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The role of osteoprotegerin (OPG) in fibrosis: its potential as a biomarker and/or biological target for the treatment of fibrotic diseases

Habibie Habibie [a,b,c,1], Adhyatmika Adhyatmika [d,1], Dedmer Schaafsma [e], Barbro N. Melgert [a,b,*]

[a] University of Groningen, Department of Molecular Pharmacology, Groningen Research Institute of Pharmacy, Groningen, the Netherlands
[b] University of Groningen, University Medical Center Groningen, GRIAC Research Institute, Groningen, the Netherlands
[c] Hasanuddin University, Faculty of Pharmacy, Makassar, Indonesia
[d] Gadjah Mada University, Department of Pharmaceutics, Faculty of Pharmacy, Yogyakarta, Indonesia
[e] Science Impact, Winnipeg, Canada

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ABSTRACT

Fibrosis is defined by excessive formation and accumulation of extracellular matrix proteins, produced by myofibroblasts, that supersedes normal wound healing responses to injury and results in progressive architectural remodelling. Fibrosis is often detected in advanced disease stages when an organ is already severely damaged and can no longer function properly. Therefore, there is an urgent need for reliable and easily detectable markers to identify and monitor fibrosis onset and progression as early as possible; this will greatly facilitate the development of novel therapeutic strategies. Osteoprotegerin (OPG), a well-known regulator of bone extracellular matrix and most studied for its role in regulating bone mass, is expressed in various organs and functions as a decoy for receptor activator of nuclear factor kappa-B ligand (RANKL) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Recently, OPG has been linked to fibrosis and fibrogenesis, and has been included in a panel of markers to diagnose liver fibrosis. Multiple studies now suggest that OPG may be a general biomarker suitable for detection of fibrosis and/or monitoring the impact of fibrosis treatment. This review summarizes our current understanding of the role of OPG in fibrosis and will discuss its potential as a biomarker and/or novel therapeutic target for fibrosis.

Contents

Abbreviations: IPF, idiopathic pulmonary fibrosis; LAP-β1, latency-associated peptide β1; LTBP-1, latent TGFβ binding protein 1; miRNAs, microRNAs; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kappa-B ligand; SLC, small latent complex; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TGFβ, transforming growth factor beta; TGFβ1, transforming growth factor beta 1.

* Corresponding author at: University of Groningen, Groningen Research Institute of Pharmacy, Department of Molecular Pharmacology, Antonius Deusinglaan 1, 9713 AV Groningen, the Netherlands.
E-mail address: b.n.melgert@rug.nl (B.N. Melgert).

[1] Authors contributed equally.

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1. Introduction

Fibrosis is linked to abnormal wound healing and is associated with many chronic inflammatory diseases. It is characterized by overproduction and accumulation of extracellular matrix proteins by (myo)fibroblasts, resulting in progressive and destructive remodelling of affected organs. Extracellular matrix production is a physiological process necessary to maintain tissues, especially in response to tissue damage. However, chronic or severe (repetitive) injury can lead to dysregulation of extracellular matrix metabolism, exceeding normal wound healing responses, and result in permanent scarring (Distler et al., 2019; Weiskirchen, Weiskirchen, & Tacke, 2019; Wynn, 2008, 2011).

Progressive fibrosis with severe destructive architectural remodelling and physiological changes leads to organ malfunction and can ultimately be the cause death. Current available treatments delay the fibrotic process at most and cannot reverse or block fibrosis progression, rendering organ transplantation as the only remaining option for patients with advanced stages of fibrosis (Caminati, Cassandro, Torre, & Harari, 2017; Fan & Guan, 2016; Thabut et al., 2003). In addition, fibrosis is often diagnosed in a late phase when an organ has already lost most of its function due to excessive scarring. This late detection is one of the main causes of the poor prognosis for and the high mortality rate of patients with fibrosis. Therefore, there is an urgent need for novel antifibrotic therapies along with reliable and readily applicable diagnostic tools to detect the development of fibrosis in earlier phases, to be able to test novel treatments in an easier way, and to monitor treatment efficacy more accurately (Martinez et al., 2017; Robbie, Daccord, Chua, & Devaraj, 2017; Schuppan, 2015).

As fibrosis is characterized by aberrant accumulation of extracellular matrix and several studies have demonstrated that extracellular matrix is not only a consequence but also an important driver of fibrosis (Herrera, Henke, & Bitterman, 2018; Liu et al., 2016; Liu et al., 2019), extracellular matrix and associated (signalling) proteins may represent likely candidates to be indicators and/or therapeutic targets for fibrosis (Burgess, Mauad, Tjin, Karlsson, & Westergren-Thorsson, 2016; Karsdal et al., 2015; Toba & Lindsey, 2019). Osteoprotegerin (OPG) is an extracellular matrix-associated protein traditionally well-recognized as an important regulator of bone matrix homeostasis (Boyce & Xing, 2007, 2008). More recently, several studies indicated the involvement of OPG in several other physiological and pathological processes, including tissue fibrosis (Adhyatmika, Putri, et al., 2020; Adhyatmika, Beljaars, et al., 2020; Boorsma et al., 2014; Habibie et al., 2020; Infante et al., 2019; Rochette et al., 2018, 2019). Relatively high levels of OPG were found locally in fibrotic lung, liver, heart and vascular tissue, which suggests a role for OPG in fibrosis development (Adhyatmika, Beljaars, et al., 2020; Adhyatmika, Putri, et al., 2020; Boorsma et al., 2014; Habibie et al., 2020; Rochette et al., 2018, 2019; Ueland et al., 2005). In this review, we summarize our current understanding of how OPG affects fibrosis development and severity and evaluate its potential as a biomarker and/or novel therapeutic target for fibrotic diseases.

2. Osteoprotegerin structure and known physiological functions

OPG is the trivial name of tumor necrosis factor receptor superfamily member 11B, based on its main function to counteract bone resorption (Boyce & Xing, 2007; Udagawa et al., 2000). Structurally, OPG is produced as a 60 kDa-monomer consisting of 401 amino acids. The monomers may also be assembled at the cys-400 residue to form 120 kDa disulfide-linked dimers. Both mono- and dimer proteins harbour a signal peptide, which is cleaved prior to secretion to form active OPG. The structure of OPG also consists of four cysteine-rich pseudo repeats located in the N-terminal and these are responsible for its binding activity to receptor activator of nuclear factor kappa-B ligand (RANKL, Fig. 1A) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, Fig. 1B). However, OPG lacks a trans-membrane domain for attachment to cell membranes and is thus only biologically available as a soluble protein, which increases its effectiveness to scavenge available RANKL and TRAIL (Anderson et al., 1997; Vitovski, Phillips, Sayers, & Croucher, 2007; Wright, McCarthy, Middleton, & Marshall, 2009).

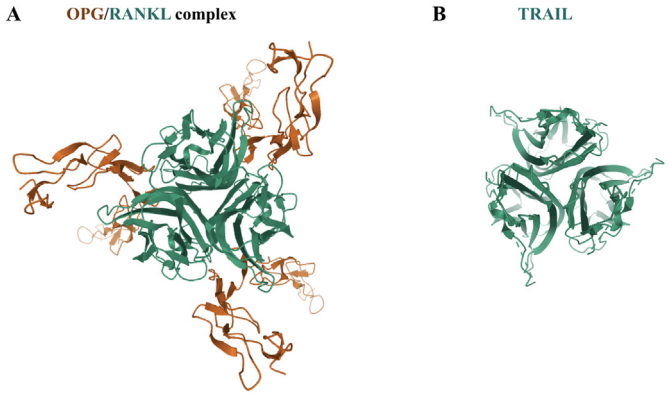

Fig. 1. A: 3D structure of trimeric human RANKL (green) in complex with human OPG (orange). Structures generated on wwPDB (wwpdb.org) (Berman, Henrick, & Nakamura, 2003; Berman, Henrick, Nakamura, & Markley, 2007; wwPDB consortium, 2019) based on data from (Luan et al., 2012). B: 3D structure of trimeric human TRAIL. Structure generated on wwPDB based on data from (Cha et al., 1999). Note the similarities between trimeric RANKL and TRAIL, explaining why OPG can bind to both.

OPG is widely recognized for its biological function as a soluble decoy receptor for RANKL (also known as TNFSF11) (Boyce & Xing, 2008; Tobeiha, Moghadasian, Amin, & Jafarnejad, 2020). RANKL will induce formation of multicellular bodies upon binding to receptor activator of nuclear factor kappa-B (RANK, also known as TNFRSF11A) on the surface of osteoclasts. The multicellular bodies will then degrade the organic extracellular bone matrix, ossein, which primarily consists of collagen and hydroxyapatite (Park, Lee, & Lee, 2017). By scavenging RANKL, less degradation of ossein will take place through RANKL/RANK interaction and bone matrix integrity is maintained (Jones, Kong, & Penninger, 2002; Ono, Hayashi, Sasaki, & Nakashima, 2020).

The production of OPG and RANKL by osteoblasts is regulated, among others, through transforming growth factor beta (TGFβ) released from bone matrix upon degradation (Fennen, Pap, & Dankbar, 2016; Janssens, ten Dijke, Janssens, & Van Hul, 2005; Lamora et al., 2016; Weivoda et al., 2016). TGFβ binds to its receptors on osteoclasts to activate a pathway that induces the secretion of proto-oncogene protein Wnt-1 by the multi-cellular body. Subsequently, Wnt-1 upregulates OPG as well as downregulates RANKL produced by osteoblasts via the canonical β-catenin-dependent Wnt signalling pathway (Duan & Bonewald, 2016; Kobayashi, Uehara, Udagawa, & Takahashi, 2016; Kovacs, Vajda, & Nagy, 2019; Kramer et al., 2010). OPG production is also well-known to be induced by the steroid hormone estradiol (Bord, Ireland, Beavan, & Compston, 2003; Jia, Zhou, Zeng, & Feng, 2017; Saika, Inoue, Kido, & Matsumoto, 2001), which explains the higher risk of developing osteoporosis in elderly women, in whom the production of estradiol is dramatically decreased.

OPG has also emerged as a decoy receptor for TRAIL (Sandra, Hendarmin, & Nakamura, 2006). TRAIL is an apoptotic factor and known to bind to decoy receptor 1 (Bernard, Quatannens, Vandenbunder, & Abbadie, 2001; Gottwald et al., 2013), death receptor 4, and death receptor 5 expressed by a wide range of cells (S. Chen et al., 2010; Gomez-Benito, Martinez-Lorenzo, Anel, Marzo, & Naval, 2007; Rizzardi et al., 2009). Because of its association with cell death, OPG has been studied in the context of cancer/tumorigenesis and vascularization (Bernardi, Bossi, Toffoli, & Fabris, 2016; Rochette et al., 2019). For instance, OPG was shown to be produced by cancer cells to intercept TRAIL to prevent TRAIL-receptor mediated apoptosis (Lane et al., 2013; Lane, Matte, Rancourt, & Piche, 2012; Rachner et al., 2009).

Inflammation can be an early initiating event of fibrosis and the levels of both OPG and TRAIL were reported to be positively correlated

with inflammation. As reported by Dumnicka et al. (2014) in patients with acute pancreatitis, OPG and TRAIL were elevated. The level of OPG correlated positively with the severity of the disease and the duration of hospitalization; however, this was not the case for TRAIL (Dumnicka et al., 2014). These findings suggest that TRAIL is expressed earlier during the inflammatory phase, whereas OPG follows as a feedback and is more important for the remodelling phase and therefore involved in fibrosis. Kuzniewsky et al. reported that in patients with chronic kidney disease, the OPG/TRAIL ratio correlated with mortality of long-term disease, with OPG levels increasing over time which was not mirrored by TRAIL. This result also implies that OPG may play an important role in the chronicity of the disease (Kuzniewski et al., 2016).

In addition to RANKL and TRAIL, some information is available on other OPG-binding ligands, which include extracellular matrix constituents like glycosaminoglycans and proteoglycans, and vascular factors von Willebrand factor and its complex with factor VIII (Baud'huin et al., 2009). In addition, OPG has been reported to bind to syndecan-1 on myeloma cells, resulting in OPG internalization and degradation (Standal et al., 2002). OPG can also stimulate chemotaxis of monocytes by binding to syndecan-1 and stimulating migration towards high concentrations of OPG (Mosheimer et al., 2005). Moreover, in endothelial cells, OPG stimulated a dose-dependent increase in the expression of adhesion molecules in the presence of tumor necrosis factor α, which was reflected by enhanced binding of monocytes (Mangan, Van Campenhout, Rush, & Golledge, 2007). Thus, OPG appears to modulate monocyte chemotaxis and migration on multiple levels.

As a soluble glycoprotein, OPG can be found in most organs in various levels (W. Liu & Zhang, 2015). It is produced by a multitude of cells, including osteoblasts, epithelial cells (Ariyasu et al., 2002; Castellanos-Gonzalez et al., 2008; Labovsky, Vallone, Martinez, Otaegui, & Chasseing, 2012; Sakata et al., 1999; Vidal et al., 2004), vascular endothelial cells (Rimondi et al., 2012; Secchiero et al., 2008; Zannettino, et al., 2005), smooth muscle cells (Callegari, Coons, Ricks, Rosenfeld, & Scatena, 2014; Davenport et al., 2018; Nakahara et al., 2016), fibroblasts (Harashima, Tsukamoto, & Horiuchi, 2004; Miyashita et al., 2004; Tunyogi-Csapo et al., 2008), and cancer cells (Holen et al., 2005; Katopodis et al., 2009; Schubert, Schulz, Emons, & Grundker, 2008; Vandyke, Jackson, Rowe, Russell, & Blair, 2007; Weichhaus, Segaran, Renaud, Geerts, & Connelly, 2014). In addition to the regulation of bone integrity, OPG has been shown to play a significant role in arthritis (Liu et al., 2019; P. Wang et al., 2017), cancer (Geerts, Chopra, & Connelly, 2020; Mielczarek-Palacz, Kondera-Anasz, & Smycz-Kubanska, 2020), and vascularization (McGonigle, Giachelli, & Scatena, 2009). Furthermore, OPG levels can be induced by several factors other than estradiol and TGFβ, such as vascular endothelial growth factor and stromal cell-derived factor-1 in endothelial colony forming cells, and interleukin-13 in fibroblasts (Adhyatmika, Putri, et al., 2020; Benslimane-Ahmim et al., 2011). These findings indicate that OPG is subject to regulation by various factors derived from a vast number of different cell types, which makes local/targeted modification of OPG expression and/or associated effects a difficult task.

3. Potential contributions of osteoprotegerin to fibrosis development

Although OPG has been found to be upregulated in a multitude of fibrotic organs (see below for more detailed information) little is known about its role in fibrosis development. Many effects of OPG are related to its function as a decoy receptor for RANKL and TRAIL; therefore, the impact of these ligands during fibrosis development needs to be considered as well.

Several hypotheses regarding the role of OPG in fibrosis development exist, but none have been definitively validated. It has been suggested that RANKL may stimulate the production of extracellular matrix-degrading enzymes, like cathepsins and metalloproteinases, in tissue macrophages in a similar way as it does in osteoclasts. In this way, RANKL could contribute to degradation of extracellular matrix and resolving of fibrosis (Boorsma et al., 2014; Corisdeo, Gyda, Zaidi, Moonga, & Troen, 2001; Giannandrea & Parks, 2014). However, matrix metalloproteinases are also involved in regulation of other cellular activities, such as stimulating cell proliferation (I. Herrera et al., 2013; Newby, 2006) and migration (P. Chen & Parks, 2009), and inducing inflammatory processes (Fingleton, 2017) and apoptosis (Mannello, Luchetti, Falcieri, & Papa, 2005). These activities could contribute to the development or progression of fibrosis (Giannandrea & Parks, 2014). If a key role for OPG in fibrosis is through inhibiting RANKL-induced matrix metalloproteinase production, the outcome in fibrosis is still unclear due to the multiple functions of matrix metalloproteinases.

High levels of OPG may prevent RANKL-induced macrophage-assisted degradation of matrix and contribute to the pathogenesis of fibrosis. We tested this hypothesis in both lung and liver fibrosis but did not find any supporting evidence (Adhyatmika, Putri, et al., 2020; Habibie et al., 2020). Interestingly, Corisdeo et al. (2001) suggested that RANKL stimulates the production of cathepsin K, a collagen-degrading protease, in bone marrow cultures and macrophages (Corisdeo et al., 2001). Moreover, OPG has been reported to directly inhibit the production of matrix metalloproteinase-13 (Meng, Bai, Yu, Wang, & Guo, 2017), an important anti-fibrotic metalloproteinase in liver fibrosis (Kim et al., 2011; Nkyimbeng et al., 2013). However, in the early development of cholestasis-induced liver fibrosis, matrix metalloproteinase-13 acts as a pro-fibrotic factor by stimulating the initial inflammation in the liver (Uchinami, Seki, Brenner, & D'Armiento, 2006). These findings suggest that high levels of OPG can directly and/or indirectly prevent the expression of matrix metalloproteinase-13 and cathepsin K, respectively, and may thus inhibit or accelerate fibrogenesis depending on the stage of fibrosis. In addition, OPG could promote vascular fibrosis by inducing TGFβ1 production *in vitro* and *in vivo* (Toffoli et al., 2011). We recently showed that OPG had similar effects in liver tissue, promoting fibrogenesis through TGFβ, which could be blocked by the presence of the TGFβ receptor kinase inhibitor galunisertib (LY2157299) (Adhyatmika, Beljaars, et al., 2020). Therefore, the effects of OPG on extracellular matrix remodelling may be regulated independently of its ability to neutralize RANKL.

Neutralizing TRAIL represents another mechanism through which OPG can affect fibrosis development. OPG prevents binding of TRAIL to death receptors 4 and 5 (S. Wang & El-Deiry, 2003). Fibroblasts have been reported to express these receptors and are therefore susceptible to TRAIL-induced apoptosis (Crowder, Dicker, & El-Deiry, 2016). When fibroblasts are activated and transformed into myofibroblasts after exposure to TGFβ1, they may start producing high levels of OPG as a strategy to protect themselves from undergoing TRAIL-induced apoptosis. We recently showed that OPG and alpha smooth muscle actin, a marker for myofibroblasts, colocalized in human cirrhotic liver tissue (Adhyatmika, Beljaars, et al., 2020). In addition, we demonstrated that *in vitro* cultures of human primary (myo)fibroblasts produce high quantities of OPG (Adhyatmika, Beljaars, et al., 2020; Habibie et al., 2020). These findings imply that myofibroblasts have the ability to generate OPG *in vivo*, which may prevent TRAIL-induced apoptosis of activated myofibroblasts leading to excessive formation of extracellular matrix. This hypothesis, however, needs further investigation.

Lesser-known functions of OPG include its ability to bind to cell surface molecules, such as heparan sulphate, syndecan-1, and αV integrins (Goswami & Sharma-Walia, 2016; Jia et al., 2017). Especially the latter is of interest with respect to profibrotic effects as integrins can activate latent TGFβ1. TGFβ1 is normally secreted as a complex of three proteins: bioactive TGFβ1, latency-associated peptide β1 (LAP-β1), and latent TGFβ binding protein 1 (LTBP-1). TGFβ1 forms a noncovalent complex with LAP-β1 called the small latent complex (SLC), rendering TGFβ1 unable to bind to TGFβ receptors. LTBP-1 can bind to SLC resulting in the complex of all three proteins called the large latent complex (Annes, Munger, & Rifkin, 2003). Various studies have demonstrated that cell surface molecules or secreted extracellular molecules can activate latent

TGFβ1 in this complex. Two mechanisms have been reported to date: the first is by proteolysis of LAP-β1, which results in the release of active TGFβ from SLC. The second involves a conformational change of LAP-β1, leading to the release of active TGFβ1 from SLC. This nonproteolytic process is thought to be dependent on the intrinsic ability of LAP-β1 to adopt different conformations (McMahon, Dignam, & Gentry, 1996). LAP-β1 contains an Arg-Gly-Asp (RGD) motif that is recognized by αv-containing integrins, including αvβ1, αvβ3, αvβ5, αvβ6, and αvβ8 (Ludbrook, Barry, Delves, & Horgan, 2003; Mu et al., 2002; Munger et al., 1999; Munger, Harpel, Giancotti, & Rifkin, 1998), which therefore have the potential to modulate the localization and possible activation of SLC by binding to LAP-β1 (Asano et al., 2005; Mu et al., 2002; Munger et al., 1999). As OPG has been shown to interact with integrin αvβ3 on endothelial cells (Jia, Zhou, et al., 2017), such an interaction with integrin αvβ3 on fibroblasts is conceivable too and may lead to the release of active TGFβ1 from the SLC and subsequent activation of fibrosis-associated genes. Interestingly, activation of TGFβ1 by αvβ3 also requires mechanical resistance from the extracellular matrix (Wipff & Hinz, 2008) and fibrogenesis has been shown to be directly affected by changes in tractional forces. This was demonstrated for myocardial infarction with loss of cardiomyocytes (Hinz, 2009; Münch & Abdelilah-Seyfried, 2021). Preliminary data from our lab showed that OPG production by primary human lung fibroblasts was higher on a stiffer matrix compared to culture on a soft matrix and demonstrated that OPG production correlated with (increased) levels of other fibrosis-related proteins (unpublished observations). These data suggest that the role of OPG in fibrogenesis may be associated with changes in localized tractional forces that can occur in affected tissues during disease. Future in-depth studies are warranted to conclusively identify and validate this process and the involvement of OPG.

These potential mechanisms could contribute in varying degrees to fibrosis development in different organs. Therefore, a more detailed discussion on the associations of OPG with fibrosis in several specific organs follows below.

4. Osteoprotegerin in liver fibrosis

The pathology of liver fibrosis is characterized by the accumulation of extracellular matrix preventing regeneration of new functional hepatocytes (Hernandez-Gea & Friedman, 2011). Liver fibrosis is thought to be the result of an unbalanced wound healing process that can be caused by several chronic factors, such as hepatitis infection, alcohol abuse, or long-term use of hepatotoxic drugs (Pellicoro, Ramachandran, Iredale, & Fallowfield, 2014).

Fibrotic sites in the liver are dominated by activated hepatic stellate cells producing cytokines and growth factors that promote fibrosis (Hernandez-Gea & Friedman, 2011). The early stage of liver fibrosis is difficult to diagnose since symptoms of disturbed liver function only occur when the liver is already moderately to severely compromised (Tsochatzis, Bosch, & Burroughs, 2014). Although it is considered possible to reverse the fibrotic state towards resolution (Ismail & Pinzani, 2009), to date there is no effective medical treatment available to accomplish this, especially in cases of full-blown cirrhosis (Ge & Runyon, 2016). Unfortunately, liver transplantation is currently the only option to restore organ functionality.

The pathogenesis of liver fibrosis includes, but is not limited to, three main contributing factors: (1) persistent inflammatory and immune responses by macrophages and lymphocytes from long-term injury (Robinson, Harmon, & O'Farrelly, 2016), (2) hepatocyte death either by apoptosis or necrosis and replacement by fibroblasts (Guicciardi, Malhi, Mott, & Gores, 2013), and (3) high oxidative stress that triggers stellate cell activation; this is especially the case for fibrosis caused by infections and chemical injury (K. S. Lee et al., 2001). The process of liver fibrosis is generally thought to start with continuous inflammation that activates Kupffer cells, liver resident macrophages, resulting in the release of various proinflammatory and profibrotic cytokines, such as tumor necrosis factor-α, interleukin-1β, TGFβ, and platelet-derived growth factor-BB (Ramachandran & Iredale, 2012). This is followed by the activation of hepatic stellate cells and their transformation into myofibroblasts, producing the excess extracellular matrix that characterizes fibrosis (U. E. Lee & Friedman, 2011). The activation of hepatic stellate cells is prominent and represents the most studied event for drug development purposes (Bataller & Brenner, 2001; Wu & Zern, 2000).

High serum OPG levels were reported in patients with liver fibrosis (Bosselut et al., 2013; García-Valdecasas-Campelo et al., 2006; Guañabens et al., 2009; Yilmaz et al., 2010). Accordingly, the inclusion of OPG in a panel of existing biomarkers to diagnose liver fibrosis was introduced in 2013 and has proven to increase diagnostic accuracy (Bosselut et al., 2013). Higher serum OPG was shown in patients with alcoholic liver fibrosis (Fábrega et al., 2005; García-Valdecasas-Campelo et al., 2006; Prystupa et al., 2016), end-stage cirrhosis (Monegal et al., 2007), chronic liver disease (Moschen et al., 2005), viral cirrhosis (González-Calvin, Mundi, Casado-Caballero, Abadia, & Martin-Ibañez, 2009; Nanda et al., 2012), and primary biliary cirrhosis (Guañabens et al., 2009; Szalay et al., 2003) compared to respective controls. These relatively high levels of OPG in several cohorts and different types of fibrotic liver diseases indicate that OPG is a suitable biomarker for liver fibrosis.

With respect to the development of liver fibrosis and the prediction of disease severity, a cohort study in patients with primary biliary cirrhosis indicated that circulating OPG increased with a more advanced disease state (Guañabens et al., 2009). Similar observations were made in patients with alcoholic liver disease, with elevated OPG in alcoholics and even higher levels in alcoholics with cirrhosis (García-Valdecasas-Campelo et al., 2006). These findings imply that OPG levels could potentially be used to assess disease severity. Moreover, it has been demonstrated that combining several blood-based biomarkers, including OPG, into a panel of diagnostic tools could further improve the accuracy in the diagnosis of liver fibrosis (Bosselut et al., 2013) and non-alcoholic steatohepatitis (Yang et al., 2015).

Our recent work has demonstrated that OPG is expressed in quiescent human hepatic stellate cells and more so in activated hepatic stellate cells (Adhyatmika, Beljaars, et al., 2020). However, OPG expression was downregulated upon TGFβ1 stimulation of these cells *in vitro*, suggesting highly complex regulatory mechanisms. Future studies are aimed at more specifically assessing the regulation of OPG production and the fibroblast subpopulations in which OPG is expressed by looking at co-expression of specific markers. Our previous work also showed that OPG is strongly expressed by scar tissue-associated fibroblasts in fibrotic tissue in both human and mouse liver, but expression in other (myo)fibroblast subpopulations could not be excluded. OPG production appeared to be exclusive to quiescent hepatic stellate cells as single cell RNA sequencing of murine liver cells showed no production of OPG by liver endothelial cells, Kupffer cells, or monocyte-derived macrophages. (Adhyatmika, Beljaars, et al., 2020). Our studies also indicated that OPG promoted fibrosis by enhancing TGFβ production in liver tissue. Upon inhibition of TGFβ1 receptor signalling with galunisertib (Herbertz et al., 2015), this profibrotic effect of OPG was inhibited (Adhyatmika, Beljaars, et al., 2020). These findings are in line with those found for OPG in vascular fibrosis (Toffoli et al., 2011). Of note, how OPG induces TGFβ expression in liver tissue remains an open question, but it may involve the previously mentioned release of active TGFβ1 from the SLC as a result of OPG binding to integrins (as proposed in Fig. 2).

5. Osteoprotegerin in pulmonary fibrosis

The pathology of pulmonary fibrosis is characterized by formation of scar tissue in interstitial areas of the lung due to exaggerated deposition of extracellular matrix by activated (myo)fibroblasts. This results in irreversible changes to lung morphology leading to impaired pulmonary function with high mortality rates (Strieter, 2008; Wilson & Wynn, 2009). Idiopathic pulmonary fibrosis (IPF) is the most severe form of pulmonary fibrosis, with a median survival of less than 5 years after

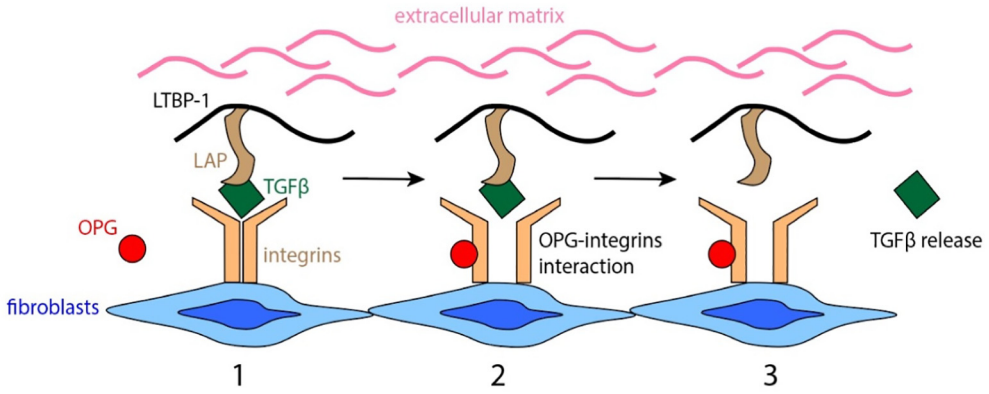

Fig. 2. Proposed interaction of OPG with integrin (αvβ3) on the surface of fibroblasts that may lead to the release of active TGFβ from SLC and subsequent activation of fibrosis-associated genes. TGFβ release upon LAP cleavage has been reported previously by Costanza et al. (Costanza, Umelo, Bellier, Castronovo, & Turtoi, 2017). LAP: latency associate peptide; LTBP-1: latent TGFβ binding protein 1; SLC: small latent complex; OPG: osteoprotegerin; TGFβ: transforming growth factor beta.

diagnosis (Demedts et al., 2001; Raghu, Weycker, Edelsberg, Bradford, & Oster, 2006). Pulmonary fibrosis is thought to be driven by repetitive epithelial injury that continuously activates stromal lung (myo)fibroblasts to produce extracellular matrix to facilitate repair. Proliferation and differentiation of alveolar epithelial type 2 cells is then required to replace damaged epithelium. However, repetitive injury may dampen epithelial regeneration properties and thereby induce abnormal activation of fibroblasts resulting in restriction of alveolar regeneration and excessive accumulation of extracellular matrix (Chanda et al., 2019).

We recently reported higher OPG levels in lung tissue, but not in serum, obtained from IPF patients compared to controls (Habibie et al., 2020). OPG levels in serum from these patients negatively correlated with diffusion capacity for carbon monoxide, a measure for lung function and severity of the disease, and we further showed that serum OPG levels exceeding 1234 pg/mL associated with rapid disease progression and shorter survival time. Interestingly, in patients with stage II sarcoidosis, who in some cases develop fibrosis, OPG levels in bronchoalveolar lavage also negatively correlated with diffusion capacity for carbon monoxide (Naumnik, Naumnik, Niklinska, Ossolinska, & Chyczewska, 2017). Moreover, another recent study found higher OPG levels in bronchoalveolar lavage fluid from patients with IPF compared to those with sarcoidosis (Majewski et al., 2021).

Previously, Brass et al. also reported higher OPG levels in the lungs of mice exposed to either silica or bleomycin (Brass et al., 2008; Brass et al., 2010). In bleomycin-induced fibrosis, OPG levels and collagen expression increased soon after bleomycin administration and waned over time in a similar fashion (Brass et al., 2008), suggesting OPG is associated with fibrosis development and lung recovery. Our recent work using murine precision-cut lung slices revealed OPG is produced locally in lung tissue and that its mRNA expression correlated with several fibrosis-associated markers (collagen 1α1, fibronectin and plasminogen activator inhibitor-1) after induction of fibrosis by TGFβ (Habibie et al., 2020). Furthermore, we found that OPG is produced by human lung fibroblasts and that lung fibroblasts isolated from IPF patients secreted more OPG compared to those from control patients. However, as with hepatic stellate cells, OPG expression was downregulated upon TGFβ1 stimulation of lung fibroblasts *in vitro*, suggesting highly complex regulatory mechanisms. Together, these findings suggest that OPG may be produced by (activated) fibroblasts in lung tissue in response to fibrotic stimuli and may have potential as a marker for establishing disease severity and prognosis.

6. Osteoprotegerin in cardiac, vascular, and renal fibrosis

6.1. Cardiac fibrosis

The pathology of cardiac fibrosis is characterized by excessive formation of scar tissue in the interstitial part of the heart. Cardiomyocyte death or damage stimuli (myocardial inflammation or pressure overload) are responsible for the initiation of fibrogenic signals and pathways leading to fibroblast activation. These subsequently produce abundant extracellular matrix resulting in fibrotic remodelling of the heart (Kong, Christia, & Frangogiannis, 2014).

Evidence for the involvement of OPG in fibrosis of components of the cardiovascular system is abundant. In a rat model of experimental autoimmune myocarditis, OPG mRNA levels steeply increased over four weeks while interstitial fibrosis developed as marked by increased expression of total cardiac fibrillar collagen (W. Liu et al., 2008). Ageing $OPG^{-/-}$ mice showed a decrease in interstitial fibrosis in parallel with an activation of matrix metalloproteinase-2 (Hao et al., 2016). Due to the fact that matrix metalloproteinase-2 is a matrix-degrading enzyme associated with fibrosis resolution (Giannandrea & Parks, 2014), OPG may promote cardiac fibrosis, at least in part, by preventing activation of matrix metalloproteinase-2. Furthermore, in an *in vivo* setup using $OPG^{-/-}$ mice and wildtype littermates, treatment with angiotensin II was shown to induce OPG mRNA expression as well as type 1 collagen in wildtype mice. In $OPG^{-/-}$ mice, however, angiotensin II stimulation resulted in significantly less interstitial fibrosis and pro-collagen-α1 mRNA expression. This study suggested that OPG facilitates cardiac fibrosis by mediating the profibrotic effects of angiotensin II (Tsuruda et al., 2016). Contrary to the aforementioned findings, in Wistar-Kyoto rats with isoproterenol-induced heart failure, anti-interleukin 17 treatment showed antifibrotic effects in myocardial tissue in parallel with a surprising increase of OPG and concomitant decrease in RANKL expression levels (Feng et al., 2009). The apparent discrepancy between these results regarding OPG in cardiac fibrosis could be related to the models and profibrotic stimuli studied or different OPG response/feedback mechanism(s), and emphasizes the intricate and complex role of OPG in cardiac fibrosis.

OPG has been investigated in myocardial fibrosis in patients with aortic stenosis (Loudon et al., 2018). This study revealed that higher OPG levels were evident in patients with chronic fibrosis after myocardial infarction compared to patients with no fibrosis, that OPG levels increased with aortic stenosis severity, and that increased serum OPG levels were associated with reduced survival. In addition, in patients with a preserved ejection fraction, higher OPG levels were associated with increased left ventricular mass index and myocardial stiffness (Kamimura et al., 2017). In a recent study in heart failure patients, plasma biomarkers were measured to predict the risk of all-cause death or heart failure-related hospital admission. Results revealed that OPG was among the best predictive biomarkers alongside fibroblast growth factor 23 and matrix metalloproteinase-7 (Chirinos et al., 2020). Furthermore, in a systematic review (2014) on biomarkers for post-infarction heart failure, a close relationship between OPG serum levels and new-onset myocardial infarction was described (Lippi &

Cervellin, 2014). Considering one of the risk factors for myocardial infarction is a gradual development of fibrous tissue on the inner wall of the coronary arteries, further investigation of OPG as a key mediator/biomarker in this process is warranted (Shetelig et al., 2017).

6.2. Vascular fibrosis

The pathology of vascular fibrosis is characterized by excessive deposition of extracellular matrix in vessel walls, including arterial wall thickening, resulting in a reduction of the lumen diameter. Hypertension, hyperglycemia, dyslipidemia and hyperhomocysteinemia are known as initiation factors that lead to activation of the fibrogenic process characterized by proliferation and activation of vascular smooth muscle cells, accumulation of extracellular matrix, and inhibition of matrix degradation. Vascular fibrosis is linked to many pathological processes and believed to be one of the primary causes of the development of atherosclerosis (Lan, Huang, & Tan, 2013).

OPG has been proposed as a biomarker for atherosclerosis because serum OPG levels were significantly higher in patients with metabolic syndrome-driven atherosclerosis (Musialik, Szulinska, Hen, Skrypnik, & Bogdanski, 2017). Similar observations were made in patients with coronary artery disease, in whom serum OPG was elevated (Maniatis et al., 2020). These more recent findings validate a previous review on biomarker functions of OPG in vascular diseases, which proposed that OPG levels are associated with the prevalence and severity of atherosclerosis-related diseases (Hosbond et al., 2012).

However, counterintuitive findings have been reported in blood vessels: by inhibiting RANKL-induced osteoclastogenesis, OPG prevented vascular calcification and as such protected vessel integrity (Cao et al., 2017; G. L. Lee et al., 2019). Accordingly, Di Bartolo et al. (2013) reported higher RANKL expression during vascular calcification. Intriguingly, they also demonstrated that TRAIL dose-dependently inhibited vascular calcification in human vascular smooth muscle cells *in vitro* and that vascular smooth muscle cells isolated from TRAIL$^{-/-}$ mice developed calcification more rapidly (Di Bartolo et al., 2013). Yet, another study demonstrated that TRAIL promoted atherosclerosis by inducing apoptosis of human microvascular endothelial cells (Pritzker, Scatena, & Giachelli, 2004). In the early atherosclerotic lesion, apoptosis of endothelial cells will increase permeability of the vessel wall resulting in increased inflammatory cell and lipid penetration (Kavurma, Tan, & Bennett, 2008). Furthermore, TRAIL has also been reported to initiate apoptosis of vascular smooth muscle cells, which may trigger the transformation of stable to vulnerable plaques since matrix proteins produced by vascular smooth muscle cells are important for plaque stabilization (Sato et al., 2006). These data suggest that OPG via TRAIL and/or RANKL neutralization may have pro- or anti-atherosclerotic properties depending on the stage of the atherosclerotic lesion.

6.3. Renal fibrosis

The pathology of kidney (renal) fibrosis is mainly initiated by prolonged injury followed by chronic inflammation (S. B. Lee & Kalluri, 2010). Chronic kidney inflammation and leukocyte infiltration induce resident kidney cells to produce large amounts of profibrotic cytokines and growth factors. These cytokines and growth factors activate (myo)fibroblasts and promote interstitial fibrosis (X.-M. Meng, Nikolic-Paterson, & Lan, 2014). High serum OPG levels were observed in patients with chronic kidney disease receiving haemodialysis (Kazama et al., 2002). Furthermore, elevated serum OPG was found to predict decline of kidney function in elderly women (Lewis et al., 2014). Interestingly, serum OPG levels were higher in hypertensive patients with chronic kidney disease and negatively associated with kidney function (Bernardi et al., 2017). These findings suggest that OPG is associated with kidney fibrosis development considering renal fibrosis is regarded as the advance-stage of chronic kidney disease (Zhong, Yang, & Fogo, 2017).

7. Osteoprotegerin as a biomarker for organ fibrosis

Most patients with fibrosis are diagnosed in an advanced stage when an organ is already severely damaged and has lost (most of) its physiological function. Another problem is that to date no sufficiently accurate biomarkers reflecting the activity/status of the fibrotic process have been identified; therefore, the right tools to effectively and easily evaluate novel antifibrotic drugs are not available. Thus, specific biomarkers are desperately needed to timely diagnose fibrotic diseases, improve clinical treatment, and accelerate the development of effective therapeutic strategies (including novel drug discoveries) (Marshall, Simpson, & Lukey, 2013). In addition, specific biomarkers could help to identify high-risk individuals, determine fibrosis severity, closely monitor fibrosis progression, develop tailored therapies most suitable for a specific subgroup of fibrosis patients, and assess the effectiveness and outcome of disease treatment.

Elevated levels of serum OPG have been detected in various fibrotic diseases and its value as a prognostic biomarker for different clinical parameters has been extensively investigated in several cohorts (as summarized in Table 1), especially in patients with liver fibrosis. Relatively high levels of OPG in different types of fibrotic organs implicate OPG as a potential biomarker for fibrosis. However, generally speaking, it is unlikely that one single biomarker can distinguish and predict the prognosis of fibrosis since numerous factors, which vary between patients and diseases, are known to initiate and be involved in fibrosis development. Therefore, the use of a panel of several biomarkers is more likely to increase the accuracy of fibrosis diagnosis and inclusion of OPG may provide crucial information in this regard. This is exemplified by the observation that the addition of OPG as a biomarker increased the accuracy of a panel of serum markers for assessment of the severity of liver fibrosis (Bosselut et al., 2013). This approach could conceivably be more widely applied, for instance in the evaluation of lung and cardiovascular fibrosis. Importantly, several other extracellular matrix-related proteins have been reported to play a role in fibrosis and could potentially serve as additional biomarkers in combination with OPG to further enhance the accuracy of the identification and diagnosis of fibrosis. These proteins include periostin (Landry, Cohen, & Dixon, 2017; Okamoto et al., 2011; Okamoto, Izuhara, Ohta, Ono, & Hoshino, 2019), extra-domain-A fibronectin (Hackl et al., 2010; Malara et al., 2019; Muro et al., 2008; van der Straaten et al., 2004; Ziffels et al., 2016) and fibulin-1 (Jaffar et al., 2014; G. Liu, Mao, et al., 2019). Interestingly, periostin, a matricellular protein critically involved in collagen organization (González-González & Alonso, 2018), has also been shown to regulate the expression of OPG (Bonnet, Garnero, & Ferrari, 2016; Galli et al., 2014). This suggests that a combination of periostin and OPG may be particularly suitable for inclusion in a biomarker panel.

Because few antifibrotic drugs are available, the use of OPG as a proxy for treatment success is promising but still needs to be investigated in more detail. Our own *in vitro* studies on liver and lung fibrosis have indicated that reduced OPG production is an early response to antifibrotic drug treatment; thus, a decrease in OPG preceded reduced expression of definitive markers of fibrosis such as collagen (unpublished observations). Additional studies are needed to validate these findings and conclusively assess the use of OPG as a biomarker for treatment success of experimental antifibrotic therapies.

8. Osteoprotegerin as a therapeutic target in organ fibrosis

As described in this review, in addition to serving as a biomarker, OPG itself may contribute to the pathology of fibrosis and may therefore represent a potential therapeutic target. In this context, we will consider and discuss two strategies: (1) neutralizing OPG, and (2) targeting the OPG/RANKL/TRAIL-axis (as depicted in Fig. 3). Of note, the OPG/RANKL/TRAIL-axis is involved in a multitude of biological events in a wide range of tissues. Complete knock-out of one of these proteins results in a disbalance of systemic homeostasis with various undesirable

Table 1
Osteoprotegerin in fibrotic disease.

Organ	Species	Level	Expression by	Mechanism	References
Liver	Human	↑ Serum of patients with primary biliary cirrhosis, alcoholic liver fibrosis, end-stage cirrhosis, chronic liver disease and viral cirrhosis.	NA	NA	(Fábrega et al., 2005; García-Valdecasas-Campelo et al., 2006; González-Calvin et al., 2009; Guañabens et al., 2009; Monegal et al., 2007; Moschen et al., 2005; Nanda et al., 2012; Prystupa et al., 2016; Szalay et al., 2003)
		NA	↑ Activated hepatic stellate cells	NA	(Adhyatmika, Beljaars, et al., 2020)
	Mouse	NA	↑ (myo)fibroblasts	NA	(Adhyatmika, Putri, et al., 2020)
		NA	NA	Induced fibrosis by enhancing TGFβ production; this profibrotic effect was inhibited by galunisertib	(Adhyatmika, Beljaars, et al., 2020)
Lung	Human	↑ Lung tissue of patients with IPF	NA	NA	(Habibie et al., 2020)
		↑ BAL of patients with stage II sarcoidosis and IPF	NA	NA	(Majewski et al., 2021; Naumnik et al., 2017)
		NA	↑ Lung fibroblasts derived from patients with IPF	NA	(Habibie et al., 2020)
	Mouse	↑ Lung tissue of mice exposed to silica or bleomycin	NA	NA	(Brass et al., 2008; Brass et al., 2010; Habibie et al., 2020)
		↑ Lung slices after TGFβ stimulation	NA	NA	(Habibie et al., 2020)
Heart	Human	↑ Serum of patients with chronic myocardial infarction fibrosis	NA	NA	(Loudon et al., 2018)
	Rat	↑ OPG mRNA in rat model of autoimmune myocarditis	NA	NA	(W. Liu et al., 2008)
		↑ Heart tissue of rat model of isoproterenol-induces heart failure treated with anti IL-17	NA	NA	(Feng et al., 2009)
	Mouse	NA	NA	Promoted fibrosis by preventing activation of matrix metalloproteinase-2	(Giannandrea & Parks, 2014; Hao et al., 2016)
		NA	NA	Facilitated fibrosis by mediating the profibrotic effect of angiotensin II	(Tsuruda et al., 2016)
Vascular	Human	↑ Serum of patients with metabolic syndrome-driven atherosclerosis and coronary artery disease	NA	NA	(Maniatis et al., 2020; Musialik et al., 2017)
	Mouse	NA	NA	Prevented vascular calcification via inhibiting RANKL-induced osteoclastogenesis	(Cao et al., 2017)
		NA	NA	Promoted vascular fibrosis by inducing TGFβ1 production	(Toffoli et al., 2011)
	Human/mouse	NA	NA	Prevented or promoted atherosclerosis depending on the phase of the atherosclerotic lesion via neutralization of TRAIL-induced endothelial and/or vascular smooth muscle cell apoptosis	(Di Bartolo et al., 2013; Pritzker et al., 2004; Sato et al., 2006)
Renal	Human	↑ Serum of patients with chronic kidney disease	NA	NA	(Bernardi et al., 2017; Kazama et al., 2002)

manifestations. For example, the OPG-RANKL ratio is involved in bone metabolism, while the OPG-TRAIL ratio plays an important role in the cardiovascular system (Bernardi et al., 2016). Therefore, interfering with this axis needs to be approached carefully as for instance neutralizing systemic OPG would undoubtedly promote osteoporosis.

The use of a therapeutic antibody against OPG has recently been tested as a treatment for pulmonary arterial hypertension in multiple rodent models (Arnold et al., 2019). The idea was that OPG can promote the proliferation and migration of pulmonary artery smooth muscle cells via the Fas receptor. Experimental treatment effectively neutralized OPG and it was confirmed that OPG was indeed able to bind to Fas receptors to initiate migration of (primarily) smooth muscle cells that contribute to fibrotic events by releasing proteins relevant to extracellular matrix formation. Therefore, the use of an OPG antibody could represent a novel approach to target fibrosis, particularly when smooth muscle cells are prominently involved, as is the case for cardiovascular and muscular fibrosis (Arnold et al., 2019).

Another potential approach to (directly) target OPG is by using microRNAs (miRNAs). Stimulation of primary lung fibroblasts with TGFβ resulted in the upregulation of several miRNAs including those that target the TNFRSF11B (OPG) gene (Ong et al., 2017; Wong & Wang, 2015). This suggests a negative feedback mechanism limiting

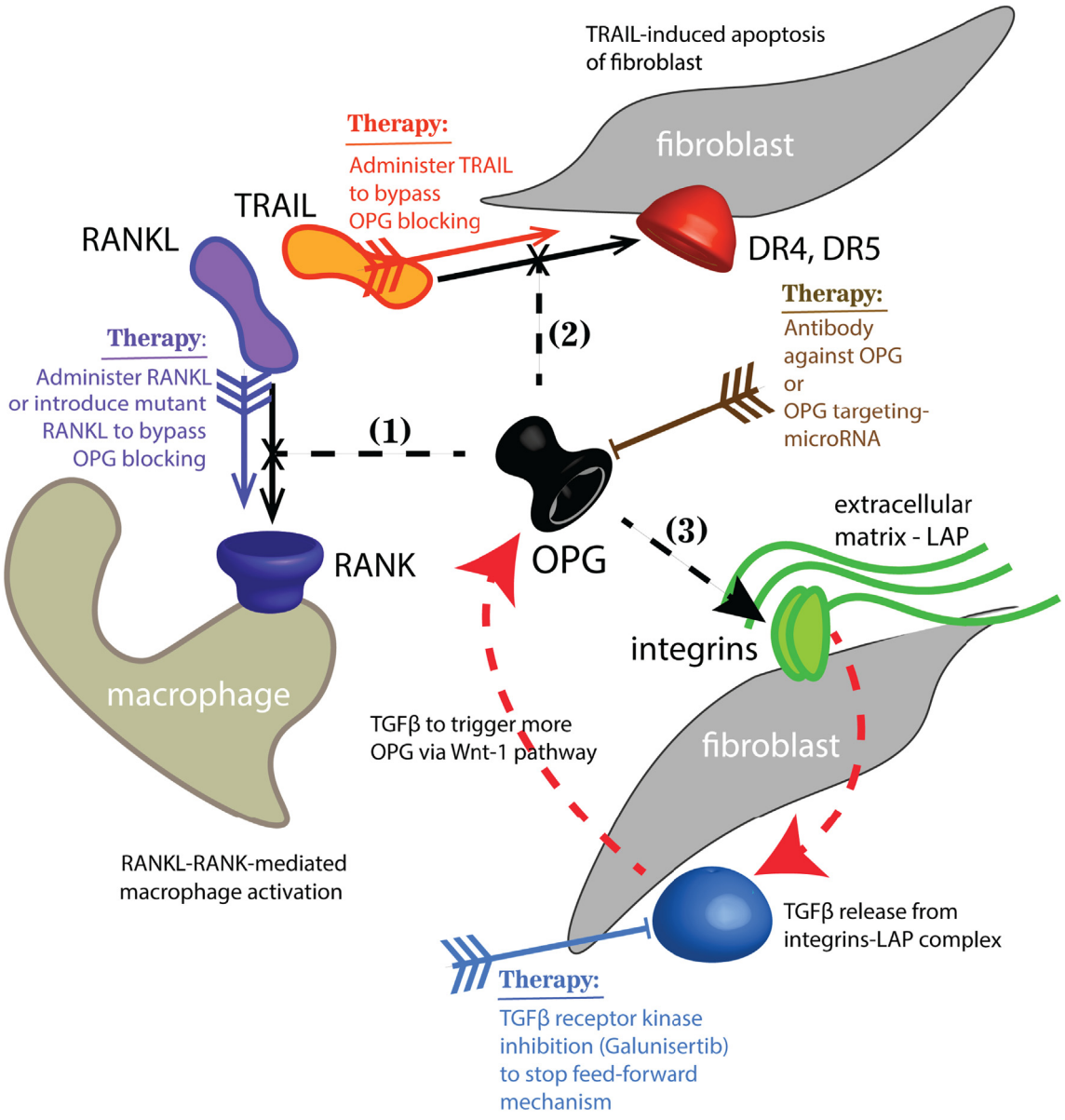

Fig. 3. OPG may contribute to profibrotic events in various ways, including, but not limited to, three main mechanisms: (1) as a RANKL decoy receptor to prevent RANKL from activating antifibrotic macrophages by binding to RANK; (2) as a TRAIL decoy receptor to prevent TRAIL-mediated apoptosis of (myo)fibroblasts; and (3) by binding to integrins on the fibroblast surface to release TGFβ resulting in the TGFβ-OPG feed-forward loop. Therapeutic approaches targeting OPG signalling to reduce and/or (further) prevent fibrosis can be achieved by reducing free OPG using a therapeutic antibody or OPG-targeting microRNAs, or by the administration of RANKL (or mutant RANKL) and TRAIL creating a surplus of these ligands to bypass the blocking effects of OPG. An indirect strategy could consist of inhibiting TGFβ receptor kinase (e.g., by galunisertib) to inhibit feed-forward production of OPG by TGFβ.

the production of OPG after TGFβ stimulation. Although this needs to be studied in more depth, it is conceivable that such a negative feedback mechanism is lost or impaired in fibroblasts from patients with fibrosis. Reconstituting these TNFRSF11B-targeting miRNAs would be another way to limit OPG production with possible therapeutic benefit.

Examples of indirectly targeting OPG include the use of RANKL mutants with low affinity for OPG that could be used if the RANKL-neutralizing activity of OPG is part of the pathological problem (Y. Wang et al., 2019); a similar approach could be attempted for TRAIL. In addition, targeting the TGFβ-OPG feed-forward loop that seems to exist at least for liver fibrosis represents another strategy to indirectly impact OPG functional dynamics. In our recent study, we found that TGFβ induced OPG expression in liver tissue and that OPG on its own could also induce expression of fibrosis-associated markers through upregulation of TGFβ expression. This was confirmed by the observation that galunisertib blocked the profibrotic effects of OPG (Adhyatmika, Beljaars, et al., 2020). These results indeed suggest a feed-forward loop between TGFβ and OPG that could be interfered with by either targeting TGFβ or OPG. However, inhibiting OPG or TGFβ systemically will need to be approached with caution, as it will trigger a wide range of systemic side effects, among which the development of osteoporosis by inhibiting OPG. A targeted, organ-specific therapy (e.g., inhalation for lung-specific treatment) may be needed to circumvent such problems.

9. Conclusion

OPG expression and serum levels are typically elevated when organs, including the liver, lungs, heart, kidneys, and the vasculature become fibrotic. Upregulation of OPG seems to promote fibrotic processes by inhibiting the biological (anti-fibrotic) functions of its ligands, which validates the therapeutic potential of targeting OPG.

However, such a strategy should be approached with caution as direct inhibition of OPG may have severe side effects such as the development of osteoporosis.

Considering OPG as a biomarker rather than a therapeutic target may have more potential. Several studies focusing on different types of fibrosis in large cohorts have demonstrated that (increased) serum levels of OPG are associated with fibrosis and could serve as a biomarker to identify patients with fibrosis and determine the severity and prognosis of the disease. A critical problem in evaluating novel treatments against fibrosis is the lack of available biomarkers that accurately reflect the state of fibrogenesis. In this regard, OPG should be considered as its levels reflect early onset of fibrosis and ongoing fibrogenesis. Implementing OPG as a biomarker on its own or as part of a panel may also facilitate the evaluation of potential therapies against fibrosis.

In conclusion, in this review we present evidence that OPG may represent a novel biomarker and therapeutic target for fibrosis. Larger cohort and further in-depth mechanistic studies are warranted to validate the clinical relevance of OPG levels and the potential therapeutic effectiveness of interfering with OPG function in fibrotic diseases.

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Declaration of Competing Interest

H. Habibie has nothing to disclose; Dr. Adhyatmika has nothing to disclose; Dr. Schaafsma has nothing to disclose; Dr. Melgert reports grants from Boehringer Ingelheim, outside the submitted work.

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