	Latent membrane protein 1
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MFGE8	Milk fat globule-EGF factor 8 protein
MHC	Major histocompatibility complex molecules
miRNA	MicroRNA
MVE	Multivesicular endosome
MyD88	Myeloid differentiation primary response protein 88
NEF	Negative regulatory factor
NF-κB	Nuclear factor-kappa B
NK	Natural killer
NKG2D	Natural killer group 2 member D receptor
OVA	Ovalbumin
PA	Protective antigen
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PfPTP2	Plasmodium falciparum tyrosine phosphatase 2
RANTES	Regulated on activation, normal T cell expressed and secreted
SNARE	Soluble *N*-ethylmaleimide-sensitive fusion attachment protein (SNAP) receptors
TAR	Transactivating response
TGF-β	Tumor growth factor beta
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
tRNA	Transfer RNA
TSG101	Tumor susceptibility gene 101
vtRNA	Vault RNA

1 Introduction

Cell-to-cell communication is crucial for maintaining homeostasis within a multicellular organism. In particular, this communication is fundamental in innate and acquired immunities to trigger well-orchestrated immune responses. Among identified mediators, extracellular vesicles (EVs) have achieved a growing interest and are the subject of an increasing number of studies. Several types of EVs have been described to date that have been given different names throughout literature

such as microvesicles (also called microparticles or ectosomes) to designate EVs directly released from the plasma membrane, membrane particles, microvesicles, nanoparticles, "exosome-like" microvesicles, tolerosomes, prostasomes, or exosomes to refer to EVs released upon fusion of multivesicular endosomes (MVEs) with the plasma membrane. EVs are traditionally classified according to their intracellular origin, their physical properties, or their protein content. Specific isolation tools and techniques to distinguish EVs from different origins in order to establish a reliable classification are lacking. Therefore, Kowal and coauthors have recently compared the protein content of heterogeneous populations of EVs in order to establish a reliable classification (Kowal et al. 2016). According to the authors, EVs can be firstly classified according to their sedimentation speed, and then EV subpopulations can be distinguished according to their floatation density on iodixanol gradient and their protein content (Kowal et al. 2016).

Exosomes are defined as small EVs (30–100 nm in diameter) pelleting at high speed (ultracentrifugation at 100,000 g) and released upon fusion of MVEs with the plasma membrane (Colombo et al. 2014). In the 1980s, P. Sthal's and R. Johnstone's groups originally identified exosomes by their role in elimination of the transferrin receptor via secretion during reticulocyte maturation (Harding et al. 1983; Pan et al. 1985). Since their first description, exosomes have been well-characterized and were shown to be nanovesicles of endocytic origin. Exosomes have been successfully purified from most of body fluids (i.e., serum, saliva, urine, breast milk, etc.) and from cell culture medium (Théry et al. 2006). Analysis of molecular composition of exosomes allowed identification of a rich content with numerous proteins as well as lipids and nucleic acids (Théry et al. 2009). In addition to the molecular composition, numerous groups have been interested in studying the functions of exosomes either in physiological or in pathological states. To date, the most widely documented function of exosomes is their role in immunoregulation. Indeed, exosomes act as crucial regulators in innate immunity since exosomes released from immune cells were shown to be able to stimulate activation, proliferation, and inflammatory responses in various immune recipient cells (Théry et al. 2009). In addition, increasing evidence supports the involvement of exosomes in acquired immunity and particularly in antigen presentation (Théry et al. 2009). The wide range of functions of exosomes in immunoregulation attracts the attention of scientists in fields of research of pathologies such as infectious disorders. As such, exosomes have been shown to be involved in immunoregulation during fungal, parasitic, viral, and bacterial infections, and they can be beneficial either for host defense or for virulence and spread of pathogens. Due to their accessibility in various body fluids and their capacity to convey a complex molecular content even to distant cells, exosomes have been proposed as potential diagnostic, vaccine, and therapeutic tools. However, only a few experiments have been performed to date, in which exosomes were used to diagnose disease, vaccinate, and convey therapeutic molecules.

In this review, we introduce current understandings of biogenesis, secretion, and composition of exosomes. We will then highlight the function of exosomes as immunomodulators in pathological states such as in infectious disorders. The

potential of using exosomes as diagnostic, vaccine, and therapeutic tools will also be discussed. It is worthy to note that in several publications cited in this review, other terms rather than "exosomes" were used, which correspond to a mixture of vesicles from different origins.

2 Exosome Biogenesis and Secretion

Exosomes have been isolated from various body fluids such as urine, saliva, bile, breast milk, or blood (Yáñez-Mó et al. 2015). Exosomes are actively secreted by most cell types, in particular, immune cells such as B cells (Clayton et al. 2005), T cells (Nolte-'t Hoen et al. 2009), dendritic cells (DCs) (Théry et al. 1999; Zitvogel et al. 1998), macrophages (Bhatnagar et al. 2007), platelets (Heijnen et al. 1999), and mast cells (Raposo et al. 1997) and from other cell types such as neurons (Fauré et al. 2006), epithelial (Marzesco et al. 2005), endothelial (Song et al. 2014), and mesenchymal stem cells (Lai et al. 2015).

The unique property of exosomes is attributed to their endocytic origin. During exosome biogenesis, extracellular components and membrane receptors are endocytosed in an early endosome (Fig. 1). Then, early endosomes mature into late endosomes (Stoorvogel et al. 1991) and, during this process, small intraluminal vesicles (ILVs) accumulate into MVEs upon budding of the inner membrane of late endosomes, leading to sequestration of proteins, lipids, and cytosolic components. Although MVEs can subsequently fuse with the lysosome to induce cargo degradation (Woodman and Futter 2008), some MVEs can fuse with the plasma membrane, resulting in the release of ILVs as exosomes (Denzer et al. 2000).

Although exosome biogenesis is still being defined, a well-described mechanism for ILV formation is driven by the endosomal sorting complexes required for transport (ESCRT), which is composed of four ESCRT complexes (ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III) with associated proteins (e.g., ALIX, VPS34) (Hanson and Cashikar 2012). Firstly identified in endosomal sorting and degradation of ubiquitinated proteins (Davies et al. 2009; Metcalf and Isaacs 2010), ESCRT proteins have been shown to mediate membrane invagination process and ILV formation (Davies et al. 2009; Hurley 2010; Metcalf and Isaacs 2010). Thus, ESCRT-0 binds ubiquitinated proteins, allowing their delivery to MVEs (Raiborg and Stenmark 2002). Then, ESCRT-0 recruits ESCRT-I, which consequently recruits ESCRT-II and ESCRT-III (Babst et al. 2002; Katzmann et al. 2001). By triggering membrane invagination and scission, ESCRT-III enables ILV formation (Wollert et al. 2009). Several studies support the involvement of ESCRT proteins in exosome biogenesis since knockdown of ESCRT proteins has been shown to abolish ILV formation and exosome secretion (Stuffers et al. 2009; Tamai et al. 2010).

Another ESCRT-independent mechanism in exosome biogenesis has been raised, in which siRNA-mediated silencing of *ESCRT* genes did not abrogate totally exosome release (Stuffers et al. 2009). First, analysis of exosome secretion from

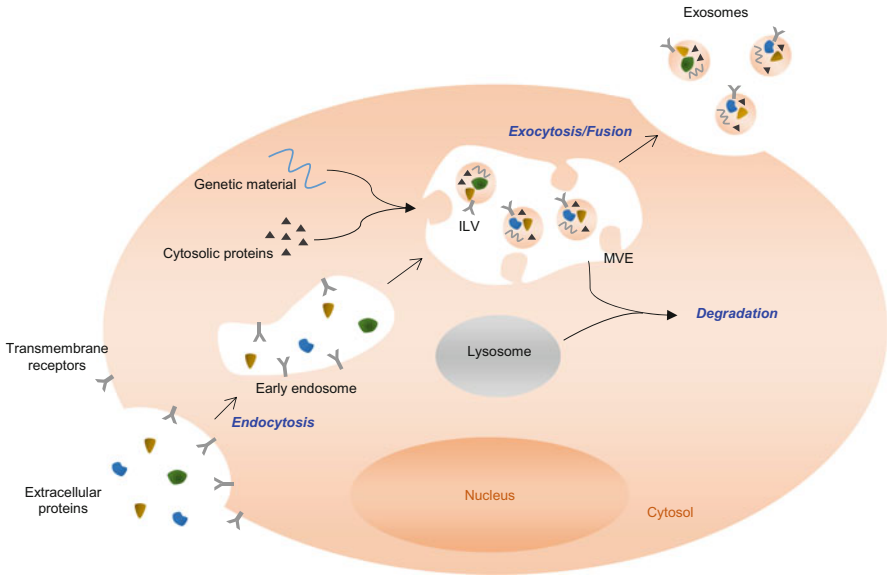

Fig. 1 Exosome biogenesis. Extracellular proteins and transmembrane receptors are endocyted in an early endosome. Early endosomes mature into late endosomes, and small intraluminal vesicles (ILVs) accumulated into their lumen upon budding of the inner membrane of late endosomes, leading to sequestration of cytosolic components and genetic material. Multivesicular endosomes (MVEs) will then fuse with either lysosomes, leading to the degradation of their content, or the plasma membrane, a process involving SNARE proteins and RAB GTPases, leading to the release of ILVs called "exosomes" into extracellular environment

oligodendrocytes, central nervous system cells, showed that exosome secretion requires the sphingolipid ceramide (Trajkovic et al. 2008). Another study reported that tetraspanins might be also involved in exosome biogenesis since depletion of the CD63-coding gene in vitro in melanocytes or in vivo in *cd63*$^{-/-}$ mice led to a reduction of ILV formation (van Niel et al. 2011).

Once MVEs are formed, they can either fuse with the lysosome to mediate cargo degradation (Woodman and Futter 2008) or with plasma membrane, a process mediated by the cytoskeleton, small GTPases, and fusion machinery (Colombo et al. 2014). Among the GTPases involved in ILV exocytosis, several RAB GTPases, which are members of the Ras GTPase superfamily, have been identified in exosomes such as RAB5, RAB11, RAB27, and RAB35. Indeed, RAB11 inhibition by overexpressing a dominant negative mutant in K562 erythroleukemia cells decreased exosome release (Savina et al. 2002). Moreover, inhibition of RAB35 function resulted in an impaired exosome secretion and accumulation of ILVs (Hsu et al. 2010). Furthermore, shRNA-mediated silencing of RAB27A and RAB27B in HeLa cells decreased exosome secretion (Ostrowski et al. 2010). Recently, it was reported that inhibition of RAL-1 GTPase resulted in a hampered fusion of MVEs with the plasma membrane and consequently in a decreased exosome secretion (Hyenne et al. 2015). The fusion machinery, involving soluble *N*-ethylmaleimide-

sensitive fusion attachment protein (SNAP) receptors (SNARE), has been shown to mediate exosome secretion. SNARE proteins form complexes between vesicular v-SNARE (vesicular-associated membrane proteins or VAMP) proteins and cell membrane t-SNARE proteins (Zylbersztejn and Galli 2011). It was reported that overexpression of the SNARE protein VAMP7 in K562 cells led to an impaired exosome secretion (Fader et al. 2009).

3 Exosome Molecular Composition

Exosomes have been shown to contain proteins, lipids, and nucleic acids (Fig. 2). Protein content of exosomes has been extensively analyzed by several techniques including Western blotting, immune-electron microscopy (immuno-EM), fluorescence-activated cell sorting (FACS), and mass spectrometry (Colombo et al. 2014). Exosomal protein content varies depending on the cell type of origin: for example, B-cell-derived exosomes contain the B-cell receptor (BCR), and DC-derived exosomes contain MCH-II, CD86, and ICAM-1 proteins (Théry et al. 2009). Furthermore, exosomes contain some common proteins such as adhesion molecules [milk fat globule-EGF factor 8 (MFGE8), integrins, and tetraspanins (CD63, CD81, and CD9)], chaperones [heat-shock cognate protein 70 (HSC70), heat-shock protein 90 (HSP90)], proteins involved in membrane trafficking (e.g., RAB GTPases, annexins) and in MVE biogenesis (e.g., clathrin, ALIX, TSG101), etc. (Fig. 2) (Colombo et al. 2014). Recently, proteomic analysis of heterogenous populations of small EVs, separated by a combinatorial approach using differential ultracentrifugation, floatation in a density gradient, and immuno-isolation, confirmed that exosomes can be distinguished from other subpopulations as they are co-enriched in CD63, CD9, and CD81 tetraspanins and endosomal markers (Kowal et al. 2016). Interestingly, proteomic analyses revealed that exosomes contain proteins from different cell compartments such as the plasma membrane, cytosol, or endosomes, while proteins from the nucleus, the mitochondria, the endoplasmic reticulum or the Golgi apparatus are almost missing in exosomes (Lundholm et al. 2014; Théry et al. 2009). These data confirm that exosomes arise from specific subcellular compartments and not from cell fragmentation. The identified exosomal proteins are listed in the online databases ExoCarta (http://www.exocarta.org) (Mathivanan et al. 2012) and Vesiclepedia (http://microvesicles.org/).

Exosomal lipid composition has been characterized, mainly using mass spectrometry or high-performance liquid chromatography (Laulagnier et al. 2004a, b; Llorente et al. 2013; Trajkovic et al. 2008; Wubbolts et al. 2003). As such, exosomes have been shown to be enriched in sphingomyelin, phosphatidylserine, cholesterol, and fatty acids, as compared to plasma membrane (Record et al. 2014). Moreover, exosomes are enriched in GM3 ganglioside (Llorente et al. 2013; Wubbolts et al. 2003), ceramide, and derivatives (Laulagnier et al. 2005; Llorente et al. 2013; Trajkovic et al. 2008). However, lysobisphosphatidic acid (LBPA), a lipid enriched in endosomal compartments and thought to be found in ILVs

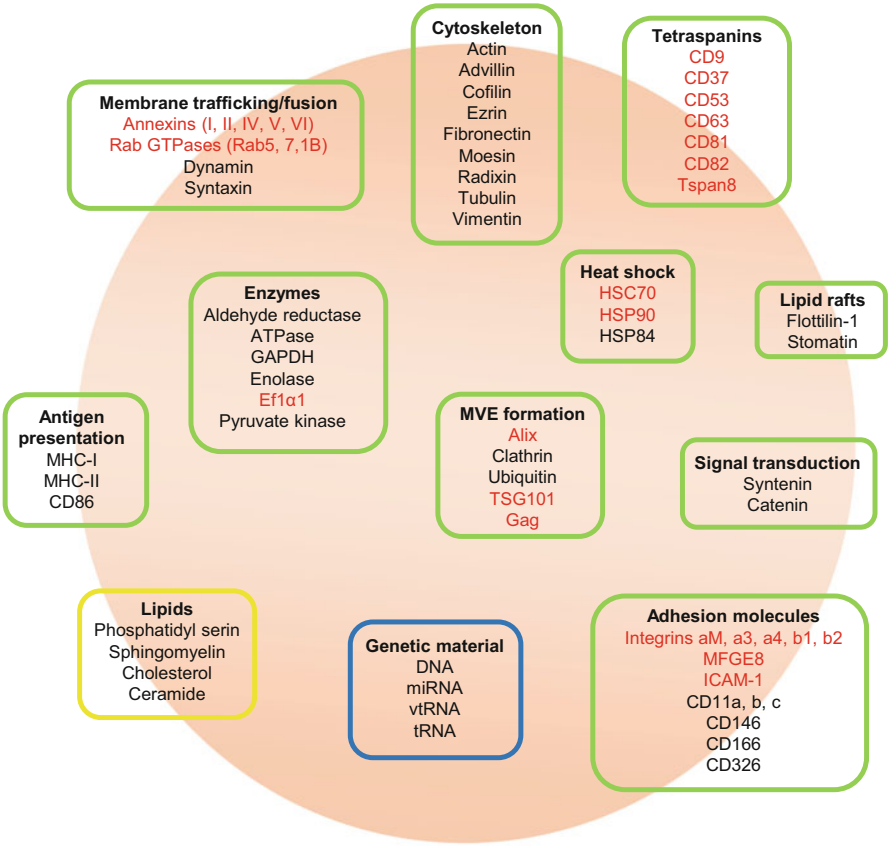

Fig. 2 Molecular composition of a typical exosome. Common composition including genetic material (in *blue box*), proteins (in *green boxes*), and lipids (in *yellow box*) found in a typical exosome is depicted. Proteins shown in *red* have been considered as exosomal markers. *EF1α1* elongation factor 1-alpha 1, *HSC* heat-shock cognate, *HSP* heat-shock protein, *ICAM-1* intercellular adhesion molecule 1, *MFGE8* milk fat globule EGF factor 8 protein, *MHC* major histocompatibility complex molecules, *MVE* multivesicular endosome, *TSG101* tumor susceptibility gene 101

(Matsuo et al. 2004), is not enriched in exosomes (Laulagnier et al. 2004b; Wubbolts et al. 2003). According to these studies, exosomes seem to display a specific lipid composition (enriched in cholesterol, sphingomyelin, and GM3 ganglioside) similar to that of lipid raft microdomains on plasma membranes. Thus, Tan and colleagues suggested and confirmed that lipid rafts are endocytosed into MVEs and released on exosomes (Tan et al. 2013). Interestingly, it has been shown that exosome biogenesis mechanisms evolve during cell maturation since the lipid content of exosomes derived from reticulocytes is similar to that of donor reticulocytes (enriched in ceramide) but is modified in erythrocytes (Carayon et al. 2011).

Exosomal lipids have been included in ExoCarta and Vesiclepedia databases as well.

Numerous groups have analyzed the genetic material in exosomes after the first description of nucleic acids in exosomes by Valadi and colleagues (Valadi et al. 2007). In this pioneer study, exosomes derived from human HMC-1 mast cells and murine MC/9 mast cells were shown to contain multiple and heterogenous RNA species including mRNAs and microRNAs (miRNAs), which were efficiently transferred to recipient cells and biologically active (Valadi et al. 2007). Then, exosomes derived from immune cells have been shown to hold a specific set of miRNAs that can be transferred to recipient cells (Mittelbrunn et al. 2011; Montecalvo et al. 2012). For example, exosomes derived from human THP-1 macrophages convey the miRNA 150, which is handled by recipient endothelial HMEC-1 cells and inhibits the expression of its target gene *c-Myb* (Zhang et al. 2010). Moreover, high-throughput next-generation sequencing techniques have allowed the identification of other small RNAs in exosomes such as small noncoding RNAs [vault RNA (vtRNA), Y-RNA, transfer RNA (tRNA)] but limited amounts of DNA and ribosomal RNA (Nolte-'t Hoen et al. 2012; van den Boorn et al. 2013).

4 Exosome as Immunomodulators

4.1 Exosomes and Innate Immunity

Exosomes have been involved in modulating innate immune responses (Fig. 3). Raposo and colleagues have reported the release of exosomes from B lymphocytes, suggesting the involvement of exosomes in immune responses (Raposo et al. 1996). Natural killer (NK) cells can be activated through the binding to its surface receptor of HLA-B-associated transcript 3 (BAT3), which is expressed on DC-derived exosomes (Simhadri et al. 2008). Exovesicles derived from mature DCs induce a pro-inflammatory response in intestinal epithelial cells which in turn secrete pro-inflammatory cytokines and chemokines [tumor necrosis factor alpha (TNF-α), regulated on activation, normal T cell expressed and secreted (RANTES), interleukin 8 (IL-8), monocyte chemoattractant protein 1 (MCP-1)] in a TNF-α-dependent pathway (Obregon et al. 2009). Although the ultracentrifugation-based purification method used in this study is consistent with exosome purification, an involvement of other types of EVs cannot be excluded. Moreover, exosomes released from mouse DCs express on their surface TNF superfamily members [TNF, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and Fas ligand (FasL)], which directly bind to their receptors on NK cells to enhance their cytotoxic activity (Munich et al. 2012). Intradermal injection of wild-type mice with mouse DC-derived exosomes increased the amount of NK cells in the draining lymph node, and this required activation of

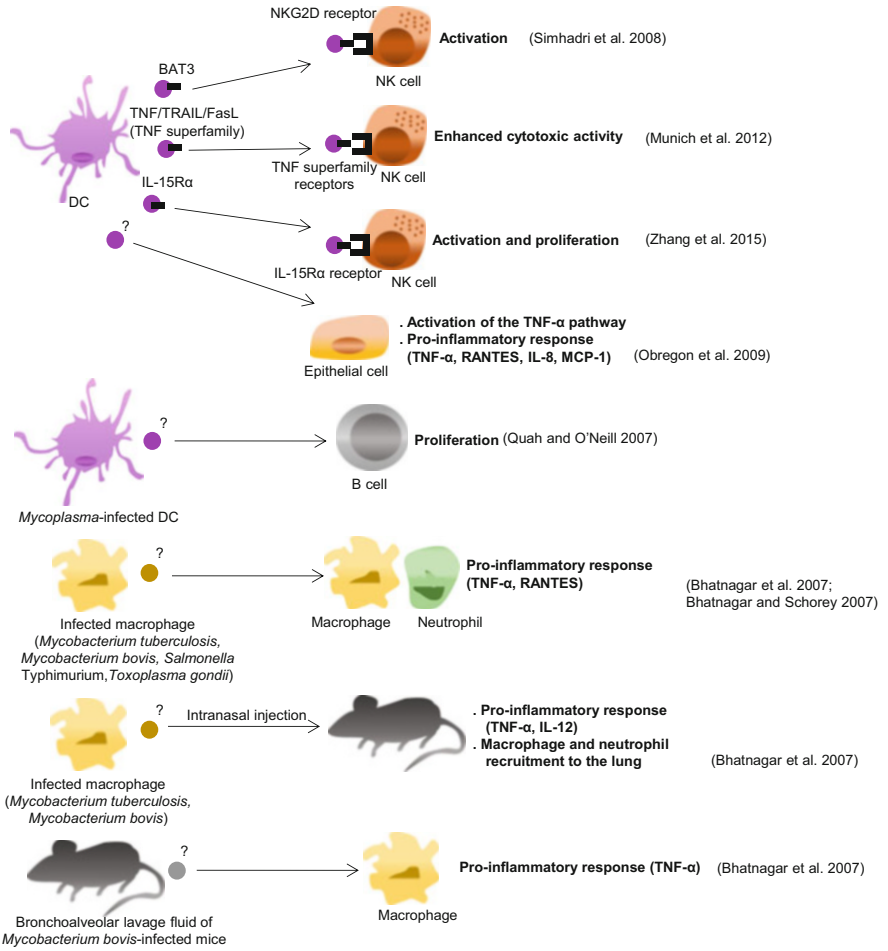

Fig. 3 Exosomes in innate immunity. Here are summarized main functions of exosomes in innate immunity. *BAT3* HLA-B-associated transcript-3, *DC* dendritic cell, *FasL* Fas ligand, *IEC* intestinal epithelial cell, *TNF* tumor necrosis factor, *TRAIL* tumor necrosis factor-related apoptosis-inducing ligand, *NK* natural killer, *NKG2D* natural killer group 2 member D *RANTES* regulated on activation, normal T cell expressed and secreted, *IL* interleukin MCP-1, monocyte chemoattractant protein-1, *?* exosomal content undetermined

the natural killer group 2 member D (NKG2D) receptor on NK cells (Viaud et al. 2009). A recent study reported that exosomes released from mouse DCs carry on their surface IL-15Rα, a NKG2D receptor ligand (Viaud et al. 2009). Upon activation, NKG2D induces activation and proliferation of NK cells (Zhang et al. 2015).

Exosomes released from macrophages infected with intracellular pathogens, when exposed to uninfected macrophages, induce secretion of pro-inflammatory mediators such as TNF-α and RANTES (Bhatnagar et al. 2007; Bhatnagar and

Schorey 2007). Moreover, intranasal injection of mice with exosomes released from mycobacteria-infected macrophages increases secretion of pro-inflammatory mediators (TNF-α and IL-12) and neutrophil and macrophage recruitment in the lung (Bhatnagar et al. 2007). In vitro stimulation of macrophages with exosomes purified from bronchoalveolar lavage fluid of mycobacteria-infected mice provokes an increase of TNF-α secretion (Bhatnagar et al. 2007). It has also been shown that *Mycoplasma*-infected DCs release exosomes that trigger B-cell proliferation, and this is independent of any antigen presentation (Quah and O'Neill 2007).

4.2 Exosomes and Acquired Immunity

Increasing evidence supports the involvement of exosomes in adaptive immune responses and particularly in antigen presentation (Fig. 4). During antigen presentation, antigen-presenting cells (APCs) such as DCs or B lymphocytes present antigen-MHC complexes to T lymphocytes that are consequently activated. Identification of MHC classes I and II and T-cell co-stimulatory molecules on exosomes released from immune cells (Colombo et al. 2014) led scientists to consider exosomes as new mediators of antigen presentation. Moreover, exosomes have been shown to contain antigens. Exosomes derived from tumor cell lines (Napoletano et al. 2009; Wolfers et al. 2001) or ascites from cancer patients (Andre et al. 2002) carry tumor antigens. Furthermore, macrophages infected with *Mycobacterium tuberculosis* or *Mycobacterium bovis* can release exosomes containing bacterial antigens (Giri et al. 2010; Giri and Schorey 2008).

Several works highlighted the capacity of exosomes to perform indirect antigen presentation or cross-presentation (Fig. 4). Antigens conveyed by exosomes are handled by APCs, which complex antigens with their own MHC molecules to present these antigen peptides to T lymphocytes. Stimulation of T lymphocytes with antigen-containing exosomes in the presence of naïve recipient DCs resulted in activation of T cells (Andre et al. 2002; Napoletano et al. 2009; Wolfers et al. 2001). Another study reported that injection of antigen- or peptide-bearing exosomes induced antigen-specific naïve $CD4^+$ T-cell activation in vivo, but in vitro, these exosomes failed to induce antigen-dependent T-cell stimulation unless intermediate DCs were present (Théry et al. 2002). Similarly, exosomes released from mouse mast cells carry antigens and can activate naïve DCs, which in turn activate T cells in vitro (Skokos et al. 2001). Skokos and colleagues injected ovalbumin (OVA)-bearing exosomes released from mast cells, recovered DCs, and showed their ability to activate OVA-specific T-cell hybridomas (Skokos et al. 2003). Moreover, exosomes released from macrophages infected with *Mycobacterium tuberculosis* or *Mycobacterium bovis* carry bacterial antigens that, in the presence of intermediate APCs, can activate $CD4^+$ and $CD8^+$ lymphocytes isolated from mycobacteria-immunosensitized mice (Giri and Schorey 2008).

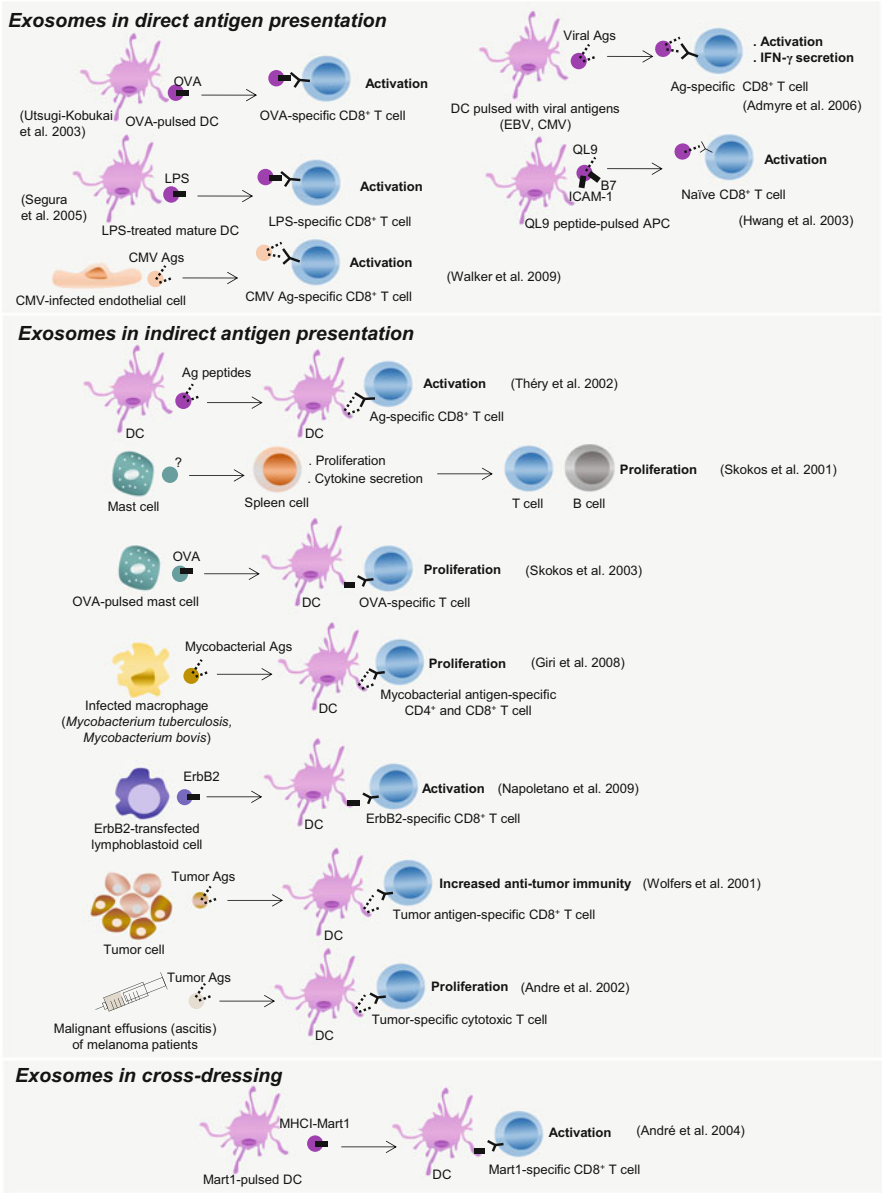

Fig. 4 Exosomes in adaptive immunity. Here are summarized main functions of exosomes in adaptive immunity (i.e., in direct antigen presentation, indirect antigen presentation and in cross-dressing). *Ag* antigen, *APC* antigen-presenting cell, *CMV* cytomegalovirus, *DC* dendritic cell, *EBV* Epstein-Barr virus, *LPS* lipopolysaccharide, *OVA* ovalbumin, *?* exosomal content undetermined.

Increasing evidence has shown that exosomes can be involved in direct antigen presentation (Fig. 4). APC-derived exosome-like microvesicles can directly activate naïve $CD8^+$ T cells in vitro (Hwang et al. 2003). Monocyte-derived DCs secrete exosomes containing viral antigens which can activate T lymphocytes in vitro without the presence of DCs (Admyre et al. 2006). Similarly, OVA-containing exosomes derived from mouse OVA-pulsed DCs can directly activate OVA-specific $CD8^+$ T-cell hybridomas (Utsugi-Kobukai et al. 2003). Another study reported that exosomes derived from lipopolysaccharide (LPS)-treated DCs induced a strong antigen-specific T-cell activation both in vitro and in vivo (Segura et al. 2005).

Some evidence showed that pre-formed antigen-MHC complexes carried in exosomes can be directly handled by APCs and presented to T lymphocytes, in a process named "cross-dressing" (Fig. 4) (Yewdell and Dolan 2011). Montecalvo et al. showed that DCs can secrete exosomes containing antigen-MHC complexes (Montecalvo et al. 2008). These exosomes can be internalized, and the antigen-MHC complexes can be directly presented by DCs to activate $CD8^+$ T lymphocytes (André et al. 2004). However, these results are debatable since some studies have shown the disability of exosomes bearing antigen-MHC complexes to perform "cross-dressing" (Coppieters et al. 2009; Wakim and Bevan 2011).

5 Exosomes in Host-Pathogen Interactions

Exosomes secreted during host responses to infection with several pathogen classes including fungi, parasites, viruses, and bacteria have been isolated and characterized. The content and activity of these exosomes, which can be derived from infected host cells or from pathogens, have been analyzed.

5.1 Exosomes in Fungal Infection

Only few studies concerning the involvement of exosomes in fungal infection are available, and these are limited to the analysis of exosomes derived directly from the fungi but not from fungus-infected cells (Fig. 5). EVs released from the yeast *Cryptococcus neoformans* strain induce cytokine secretion by recipient macrophages as shown by increased TNF-α and tumor growth factor beta (TGF-β) secretion, leading to a restricted fungal infection (Oliveira et al. 2010). It should be noted that the ultracentrifugation-based purification method was used in this study, thus, an involvement of other EVs rather than exosomes cannot be excluded. On another hand, exosomes have been proposed to promote fungal virulence. Indeed, blocking export of exosomes from *C. neoformans* by knocking down *SEC6* (involved in fusion of exocytic vesicles with the plasma membrane) diminished virulence of the yeast in vivo (Panepinto et al. 2009). This decreased

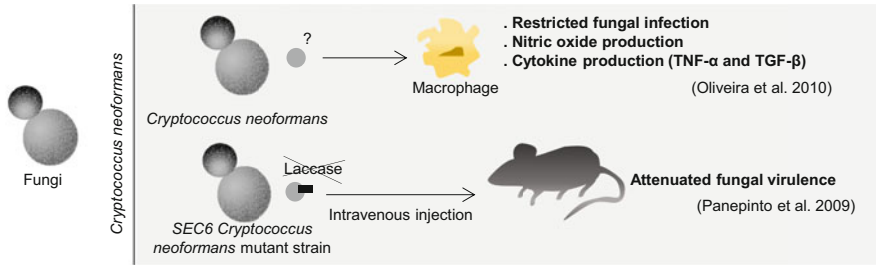

Fig. 5 Exosomes in fungal infection. This figure summarizes the known functions of exosomes in fungal infection. Exosome source and their functional impacts on recipient cells/organisms with underlying mechanism are presented. *TNF* tumor necrosis factor, *TGF-β* tumor growth factor beta, *?* exosomal content undetermined

virulence was shown to be due to the inability of the yeast to export crucial virulence factors such as laccase (Panepinto et al. 2009).

5.2 *Exosomes in Parasitic Infection*

The involvement of exosomes in parasitic infection, including those released from infected host cells and from the parasite, has been analyzed (Fig. 6). The first study of exosomes in parasitic infection is performed by Bhatnagar and colleagues, which showed that exosomes released from macrophages infected with the intracellular protozoan *Toxoplasma gondii* triggered a pro-inflammatory response in naïve macrophages with an increased secretion of TNF-α (Bhatnagar et al. 2007).

Exosomes have also been studied in the context of *Plasmodium* infection. Red cells infected with the malaria-causative parasite *Plasmodium falciparum* release exosome-like vesicles and microvesicles that contain parasite components (Mantel et al. 2013) and particularly the parasitic protein *Plasmodium falciparum* tyrosine phosphatase 2 (PfPTP2), which promotes sexual differentiation of the parasite (Regev-Rudzki et al. 2013). An in vivo study reported that microvesicles isolated from the plasma of malaria-infected mice induce a pro-inflammatory response in macrophages in vitro with increased TNF-α secretion (Couper et al. 2010).

Moreover, infection of blood cells with *Trypanosoma cruzi* provokes the release of microvesicles which, by forming a complex with the complement C3 convertase on the parasite surface, protect the parasite against complement-mediated lysis, resulting in increased parasite survival (Cestari et al. 2012). It was also reported that *T. cruzi* release EVs carrying parasitic small RNAs which confer susceptibility to infection upon uptake by mammalian epithelial cells (Garcia-Silva et al. 2014). In this study, EVs were isolated using ultracentrifugation method, thus, an involvement of exosomes cannot be excluded. Using the same techniques, it has also been shown that small EVs (e.g., exosomes) released from *T. cruzi* display a phosphatase

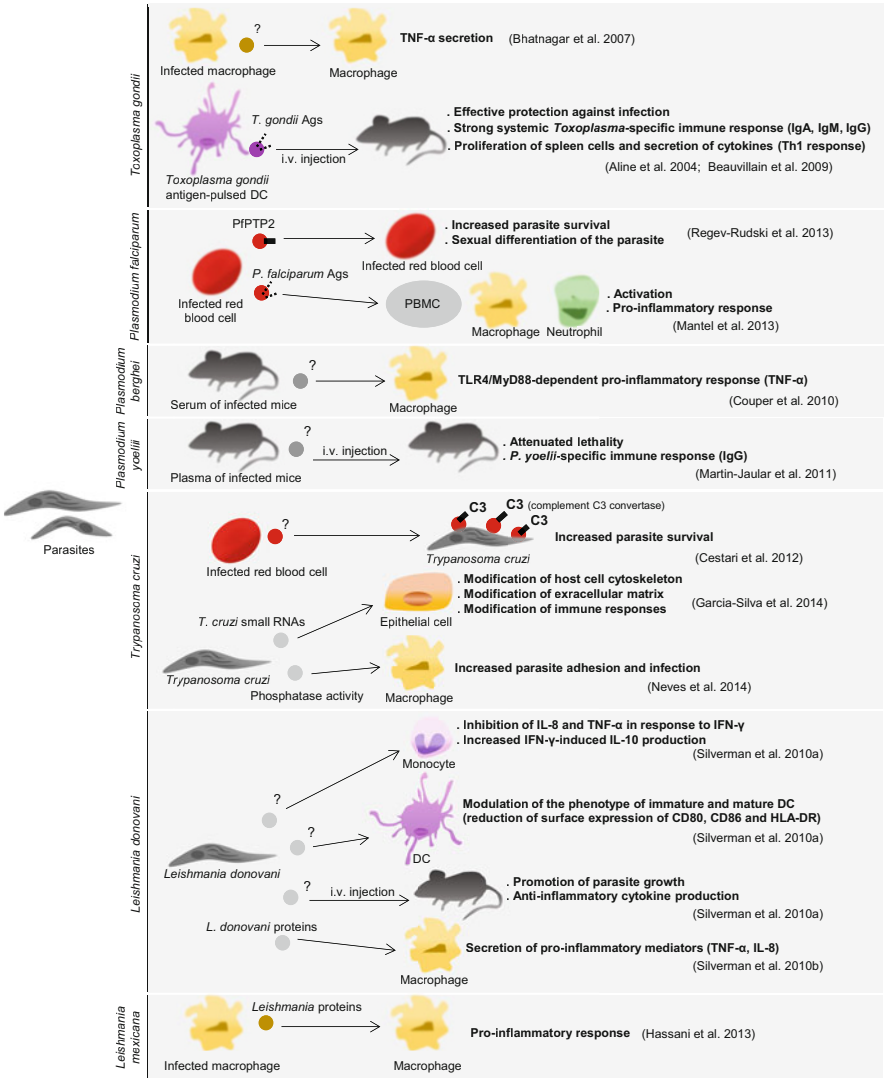

Fig. 6 Exosomes/extracellular vesicles in parasitic infection. Here are summarized known functions of exosomes in parasitic infection. Exosome source and their functional impacts on recipient cells/organisms with underlying mechanism are presented. *DC* dendritic cell, *HLA* human leukocyte antigen, *IFN* interferon, *Ig* immunoglobulin, *i.v.* intravenous, *PBMC* peripheral blood mononuclear cells, *PfPTP2 Plasmodium falciparum* tyrosine phosphatase 2, *?* exosomal content undetermined

activity resulting in increased adhesion and invasion abilities of the parasite in host cells (Neves et al. 2014).

The involvement of exosomes in the context of *Leishmania* infection has also been studied. Exosomes were proposed to mediate the delivery of *Leishmania* into macrophages. Indeed, it has been shown that *Leishmania* spp. release exosomes to

deliver proteins to recipient macrophages, inducing a pro-inflammatory response (Silverman et al. 2010b). Similarly, *Leishmania*-derived exosomes induce secretion of pro-inflammatory cytokines by recipient monocytes (Silverman et al. 2010a). The HSP100 protein has a crucial role in the packaging of *Leishmania*'s proteins into exosomes since its absence resulted in a modification of exosome content and an impaired pro-inflammatory activity in recipient cells (Silverman et al. 2010b). *L. major*- and *L. donovani*-derived exosomes have also been shown to suppress the immune response in vivo since mice pretreated with these exosomes prior to infection showed higher parasite burden compared with untreated mice (Silverman et al. 2010b). Proteomic analyses revealed that exosomes released from *Leishmania mexicana*-infected macrophages contain GP63 protein, an essential virulence factor (Hassani and Olivier 2013).

5.3 Exosomes in Viral Infection

Exosomes in the context of viral infection have been extensively studied (Fig. 7). The hypothesis of an involvement of exosomes in viral infection resulted from several observations. Numerous viruses such as hepatitis B, hepatitis C, and herpesviruses use the ESCRT machinery (Hurley 2010), to leave the infected host cell (Chen and Lamb 2008; Mori et al. 2008). Moreover, Fang and colleagues reported that human immunodeficiency virus (HIV) budding seems to result from a similar pathway to the exosome biogenesis pathway (Fang et al. 2007). This was later supported by another study showing the involvement of TSG101 and ALIX proteins in virus budding, two major proteins acting in exosome biogenesis (Usami et al. 2009).

A role for exosomes released from infected host cells in viral spread and in immunoregulation, which result in an increased infectivity of viruses, has been raised. For instance, exosomes in the context of HIV infection and diffusion has been extensively studied. Several groups reported that CD63 and CD81 tetraspanins, enriched in exosomes, participate in viral budding, viral spread, and in HIV infectivity (Grigorov et al. 2009; Izquierdo-Useros et al. 2009; Jolly and Sattentau 2007; Sato et al. 2008). Particularly, Grigorov and colleagues showed that HIV-1 structural Gag and Env proteins interact with the CD81 tetraspanin in tetraspanin-enriched microdomains on T-cell surface (Grigorov et al. 2009). Furthermore, CD81 expression is crucial for viral replication since shRNA-mediated inhibition of CD81 resulted in an impaired HIV-1 release (Grigorov et al. 2009). It has also been shown that the CD63 tetraspanin is eliminated from the plasma membrane of HIV-1-infected and virion-producing T cells and is embedded on the membrane of released virions (Sato et al. 2008). Interestingly, virion-incorporated CD63 was shown to inhibit HIV-1 infection (Sato et al. 2008). Exosomes have also been shown to convey HIV-1 proteins involved in viral replication cycle such as GAG (Fang et al. 2007) and negative regulatory factor (NEF; (de Carvalho et al. 2014; Lenassi et al. 2010). NEF-containing exosomes

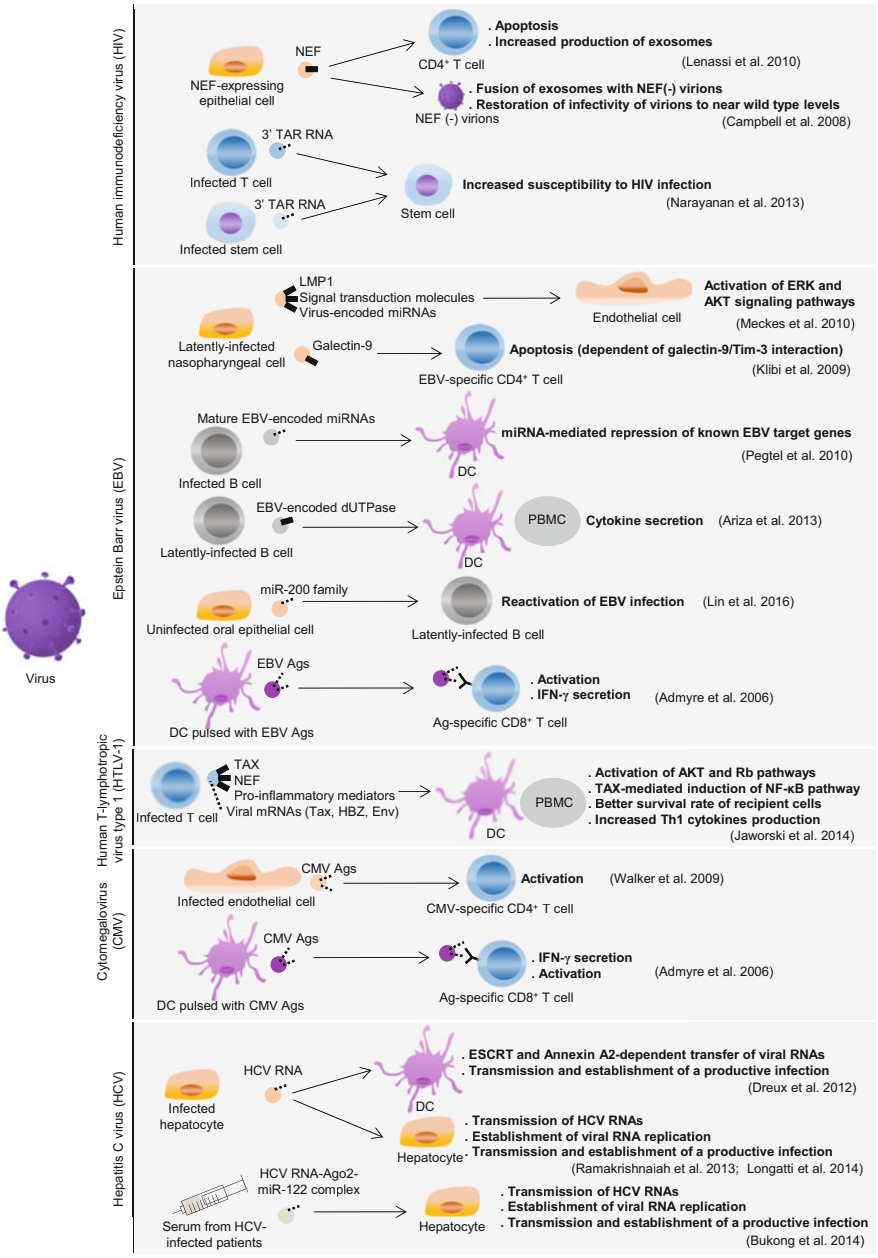

Fig. 7 Exosomes in viral infection. This figure summarizes the known functions of exosomes in viral infection. Exosome source and their functional impacts on recipient cells/organisms with underlying mechanism are presented. *Ag* antigen, *CMV* cytomegalovirus, *DC* dendritic cell, *EBV* Epstein-Barr virus, *ERK* extracellular signal-regulated kinase, *ESCRT* endosomal sorting complexes required for transport, *HCV* hepatitis C virus, *HIV* human immunodeficiency virus, *HTLV-1* human T-lymphotropic virus type 1, *IFN* interferon, *LMP-1* latent membrane protein 1, *NEF* negative regulatory factor, *NF-κB* nuclear factor-kappa B, *PBMC* peripheral blood mononuclear cells, *TAR trans*-activation response element, *?* exosomal content undetermined

induce T-cell apoptosis in vitro, a key feature of HIV infection (Lenassi et al. 2010). Narayanan and colleagues showed that the HIV-1 RNA named transactivating response (TAR) is released into exosomes from infected host cells in vitro and from sera of HIV-infected patients (Narayanan et al. 2013). Moreover, pretreatment of host cells with exosomes derived from HIV-infected cells increased susceptibility of treated cells to HIV infection (Narayanan et al. 2013).

Exosomes have also been studied in the context of infection with other viruses. The latent membrane protein 1 (LMP1), an immunosuppressive protein important in Epstein-Barr virus (EBV) infection (Dukers et al. 2000), was found in exosomes released from EBV-infected B cells, suggesting that exosomes can mediate the immunosuppressive effect of LMP1 during EBV infection (Verweij et al. 2011). Exosomes released from EBV-infected cells also contain the dUTPase enzyme which triggers pro-inflammatory and antiviral responses in recipient DCs and peripheral blood mononuclear cells (PBMCs) (Ariza et al. 2013). Exosomes have also been shown to mediate a functional delivery of viral miRNAs. Indeed, exosomes released from EBV-infected B cells secrete exosomes containing EBV miRNAs which can induce inhibition of known EBV target genes in recipient cells such as C-X-C motif chemokine 11 (*CXCL11*), a cytokine involved in antiviral responses (Pegtel et al. 2010). Moreover, Jaworski et al. reported the incorporation of the human T-cell leukemia virus type 1 (HTLV-1) TAX protein which is crucial for viral replication (Jaworski et al. 2014). Hepatitis C virus (HCV)-infected cells secrete exosomes containing viral RNAs which can be transferred to recipient DCs, inducing DC activation and secretion of interferon-α (IFN-α) (Dreux et al. 2012). It was also reported that the HCV envelop glycoprotein interacts with the CD81 cell membrane protein and that this complex is released within exosomes (Masciopinto et al. 2004). HCV structural proteins have also been identified in exosomes purified from HCV-infected patients' plasma (Masciopinto et al. 2004).

5.4 Exosomes in Bacterial Infection

The involvement of exosomes during bacterial infection has been largely studied (Fig. 8). Particularly, the role of exosomes has been extensively analyzed in the context of mycobacterial infection. Exosomes derived from *Mycobacterium avium*-infected macrophages trigger a pro-inflammatory response in naïve recipient macrophages (Bhatnagar and Schorey 2007). Using antibody-based techniques, these exosomes were shown to contain glycopeptidolipids, a major mycobacterial cell wall constituent (Bhatnagar and Schorey 2007). Wang and colleagues reported that exosomes released from *Mycobacterium avium* subspecies *tuberculosis*-infected macrophages induce an increased release of the pro-inflammatory cytokines IFN-γ and TNF-α in naïve recipient macrophages (Wang et al. 2014). Similarly, *Mycobacterium tuberculosis*- and *Mycobacterium bovis*-infected macrophages release exosomes inducing a pro-inflammatory response in naïve recipient macrophages (Bhatnagar et al. 2007). The authors highlighted the presence of a mycobacterial

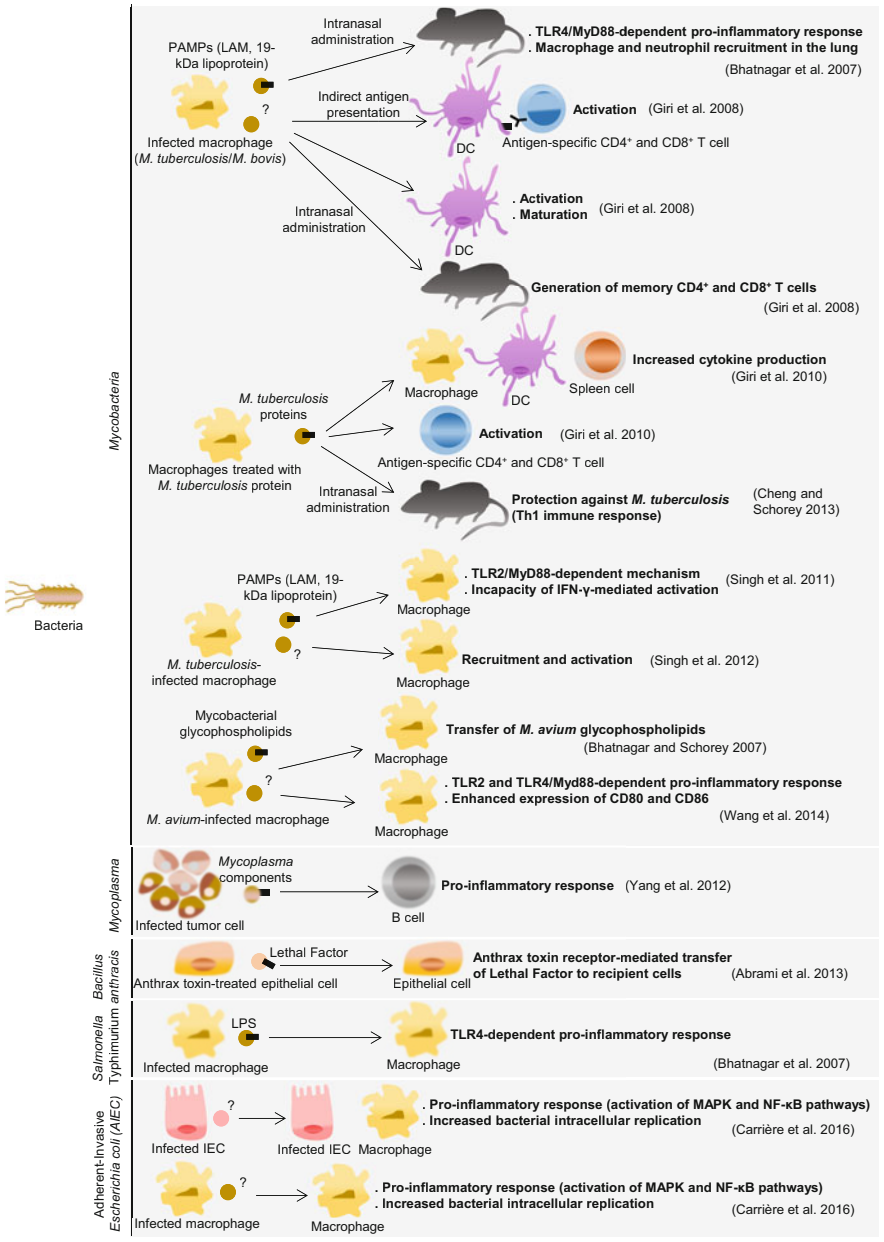

Fig. 8 Exosomes in bacterial infection. This figure summarizes the known functions of exosomes in bacterial infection. Exosome source and their functional impacts on recipient cells/organisms with underlying mechanism are presented. *DC* dendritic cell, *IEC* intestinal epithelial cell, *LAM* lipoarabinomannan, *LPS* lipopolysaccharide, *MAPK* mitogen-activated protein kinase, *MyD88* myeloid differentiation primary response protein 88, *NF-κB* nuclear factor-kappa B, *PAMP* pathogen-associated molecular pattern, *TLR* Toll-like receptor, *?* exosomal content undetermined

lipoprotein mediating this pro-inflammatory message through a Toll-like receptor (TLR)/myeloid differentiation primary response protein 88 (MyD88)-dependent pathway (Bhatnagar and Schorey 2007). These results were confirmed in vivo since exosomes purified from bronchoalveolar lavage fluid of *Mycobacterium bovis*-infected mice contain the mycobacterial components lipoarabinomannan and the 19-kDa lipoprotein and can trigger a pro-inflammatory response in vitro (Bhatnagar et al. 2007). Similarly, exosomes released from *Mycobacterium bovis*- or *Mycobacterium tuberculosis*-infected macrophages in vitro can, when intranasally injected into mice, induce an increased TNF-α and IL-12 secretion as well as neutrophil and macrophage recruitment in the lung (Bhatnagar et al. 2007). Moreover, exosomes released from *Mycobacterium tuberculosis*-infected macrophages can partially inhibit the ability of naïve macrophages to be activated by IFN-γ (Singh et al. 2011), which is crucial in host response to mycobacterial infection since activated macrophages control intracellular bacterial replication (Flynn et al. 1993).

The involvement of exosomes in infection with other bacteria has been also analyzed. It was shown that exosomes released from *Salmonella* Typhimurium-infected macrophages induced a pro-inflammatory response in naïve recipient macrophages (Bhatnagar et al. 2007). The authors showed that the released exosomes contain bacterial LPS (Bhatnagar et al. 2007), which was responsible for this pro-inflammatory response since no inflammatory response was observed in *tlr4*$^{-/-}$ macrophages depleted for the LPS receptor TLR4 (Bhatnagar et al. 2007). Furthermore, *Mycoplasma*-infected cells release exosomes that induced increased IFN-γ secretion in recipient B cells (Yang et al. 2012). Exosomes have also been shown to convey bacterial toxins. In fact, Abrami et al. reported that upon treatment of epithelial cells with the two components of the lethal anthrax toxin, protective antigen (PA) and lethal factor (LF), PA induced the formation of a channel allowing the translocation of LF in the cytosol and in ILVs (Abrami et al. 2013). LF persists in ILVs and is then released in exosomes that can be internalized and consequently delivered in recipient epithelial cells (Abrami et al. 2013).

Our group recently deciphered a previously unknown function of exosomes in the interaction between host cells and Crohn's disease (CD)-associated adherent-invasive *Escherichia coli* (AIEC). Increased abundance of invasive *E. coli* strains in intestinal mucosa of CD patients comparatively to control subjects have been reported (Baumgart et al. 2007; Conte et al. 2006; Darfeuille-Michaud et al. 1998; Martin et al. 2004; Martinez-Medina et al. 2009; Neut et al. 2002; Sasaki et al. 2007; Swidsinski et al. 2002), and these strains were later named AIEC (Darfeuille-Michaud et al. 2004). We showed that AIEC infection induced the release of exosomes by human intestinal epithelial cells and macrophages (Carrière et al. 2016). Characterization of the exosomes released from AIEC-infected cells showed that they are able to trigger a pro-inflammatory response in naïve intestinal epithelial and macrophagic cells with activation of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways and increased pro-inflammatory cytokine secretion (Carrière et al. 2016). In addition, stimulation of human intestinal epithelial cells and macrophages with exosomes released from

AIEC-infected cells increased bacterial intracellular replication compared with stimulation with exosomes secreted by uninfected cells (Carrière et al. 2016). Our findings suggest that exosomes are involved in the activation of host innate immune responses upon AIEC infection and in bacterial intracellular replication, two key features of host-AIEC interaction.

6 Exosomes in Disease States: Applications in Diagnostic, Vaccine, and Therapeutic Approaches

6.1 Exosomes: Promising Diagnostic Tools

Exosomes have been successfully purified from numerous body fluids such as blood, urine, bronchoalveolar lavage fluid, and saliva (Admyre et al. 2003; Caby et al. 2005; Pisitkun et al. 2004). Due to their easy recovery and their rich content, exosomes have been proposed as a new diagnostic tool in numerous diseases. Thus, the exosomal Fetuin-A, a protein which is synthesized in the liver and secreted into the blood, has been reported as a novel urinary biomarker for detecting acute kidney injury (Zhou et al. 2006). Especially, the content of tumor-derived exosomes has been extensively analyzed and proposed to diagnose cancers. Szajnik et al. reported that the plasma collected from ovarian cancer patients contains higher levels of exosomal proteins compared to control individuals, and that ovarian cancer patients can be distinguished from healthy individuals by the presence of TGF-β1 and MAGE3/6 in plasma-derived exosomes (Szajnik et al. 2013). Some new proteins previously undescribed have been identified in exosomes isolated from malignant pleural effusions of patients suffering from mesothelium, ovarian, breast and non-small cell lung cancers (Bard et al. 2004). Recently, Melo et al. showed using mass spectrometry analysis that exosomes purified from serum of pancreas cancer patients are enriched in glypican-1 (GPC1), a cell surface proteoglycan (Melo et al. 2015). Using flow cytometry, the authors observed that $GPC1^+$ exosomes enabled distinction between cancer patients from healthy individuals, even in early stages of the disease (Melo et al. 2015). Furthermore, another group developed a powerful multiplex detection chip for blood-based diagnosis of ovarian cancer by multiplexed measurement of three exosomal tumor markers CA-125, EpCAM, and CD24 (Zhao et al. 2015).

Exosomal miRNAs have also been proposed as diagnostic biomarkers since altered miRNA expressions have been reported in numerous diseases (Hu et al. 2012). Indeed, modified miRNA and long noncoding RNA profiles have been identified in exosomes isolated from peritoneum lavage fluid and plasma of patients suffering from gastric cancer (Li et al. 2015; Tokuhisa et al. 2015; Zhou et al. 2015; Zöller 2016). Modified miRNA profiles have also been identified in circulating exosomes derived from patients suffering from glioblastoma (Rabinowits et al. 2009), lung cancer (Rabinowits et al. 2009), and ovarian cancer

(Taylor and Gercel-Taylor 2008). The RNA content of exosomes isolated from the blood of patients with dental and neurologic disorders has been analyzed (De Smaele et al. 2010; Miranda et al. 2010; Palanisamy et al. 2010; Rabinowits et al. 2009), and the potential use of exosomal miRNAs as powerful diagnostic biomarkers for Alzheimer's disease has been raised (Van Giau and An 2016).

Finally, exosomes could be used as diagnostic tools in infectious diseases. The amount of exosomes in the serum of *Mycobacterium bovis*-infected mice increased proportionally to the bacterial burden (Singh et al. 2012). Moreover, *Mycobacterium tuberculosis*-infected cells secrete exosomes carrying mycobacterial proteins, suggesting the use of exosomes to diagnose tuberculosis (Kruh-Garcia et al. 2014).

6.2 *Exosome-Based Vaccination: An Encouraging Approach*

With their immunoregulatory property, exosomes have been proposed and tested as vaccines in cancer and in infectious diseases in order to mobilize the immune system against tumor cells or pathogens.

Dai and colleagues genetically modified human colon adenocarcinoma cells with a recombinant adenovirus encoding human IL-18 and showed that exosomes derived from these cells exhibited more potent capability to induce antitumor immunity compared with exosomes derived from nongenetically modified cells, suggesting that modification of exosomes could be an approach to develop exosome-based tumor vaccines (Dai et al. 2006). A phase I clinical trial reported that ascite-derived exosomes in combination with the granulocyte-macrophage colony-stimulating factor (GM-CSF) used as an adjuvant are safe, well tolerated, and induce a specific antitumor immunity in patients with colorectal cancer (Dai et al. 2008). Several studies reported that murine DC-derived exosomes are able to induce antigen-specific $CD4^+$ and $CD8^+$ T-cell responses both in vitro and in vivo and to enhance antitumor immunity in vivo (Damo et al. 2015; Luketic et al. 2007; Näslund et al. 2013a, b; Segura et al. 2005; Théry et al. 2002; Zitvogel et al. 1998). However, several phase I clinical trials using exosomes released from antigen-loaded DCs from cancer patients for treatment of non-small cell lung cancer and melanoma showed that the use of exosomes in vaccination is safe but does not exhibit a significant impact on tumor growth or in cancer regression (Escudier et al. 2005; Morse et al. 2005; Viaud et al. 2010). Nevertheless, a recent clinical trial revealed a modification of the protein and mRNA composition of exosomes released in glioma patients' plasma after receiving antitumor vaccines (Muller et al. 2015). This modification has been shown to be correlated with immunological and clinical responses as well as survival, providing a promising approach to evaluate glioma patients' response to antitumor vaccination (Muller et al. 2015).

Regarding the use of exosomes as vaccines in infectious conditions, it was shown that intravenous injection of exosomes released from DCs infected with the parasite *Leishmania major* conferred vaccinated mice an effective protection against infection, as shown by a decrease in the number of infected cells in draining nymph lodes (Schnitzer et al. 2010). Moreover, exosomes released from macrophages infected with *Mycobacterium bovis* or *Mycobacterium tuberculosis* can activate antigen-specific $CD4^+$ and $CD8^+$ T cells isolated from mycobacteria-immunosensitized mice and promote activation and maturation of DCs (Giri and Schorey 2008). Macrophages treated with *Mycobacterium tuberculosis* proteins released exosomes that, upon intranasal injection into mice, activated DCs and $CD4^+$ and $CD8^+$ T cells isolated from *Mycobacterium tuberculosis*-infected mice (Giri et al. 2010). Furthermore, DCs treated with the highly immunogenic diphtheria toxoid (DT) protein secrete exosomes that, once injected intraperitoneally in mice, stimulate a specific DT IgG response (Colino and Snapper 2006). Exosomes have also been suggested to be used as vaccines in parasitosis. Indeed, upon intravenous injection of exosomes released from DCs pulsed with *Toxoplasma gondii* antigens, anti-*Toxoplasma gondii* IgM antibodies were detected in the serum of mice (Aline et al. 2004). Moreover, mice were subcutaneously vaccinated before pregnancy with exosomes released from DCs pulsed with *Toxoplasma gondii*-derived antigens and infected with the parasite during pregnancy (Beauvillain et al. 2009). The results showed that vaccination resulted in effective protection of pups against congenital infection (Beauvillain et al. 2009). Another study showed that infection of epithelial cells with the protozoan parasite *Cryptosporidium parvum* results in an increased luminal secretion of exosomes (Hu et al. 2013). These exosomes were shown to contain antimicrobial peptides such as cathelicidin-37 and beta-defensin-2 that affect survival of the parasite (Hu et al. 2013). Recently, using proteomic analysis, the parasite *Schistosoma mansoni* has been shown to secrete exosomes carrying potential virulence factors as well as known vaccine candidates (Sotillo et al. 2015). The use of exosomes as vaccine in *Cryptococcus* infection has been proposed since extracellular vesicles of the *Cryptococcus neoformans* yeast strain induce activation of recipient macrophages, improving their abilities to perform phagocytosis and to secrete microbicidal components (Oliveira et al. 2010). Finally, exosomes can constitute a defense mechanism in viral infection. Indeed, Khatua and colleagues identified the secretion in exosomes of the viral cytidine deaminase apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) (Khatua et al. 2009), a protein known to control the replication of several enteroviruses (Chiu and Greene 2008). The authors reported that APOBEC3G-containing exosomes confer recipient epithelial cells protection against HIV-1 infection (Khatua et al. 2009).

6.3 *Exosomes: Promising New Conveyors of Therapeutic Molecules*

Cells use exosomes as cargos to deliver proteins and genetic material to neighboring or distant recipient cells. In addition to the use of antigen-pulsed DC-derived exosomes to induce antitumor immune responses (Damo et al. 2015; Escudier et al. 2005; Luketic et al. 2007; Morse et al. 2005; Näslund et al. 2013a, b; Segura et al. 2005; Théry et al. 2002; Viaud et al. 2010; Zitvogel et al. 1998), different strategies for delivering therapeutic molecules using exosomes have been proposed and developed. When being directly incubated with exosomes, curcumin, an anti-inflammatory agent, and antitumor agents such as doxorubicin and paclitaxel have been successfully incorporated into exosomes and have been shown to be effective in vitro and in vivo (Sun et al. 2010; Tian et al. 2014; Yang et al. 2015; Zhuang et al. 2011). Indeed, in a mouse model of sepsis, intraperitoneal injection of curcumin-containing exosomes resulted in the protection of mice against a LPS-induced septic shock (Sun et al. 2010). Similarly, in mouse models of brain inflammation, intranasal administration of curcumin-carrying exosomes led to the uptake of exosomes by microglia cells and consequently to an effective curcumin-mediated anti-inflammatory effect (Zhuang et al. 2011). Moreover, administration of doxorubicin or paclitaxel-containing exosomes to tumor-bearing mice or zebra fishes induced antitumor effects (Tian et al. 2014; Yang et al. 2015). As exosomes can convey genetic material, they have been proposed for the delivery of exogenous RNA in disease states. Using electroporation, a siRNA against gluceraldehyde-3 phosphate dehydrogenase was incorporated into DC-derived exosomes and effectively delivered in vivo, leading to the loss of expression of its target gene (Alvarez-Erviti et al. 2011). Some years later, Ohno and colleagues used this technique to deliver miRNAs in breast cancer (Ohno et al. 2013). Breast cancer is associated with an increased expression of the epidermal growth factor receptor (EGFR) in cancer cells (Woodburn 1999). The authors first transfected an EGF-encoding plasmid into human embryonic kidney HEK293 cells and purified secreted exosomes expressing EGF on their surface. By transfecting the antitumor miRNA let-7a in the EGF-expressing exosomes, they were then successful to deliver let-7a miRNA specifically to EGFR-expressing xenograft breast cancer tissue in immunodeficient *rag2*$^{-/-}$ mice (Ohno et al. 2013).

Another strategy based on treating donor cells with drugs has been developed in order to incorporate drugs inside exosomes. This approach enabled the incorporation of antitumor agents such as paclitaxel, etoposide, or carboplatin in HepG2 hepatoma cell line-derived exosomes (Lv et al. 2012). Furthermore, in vitro treatment of NK cells with these exosomes led to an increase of their cytotoxic activity toward cancer cells (Lv et al. 2012).

Finally, two groups have transfected macrophages with plasmids encoding therapeutic proteins such as catalase or glial cell line-derived neurotropic factor (Haney et al. 2013; Zhao et al. 2014). By injecting these macrophages to a mouse model of Parkinson's disease, the authors observed the release of exosomes

carrying modified genetic material and an improvement of motor functions with disease-associated neurodegeneration and neuroinflammation (Haney et al. 2013; Zhao et al. 2014).

7 Conclusion

Although exosomes have become the focus of exponentially growing interest since their first description about 30 years ago, our knowledge of these nanovesicles has only just begun. Working with exosomes remains challenging because of their small size and the fact that other extracellular vesicles (i.e., microvesicles, microparticles, etc.) or biofluid components can be co-extracted with exosomes. Although numerous studies have reported an important role of exosomes in immunoregulation, most of the time, the exosomal component responsible for the functional impact on recipient cells has not been identified. This might be due to the difficulty to identify a relevant candidate among the numerous exosomal proteins, nucleic acids, and lipids. Consequently, the mechanisms underlying the exosome-mediated immune responses observed in recipient cells (i.e., activated or inhibited signaling pathways, etc.) are not always elucidated. Moreover, the role of exosomes as an immunoregulator has been shown only in pathological states, and their functions in homeostasis remain to be elucidated. Finally, although only few experiments and clinical trials have been performed to date, the accessibility of exosomes in various body fluids, the proved safety and feasibility of the use of exosomes in clinical experiments, and the first promising results suggest that exosomes might become a future powerful diagnostic, vaccine, and drug delivery tool for numerous diseases.

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The Stress-Response MAP Kinase Signaling in Cardiac Arrhythmias

Xun Ai, Jiajie Yan, Elena Carrillo, and Wenmao Ding

Abstract Stress-response kinases, the mitogen-activated protein kinases (MAPKs) are activated in response to the challenge of a myriad of stressors. c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinases (ERKs), and p38 MAPKs are the predominant members of the MAPK family in the heart. Extensive studies have revealed critical roles of activated MAPKs in the processes of cardiac injury and heart failure and many other cardiovascular diseases. Recently, emerging evidence suggests that MAPKs also promote the development of cardiac arrhythmias. Thus, understanding the functional impact of MAPKs in the heart could shed new light on the development of novel therapeutic approaches to improve cardiac function and prevent arrhythmia development in the patients. This review will summarize the recent findings on the role of MAPKs in cardiac remodeling and arrhythmia development and point to the critical need of future studies to further elucidate the fundamental mechanisms of MAPK activation and arrhythmia development in the heart.

Keywords Aging • Arrhythmia • Cardiovascular diseases • Heart • Mitogen-activated protein kinases • Stress

Contents

X. Ai (✉), J. Yan, E. Carrillo, and W. Ding
Department of Molecular Biophysics and Physiology, Rush University Medical Center, 1750 W Harrison St, Chicago, IL 60612, USA
e-mail: xun_ai@rush.edu

Non-standard Abbreviations and Acronyms

AERP	Atrial effective refractory period
AF	Atrial fibrillation
APD	Action potential duration
Ca^{2+}	Calcium
CABG	Coronary artery bypass graft
CaMKII	Calcium/calmodulin dependent protein kinase II
CDC42	Cell division protein 42 homolog
CL	Cycle length
CVD	Cardiovascular disease
DAD	Delayed afterdepolarizations
EAD	Early afterdepolarizations
Egr-1	Early growth response protein
ERK	Extracellular signal regulated kinase
HF	Heart failure
HL-1	Mouse atrial myocyte line from Louisiana State University
I/R	Ischemia reperfusion
ICa	L-type calcium channel
JNK	c-Jun N-terminal kinase
LA	Left atrium
LAD	Left anterior descending
MAPK	Mitogen-activated protein kinase
MI	Myocardial infarction
MKK	Mitogen-activated protein kinase kinase
NCX	Sodium calcium exchange channel
p38	Member of MAPK family
PLB	Phospholamban
Pro	Proline amino acid residue
Rac1	Ras-related C3 botulinum toxin substance 1
RyR	Calcium-triggered calcium release channel
Ser	Serine amino acid residue
SERCA	Sarcoplasmic reticulum calcium ion-ATPase
SP-1	Transcription factor SP-1
SR	Sarcoplasmic reticulum
TAC	Transverse aortic constriction
Thr-Gly-Tyr	Threonine, glycine, tyrosine amino acid sequence

1 Introduction

Accumulating evidence suggests that intrinsic stress (e.g., oxidative stress and chronic inflammatory stress) are markedly enhanced in aging and cardiovascular diseases (CVDs), while the aged and pathologically altered hearts (such as by ischemia and heart failure) have also been shown to exhibit a higher susceptibility to extrinsic stress stimuli (Beckman and Ames 1998; Belmin et al. 1995; He et al. 2011; Ismahil et al. 2014; Juhaszova et al. 2005; Neuman et al. 2007). Mitogen activated protein kinase (MAPK) cascades act as critical regulators of cell survival and growth in response to both intrinsic and extrinsic stress challenges. The MAPK family is composed of c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinases (ERKs), and p38 MAPKs. These three subfamilies have been the focus of extensive studies to uncover their roles in cardiac disease development (Davis 2000; Karin and Gallagher 2005; Ramos 2008; Rose et al. 2010). Recently, the impacts of these stress-response MAPKs on cardiac arrhythmic remodeling have begun to be revealed (Hagiwara et al. 2007; Ho et al. 1998, 2001; Huang et al. 2014; Scharf et al. 2013; Takahashi et al. 2004; Yan et al. 2013).

Cardiac arrhythmias, a disturbance in the regular rhythm of the heartbeat, are commonly associated with aging and CVDs. While ventricular arrhythmias can be life-threatening arrhythmias, atrial arrhythmia, especially atrial fibrillation (AF) is the most common arrhythmias and a significant health problem, AF is associated with significant morbidity and mortality including a 5-fold increased risk for stroke (Krahn et al. 1995; Wolf et al. 1978), tripled risk for heart failure (HF), and 40–90% increased all-cause mortality (Benjamin et al. 1998, 2009; Krahn et al. 1995). In the Framingham Cohort Study, the risk of stroke is 5.6 times higher in AF patients compared to those with sinus rhythm (Kannel et al. 1983) and AF patients present a poorer prognosis and higher mortality (Lip 2013). The comorbidity of AF aggravates existing CVDs such as HF and accelerates the progression of ventricular dysfunction, all-cause hospitalization, and increased mortality (Aleong et al. 2014; Carson et al. 1993; Clark et al. 1997; Dries et al. 1998). To date, nearly 15 million people worldwide and 5 million people in the USA are affected by HF (Cowie et al. 1997; Hershberger et al. 2003; January et al. 2014). It has been shown that CVDs and aging are also the independent risk factors for cardiac arrhythmias (Benjamin et al. 1994; Ehrlich et al. 2002; Kannel et al. 1998; Neuberger et al. 2007). By 2050, the prevalence of both AF and HF will more than double as the population ages (Di Lenarda et al. 2003; Kannel and Benjamin 2008; Linne et al. 2000). This accounts for more than 350,000 hospital admissions annually and costs the health care system approximately $26 billion each year (Calkins et al. 2012; Kannel et al. 1982; Kannel and Benjamin 2008; Wattigney et al. 2003). Thus, this review focuses on the recent progress in understanding the role of stress-response MAP kinases in the development of cardiac arrhythmias, especially atrial arrhythmias.

2 Electrical Remodeling and Arrhythmia Development

It is generally believed that abnormal triggers initiate arrhythmias. Reentry circuits form and are sustained by an arrhythmogenic substrate, due to both molecular and structural remodeling (Allessie et al. 1976; Mandapati et al. 2000; Nattel et al. 2008). Studies suggest that electrical remodeling of membrane ion channels (e.g., Ca^{2+} and potassium channels) leads to altered action potential duration (APD) and atrial effective refractory period (AERP); both have been found to be associated with the development of AF (Christ et al. 2004; Marx et al. 2000; Nattel et al. 2007). Before the onset of AF, shorter AERPs have been associated with a higher inducibility of AF, while longer AERPs and slowing atrial conduction velocity, which may cause a pro-arrhythmogenic shortening of the conduction wavelength (Rensma et al. 1988), have been found to be linked to AF development in HF patients and animals (Huang et al. 2003; Sanders et al. 2003). In aged rabbit left atrium, we found that a slight reduction in AERP and unchanged APD were associated with slowed conduction velocity and a markedly increased pacing-induced AF compared to that of young controls (Yan et al. 2013). Although similar results of slightly altered APD and AERP were also reported in aged canine and rat atria (Anyukhovsky et al. 2005; Huang et al. 2006), studies from coronary artery bypass graft (CABG) surgery patients suggest that AERP was positively correlated with age (Sakabe et al. 2003). However, the molecular and electrophysiological properties of human hearts are varied and complicated, especially when co-existing pathological conditions (such as HF or myocardial infarction) are present.

Extensive studies in ventricular myocytes have shown that ectopic activities can occur by prolonged APD causing early afterdepolarizations (EADs) and by spontaneous SR Ca^{2+} releases leading to delayed afterdepolarizations (DADs) (Bers 2000; Nattel et al. 2008; Venetucci et al. 2008). EADs normally occur with abnormal depolarization during phase 2 or phase 3 of the action potential. While ventricular myocytes can only develop phase 2 EADs, atrial myocytes do not produce phase 2 EADs but may produce late phase 3 EADs with an abbreviation of the atrial APD (Burashnikov and Antzelevitch 2003). These late phase 3 EADs have only been shown to be responsible for the immediate initiation of AF following termination of paroxysmal AF, but not in the case of new-onset AF or recurrence of AF that has been terminated for a long time (Oral et al. 2003; Timmermans et al. 1998). Thus, other features of the arrhythmogenic substrate such as sarcoplasmic reticulum (SR) Ca^{2+} handling dysfunction, a generally acknowledged arrhythmogenic factor of generating DADs, could play an important role in heart failure or age-related enhancement of atrial arrhythmogenicity.

3 Excitation–Contraction Coupling

An action potential is essential for triggering myocyte contraction. Excitation–contraction coupling is the link between myocyte excitation (depolarization of the action potential) and Ca^{2+} release from the SR for myocyte contraction. Ca^{2+} entry via L-type Ca^{2+} channels along with a much smaller amount of Ca^{2+} influx via Na^{+} and Ca^{2+} exchanger (NCX) activates SR Ca^{2+} release via Ca^{2+} triggered Ca^{2+} release channel (RyR) in ventricular myocytes. During systole, this Ca^{2+} triggered SR Ca^{2+} release produces a large intracellular Ca^{2+} ($[Ca]_i$) transient that drives cell contraction (Bers 2000). During diastole, RyRs usually remain closed and excess cytosolic Ca^{2+} ions are removed from cytosol either by pumping Ca^{2+} back to SR (via SERCA2 function) or extruding Ca^{2+} from the cell (mostly via NCX function) (Bers 2000). However, RyRs can (albeit rarely) spontaneously open during diastole. Individual diastolic RyR openings represent non-spark SR Ca^{2+} leak. Diastolic RyR openings that drive local inter-RyR Ca^{2+} induced Ca^{2+} release (CICR) may produce a Ca^{2+} spark (i.e., spark-mediated SR Ca^{2+} leak). Unusually large/frequent sparks may trigger propagating diastolic Ca^{2+} waves. Abnormally high SR Ca^{2+} leak will reduce SR Ca^{2+} content and consequently reduce systolic fractional SR Ca^{2+} release ($[Ca]_{FR}$) for a given L-type voltage-gated Ca^{2+} current (I_{Ca}) trigger (Ai et al. 2005; Bers 2014; Respress et al. 2012). Propagating Ca^{2+} waves will result in excess diastolic NCX function, which is electrogenic (three Na in, one Ca^{2+} out), and thus may produce abnormal triggered activities (e.g., DADs) and initiate arrhythmias (Bers 2000, 2014).

Although Ca^{2+} handling in atrial myocytes is similar to that of ventricular myocytes, there are some important structural and cellular signal differences between atrial and ventricular myocytes. Atrial myocytes are thinner and longer (Walden et al. 2009), which may lead to a longer delay between APs and Ca^{2+} transients at the center of the cells. This property of the atrial cell can increase the instability of Ca^{2+} propagation, which is pro-arrhythmogenic. Compared to the ventricles, atria have a smaller Ca^{2+} transient amplitude and a higher rate of intracellular Ca^{2+} decay (Freestone et al. 2000; Walden et al. 2009). This is due to an increased SERCA uptake and enhanced function of NCX to remove cytosolic Ca^{2+} during the diastolic phase (Walden et al. 2009). The increased SERCA-dependent intracellular Ca^{2+} removal is attributed to the greater amount of SERCA2 and decreased expression of SERCA inhibitory protein phospholamban (PLB) (Freestone et al. 2000; Walden et al. 2009). Also, atrial myocytes have higher SR Ca^{2+} content compared to ventricular myocytes (Walden et al. 2009). The greater SR Ca^{2+} content makes atrial myocytes prone to spontaneous diastolic SR Ca^{2+} release when RyRs are pathologically sensitized, such as during AF (Bers 2014; Chelu et al. 2009; Neef et al. 2010; Venetucci et al. 2008).

In addition, atrial myocytes exhibit a different structural pattern of the Transverse tubules (T-tubules) compared to ventricular myocytes. T-tubules are an important subcellular network involved in SR Ca^{2+} dynamics in ventricular myocytes (Brette and Orchard 2003; Franzini-Armstrong et al. 2005; Ibrahim

et al. 2010; Wang et al. 2001). T-tubules are located at the z-line of the myocyte and provide close coupling of L-type Ca^{2+} channels to ryanodine receptors (RyRs) on the SR membrane. This structure allows rapid intracellular Ca^{2+} triggered SR Ca^{2+} release in response to electrical excitation (Franzini-Armstrong et al. 2005). Emerging evidence suggests that an atrial T-tubule network is present in large mammalian species including humans, sheep, dogs, cows, and horses although atrial T-tubular networks are less abundant and less organized compared to that in the ventricles (Dibb et al. 2009; Lenaerts et al. 2009; Richards et al. 2011; Wakili et al. 2010). While it was previously believed that atrial T-tubules were virtually absent in the small rodents (Berlin 1995; Forbes et al. 1990), a recent report by Frisk et al. (2014) showed similar structural organization and density of the T-tubules in pig and rat atria. A disorganized T-tubule network has been found to contribute to SR Ca^{2+} release dysfunction in failing ventricular myocytes from both human and HF animal models (Balijepalli et al. 2003; Heinzel et al. 2008; Louch et al. 2006; Lyon et al. 2009). In rapid pacing-induced failing dog atria, reduced T-tubular abundance was also found to be linked to altered subcellular Ca^{2+} dynamics and AF development (Dibb et al. 2009; Lenaerts et al. 2009; Yeh et al. 2008). While accumulating evidence suggests that atrial T-tubular structure is present in most mammalian species, its functional role in arrhythmia development requires further investigation.

4 Arrhythmia Initiation and Abnormal Ca^{2+} Triggered Activities

Arrhythmia initiation stems from DADs that are caused by SR Ca^{2+} handling dysfunction. Others and we have previously discovered that increased diastolic SR Ca^{2+} release causes abnormal ectopic activities, which lead to ventricular arrhythmogenesis in the failing heart (Ai et al. 2005; Respress et al. 2012; Yeh et al. 2008). During the diastolic phase, SR Ca^{2+} release normally shuts off almost completely (~99%). However, increased diastolic RyR Ca^{2+} release could be responsible for increased diastolic SR Ca^{2+} leak and reduced systolic $[Ca]_{FR}$ for a given L-type voltage-gated Ca^{2+} current (I_{ca}) as the release trigger (Bassani et al. 1995; Bers 2014; Shannon et al. 2000). The increased diastolic SR Ca^{2+} leakage along with an impaired function of Ca^{2+} uptake due to altered SERCA2 elevates the amount of $[Ca]_i$ and prolongs the $[Ca]_i$ decay phase in HF (Bers 2000, 2014). Then, increased Na influx via NCX for $[Ca]_i$ removal can produce abnormal triggered activities (e.g., DADs) and initiate atrial arrhythmias (Bers 2000, 2014). Studies suggest that alterations of Ca^{2+} handling proteins including RyR2, PLB, and Cav1.2 contribute to changed intracellular Ca^{2+} transients and diastolic SR Ca^{2+} release (DeSantiago et al. 2002; Schulman et al. 1992; Wu et al. 1999).

Others and we have previously demonstrated that activated CaMKII, a pro-arrhythmic signaling molecule, is critically involved in phosphorylation of

RyR2–2815 and PLB-Thr17 (RyR2815-P, PLB17-P), which results in sensitized RyR channels that in turn leads to triggered activities and arrhythmia initiation due to diastolic SR Ca^{2+} leak in pathologically altered ventricles (Ai et al. 2005; Greiser et al. 2009; Hoch et al. 1999; Maier et al. 2003; Respress et al. 2012; Sossalla et al. 2010; Yeh et al. 2008; Zhang et al. 2003). Recent studies indicate that alterations of CaMKII-dependent RyR phosphorylation are also exhibited in the atrium of chronic AF patients (Chelu et al. 2009; Neef et al. 2010). Results from several animal models have shown that these altered SR Ca^{2+} handling proteins contribute to enhanced SR Ca^{2+} leak and AF development (Chelu et al. 2009; Chiang et al. 2014). Alteration of I_{Ca} could also contribute to abnormal SR Ca^{2+} release, and studies indicate that reduced I_{Ca} is a hallmark of AF-induced electrical remodeling (Christ et al. 2004; Van Wagoner et al. 1999). These results indicate that SR Ca^{2+} mishandling could be the major cause of arrhythmias in HF and chronic AF (Ai et al. 2005; Respress et al. 2012; Yeh et al. 2008). CaMKII inhibition has been shown to improve the function of L-type Ca^{2+} channel in mouse ventricular myocytes and cultured HL-1 atrial myocytes, which could be due to up-regulated expression of L-type Ca^{2+} channel proteins (Ronkainen et al. 2011; Zhang et al. 2005). However other studies have reported inconsistent results of increased, reduced, or unchanged I_{ca} preceding the onset of AF in postoperative patients compared to that of patients at low risk for AF (Christ et al. 2004; Dinanian et al. 2008; Van Wagoner et al. 1999; Workman et al. 2009). Thus, the underlying mechanisms of abnormal Ca^{2+} handling in AF onset and maintenance in the pathologically altered heart require further investigation.

5 Arrhythmia Stabilization

Under normal conditions, the initiation of the cardiac impulse (generated from the sinus node) and impulse propagation (through gap junctional channels – specialized membrane structures that directly connect adjacent cells by providing chemical and electrical communication) across the cardiac tissue are critical for maintaining normal heart rhythm and impulse conduction.

AF is a heart rhythm disorder (termed an arrhythmia) with fast and irregular beats in the upper chambers (atria) of the heart. Abnormal triggered activities initiate AF, while arrhythmogenic substrates sustain it. The area of the pulmonary veins located adjacent to the left atrium (LA) has been shown to be the most common place for AF initiation, but the arrhythmogenic substrates include the structural and electrical remodeling of the atrium and are required for the maintenance of AF (Burstein and Nattel 2008). Both shortened atrial effective refractory period and reduced conduction velocity have been linked to the maintenance of AF (Wijffels et al. 1995). Although electrical remodeling of cardiac membrane ion channels (such as calcium and potassium channels) leads to shortened duration of action potentials and shortened refractory periods during AF, these alterations alone might not be sufficient to provide a substrate for sustained reentry in single or

multiple circuits (Nattel et al. 2007). As such, AF has been considered as a reentrant arrhythmia (Janse and Wit 1989). There is accumulated evidence suggesting that ischemia and HF as well as elderly myocardium, all undergo slowed conduction of the cardiac action potential, and this is likely due to the electrical and structural remodeling correlating with increased age (Anyukhovsky et al. 2005). Although cardiac conduction is determined by the active membrane properties of each cell (largely a function of sodium channels) and tissue resistivity (gap junction channels) (Walton and Fozzard 1983), expression and activity of sodium channels have been shown to be unchanged in aging LA (Baba et al. 2006). This disruption of electrical coupling has been associated with the age-related increase in fibrosis and the remodeling of the gap junctions in aging sinus node and aortic endothelium (Jones et al. 2004; Yeh et al. 2000). Thus, gap junction proteins could also play a critical role in slowed conduction in diseased and aging hearts.

6 MAPKs and Arrhythmia Development

Studies suggest that aged and diseased hearts exhibit increased intrinsic stress and higher susceptibility to extrinsic stress stimuli (Beckman and Ames 1998; Belmin et al. 1995; He et al. 2011; Ismahil et al. 2014; Judge and Leeuwenburgh 2007; Juhaszova et al. 2005; Li et al. 2005a; Neuman et al. 2007; Yang et al. 2005). In response to stress stimuli, the mitogen-activated protein kinases (MAPKs) are activated (Rose et al. 2010). The MAPK family is composed of three major members, c-Jun NH2-terminal kinases (JNK), extracellular signal-regulated kinases (ERK1/2), and p38 kinase. MAPK activation has been found to be critical in the development of various diseases such as diabetes (Brozzi and Eizirik 2016), cancer (Xiao et al. 2016), Alzheimer's disease (Yarza et al. 2015) as well as various cardiac diseases such as cardiac hypertrophy and heart failure (Rose et al. 2010).

Our laboratory recently discovered and reported for the first time (Yan et al. 2013) that activated JNK leads to enhanced atrial arrhythmogenicity. In aged animals, JNK activation leads to reduced gap junction channels and impaired action potential conduction velocity. Young animals subjected to an in vivo JNK activator (anisomycin) (Hazzalin et al. 1998; Petrich et al. 2004) challenge results in a dramatically increased incidence and duration of pacing-induced atrial arrhythmias, which is consistent with that found in aged hearts. While a significantly increased propensity for AF in aged humans has been well recognized (Benjamin et al. 1994; Go et al. 2001; Rich 2009), our recent observations (Wu et al. 2014) suggest an increase in activated JNK in aging human atrium from healthy donor hearts (which were rejected for heart transplant due to technical reasons). Moreover, we demonstrated that JNK-induced gap junction remodeling impairs atrial conduction and causes formation of reentrant circuits in cultured atrial myocytes (Yan et al. 2013, 2014). However, previous studies have suggested that gap junction remodeling was most likely to contribute to stabilization and maintenance of AF (Dupont et al. 2001; Elvan et al. 1997; Kanagaratnam et al. 2004; Kostin et al. 2002; Nao et al.

2003; Nattel et al. 2008; Polontchouk et al. 2001; Sakabe et al. 2004; van der Velden et al. 1998, 2000; Wetzel et al. 2005). Therefore, other mechanisms such as SR Ca^{2+} handling dysfunction could be responsible for the initiation of atrial arrhythmias in aged hearts. A computer simulation study (Xie et al. 2010) suggested that generating an ectopic beat in heart tissue with poorly coupled neighboring myocytes (slowed action potential conduction) requires much fewer EAD or DAD-producing myocytes than in normal tissue composed of well-coupled cells. In another words, impaired intercellular coupling could make cardiac tissue more vulnerable to generating ectopic triggers that may initiate arrhythmias. Therefore, JNK-induced slowed conduction may create a favorable environment for JNK-induced abnormal Ca^{2+} activities to form ectopic beats and even to initiate AF. Many questions regarding the underlying mechanisms of JNK-induced AF genesis remain unanswered. Further investigations are clearly needed in this important research area.

Like JNK, both ERK and p38 are involved in various pathologies such as CVDs, diabetes, and cancers (Davis 2000; Karin and Gallagher 2005; Kyoi et al. 2006; Kyriakis and Avruch 2001; Rose et al. 2010; Yoon and Seger 2006). Although enhanced activity of ERK or p38 alone may or may not be required or sufficient for facilitating cardiac hypertrophy, both ERK and p38 were found to be activated in HF and these activated stress kinases are involved in pathological remodeling and AF development in the failing heart (Cardin et al. 2003; Li et al. 2001, 2005b; Nishida et al. 2004; Purcell et al. 2007; Wang et al. 1998; Zechner et al. 1997). Hypertrophic stimuli lead to an increase in I_{ca} and down-regulation of SERCA2 expression via activated ERK (Hagiwara et al. 2007; Huang et al. 2014; Takahashi et al. 2004). Ras, a GTPase, is able to activate ERK through a Ras-Raf-MEK cascade (Avruch et al. 2001). However, Ras signaling activated ERK was found to contribute to down-regulation of L-type Ca^{2+} channels and reduced channel activity along with reduced SERCA2 protein expression in cultured myocytes (Ho et al. 1998, 2001). It was also found that Ras-ERK-modulated molecular remodeling led to decreased intracellular Ca^{2+} transients and impaired SR Ca^{2+} uptake, which could lead to enhanced arrhythmogenicity (Zheng et al. 2004). Moreover, recent work reported by Scharf et al. (2013) suggests that p38 directly regulates SERCA2 mRNA and protein expression via transcription factors Egr-1 and SP1. Taken together, emerging evidence indicates that the stress-response MAP kinases signaling cascades could be involved in cardiac Ca^{2+} handling and arrhythmia development. Further investigation is needed for understanding the underlying molecular and electrophysiological mechanisms of altered stress signaling cascades and their cross talking in arrhythmia development under pathological conditions.

7 MAPKs Activation and Pathological Remodeling in the Heart

Extensive studies suggest that pathologically remodeled hearts exhibit a higher propensity to arrhythmias (Ai et al. 2005; Ai and Pogwizd 2005; Desantiago et al. 2008; Guo et al. 2007; Packer 1985; Respress et al. 2012). MAPKs activation has been observed in various cell types and stress conditions. Although all the MAPK family members play important roles in the development of diabetes and CVDs (Davis 2000; Karin and Gallagher 2005; Kyoi et al. 2006; Kyriakis and Avruch 2001; Rose et al. 2010), their actions are dependent on cellular context and type (Maruyama et al. 2009). For instance, in cellular senescence JNK and p38 have opposite functions (activation or suppression) (Das et al. 2007; Wada et al. 2008). In a pressure-overload mouse model induced with transverse aortic constriction (TAC), all the three MAPKs members were activated, however JNK was activated at the early phase (7 h post-TAC) followed by the activation of p38 (3 days post-TAC) and ERK (7 days post-TA) (Esposito et al. 2001). While this activation of JNK/p38 was confirmed by other groups using the same TAC mouse model (Satomi-Kobayashi et al. 2009; Villar et al. 2013), JNK/p38 inhibition alleviated the cardiac remodeling (Zhang et al. 2014). In contrast, ERK null mice predisposed the hearts to decompensation after long-term pressure overload (Purcell et al. 2007).

The MAPK activation has also been intensively studied in MI animal models (Liu et al. 2013; Ren et al. 2005; Sun et al. 2015; Yeh et al. 2010). Studies suggest that the MAPK activation profile changes dramatically at different time points after the MI-injury. For instance, Ren et al. found that minutes after LAD-ligation in a mouse model of MI, JNK, p38α, and ERK phosphorylation were all enhanced, while ablating p38 signaling with dominant negative expression of p38α decreased infarct size. They also found that p38α activation promoted a decrease in anti-apoptotic proteins (such as Bcl-2 and Bcl-XL) which further elucidated the role of p38 in MI injury and remodeling (Ren et al. 2005). In a rat model of acute MI (6 h post-MI), the activation of JNK and p38 was increased, while that of ERK was decreased and was accompanied by increased activity of caspase-3 which is pivotal in apoptosis. Further, a pharmacological reagent that decreased the activation of JNK and p38 and augmented the activation of ERK reduced the infarct size (Liu et al. 2013). Time-dependent MAPK activation has also been reported by Yeh et al., wherein the infarct or infarct border zone of post-MI mice, p38 phosphorylation is increased initially while JNK phosphorylation increased approximately 2 weeks post-MI; on the other hand, ERK phosphorylation increased after about 4 weeks post-MI, likely contributing to the post-MI ventricular remodeling (Yeh et al. 2010). In a rat model of ischemia-reperfusion (I/R) model, p38 was increased while ERK remained unchanged, during the ischemia phase, while during the reperfusion phase JNK was increased (Bogoyevitch et al. 1996). Similar findings were reported in a rat model of I/R injury (Li et al. 2011; Zhang et al. 2015). The enhanced phosphorylation of p38 and JNK after ischemia/reperfusion injury further

decreased the cell viability and promoted cardiac cell apoptosis (Song et al. 2016) via decreasing Bcl-2 and increasing Bax (Li et al. 2016). Moreover, it has been recently discovered that inhibiting JNK/p38 while enhancing ERK alleviates ischemic injury-related cardiac function loss and infarct formation (Jeong et al. 2012; Milano et al. 2007). Finally, activation of JNK, p38, and ERK was also elevated in ischemic failing human hearts, while the expression level of JNK, p38, and ERK proteins remained unaltered (Cook et al. 1999; Haq et al. 2001). All these findings suggest that MAPKs are critically involved in cardiac pathological remodeling. Although this activation of MAPKs has been found in the ischemic and failing hearts, the contribution of MAPKs in the development of cardiac arrhythmias in these pathological settings requires further investigation. Modulation of MAPK activity could be a novel therapeutic strategy to promote post-MI recovery and prevent the development of cardiac arrhythmias.

8 MAPK Family

MAPKs are serine/threonine kinases that phosphorylate serine or threonine residues in a consensus sequence of Pro-X-Thr/Ser-Pro on the target protein (Bogoyevitch and Kobe 2006). The MAPKs cascade controls the activity of numerous transcription factors and enzymes, through regulating binding partners, conformational changes, subcellular localization, and protein stability (Widmann et al. 1999).

JNK, a family member of the MAPKs, was discovered by Davis in the early 1990s (Davis 2000). JNK has three major isoforms (JNK1, JNK2, and JNK3), while the major cardiac isoforms are JNK1 and JNK2. JNK is activated by dual phosphorylation of a specific threonine-X-tyrosine motif by upstream kinases MKK4 and MKK7 (Bogoyevitch and Kobe 2006; Davis 2000). In response to stress challenges, it was found that JNK was activated to regulate cell proliferation, differentiation, apoptosis, cell survival, cell mobility, and cytokine production (Bogoyevitch and Kobe 2006; Davis 2000; Raman et al. 2007). Evidence suggests that the JNK signaling pathway is critical in the development of cancer, diabetes, and cardiovascular diseases (CVD; e.g. HF, myocardial infarction, atherosclerosis) (Davis 2000; Karin and Gallagher 2005; Rose et al. 2010). Enhanced JNK activation is also linked to significantly elevated intrinsic stress (e.g., oxidative stress or inflammatory stress) (Liu et al. 2014; Sun et al. 2014). Studies have also shown that rapid transient JNK activation appears in cultured myocytes and animals that are subjected to exercise or severe pressure overload (Boluyt et al. 2003; Nadruz et al. 2004, 2005; Pan et al. 2005), while 24 h mechanically stretched myocytes or exercise trained animals showed reduced or unchanged JNK activity (Boluyt et al. 2003; Miyamoto et al. 2004; Roussel et al. 2008). These results support the hypothesis that JNK activation could be a dynamic response to the stress stimuli.

ERKs and p38 MAPKs are the other two important stress-response signaling pathways in cellular biology (Ramos 2008; Rose et al. 2010). Through investigating the effect of mutated Thr-Gly-Tyr motif of p38 on its phosphorylation status, it is

now understood that canonical activation of p38 MAPK is achieved through dual-phosphorylation of the Thr-Gly-Tyr motif in the activation loop (Raingeaud et al. 1995). Phosphorylation of the Threonine-Tyrosine motif is mediated by upstream MAPK kinases specific to p38, MKK3 and MKK6 (Kyriakis and Avruch 2001; Moriguchi et al. 1996; Whitmarsh and Davis 1996). More upstream MAPK regulators include low molecular weight GTP-binding proteins in the Rho family (i.e., Rac-1, cdc42, Rho and Rif) and heterotrimeric G-protein coupled receptors (Marinissen et al. 1999; Zarubin and Han 2005; Zhang et al. 1995). There are four identified genes of the p38 MAPK: p38α, p38β, p38γ, and p38δ. Studies suggest that the isoforms present in the heart are p38α-γ (Court et al. 2002; Dingar et al. 2010; Seta and Sadoshima 2002), however Dingar et al. demonstrated that p38δ is also expressed in the heart at a protein level comparable to p38β, while p38α is the most abundant, followed by p38γ (Dingar et al. 2010). P38α shares sequence homology with p38β (~75%), p38γ (~62%), and p38δ (~61%), while p38γ and p38δ are ~70% identical (Cuenda and Rousseau 2007; Remy et al. 2010). Some studies suggest that the different isoforms require differential activation of MAPK kinases for full activation, one such example is p38α, which needs both MKK6 and MKK3 activation to be phosphorylated in response to cytokines, as p38δ is activated by MKK6, but negatively regulated by MKK3 (Remy et al. 2010).

ERK is also a Thr/Ser kinase that is normally located in the cytoplasm and translocated into the nucleus when activated (Chen et al. 2001; Widmann et al. 1999). Among the discovered eight ERK isoforms to date, ERK1/2 (44 kDa and 42 kDa, respectively) are the most extensively investigated isoforms within the ERK family (Kohno and Pouyssegur 1986; Sturgill et al. 1988). ERK also can be activated by tyrosine kinase receptors and Gi/o-, Gq-, and Gs-coupled receptors via a range of different signaling pathways (Lowes et al. 2002). The most characterized MKKK to activate ERK is Raf-1, a Ser/Thr protein kinase (Wellbrock et al. 2004), which binds directly to activated GTP-bound Ras leading to full kinase activation (Muslin 2005). Activated ERK1/2 (phosphorylated ERK1/2) undergoes nuclear translocation to regulate cell proliferation, differentiation, adhesion, migration, and survival (Blasco et al. 2011; Hatano et al. 2003; Saba-El-Leil et al. 2003; Yao et al. 2003). Once fully activated, Raf-1 phosphorylates and activates MKK1 or MKK2. MKK1/2, which in turn phosphorylates ERK1 or ERK2 (ERK1/2) on a Threonine and a Tyrosine residue in its activation loop, thus leading to kinase activation. Fully activated ERK1/2 has a variety of substrates at the plasma membrane, in the cytosol and in the nucleus that regulates important aspects of cell physiology and are involved in the cellular pathological remodeling process.

9 Conclusions and Implications

The stress-response MAPK family serves an integral role in cardiac development, function, pathological remodeling, and arrhythmia development in the heart. Emerging evidence suggests a link between altered stress signaling cascades and

cardiac pathological alteration and arrhythmic remodeling in pathologically altered hearts. The activation of the different MAPK members is dependent on cellular context, time, and pathological conditions. Thus, the functional impact of these MAPKs in the heart under stress conditions is likely complex and dynamic during the progression of CVDs and the development of cardiac arrhythmias. Further understanding of the underlying mechanisms of stress-induced cardiac remodeling and arrhythmia development could reveal potential effective therapeutic strategies for improving cardiac function and prevention and treatment of cardiac arrhythmias in the elderly and in patients with cardiovascular diseases.

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